

Marteilia sp. and other parasites and pathological conditions in *Solen marginatus* populations along the Galician coast (NW Spain)

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ABSTRACT: This paper reports the results of the survey developed after the first detection of protozoan *Marteilia* sp. infection of the grooved razor shell *Solen marginatus* (Pulteney, 1799) from Galicia (NW Spain) in 2006. Furthermore, we analysed other parasites and pathological conditions found in grooved razor shell populations throughout this survey, such as metacercariae of trematodes, prokaryotic infections and disseminated neoplasms, some of which could cause moderate or severe damage to the host depending on the intensity of infection. A total of 17 natural beds distributed along the Galician coast were analysed, and *Marteilia* sp. was detected in 6 of them with low prevalence, moderate intensity and no negative effects over the populations.

KEY WORDS: Grooved razor shell · Prokaryote · Protozoa · Metacercaria · Disseminated neoplasm · Histopathological survey

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INTRODUCTION

The Solenoidea are a group of infaunal bivalves which live buried in soft sea beds and have an elongated and compressed shell that facilitates penetration into the sediment. Species commercially important in Galicia (NW Spain) are the sword razor shell *Ensis magnus* (= *E. arcuatus*), pod razor clam *E. siliqua* and grooved razor shell *Solen marginatus*. Galicia is responsible for over 95% of the Spanish pod and sword razor shell production, making the Solenoidea an appreciated resource for their commercial value. Different parasites and pathological conditions have been reported in Solenoidea. General histopathological studies were published by Bilei et al. (1997), Ceschia et al. (2001), Fahy et al. (2002), Darriba et al. (2010), López et al. (2011), Montes (2011), and Ruiz et al. (2013). Other studies that

focused on a specific parasite or pathological conditions include: unidentified intranuclear inclusions associated with high mortality in *Siliqua patula* from the USA (Elston 1986), metacercariae stages of *Acanthoparyphium tyosenense* (Digenea) in *Solen grandis* in the Republic of Korea (Chai et al. 2001), germinoma in *E. magnus* (= *E. arcuatus*) from Galicia (Darriba et al. 2006), the detection of *Marteilia* sp. in *S. marginatus* from Galicia (López & Darriba 2006) and the identification, by molecular techniques, of *M. refringens* in *S. marginatus* from Andalucía, southern Spain (López-Flores et al. 2008a).

Among the parasites and other pathological conditions previously mentioned, the most relevant is the protozoan *Marteilia* sp. because of the negative effects and mass mortality events associated with species of the genus *Marteilia* in other bivalves (reviewed by Berthe et al. 2004, Carrasco et al. 2011,

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Villalba et al. 2014). In fact, infection by *M. refringens* is a notifiable disease listed by the World Organisation for Animal Health (2013) and under European legislation (Directive 2006/88). The aim of the present study was to survey for *Marteilia* sp. in *S. marginatus* and to characterize other parasites and pathological conditions affecting grooved razor shell along the Galician coast.

MATERIALS AND METHODS

Samples of 20 to 30 commercial grooved razor shells (>80 mm length) were collected by hand using salt (spread in burrow entrances, causing the razor shells to leave the burrow) in 17 intertidal beds in northern and western rías along the Galician coast (Fig. 1). Samplings were conducted seasonally, during the years 2008 to 2011 (previous data from the Redondela bed between the years 2006 and 2008 were also included), except in the Barraña and Mañóns beds, where samples were collected monthly and in the Cariño bed, where they were collected every 2 mo.

In the laboratory, valves were separated, and gills, mantle and visceral mass were examined macroscopically for evidence of macroparasites, lesions, abnormal colouration and other malformations. A section including digestive gland, gonad, kidney, foot, mantle lobes, labial palps and gills was taken from every specimen, fixed in Davidson's solution (Shaw & Battle 1957) and embedded in paraffin. Paraffin blocks were sectioned at 5 μ m with a rotary microtome. Tis-

sue sections were deparaffinised, stained with Harris' haematoxylin and eosin and examined by light microscopy for symbionts and pathological conditions such as haemocyte infiltrations and neoplasms.

To further characterize the prokaryotic colonies detected in histology sections stained with Harris' haematoxylin and eosin, additional staining techniques were used: Gram's Brown and Hopps method for Gram-positive and Gram-negative bacteria, the Pinkerton method for rickettsial organisms, the Ziehl-Neelsen method for acid-fast bacteria and the Feulgen method for DNA (Howard & Smith 1983).

Some samples were selected for ultrastructural analysis for better identification of particular parasites. The portion of the affected tissue was taken out of the paraffin block. Paraffin was removed by several rinses in xylene with agitation. Tissue was placed in 2.5% glutaraldehyde, postfixed in 2% OsO₄ and embedded in Epon. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined under a JEOL JEM 1010 transmission electron microscope at 80 kV.

Overall prevalence with 95% confidence intervals was determined for each parasite or pathological condition in each bed. Paired comparisons of prevalence between study sites were performed using a critical ratio (Z) test on the difference between 2 independent proportions given the proportion and sample size in each sample. A critical level of 0.05 was considered for the rejection of the hypothesis tested. These calculations were performed using Epi-Calc 2000 software Version 1.02 (Gilman & Myatt 1998). In addition, the Marascuilo procedure for non-equal proportions was performed to simultaneously test the differences of all pairs of proportions (Prins et al. 2013). Bar graphs were created with OriginPro 7 software to summarize the most significant results (Fig. 2).

Furthermore, specific scales to assess the intensity of infection were used for the most relevant parasites, viz. *Marteilia* sp. and cysts of prokaryotic organisms (CPO) (Table 1).

RESULTS AND DISCUSSION

Examination of the histological sections revealed the occurrence of different prokaryotic-like organisms, protozoan and metazoan parasites and other pathological conditions. Here we focus on those with pathogenic potential and/or presence at high prevalence and intensity. The protozoan *Marteilia* sp. was highlighted for this reason, as well as colonies of

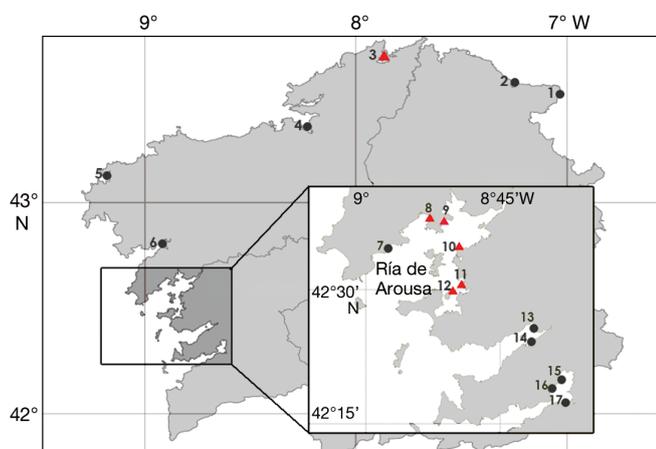


Fig. 1. *Solen marginatus* beds on the Galician coast (NW Spain). 1: Ribadeo, 2: Foz, 3: Cariño, 4: Miño, 5: Camariñas, 6: Noia, 7: Palmeira, 8: Barraña, 9: Mañóns, 10: Vilanova, 11: Sarrido, 12: O Grove, 13: Combarro, 14: Lourizán, 15: Cunchido, 16: Rio Maior, 17: Redondela. Red triangles represent the beds in which *Marteilia* sp. was detected

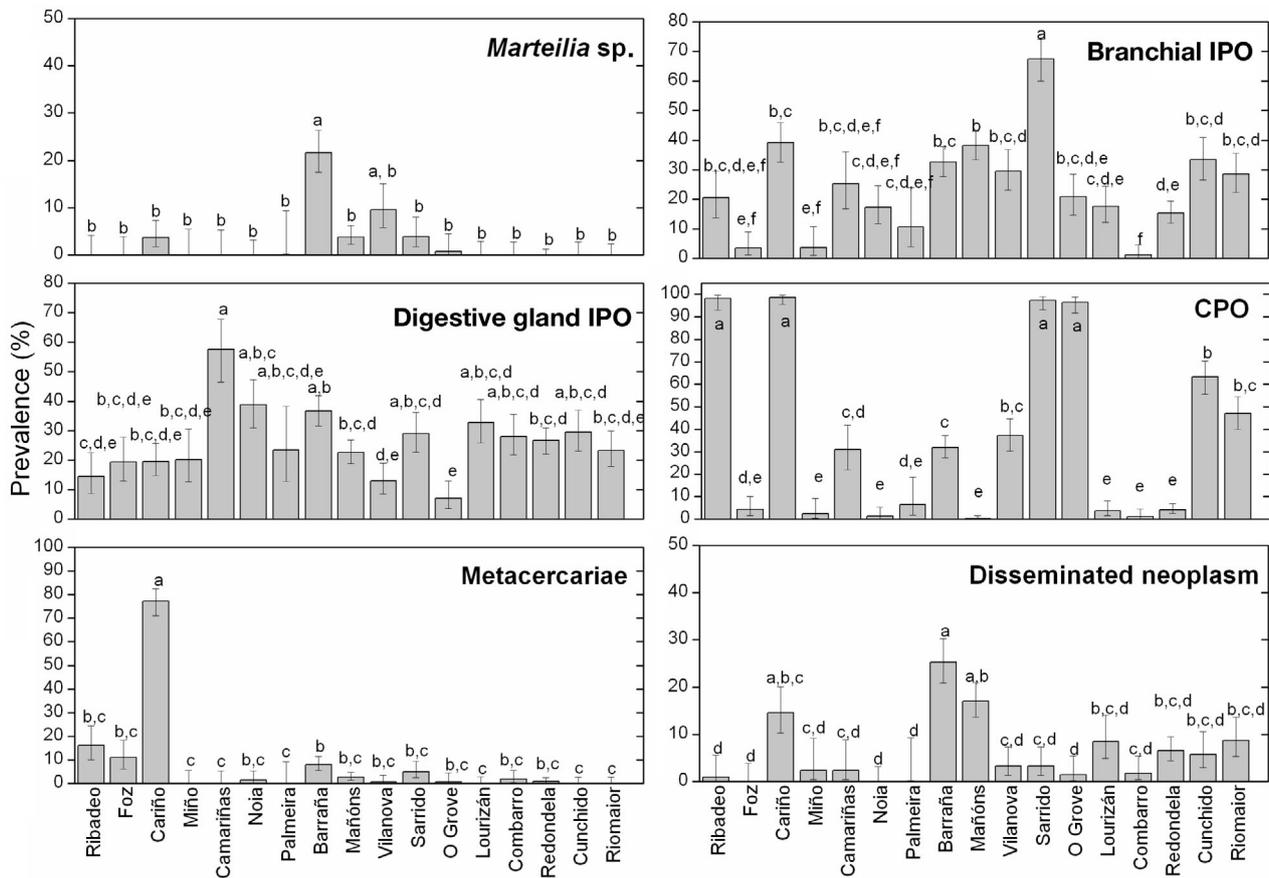


Fig. 2. Prevalence of parasites and other pathological conditions and results of pairwise comparisons, where different letters indicate statistically significant differences between *Solen marginatus* beds (see Fig. 1 for locations); IPO: intracellular prokaryotic-like organisms, CPO: cysts of prokaryotic organisms

Table 1. Specific scales to assess the intensity of infection by *Marteilia* sp. and cysts of prokaryotic organisms (CPO) in *Solen marginatus*. For the *Marteilia* sp. scale, each specimen was scored by examining a single histological section. The entire digestive gland section was observed, taking into account the presence of initial stages, low advanced stages (spores not fully developed) and/or advanced stages (mature refringent spores) and their spread over the digestive gland tubules

Stage	<i>Marteilia</i> sp. scale	CPO scale (×100)
Initial infection (II)	Presence of only initial stages	At least 1 cyst
Light infection (LI)	Most initial stages and few advanced stages	In the 10 fields observed, at least 4 were positive and 1 had >3 cysts
Moderate infection (MI)	Most advanced stages in a limited number of tubules	In the 10 fields observed, at least 8 were positive and 4 had >5 cysts
Advanced infection (AI)	Majority of advanced stages in many tubules	All fields observed were positive and at least 5 had >10 cysts

prokaryotic-like organisms, trematode metacercariae and neoplasms.

***Marteilia* sp. (Phylum Paramyxea)**

Early and advanced developmental stages of the protozoan *Marteilia* sp. were observed in the epithe-

lial cells of the digestive ducts and tubules (Fig. 3). With reference to the geographic distribution, this parasite was mainly present in the western rías. We observed the parasite in 5 beds in the western rías, all of them located in Ría de Arousa (Fig. 1), while in the northern rías, we only detected *Marteilia* sp. in the Cariño bed (Fig. 1). Overall prevalence was generally low, except at Barraña, where prevalence was

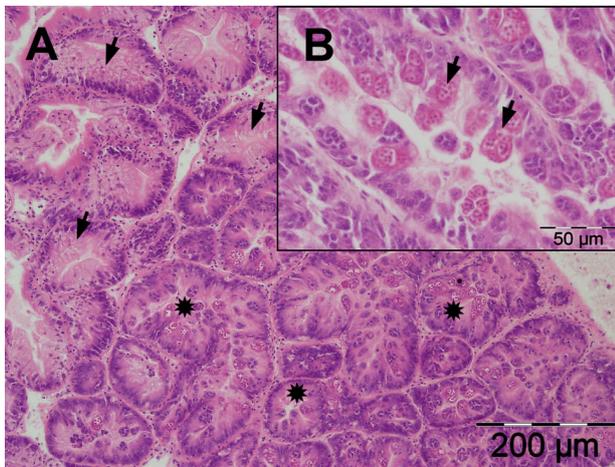


Fig. 3. (A) Histological section of *Solen marginatus* digestive gland showing normal digestive tubules (arrows) and tubules infected by *Marteilia* sp. (asterisks). (B) Detail of the epithelium of a digestive tubule with advanced stages of *Marteilia* sp. (arrows)

significantly higher than in all other beds apart from Vilanova (Fig. 2). With reference to the intensity of infection, Mañóns showed the highest value, followed by Barraña and Vilanova, although the intensity levels were light to moderate in all studied beds. An associated haemocytic response was occasionally observed.

Marteilia sp. infecting *Solen marginatus* had already been reported in Galicia (López & Darriba 2006). The ultrastructural analysis carried out by those authors showed differences with respect to other species of *Marteilia*. On the other hand, *M. refringens* (type M) was reported in *S. marginatus* from Andalucía (López-Flores et al. 2008a). In Spain, protozoae of this genus have been recently reported infecting Mediterranean striped Venus clams *Chamelea gallina* and cockles *Cerastoderma edule* (López-Flores et al. 2008b, Carrasco et al. 2011, Villalba et al. 2014), in the 2 last cases associated with mortality outbreaks.

Regarding the temporal trend of this disease, a pattern in the prevalence distribution was not observed (data not included), as in the case of mussel populations from Galicia affected by *M. refringens* (Villalba et al. 1993). However, in the case of oysters from France, a temporal trend of the prevalence was observed (Grizel 1985).

Molecular analyses are required to assess whether the species infecting *S. marginatus* from Galicia is *M. refringens*, as is the case for Andalusian grooved razor shells.

Colonies of intracellular prokaryotic-like organisms (IPO) in the epithelium of branchial filaments

Branchial IPO colonies were oval to round (Fig. 4A) and were detected in individuals from all beds, although the highest prevalence was observed at Sarrido, where prevalence was significantly higher than in the other beds (Fig. 2). Ultrastructural analysis of a sample showing a large amount of this type of IPO confirmed the presence of intracellular colonies composed of pleomorphic prokaryotic organisms within the epithelial cells of the branchial filaments (Fig. 4B). Prokaryotic organisms had a cell wall with a rippled appearance separated from a plasma membrane by an electron-lucent zone. They showed the typical prokaryotic nucleoid and densely stained ribosomes at the periphery. The morphological features observed by transmission electron microscopy are consistent with *Rickettsia*-like species because they are pleomorphic, unlike chlamydial species, which are coccoids (Fryer & Lannan 1994).

Similar colonies have been observed in gills of different bivalve species (Fries & Grant 1991, Fries et al. 1991, Villalba et al. 1999, Hine & Diggles 2002, Darriba et al. 2012). Sometimes, they were associated with mortality (Gulka & Chang 1984a, Le Gall et al. 1988, Norton et al. 1993, Villalba et al. 1999, Wu & Pan 2000, Sun & Wu 2004, Darriba et al. 2012). In this study, the presence of branchial IPO was light, and these colonies of prokaryotes did not cause significant adverse effects on the host.

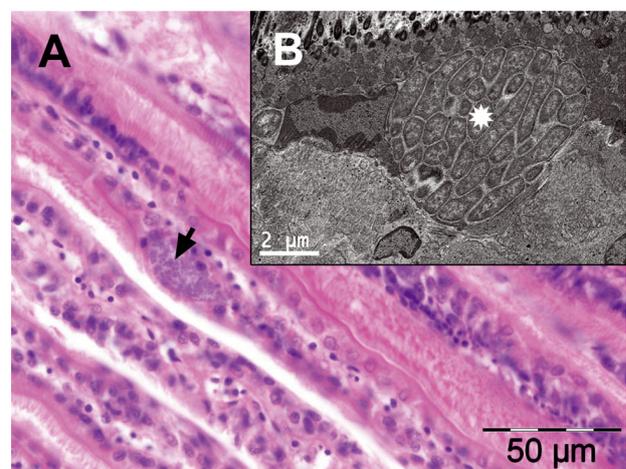


Fig. 4. (A) Histological section of *Solen marginatus* gill showing an intracellular prokaryotic-like organism colony (arrow) in the epithelium of branchial filaments. (B) Ultra-thin section of pleomorphic prokaryotic organisms (white asterisk) showing a typical nucleoid and densely stained ribosomes at the periphery

Colonies of IPO in the tubules of the digestive gland

These colonies were basophilic and rounded (Fig. 5). Digestive gland IPO were observed in all studied beds with similar prevalence (Fig. 2), their presence was light, and infiltration was not observed. These colonies are similar to those observed in *Ensis siliqua* from Galicia in the same tissues (Ruiz et al. 2013) without a clear assignment to the orders *Chlamydiales* or *Rickettsiales*.

Cysts of prokaryotic organisms (CPO)

Oval to rounded cysts were found in the branchial water tubules (Fig. 6A). Cysts ranged between 6 and 34 μm in diameter (mean \pm SD = 22 \pm 6 μm , n = 15) and contained granular material within a characteristic eosinophilic envelope. The morphological characteristics observed by optical microscope were similar to the branchial cysts observed in other bivalves (Gulka & Chang 1984b, Goggin & Lester 1990, Villalba et al. 1999, Carballal et al. 2001, Costa et al. 2012). Gulka & Chang (1984b) suggested a possible host origin of the eosinophilic wall in response to the rickettsial organisms. Ultrastructural analysis of an *S. marginatus* sample with high intensity of CPO confirmed the presence of prokaryotic organisms inside, with the same ultrastructural characteristics as those observed in branchial IPO. Nevertheless, they seemed to have an extracellular location (Fig. 6B). Molecular techniques will be an important tool in the study of these prokaryotes; for example, Costa et al. (2012) used PCR to associate the pro-

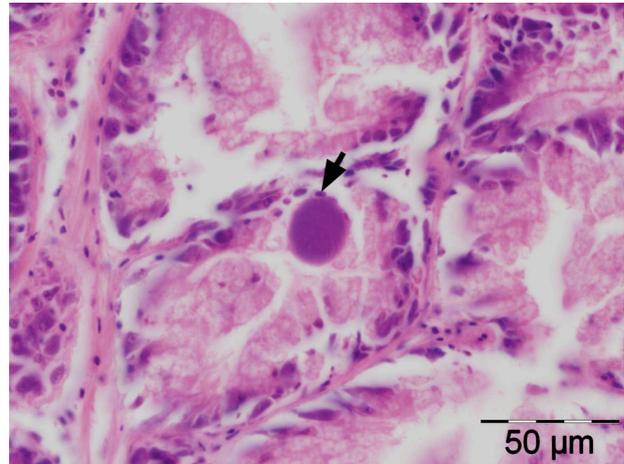


Fig. 5. Histological section of *Solen marginatus* with an intracellular prokaryotic-like organism colony (arrow) in an epithelial cell of the digestive gland

karyotes infecting gills of *Ruditapes decussatus* from Portugal with the *Spongiobacter/Endozoicomonas* group.

CPO were present in all beds, in some of them (Ribadeo, Cariño, Sarrido and O Grove) with a prevalence near 100% (Fig. 2). Intensity was generally light (stages II and LI; Table 1) in most of the beds, although in the most prevalent beds (Ribadeo, Cariño, Sarrido and O Grove), moderate and advanced intensity stages were observed. In some cases, associated haemocytic response was observed. Different studies carried out in different mollusc populations from Galicia referred to these colonies, although important damage to the host was not reported (Carballal et al. 2001, López et al. 2011, 2012). Neverthe-

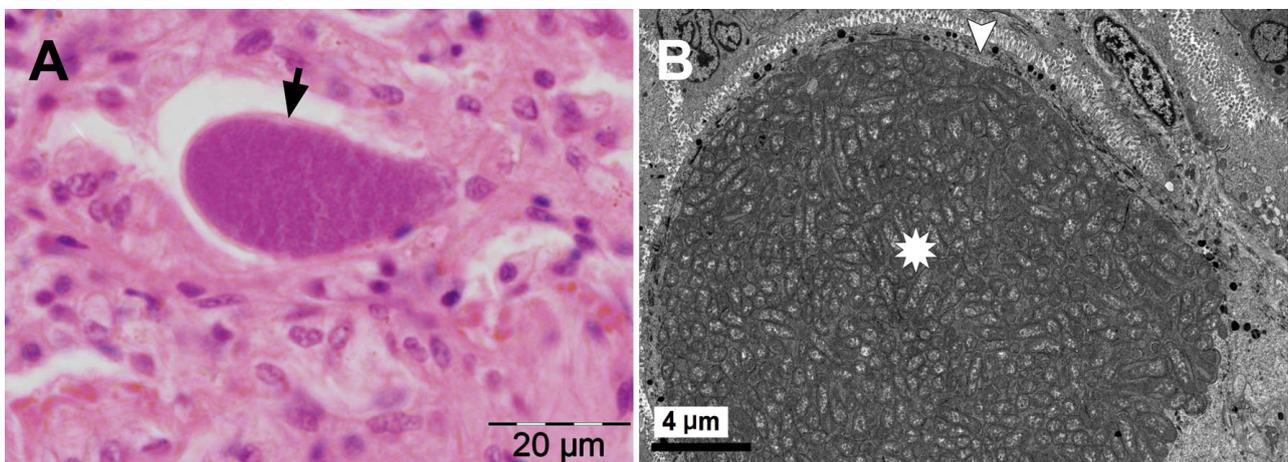


Fig. 6. (A) Histological section of *Solen marginatus* gill with an extracellular cyst of prokaryotic organisms and a characteristic eosinophilic envelope (arrow). (B) Ultrathin section of the cyst showing pleomorphic prokaryotic organisms (asterisk) enveloped by electron-dense material and a thick wall surrounding the cyst (arrowhead)

less, it is possible that high intensity provokes structural and functional damage due to the size and location of CPO in the gills.

All prokaryote colonies observed in this study were Gram-negative, acid-fast negative, Feulgen-positive and Pinkerton-positive (like rickettsial colonies). Similar results have been reported by several authors (Wen et al. 1994, Gardner et al. 1995, Wu & Pan 2000, Darriba et al. 2012, Ruiz et al. 2013).

Metacercariae of trematodes

Throughout this survey, trematode metacercariae encysted in connective tissue, gills and foot were detected. In the Cariño bed, we also observed metacercariae encysted in pericardial glands (Fig. 7). Prevalence and intensity of metacercariae in grooved razor shell tissues were low in general, with the exception of those encysted in the pericardial gland, in the Cariño bed. In this case, prevalence was high (Fig. 2), as was the intensity of infection (up to 35 cysts per histological section), and a haemocyte infiltration response occurred in the affected tissues. Although histological sections are not the best diagnostic technique for identifying species of metacercariae, the wide range of cyst diameter observed and the variety of microhabitats occupied within the host suggest that different digenean trematode species may be using *S. marginatus* as a second intermediate host. The isolation and excystation of the metacercariae *in vivo* could help in their taxonomic classification. High loads of metacercariae have been reported to cause damage to bivalve molluscs, and the effects

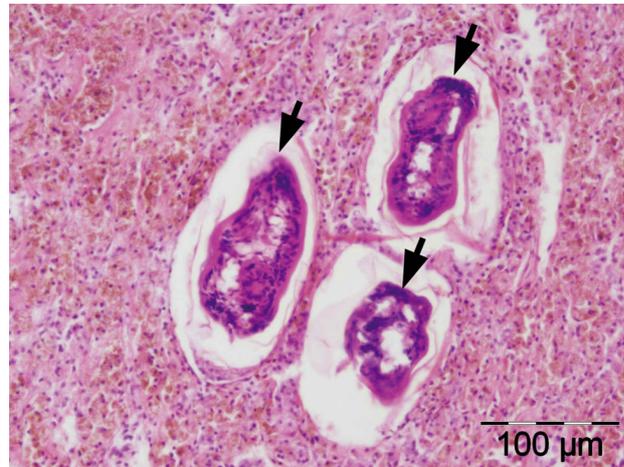


Fig. 7. Histological section through metacercariae (arrows) encysted in the pericardial gland of *Solen marginatus*

provoked by them include, among others, impaired growth rate, reduced capacity to burrow and increased mortality (Desclaux et al. 2002, Thieltges 2006, Bower 2010).

Disseminated neoplasms

An abnormal proliferation of circulating cells was observed in the connective tissue of gills (Fig. 8A) and other organs (digestive gland, pericardial gland and kidney). Neoplastic cells had a high nucleus-to-cytoplasm ratio and high frequency of mitotic figures, indicating the high rate of proliferation (Fig. 8B). The highest prevalence was observed in the Barraña, Mañóns and Cariño beds (Fig. 2). The intensity of

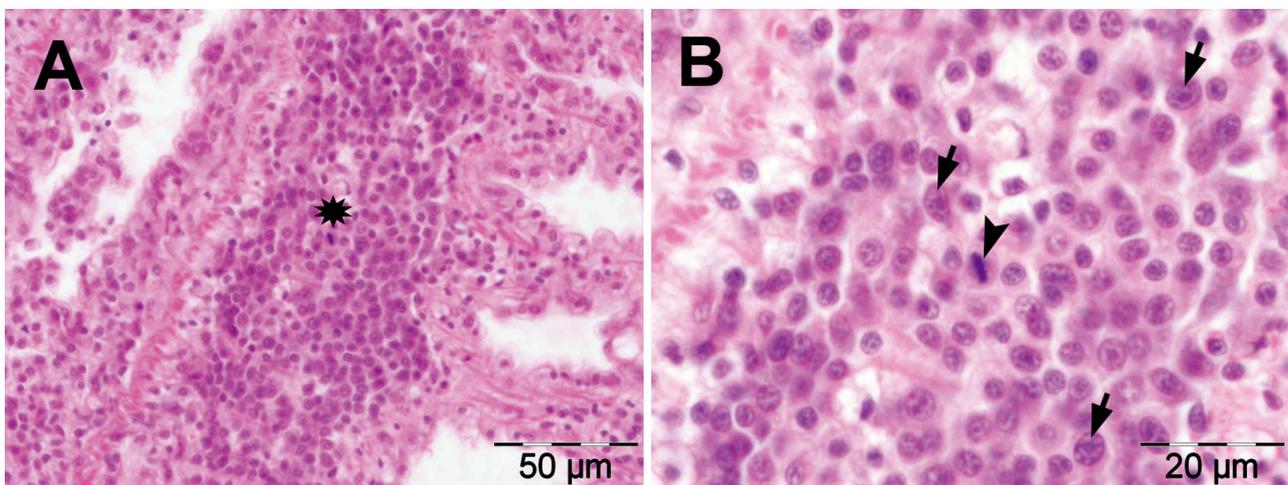


Fig. 8. (A) Histological section of a focus of disseminated neoplasm (asterisk) in the gill of *Solen marginatus*. (B) Detail of neoplastic cells (arrows), characterized by a high nucleus-to-cytoplasm ratio and presence of a mitotic figure (arrowhead)

infection was light in all beds, showing one to a few foci of neoplastic cells per section.

Disseminated neoplasm is the most common neoplastic disease in marine invertebrate animals. This condition has frequently been described as causing a fatal outcome (reviewed by Barber 2004, Bower 2010), although some individuals can effect remission (Barber 2004). The aetiology of the disseminated neoplasm is controversial, and several hypotheses have been proposed involving different factors such as anthropogenic pollutants, genetic alterations induced by some environmental toxins, a viral agent or an interaction of several factors. However, none of the studies has been successful in demonstrating a repeatable single-factor aetiology or the sequence of events operative in a multifactorial aetiology (Elston et al. 1992). The tissue from which neoplastic cells are derived remains unknown. Most authors suggest a haematopoietic origin because of morphological similarities between neoplastic cells and haemocytes and the consistent observation that neoplastic cells are first seen in the circulatory system (Barber 2004). However, confirmation of this hypothesis is difficult due to the lack of knowledge regarding bivalve haematopoiesis. In recent years, work has focused on the study of the molecular bases in the development of neoplastic cells, such as the study of differences in expression of gene families involved in the regulation of cell cycles and the discovery of the mutant protein p53 and other homologues known to play a role in carcinogenesis (Farcy et al. 2008, Ruiz & López 2012, Martín-Gómez et al. 2013).

In summary, prokaryotic organisms appear as the most prevalent group, being present in all beds, with moderate prevalence except in the case of CPO, where prevalence was close to 100% in 4 beds. If the intensity of CPO increased, they could compromise the function of the gills, particularly when the host is under stress, as indicated by Goggin & Lester (1990). Furthermore, neoplasms were also detected in nearly all surveyed beds; however, intensity data indicate that this condition was not a threat to grooved razor shell populations. In the case of metacercariae, most did not cause adverse effects in grooved razor shells, with the exception of metacercariae observed in the Cariño bed, where they reached epidemic prevalence and high intensity, provoking inflammatory responses in the affected tissues. With regard to *Marteilia*, the low prevalence and moderate intensity may indicate that it does not involve a risk for Galician *S. marginatus* populations. Nevertheless, it is important to be on the alert for these parasites and conditions because of the pathogenic potential of

some of them. For example, *M. cochillia* was recently associated with a mortality outbreak in cockle *Cerastoderma edule* populations from the Galician coast (Villalba et al. 2014).

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