

Study of perkinsosis in the carpet shell clam *Tapes decussatus* in Galicia (NW Spain). II. Temporal pattern of disease dynamics and association with clam mortality

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ABSTRACT: Temporal dynamics of the infection by *Perkinsus olseni* in a clam (*Tapes decussatus*) bed was studied over 5 yr (March 1996 to December 2000). Diagnostic techniques were compared to assess their suitability for epizootiological purposes. A technique based on incubation of 2 gill lamellae in Ray's fluid thioglycollate medium (RFTM) was more sensitive, quicker and cheaper than examination of histological sections. Incubation of the whole-clam soft tissues in RFTM allowed detection of very light infections that were not detected with incubation of only 2 gill lamellae. Nevertheless, the correlation between the infection intensity estimated by both RFTM incubations was high. Infection intensity was significantly and positively correlated with clam size/age. No infected clam smaller than 20 mm was found. There was an annual pattern of infection involving lower mean infection intensity and prevalence in winter and higher values for both variables from spring to autumn, with 2 main annual peaks in spring and late summer–early autumn. This temporal pattern was significantly associated with the seawater temperature. The annual spring peak of infection intensity occurred when seawater temperature was around 15°C. Monthly mortality in the clam bed peaked in spring and summer—after peaks of *P. olseni* infection intensity and concurrently with high seawater temperature. A comparison of percentage mortality between clams from 2 sources (a perkinsosis-affected and a non-affected area) placed in the same clam bed revealed significantly higher mortality in the clams originating from the perkinsosis-affected area.

KEY WORDS: *Perkinsus olseni* · *Tapes decussatus* · Infection dynamics · Epizootiology · Diagnosis · Disease · Mortality · Temperature

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INTRODUCTION

The genus *Perkinsus* includes protistan parasites infecting marine molluscs throughout the world. *P. marinus*, the most thoroughly studied species of the genus, has affected Eastern oyster *Crassostrea virginica* populations of the USA for more than 50 yr, causing mass mortalities and dramatic economic losses (Andrews 1996, Burreson & Ragone Calvo 1996). *P. atlanticus* was blamed for mass mortalities of clams *Tapes decussatus* in Southern Portugal (Azevedo 1989) and the clams *T. philippinarum* and *T. decussatus* in Catalonia (NE Spain) (Sagristà et al. 1991,

1996, Santmartí et al. 1995). Because of the earlier association of perkinsosis with shellfish mortality, the detection of *Perkinsus*-like parasites in clams *T. decussatus* from Galicia (NW Spain) in the late 1980s (Figueroas et al. 1992) was considered a threat to the clam industry of the region. Perkinsosis has also been detected in other venerid clams on the Pacific coasts, including the Manila clam *T. philippinarum* in South Korea (Choi & Park 1997, Park et al. 1999, Park & Choi 2001), Japan (Hamaguchi et al. 1998, Maeno et al. 1999, Choi et al. 2002) and China (Liang et al. 2001), and the surf clam *Paphia undulata* in Thailand (Leethochavalit et al. 2003).

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The parasite affecting Galician clams was identified as *Perkinsus atlanticus* (Robledo et al. 2000, Casas et al. 2002b). Genetic studies suggested that *P. atlanticus* is also the etiologic agent of perkinsosis in *Tapes philippinarum* from Japan (Hamaguchi et al. 1998) and South Korea (Park et al. 2002) and that of *P. undulata* from Thailand (Leethochavalit et al. 2003). Molecular taxonomy supports close proximity between *P. olseni* and *P. atlanticus* (Goggin 1994, Robledo et al. 2000, Casas et al. 2002a,b) and both were recently considered to be conspecific, with *P. olseni* taking precedence (Murrell et al. 2002). Therefore, *P. atlanticus* is referred to herein as *P. olseni*. *P. olseni* was originally described as a parasite of the abalone *Haliotis ruber* in the south of Australia (Lester & Davis 1981). *P. olseni* was blamed for severe mortalities in *H. laevigata* (O'Donoghue et al. 1991, Goggin & Lester 1995) and was detected in the pearl oyster *Pinctada maxima* (Norton et al. 1993). Furthermore, *Perkinsus*-like parasites occur in numerous molluscan species in Australian waters (Goggin & Lester 1987, Hine & Thorne 2000). 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 confirms that this *Perkinsus* species has a wide host range (Goggin et al. 1989, Rodríguez et al. 1994).

Accurate risk assessment of perkinsosis for the clam industry of different regions requires epizootiological knowledge of the disease. Most epizootiological studies on perkinsosis have been focused on the infection of *Crassostrea virginica* by *Perkinsus marinus*, and have demonstrated that temperature and salinity have a marked influence on the disease dynamics. This results in a seasonal pattern of variation in both prevalence and intensity of infection by *P. marinus* (reviews by Andrews 1988, Bureson & Ragone Calvo 1996, Soniat 1996). If environmental parameters also have a marked influence on *P. olseni* dynamics, the effects of this parasite on Galician clams could differ from those in warmer areas where clam mortality associated with perkinsosis had been reported. A research programme was started in 1996 in our laboratory to evaluate the potential effect of perkinsosis on Galician populations of *Tapes decussatus*, the clam species with the highest market value regionally. The programme included the epizootiological study described herein. An important decision was the choice of the diagnostic technique. Incubation in Ray's fluid thioglycollate medium (RFTM, Ray 1966) has been widely used to diagnose *P. marinus*, with variants depending on the incubated organs (Choi et al. 1989, Gauthier & Fisher 1990, Fisher & Oliver 1996, Nickens et al. 2002). The RFTM technique was the recommended choice for epizootiological studies on infection of *C. virginica* by *P. marinus*, as

opposed to classic histology (Ray 1954, Bushek et al. 1994, Ford & Tripp 1996). At the beginning of the present research programme, the RFTM method was tested for *P. olseni* (Rodríguez & Navas 1995); molecular diagnostic procedures that have been developed since (de la Herrán et al. 2000, Robledo et al. 2000, Elandaloussi et al. 2004) were not at that time available for this parasite.

The study reported herein was conducted to (1) evaluate the suitability of RFTM as a diagnostic technique of clam perkinsosis for epizootiological purposes, (2) assess the occurrence of patterns of temporal variation in the disease dynamics and evaluate the role of host age and environmental parameters, and (3) evaluate the association of the disease with clam mortality.

MATERIALS AND METHODS

Field studies. All field studies were performed in an intertidal natural bed of clams *Tapes decussatus* located in Enseada do Grove, an inlet on the south side of the outer area of the Ría de Arousa (8.84774° W, 42.46067° N, Galicia, NW Spain) from March 1996 to December 2000. The bed was chosen because it was one of the zones most heavily affected by perkinsosis in a previous survey along the Galician coast (internal report of the 'Consellería de Pesca, Marisqueo e Acuicultura', Santiago de Compostela). Sampling and field work were performed at low tide when the bed was uncovered. From March 1996 to February 2000, the Centro de Control do Medio Mariño provided seawater temperature and salinity data, recorded weekly 1 m deep in the water column, at a sampling station (A7) of its monitoring net located approximately 500 m from the study bed. In addition, measurements of salinity and temperature of the surface seawater close to the bed and the interstitial water in the bed sediment were performed with a portable device (ISY 30/10 FT) from March 1999 to December 2000.

Comparison of diagnostic techniques. A total of 858 clams were processed to compare a diagnostic procedure based on incubation in RFTM (Ray 1966) with that based on classic histology. From March 1996 to October 1997, market sized (≥ 40 mm long) clams were taken monthly from the study bed of the study area. Monthly sample size varied from 25 to 150. Each clam was shucked and the 2 branchial lamellae of 1 side were excised. The lamellae were incubated in RFTM for 7 d in the dark at room temperature. In addition, a transversal, approximately 5 mm thick section of meat containing the remaining gills, visceral mass, foot and mantle lobes was excised and fixed in Davidson's solution and embedded in paraffin; 5 μ m thick sections were stained with Harris' hematoxylin and eosin

(Howard & Smith 1983). Gill tissues incubated in RFTM were removed after 7 d, set on a slide, chopped, flooded with Lugol's solution and examined by light microscopy for dark-stained *Perkinsus olseni* hypnospores. Each clam was rated using an infection intensity scale proposed by Mackin (Ray 1954): 0 = null infection, with no hypnospore observed on the whole slide; 1 = very light infection, with at least 1 hypnospore observed on the whole slide; 2 = light infection, with at least 1 hypnospore observed in each of 10 microscope fields (40 \times) scattered over the slide; 3 = moderate infection, with at least 10 hypnospores observed in each of 10 microscope fields (100 \times) scattered over the slide; 4 = heavy infection, with at least 50 hypnospores observed in each of 10 microscope fields (100 \times) scattered throughout the slide; and 5 = very heavy infection, with the number of hypnospores so high that more than half the tissue volume was occupied by hypnospores (all microscope fields appeared almost or completely black through the stained hypnospore colour).

Histological sections were examined for the presence of *Perkinsus olseni* under light microscopy. Each clam was rated according to the infection intensity as follows: N = null infection, with no parasite detected; B = branchial infection, with the parasites confined to the gills; LS = light systemic infection, with a few parasites in no more than 3 foci in the whole section in organs other than the gills; MS = moderate systemic infection, with parasites spread through various organs or concentrated in a single large focus outside the gills; and HS = heavy systemic infection, with numerous parasites in large foci of haemocytic infiltration occupying more than 50% of the surface of the histological section.

Additionally, 283 large (≥ 40 mm) clams were processed from December 1997 to October 2000 to compare the semiquantitative diagnostic procedure based on the incubation of 2 gill lamellae in RFTM with a quantitative procedure designed to estimate the total parasite body burden. Briefly, each clam was shucked, the whole meat was weighed and the 2 branchial lamellae of 1 side were excised, incubated in RFTM, and processed as described above to estimate infection intensity. The remaining soft tissues were weighed and then incubated in RFTM for 7 d in the dark at room temperature. Tissues were then removed and processed as described by Fisher & Oliver (1996) to isolate *Perkinsus olseni* hypnospores from host tissues and to estimate the total parasite body burden (number of hypnospores g^{-1} host wet meat).

Disease dynamics in clam bed. From March 1996 to December 2000, samples of 25 large (≥ 40 mm long) clams were collected monthly from the natural bed. The size range (shell length, SL) of the clams taken

from January 1998 to December 2000 was 42 to 48 mm, to minimise the effect of size dispersion on mean infection intensity (Table 1). In addition, smaller clams were taken to evaluate the influence of host age in infection dynamics. We collected 25 medium-sized (32 to 39 mm SL) clams monthly from December 1996 to December 1997, and 25 small-sized (22 to 28 mm SL) clams monthly from January 1998 to December 2000. Furthermore, 57 clams in the range 13 to 21 mm SL were taken from March 1998 to November 1998. We incubated 2 branchial lamellae of each sampled clam in RFTM and estimated infection intensity as described in the foregoing subsection. Monthly prevalence was calculated as the percentage of infected clams in each monthly sample of large clams. Monthly mean infection intensity was calculated after estimating individual infection intensity of large clams.

Additionally, 29 clams 15 to 21 mm SL were collected in July 1999. Whole soft tissues of each clam were processed as described in foregoing subsection for estimation of the total parasite body burden to establish the minimum size at which the parasite could be detected with the most sensitive technique among those used in the study.

Estimation of percentage mortality in clam bed affected by perkinsosis. We randomly harvested 150 clams each month from the study bed, between February 1999 and March 2000. Each clam was marked by filing down a small area of the shell and colouring it with indelible red ink. Then the clams were placed in a 3 m square plot. A shallow hole was dug in the sediment for each clam to facilitate burrowing and to avoid the clam's removal by tidal currents. Both live and dead marked clams were collected from the plot during the next monthly sampling, i.e. approximately 1 mo after deployment. Collection ceased when at least 70 marked clams had been recovered, assuming equal probability for finding dead and live clams. Recovery of all marked clams was considered impracticable, and previous sampling of the bed had shown that by using a sample size of 70 clams, the proportion of dead clams could be estimated with a margin error of 8.6%, with a probability $\alpha = 0.10$ of not achieving this margin error

Table 1. *Tapes decussatus*. Size range, sampling periods, and sample sizes (N) of clams collected from an intertidal natural clam bed, NW Spain, to ascertain perkinsosis dynamics

Clam length range (mm)	Sampling period	N
≥ 40	Mar 1996–Dec 1997	25 each month
42–48	Jan 1998–Dec 2000	25 each month
32–39	Dec 1996–Dec 1997	25 each month
22–28	Jan 1998–Dec 2000	25 each month
13–21	Mar 1998–Nov 1998	57 in all

(Krebs 1989). The shell length of each recovered clam was measured. Monthly percentage mortality was calculated after counting the number of dead and live clams and then extrapolating to a 30 d period. Recovered live clams were not re-used and a different plot was marked each month to avoid collecting marked clams not recovered in previous months.

Assessment of association of perkinsosis with mortality. From June 2000 to November 2000, we harvested 75 clams monthly from each of 2 intertidal beds, one of which was affected by perkinsosis. The perkinsosis-affected bed comprised the study bed (Enseada do Grove), and the non-affected bed located at the beach of Baraña on the north side of the Ría de Arousa (8.88280° W to 42.63867° N) served as a control. Only large (≥ 40 mm SL) clams were used, since our aim was to evaluate the influence of perkinsosis on mortality and early results of the study had shown that the older the clam the higher the infection intensity. Clams were marked and placed in 10 plastic netting trays (56.5 \times 36.5 \times 16.5 cm), which had been buried at ground level and filled with sediment, whereby 5 replicates (trays) were prepared for each of the 2 beds, with 15 clams per tray. Each tray was dug out at the next monthly sampling, i.e. approximately 1 mo after deployment. Both live and dead marked clams were counted and the monthly percentage mortality was calculated, extrapolating for a 30 d period. Marked clams were only used once.

Statistical analysis. The Spearman rank correlation coefficient (r_s) was calculated to estimate the association between diagnostic techniques and the association of infection intensity with clam size, infection prevalence and seawater temperature. To test association between size and mortality, all clams recovered from the 3 m square plots were sorted into 1 mm length classes. The percentage of dead clams corresponding to each length class was calculated, and the Spearman rank correlation coefficient between size and percentage of dead clams. Differences in the percentage mortality between clams from the affected and the non-affected bed in the experiment with trays were analysed by 2-way ANOVA, in which one factor was origin (either affected or non-affected bed) and the other sampling date. The variable was arcsine transformed to fulfil test requirements. MINITAB 13 software was used in every statistical test.

RESULTS

Comparison of diagnostic techniques

Incubation of 2 gill lamellae in RFTM achieved higher sensitivity than examination of histological sec-

Table 2. *Tapes decussatus* (N = 858). Distribution and intensity of infection by *Perkinsus olseni* determined using 2 diagnostic techniques: incubation of 2 gill lamellae in Ray's fluid thioglycollate medium (RFTM) and examination of histological sections. Data represent number of clams corresponding to each pair of infection categories. For RFTM: 0 = null, 1 = very light, 2 = light, 3 = moderate, 4 = heavy and 5 = very heavy infections; for histological sections, N: null, and B: branchial infection, and LS: light, MS: moderate and HS: heavy systemic infection

RFTM	N	Histological section			
		B	LS	MS	HS
0	82	2	5	2	0
1	132	12	18	1	0
2	97	45	80	23	1
3	23	14	83	50	2
4	0	0	37	60	15
5	0	0	6	44	24

Table 3. *Tapes decussatus*. Correspondence between scale of intensity of infection by *Perkinsus olseni* based on incubation of 2 gill lamellae in RFTM and on total parasite body burden (number of hypospores g^{-1} of host wet meat)

RFTM	N	No. hypospores g^{-1}	
		Mean \pm SE	Range
0	48	581 \pm 178	0–6667
1	85	8877 \pm 1705	2–95529
2	74	46947 \pm 5862	971–210382
3	40	161434 \pm 25824	847–765756
4	21	551662 \pm 76387	62844–1359080
5	15	643611 \pm 115589	126429–1696788

tions (Table 2). In histological sections, 76% of null infections were classed as positive by the RFTM incubation method. False negatives from histological sections were mostly for clams with an infection intensity score of '1' by the RFTM method, but also for some clams classed as '2' and '3' by the RFTM method. In contrast, 10% of the clams shown to be non-infected by the RFTM technique were revealed as infected in histological sections. Both procedures correlated significantly ($r_s = 0.75$; $p < 0.001$).

A comparison between the infection intensity based on incubation of gill lamellae in RFTM and based on the total parasite body burden revealed that 94% of the negative results for gill lamellae were false, since the parasite was detected within the whole body. The number of hypospores g^{-1} wet tissue was < 1000 in 81% of these false negatives. Significant correlation between both procedures was observed (Table 3; $r_s = 0.85$; $p < 0.001$).

Disease dynamics in clam bed

Grouping all clams sampled in the study, infection intensity estimated by the incubation of gill lamellae

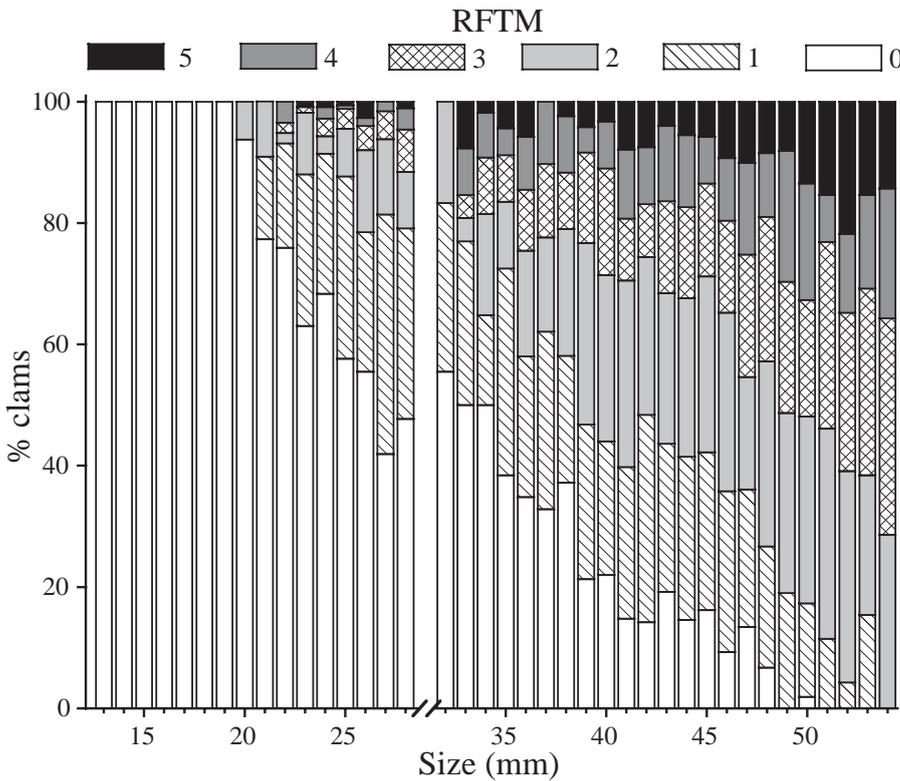


Fig. 1. *Tapes decussatus*. Intensity of infection by *Perkinsus olseni* as a function of clam size, estimated after incubation of 2 gill lamellae in RFTM. Infection intensity categories as in legend to Table 2

in RFTM was correlated with clam size ($r_s = 0.49$; $p < 0.001$). The minimum size of infected clams was 20 mm. Each sampled clam > 50 mm was infected (Fig. 1). Only 1 clam (21 mm SL) was infected among the 29 small-sized (15 to 21 mm) clams whose whole soft tissues had been incubated in RFTM.

The temporal variation in mean infection intensity in large clams is shown in Figs. 2 & 3. There were inter-annual differences, although a common annual pattern was apparent. The lowest annual values (around 1.0) were in winter. Spring and late summer–early autumn peaks were detected every year. In addition, 1 mid-summer peak was recorded in some years. The spring peak was the highest annual peak in the period 1996 to 1998, whereas the late summer–early autumn peak was the highest annual peak in 1999 to 2000. The highest monthly mean infection intensity recorded during the study was 3.0. Temporal variation in prevalence matched that in mean infection intensity, and ranged from 46 to 100% (Fig. 2). Both variables were significantly correlated ($r_s = 0.84$; $p < 0.001$). Variation in mean infection intensity followed a pattern similar to that in seawater temperature throughout most of the study period. Midsummer and late summer–early autumn peaks of infection intensity were associated with temperature peaks. Spring peaks of infection

intensity occurred when seawater temperature was increasing but not peaking. Seawater temperature was slightly above 15°C when spring peaks of infection intensity occurred in 1996 to 1998, whereas the year with the lowest temperature in spring (1999) showed the lowest spring peak of infection intensity (Fig. 2). The correlation of mean infection intensity with the seawater temperature recorded on the sampling day was higher than its correlation with temperature records made prior sampling the clams. The earlier the temperature records, the lower the correlation with mean infection intensity (Table 4). The temporal variation in the mean infection intensity showed a weaker association with that of salinity (Fig. 2). The correlation coefficient between both variables was low but significantly different from zero ($r_s = 0.32$; $p = 0.024$).

The pattern of temporal variation in the mean infection intensity in clams 32 to 39 mm SL was similar to that in large clams, but with lower values ($r_s = 0.78$; $p = 0.001$) (Fig. 4). However, the pattern of variation for clams 22 to 28 mm SL differed from that of large clams ($r_s = 0.21$; $p = 0.23$) (Fig. 4).

Estimation of mortality in a perkinsosis-affected clam bed

Monthly percentage mortalities estimated in the 3 m square plots are shown in Fig. 5. Mortality peaked in June 1999 (10.2%) and September 1999 (7.2%). The

Table 4. *Tapes decussatus*. Spearman rank correlation coefficients (r_s) between monthly mean intensity of infection by *Perkinsus olseni* and seawater temperature recorded on day of clam sampling and at different times prior to clam sampling. Significance levels = ns (not significant); $p > 0.05$; * $0.05 \geq p > 0.01$; ** $0.01 \geq p > 0.001$

Temperature recorded	Temperature vs. infection intensity (r_s)
On sampling day	0.38**
7 d before sampling	0.31*
15 d before sampling	0.25 ns
1 mo before sampling	0.21 ns
2 mo before sampling	0.03 ns
3 mo before sampling	-0.19 ns

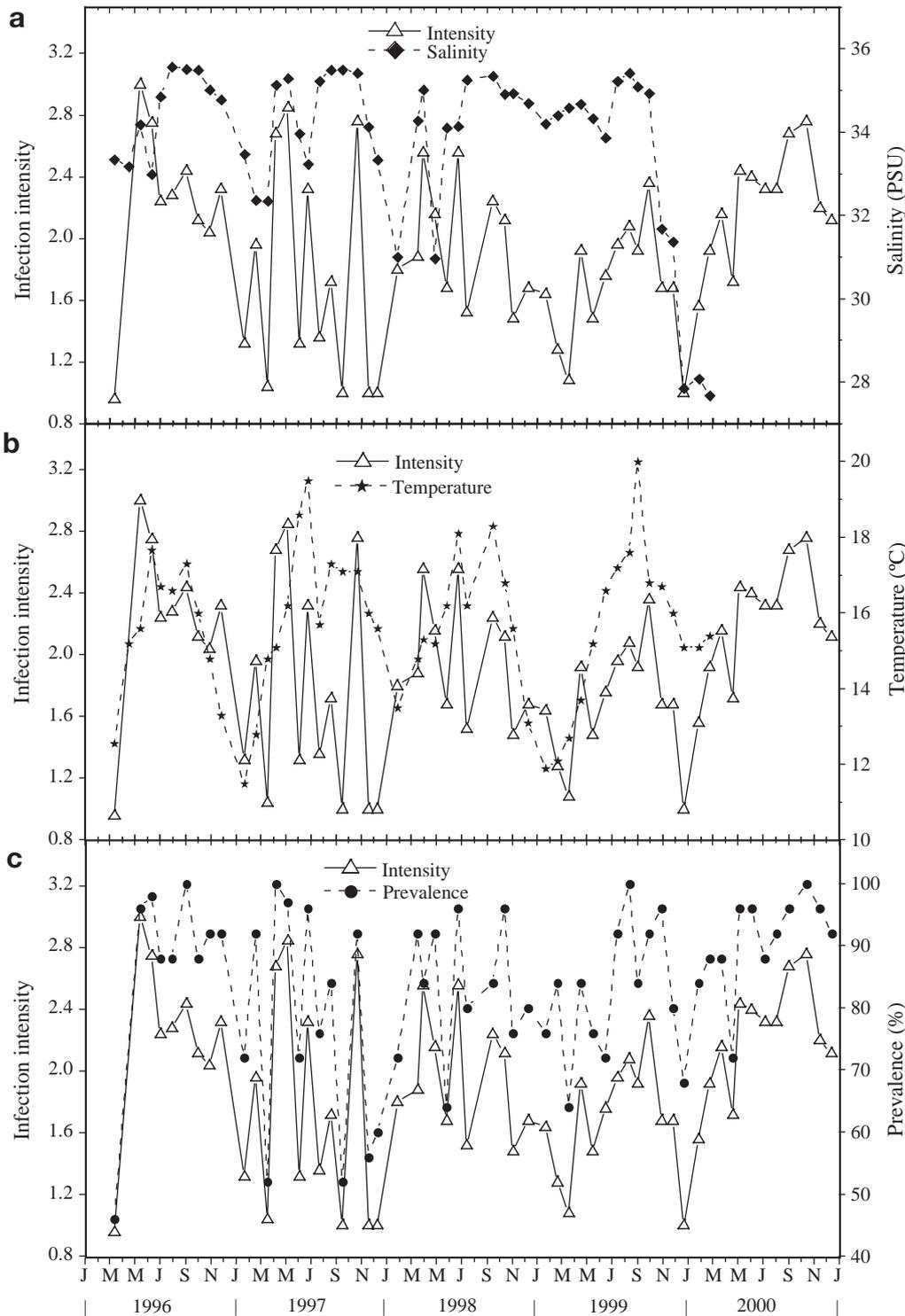


Fig. 2. *Tapes decussatus* infected by *Perkinsus olseni*. Temporal dynamics of infection as a function of environmental conditions. (a) Monthly variation in mean infection intensity in large clams and in seawater salinity. (b) Monthly variation in mean infection intensity in large clams and in the mean of last 3 weekly temperature records prior to each monthly sampling. (c) Monthly variation in mean infection intensity and prevalence in large (≥ 40 mm) clams

June peak in mortality occurred after a spring peak of infection intensity, and the September peak of mortality occurred after a midsummer peak of infection intensity. Both mortality peaks occurred when seawater temperature was at their highest annual values and coincided with temperature peaks of the inter-

stitial water (Fig. 5). The cumulative mortality for an average 1 yr period inferred from monthly mortality data would be 41.2%. Fig. 6 shows the percentage of dead clams in each size class. The correlation between length and percentage of dead clams was not significant ($r_s = -0.25$; $p = 0.25$; $N = 1100$).

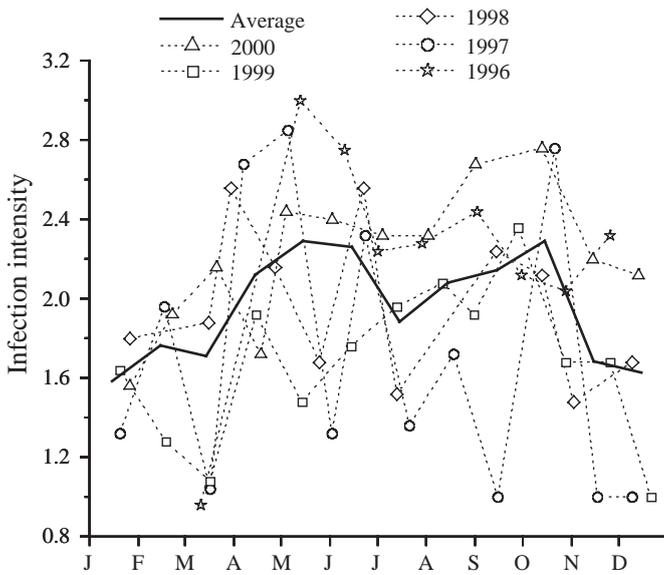


Fig. 3. *Tapes decussatus*. Annual cycle in mean intensity of infection by *Perkinsus olseni* in large (≥ 40 mm) clams. Dotted lines: year-to-year variability for years 1996 to 2000; continuous line: average of all years (1996 to 2000)

Association of perkinsosis with mortality

Mortality of clams taken from the perkinsosis-affected bed and kept in buried trays for 1 mo was higher than that of clams taken from the non-affected bed (Fig. 7). The highest percentage mortality was recorded in early September in clams from both sources (7.0 and 2.8%, respectively), when the seawater temperature was close to the annual maximum (Fig. 7). Differences due to clam origin and sampling date were significant ($p = 0.001$ and $p = 0.017$, respectively).

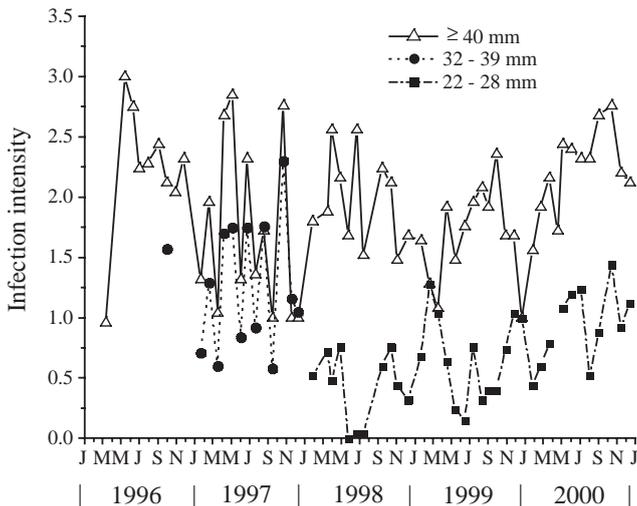


Fig. 4. *Tapes decussatus*. Monthly variation in mean intensity of infection by *Perkinsus olseni* in 3 size groups of clams: large (≥ 40 mm), 32 to 39 mm and 22 to 28 mm shell length

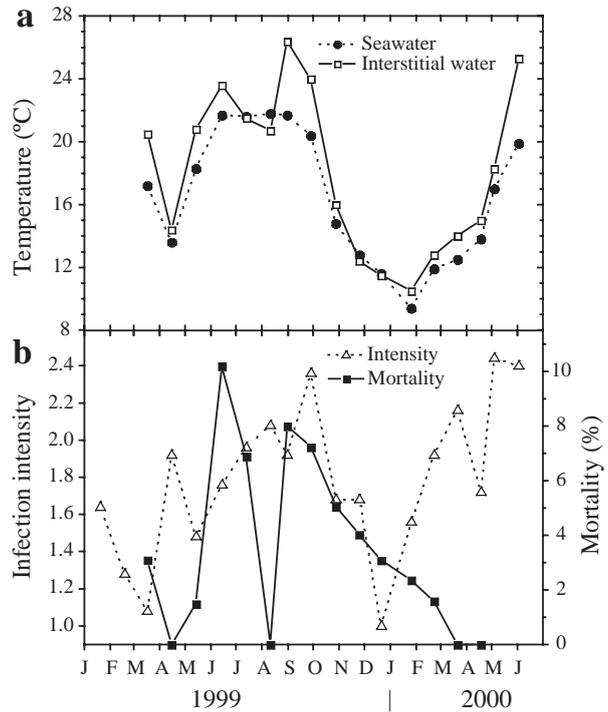


Fig. 5. *Tapes decussatus*. Mortality in clam bed affected by *Perkinsus olseni*. (a) Variation in monthly records of seawater temperature approximately 500 m from clam bed and that of interstitial water in clam bed sediment. (b) Variation in monthly percentage mortality estimated after leaving clams in plots for 1 mo and variation in mean infection intensity recorded in bed samples of large (≥ 40 mm) clams

DISCUSSION

This study has confirmed that the technique based on incubation of gill lamellae in RFTM is more sensitive than examination of histological sections for diagnosis of perkinsosis in *Tapes decussatus*, as reported

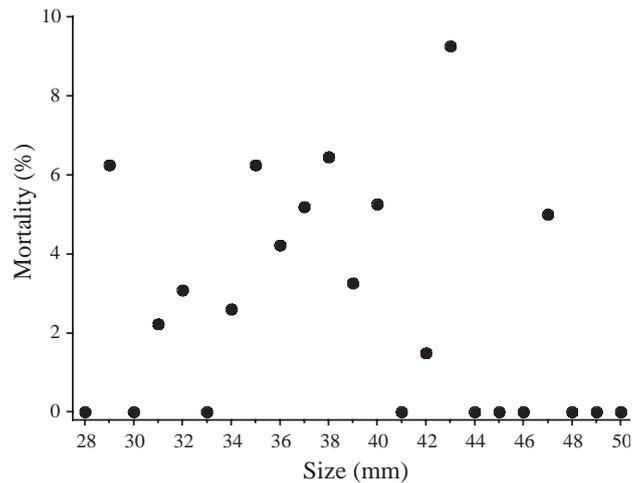


Fig. 6. *Tapes decussatus*. Percentage of dead clams in each 1 mm size class

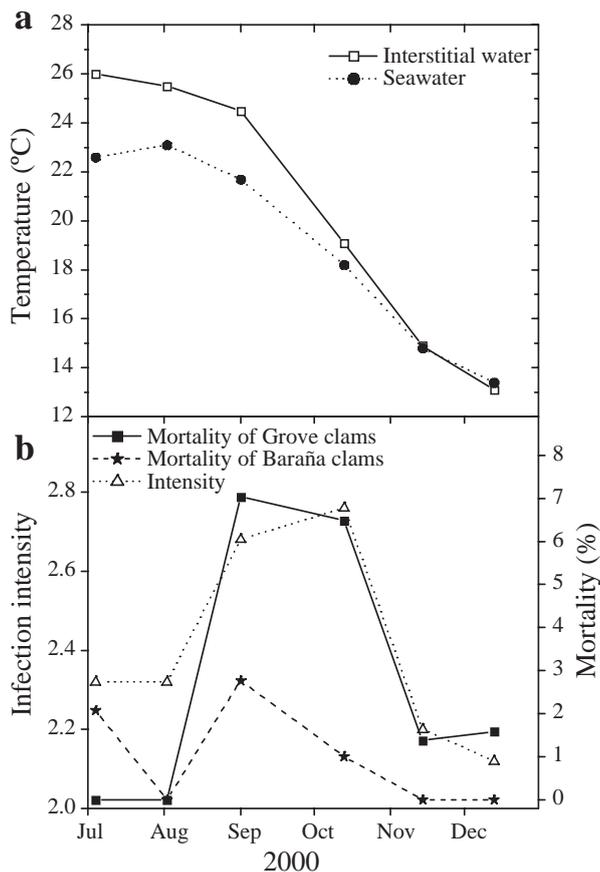


Fig. 7. *Tapes decussatus*. (a) Variation in monthly temperature records of seawater approximately 500 m from clam bed and that of interstitial water in clam bed sediment. (b) Monthly variation in mean percentage of mortality of clams taken from a perkinsosis-affected bed (Enseada do Grove) and a non-affected bed (beach of Baraña) and kept for 1 mo in buried trays

by Rodríguez & Navas (1995). The RFTM involved examination of a much higher volume of host tissue. Similarly, the technique based on incubation of tissue (rectum, mantle, gills) pieces in RFTM is considered more sensitive than histology in diagnosing infection by *Perkinsus marinus* in *Crassostrea virginica* (Ray 1954, Bushek et al. 1994, Ford & Tripp 1996). The incubation of the whole soft tissues in RFTM resulted in higher sensitivity than incubation of 2 gill lamellae. Similar results were reported for *P. marinus* (Bushek et al. 1994) and *P. olseni* infections (Rodríguez & Navas 1995, Almeida et al. 1999). Most false negatives recorded for incubation of gill lamellae in RFTM occurred when the total parasite body burden was <1000 hyphospores g^{-1} wet tissue, which agrees with reports for *P. marinus* infections (Bushek et al. 1994). Correlation between the infection intensity estimated after incubation of gill lamellae in RFTM and the total parasite body burden in the whole soft tissues was

high. Therefore, the incubation of 2 gill lamellae in RFTM is cheaper and simpler than the other tested techniques, and is thus suitable for epizootiological studies, which usually require the analysis of high numbers of samples. Whereas the actual prevalence in sampled populations could be higher than that estimated after incubation of gill lamellae in RFTM because of false negatives, the estimation of mean infection intensity should closely reflect the actual values, since here, false negatives correspond to very light infections, as demonstrated by the whole clam incubation procedure. This technique of incubation of gill lamellae cannot therefore be used to certify lack of perkinsosis in clams.

A significant association between infection intensity and clam age/size was observed. No infection was found in clams <20 mm SL (i.e. <1 yr old) by any technique in the study bed. Choi & Park (1997) did not find infection in *Tapes philippinarum* <15 mm SL and detected nearly 100% infection prevalence in clams >20 mm SL. Infection of *T. decussatus* by *Perkinsus olseni* is probably dosage dependent (Rodríguez et al. 1994), as in *P. marinus* (Chu 1996, Ragone Calvo et al. 2003). Thus, the size threshold for detecting infection may arise from the limited filtering capacity of small clams, which do not filter a water volume large enough to acquire sufficient infective particles. This hypothesis was also proposed for the infection of *Crassostrea virginica* by *P. marinus* (Andrews & Hewatt 1957). In addition, the longer a clam stays in an affected bed the higher the probability of its becoming infected. Association of perkinsosis intensity with clam length was also observed in *T. philippinarum* from Korea (Park et al. 1999, Park & Choi 2001). Andrews & Hewatt (1957) reported that the intensity of infection by *P. marinus* increases with increasing age until *C. virginica* are 3 yr old. Calvo et al. (1996) found that the prevalence of infection by *P. marinus* in large (50 to 200 mm shell height) *C. virginica* was twice that of small (20 to 50 mm shell height) oysters; they also observed that infection intensity stages of 4 and above were less frequent in small (20 to 50 mm shell height) oysters and very large (100 to 100 mm shell height) oysters than in medium (50 to 100 mm shell height) oysters, although the correlation between oyster size and infection stage was not statistically significant.

According to reports on growth in *Tapes decussatus* (Vilela 1950, Pérez-Camacho 1979, Guelorget et al. 1980, Pérez-Camacho & Cuña 1987), which revealed a size-age relationship, a hypothetical pattern of progression of perkinsosis in our study bed associated with clam age could be deduced: Infection is not detectable before clams are 1 yr old (<20 mm SL); it becomes detectable during the second year in some clams; infection progresses (intensity and prevalence

increase) after the clams are 2 yr old; infections are heavier and most (>80%) clams are infected when they are >3 yr old (>40 mm SL).

Perkinsosis dynamics showed an annual pattern involving lower mean infection intensity in winter and higher values from spring to autumn, with 2 main annual peaks (spring and late summer–early autumn). The decrease in the percentage of clams with heavy infection after peaks of mean infection intensity could be due to death of heavily infected clams or regression of infection. Seasonality of perkinsosis has also been detected in the clam *Tapes decussatus* from a culture bed in the inner side of Ría de Arousa (López 1995, López et al. 1998). The annual pattern in our study was similar to that of infection of *Crassostrea virginica* by *Perkinsus marinus* in Chesapeake Bay (USA), although only 1 annual peak of infection intensity occurs in the latter, i.e. in late summer–early autumn (Andrews & Hewatt 1957, Andrews 1988, Burrenson & Ragone Calvo 1996). Mean infection intensity was significantly correlated with the seawater temperature in our study: the longer the interval between temperature recording and clam sampling, the lower the correlation between seawater temperature and mean intensity of infection by *P. olseni*. However, Burrenson & Ragone Calvo (1996) reported higher correlation between temperature and both mean infection intensity and prevalence of infection by *P. marinus* in *C. virginica* when temperature was recorded 3 mo earlier. The effect of seawater temperature on *P. olseni* infection in clams seems to be faster. Seawater temperature was around 15°C when spring peaks in mean infection intensity of *P. olseni* occurred, whereas temperature favourable to *P. marinus* proliferation is above 20°C (Andrews 1988, Burrenson & Ragone Calvo 1996). La Peyre et al. (2002) compared *in vitro* proliferation and metabolic activity between *P. olseni* and *P. marinus* at 4, 15 and 28°C. Both species were able to proliferate at 15 and 28°C, but *P. olseni* showed higher metabolic activity at every temperature tested, with the greatest difference at 15°C. These *in vitro* results were consistent with the field records of temperature and infection intensity in the present study: infection by *P. olseni* increased when seawater temperature was above 15°C (April to November) and prevalence and infection intensity were lower in winter (temperature below 15°C). These results suggest that *P. olseni* could proliferate in colder waters than *P. marinus*.

The temporal pattern of perkinsosis dynamics showed weak association with seawater salinity. Low salinity was rarely recorded through the study period; therefore, its inhibitory effect demonstrated for infection of *Crassostrea virginica* by *Perkinsus marinus* (Andrews & Hewatt 1957, Ragone & Burrenson 1993, Burrenson & Ragone Calvo 1996) could not be assessed in the

case of clam infection by *P. olseni*. Nevertheless, a significant influence of seawater salinity on *in vitro* vegetative multiplication (S. M. Casas pers. obs.) and *in vitro* zoosporulation of *P. olseni* had been reported (Casas et al. 2002b).

The annual cumulative mortality (41.2%) estimated for the clam bed in our study corresponded to high values within mortality ranges reported for this clam from other areas. Walne (1976) estimated that annual mortality of *Tapes decussatus* cultured from seed to market size along the coast of Wales ranged from 10 to 40% (23% average). Cumulative mortality of 45% in 20 mo was reported for *T. decussatus* cultured in Italy from an initial size of 15 mm (Pastore et al. 1996). Cumulative mortality of *T. decussatus* cultured in the Ría de Arousa for 8 mo ranged from 21 to 61% (Pérez-Camacho & Cuña 1987). Monthly percentage mortality in the clam bed peaked immediately after spring and midsummer peaks of *Perkinsus olseni* infection intensity, concurrently with high temperature of the water in and over the bed. Was perkinsosis the main cause of clam mortality? The distribution of clam percentage mortality as a function of size showed that the highest mortality did not correspond to the largest (oldest) clams, despite the fact that larger the clam the heavier the infection. Therefore, other causes contributed to clam mortality. Comparison of clam percentage mortality between the clams from an affected and a non-affected bed revealed significantly higher mortality in the clams from the perkinsosis-affected bed. Therefore, perkinsosis does contribute to clam mortality. Nevertheless, mortality in the study bed was much lower than that (50 to 80%) reported for *T. decussatus* beds affected by *P. olseni* in Algarve (S. Portugal) (Ruano & Cachola 1986) and that (up to 100%) for *T. philippinarum* cultures in Catalonia (NE Spain) (Santmartí et al. 1995), where seawater temperature is higher than in the Galician rías in summer–early autumn. In Korea, where the water temperature is also higher than in Galicia in summer, perkinsosis was associated with an 80% decrease in *T. philippinarum* landings (Park & Choi 2001).

Transmission of *Perkinsus marinus* may be associated with host-spawning, excretory activities, alternate host or vector activities, heterotrophic parasite proliferation, or periodic resuspension of parasite cells present in sediments (Bushek et al. 2002, Ragone Calvo et al. 2003); however, the primary mode of *P. marinus* transmission occurs via the direct dissemination of parasite cells released from dead oysters (Bushek et al. 2002, Ragone Calvo et al. 2003). Probably, transmission of *P. olseni* is favoured after the death of heavily infected clams in the period with the highest mortality, since the dead clams may comprise a source of infective parasite stages. Clam mortality was

negligible in spring. Therefore, if dead infected clams were the main source of infective parasite stages, the spring peaks in mean infection intensity and prevalence could derive from overwintering infections (some undetected), rather than new infections. Nevertheless, transmission of *P. olsenii* in the field requires further study.

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LITERATURE CITED

- Almeida M, Berthe F, Thébault A, Dinis MT (1999) Whole clam culture as a quantitative diagnostic procedure of *Perkinsus atlanticus* (Apicomplexa, Perkinsea) in clams *Ruditapes decussatus*. *Aquaculture* 177:325–332
- Andrews JD (1988) Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effects on the oyster industry. *Am Fish Soc Spec Publ* 18:47–63
- Andrews JD (1996) History of *Perkinsus marinus*, a pathogen of oyster in Chesapeake Bay 1950–1984. *J Shellfish Res* 15:13–16
- Andrews JD, Hewatt WG (1957) Oyster mortality studies in Virginia. II. The fungus disease caused by *Dermocystidium marinum* in oysters of Chesapeake Bay. *Ecol Monogr* 27:1–26
- 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
- Burreson EM, Ragone Calvo LM (1996) Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. *J Shellfish Res* 15:17–34
- Bushek D, Ford SE, Allen SK (1994) Evaluation of methods using Ray's fluid thioglycollate medium for diagnosis of *Perkinsus marinus* infection in the eastern oyster, *Crassostrea virginica*. *Annu Rev Fish Dis* 4:201–217
- Bushek D, Ford SE, Chintala MM (2002) Comparison of *in vitro*-cultured and wild-type *Perkinsus marinus*. III. Fecal elimination and its role in transmission. *Dis Aquat Org* 51: 217–225
- Calvo LM, Fagan RJ, Greenhawk KN, Smith GF, Jordan SJ (1996) Spatial distribution and intensity of *Perkinsus marinus* in oyster recovery areas in Maryland. *J Shellfish Res* 15:381–389
- Casas SM, La Peyre JF, Reece KS, Azevedo C, Villalba A (2002a) Continuous *in vitro* culture of the carpet-shell clam *Tapes decussatus* protozoan parasite *Perkinsus atlanticus*. *Dis Aquat Org* 52:217–231
- Casas SM, Villalba A, Reece KS (2002b) 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
- Choi KS, Park KI (1997) Report on the occurrence of *Perkinsus* sp. in the Manila clams, *Ruditapes philippinarum* in Korea. *J Aquacult* 10:227–237
- Choi KS, Wilson EA, Lewis DH, Powell EN, Ray SM (1989) The energetic cost of *Perkinsus marinus* parasitism in oysters: quantification of the thioglycollate method. *J Shellfish Res* 8:125–131
- Choi KS, Park KI, Lee KW, 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 FLE (1996) Laboratory investigations of susceptibility, infectivity and transmission of *Perkinsus marinus* in oysters. *J Shellfish Res* 15:57–66
- de la Herrán R, Garrido-Ramos MA, Navas JI, 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
- Elandalloussi LM, Leite RM, Afonso R, Nunes PA, Robledo JAF, Vasta GR, Cancela ML (2004) Development of a PCR-ELISA assay for diagnosis of *Perkinsus marinus* and *Perkinsus atlanticus* infections in bivalve molluscs. *Mol Cell Probes* 18:89–96
- Figueras A, Robledo JAF, Novoa B (1992) Occurrence of haplosporidian and *Perkinsus*-like infection in carpet-shell clams, *Ruditapes decussatus* (Linnaeus, 1758), of the Ria de Vigo (Galicia, NW Spain). *J Shellfish Res* 11:377–382
- Fisher WS, Oliver LM (1996) A whole-oyster procedure for diagnosis of *Perkinsus marinus* disease using Ray's fluid thioglycollate culture medium. *J Shellfish Res* 15:109–117
- Ford SE, Tripp MR (1996) Diseases and defense mechanisms. In: Kennedy VS, Newell RIE, Eble AF (eds) *The eastern oyster Crassostrea virginica*. Maryland Sea Grant College, College Park, MD, p 581–660
- Gauthier JD, Fisher WS (1990) Hemolymph assay for diagnosis of *Perkinsus marinus* in oysters *Crassostrea virginica*. *J Shellfish Res* 9:367–371
- Goggin CL (1994) Variation in the two internal transcribed spacers and 5.8 S ribosomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). *Mol Biochem Parasitol* 65:179–182
- Goggin CL, Lester RJG (1987) Occurrence of *Perkinsus* species (Protozoa, Apicomplexa) in bivalves from the Great Barrier Reef. *Dis Aquat Org* 3:113–117
- Goggin CL, Lester RJG (1995) *Perkinsus*, a protistan parasite of abalone in Australia: a review. *Mar Freshw Res* 46: 639–646
- Goggin CL, Sewell KB, Lester RJG (1989) Cross-infection experiments with Australian *Perkinsus* species. *Dis Aquat Org* 7:55–59
- Guelorget O, Mayer C, Amanieu M (1980) Croissance, biomasse et production de *Venerupis decussata* et *Venerupis aurea* dans une lagune méditerranéenne, l'étang du Pré-vost à Palavas (Hérault, France). *Vie Mar* 2:25–38
- 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
- Hine PM (2001) Problems of applying risk analysis to aquatic

- organisms. In: Rodgers CJ (ed) Risk analysis in aquatic animal health. Office Internationale des Epizooties, Paris, p 71–82
- Hine PM, Thorne T (2000) A survey of some parasites and diseases of several species of bivalve mollusc in northern Western Australia. *Dis Aquat Org* 40:67–88
- Howard AW, Smith CS (1983) Histological techniques for marine bivalve molluscs. NOAA Tech Memo NMFS F/NEC 25:1–97
- Krebs CJ (1989) Ecological methodology. Harper Collins, New York
- La Peyre JF, Nickens AD, Casas SM, Villalba A (2002) Viability and growth of *Perkinsus marinus* and *Perkinsus atlanticus* at three temperatures. *J Shellfish Res* 21:374
- Leethochavalit, S, Upatham ES, Choi KS, Sawangwong P, Chalermwat K, Kruatrachue (2003) Ribosomal RNA characterization of non-transcribed spacer and two internal transcribed spacers with 5.8 s ribosomal RNA of *Perkinsus* sp. found in undulated surf clams (*Paphia undulata*) from Thailand. *J Shellfish Res* 22:431–434
- Lester RJG, Davis GHG (1981) A new *Perkinsus* species (Apicomplexa, Perkinsea) from the abalone *Haliotis ruber*. *J Invertebr Pathol* 37:181–187
- Liang YB, Zhang XC, Wang LJ, Yang B, Zhang Y, Cai CL (2001) Prevalence of *Perkinsus* sp. in the Manila clam, *Ruditapes philippinarum*, along the Northern coast of the Yellow Sea in China. *Oceanol Limnol Sin* 32:502–511
- López C (1995) Estudio patológico de la almeja *Ruditapes decussatus* (Linnaeus, 1758) cultivada en Galicia y sus mecanismos de defensa. PhD dissertation, Universidad de Santiago de Compostela
- López C, Villalba A, Carballal MJ (1998) Estudio patológico de la almeja fina (*Ruditapes decussatus*) cultivada en Carril (Galicia). In: Penas X (ed) Marisqueo en Galicia. Edición do Castro, Sada, A Coruña, p 225–237
- 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
- Murrell A, Kleeman SN, Barker SC, Lester RJG (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
- Nickens AD, La Peyre JF, Wagner SE, Tirsch TR (2002) An improved procedure to count *Perkinsus marinus* in Eastern oyster haemolymph. *J Shellfish Res* 21:725–732
- Norton JH, Shepherd MA, Perkins FP, Prior HC (1993) *Perkinsus*-like infection in farmed golden-lipped pearl oyster *Pinctada maxima* from the Torres Strait, Australia. *J Invertebr Pathol* 62:105–106
- O'Donoghue, PJ, Phillips PH, Shepherd SA (1991) *Perkinsus* (Protozoa: Apicomplexa) infections in abalone from South Australian waters. *Trans R Soc S Aust* 115:77–82
- Park KI, Choi KS (2001) Spatial distribution of the protozoan parasite *Perkinsus* sp. found in the Manila clams, *Ruditapes philippinarum*, in Korea. *Aquaculture* 203:9–22
- Park KI, Choi KS, Choi JW (1999) Epizootiology of *Perkinsus* sp. found in the Manila clam *Ruditapes philippinarum* in Komsoe Bay, Korea. *J Korean Fish Soc.* 32:303–309
- Park KI, Park YM, Lee J, Choi KS (2002) Development of a PCR assay for detection of the protozoan parasite *Perkinsus*. *Korean J Environ Biol* 20:109–117
- Pastore M, Prato E, Palagiano B (1996) Allevamento in sospensione di *Tapes decussatus* (L.) (Mollusca, Veneridae) nel Mar Piccolo di Taranto. *Quad Ist Idrobiol Acquacolt Brunelli* 15:19–40
- Pérez-Camacho A (1979) Biología de *Venerupis pullastra* (Montagu, 1803) y *Venerupis decussata* (Linné, 1767) (Mollusca, Bivalvia), con especial referencia a los factores determinantes de la producción. *Bol Inst Esp Oceanogr* 5: 43–77
- Pérez-Camacho A, Cuña A (1987) Cultivo experimental de *Venerupis decussata* y *Ruditapes philippinarum*. *Cuad Marisq Publ Téc* 12:353–358
- Ragone LM, Bureson EM (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 Calvo LM, Dungan CF, Roberson BS, Bureson EM (2003) Systematic evaluation of factors controlling *Perkinsus marinus* transmission dynamics in lower Chesapeake Bay. *Dis Aquat Org* 56:75–86
- Ray SM (1954) Biological studies of *Dermocystidium marinum* a fungus parasite of oysters. Rice Institute pamphlet (Monogr Biol Spec Ser Iss). Rice Institute, Washington, DC
- Ray SM (1966) A review of the culture method for detecting *Dermocystidium marinum*, with suggested modifications and precautions. *Proc Natl Shellfish Assoc* 54:55–69
- Robledo JAF, Coss CA, Vasta GR (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
- Rodríguez F, Navas JI (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 et Reeve). *Aquaculture* 132:145–152
- Rodríguez F, Godoy T, Navas JI (1994) Cross-infection with *Perkinsus atlanticus* in *Ruditapes decussatus*, *Ruditapes philippinarum* and *Venerupis pullastra*. *Bull Eur Assoc Fish Pathol* 14:24–27
- Ruano F, Cachola R (1986) Outbreak of a severe epizootic of *Perkinsus marinus* (Levin - 78) at Ria de Faro clam's culture beds. In: Proc 2nd Int Colloq Pathol Mar Aquac (PAMAQ II). Porto, p 41–42
- Sagrístá E, Durfort M, Azevedo C (1991) Ultrastructural study of the life cycle of *Perkinsus* sp. (phylum Apicomplexa), parasite of a Mediterranean clam. In: I Coloquio Franco-Ibérico de Microscopía Electrónica. Barcelona, p 145–146
- Sagrístá 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
- Santmartí MM, 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. Universidad de Barcelona, Barcelona, p 260–265
- Soniat TM (1996) Epizootiology of *Perkinsus marinus* disease of Eastern oysters in the Gulf of Mexico. *J Shellfish Res* 15: 35–44
- Vilela H (1950) Vida bentónica de '*Tapes decussatus*' (L.). PhD dissertation, Universidade de Lisboa, Lisbon
- Walne PR (1976) Experiments on the culture in the sea of the butterfish *Venerupis decussata* L. *Aquaculture* 8:371–381