A mortality event of the venerid bivalve *Callista chione* (Linnaeus, 1758) in a hatchery system - A case study

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Abstract

Abnormal mortality of the smooth venus clam (*Callista chione*) was encountered when conditioning these clams in a hatchery system. A histopathological analysis was performed to establish the causes of this mortality episode. Our results showed an increase in rickettsia-like bacteria infection intensity between the individuals collected at the start of the conditioning in the hatchery and those collected during the mortality episode. Husbandry stress most likely increased disease susceptibility and progression in these clams. Rickettsia-like colonies were observed in large numbers in the gills of all individuals examined. *Nematopsis* sp. spores and rod-shaped basophilic bacteria could also been seen in some of the individuals examined. Microbiological analysis of clam tissue did not reveal the presence of any potentially pathogenic bacteria and all the clams were shown to be free of *Perkinsus* sp. parasites. The conditioning protocol was adapted from those used for other venerid clams due to the lack of data on this species. These findings highlight the need to perform further studies to evaluate the optimal parameters for *C. chione* broodstock conditioning.

Introduction

The venerid bivalve *Callista (=Cytherea) chione* (Linnaeus, 1758), or smooth clam, is distributed on sandy bottoms over 0-180 m depth range along the Mediterranean and European Atlantic coasts. Overexploitation in Mediterranean natural beds is a major concern for natural population of bivalves including the commercially important *C. chione*. Spain leads both bivalve consumption and production in Europe and there is a growing interest in diversifying shellfish aquaculture in the western Mediterranean coast and developing commercial clam farms. Within this scope, knowledge of the physiology and health status of *C. chione* appears crucial for the development of hatchery-reared smooth clam and seed production.

Limited information is available on its ecology (Forster, 1981; Metaxatos, 2004; Leontarakis & Richardson, 2005) or reproductive cycle (Cano, 1981; Valli et al., 1983, 1994; Tirado et al., 2002; Galimany, 2006). In addition, very little is known on the pathology of these clams (Canestri-Trotti et al., 2000) and specific diseases that could occur under hatchery or stress conditions. To date, there is no published protocol for the growth of smooth clam under hatchery conditions.

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Rickettsia-like infections have been reported in a number of marine bivalves (Elston & Peacock, 1984; Elston, 1986; Hine & Diggles, 2002; Marshall et al., 2003). However, infections usually induce mild or undetectable host response that is not associated with disease (Otto et al., 1979; Elston & Peacock, 1984; Elston, 1986). Nevertheless, heavy infections have occasionally been found under intensive culture (Gulka et al., 1983) and linked to bivalve mortality (Norton et al., 1993 a & b; Le Gall et al., 1988; Villalba et al., 1999).

This study aimed to determine the causes of a mass mortality episode in a hatchery system during the conditioning of *C. chione* individuals. Results of this study suggest that stress and branchial rickettsia-like infection could be responsible for the mortality observed.

Material and methods
Design and experimental conditions
The conditioning experiment was carried out using adult specimens of *C. chione* (110 individuals), with a mean initial length of 68.5±7.6 mm. Clams were collected during February 2006 at a depth of 10m from Maresme, Spain (Catalan coast, NW Mediterranean Sea).

The experiment was performed in 20-litre plastic tanks (4 replicates), containing sea-water filtered through 1 μm in a flow through circuit of between 20 and 24 l/hr. Temperature was maintained at 19 ± 1° C and salinity at 34‰. Cultured microalgae were continuously supplied to the clams system by means of a variable flow peristaltic pump (Dinko D-21V). Microalgae entered the tanks at mid-depth, the outflow being located at the top of the tanks.

Microalgae offered consisted of a mixture of: *Isochrysis galbana* clone T-ISO (40%), *Tetraselmis suecica* (30%) and *Chaetoceros gracilis* (30%). The daily food ration was 0.2% (percentages corresponding to the organic weight of food supplied as a proportion of the live weight of the clams). The microalgae were initially cultured in 6 l jars and then transferred to 150 l tanks. Walne medium (Walne, 1966) and industrial fertilizer were used for the jar and tank culture, respectively. The microalgae were harvested during the stationary growth phase.

The total experimental period was 70 days. Samples were taken twice: first at the beginning of the conditioning period (08.02.06), and then when mortality was evident (55 days afterwards). Histo-pathological analysis and Ray's Fluid Thioglycollate Medium (RFTM) assay for *Perkinsus* sp. diagnosis were performed for each sample. A bacteriology study was carried out for the sample on day 55.

Histopathology
Ten individuals from the initial sampling were analyzed in order to establish the pathological status of the experimental clams. When mortality occurred, twelve individuals were also processed for histological examination (day 55). A section of around 5 mm with the representation of all tissues was fixed in 10 % formaldehyde in sterile filtered seawater for 24-48 hours, and afterwards conserved in 70% ethanol for histological studies in order to detect the possible presence of parasites. Samples were dehydrated and embedded in paraffin. Paraffin blocks were cut at 3 μm and hematoxylin-eosin stained. Slides were

studied under light microscopy (Leyca DMLB).

The mean infection intensity for rickettsia-like colonies was calculated from the record of the number of colonies observed in gills at 20X (light microscopy) in 5 fields of each individual (equivalent area: 0.74mm^2). This procedure was followed for all the rickettsia-like parasitized individuals. Ten colonies from each sample and some basophilic rod-shaped colonies were also measured by the microscope software AnalySIS (Olympus).

Perkinsus diagnosis
Perkinsus sp. presence was examined by the RFTM diagnostic method. Thirty whole clams from the initial sampling were individually incubated in 20 ml fluid thioglycollate medium (Sigma) supplemented with 500 U ml^{-1} penicillin G and 500 µg ml^{-1} Streptomycin sulphate (Sigma) at room temperature for 7 days in the dark (Ray, 1966; Almeida et al., 1999). After incubation, FTM was discarded after centrifugation (10 min at 1500 g, room temperature) and the remaining tissues digested in 20 ml of 2M NaOH at 60°C for 3h. The samples were then washed three times with filtered seawater and stained with Lugol's iodine solution for observation under a light microscope.

During the mortality episode (day 55), five entire clams and gill fragments from five other specimen of *C. chione* were also processed for RFTM diagnosis.

Bacteriology analysis
During the mortality event (day 55) one sample of five whole individuals were macerated in sterile saline solution (2.5% NaCl) and 100 µl of this macerate were inoculated onto marine agar plates (Scharlab, Spain), Trypticase Soy Agar (TSA; Scharlab, Spain) + 2%NaCl plates and in tubes containing 10 ml of Trypticase Soy Broth (TSB; Scharlab, Spain) + 2% NaCl. Plates and tubes were incubated at room temperature (22°C) for up to 72h. After this period of time, different type colonies were recultured in TSA+ 2%NaCl and tubes where growth was present were also re-inoculated on TSA+2% NaCl. Individual colonies formed were picked and re-streaked onto agar plates. Once all types of colonies were pure, isolates were characterised and the following tests performed on them: presence of catalase, oxidase, gram staining, mobility (Frerichs and Millar, 1993) and the tests of the API20E strip (Biomérieux, France).

Results

After the first 20 days of conditioning, *C. chione* individuals started to die. Mortality rate increased with time and after 55 days 70% of the population was dead (table 1).

Histopathology
Some parasites, such as rickettsia-like and *Nematopsis* sp. spores were detected in the individuals from the initial sample. Rickettsia-like colonies were detected in the gills of all the individuals, with a mean infection intensity of 3 colonies/area and a mean colony size of 21.3 µm (Table 1).

Spores of an Apicomplexan similar to *Nematopsis* sp. were observed in the gills of one out of the 10 individuals analyzed from the initial sample.

In the individuals from the mass mortalities sample, rickettsia-like and *Nematopsis* sp.
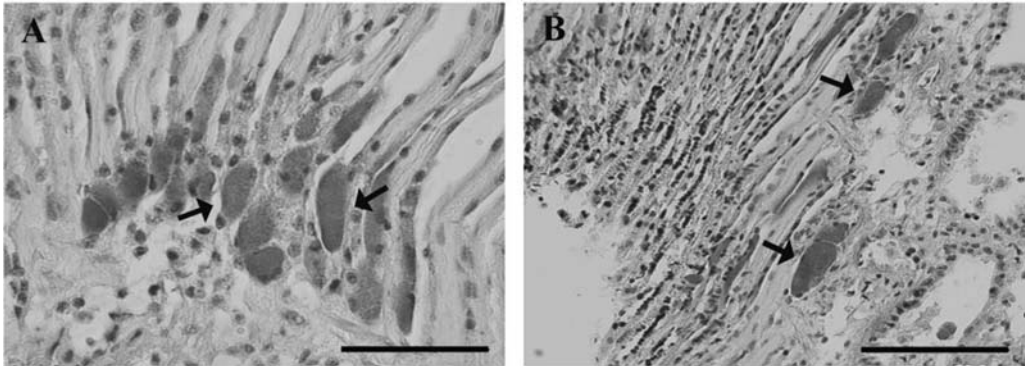
Figure 1. A and B, *Rickettsia*-like bacteria inclusions (arrows) in the gills of *Callista chione* during the mass mortalities period. Scale bar = 50 μm.

Nº days	Colonies no.	Colonies size (μm)	% Mortality
0	3	21.3	0
20	-	-	2
50	-	-	47
55	30	21.3	70
70	-	-	97

Table 1. Number and mean size of rickettsia-like colonies in the gills, and mortality (%) of *C. chione* during the experimental period (days).

spores were also observed (day 55). Rickettsia-like bacteria colonies were observed in the gills of all analyzed individuals as well, however, in this case the infection intensity was increased to 30 colonies/area (Table 1; Figure 1). *Nematopsis* sp. spores were observed in the gills of two out of the 12 individuals (Figure 2).

Inclusions of rod-shaped basophilic bacteria colonies were observed (Figure 3A), in low intensity, in the gills of 6 out of the 12 analyzed individuals when mass mortality occurred after 55 days of conditioning. These inclusions were usually situated close to rickettsia-like colonies (Figure 3A) and in some cases appeared to be on top of the rickettsia-like colonies (Figure 3B). The mean size of rod-shaped basophilic colonies was 4.6 μm length.

Perkinsus diagnosis

No *Perkinsus* sp. hypnospore could be observed by microscopical examination of the RFTM cultures.

Bacteriology analysis

Twelve isolates were purified and characterised preliminarily. Four were isolated from TSA, four from TSB and four from Marine Agar. They all were gram negative. Isolates 1, 6, 8 and 9 were oxidase negative and the remaining positive. Only isolates 10 and 11 were catalase negative. Isolates 1, 2, 3, 4 and 6 were not mobile. API profiles were obtained for isolates 1 to 11 and though this test was repeated for isolate 12 all tests in the strip were negative for this isolate. API profiles were different for all the isolates except numbers 8 and 9 which gave the same responses. These two isolates came from the same organism, though one was initially purified from MA and the other from TSA+2%NaCl. The profiles for the strip tests itself integrated with oxidase test are shown in Table 2.

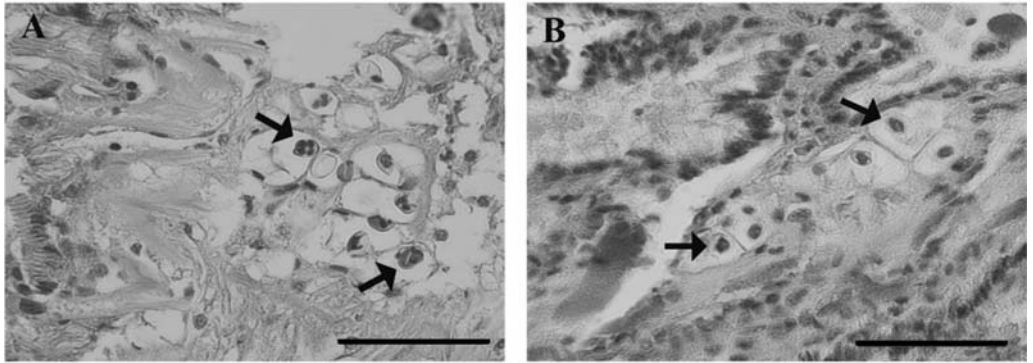Further characterization was not performed since there was no repetitive pattern and

Figure 2. A and B, *Nematopsis* sp. spores (arrows) in the gills of *Callista chione*. Scale bar = 50 μm.
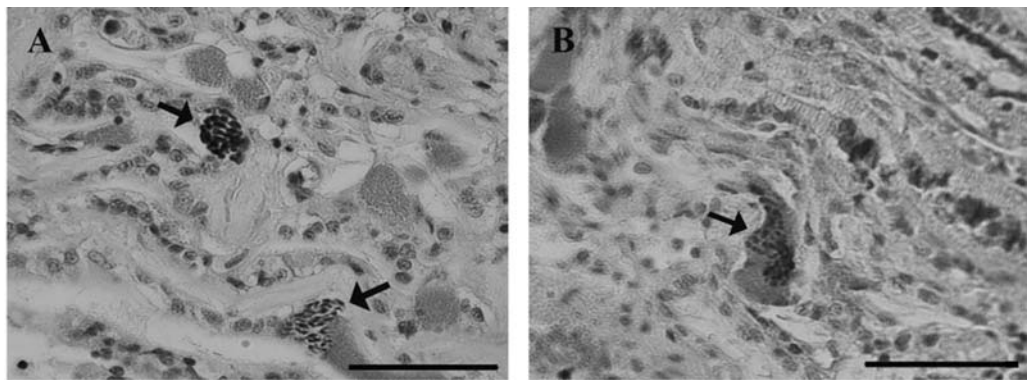

Figure 3. Rod-shaped basophilic bacteria colonies inclusions (arrows) in the gills of *Callista chione*: A, rod-shaped basophilic bacteria situated close to *Rickettsia*-like colonies; B, rod-shaped basophilic bacteria situated on the top of *Rickettsia*-like colonies. Scale bar = 50 μm.

Isolate no.	Oxidase	API profile no.
1	Negative	0014046
2	Positive	0014042
3	Positive	0205042
4	Positive	0005573
5	Positive	2105573
6	Negative	0202046
7	Positive	1204072
8	Negative	1005576
9	Negative	1005576
10	Positive	0007572
11	Positive	2006520
12	Positive	No results*

*Twice the test strip was negative for all tests

Table 2. API profiles of the bacteria isolated from *C. chione* on day 55.

therefore it was assumed that mortalities were not due to the presence of specific bacteria.

Discussion

Rickettsia-like bacteria infections were reported in bivalves for the first time by Harsherbarger et al. (1977). Since then, several authors have described these bacteria in a variety of bivalve species (Elston & Peacock, 1984; Elston, 1986; Hine & Diggles, 2002; Marshall et al., 2003). In this study, histopathological examination of tissues from smooth venus clams revealed the presence of a large number of rickettsia-like organisms,

especially in the individuals collected during the mortality episode. Rod-shaped basophilic bacteria colonies and spores of *Nematopsis* sp. were also occasionally observed. *Nematopsis* sp. had been previously found in *C. chione* from the Adriatic Sea (Canestri-Trotti *et al.*, 1998 & 2000) whereas inclusions of rod-shaped basophilic bacteria colonies have been reported in some clam species (Marshall et al., 2003). These authors described colonies of similar size and shape to those observed in the present study, and also reported a weak correlation between moderate and high intensity of rickettsia-like colonies and presence of rod-shaped basophilic bacteria.

The presence of *Perkinsus* sp. has already been documented in the smooth venus clam from the North-Western Adriatic sea, Italy (Canestri-Trotti et al., 2000). However, no *Perkinsus* infection could be diagnosed by the RFTM assay in any of the sample tested in the present study. In addition, bacteriological analysis did not detect any potentially pathogenic bacteria.

Infections by rickettsia-like organisms are often seen in molluscs and are usually believed to be innocuous. In this sense, Villalba et al. (1993) described intracytoplasmic basophilic inclusions identified as rickettsia-like colonies in *Ruditapes decussatus* and *Venerupis pullastra* clams from the Galician coast (Spain), that were not linked to mortality events. Nevertheless, heavy infections with these bacteria have been shown to induce mortalities (Leiboutz, 1989) by reducing the metabolic efficiency and nutritional status of the host (Otto et al., 1979; Elston, 1986). Villalba et al. (1999) reported mortality of

Venerupis romboides from the Spanish coast (Galicia) associated with branchial rickettsia-like infection. Similarly, high mortality due to the presence of branchial rickettsiae in sea scallops *Placopecten magellanicus* (Gulka & Chang, 1984), razor clams *Siliqua patula* (Elston, 1986), scallops *Pecten maximus* (Le Gall et al., 1988), giant clams *Hippopus hipppopus* and *Tridacna gigas* (Norton et al., 1993 a & b) have been reported. In the present study, rickettsia-like organisms were already found in clams collected during the conditioning in the hatchery. Their increase in number of colonies over time, together with the absence of *Perkinsus* sp and potentially pathogenic bacteria, suggests that rickettsia-like organisms may have been responsible for the episode of mass mortality of *C. chione* observed in the hatchery. For heavy infections, gill lesions are likely to occur and consequently alter respiratory and digestive functions. Parasites other than *Perkinsus* sp. were also occasionally detected; however these were at a prevalence and infection intensity that could not explain this mortality event.

Critical parameters to be considered when conditioning marine bivalves in hatcheries include the food, water flow rate, water temperature and salinity. To our knowledge, there is no protocol available for the hatchery conditioning of *C. chione* broodstock. Due to the lack of information for this species, clams were conditioned under temperature and nutritional parameters optimized for other venerid species (Delgado & Pérez-Camacho, 2005). Norton et al. (1993a) suggested that several factors could influence the pathogenicity of rickettsia-like bacteria under intensive culture conditions. For example,

overcrowding and low turnover rates of water in culture tanks would favour the proliferation of rickettsia-like bacteria and the presentation of clinical diseases that would eventually lead to the death of the animals. In the present study, high turnover rate of water (1 complete renewal/h) and low culture density of clams were applied when compared with available data from other venerid species. The initial culture density was 0.07 kg/l in our experiment versus 0.1 kg/l for *R. decussatus* (Delgado & Pérez-Camacho, 2005). In January, the seawater temperature from the natural habitat of *C. chione* is around 13°C (Galimany, 2006). The relatively high temperature applied to the clams during the experimental period (19ºC) most likely caused physiological stress, thus reducing the clam resistance to disease and enhancing the proliferation of rickettsia-like organisms in the host. Nevertheless, other factors like the absence of adequate sandy bed, or nutritional deficiencies (caused by the lack of suitable species of microalgae, bacteria, detritus or dissolved organic matter) can also cause stress in the clams. With regard to the salinity, both the field and the hatchery water remaining at about 34‰ throughout the observation period.

In conclusion, husbandry conditions for *C. chione* broodstock, most likely triggered the proliferation of rikettsia-like organisms initially present in the individuals. Severe infections might have affected the respiratory and digestive functions of *C. chione* leading to the death of the animals. Further work is necessary to evaluate the optimal parameters for conditioning of *C. chione* and to assess the reproductive cycle of this bivalve under experimental conditions.

References

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

Canestri-Trotti G, Baccarani EM & Paesanti F (1998). Gregarine del genere *Nematopsis* (Apicomplexa: Porosporidae) in molluschi bivalve del mar Adriatico. *Bolletino della Societa Italiana di Patologia Ittica* **23**, 58-66

Canestri-Trotti G, Baccarani EM, 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). *Diseases of Aquatic Organisms* **42**, 157-161.

Cano J (1981). Biología y crecimiento de *Callista chione* (L., 1758). *Iberus* **1**, 67-78.

Delgado, M & Pérez-Camacho A (2005). Histological study of the gonadal development of *Ruditapes decussatus* (L.) (Mollusca: Bivalvia) and its relationship with available food. *Scientia Marina* **69 (1)**, 87-97.

Elston R & Peacock MG (1984). A rickettsiales-like infection in the pacific razor clam, *Siliqua patula*. *Journal of Invertebrate Pathology* **44**, 84-96.

Elston R (1986). Occurrence of branchial rickettsiales-like infections in two bivalve molluscs, *Tapes japonica* and *Patinopecten yessoensis*, with comments on their significance. *Journal of Fish Diseases* **9**, 69-71.

Forster GR (1981). The age and growth of *Callista chione*. *Journal of Marine Biology Association U.K.* **61**, 881-883.

Frerichs GN & Millar SD (1993). "Manual for the isolation and identification of fish bacterial pathogens". Pisces Press. Stirling. 60 pp.

Galimany E (2006). Cicle reproductor del mollusc bivalve *Callista chione* (Linnaeus, 1758) a la costa catalana. *Diploma de Estudios Avanzados*. University of Barcelona.

Gulka G, Chang PW & Marti KA (1983). Prokaryotic infection associated with mass mortality of the sea scallop *Placopecten magellanicus*. *Journal of Fish Diseases* **6**, 355-364.

Gulka G & Chang PW (1984). Pathogenicity and infectivity of a rickettsia-like organism in the sea scallop, *Placopecten magellanicus*. *Journal of Fish Diseases* **8**, 309-318.

Harsherbarger JC, Chang SC & Otto SV (1977). Chlamydiae (with phages), mycoplasmas, and Rickettsiae in Chesapeake Bay bivalves. *Science* **196**, 666-668.

Hine PM & Diggles BK (2002). Prokaryote infections in the New Zeland scallops *Pecten novaezelandiae* and *Chlamys delicatula*. *Diseases of Aquatic Organisms* **50**,137-144.

Le Gall G, Chagot D, Mialhe E & Grizel H (1988). Branchial rickettsiales-like infection associated with a mass mortality of sea scallop *Pecten maximus*. *Diseases of Aquatic Organisms* **4**, 229-232.

Leiboutz L (1989). Chlamydiosis: a newly reported serious disease of larval and postmetamorphic bay scallops, *Argopecten irradians* (Lamarck). *Journal of Fish Diseases* **12**, 125-136.

Leontarakis PK & Richardson CA (2005). Growth of the smooth clam, *Callista chione* (Linnaeus, 1758) (Bivalvia: Veneridae) form the Thracian Sea, northeastern Mediterranean. *Journal of Molluscan Studies* **71**, 189-198.

Marshall WL, Bower SM & Meyer GR (2003). A comparison of the parasite and symbiont fauna of cohabiting native (*Protothaca staminea*) and introduce (*Venerupis philippinarum* and *Nuttalia obscurata*) clams in British Columbia. *Journal of Shellfish Research* **22(1)**, 185-192.

Metaxatos A (2004). Population dynamics of the venerid bivalve *Callista chione* (L.) in a coastal area of the eastern Mediterranean. *Journal of Sea Research* **52**, 293-305.

Norton JH, Shepherd MA, Abdon-Naguit MR & Lindsay S (1993a). Mortalities in the giant clam *Hippopus hippopus* associated with Rickettsiales-like organisms. *Journal of Invertebrate Pathology* **62**, 207-209.

Norton JH, Shepherd MA & Prior HC (1993b). Intracellular bacteria associated with winter mortality in juvenile giant clams, *Tridacna gigas*. *Journal of Invertebrate Pathology* **62**, 204-206.

Otto SV, Harsherbarger JC & Chang SC (1979). Status of selected unicellular eukaryote pathogens, and prevalence and histopathology of inclusions containing obligate prokaryote parasites, in commercial bivalve molluscs from Maryland estuaries. *Haliotis* **8**, 285-295.

Ray SM (1966). A review of the culture method for detecting *Dermocystidium marinum*, with suggested modifications and precautions. *Proceedings of National Shellfish Association* **54**, 55-69.

Tirado C, Salas C & López JI (2002). Reproduction of *Callista chione* L., 1758 (Bivalvia: Veneridae) in the littoral of Málaga (Southern Spain). *Journal of Shellfish Research* **21 (2)**, 643-648.

Valli G, Bidoli E & Marussi C (1983). Osservazioni preliminari sulla riproduzione e sulla biometría di *Callista chione* (L) (Mollusca, Bivalvia) del Golfo di Trieste. *Nova Thalassia* **6**, 97-103.

Valli G, Marsich N & Marsich MG (1994). Riproduzione, biometría e contenuto di metalli in *Callista chione* (L.) (Mollusca: Bivalvia) del Golfo di Trieste nel corso di un ciclo annuale. *Bolletino de la Societa Adriatica di Scienze* **LXXV (II)**, 441-464.

Villalba A, López MC & MJ Carballal (1993). Parásitos y alteraciones patológicas de tres especies de almeja, *Ruditapes decussatus*, *Venerupis pullastra* y *Venerupis rhomboides*, en las rías gallegas. *In* **"Actas del IV Congreso Nacional de Acuicultura"**, pp. 551-556. Vilagarcía de Arousa, Spain.

Villalba A, Carballal MJ, López C, Cabada A, Corral L & Azevedo C (1999). Branchial rickettsia-like infection associated with clam *Venerupis romboides* mortality. *Diseases of Aquatic Organisms* **36**, 53-60.

Walne PR (1966). Experiments in the large-scale culture of the larvae of *Ostrea edulis* (L.). *Fishery Investigations* Series II, **XXIV (1)**, 1-45.