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Perkinsosis in molluscs: A review

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Abstract – The genus *Perkinsus* includes protistan parasites infecting marine molluscs throughout the world, some of which are associated with mass mortalities. Life cycle involves vegetative proliferation within the host, by which a cell named trophozoite undergoes successive bipartitioning. Other stages have been observed in vitro or in vivo, depending on the species: hypnospore, zoosporangium and zoospore. Molecular taxonomy supports a close affinity between dinoflagellates and *Perkinsus* spp. Six species of *Perkinsus* are currently considered valid: *P. marinus*, *P. olseni*, *P. qugwadi*, *P. chesapeaki*, *P. andrewsi* and *P. mediterraneus*. Histology and, above all, incubation of host tissues in Ray's fluid thioglycollate medium (RFTM) are classic diagnostic methods. In addition, more sensitive and quicker molecular diagnostic techniques based on either immunoassays or PCR have been developed for *Perkinsus* spp. Epizootiological studies have shown a marked influence of water temperature and salinity on *P. marinus* infection in oysters *Crassostrea virginica*, thus determining parasite geographical range and temporal disease dynamics (seasonality). In vitro cultures have been established for four *Perkinsus* spp. Immune response to infection varies depending on host and involves phagocytosis or encapsulation of the parasite cells by host haemocytes. A polypeptide is secreted by clam *Tapes philippinarum* haemocytes that could kill the parasite. In vitro cultured *P. marinus* cells secrete proteases that are likely involved in degradation of host tissues. *P. marinus* can suppress the toxic oxygen radicals produced by host haemocytes. In addition to host death, sublethal effects caused by *Perkinsus* spp. (reduction of fecundity, growth, and condition) may have significant ecological and economic implications. Various strategies have been assayed to mitigate the consequences of *P. marinus* epizootics on the oyster industry: modifications of management/culture procedures, selective breeding to obtain resistant oyster strains, and the use of triploid oysters and allochthonous oyster species. Some chemotherapeutants have been proved to inhibit or kill parasite cells in vitro.

Key words: *Perkinsus* / Bivalve molluscs / Taxonomy / Epizootiology / In vitro culture / Host-parasite interaction / Prophylaxis

1 Historical perspective

Description of the first species in the genus *Perkinsus* resulted from extensive studies that started in 1946 to determine the cause of massive oyster mortalities in Louisiana (Gulf of Mexico, USA) (Ray 1996). Due to the great economic importance of this molluscan species, various research groups were organised to study the problem. At first, crude spills into the water derived from the activity of oil companies close to the coast were thought to be responsible for the mortalities. Thus, initial research focused primarily on this issue. However, two years later, A.W. Collier and, at the same time, J.G. Mackin and H.M. Owen, discovered a spherical organism in the diseased oysters that was absent in the healthy ones. Collaboration among the three researchers resulted in description of this unknown agent as *Dermocystidium marinum* (Dermo), which is now called *Perkinsus marinus*. To date, this pathogen has been identified in native oysters, *Crassostrea virginica*, along the Atlantic coast of the United States and Mexico, from Maine (Ford 1996) to Tabasco, in the Gulf of Mexico (Burreson et al. 1994a; Andrews 1988; Soniat 1996).

Since the description of *P. marinus*, several new species have been added to this genus, whose taxonomic position is a controversial matter, as discussed below. In total, eight different *Perkinsus* species have been described; however, currently only six are still considered valid. The second described species was *Perkinsus olseni*, a parasite of the abalone *Haliotis ruber* in the South of Australia (Lester and Davis 1981). *P. olseni* was blamed for severe mortalities in *Haliotis laevigata* (O'Donoghue et al. 1991; Goggin and Lester 1995) and was detected in the pearl oyster *Pinctada maxima* (Norton et al. 1993). *Perkinsus*-like parasites have been reported from a large number of molluscan species from Australian waters (Goggin and Lester 1987; Hine and Thorne 2000). In the Pacific Ocean, *Perkinsus*-like parasites have been associated with mass mortalities of the Manila clam *Tapes philippinarum* in South Korea (Choi and Park 1997; Park et al. 1999; Park and Choi 2001) and have been detected in clams from Japan (Hamaguchi et al. 1998; Maeno et al. 1999; Choi et al. 2002) and China (Liang et al. 2001)

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In 1989, Azevedo described the species *Perkinsus atlanticus* in the carpet shell clam (*Tapes decussatus*) blaming it for mass mortalities of its host species in Southern Portugal. The occurrence of this parasite has been reported in the clams *T. decussatus*, *T. philippinarum*, *Venerupis pullastra* and *Paphia aurea* from different areas of the Atlantic and Mediterranean coasts of Spain (Navas et al. 1992; Sagristà et al. 1996; Ordás et al. 2001; Casas et al. 2002a). In addition, the occurrence of *Perkinsus*-like parasites has been reported in various bivalve species from several European countries, including France (Goggin 1992), Italy (Da Ros and Canzonier 1985; Ceschia et al. 1991; Canestri-Trotti et al. 2000) and Spain (Figueras et al. 1992; Santmartí et al. 1995; Figueras et al. 1996; Cigarría et al. 1997). *Perkinsus*-like parasites have also been observed in clams *T. decussatus* and *T. philippinarum* from the Mediterranean coast of Northern Africa (Navas pers. comm., AV unpublished data). Based largely on molecular data, *P. olseni* and *P. atlanticus* were recently found to be conspecific, with *P. olseni* having priority (Murrell et al. 2002). Synonymy of *P. olseni* and *P. atlanticus* supports the hypothesis that *P. olseni* was transported from Asia to Europe by movement of the clam host *T. philippinarum* (Hine 2001) and further confirms that this *Perkinsus* species has a wide host range (Goggin et al. 1989).

McGladdery, Cawthorn and Bradford described *Perkinsus karlssoni* from *Agropecten irradians* in 1991. A subsequent study on this parasite, however, concluded that it was not a valid *Perkinsus* species (Goggin et al. 1996).

In 1998, Blackbourn, Bower and Meyer described *Perkinsus qugwadi* as the causative agent of high mortalities among cultured Japanese scallops *Patinopecten yessoensis* in British Columbia, Canada (Bower et al. 1992). As discussed below, this parasite has important morphological, molecular, ecological and life cycle-related differences with the other species of the genus.

Since the first description of *P. marinus*, *Perkinsus*-like organisms have been found in various bivalve species along the Atlantic coast of North America (Perkins 1968, 1988). Two new species of the genus *Perkinsus* have been described from this region. *Perkinsus chesapeaki* was described parasitising soft shell clams *Mya arenaria* in the Chesapeake Bay (McLaughlin et al. 2000). *Perkinsus andrewsi* (Coss et al. 2001b) was isolated from the Baltic clam *Macoma balthica* also from the Chesapeake Bay. This species was also found in oysters *C. virginica* and other clam species, *Macoma mitchelli* and *Mercenaria mercenaria,* from the same area and could coexist with *P. marinus* in the four bivalve species just mentioned (Coss et al. 2001b).

Recently, the new species *Perkinsus mediterraneus* (Casas et al. 2004) has been described parasitising European oysters *Ostrea edulis* from Balearic Islands (Spain, Mediterranean Sea).

Perkinsosis affects a wide variety of molluscs (oysters, abalones, clams, scallops, pearl oysters, cockles, mussels, etc.) in the five continents with dramatic consequences in many areas. Among molluscan diseases, it is likely that this disease has resulted in the most severe economic losses and therefore, researchers from around the world have focused considerable effort on studying the *Perkinsus* parasites.

2 Life cycle and transmission

Perkinsus spp. have in common three main life stages, some of which have received multiple names as a result of placement of these parasites into various phyla over the years.

The ***trophozoite*** is the stage occurring in the tissues of the live host. It is a spherical cell with a large slightly eccentric vacuole occupying most of cell volume and a peripheral nucleus, thus having a "signet ring" appearance (Plate 1(1)). In some of the species, the vacuole contains a large polymorphic inclusion body termed a vacuoplast. The range of cell size varies among species. Vegetative proliferation (palintomy) within the host involves the trophozoite undergoing successive bipartitioning (cycles of karyokinesis followed by cytokinesis) to yield up to 32 daughter cells that stay together in a rosette-like arrangement inside a wall (Plate 1(2)). Wall rupture allows liberation of cuneiform to spherical daughter cells or immature trophozoites, which gradually enlarge and form a vacuole inside becoming mature trophozoites (Goggin and Lester 1995; Perkins 1996; Blackbourn et al. 1998).

Ray (1952) observed that when host tissues infected by *P. marinus* were incubated in fluid thioglycollate medium (FTM), trophozoites enlarged (keeping the spherical shape) and developed a thick wall, thus becoming a new stage he called the ***hypnospore***. This process also has been observed in all of the other *Perkinsus* spp. except for *P. qugwadi* (Blackbourn et al. 1998). When hypnospores produced in FTM are isolated and transferred into seawater, zoosporulation begins and progresses with successive karyokinesis and cytokinesis.

Finally, hundreds of ***zoospores*** are formed within the original cell wall (Plates 1(3) and 1(4)). Biflagellated ellipsoidal zoospores (Plates 1(4) and 1(5)) leave the zoosporangium through a single (occasionally two) discharge tube, which appears on each hypnospore before any cell division has occurred. (Perkins and Menzel 1966; Lester and Davis 1981; Azevedo 1989; McLaughlin et al. 2000; Casas et al. 2002a, 2004). Hypnospores are precursors of zoosporangia, and therefore, have been referred to as prezoosporangia, as well (Perkins 1996). Enlargement of trophozoites also was observed in moribund hosts (Ray 1954; Mackin 1962; Perkins 1968; Valiulis and Mackin 1969). The observations of Perkins (1968) and Valiulis and Mackin (1969), who induced production of zoospores by isolating *Perkinsus* sp. hypnospores from decaying tissues of *Macoma balthica* and placing the parasite cells in sea water, led to the hypothesis that transformation of *Perkinsus* spp. trophozoites into hypnospores occurs naturally in moribund hosts and that the latter begin zoosporulating when they are released into sea water. However, it is unclear whether hypnospores released into sea water from moribund and deceased oysters would zoosporulate in nature (Chu 1996). In vitro zoosporulation of *Perkinsus* spp. is modulated by temperature and salinity, with threshold values of both environmental variables under which the process does not progress (Chu and Greene 1989; Auzoux-Bordenave et al. 1995; Ahn and Kim 2001; Casas et al. 2002a). Nevertheless, hypnospores can survive for a long time under environmental conditions that prevent zoosporulation without loosing the ability to zoosporulate and, when conditions become favourable, these hypnospores can then undergo

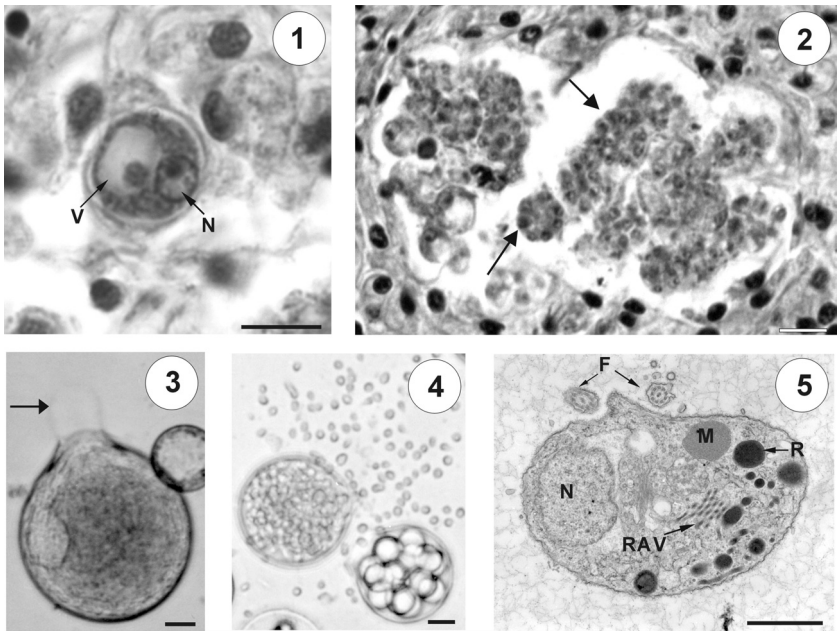

Plate 1. (1) Light micrograph of an histological section of an oyster *Ostrea edulis* showing a trophozoite of *Perkinsus mediterraneus* in the connective tissue of the visceral mass; N: nucleus; V: vacuole. Bar = 10 μm. (2) Light micrograph of an histological section of a clam *Tapes decussatus* showing multicellular stages of *Perkinsus olseni/Perkinsus atlanticus* (arrows) resulting from vegetative multiplication of mother cells with daughter cells in a rosette-like arrangement. Bar = 10 μm. (3) Light micrograph of a zoosporangium of *Perkinsus olseni/Perkinsus atlanticus* at single cell stage, showing the discharge tube (arrow). Bar = 10 μm. (4) Light micrograph showing two zoosporangia of *Perkinsus olseni/Perkinsus atlanticus*, one of them enclosing 16 cells and the other one discharging zoospores. Free zoospores appear swimming. Bar = 10 μm. (5) Transmission electron micrograph of a *Perkinsus olseni/Perkinsus atlanticus* zoospore showing flagella (F), mitochondrion (M), nucleus (N), rhoptries (R) and ribbon associated vesicles (RAV). Bar = 1 μm.

zoosporulation (Chu and Greene 1989; Casas et al. 2002a). The high tolerance of hypnospores to chlorine (Goggin et al. 1990; Bushek et al. 1997a; Casas et al. 2002a), low temperature, and low pH (Auzoux-Bordenave et al. 1995) exposure, suggests that the hypnospore may be a dormant stage in the life cycle of *Perkinsus* spp., which allows it to endure unfavourable conditions (Casas et al. 2002a).

Transmission of *Perkinsus* spp. occurs without intermediate hosts (Ray 1954; Goggin and Lester 1995; Chu 1996; Blackbourn et al. 1998). White et al. (1987) reported transmission of *P. marinus* to healthy oysters from the gastropod ectoparasite *Boonea impressa* that had been fed infected oysters. Nevertheless, transmission occurs in places lacking the gastropod, thus the latter is not required for transmission (Burreson and Ragone Calvo 1996). Trophozoites, hypnospores and zoospores of *Perkinsus* spp. all have been shown experimentally to cause infection in oysters (Goggin et al. 1989; Volety and Chu 1994a; Rodríguez et al. 1994; Chu 1996; Bushek et al. 1997b; Chintala et al. 2002; Ford et al. 2002; Bushek et al. 2002b). Oysters inoculated with *P. marinus* trophozoites resulted in higher infection prevalence and intensity than oysters inoculated with hypnospores (Volety and Chu 1994a). However, it is not known which stage is most effective or the principal stage for transmitting the disease in the natural environment (Chu 1996). Viable *P. marinus* cells are released from live infected oysters through diapedesis and in faeces (Bushek et al. 1997b; Scanlon et al. 1997; Bushek et al. 2002b). Therefore, infected live hosts are a continuous source of infective stages (trophozoites) and, if the hypothetical cycle described above occurs in the natural environment, host death does not prevent transmission since hypnospore formation would allow further transmission either through the hypnospore itself or by it giving rise to infective zoospores. Recently, Ragone Calvo et al. (2003b) have reported that maximum transmission rates of *P. marinus* are observed during maximum *C. virginica* mortality caused by this parasite, although transmission can occur when host mortality is low or absent, which suggests the involvement of mortality-independent dissemination mechanisms.

Morphology of the different stages of *P. marinus* had been thoroughly studied (zoosporulation: Perkins and Menzel 1967; Perkins 1976; trophozoites and vegetative proliferation: Perkins 1969; reviewed by Perkins 1996) before any other species of the genus were described. Thus, a solid morphological reference was available for comparison as new species were being described. Ultrastructural studies of the latter (*P. atlanticus:* Azevedo 1989; Azevedo et al. 1990; Sagristà et al. 1996; *P. qugwadi*: Blackbourn et al. 1998; *P. cheasapeaki*: McLaughlin et al. 2000; *P. andrewsi*: Coss et al. 2001a; *P. mediterraneus*: Casas et al. 2004) have revealed only minor deviations from the characters of the type species.

Ultrastructural study showed that most of the organelles occurring in the trophozoites were similar to those of many other eukaryotic protists. The formation and structure of the wall (with lomasomes) surrounding the cell and the nature of the vacuoplast suggested affinities with the Labyrinthulomycota and the Phycomycetes. In addition, there are micropores associated with the cell surface resembling

those of Apicomplexa (Perkins 1969). Most ultrastructural studies on *Perkinsus* spp. have been focused on the zoosporulation process. Zoospores are uninucleated, with several vacuoles in the cytoplasm, mitochondria (or mitochondrion) in a lateral position. They posses two flagella with lateral insertion, the anterior one having mastigonemes. Remarkably, the occurrence of an apical complex inside the zoospore resembling (although with some differences) that of Apicomplexa was first noted by Perkins (1976) (Plate 1(5)).

3 Molecular taxonomy

There has been considerable debate and confusion concerning the taxonomic affinities and phylogenetic placement of the genus *Perkinsus*. As mentioned above, the type species *Perkinsus marinus* was originally described as a fungus *Dermocystidium marinum* (Mackin et al. 1950). Subsequent morphological analyses led to various reclassifications including placement in the Ascomycetales, Entomophthorales, Saprolegnales, the Haplosporidia and with the labyrinthuloides (Mackin 1951; Sprague 1954; Mackin and Boswell 1956; Mackin 1962; Mackin and Ray 1966). Perkins (1976) argued to place this organism in the phylum Apicomplexa based on the presence of a conoid apical structure, a subpellicular membrane and micropores and in 1978 Levine established the genus *Perkinsus*, formally placing it in Apicomplexa (Levine 1978). Vivier (1982), however, argued that the membrane structure and micropores were not unique to apicomplexans, but were also common to ciliates and dinoflagellates. In addition, the conoid structure is incomplete suggesting that the apicomplexan affinity was tenuous.

Development of relatively rapid and efficient DNA sequencing technology has facilitated examination of taxonomic affiliations based on molecular characters. Regions in the *Perkinsus* genome can be sequenced and compared to the corresponding sequences in other protists for molecular phylogenetic analyses. Fong et al. (1993) and Goggin and Barker (1993) published the first DNA sequences for a *Perkinsus* species. Results of their phylogenetic analyses with the small subunit ribosomal RNA (SSU rRNA) gene sequence –using the relatively small protistan taxonomic dataset that was available– suggested that *Perkinsus* species were more closely related to dinoflagellates than the apicomplexans within the group of taxa referred to as alveolates, which includes the ciliates, apicomplexans and dinoflagellates (Cavalier-Smith 1993). In a few molecular phylogenetic studies, however, *Perkinsus* has grouped with apicomplexans (Nóren et al. 1999). In the last decade much more molecular sequence data has become available for the Alveolata, as well as for other protistan taxa. Most analyses using these larger taxonomic datasets with the SSU rRNA gene sequences, as well as those with actin and tubulin gene sequences, have continued to support a close affinity between dinoflagellates and *Perkinsus* species with *Perkinsus* falling at the base of the dinoflagellate clade (Escalante and Ayala 1995; Reece et al. 1997b; Siddall et al. 1997; de la Herrán et al. 2000; Murrell et al. 2002; Saldarriaga et al. 2003). Nóren et al. (1999) described a new alveolate *Parvilucifera infectans* that they found was closely related to *Perkinsus* and they proposed a new phylum, Perkinsozoa, to include those two genera, as well as *Colpodella*. This new phylum, however, has not yet received wide acceptance and some recent analyses have failed to group all of these genera together (Kuvardina et al. 2002; Saldarriaga et al. 2003; Reece et al. 2004).

4 Species designations

Typically, descriptions of various *Perkinsus* species have relied largely on differences in geographic and host range as a basis for species discrimination. Although slight variations in morphology have been observed among the *Perkinsus* species, it is difficult to discriminate among species based on morphology alone, and in some cases difficult to distinguish *Perkinsus* species from other protists such as *Colpodella*, which has similar morphological features. Trophozoite morphology does not have taxonomic value, because it can be influenced by the host (Goggin and Lester 1995), the time of the year (Ray and Chandler 1955; Bushek et al. 1994) and nutrient availability (La Peyre et al. 1993; La Peyre and Faisal 1996; Casas et al. 2002b). Some differences have been reported in hypnospore/zoosporangium morphology, such as hypnospore size, ratio between discharge tube length and zoosporangial body diameter, number of discharge tubes and fine structure of the zoosporangial wall. In the case of zoospores, several characters have received taxonomic attention, such as the size, the angle forming the flagella at their insertion, the occurrence of an electron-dense body in the flagellum basal body, and distribution of cortical alveoli on the surface of the zoospore. Therefore, individual *Perkinsus* species cannot be differentiated using conventional diagnostic techniques such as histological examination and the Ray's fluid thioglycollate assay (Ray 1966), described below. Nevertheless, in addition to morphological characters of zoospores, *P. qugwadi* differs from the other *Perkinsus* spp. in that all the known stages (trophozoites, hypnospores and zoospores) can be observed in host tissues (Blackbourn et al. 1998), it multiplies and is pathogenic at lower temperatures, and the Ray's fluid thioglycollate assay is negative for this species. Experimental transmission of *P. qugwadi* by cohabitation was only possible when zoospores were present in the "donors" (Bower et al. 1998).

As mentioned above, six species of *Perkinsus* are currently considered valid (Table 1). There is now evidence from DNA sequence data that, as was found with *P. olseni* and *P. altanticus*, *P. chesapeaki* and *P. andrewsi* are the same species and should be synonymised. Distinct internal transcribed spacer (ITS) sequences that were previously attributed to the two different species were found together in several clonal cultures isolated from multiple hosts suggesting that the observed polymorphisms demonstrated intra-specific, rather than inter-specific, variation (Dungan et al. 2002). This result suggests that the two species are synonymous and, like *P. olseni*, this species also has a wide host range. In general, it is now clear that molecular sequence data is playing an increasingly important role in the identification of *Perkinsus* species.

Although SSU rRNA gene sequences cannot be used to confidently resolve *Perkinsus* species (see Casas et al. 2004), ITS region sequences can discriminate among most *Perkinsus*

Table 1. *Perkinsus* spp. and hosts.

***Perkinsus* species**	**Type Host**	**Type Location**	**Other Hosts**
P. marinus	*Crassostrea virginica*	East and Gulf Coasts of USA	*Crassostrea rhizophorae*
P. olseni/P. atlanticus	*Haliotis rubra*	Australia	*Anadara trapezia* *Austrovenus stutchburyi* *Chamus pacificus* *Haliotis laevigata* *Tapes decusatus* *Tapes philippinarum*
P. qugwadi	*Patinopecten yessoensis*	British Columbia, Canada	
P. chesapeaki	*Mya arenaria*	Chesapeake Bay, USA	*Crassostrea virginica* *Mya arenaria* *Macoma balthica* *Mercenaria mercenaria* *Tagelus plebeius* *Tridacna maxima* *Tridacna crocea*
P. andrewsi	*Macoma balthica*	Delaware Bay, USA	
P. mediterraneus	*Ostrea edulis*	Balearic Islands, Spain	

species, except as discussed above, and have been used to examine relationships within the genus (Goggin 1994; Kotob et al. 1999; Casas et al. 2002a,b; Dungan et al. 2002; Brown et al. 2004; Casas et al. 2004). ITS region sequences suggest that *P. mediterraneus* is a close sister group to *P. olseni/P. atlanticus*, with *P. marinus* the next most similar species. The *P. qugwadi* ITS sequence is most divergent and the *P. chesapeaki/P. andrewsi* ITS sequences fall intermediate to those of *P. qugwadi* and the other *Perkinsus* species, but are much less similar to *P. qugwadi* (Fig. 1). Although less data are available, the within genus relationships based on non-transcribed spacer (NTS) region sequences (Murrell et al. 2002) are in agreement with those hypothesized by the ITS sequence data.

We recommend that before designation of new *Perkinsus* species, hosts from a wide geographic range be examined using RFTM and histology as initial diagnostic methods. Subsequently, whenever possible multiple clonal cultures from infected hosts throughout the range should be established so that the parasite can be described from histology, culture, transmission electronic microscopy, TEM, if possible, and using molecular sequence data. It is important to obtain sequence data from multiple loci (e.g. SSU, ITS, NTS) and from multiple cultures (hosts) to allow thorough phylogenetic analyses for documentation of a distinct clade for the proposed species. Deposition of type material, including a type culture and a type histological section from the same individual as the type culture isolation, is very important for validation and future studies on a new species.

5 Intra-specific variation

Within the last few years more data on the amount of genetic variation within some of the *Perkinsus* species has become available. Currently there are over 200 *Perkinsus* spp. sequences deposited in GenBank (Table 2). Most of these are DNA sequences for regions of the ribosomal RNA gene complex, particularly the ITS region. As shown in Table 2, ITS sequences from multiple isolates and/or hosts are now available for *P. marinus*, for *P. olseni*, including those sequences deposited as *P. atlanticus*, for the *P. chesapeaki/P. andrewsi* group, and for *P. mediterraneus*. These numerous ITS sequences have afforded excellent opportunities to assess intra-specific variation within the ITS region (Casas et al. 2002a,b; Dungan et al. 2002), and to compare that variation to differences observed among the *Perkinsus* species. Overall, the ITS region has been found to be useful for species discrimination with intra-specific variation among sequences within a *Perkinsus* species ranging from 0 to approximately 3% and among species differences ranging from approximately 4−14% (Brown et al. 2004). Sequences for other genes in *P. marinus* including actins (Reece et al. 1997b; Saldarriaga et al. 2003), tubulins (Saldarriaga et al. 2003), superoxide dismutases (Wright et al. 2002) and serine proteases (Brown and Reece 2003) have been determined and numerous additional gene sequences will undoubtedly soon become available for this parasite, as well as for the other *Perkinsus* species. These data provide additional information for phylogenetic studies, species comparisons and assessments of intra-specific genetic variation.

Within the *Perkinsus* genus, genetic strain variation has been most comprehensively examined in *P. marinus* for which isolate cultures have been available since 1993 (Gauthier and Vasta 1993; Kleinschuster and Swink 1993; La Peyre et al. 1993). Reece et al. (1997a, 2001) developed a series of PCR primers to amplify polymorphic regions of the *P. marinus* genome. These markers were used to examine the geographic distribution of genetic strains along the US Atlantic and Gulf coasts (Reece et al. 2001). They surveyed 76 primary and

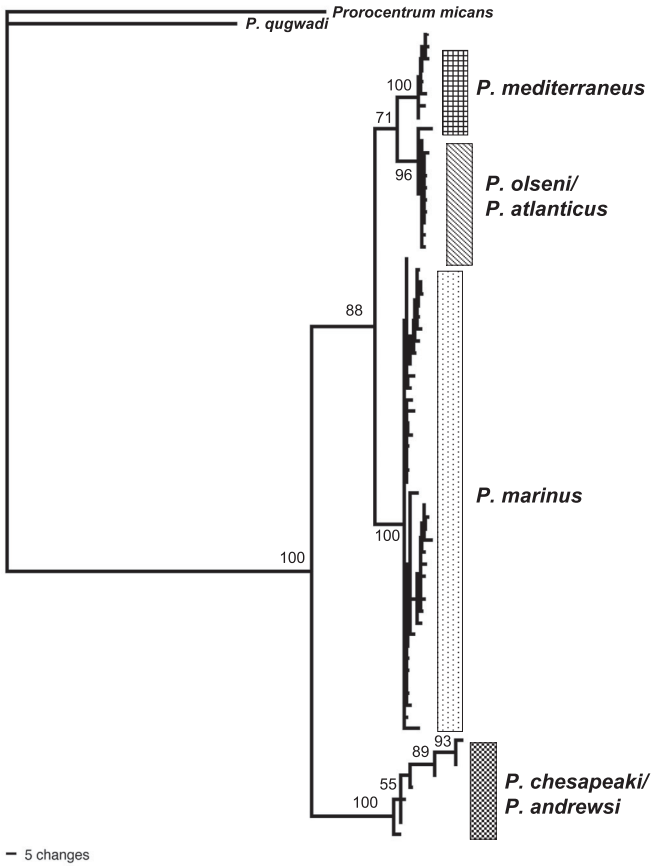

Fig. 1. Representative phylogram resulting from parsimony analyses conducted with rRNA internal transcribed spacer (ITS) region sequences from *Perkinsus* spp. and *Prorocentrum micans*. More than 100 equally parsimonious trees resulted from this analysis, although the composition of sequences within each major clade did not vary. Bootstrap support values for each clade are given at the nodes. Analysis was conducted with 100 replicates of 10 random additions. 820 total characters were included. 303 characters were constant, 302 were variable, but uninformative and 215 were parsimony informative. Tree statistics: Tree length = 785, Consistency index = 0.848, Retention index = 0.964.

86 clonal isolate cultures established from infected oysters collected from Massachusetts to Texas and found 12 different genetic strains based on eight polymorphic loci. The geographic distribution of *P. marinus* strains was not uniform with unique compositions of strains found in the Northeast, Southeast and Gulf coast regions of the US. The predominant strain isolated from north eastern oysters was not found along the Gulf coast, and conversely one of the two strains that dominated in the Gulf was absent along the Atlantic coast. In addition, there was evidence that oysters can be infected by multiple genetic strains of the parasite and that at least some of the *P. marinus* cells in culture were diploid (Reece et al. 1997a; Reece et al. 2001). As discussed further below, these data strongly support the need to obtain adequate DNA sequence data at the targeted loci from multiple strains and from related species in order to develop reliable, accurate and sensitive molecular diagnostics.

6 Diagnostic techniques

6.1 Ray's fluid thioglycollate assay

The standard diagnosis method for *Perkinsus* is the incubation of host tissues in fluid thioglycollate medium (FTM). This technique was fortuitously developed in the 1950s when researchers were trying to establish an in vitro culture of this pathogen, then thought to be a fungus (Ray 1996). Since it did not grow in the usual fungus media, it was thought to be an obligatory parasite. Therefore, in order to establish bivalve tissue cultures in which the pathogen could grow, oyster gill tissues were treated with antibiotics and incubated in FTM to test their sterility. After an incubation time of 48h, no bacteria were detected but large spherical bodies that, in subsequent studies, were identified as enlarged parasite cells were observed (Ray 1996). Due to the rapid staining of these bodies with Lugol's iodine and the ease of visualization under light microscopy as blue-black spheres, this method has been adopted as the standard technique for *Perkinsus* diagnosis since it was published by Ray (1952). The addition of nystatin to the FTM, not only for prohibiting fungal growth, but also for enhancing enlargement of the parasite, completed the final formulation of the Ray's fluid thioglycollate medium (RFTM) (Ray 1966). Although the incubation time needed for the enlargement of all of the parasitic forms was first proposed to be 48 hours (Ray 1954), the low infection rates found in field studies prompted Ray (1966) to increase incubation time to one week. These enlarged parasitic forms are known to be hypnospores, a relatively resistant stage for organisms in the genus *Perkinsus*.

To detect light *P. marinus* infections by the RFTM technique, incubation of oyster heart, rectum, and mantle and gill fragments, was necessary (Ray 1952, 1954). For routine diagnosis, gills were incubated in RFTM, the number of dark blue hypnospores were counted, and the determination of the infection rate was made according to the scale proposed by Mackin (Mackin, in Ray 1954) in which a numerical value of 0.5, 1, 2, 3, 4 or 5 was assigned for infections ranging from very light to heavy, respectively. In addition to the simplicity of the RFTM technique, it has been found to be more sensitive, if rectum and gill tissues are incubated in thioglycollate medium, than direct observation in the light microscope of paraffin-embedded host tissues (McLaughlin and Faisal 1999). Due to the differential distribution of *Perkinsus* spp. in molluscan tissues, as assessed by selective incubation of the host organs in RFTM (Choi et al. 1989; McLaughlin and Faisal 1999; Oliver et al. 1998), the technique has been modified for *P. marinus* in oyster (Fisher and Oliver 1996) and for *P. olseni/P. atlanticus* in carpet shell clam (Almeida et al. 1999) to include the whole bivalve body in the assay. In addition, Gauthier and Fisher (1990) suggested the incubation of haemolymph in RFTM as an alternative to the classical tissue RFTM to allow non-lethal sampling of individuals. This procedure has been improved by Nickens et al. (2002). The different RFTM assays for *P. marinus* and *P. olseni/P. atlanticus* have been revised and compared (Bushek et al. 1994; Rodríguez and Navas 1995), and the whole host body incubation in thioglycollate medium has been found to be the most sensitive method. Recently, Novoa et al. (2002) reported the lack of specificity of the RFTM by DNA sequence analysis of SSU rRNA genes

Table 2. Perkinsus spp. DNA sequence data summary.

Species	Gene or Region	# of Sequences	GenBank Accession Numbers
P. marinus	SSU	5	L07375, X75762, AF042708, AF497479, AF126013, AF324218
	ITS (+ 5.8S)	33	AF497479, AF149876, AF126022, AF150985-AF150990, U07700, AY295177- AY295199
	NTS (+ 5S)	2	S78416, AF497479
	actin	5	U84287, U84288, AY436363- AY436365
	$\alpha-$ and β-tubulin	3	AF482399, AF482400, AF482401
	Misc. seqs	44	AR179760-AR179774, AY299656-AY299683, AY527398
	Serine protease	13	AY340222-AY340234
	Superoxide dismutase	3	AY095212, AY095213, AY137780
	Divalent cation transporter	1	AY324383
	Heat shock protein 90	1	AY391259
P. olseni/ P. atlanticus	SSU	2	AF509333, AF295120
	ITS (+ 5.8S)	40	AF472517-AF472523, AF509333, AF473840, AF369967-AF369979, U07701, U07697-U07699, AY435092, AF441207-AF441218, AF522321
	NTS (+ 5S)	16	AJ238400, AF509333, AF438150, AF466527-AF466540
	LSU	1	AF509333
P. chesapeaki/ P. andrewsi group	SSU	4	AF252288, AF042707, AF102171, AY305326
	ITS (+ 5.8S)	12	AF440464-AF440471, AF252288, AF102171, AF091541, AY305326
	NTS (+ 5S)	2	AF252288, AF102171
	Intergenic spacer	1	AY305328
	LSU	1	AY305327
P. mediterraneus	SSU	6	AY517645-AY517647, AY487831-AY487833
	ITS (+ 5.8S)	10	AY487834-AY487843
P. qugwadi	ITS (+ 5.8S)	1	AF151528

SSU Small subunit gene, ITS Internal transcribed spacer region, NTS Non transcribed spacer, LSU Large subunit gene.

from hypnospores obtained through incubation of clam gills in RFTM. The results showed that the assay was not specific to members of the genus *Perkinsus*, and that other organisms including a *Perkinsus*-like organism (*Pseudoperkinsus tapetis*, Mesomycetozoa), also enlarged to hypnospores when incubated in thioglycollate medium.

6.2 Molecular diagnostic assays

Over the last several years, new immunological and molecular diagnosis techniques have been developed as an alternative to the RFTM assay and they were intended to specifically target the members of the genus *Perkinsus* or even particular species. Choi et al. (1991) developed polyclonal antibodies against *P. marinus* hypnospores that did not react against other parasite stages, pointing out a change in the antigenic properties of the parasite membrane during the maturation of the hypnospore cellular wall. Dungan and Robertson (1993) obtained similar results with monoclonal antibodies. In contrast, the polyclonal antibodies developed by these authors bound to every cellular stage of *P. marinus*, and even recognized particulate substances localized in host tissues in areas surrounding focal lesions. These antibodies also recognized *P. olseni/P. atlanticus* and several *Perkinsus* sp., but not some *Dermocystidium* species. Recently, however, these antibodies have been shown to cross-react with several dinoflagellate species, particularly many of the parasitic dinoflagellates (Bushek et al. 2002a). An ELISA assay using polyclonal antibodies raised against in vitro produced *P. marinus*

extracellular products was shown to be more sensitive than the RFTM assay as diagnostic tool (Ottinger et al. 2001); however, specificity was not rigorously examined. Polyclonal antisera specific against polypeptides obtained from *P. olseni/P. atlanticus* hypnospores have been produced by Montes et al. (2002).

Several PCR primer pairs that specifically target *P. marinus* sequences have been developed. These include primers that target portions of the ribosomal RNA gene complex (Marsh et al. 1995; Robledo et al. 1998; Yarnall et al. 2000), as well as primers that target other regions of the genome (Reece et al. 1997a; Reece et al. 2001). A PCR assay that targets the non-transcribed spacer (NTS) region of *P. marinus* (Marsh et al. 1995; Robledo et al. 1998), amplifying a 307 bp fragment, was found to be more sensitive and specific than the RFTM assay, discriminating *P. marinus* from other *Perkinsus* species. Many of the *P. marinus* assays and PCR primers, however, were not designed specifically for the purpose of species discrimination and have not been extensively tested for cross-reactivity to other species within the genus or to closely related taxa. In order to quantify the amount of parasite present in the host, Marsh et al. (1995) and Yarnall et al. (2000) developed semi-quantitative and quantitative assays for *P. marinus*, respectively. The quantitative assay was based on amplification of a 1210 bp fragment of the parasite SSU rRNA gene- ITS region, and the comparison of its band intensity with that obtained for a competitor plasmid with the same sequence excepting a 13 bp deletion. As with the standard PCR assay, the QCPCR was more sensitive than RFTM of tissues and haemolymph, although the body burden incubation in thioglycollate described by Fisher and Oliver (1996) detected the presence of *P. marinus* in the same number of individuals as the new technique (Yarnall et al. 2000). This PCR assay allowed the quantification of the parasite DNA present in the host, and detected as few as 0.01 fg of *P. marinus* DNA in 1.0 μg of oyster tissue. In addition, a multiplex PCR assay has been developed that allows simultaneous and differential detection of *Haplosporidium nelsoni*, *H. costale* and *P. marinus*, all parasites of the eastern oyster *C. virginica* (Penna et al. 2001).

New diagnosis tools based on specific PCRs have also been developed for other species of the genus *Perkinsus*. PCR primers based on ITS region sequences were recently designed to detect all described *Perkinsus* species except *P. qugwadi* (Casas et al. 2002a). Nested ITS region primers were developed to diagnose the *Perkinsus* sp. infecting Japanese short-necked clams (Hamaguchi et al. 1998), which based on the ITS sequence is likely to be *P. olseni*. Several PCR primer pairs have been designed to target specifically the NTS region of *P. olseni/P. atlanticus* (de la Herrán et al. 2000; Robledo et al. 2000; Murrell et al. 2002). The same genomic region was selected by Coss et al. (2001b) to develop a specific PCR assay for *P. andrewsi* to discriminate this species from *P. marinus* and *P. olseni/P. atlanticus*. When tested for specificity, these NTS region primers generally have demonstrated good species-specificity, as this region displays relatively high inter-specific variation (Robledo et al. 1998; Coss et al. 2001b). The intra-specific sequence variation within the NTS region, however, has not been broadly assessed and there is a risk of false-negatives due to polymorphisms among the NTS sequences of genetic strains within each species that may result in a PCR primer being unable to efficiently bind the target sequence in all strains. Intra-specific variation in the NTS region needs to be comprehensively examined, as has been done for the ITS region, in order to confidently employ these assays for projects that require extensive monitoring and/or application across a wide geographic range.

Over time, the molecular diagnostic techniques based on the PCR assay will probably largely replace the classical RFTM method. A primary benefit of the PCR technique is the rapidity: the presence of *Perkinsus* can be assessed in 200 individuals in just one day, whereas the RFTM assay typically requires at least five days to one week (Robledo et al. 1998). This is an important advantage for disease prevention and control. In conclusion, although molecular detection methods have the potential to greatly facilitate detection and discrimination of *Perkinsus* species, as discussed above, it is important that intra- as well as inter-specific sequence variation at targeted loci be examined and adequately characterized, in order to develop the most powerful molecular assays.

7 Epizootiology

Many factors can influence spatial and temporal variations in prevalence and parasitosis intensity, either by affecting the parasite, the host or their interaction. Most epizootiological studies on perkinsosis have focused on the geographical range of the disease and the effects of temperature and salinity on parasite and disease dynamics. Considerable epizootiological information is available for *P. marinus* whereas it is rather scarce on the other *Perkinsus* spp. *P. marinus* has been found in *C. virginica* oysters from as far north as Damariscotta River ME, throughout the bays and estuaries along the east coast of the United States and through the Gulf of Mexico to as far south as Tabasco, Mexico (Burreson and Ragone Calvo 1996; Ford 1996; Soniat 1996). Field studies in which the prevalence and intensity of infection by *P. marinus* were recorded along salinity gradients, as well as for multiple years –involving annual cycles of temperature and salinity variation– demonstrated the marked influence of both environmental parameters on the disease dynamics (Ray 1954; Mackin 1956; Andrews and Hewatt 1957; Mackin 1962; Quick and Mackin 1971; Soniat 1985; Andrews 1988; Craig et al. 1989; Soniat and Gauthier 1989; Crosby and Roberts 1990; reviewed by Burreson and Ragone Calvo 1996; Soniat 1996). Prevalence and intensity of *P. marinus* infections in the natural environment increase with increasing salinity. Historically, *P. marinus* was absent from areas with salinities below 12–15 ppt, which were considered disease-free "sanctuaries". However, in drought years, salinity raised beyond that limit in many of those areas, leading to the establishment of the parasite into them. Once established in a low salinity area the parasite persists and has been observed to tolerate salinities below 5 ppt for longer than 3 months. When salinity returns to more favourable conditions, the parasite responds through increases in prevalence and intensity.

Prevalence and intensity of *P. marinus* infections in the natural environment also increase with increasing temperature.

The annual temperature cycle determines a seasonal pattern of variation of prevalence and intensity. Temperature has more influence in places with broad fluctuations (Chesapeake Bay and northwards) than in places where the temperature remains relatively warm year-round (Gulf of Mexico). The seasonal pattern of *P. marinus* parasitism in Chesapeake Bay begins with the prevalence and intensity increasing in late spring, when the temperature increases above 20 °C, as a result of proliferation of overwintering infections. Maximum values of both variables usually occur in late summer. Oyster mortality is mostly concentrated in late August and September. Regression of infection in oysters that did not die takes place during the winter and spring as temperature decreases. The decline in prevalence observed over the winter is primarily due to infection regression and persistence of the parasite at an undetectable level by standard diagnostic techniques, rather than to complete elimination of the parasite (Ragone and Burreson 1994). New infections are acquired mainly in late summer, coinciding with maximal mortality, although transmission can also occur when host mortality is low or absent (Ragone Calvo et al. 2003b). Unusually warm springs and falls allow longer periods of parasite proliferation, thus resulting in greater oyster mortality. Likely, temperature is the most important environmental factor affecting the large-scale geographic distribution of *P. marinus*. The range extension by *P. marinus* northwards in the early 1990s was probably due to above average winter temperatures linked to a warming trend (Ford 1996).

Laboratory experiments performed in vitro have confirmed the marked influence of temperature and salinity on the *P. marinus* cell activity and viability, both of trophozoites (Burreson et al. 1994b; Dungan and Hamilton 1995; Gauthier and Vasta 1995; O'Farrell et al. 2000) and zoosporulation stages (Chu and Greene 1989). In vivo laboratory experiments also demonstrated the influence of both environmental factors on infectivity and disease progression (Fisher et al. 1992; Chu and La Peyre 1993a,b; Chu et al. 1993; Ragone and Burreson 1993; Chu 1996; Chu et al. 1996; Chu and Volety 1997; La Peyre et al. 2003). Environmental factors impact disease progression/regression by modulating the host immune system (Chu and La Peyre 1993a,b; Anderson 1996) as well as parasite activity (see discussion below). If temperature and salinity influence *P. marinus* infection, their interaction is particularly significant (Burreson and Ragone Calvo 1996; Soniat 1996; Chu 1996).

Epizootiological knowledge on *P. marinus* is a reference for the other *Perkinsus* spp. Five years of monthly sampling of a *T. decussatus* clam bed affected by *P. olseni/P. atlanticus* revealed an annual pattern of perkinsosis dynamics influenced by temperature (Casas 2002). Prevalence and intensity peaked in spring coupled with increasing temperature (above 15 °C). Intensity stayed high through the summer and peaked again in late summer-early autumn when temperature was maximal (19–21 °C). Infection regression occurred in late autumn to early spring, coinciding with lower temperature (annual minimum of 9–10 °C). The highest clam mortality percentage was recorded in early autumn. Salinity stayed above 15 ppt throughout the study period, thus the effect of low salinity could not be observed in that field study. Laboratory experiments performed in vitro have confirmed the marked influence of temperature and salinity on *P. olseni/P. atlanticus* cell activity and viability of zoosporulation stages (Auzoux-Bordenave et al. 1995; Ahn and Kim 2001; Casas et al. 2002a). Infection intensity and mortality caused by *P. olseni* in Australian abalones was also enhanced by increasing temperature (Goggin and Lester 1995). In the case of *P. qugwadi*, no apparent seasonality was detected, however, and it remained pathogenic and was able to produce zoospores at temperatures as low as 10 °C (Bower et al. 1998).

As mentioned above, *Perkinsus* spp. can be directly transmitted between molluscan hosts without any intermediate host. Therefore, proximity among individuals of a susceptible species (population density) enhances disease transmission (Andrews 1965). Laboratory experiments on transmission demonstrated the importance of dosage of infective cells (Chu and La Peyre 1993b; Chu et al. 1993; Chu and Volety 1997). In the natural environment, the longer the distance between infected and healthy individuals, the higher dilution of infective particles. Higher filtration rates and longer exposure to infective particles could explain why *P. marinus* prevalence is higher in adult than in juvenile oysters living in the same area (Burreson and Ragone Calvo 1996; Soniat 1996; Powell et al. 1996). Similarly, intensity of infection by *P. olseni/P. atlanticus* is highly correlated with clam *T. decussatus* age (Casas 2002).

Other stressing factors can increase host susceptibility to infection. *P. marinus* disease progression is enhanced by pollution (Anderson et al. 1996; Chu and Hale 1994). Synergic effects of *P. olseni/P. atlanticus* infection (Azevedo 1989), pollution (Bebianno 1995), and hypoxia (Sobral and Widdows 1997) could be responsible of mass mortalities of the clam *T. decussatus* in southern Portugal. Anderson et al. (1998) also suggested that synergistic effects of TBT pollution, hypoxia and *P. marinus* infection could increase oyster mortality. The role of factors governing oyster nutritional stage (food availability and other factors affecting food intake by the oyster) in *P. marinus* infection progression has been emphasised by Hofmann et al. (1995) and Powell et al. (1996). The weakened condition of post-spawning stage animals may also facilitate disease progression.

Various epizootiological simulation models have been developed taking into account the known factors influencing *P. marinus* epizootic dynamics (Hofmann et al. 1995; Powell et al. 1996; Soniat and Kortright 1998; Ragone Calvo et al. 2000). They may be useful predictive tools that will aid in designing strategies to manage affected oyster beds.

8 In vitro proliferation

The development of in vitro cultures of parasites has enabled and/or greatly facilitated much of the research that is essential to understanding parasite biology and the diseases that they may cause. In vitro cultures have been established for four *Perkinsus* spp. The procedures that were used varied among researchers in the type of medium used, the particular culture conditions and the method used for the establishment of primary cultures (La Peyre 1996). The culture medium JL-ODRP-1 (La Peyre et al. 1993) originally formulated to resemble the composition of eastern oyster plasma was used to

propagate *P. marinus* and *P. chesapeaki* (La Peyre et al. 1993; McLaughlin and Faisal 1998). The culture medium JL-ODRP-2A (resulting from modifying JL-ODRP-1) was used to establish cultures of *P. olseni/P. atlanticus* (Casas et al. 2002b). The commercial medium DME:Ham's F-12 with various nutritional supplements was used to culture *P. marinus* (Gauthier and Vasta 1993, 1995; Gauthier et al. 1995), *P. andrewsi* (Coss et al. 2001a) and *P. olseni/P. atlanticus* (Casas et al. 2002b; Robledo et al. 2002). The former also was cultured with another commercial medium, Leibowitz's L-15, with supplements (Kleinschuster and Swink 1993). Dungan and Hamilton (1995) compared proliferation of *P. marinus* in three commercial media (DME/Ham's F-12, NCTC-135, RPMI-1640) and in a modified version of JL-ODRP-1. The authors observed faster growth with the first commercial medium. Because the occurrence of proteins in the culture medium could interfere with studies on the proteins secreted by cultured cells, protein deficient media were designed *ad hoc* and were shown to efficiently support *P. marinus* proliferation (La Peyre and Faisal 1996, 1997). Different host organs have been used as the parasite source to establish in vitro cultures of *Perkinsus* spp.: hearts, visceral ganglia, haemolymph and gills of infected bivalves (La Peyre et al. 1993; Gauthier and Vasta 1993; Kleinschuster and Swink 1993; McLaughlin and Faisal 1998; Coss et al. 2001a; Casas et al. 2002b; Robledo et al. 2002). Parasite hypnospores isolated from infected bivalve tissues that had been incubated in Ray's fluid thioglycollate medium (RFTM) also have been used as a culture source (La Peyre and Faisal 1995; Bushek and Allen 1996b; Casas et al. 2002b; Robledo et al. 2002). Different criteria can be used to choose the source of parasite cells, such as the success rate of establishing cultures, the number of cultures that can be established and the time required to establish the culture. In addition, the source can influence some of the cultured cell characteristics, such as the cell size and the number of daughter cells produced by each cell through vegetative division (Casas et al. 2002b) and the secretion of proteins (La Peyre and Cooper 1997; Casas et al. 2002c). The cultured cells can be cryopreserved (Dungan and Hamilton 1995; Gauthier and Vasta 1995). There are a number of cryopreserved *Perkinsus* spp. isolates deposited at the American Type Culture Collection (ATTC, Rockville, MD, USA) that are available to researchers.

Morphology of cultured cells varies depending on nutrient availability and culture stage. Initially cultured cells are primarily spherical, with the cytoplasm enclosing one or more refractile bodies (lipid droplets) and a prominent vacuole. Cells enlarge, accumulate lipid droplets in the cytoplasm and usually occur in clumps. Several days after seeding, cell enlargement is followed by division. When cultures are kept without a change of medium for extended periods and nutrients are no longer available, cells develop a large vacuole and lose their lipid droplets. Three types of in vitro multiplication have been described. One type involves multiple karyokinesis followed by cytoplasmic cleavage, giving rise to tight clusters of daughter cells surrounded by the mother cell wall. Daughter cells enlarge and are freed following rupture of the mother cell wall. Cytoplasmic cleavage takes place either simultaneously throughout the cytoplasm or progressively in that it starts at one end of the cell and advances to encompass the rest of the mother cell. This type of division, multiple fission, was observed in cultures of *P. marinus* (La Peyre et al. 1993; La Peyre and Faisal 1995; McLaughlin and Faisal 1998; Sunila et al. 2001), *P. chesapeaki* (McLaughlin and Faisal 1998) and *P. olseni/P. atlanticus* (Casas et al. 2002b; Robledo et al. 2002). Another type of division involves binary fission, in which a daughter cell "buds" from a mother cell and has been observed in cultures of *P. marinus* (La Peyre et al. 1993; Sunila et al. 2001), *P. andrewsi* (Coss et al. 2001a) and *P. olseni/P. atlanticus* (Robledo et al. 2002). Finally, palintomic zoosporulation yielding motile zoospores was also observed in *P. marinus* (La Peyre and Faisal 1995), *P. chesapeaki* (McLaughlin and Faisal 1998), *P. andrewsi* (Coss et al. 2001a) and *P. olseni/P. atlanticus* (Casas et al. 2002b) cultures. Transformation of zoospores into trophozoites and the subsequent growth has been observed in some cultures of *Perkinsus* spp. (Kleinschuster et al. 1994; La Peyre and Faisal 1995; Coss et al. 2001a), but not in *P. olseni/P. atlanticus* (Casas et al. 2002b).

As mentioned above, intra-specific genetic variation of *Perkinsus* spp. has been shown and even multiple genetic strains can exist within a single host. In addition, genotypic alterations may occur in cultures (La Peyre 1996). Therefore, mixed cell populations could occur in in vitro cultures and the ability to clone *Perkinsus* spp. may be essential when genetically identical cells are required. Clonal cultures have been established through isolation of individual cells by a standard limiting dilution method (Gauthier and Vasta 1995). Another method involves isolating individual cells by micromanipulation and maintaining them on a membrane above a layer of actively dividing cells. Extracellular products of the latter diffuse across the membrane and stimulate proliferation of the isolated cell (Bushek et al. 2000). Since the establishment of culture procedures, the availability of a large number of *Perkinsus* spp. cells has enabled and will continue allowing more extensive studies on morphology, environmental tolerance, virulence, genetic variability, metabolism and drug sensitivity. Nevertheless, the use of cultured cells can have some limitations for particular experimental approaches because it has been found that the virulence of parasites freshly isolated from infected hosts is much higher than those propagated in culture (Bushek et al. 1997b; Ford et al. 2002; Bushek et al. 2002b).

9 Host-parasite interactions: Progression of infection and immune response

Perkinsus spp. must surpass physical and chemical host barriers to gain entry into host tissues. The interaction between *P. marinus* and its host *C. virginica* is the most extensively studied of these host (mollusc)-parasite (*Perkinsus* spp.) interactions and can be used as a model for this group of parasites. Nevertheless, differences among species have been found in the host-parasite interaction due to specific parasite characteristics and/or to differential host response.

After histological observations by Mackin (1951), it was thought that the primary portal of entry of *P. marinus* was gut epithelium of the oyster. However, experimental infections support that epithelia of the gill, labial palps and mantle are equally, if not more, important (Chintala et al. 2002).

It is not well understood how *P. marinus* cells go beyond those epithelia, but the parasite multiplies and colonises new organs. Infection evokes haemocytic infiltration of tissues where the parasite is present and haemocytes actively phagocytose the parasite cells; however, the latter are not destroyed but rather divide inside haemocytes and can cause their rupture. Heavy infections are characterised by massive haemocytic infiltration of epithelia, connective tissue, muscle fascicles and blood spaces, with parasite cells occurring both inside haemocytes and free. *Perkinsus* cells are associated with tissue destruction and the loss of normal structure (Mackin 1951; La Peyre et al. 1995a), which likely involves organ dysfunction.

Conclusive studies on infection routes for the other *Perkinsus* spp. are lacking. The fact that most light infections by *P. olseni/P. atlanticus* in *T. decussatus* are confined to gill and labial palps suggests that these organs are the primary portals of entry (Casas 2002). The inflammatory reaction evoked by perkinsosis in hosts other than oysters is different. Thus, the reaction in venerid clams *T. decussatus*, *T. phillippinarum*, *V. pullastra* and *P. aurea* (Chagot et al. 1987; Navas et al. 1992; Montes et al. 1995a; Sagristà et al. 1995; Montes et al. 1996; Ordás et al. 2001; Park and Choi 2001; Casas 2002; Choi et al. 2002), abalone species *Haliotis* spp. (Goggin and Lester 1995), Japanese scallops *P. yessoensis* (Bower et al. 1998) and gaper clams *M. arenaria* (McLaughlin and Faisal 1998) primarily involves encapsulation of the parasite cells (up to 10–20 cells) by haemocytes. Heavy infections are characterised by the occurrence of multiple large foci where free and encapsulated parasites occur; this condition involves tissue destruction and loss of the normal structure of affected organs. Inflammatory foci in these molluscs become macroscopically evident as pustules or abscesses of varying size (up to 10 mm, Bower et al. 1992) and colour (white, creamy or yellowish). Encapsulated *P. olseni/P. atlanticus* cells in *T. decussatus* (Chagot et al. 1987) and *T. philippinarum* (Montes et al. 1995a; Sagristà et al. 1995), and *P. chesapeaki* cells in *M. arenaria* (McLaughlin and Faisal 1998) are embedded/encysted in a substance (PAS + in *T. decussatus* and *M. arenaria*) secreted by the surrounding haemocytes. Some of the parasite cells within the capsules appear dead. Montes et al. (1995b) demonstrated that a non-glycosilated polypeptide is the main component of the embedding material. The polypeptide is secreted by the surrounding haemocytes and is likely to destroy the parasite. A similar polypeptide was detected in *T. decussatus* infected by *P. olseni/P. atlanticus* (Montes et al. 1996).

In the last decade much research effort has been directed toward understanding how *Perkinsus* spp. (especially *P. marinus*) overcome the host immune system. Implications of known components of molluscan immunity have been studied. Molluscan resistance to diseases is greatly dependent on the increase in haemocyte activity (Fisher and Newell 1986a). One of the most important cellular defence mechanisms, phagocytosis (Feng 1988), has been found to decrease upon infection by *P. marinus* (La Peyre et al. 1995b) and *Perkinsus* sp. (Ordás et al. 2000). Interestingly, the natural *P. marinus* host, *C. virginica*, is able to phagocytose more *P. marinus* cells than *C. gigas*, an oyster which is not naturally infected by the parasite (Gauthier and Vasta 2002). This could be due to a temporal activation of the oyster defence system caused by previous exposure to the pathogen, as has been suggested for molluscs exposed to foreign material (Chu 1988; Ford 1988; Gaffney and Bushek 1996). Extracellular products (ECP) of *P. marinus,* and particularly proteases present in those ECPs, are capable of inhibiting haemocyte motility, a parameter closely related to phagocytic activity (Garreis et al. 1996). Moreover, secretory products of *P. marinus* can inhibit the production of superoxide anions by the oyster haemocyte, modulating the respiratory burst activity (Anderson 1999a). Characterisation of *Perkinsus* spp. secretion products and their role in the pathogenicity of the parasite will be discussed later.

The haemocyte number in molluscs varies among species and individuals, and depends on the age and heart rate of the organisms, as well as the bleeding technique employed (Adema et al. 1991) for analysis. Changes in environmental conditions, such as water temperature variation or air exposure, also alter the number of circulating haemocytes (Thompson et al. 1978). It is difficult, therefore, to assess the effect of *Perkinsus* infection on the bivalve haemocyte concentration. Oysters infected with *P. marinus* tend to show an increase in haemocyte concentration in the haemolymph (Anderson et al. 1992, 1995; Chu and La Peyre 1993b; La Peyre et al. 1995a,b), while a decreasing trend in haemocyte number has been reported in clams heavily infected with *Perkinsus* sp. (Ordás et al. 2000; Casas 2002). *Perkinsus* cells are able to grow and divide by palintomy inside host haemocytes, finally resulting in rupture of the blood cells (Perkins 1996). Therefore, the first host reaction to the *Perkinsus* infection is, as reported for *P. marinus*, an increase in circulating haemocytes, but they quite possibly may decrease in number when the infection rate is high because either haemocytes are lysed by the parasite (*Perkinsus* sp., Ordás et al. 2000) or they are massively channelled to the affected tissues for encapsulation and destruction (*P. olseni/P. atlanticus*, Casas 2002).

The effect of infection by *Perkinsus* spp. on the haemolymph lysozyme concentration is uncertain. Some authors have reported no difference in this parameter among *P. marinus* infected and uninfected oysters although seasonal variations and variation correlated with salinity and temperature differences was observed (Chu and La Peyre 1989, 1993a,b; Chu et al. 1993). A similar result was obtained for *P. olseni/P. atlanticus*-infected vs. uninfected carpet shell clams (Ordás et al. 2000; Casas 2002). However, a slight decrease in lysozyme activity has been seen in infected oysters (La Peyre et al. 1995b) or in samples of serum treated with *P. marinus* extracellular products (Garreis et al. 1996) whereas increasing levels of serum lysozyme were reported in *P. marinus*-infected oysters (Chu and La Peyre 1993a). It has been suggested, therefore, that observed changes in molluscan haemolymph composition (Fisher and Newell 1986b) and, particularly in lysozyme concentrations and activity, may be due largely to environmental factors (Steinert and Pickwell 1985; Chu 1988). As a result, in vivo changes in lysozyme activity may be attributed not only to the triggering of a defence response in the mollusc, but also to environmental parameters. In vitro, serum lysozyme was suggested as responsible for inhibition of *P. marinus* growth when mussel plasma was added to the media. Anderson and Beaven (2001a) proposed that the

lack of such growth suppression by *C. virginica* serum could be related to the high pathogenicity of *P. marinus* for its natural host, the oyster.

Two mussel species *Mytilus edulis* and *Geukensia demissa* that do not develop *P. marinus* disease demonstrated anti-*P. marinus* activity in their sera at levels many hundred-fold higher than that of *C. virginica*, whereas the lysozyme levels were relatively low in the mussels as compared to the oyster (Anderson and Beaven 2001b). The authors stated that anti-*P. marinus* activity of mussel sera may depend on cytotoxic molecules other than, or in addition to, lysozyme or antimicrobial peptides. Gauthier and Vasta (2002) also showed that plasma from bivalves that are less susceptible to *P. marinus* infections, *Mercenaria mercenaria*, *Anadara ovalis* and, especially, *M. edulis*, inhibited *P. marinus* in vitro proliferation more effectively than that of *C. virginica*.

Studies on the serum protein concentration in *Perkinsus* infected molluscs have resulted in some contradictory results. Chu et al. (1993) and La Peyre et al. (1995b) reported slightly lower protein concentrations in the serum of *P. marinus* infected oysters than in healthy bivalve serum, although the differences that they observed were not statistically significant. Ordás et al. (2000), however, recorded higher protein concentrations for *P. olseni/P. atlanticus*-infected clams, as compared to uninfected clams. There are also several studies in which no differences in protein concentration were found between *P. marinus* infected and uninfected oysters (Chu and La Peyre 1989, 1993a). As with lysozyme concentration and activity, this lack of concordance is probably due to the influence of external factors, such as season, habitat, salinity or temperature, on the serum protein concentration (Chu and La Peyre 1989; Fisher and Newell 1986b). The increase of this parameter in *P. olseni/P. atlanticus* infected clams could be partially due to the synthesis of a specific polypeptide against the parasite as reported by Montes et al. (1996, 1997).

Agglutinins may increase phagocytosis by acting as opsonins (Olafsen et al. 1992). In fact, agglutination of non-self particles is a first step in the process of recognition and subsequent internalisation of the agglutinated material by host haemocytes (Chu 1988). In spite of the inhibitory effect that *P. marinus* ECP can have on the oyster haemagglutination titer (Garreis et al. 1996), serum agglutinin levels in these bivalves were not dependent, in vitro (Fisher et al. 1992) or in vivo (Chu and La Peyre 1993b), on the *P. marinus* infection intensity. Chintala et al. (1994) concluded that serum agglutinins play no role in oyster defence against *P. marinus*. In contrast, carpet shell clams infected with *P. olseni/P. atlanticus* (Ordás et al. 2000) and Pacific oyster *C. gigas* infected with *P. marinus* (La Peyre et al. 1995b) showed higher agglutination titer than uninfected molluscs.

The release of lytic enzymes by *P. marinus* was suggested soon after the pathogen description (Ray 1954). However, rigorous studies on the extracellular products of this parasite were not initiated until forty years later. Modulation of the oyster immune response in vitro, as well as the increase of the infection intensity in vivo by *P. marinus* secretory products, has been demonstrated (Garreis et al. 1996; La Peyre et al. 1996; Anderson 1999a). This confirms the important, but as yet largely uncharacterised, role of the products secreted by the parasite in the infection process of the host.

As noted before, *P. marinus* ECP and the proteases that they contain inhibit oyster haemocyte motility, while *P. marinus* cells themselves produce the opposite effect (Garreis et al. 1996). This apparent contradiction is probably due to surface determinants that facilitate the recognition and entry of the parasite into the haemolymph cells, where proliferation can occur (Vasta et al. 1995). In fact, it has been recently demonstrated that *P. marinus* trophozoites can remain alive inside oyster haemocytes (Gauthier and Vasta 2002). In this context, it has been also shown that oyster haemocytes have a higher phagocytic affinity for live *P. marinus* cells than for fixed parasite cells (Gauthier and Vasta 2002).

Parasite proteolytic enzymes may participate in necrotic reactions in *Perkinsus*-infected molluscs because they are capable of degrading extracellular matrix components of the stroma and basal membranes, facilitating invasion of host tissues by the parasite. The addition of homogenate extracts or plasma from *C. virginica* to in vitro *P. marinus* cultures enhances secretion of proteases from the cultured cells, which does not happen with extracts or plasma from other less susceptible oyster species (MacIntyre et al. 2003). Characterization of the proteases present in ECP of in vitro cultured *P. marinus* (derived both from oyster *C. virginica* and soft shell clam *M. arenaria* isolates) were primarily serine-type proteases, which were released from the cells soon after culture initiation and showed stability at very high pHs (La Peyre et al. 1995a; Faisal et al. 1999c). In contrast, ECP of in vitro cultured *P. chesapeaki* did not show any proteolytic activity under the same conditions as those of *P. marinus* ECP analysis; the production of inactive protease forms and the possible suboptimal conditions of the assay could be responsible for this result (McLaughlin et al. 2000). Significant differences in enzyme activities were found in ECP of cultures of *P. olseni/P. atlanticus* initiated from different host tissue sources. Surprisingly, no activity of the serine proteases trypsin and α-chymotrypsin were detected in ECP of *P. olseni/P. atlanticus* cultures, whereas high α-chymotrypsin and low trypsin activity were detected in *P. marinus* cultures under the same conditions (Casas et al. 2002c). Serine proteases are involved in the life cycle development of protozoa and in blood cell invasion (Rosenthal et al. 1987; Braun-Breton and Pereira da Silva 1988). Moreover, they can be lethal for fish, as has been demonstrated for the proteases secreted by *Aeromonas hydrophila* (Rodriguez et al. 1992). This could explain why some proteases purified from *P. marinus* secretion products, especially those of the serine class (La Peyre et al. 1995a), affect several immune parameters of oysters (Garreis et al. 1996), and also why *P. marinus* ECP that contain serine proteases, degrade certain host serum proteins (Oliver et al. 1999) promoting infection in vivo (La Peyre et al. 1996). Indeed, *P. marinus* serine proteases suppress the vibriocidal capability of oyster haemocytes, thus making the host more susceptible to secondary infections (Tall et al. 1999). The lack of (or undetected) production of serine proteases by *P. olseni/P. atlanticus* in vitro could support the hypothesis of lower virulence of *P. olseni/P. atlanticus* in *T. decussatus* than that of *P. marinus* in *C. virginica* (Casas et al. 2002c).

Antiproteases have been found in the serum of several animal species, including molluscs (Armstrong and Quigley 1992; Bender et al. 1992; Thogersen et al. 1992; Elsayed et al. 1999). Protease inhibitors can constitute humoral defence factors, protecting the host cells against entry of the protozoan parasites (Fuller and McDougald 1990). In particular, the serine protease inhibitor family (serpin superfamily) is involved in defending insects against pathogen proteases (Polanowski and Wilusz 1996). *P. marinus*-infected oysters have antiproteases in their serum with specific activity against this parasite's ECP (Faisal et al. 1998; Oliver et al. 1999). Disease intensity is negatively correlated with protease inhibitory activity (Oliver et al. 2000). In fact, the highest antiprotease concentration was observed just before parasite elimination, suggesting that oyster antiproteases play a role in protecting haemagglutinins from degradation by parasite proteases (Romestand et al. 2002). The study of interaction between oyster serum and parasite proteases is providing insights into the role of proteases in the pathogenesis (Muñoz et al. 2003). *C. gigas*, which is much less susceptible to *P. marinus,* possesses protease inhibitors with significantly higher specific activities than those in *C. virginica* (Faisal et al. 1999b). For *P. olseni/P. atlanticus*-infected carpet shell clams, the specific production of proteins by haemocytes as a defensive response against the parasite has been demonstrated (Montes et al. 1995b, 1996, 1997)

The role of parasite acid phosphatase activity in the host immune response has been described for several parasites (Remaley et al. 1984; Hervio et al. 1991), including *P. marinus* (Volety and Chu 1997). This enzyme might alter the bivalve cellular defence activity by the disruption of phosphoproteins and inhibition of superoxide anion production (Anderson 1996). Dead *P. marinus* cells do not show a negative effect on the haemocyte respiratory burst activity, as measured by chemiluminescence, while living cells inhibit this mechanism (La Peyre et al. 1995c; Volety and Chu 1995; Anderson 1999a,b; Schott et al. 2003a). This demonstrates that this pathogen, as for some bacterial species (Bramble and Anderson 1997, 1998; Lambert and Nicolas 1998), can actively suppress the active oxygen radicals. By interfering with host oxygen-dependent killing mechanisms, acid phosphatase helps the parasite to survive in the host (Hervio et al. 1991). Recently, two iron-containing superoxide dismutases (Fe- SOD), also antioxidative enzymes, have been described and characterized in in vitro cultured *P. marinus* (Wright et al. 2002; Ahmed et al. 2003; Schott et al. 2003b). They were highly divergent in their genomic organization (Schott et al. 2003b) and cellular localization (Schott and Vasta 2003), thus suggesting the complexity of the antioxidant activity of this parasite. In vitro assays showed that *P. marinus* also caused decrease of phenoloxidase activity in the haemolymph of *C. virginica* and the non susceptible mussel *G. demissa* (Deaton and Jordan 2002). The involvement of phenoloxidase in arthropod immune response is well known but its role in mollusc hemocytes is poorly understood.

Studies on lipid metabolism of *Perkinsus* spp. (Soudant and Chu 2001; Lund and Chu 2002; Chu et al. 2003) have recently been initiated and will contribute to our understanding of the host-parasite interactions.

10 Effects on the host

As has been extensively documented, progression of *Perkinsus* spp. infections through the host tissues causes lesions that may lead to host death. Mass mortalities in molluscan populations due to perkinsosis have obvious ecological and socio-economic consequences. In addition, however, sublethal effects may also have significant ecological and economic implications. Interference with host energy fluxes may slow growth and infection may result in poor condition of market size individuals. In addition, there may be a deficiency of energy reserves for successful gametogenesis. Negative effects on host fecundity could result in lower recruitment and scarcity of seed for grow out purposes. In general terms, infection causes host weakening so that it becomes increasingly difficult for affected individuals to overcome any other adverse conditions.

Early studies on the effects of *P. marinus* on eastern oysters showed that the infection reduces host growth (Andrews 1961). Oyster meat content, evaluated through condition indices, also seems to be reduced by infection, although the effect of infection can be masked because the period of the year in which infection intensity is highest usually coincides with postspawning condition. Nevertheless, contradictory results were obtained by different authors (reviewed by Paynter 1996). Meat content is moderately, although significantly, reduced in clams *T. decussatus* heavily infected by *P. olseni/P. atlanticus*, during gametogenesis through gonadal ripening, but that effect is not significant during the postspawning period (Casas 2002). Meat content in bivalve molluscs is closely associated with gonad condition. Dittman et al. (2001) showed that *P. marinus* can have significant negative effects on oyster reproduction, although this effect varied with the stage of gametogenic cycle. Both gonad size and germinal tissue in the gonad were most strongly reduced during the spring in infected hosts, when gametogenesis progresses, whereas no significant effect was detected in the period of gonad ripeness. In the case of the infection of *T. decussatus* by *P. olseni/P. atlanticus*, gametogenesis is inhibited in heavily infected clams in the active gametogenic period (February-March) and the number of heavily infected clams with ripe gonad is significantly reduced throughout the ripeness-spawning period (April-August). Heavy infection causes a significant decrease of storage tissue and gametes whereas haemocytic infiltration of the gonad increases (Casas 2002).

The negative effect of infection on growth and condition is likely a consequence of a decrease in the available energy in infected hosts. According to Mackin and Ray (1955) the feeding rate of oysters decreased with intensity of infection by *P. marinus*. However, other studies have shown that *P. marinus* has little or no effect on clearance and assimilation rates (energy acquisition) and on oxygen consumption (energy consumption) (Newell et al. 1994; Paynter 1996; Willson and Burnett 2000). Clearance rate tended to decrease and oxygen consumption to increase in clams *T. decussatus* heavily infected by *P. olseni/P. atlanticus*, although the effects were not statistically significant, whereas no effect was found on absorption efficiency (Casas 2002).

Choi et al. (1989) estimated the energy consumption of *P. marinus*, through production and metabolism, based on

measurements of doubling time and parasite loads in oyster tissues along with energetic physiology measurements obtained from other protozoans of similar size. The authors concluded that the *P. marinus* cells in large oysters with heavy infections consume more energy than the oyster would have available after meeting its own metabolic demand. In smaller oysters, respiration consumes a smaller fraction of the total energy budget; hence the impact of *P. marinus* infection on oyster net productivity is lower. Using those estimations of parasite energy consumption for *P. olseni/P. atlanticus* and the clam *T. decussatus*, parasite energy consumption in market-sized clams with heavy infections would exceed the energy available in the clam for growth, especially under conditions of high temperature and low food availability (Casas 2002). This type of situation would not be unusual during the summer months when infection intensity is often found to be high. The negative energy balance caused by progression of perkinsosis in hosts helps to explain lethal and sublethal detrimental effects.

11 Management strategies

Different strategies have been developed and assayed to mitigate the dramatic consequences of *P. marinus* epizootics on the eastern oyster industry, with varying degrees of effectiveness. Earlier strategies involved **modifications of management/culture procedures** for oyster beds and reefs. Those procedures were adapted according to the epizootiological knowledge of the disease (reviewed by Andrews and Ray 1988 and Krantz and Jordan 1996). Three main factors governed the procedure changes. 1) Low salinity areas are characterised by null or low infection levels, but very slow oyster growth, whereas infection incidence is generally much higher and oyster growth faster in high salinity areas. 2) Temperature has a large impact on infection transmission, infection intensification and mortality as disease occurs primarily in warm months (length of warm period changes latitudinally). 3) Infected oysters are the source of infective particles. These three issues were the basis for some of the recommendations made to oystermen. On the basis of the first point, management procedures involved taking oyster seed from low salinity areas (areas with uninfected seed) and transplanting them into high salinity areas for ongrowing to market size. The second point suggested that seed should be transplanted immediately after the infective period ends and later harvested immediately before mass mortality occurs, thus allowing the oysters to grow through a period without infection followed by a period of infection progression without high mortality. In lower latitude areas, warm weather lasts longer and therefore, the time period to avoid losses by *P. marinus* in high salinity areas is shorter. This procedure also involved lowering the market size for oysters. On the basis of the third point, harvesting should be made thoroughly so that infected oysters do not stay on oyster grounds and serve as a source for the infective stages when seed are transplanted to the areas. In addition, the sites for oyster outgrowth should be separated to make disease transmission between sites difficult. However, over time, it became increasingly difficult to follow these general recommendations. Several successive years of winter and spring drought resulted in abnormally high salinity in areas usually characterised by low salinity, thus allowing the invasion of *P. marinus* with the scarcity of uninfected seed. Lowering legal market size and the complete removal of large oysters from some areas (especially public oyster grounds) was in conflict with legislation intended to ensure the persistence of broodstock and subsequent oyster recruitment. A further complication was epizootic outbreaks of the oyster protozoan parasite *Haplosporidium nelsoni* in areas that were also affected by *P. marinus*. These difficulties required searching for new areas for oyster grow-out and also the use of low salinity areas, in spite of the long time required for oysters to reach market size in those areas. Intense monitoring of infection intensity and mortality at critical times helped to improve recommendations for the appropriate transplantation and harvest times. In addition, accumulation of long series of data involving infection intensity, mortality and environmental conditions from various areas allowed modelling infection dynamics in oyster populations (Hofmann et al. 1995; Powell et al. 1996, 1997; Soniat and Kortright 1998; Ragone Calvo et al. 2000). These simulation models can help to predict disease evolution and therefore, will also result in improved management recommendations.

Traditional ***selective breeding*** procedures have been employed in attempts to obtain ***resistant strains*** of oysters. Groups of oysters exhibiting low mortality in areas of heavy *P. marinus* pressure were used as broodstock to obtain descendents over multiple generations (Andrews and Ray 1988; Krantz and Jordan 1996). The success of a selective breeding programme for developing eastern oyster strains that exhibited tolerance to *H. nelsoni* (Ford and Haskin 1988) encouraged those attempts. Unfortunately, *H. nelsoni*-tolerant oysters strains were highly susceptible to *P. marinus* (Burreson 1991). Historically, the *P. marinus* endemic and *H. nelsoni* endemic areas did not significantly overlap, so that oysters were not simultaneously exposed to both diseases until relatively recently. In areas where both diseases now coexist, selective breeding programmes must take both into account in order to achieve success. Some promising advances have been reported with development of the strain "DEBY", which showed enhanced tolerance to both diseases after several generations of selective breeding at the Virginia Institute of Marine Science, USA (Ragone Calvo et al. 2003a). These programmes require genetic variability among eastern oysters with respect to *P. marinus* tolerance for success. Bushek and Allen (1996a,b) assessed difference among oyster stocks and showed that long-term exposure of oyster populations to *P. marinus* in the natural environment leads to increased tolerance through natural selection. Genetic variability among oyster families obtained through interbreeding oyster populations was also demonstrated in protease inhibitory activity against proteases of *P. marinus*, which was negatively correlated with infection intensity and mortality (Oliver et al. 2000).

Bower et al. (1999) found that the first generation progeny of Japanese scallops *Patinopecten yessoensis* that survived an epizootic outbreak of *P. qugwadi* had a significant increase in resistance to infection and resultant mortality. A similar resistance was observed in hybrid scallops obtained from a cross between *Patinopecten carinus* and *P. yessoensis* outbreak-survivors. Therefore, production of resistant scallops

seems to be a feasible way to overcome perkinsosis in British Columbia.

An alternative to time consuming traditional selective breeding to develop a resistant strain could be ***direct genetic manipulation***. One of the procedures that has been tested was the use of ***triploid oysters***. The technology of triploid oyster production was developed looking for faster growth and better taste (higher glycogen content due to gametogenesis arrest). Hypothetically, faster growth could allow oysters to reach market size before mass mortality occurs and reduced or absent gametogenesis could allow oysters to reserve energy, thus allowing them to face infection in a more favourable condition. However, triploid *C. virginica* do not demonstrate improved tolerance to *P. marinus,* although they do grow more quickly and more individuals may reach market size before disease causes mortality (Barber and Mann 1991; Meyers et al. 1991).

Genetic engineering is a more refined approach to increase oyster resistance. Some preliminary steps are in progress: technology for gene transfer and expression in adult oysters is being developed successfully (Cadoret et al. 1997a,b; Buchanan et al. 2001a,b; Cadoret et al. 2000). Antimicrobial peptides produced by organisms other than oysters with anti-*P. marinus* activity are being identified (tachyplesin I, Morvan et al. 1997; polyphemusin, Pierce et al. 1997; bacitracin, Faisal et al. 1999a) and their encoding genes could be potential candidates for transfer into oysters. In addition, research is in progress to identify, clone and sequence genes involved in the response of *C. virginica* and *C. gigas* to *P. marinus* infection (Gómez-Chiarri and Muñoz 2002; Tanguy et al. 2004).

A more drastic option being undertaken in the Chesapeake Bay region of the USA is the testing of disease susceptibility in ***allochthonous species*** of genus *Crassostrea* other than *C. virginica* as potential candidates to restore oyster populations. Field tests have required the use of triploid (sterile) allochthonous oysters to avoid undesired habitat colonisation and that could bias the results. Diploid allochthonous oysters were only tested in quarantine facilities. *C. gigas* showed lower disease susceptibility than *C. virginica,* but survival and growth were equal or superior in native oysters than in *C. gigas* in Chesapeake Bay (Calvo et al. 1999). In contrast, *Crassostrea ariakensis*, native in China, showed lower disease susceptibility, higher survival and faster growth than native oysters at different salinity regimes (Calvo et al. 2001). The decision of introducing the allochthonous oyster *C. ariakensis* in American waters is under debate and many aspects have to be taken into consideration. Preliminary tests showed that the Caribbean oyster *Crassostrea rhizophorae* is susceptible to *P. marinus* but may be somewhat more tolerant to heavy infections than *C. virginica* (Bushek et al. 2002c).

In vitro and in vivo assays have been performed to test the effectiveness of ***chemotherapeutants*** to reduce *P. marinus* infection (Calvo and Burreson 1994; Krantz 1994; Dungan and Hamilton 1995). Only cycloheximide was found to be effective in reducing infections in live oysters without killing the host, although infections were not completely eliminated even after 30 days of exposure and progressed after treatment ceased. Other chemotherapeutants inhibit or kill parasite cells in vitro. The use of chemotherapeutants in open environments would raise ecological and cost-effective objections. However, their use in close systems, either to eliminate infection in oysters or to kill free infective cells, could be feasible. Treatments with chlorine (Goggin et al. 1990; Bushek et al. 1997a; Casas et al. 2002a), N-halamine disinfectant compounds (Delaney et al. 2003), freshwater (Burreson et al. 1994b; Auzoux-Bordenave et al. 1995; Casas et al. 2002a; La Peyre et al. 2003) and particle filtration followed by UV irradiation (Ford et al. 2001) can be efficiently used to kill free infective *Perkinsus* cells.

References

Adema C.M., Van der Knaap W.P.W., Sminia T., 1991, Molluscan hemocyte-mediated cytotoxicity: The role of reactive oxygen intermediates. Rev. Aquat. Sci. 4, 201-223.

Ahmed H., Schott E.J., Gauthier J.D., Vasta G.R., 2003, Superoxide dismutases from the oyster parasite *Perkinsus marinus*: purification, biochemical characterization, and development of a plate microassay for activity. Anal. Biochem. 318, 132-141.

Ahn K.J., Kim K.H., 2001, Effect of temperature and salinity on in vitro zoosporulation of *Perkinsus* sp. in Manila clams *Ruditapes philippinarum*. Dis. Aquat. Org. 48, 43-46.

Almeida M., Berthe F., Thébault A., Dinis M.T., 1999, Whole clam culture as a quantitative diagnostic procedure of *Perkinsus atlanticus* (Apicomplexa, Perkinsea) in clams *Ruditapes decussatus*. Aquaculture 177, 325-332.

Anderson R.S., 1996a, Interactions of *Perkinsus marinus* with humoral factors and hemocytes of *Crassostrea virginica*. J. Shellfish Res. 15, 127-134.

Anderson R.S., 1999a, *Perkinsus marinus* secretory products modulate superoxide anion production by oyster (*Crassostrea virginica*) haemocytes. Fish Shellfish Immunol. 9, 51-60.

Anderson R.S., 1999b, Lack of hemocyte chemiluminescence stimulation by *Perkinsus marinus* in eastern oysters *Crassostrea virginica* with Dermo disease. J. Aquat. Anim. Health 11, 179-182.

Anderson R.S., Beaven A.E., 2001, Antibacterial activities of oyster (*Crassostrea virginica*) and mussel (*Mytilus edulis* and *Geukensia demissa*) plasma. Aquat. Living Resour. 14, 343-349.

Anderson R.S., Paynter K.T., Burreson E.M., 1992, Increased reactive oxygen intermediate production by hemocytes withdrawn from *Crassostrea virginica* infected with *Perkinsus marinus*. Biol. Bull. 183, 476-481.

Anderson R.S., Burreson E.M., Paynter K.T., 1995, Defense responses of hemocytes withdrawn from *Crassostrea virginica* infected with *Perkinsus marinus*. J. Invertebr. Pathol. 66, 82-89.

Anderson R.S., Brubacher L.L, Ragone Calvo L., Unger M.A., Burreson E.M., 1998, Effects of tributyltin and hypoxia on the progression of *Perkinsus marinus* infections and host defence mechanisms in oyster, *Crassostrea virginica* (Gmelin). J. Fish. Dis. 21, 371-380.

Andrews J.D., 1961, Measurement of shell growth in oysters by weighing in water. Proc. Natl. Shellfish Assoc. 52, 1-11.

Andrews J.D., 1965, Infections experiments in nature with *Dermocystidium marinum* in Chesapeake Bay. Cheapeake Sci. 6, 60-67.

Andrews J.D., 1988, Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effects on the oyster industry. Amer. Fish. Soc. Spec. Publ. 18, 47-63.

Andrews J.D., Hewatt W.G., 1957, Oyster mortality in Virginia II. The fungus disease caused by *Dermocystidium marinum* in oysters of Chesapeake Bay. Ecol. Monogr. 27, 1-25.

Andrews J.D., Ray S.M., 1988, Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effects on the oyster industry. Amer. Fish Soc. Spec. Publ. 18, 47-63.

Armstrong P.B., Quigley J.P., 1992, Humoral immunity: α_2-Macroglobulin activity in the plasma of mollusks. Veliger 35, 161-164.

Auzoux-Bordenave S., Vigario A.M., Ruano F., Domart-Coulon I., Doumenc D., 1995, In vitro sporulation of the clam pathogen *Perkinsus atlanticus* (Apicomplexa, Perkinsea) under various environmental conditions. J. Shellfish Res. 14, 469-475.

Azevedo C., 1989, Fine structure of *Perkinsus atlanticus* n. sp. (Apicomplexa, Perkinsea) parasite of the clam *Ruditapes decussatus* from Portugal. J. Parasitol. 75, 627-635.

Azevedo C., Corral L., Cachola R., 1990, Fine structure of zoosporulation in *Perkinsus atlanticus* (Apicomplexa: Perkinsea). Parasitology 100, 351-358.

Barber B.J., Mann R., 1991, Sterile triploid *Crassostrea virginica* (Gmelin 1791) grow faster than diploids but are equally susceptible to *Perkinsus marinus*. J. Shellfish Res. 10, 445-450.

Bebianno M.J., 1995, Effects of pollutants in the Ria Formosa Lagoon, Portugal. Sci. Total Environ. 171, 107-115.

Bender R.C., Fryer S.E., Bayne C.J., 1992, Proteinase inhibitory activity in the plasma of a mollusc: evidence for the presence of a-macroglobulin in *Biomphalaria glabrata*. Comp. Biochem. Physiol. 102B, 821-824.

Blackbourn J., Bower S.M., Meyer G.R., 1998, *Perkinsus qugwadi* sp. nov. (incertae sedis), a pathogenic protozoan parasite of Japanese scallops, *Patinopecten yessoensis*, cultured in British Columbia, Canada. Can. J. Zool. 76, 942-953.

Bower S.M., Blackbourn J., Meyer G.R., Nishimura D.J.H., 1992, Diseases of cultured scallops (*Patinopecten yessoensis*) in British Columbia, Canada. Aquaculture 107, 201-210.

Bower S.M., Blackbourn J., Meyer G.R., 1998, Distribution, prevalence, and pathogenicity of the protozoan *Perkinsus qugwadi* in Japanese scallops, *Patinopecten yessoensis*, cultured in British Columbia, Canada. Can. J. Zool. 76, 954-959.

Bower S.M., Blackbourn J., Meyer G.R., Welch D.W., 1999, Effect of *Perkinsus qugwadi* on various species and strains of scallops. Dis. Aquat Org. 36, 143-151.

Bramble L.H., Anderson R.S., 1997, Modulation of *Crassostrea virginica* haemocyte reactive oxygen species production by *Listonella anguillarum*. Dev. Comp. Immunol. 21, 337-348.

Bramble L.H., Anderson R.S., 1998, A comparison of the chemiluminescent response of *Crassostrea virginica* and *Morone saxatilis* phagocytes to zymosan and viable *Listonella anguillarum*. Dev. Comp. Immunol. 22, 55-61.

Braun-Breton C., Pereira da Silva L., 1988, Activation of a *Plasmodium falciparum* protease correlated with merozoite maturation and erythrocyte invasion. Biol. Cell. 64, 223-231.

Brown G.D., Reece K.S., 2003, Isolation and characterization of serine protease genes from *Perkinsus marinus*. Dis. Aquat. Org. 57, 117-126

Brown G., Hudson K.L., Reece K.S., 2004, Genetic variation at the ITS and ATAN loci among and within cultured isolates of *Perkinsus marinus*. J. Eukaryot. Microbiol. 51, 312-320.

Buchanan J.T., Nickens A.D., Cooper R.K., Tiersch. T.R., 2001a, Transfection of eastern oysters *Crassostrea virginica* embryos. Mar. Biotechnol. 3, 322-335.

Buchanan J.T., Cheng T.C., La Peyre J.F., Cooper R.K., Tiersch T.R., 2001b, In vitro transfection of adult eastern oysters *Crassostrea virginica*. J. World Aquacult. Soc. 32, 286-299.

Burreson E.M., 1991, Effects of *Perkinsus marinus* infection in the eastern oyster, *Crassostrea virginica* (Gmelin 1791): I. Susceptibility of native and MSX-resistant stocks. J. Shellfish Res. 10, 417-423.

Burreson E.M., Ragone Calvo L.M., 1996, Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. J. Shellfish Res. 15, 17-34.

Burreson E.M., Alvarez R.S., Martínez V.V., Macedo L.A., 1994a, *Perkinsus marinus* (Apicomplexa) as a potential source of oyster *Crassostrea virginica* mortality in coastal lagoons of Tabasco, Mexico. Dis. Aquat. Org. 20, 77-82.

Burreson E.M., Ragone Calvo L.M., La Peyre J.F., Counts F., Paynter K.T. 1994b, Acute osmotic tolerance of cultured cells of the oyster pathogen *Perkinsus marinus* (Apicomplexa: Perkinsida). Comp. Biochem. Physiol. 109A, 575-582.

Bushek D., Allen Jr. S.K., 1996a, Races of *Perkinsus marinus*. J. Shellfish Res. 15, 103-107.

Bushek D., Allen Jr. S.K., 1996b, Host-parasite interactions among broadly distributed populations of the eastern oyster *Crassostrea virginica* and the protozoan *Perkinsus marinus*. Mar. Ecol. Prog. Ser. 139, 127-141.

Bushek D., Ford S.E., Allen Jr. S.K., 1994, Evaluation of methods using Ray's fluid thioglycollate medium for diagnosis of *Perkinsus marinus* infection in the eastern oyster, *Crassostrea virginica*. Ann. Rev. Fish Dis. 4, 201-217.

Bushek D., Holley R., Kelly M., 1997a, Treatment of *Perkinsus marinus*-contaminated materials. J. Shellfish Res. 16, 330.

Bushek D., Allen S.K., Alcox K.A., Gustafson R., Ford S.E., 1997b, Response of *Crassostrea virginica* to in vitro cultured *Perkinsus marinus*: preliminary comparison of three inoculation methods. J. Shellfish Res. 16, 479-485.

Bushek D., Holley R., Reece K.S., 2000, Use of micromanipulation and "feeder layers" to clone the oyster pathogen *Perkinsus marinus*. J. Eukaryot. Microbiol. 47, 164-166.

Bushek D., Dungan C.F., Lewitus A.J., 2002a, Serological affinities of the oyster pathogen *Perkinsus marinus* (Apicomplexa) with some dinoflagellates (Dinophyceae). J. Eukaryot. Microbiol. 49, 11-16.

Bushek D., Ford S.E., Chintala M.M., 2002b, Comparison of in vitro-cultured and wild-type *Perkinsus marinus*. III. Fecal elimination and its role in transmission. Dis. Aquat. Org. 51, 217-225.

Bushek D., Scarpa J., Laramore S.E., 2002c, Susceptibility to the Caribbean oyster *Crassostrea rhizophorae* to *Perkinsus marinus*. J. Shellfish Res. 21, 371-372.

Cadoret J.P., Boulo V., Gendreau S., Mialhe E., 1997a, Promoters from *Drosophila* heat shock protein and cytomegalovirus drive transient expression of luciferase introduced by particle bombardement into embryos of the oyster *Crassostrea gigas*. J. Biotechnol. 56, 183-189.

Cadoret J.P., Bachère E., Roch P., Mialhe E., Boulo V., 2000, Genetic transformation of farmed marine bivalve molluscs. In: M. Fingerman, R. Nagabhushanam (Eds.) Recent advances in marine biotechnology, Vol. 4: Aquaculture, Part A Seaweeds and invertebrates. Science Publishers. pp. 111-126.

Cadoret J.P., Gendreau S., Delecheneau J.M., Rousseau C., Mialhe E., 1997b, Microinjection of bivalve eggs: aplication in genetics. Mol. Mar. Biol. Biotech. 6, 72-77.

Calvo G.W., Burreson E.M., 1994, In vitro and in vivo effects of eight chemotherapeutants on the oyster parasite *Perkinsus marinus* (Mackin, Owen, and Collier). J. Shellfish Res. 13, 101-107.

Calvo G.W., Luckenbach M., Allen Jr. S.K., Burreson E.M., 1999, A comparative field study of *Crassostrea gigas* (Thunberg 1793) and *Crassostrea virginica* (Gmelin 1791) in relation to salinity in Virginia. J. Shellfish Res. 18, 465-473.

Calvo G.W., Luckenbach M., Allen Jr. S.K., Burreson E.M., 2001, A comparative field study of *Crassostrea ariakensis* (Fujita 1913) and *Crassostrea virginica* (Gmelin 1791) in relation to salinity in Virginia. J. Shellfish Res. 20, 221-229.

Canestri-Trotti G., Baccarani E.M., Paesanti F., Turolla E., 2000, Monitoring of infections by protozoa of the genera *Nematopsis*, *Perkinsus* and *Porospora* in the smooth venus clam *Callista chione* from the North-Western Adriatic Sea (Italy). Dis. Aquat. Org. 42, 157-161.

Casas S.M., 2002, Estudio de la perkinsosis en la almeja fina, *Tapes decussatus* (Linnaeus 1758), de Galicia. Ph.D. Thesis, University of Santiago de Compostela, Spain.

Casas S.M., Villalba A., Reece K.S., 2002a, Study of perkinsosis in the carpet shell clam *Tapes decussatus* in Galicia (NW Spain). I. Identification of the aetiological agent and in vitro modulation of zoosporulation by temperature and salinity. Dis. Aquat. Org. 50, 51-65.

Casas S.M., La Peyre J.F., Reece K.S., Azevedo C., Villalba A. 2002b, Continuous in vitro culture of the carpet shell clam *Tapes decussatus* protozoan parasite *Perkinsus atlanticus*. Dis. Aquat. Org. 52, 217-231.

Casas S.M., La Peyre J.F., Villalba A., 2002c, Extracellular enzyme activities of Perkinsus atlanticus in culture and comparison with *Perkinsus marinus*. In: K.S. Harrington (Ed.), Abstracts from the 4th International Symposium on Aquatic Animal Health. September 1-5, 2002, New Orleans, Louisiana, USA, p. 233.

Casas S.M., Grau A., Reece K.S., Apakupakul K., Azevedo C., Villalba A., 2004, *Perkinsus mediterraneus* n. sp. a protistan parasite of the European flat oyster *Ostrea edulis* (L.) from the Coast of Balearic Islands, Mediterranean Sea. Dis. Aquat. Org. 58, 231-244.

Cavalier-Smith T., 1993, Kingdom protozoa and its 18 phyla. Microbiol. Rev. 57, 953-994.

Ceschia G., Zentilin A., Giorgetti G., 1991, Presenza di *Perkinsus* in vongole veraci (*Ruditapes philippinarum*) allevate nel Nord-Est Italia. Boll. Soc. It. Patol. Ittica. 5, 101-108.

Chagot D., Comps M., Boulo V., Ruano F., Grizel H., 1987, Histological study of a cellular reaction in *Ruditapes decussatus* infected by a protozoan. Aquaculture 67, 260-261.

Chintala M.M., Ford S.E., Fisher W.S., Ashton-Alcox K.A., 1994, Oyster serum agglutinins and resistance to protozoan parasites. J. Shellfish Res. 13, 115-121.

Chintala M.M., Bushek D., Ford S.E., 2002, Comparison of in vitro-cultured and wild-type *Perkinsus marinus*. I. Dosing methods and host response. Dis. Aquat. Org. 51, 203-216.

Choi K.S., Park K.I., 1997, Report on the occurrence of *Perkinsus* spp. in the Manila clams, *Ruditapes philippinarum*, in Korea. J. Aquac. 10, 227-237.

Choi K.S., Wilson E.A., Lewis D.H., Powell E.N., Ray S.M., 1989, The energetic cost of *Perkinsus marinus* parasitism in oysters: quantification of the thioglycollate method. J. Shellfish Res. 8, 125-131.

Choi K.S., Lewis D.H., Powell E.N., Frelier P.F., Ray S.M., 1991, A polyclonal antibody developed from *Perkinsus marinus* hypnospores fails to cross react with other life stages of *P. marinus* in oyster (*Crassostrea virginica*) tissues. J. Shellfish Res. 10, 411-415.

Choi K.S., Park K.I., Lee K.W., Matsuoka K., 2002, Infection intensity, prevalence and histopathology of *Perkinsus* sp. in the Manila clam, *Ruditapes philippinarum*, in Isahaya Bay, Japan. J. Shellfish Res. 21, 119-125.

Chu F.L.E., 1988, Humoral defense factors in marine bivalves. Am. Fish Soc. Spec. Publ. 18, 178-188.

Chu F.L.E., 1996, Laboratory investigations of susceptibility, infectivity and transmission of *Perkinsus marinus* in oysters. J. Shellfish Res. 15, 57-66.

Chu F.L.E., Greene K.H., 1989, Effect of temperature and salinity on in vitro culture of the oyster pathogen, *Perkinsus marinus* (Apicomplexa: Perkinsea). J. Invertebr. Pathol. 53, 260-268.

Chu F.L.E., Hale R.C., 1994, Relationship between pollution and susceptibility to infectious diseases in the eastern oyster, *Crassostrea virginica*. Mar. Environ. Res. 38, 243-256.

Chu F.L.E., La Peyre J.F., 1989, Effect of environmental factors and parasitism on hemolymph lysozyme and protein of American oysters (*Crassostrea virginica*). J. Invertebr. Pathol. 54, 224-232.

Chu F.L.E., La Peyre J.F., 1993a, *Perkinsus marinus* susceptibility and defense-related activities in eastern oysters *Crassostrea virginica*: temperature effects. Dis. Aquat. Org. 16, 223-234.

Chu F.L.E., La Peyre J.F., 1993b, Development of disease caused by the parasite *Perkinsus marinus* and defense-related hemolymph factors in three populations of oysters from the Chesapeake Bay, USA. J. Shellfish Res. 12, 21-27.

Chu F.L.E., Volety A.K., 1997, Disease processes of the parasite *Perkinsus marinus* in eastern oyster *Crassotrea virginica*: minimum dose for infection initiation, and interaction of temperature, salinity and infective cell dose. Dis. Aquat. Org. 28, 61-68.

Chu F.L.E., La Peyre J.F., Burreson C.S., 1993, *Perkinsus marinus* infection and potential defense-related activities in eastern oysters, *Crassostrea virginica*: salinity effects. J. Invertebr. Pathol. 62, 226-232.

Chu F.L.E., Volety A.K., Constantin G., 1996, A comparison of *Crassostrea gigas* and *Crassostrea virginica*: effects of temperature and salinity on susceptibility to the protozoan parasite, *Perkinsus marinus*. J. Shellfish Res. 15, 375-380.

Chu F.L.E., Soudant P., Lund E.D., 2003, *Perkinsus marinus*, a protozoan parasite of the Eastern oyster (*Crassostrea virginica*): effects of temperature on the uptake and metabolism of fluorescent lipid analogs and lipase activities. Exp. Parasitol. 105, 121-130.

Cigarría J., Rodríguez C., Fernández J.M., 1997, Impact of *Perkinsus* sp. on Manila clam *Ruditapes philippinarum* beds. Dis. Aquat. Org. 29, 117-120.

Coss C.A., Robledo J.A.F., Vasta G.R., 2001a, Fine structure of clonally propagated in vitro life stages of a *Perkinsus* sp. isolated from the Baltic clam *Macoma balthica*. J. Eukaryot. Microbiol. 48, 38-51.

Coss C.A., Robledo J.A.F., Ruiz G.M., Vasta G.R., 2001b, Description of *Perkinsus andrewsi* n. sp. isolated from the Baltic clam (*Macoma balthica*) by characterization of the ribosomal RNA locus, and development of a species-specific PCR-based diagnostic assay. J. Eukaryot. Microbiol. 48, 52-61.

Craig A., Powell E.N., Fay R.R., Brooks. J.M., 1989, Distribution of *Perkinsus marinus* in Gulf Coast oyster populations. Estuaries 12, 82-91.

Crosby M.P., Roberts C.F., 1990, Seasonal infection intensity cycle of the parasite *Perkinsus marinus* (and absence of *Haplosporidium* spp.) in oysters from a South Carolina salt marsh. Dis. Aquat. Org. 9, 149-155.

Da Ros L., Canzonier W.J., 1985, *Perkinsus*, a protistan threat to bivalve culture in the Mediterranean basin. Bull. Eur. Ass. Fish Pathol. 5, 23-25.

Deaton L.E., Jordan P.J., 2002, Effect of *Perkinsus marinus* on phenoloxidase activity in bivalve haemolymph. J. Shellfish Res. 21, 372.

De la Herrán R., Garrido-Ramos M.A., Navas J.I., Ruiz Rejón C., Ruiz Rejón M., 2000, Molecular characterization of the ribosomal RNA gene region of *Perkinsus atlanticus*: its use in phylogenetic analysis and as a target for a molecular diagnosis. Parasitology 120, 345-353.

Delaney M.A., Brady Y.J., Worley S.D., Huels K.L., 2003, The effectiveness of N-halamine disinfectant compounds on *Perkinsus marinus*, a parasite of the eastern oyster *Crassostrea virginica*. J. Shellfish Res. 22, 91-94.

Dittman D.E., Ford S.E., Padilla D.K., 2001, Effects of *Perkinsus marinus* on reproduction and condition of the eastern oyster, *Crassostrea virginica*, depend on timing. J. Shellfish Res. 20, 1025-1034.

Dungan C.F., Hamilton R.M., 1995, Use of the tetrazolium-based cell proliferation assay to measure effects of in vitro conditions on *Perkinsus marinus* (Apicomplexan) proliferation. J. Eukaryot. Microbiol. 42, 379-388.

Dungan C.F., Robertson B.S., 1993, Binding specificities of mono- and polyclonal antibodies to the protozoan oyster pathogen *Perkinsus marinus*. Dis. Aquat. Org. 15, 9-22.

Dungan C.F., Hamilton R.M., Hudson K.L., McCollough C.B., Reece K.S., 2002, Two epizootic diseases in Chesapeake Bay commercial clams, *Mya arenaria* and *Tagelus plebius*. Dis. Aquat. Org. 50, 67-78.

Elsayed E.E., McLaughlin S.M., Faisal M., 1999, Protease inhibitors in plasma of the softshell clam *Mya arenaria*: identification and effects of disseminated sarcoma. Comp. Biochem. Physiol. 123B, 427-435.

Escalante A.A., Ayala F.J., 1995, Evolutionary origin of *Plasmodium* and other Apicomplexa based on rRNA genes. Proc. Nat. Acad. Sci. USA 92, 5793-5797.

Faisal M., MacIntyre E.A., Adham K.G., Tall B.D., Kothary M.H., La Peyre J.F., 1998, Evidence for the presence of protease inhibitors in eastern (*Crassostrea virginica*) and Pacific (*Crassostrea gigas*) oysters. Comp. Biochem. Physiol. 121B, 161-168.

Faisal M., La Peyre J.F., Elsayed E.E., 1999a, Bacitracin inhibits the oyster pathogen *Perkinsus marinus* in vitro and in vivo. J. Aquat. Anim. Health 11, 130-138.

Faisal M., Oliver J.L., Kaattari S.L., 1999b, Potential role of protease-antiprotease interactions in *Perkinsus marinus* infection in *Crassostrea* spp. Bull. Eur. Ass. Fish Pathol. 19, 269-276.

Faisal M., Schafhauser D.Y., Garreis K.A., Elsayed E., La Peyre J.F., 1999c, Isolation and characterization of *Perkinsus marinus* proteases using bacitracin-sepharose affinity chromatography. Comp. Biochem. Physiol. 123B, 417-426.

Feng S.Y., 1988, Cellular defense mechanisms of oysters and mussels. Am. Fish Soc. Spec. Publ. 18, 153-168.

Figueras A., Robledo J.A.F., Novoa B., 1992, Occurrence of haplosporidian and *Perkinsus*-like infections in carpet-shell clams, *Ruditapes decussatus* (Linnaeus 1758), of the Ría de Vigo (Galicia, NW Spain). J. Shellfish Res. 11, 377-382.

Figueras A., Robledo J.A.F., Novoa B., 1996, Brown ring disease and parasites in clams (*Ruditapes decussatus* and *R. philippinarum*) from Spain and Portugal. J. Shellfish Res. 15, 363-368.

Fisher W.S., Newell R.I.E., 1986a, Salinity effects on the activity of granular hemocytes of American oysters, *Crassostrea virginica*. Biol. Bull. 170, 122-134.

Fisher W.S., Newell R.I.E., 1986b, Seasonal and environmental variation in protein and carbohydrate levels in the hemolymph from American oysters (*Crassostrea virginica* Gmelin). Comp. Biochem. Physiol. A 85, 365-372.

Fisher W.S., Oliver L.M., 1996, A whole-oyster procedure for diagnosis of *Perkinsus marinus* disease using Ray's fluid thioglycollate culture medium. J. Shellfish Res. 15, 109-117.

Fisher W.S., Gauthier J.D., Winstead J.T., 1992, Infection intensity of *Perkinsus marinus* disease in *Crassostrea virginica* (Gmelin 1791) from the Gulf of Mexico maintained under different laboratory conditions. J. Shellfish Res. 11, 263-269.

Fong D., Rodríguez R., Koo K., Sun J., Sogin M.L., Bushek D., Littlewood D.T.J., Ford S.E., 1993, Small subunit ribosomal RNA gene sequence of the oyster parasite *Perkinsus marinus*. Mol. Mar. Biol. Biotechnol. 2, 346-350.

Ford S.E., 1988, Host-parasite interactions in Eastern oysters selected for resistance to *Haplosporidium nelsoni* (MSX) disease: survival mechanisms against a natural pathogen. Am. Fish Soc. Spec. Publ. 18, 206-224.

Ford S.E., 1996, Range extension by the oyster parasite *Perkinsus marinus* into the North Eastern United States: response to climate change? J. Shellfish Res. 15, 45-56.

Ford S.E., Haskin H.H., 1988, Management strategies for MSX (*Haplosporidium nelsoni*) disease in eastern oysters. Am. Fish. Soc. Spec. Publ. 18, 249-256.

Ford S.E., Xu Z., Debrosse G., 2001, Use of filtration and UV irradiation to prevent infection by *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo) in hatchery-reared larval and juvenile oysters. Aquaculture 194, 37-49.

Ford S.E., Chintala M.M., Bushek D., 2002, Comparison of in vitro-cultured and wild-type *Perkinsus marinus*. I. Pathogen virulence. Dis. Aquat. Org. 51, 187-201.

Fuller A.L., McDougald L.R., 1990, Reduction in cell entry of *Eimeria tenella* (Coccidia) sporozoites by protease inhibitors, and partial characterization of proteolytic activity associated with intact sporozoites and merozoites. J. Parasitol. 76, 464-467.

Gaffney P.M., Bushek D., 1996, Genetic aspects of disease resistance in oysters. J. Shellfish Res. 15, 135-140.

Garreis K.A., La Peyre J.F., Faisal M., 1996, The effects of *Perkinsus marinus* extracellular products and purified proteases on oyster defence parameters in vitro. Fish Shellfish Immunol. 6, 581-597.

Gauthier J.D., Fisher W.S., 1990, Hemolymph assay for diagnosis of *Perkinsus marinus* in oysters *Crassostrea virginica*. J. Shellfish Res. 9, 367-371.

Gauthier J., Vasta G., 1993, Continuous in vitro culture of the eastern oyster parasite *Perkinsus marinus*. J. Invertebr. Pathol. 62, 321-323.

Gauthier J., Vasta G., 1995, In vitro culture of the eastern oyster parasite *Perkinsus marinus*: optimisation of the methodology. J. Invertebr. Pathol. 66, 156-168.

Gauthier J.D., Vasta G., 2002, Effects of plasma from bivalve mollusk species on the in vitro proliferation of the protistan parasite *Perkinsus marinus*. J. Exp. Zool. 292, 221-230.

Gauthier J., Feig B., Vasta G., 1995, Effect of fetal bovine serum glycoproteins on the in vitro proliferation of the oyster parasite *Perkinsus marinus*: development of a fully defined medium. J. Eukaryot. Microbiol. 42, 307-313.

Goggin C.L., 1992, Occurrence of parasites of the genus *Perkinsus* in France. Bull. Eur. Ass. Fish Pathol. 12, 174-176.

Goggin C.L., 1994, Variation in the two internal transcribed spacers and 5.8S ribosomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). Mol. Biochem. Parasitol. 65, 179-182.

Goggin C.L., Barker S.C., 1993, Phylogenetic position of the genus *Perkinsus* (Protista, Apicomplexa) based on small subunit ribosomal RNA. Mol. Biochem. Parasitol. 60, 65-70.

Goggin C.L., Lester R.J.G., 1987, Occurrence of *Perkinsus* species (Protozoa, Apicomplexa) in bivalves from the Great Barrier Reef. Dis. Aquat. Org. 3, 113-117.

Goggin C.L., Lester R.J.G., 1995, *Perkinsus*, a protistan parasite of abalone in Australia: a review. Mar. Freshwater Res. 46, 639-646.

Goggin C.L., Sewell K.B., Lester R.J.G., 1989, Cross–infection experiments with Australian *Perkinsus* species. Dis. Aquat. Org. 7, 55-59.

Goggin C.L., Sewell K.B., Lester R.J.G., 1990, Tolerances of *Perkinsus* spp. (Protozoa, Apicomplexa) to temperature chlorine and salinity. J. Shellfish Res. 9, 1445-1448.

Goggin C.L., McGladdery S.E., Whyte S.K., Cawthorn R.J., 1996, An assessment of lesions in bay scallops *Argopecten irradians* attributed to *Perkinsus karlssoni* (Protozoa, Apicomplexa). Dis. Aquat. Org. 24, 77-80.

Gómez-Chiarri M., Muñoz P., 2002, Molecular immune responses of the eastern oyster to the parasite *Perkinsus marinus*. J. Shellfish Res. 21, 895.

Hamaguchi M., Suzuki N., Usuki H., Ishioka H., 1998, *Perkinsus* protozoan infection in short-necked clam *Tapes* (=*Ruditapes*) *philippinarum* in Japan. Fish Pathol. 33, 473-480.

Hervio D., Chagot D., Godin P., Grizel H., Mialhe E., 1991, Localization and characterization of acid phosphatase activity in *Bonamia ostreae* (Ascetospora), an intrahemocytic protozoan parasite of the flat oyster *Ostrea edulis* (Bivalvia). Dis. Aquat. Org. 12, 67-70.

Hine P.M., 2001, Problems of applying risk analysis to aquatic organisms. In: Proceedings of the International Conference on Risk Analysis in Aquatic Animal Health, Paris, France, pp. 71-82.

Hine P.M., Thorne T., 2000, A survey of some parasites and diseases of several species of bivalve mollusc in Northern Western Australia. Dis. Aquat. Org. 40, 67-78.

Hofmann E.E., Powell E.N., Klinck J.M., Saunders G., 1995, Modelling diseased oyster populations. I. Modelling *Perkinsus marinus* infections in oysters. J. Shellfish Res. 14, 121-151.

Kleinschuster S.J., Swink S.L., 1993, A simple method for the in vitro culture of *Perkinsus marinus*. Nautilus 107, 76-78.

Kleinschuster S.J., Perkins F.O., Dykstra M.J., Swink S.L., 1994, The in vitro life cycle of a *Perkinsus* species (Apicomplexa, Perkinsidae) isolated from *Macoma balthica* (Linnaeus 1758). J. Shellfish Res. 13, 461-465.

Kotob S.I., McLaughlin S.M., Van Berkum P., Faisal M., 1999, Discrimination between two *Perkinsus* spp. isolated from the softshell calm, *Mya arenaria*, by sequence analysis of two internal transcribed spacer regions and the 5.8S ribosomal RNA gene. Parasitology 119, 363-368.

Krantz G.E., 1994, Chemical inhibition of *Perkinsus marinus* in two in vitro culture systems. J. Shellfish Res. 13, 131-136.

Krantz G.E., Jordan S.J., 1996, Management alternatives for protecting *Crassostrea virginica* fisheries in *Perkinsus marinus* enzootic and epizootic areas. J. Shellfish Res. 15, 167-176.

Kuvardina O.N., Leander B.S., Aleshin V.V., Myl'nikov A.P., Keeling P.J., Simdyanov T.G., 2002, The phylogeny of the colpodellids (Alveolata) using small subunit rRNA gene sequences suggests they are the free-living sister group to apicomplexans. J. Eukaryot. Microbiol. 49, 498-504.

Lambert C., Nicolas J.L., 1998, Specific inhibition of chemiluminiscent activity by pathogenic vibrios in haemotyes of two marine bivalves: *Pecten maximus* and *Crassostrea gigas*. J. Invertebr. Pathol. 71, 53-63.

La Peyre J.F., 1996, Propagation and in vitro studies of *Perkinsus marinus*. J. Shellfish Res. 15, 89-101.

La Peyre J.F., Cooper R.K., 1997, Changes in protease expression by *Perkinsus marinus* cultures following incubation in Ray's fluid thioglycollate medium. J. Shelfish Res. 16, 331.

La Peyre J.F., Faisal M., 1995, Improved method for the initiation of continuous cultures of the oyster pathogen *Perkinsus marinus* (Apicomplexa). Trans. Am. Fish. Soc. 124, 144-146.

La Peyre J.F., Faisal M., 1996, Optimal culture conditions for the propagation of the oyster pathogen *Perkinsus marinus* (Apicomplexa) in protein deficient medium. Parasite 3, 147-153.

La Peyre J.F., Faisal M., 1997, Development of a protein-free chemically defined culture medium for the propagation of the oyster pathogen *Perkinsus marinus*. Parasite 4, 67-73.

La Peyre J.F., Faisal M., Burreson E.M., 1993, In vitro propagation of the protozoan *Perkinsus marinus*, a pathogen of the eastern oyster, *Crassostrea virginica*. J. Eukaryot. Microbiol. 40, 304-310.

La Peyre J.F., Chu F.E., Meyers J.M., 1995a, Hemocytic and humoral activities of eastern and Pacific oysters following challenge by the protozoan *Perkinsus marinus*. Fish Shellfish Immunol. 5, 179-190.

La Peyre J.F., Chu F.L.E., Vogelbein W.K., 1995b, In vitro interaction of *Perkinsus marinus* merozoites with eastern and Pacific oyster haemocytes. Dev. Comp. Immunol. 19, 291-304.

La Peyre J.F., Schafhauser D.Y., Rizkalla E.H., Faisal M., 1995c, Production of serine proteases by the oyster pathogen *Perkinsus marinus* (Apicomplexa) in vitro. J. Eukaryot. Microbiol. 42, 544-551.

La Peyre J.F., Yarnall H.A., Faisal M., 1996, Contribution of *Perkinsus marinus* extracellular products in the infection of eastern oysters (*Crassostrea virginica*). J. Invertebr. Pathol. 68, 312-313.

La Peyre M.K., Nickens A.D., Volety A.K., Tolley G.S., La Peyre J.F., 2003, Environmental significance of freshets in reducing *Perkinsus marinus* infection in eastern oysters *Crassostrea virginica*: potential management applications. Mar. Ecol. Prog. Ser. 248, 165-176.

Lester R.J.G., Davis G.H.G., 1981, A new *Perkinsus* species (Apicomplexa, Perkinsea) from the abalone *Haliotis ruber*. J. Invertebr. Pathol. 37, 181-187.

Levine N.D., 1978, *Perkinsus* gen. n. and other new taxa in the protozoan phylum Apicomplexa. J. Parasitol. 64, 549.

Liang Y.B., Zhang X.C., Wang L.J., Yang B., Zhang Y., Cai C.L., 2001, Prevalence of *Perkinsus* sp. in the Manila clam, *Ruditapes philippinarum*, along the Northern coast of the Yellow Sea in China. Oceanol. Limnol. Sinica 32, 502-511.

Lund E.D., Chu F.L.E., 2002, Phospholipid biosynthesis in the oyster protozoan parasite, *Perkinsus marinus*. Mol. Biochem. Parasitol. 121, 245-253.

MacIntyre E.A., Earnhart C.G., Kaattari S.L., 2003, Host oyster tissue extracts modulate in vitro protease expression and cellular differentiation in the protozoan parasite, *Perkinsus marinus*. Parasitology 126, 293-302.

Mackin J.G., 1951, Histopathology of infection of *Crassostrea virginica* (Gmelin) by *Dermocystidium marinum* Mackin, Owen and Collier. Bull. Mar. Sci. Gulf Carib. 1, 72-87.

Mackin J.G., 1956, *Dermocystidium marinum* and salinity. Proc. Natl. Shellfish Assoc. 46, 116-128.

Mackin J.G., 1962, Oyster disease caused by *Dermocystidium marinum* and other microorganisms in Louisiana. Publ. Inst. Mar. Sci. Univ. Texas 7, 132-229.

Mackin J.G., Boswell J.L., 1956, The lyfe cycle and relationships of *Dermocystidium marinum*. Proc. Natl. Shellfish Assoc. 46, 112-115.

Mackin J.G., Ray S.M., 1955, Studies on the effect of infection by *Dermocystidium marinum* on ciliary action in oysters (*Crassostrea virginica*). Proc. Natl. Shellfish Assoc. 45, 168-181.

Mackin J.G., Ray S.M., 1966, The taxonomic relationships of *Dermocystidium marinum* Mackin, Owen, and Collier. J. Invertebr. Pathol. 8, 544-545.

Mackin J.G., Owen H.M., Collier A., 1950, Preliminary note on the ocurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gmelin). Science 111, 328-329.

Maeno Y., Yoshinaga T., Nakajima K., 1999, Occurrence of *Perkinsus* species (Protozoa, Apicomplexa) from Manila clam *Tapes philippinarum* in Japan. Fish Pathol. 34, 127-131.

Marsh A.G., Gauthier J.D., Vasta G.R., 1995, A semiquantitative PCR assay for assessing *Perkinsus marinus* infections in the eastern oyster, *Crassostrea virginica*. J. Parasitol. 81, 577-583.

McGladdery S.E., Cawthorn R.J., Bradford B.C., 1991, *Perkinsus karlssoni* n. sp. (Apicomplexa) in bay scallops *Argopecten irradians*. Dis. Aquat. Org. 10, 127-137.

McLaughlin S.M., Faisal M., 1998, In vitro propagation of two *Perkinsus* species from the softshell clam *Mya arenaria*. Parasite 5, 341-348.

McLaughlin S.M., Faisal M., 1999, A comparison of diagnostic assays for detection of *Perkinsus* spp. in the softshell clam *Mya arenaria*. Aquaculture 172, 197-204.

McLaughlin S.M., Tall B.D., Shaheen A., Elsayed E.E., Faisal M., 2000, Zoosporulation of a new *Perkinsus* species isolated from the gills of the softshell clam *Mya arenaria*. Parasite 7, 115-122.

Meyers J.A., Burreson E.M., Barber B.J., Mann R., 1991, Susceptibility of diploid and triploid Pacific oysters, *Crassostrea gigas* (Thunberg 1793) and estern oysters, *Crassostrea virginica* (Gmelin 1791), to *Perkinsus marinus*. J. Shellfish Res. 10, 433-437.

Montes J.F., Durfort M., García Valero J., 1995a, Cellular defence mechanism of the clam *Tapes semidecussatus* against infection by the protozoan *Perkinsus* sp. Cell Tissue Res. 279, 529-538.

Montes J.F., Durfort M., García Valero J., 1995b, Characterization and localization of a Mr 225 kDa polypeptide specifically involved in the defence mechanisms of the clam *Tapes semidecussatus*. Cell Tissue Res. 280, 27-37.

Montes J.F., Durfort M., García Valero J., 1996, When the venerid clam *Tapes decussatus* is parasitized by the protozoan *Perkinsus* sp. it synthesizes a defensive polypeptide that is closely related to p225. Dis. Aquat. Org. 26, 149-157.

Montes J.F., del Río J.A., Durfort M., García Valero J., 1997, The protozoan parasite *Perkinsus atlanticus* elicits a unique defensive response in the clam *Tapes semidecussatus*. Parasitology 114, 339-350.

Montes J.F., Durfort M., Lladó A., García Valero J., 2002, Characterization and immunolocalization of a main proteinaceous component of the cell wall of the protozoan parasite *Perkinsus atlanticus*. Parasitology 124, 477-484.

Morvan A., Inagawa S., Comps M., Bachère E., 1997, In vitro activity of the *Limulus* antimicrobial peptide tachyplesin I on marine bivalve pathogens. J. Invertebr. Pathol. 69, 177-182.

Muñoz P., Vance K., Gómez-Chiarri M., 2003, Protease activity in the plasma of American oysters, *Crassostrea virginica*, experimentally infected with the protozoan parasite *Perkinsus marinus*. J. Parasitol. 89, 941-951.

Murrell A., Kleeman S.N., Barker S.C., Lester R.J.G., 2002, Synonymy of *Perkinsus olseni* Lester & Davis, 1981 and *Perkinsus atlanticus* Azevedo, 1989 and an update on the phylogenetic position of the genus *Perkinsus*. Bull. Eur. Assoc. Fish Pathol. 22, 258-265.

Navas J.I., Castillo M.C., Vera P., Ruiz-Rico M., 1992, Principal parasites observed in clams, *Ruditapes decussatus* (L.), *Ruditapes philippinarum* (Adams et Reeve), *Venerupis pullastra* (Montagu) and *Venerupis aureus* (Gmelin), from the Huelva coast (S.W. Spain). Aquaculture 107, 193-199.

Newell R.I.E., Paynter K.T., Burreson E.M., 1994, Physiological effects of protozoan parasitism on the eastern oyster *Crassostrea virginica*: feeding and metabolism. J. Shellfish Res. 13, 294.

Nickens A.D., La Peyre J.F., Wagner E.S., Tiersch T.R., 2002, An improved procedure to count *Perkinsus marinus* in eastern oyster hemolymph. J. Shellfish Res. 21, 725-732.

Nóren F., Möestrup O., Rehnstam-Holm A.S., 1999, *Parvilucifera infectans* Nóren et Möestrup gen. et sp. nov. (Perkinsozoa phylum nov.): a parasitic flagellate capable of killing toxic microalgae. Eur. J. Protistol. 35, 233-254.

Norton J.H., Shepherd M.A., Perkins F.O., Prior H.C., 1993, *Perkinsus*-like infection in farmed golden-lipped pearl oyster *Pinctada maxima* from the Torres Strait, Australia. J. Invertebr. Pathol. 62, 105-106.

Novoa B., Ordás M.C., Figueras A., 2002, Hypnospores detected by RFTM in clam (*Ruditapes decussatus*) tissues belong to two different protozoan organisms, *Perkinsus atlanticus* and a *Perkinsus*-like organism. Aquaculture 209, 11-18.

O'Donoghue P.J., Phillips P.H., Shepherd S.A., 1991, *Perkinsus* (Protozoa: Apicomplexa) infections in abalone from South Australian waters. Trans. R. Soc. South Aust. 115, 77-82.

O'Farrell C., La Peyre J.F., Paynter K.T., Burreson E.M., 2000, Osmotic tolerance and volume regulation in in vitro cultures of the oyster pathogen *Perkinsus marinus*. J. Shellfish Res. 19, 139-145.

Olafsen J.A., Fletcher T.C., Grant P.T., 1992, Agglutinin activity in Pacific oyster (*Crassostrea gigas*) hemolymph following in vivo *Vibrio anguillarum* challenge. Dev. Comp. Immunol. 16, 123-138.

Oliver J.L., Lewis T.D., Faisal M., Kaattari S.L., 1999, Analysis of the effects of *Perkinsus marinus* proteases on plasma proteins of the eastern oyster (*Crassostrea virginica*) and the Pacific oyster (*Crassostrea gigas*). J. Invertebr. Pathol. 74, 173-183.

Oliver J.L., Gaffney P.M., Allen S.K., Faisal M., Kaattari S.L., 2000, Protease inhibitory activity in selectively bred families of eastern oysters. J. Aquat. Anim. Health 12, 136-145.

Oliver L.M., Fisher W.S., Ford S.E., Ragone Calvo L.M., Burreson E.M., Sutton E.B., Gandy J., 1998, *Perkinsus marinus* tissue distribution and seasonal variation in oysters *Crassostrea virginica* from Florida, Virginia and New York. Dis. Aquat. Org. 34, 51-61.

Ordás M.C., Ordás A., Beloso, C., Figueras A., 2000, Immune parameters in carpet shell clams naturally infected with *Perkinsus atlanticus*. Fish Shellfish Immunol. 10, 597-609.

Ordás M.C., Gómez-León J., Figueras A., 2001, Histopathology of the infection by *Perkinsus atlanticus* in three clam species (*Ruditapes decussatus*, *R. philippinarum* and *R. pullastra*) from Galicia (NW Spain). J. Shellfish Res. 20, 1019-1024.

Ottinger C.A., Lewis T.D., Shapiro D.A., Faisal M., Kaattari S.L., 2001, Detection of *Perkinsus marinus* extracellular proteins in tissues of the eastern oyster *Crassostrea virginica*: potential use in diagnostic assays. J. Aquat. Anim. Health 13, 133-141.

Park K.I., Choi K.S., 2001, Spatial distribution of the protozoan parasite *Perkinsus* sp. found in the Manila clams, *Ruditapes philippinarum*, in Korea. Aquaculture 203, 9-22.

Park K., Choi K.S., Choi J.W., 1999, Epizootiology of *Perkinsus* sp. found in the manila clam *Ruditapes philippinarum* in Komsoe Bay, Korea. J. Korean Fish Soc. 32, 303-309.

Paynter K.T., 1996, The effects of *Perkinsus marinus* infection on physiological processes in the eastern oyster, *Crassostrea virginica*. J. Shellfish Res. 15, 119-125.

Penna M.S., Khan M., French R.A., 2001, Development of a multiplex PCR for the detection of *Haplosporidium nelsoni*, *Haplosporidium costale* and *Perkinsus marinus* in the eastern oyster (*Crassostrea virginica*, Gmelin 1971). Mol. Cell Probes 15, 385-390.

Perkins F.O., 1968, Fine structure of zoospores from *Labyrinthomyxa* sp. parasitizing the clam *Macoma balthica*. Chesapeake Sci. 9, 198-208.

Perkins F.O., 1969, Ultrastructure of vegetative stages in *Labyrinthomyxa marina* (=*Dermocystidium marinum*), a commercially significant oyster pathogen. J. Invertebr. Pathol. 13, 199-222.

Perkins F.O., 1976, Zoospores of the oyster pathogen, *Dermocystidium marinum*. I. Fine structure of the conoid and other sporozoan–like organelles. J. Parasitol. 62, 959–974.

Perkins F.O., 1988, Structure of protistan parsites found in bivalve molluscs. Am. Fish Soc. Spec. Publ. 18, 93-111.

Perkins F.O., 1996, The structure of *Perkinsus marinus* (Mackin, Owen and Collier 1950) Levine, 1978 with comments on taxonomy and phylogeny of *Perkinsus* spp. J. Shellfish Res. 15, 67-87.

Perkins F.O., Menzel R.W., 1966, Morphological and cultural studies of a motile stage in the life cycle of *Dermocystidium marinum*. Proc. Natl. Shellfish Assoc. 56, 23-30.

Perkins F.O., Menzel R.W., 1967, Ultrastructure of sporulation in the oyster pathogen *Dermocystidium marinum*. J. Invertebr. Pathol. 9, 205-229.

Pierce J.C., Maloy W.L., Salvador L., Dungan C.F., 1997, Recombinant expression of the antimicrobial peptide polyphemusin and its activity against the protozoan oyster pathogen *Perkinsus marinus*. Mol. Mar. Biol. Biotechnol. 6, 248-259.

Polanowski A., Wilusz T., 1996, Serine proteinase inhibitors from insect hemolymph. Acta Biochim. Polon. 43, 445-454.

Powell E.N., Klinck J.M., Hofmann E.E., 1996, Modeling diseased oyster populations. II. Triggering mechanisms for *Perkinsus marinus* epizootics. J. Shellfish Res. 15, 141-165.

Powell E.N., Klinck J.M., Hofmann E.E., Ford S.E., 1997, Varying the timing of oyster transplant: implications for management from simulation studies. Fish Oceanogr. 6, 213-237.

Quick J.A., Mackin J.G., 1971, Oyster parasitism by *Labyrinthomyxa marina* in Florida. Florida Dep. Nat. Resour. Prof. Paper Ser. 13, 1-55.

Ragone L.M., Burreson E.M., 1993, Effect of salinity on infection progression and pathogenicity of *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica* (Gmelin). J. Shellfish Res. 12, 1-7.

Ragone L.M., Burreson E.M., 1994, Characterization of overwintering infections of *Perkinsus marinus* (Apicomplexa) in Chesapeake Bay oysters. J. Shellfish Res. 13, 123-130.

Ragone Calvo L.M., Wetzel R.L., Burreson E.M., 2000, Development and verification of a model for the population dynamics of the protistan parasite, *Perkinsus marinus*, within its host, the eastern oyster, *Crassostrea virginica*, in Chesapeake Bay. J. Shellfish Res. 20, 231-241.

Ragone Calvo L.M., Calvo G.W., Burreson E.M., 2003a, Dual disease resistance in a selectively bred eastern oyster, *Crassostrea virginica*, strain tested in Chesapeake Bay. Aquaculture 220, 69-87.

Ragone Calvo L.M., Dungan C.F., Roberson B.S., Burreson E.M., 2003b, Systematic evaluation of factors controlling *Perkinsus marinus* transmission dynamics in lower Chesapeake Bay. Dis. Aquat. Org. 56, 75-86.

Ray S.M., 1952, A culture technique for the diagnosis of infection with *Dermocystidium marinum* Mackin, Owen, and Collier in oysters. Science 116, 360-361.

Ray S.M., 1954, Biological studies of *Dermocystidium marinum*. Rice Inst. Pamph. 41 (SI), 1-114.

Ray S.M., 1966, A review of the culture method for detecting *Dermocystidium marinum*, with suggested modifications and precautions. Proc. Natl. Shellfish Assoc. 54, 55-69.

Ray S.M., 1996, Historical perspective on *Perkinsus marinus* disease of oysters in the Gulf of Mexico. J. Shellfish Res. 15, 9-11.

Ray S.M., Chandler A.C., 1955, *Dermocystidium marinum*, a parasite of oysters. Exp. Parasitol. 4, 172-200.

Reece K.S., Bushek D., Graves J.E., 1997a, Molecular markers for population genetic analysis of *Perkinsus marinus*. Mol. Mar. Biol. Biotechnol. 6, 197-206.

Reece K.S., Siddall M., Burreson E., Graves J.E., 1997b, Phylogenetic analysis of *Perkinsus* based on actin gene sequences. J. Parasitol. 83, 417-423.

Reece K.S., Bushek D., Hudson K.L., Graves J.E., 2001, Geographic distribution of *Perkinsus marinus* genetic strains along the Atlantic and Gulf coasts of the USA. Mar. Biol. 139, 1047-1055.

Reece K.S., Siddall M.E., Stokes N.A., Burreson E.M., 2004, Molecular phylogeny of the Haplosporidia based on two independent gene sequences. J. Parasitol. 90, 1111-1122.

Remaley A.T., Kuhns D.B., Basford R.E., Glew G.H., Kaplan S.S., 1984, Leishmanial phosphatase blocks neutrophil O^{2-} production. J. Biol. Chem. 259, 11173-11175.

Robledo J.A.F., Gauthier J.D., Coss C.A., Wright A.C., Vasta G.R., 1998, Species- specificity and sensitivity of a PCR- based assay for *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica*: a comparison with the fluid thioglycollate assay. J. Parasitol. 84, 1237-1244.

Robledo J.A.F., Coss C.A., Vasta G.R., 2000, Characterization of the ribosomal RNA locus of *Perkinsus atlanticus* and development of a polymerase chain reaction-based diagnostic assay. J. Parasitol. 86, 972-978.

Robledo J.A.F., Nunes P.A., Cancela M.L., Vasta G.R., 2002, Development of an in vitro clonal culture and characterization of the rRNA gene cluster of *Perkinsus atlanticus*, a protistan parasite of the clam *Tapes decussatus*. J. Eukaryot. Microbiol. 49, 414-422.

Rodríguez F., Navas J.I., 1995, A comparison of gill and hemolymph assays for the thioglycollate diagnosis of *Perkinsus atlanticus* (Apicomplexa, Perkinsea) in clams, *Ruditapes decussatus* (L.) and *Ruditapes philippinarum* (Adams and Reeve). Aquaculture 132, 145-152.

Rodríguez F., Godoy T., Navas J.I., 1994, Cross-infection with *Perkinsus atlanticus* in *Ruditapes decussatus*, *Ruditapes philippinarum* and *Venerupis pullastra*. Bull. Eur. Assoc. Fish Pathol. 14, 24-27.

Rodríguez L.A., Ellis A.E., Nieto T.P., 1992, Purification and characterisation of an extracellular metalloprotease, serine protease and haemolysin of *Aeromonas hydrophila* strain B_{32}: All are lethal for fish. Microbiol. Pathog. 13, 17-24.

Romestand B., Corbier F., Roch P., 2002, Protease inhibitors and haemagglutinins associated with resistance to the protozoan parasite, *Perkinsus marinus*, in the Pacific oyster, *Crassostrea gigas*. Parasitology 125, 323-329.

Rosenthal P.J., Kim K., McKerrow J.H., Leech J.H., 1987, Identification of three stage-specific proteinases of *Plasmodium falciparum*. J. Exp. Med. 166, 816-821.

Sagristà E., Durfort M., Azevedo C., 1995, *Perkinsus* sp. (Phylum Apicomplexa) in Mediteranean clam *Ruditapes semidecussatus*: ultrastructural observations of the cellular response of the host. Aquaculture 132, 153-160.

Sagristà E., Durfort M., Azevedo C., 1996, Ultrastructural study of the parasite, *Perkinsus atlanticus* (Apicomplexa), on the clam *Ruditapes philippinarum*, in the Mediterranean. Sci. Mar. 60, 283-288.

Saldarriaga J.F., McEwan M.L., Fast N.M., Taylor F.J., Keeling P.J., 2003, Multiple protein phylogenies show that *Oxyrrhis marina* and *Perkinsus marinus* are early branches of the dinoflagellate lineage. Internat. J. System. Evol. Microbiol. 53, 355-365.

Santmartí M.M., García Valero J., Montes J., Pech A., Durfort M., 1995, Seguimiento del protozoo *Perkinsus* sp. en las poblaciones de *Tapes decussatus* y *Tapes semidecussatus* del delta del Ebro. In: Castelló F., Calderer A. (eds) Actas del V Congreso Nacional de Acuicultura, May 10-13, 1995, S. Carlos de la Rápita, Spain, Universidad de Barcelona, Barcelona, Spain, pp. 260-265.

Scanlon C.H., Ragone Calvo L.M., Burreson E.M., 1997, The potential for transmission of *Perkinsus marinus* by faecal matter from the Eastern oyster, *Crassostrea virginica*. J. Shellfish, Res. 16, 332.

Schott E.J., Vasta G.R., 2003, The PmSOD1 gene of the protistan parasite *Perkinsus marinus* complements the sod2*Δ* mutant of *Saccharomyces cerevisiae*, and directs an iron superoxide dismutase to mitochondria. Mol Biochem. Parasitol. 126, 81-92.

Schott E.J., Pecher W.T., Okafor F., Vasta G.R., 2003a, The protistan parasite *Perkinsus marinus* is resistant to selected reactive oxygen species. Exp. Parasitol. 105, 232-240.

Schott E.J., Robledo J.A.F., Wright A.C., Silva A.M., Vasta G.R., 2003b, Gene organization and homology modeling of two iron superoxide dismutases of the early branching protist *Perkinsus marinus*. Gene 309, 1-9.

Siddall M.E., Reece K.S., Graves J.E., Burreson E.M., 1997, "Total evidence" refutes the inclusion of *Perkinsus* species in the phylum Apicomplexa. Parasitol. 115, 165-176.

Sobral P., Widdows J., 1997, Influence of hypoxia and anoxia on the physiological responses of the clam *Ruditapes decussatus* from southern Portugal. Mar. Biol. 127, 455-461.

Soniat T.M., 1985, Changes in levels of infection of oysters by *Perkinsus marinus*, with special reference to the interaction of temperature and salinity upon parasitism. Northwest Gulf. Sci. 7, 171-174.

Soniat T.M., 1996, Epizootiology of *Perkinsus marinus* disease of eastern oysters in the Gulf of Mexico. J. Shellfish Res. 15, 35-43.

Soniat T.M., Gauthier J.D., 1989, The prevalence and intensity of *Perkinsus marinus* from the mid northern Gulf of Mexico, with comments on the relationship of the oyster parasite to temperature and salinity. Tulane Stud. Zool. Bot. 27, 21-27.

Soniat T.M., Kortright E.V., 1998, Estimating time to critical levels of *Perkinsus marinus* in eastern oysters, *Crassostrea virginica*. J. Shellfish Res. 17, 1071-1080.

Soudant P., Chu F.L.E., 2001, Lipid class and fatty acid composition of the protozoan parasite of oysters, *Perkinsus marinus* cultivated in two different media. J. Eukaryot. Microbiol. 48, 309-319.

Sprague V., 1954, Protozoa. Fish. Bull. Fish Wildl. Serv. U.S. 89, 243-256.

Steinert S.A., Pickwell G.V., 1985, Multiple forms of lysozyme in copper stressed mussels (*Mytilus edulis*). Mar. Environ. Res. 17, 211-214.

Sunila I., Hamilton R.M., Dungan C.F., 2001, Ultrastructural characteristics of the in vitro cell cycle of the protozoan pathogen of oysters, *Perkinsus marinus*. J. Eukaryot. Microbiol. 48, 348-361.

Tall B.D., La Peyre J.F., Bier J.W., Miliotis M.D., Hanes K.E., Kothary M.H., Shah D.B., Faisal M., 1999, *Perkinsus marinus* extracellular protease modulates survival of *Vibrio vulnificus* in Eastern oyster (*Crassostrea virginica*) hemocytes. Appl. Environ. Microbiol. 65, 4261-4263.

Tanguy A., Guo X.M., Ford S.E., 2004, Discovery of genes expressed in response to *Perkinsus marinus* challenge in Eastern (*Crassostrea virginica*) and Pacific (*C. gigas*) oysters. Gene 338, 121-131.

Thogersen I.B., Salvesen G., Brucato F.H., Pizzo S.V., Enghlid J.J., 1992, Purification and characterization of an alpha-macroglobulin proteinase inhibitor from the mollusc *Octopus vulgaris*. Biochem. J. 285, 521-527.

Thompson R.J., Baune C.J., Moore M.N., Carefoot T.H., 1978, Haemolymph volume, changes in the biochemical composition of the blood, and cytological responses of the digestive cells in *Mytilus californianus* Conrad, induced by nutritional, thermal and exposure stress. J. Comp. Physiol. 127, 287-298.

Valiulis G.A., Mackin J.G., 1969, Formation of sporangia and zoospores by *Labyrinthomyxa* sp. parasitic in the clam *Macoma balthica*. J. Invertebr. Pathol. 14, 268-270.

Vasta G.R., Gauthier J.D., Marsh A.G., 1995, Molecular and biochemical adaptations of the protozoan pathogen *Perkinsus marinus* for its host, *Crassostrea virginica*: current research and future perspectives (or "the best defense is a good offense"). J. Mar. Biotechnol. 3, 35-41.

Vivier E., 1982, Réflexions et suggestions à propos de la systématique des sporozoaires: création d'une classes des Hematozoa. Protistology 18, 449-457.

Volety A.K., Chu F.L.E., 1994a, Comparison of infectivity and pathogenicity of two life stages, meront (trophozoite) and prezoosporangia stages of the oyster pathogen *Perkinsus marinus* in Eastern oysters *Crassostrea virginica* (Gmelin 1971). J. Shellfish Res. 13, 521-527.

Volety A.K., Chu F.L.E., 1995, Suppression of chemiluminescence of eastern oyster (*Crassostrea virginica*) haemocytes by the protozoan parasite *Perkinsus marinus*. Dev. Comp. Immunol. 19, 135-142.

Volety A.K., Chu F.L.E, 1997, Acid phosphatase activity in *Perkinsus marinus*, the protistan parasite of the American oyster, *Crassostrea virginica*. J. Parasitol. 83, 1093-1098.

White M.E., Powell E.N., Ray S.M., Wilson E.A., 1987, Host to host transmission of *Perkinsus marinus* in oyster (*Crassostrea virginica*) populations by the ectoparasitic snail *Boonea impressa* (Pyramidellidae). J. Shellfish Res. 6, 1-5.

Willson L.L., Burnett L.E., 2000, Whole animal and gill tissue oxygen uptake in the Eastern oyster *Crassostrea virginica*: effects of hypoxia, hypercapnia, air exposure, and infection with the protozoan parasite *Perkinsus marinus*. J. Exp. Mar. Biol. Ecol. 246, 223-240.

Wright A.C., Ahmed H., Gauthier J.D., Silva A.M., Vasta G.R., 2002, cDNA cloning and characterization of two iron superoxide dismutases from the oyster parasite *Perkinsus marinus*. Mol. Biochem. Parasitol. 123, 73-77.

Yarnall H.A., Reece K.S., Stokes N.A., Burreson E.M., 2000, A quantitative competitive polymerase chain reaction assay for the oyster pathogen *Perkinsus marinus*. J. Parasitol. 86, 827-837.