

## ***In situ* hybridization detection of initial infective stages of *Marteilia refringens* (Paramyxea) in its host *Mytilus galloprovincialis***

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### **Short Communication**

**Keywords:** connective tissue, early stages, gills, *in situ* hybridization, *Marteilia refringens*, mussel.

*Marteilia refringens* is a protistan parasite of the phylum Paramyxea, which affects economically important bivalve species in Europe, such as the European flat oyster *Ostrea edulis*, and the mussels, *Mytilus edulis* and *M. galloprovincialis* (Lopez-Flores, de la Herran, Garrido-Ramos, Navas, Ruiz-Rejon & Ruiz-Rejon 2004). Infection with *Marteilia refringens* is listed as a notifiable disease by the World Organization for Animal Health (OIE) because of the detrimental impact of the parasite on infected mollusc cultures. However, the life cycle of the parasite is still unclear although recent research points towards a presumed zooplanktonic intermediary host (Audemard, Le Roux, Barnaud, Collins, Sautour, Sauriau, De Montaudouin, Coustau, Combes & Berthe 2002; Berthe, Le Roux, Adlard & Figueras 2004; Carrasco, Arzul, Furones, Chollet, Robert, Joly & Berthe 2005; Carrasco, Lopez-Flores, Alcaraz, Furones, Berthe & Arzul in press). *Marteilia refringens* is usually found in the digestive gland of flat oysters and mussels. Young stages of *M. refringens* have been described in the epithelia of stomach and primary digestive tubules, whereas more mature stages of the parasite can be found in the epithelium of the digestive gland tubules (Grizel, Comps, Bonami, Cousserans, Duthoit & Penneec 1974; Berthe et al. 2004) wheresporulation takes place. However, a few studies have reported the presence of young plasmodial stages of the parasite in gill tissues of *O. edulis* (Comps 1970; Grizel et al. 1974) and *M. galloprovincialis* (Robledo & Figueras 1995). This location was considered unusual and not the normal location of the parasite (Comps 1970; Grizel et al. 1974; Robledo & Figueras 1995; Berthe et al. 2004). Development of molecular detection techniques for *Marteilia*, such as *in situ* hybridization (ISH) (Le Roux, Audemard, Barnaud & Berthe 1999), may be very useful to improve the understanding of the parasite cycle within its host, and to observe early infections which are not easy to detect by routine histological observation. For example, ISH was used to describe the early development of *M. sydneyi* in its host *Saccostrea glomerata* (Kleeman, Adlard & Lester 2002). This report presents a preliminary study using ISH for the detection of all parasitic stages of *M. refringens* occurring throughout the year, paying special attention to initial infective and early stages of the parasite with the aim of improving the understanding of the life cycle of *M. refringens* and its transmission.

The delta of the River Ebro is located in N.W. Spain. Sediments of the River Ebro form and shape this delta, which is comprised of two semi-enclosed bays: Fangar Bay to the north and Alfacs Bay to the south. Both are exploited for mollusc aquaculture and fisheries. The mussel samples used in this study were obtained from experimental ropes, on a commercial raft stocked with Alfacs natural spat collected during spring 2004. Sixty mussels were randomly collected each month during the study period between October 2004 and October 2005. Half of the body of each mussel was fixed in 10% formalin in filtered sea water for 24–48 h, and afterwards preserved in 70% ethanol for histological studies, in order to detect the presence of early stages of *Marteilia*. Samples were dehydrated and embedded in paraffin wax. Paraffin blocks were cut at 3  $\mu\text{m}$  and stained with haematoxylin and eosin (H&E). Slides were studied under light microscopy at x40.

*In situ* hybridization analyses were carried out to detect the early stages of the parasite in mussel tissues. Each month, three mussels showing early stages of *M. refringens* by histology, or with suspected unknown stages, were tested by ISH. A total of 36 individuals were examined. ISH was performed as described by Le Roux et al. (1999). Paraffin sections of 7  $\mu\text{m}$  thickness were placed on aminoalkylsilane coated slides (Silane-Prep Slides; Sigma, St Louis, MO, USA) deparaffinated and then treated with proteinase K (100  $\mu\text{g mL}^{-1}$ ) in TE buffer (50 mM Tris, 10 mM EDTA) at 37 °C for 20 min. Slides were dehydrated by immersion in an ethanol series and air-dried. Sections were incubated with 50  $\mu\text{L}$  of hybridization buffer (4x SSC, 50% formamide, 1x Denhardt's solution, 250  $\mu\text{g mL}^{-1}$  yeast tRNA, dextran sulphate 10%) containing 10 ng of the digoxigenin-labelled probe specific for *Marteilia* species (Smart2). Sections were placed on a heating block at 95 °C for 5 min, cooled on ice for 1 min before 4 h hybridization at 42 °C in a humid chamber. Sections were washed twice for 5 min in 2x SSC at room temperature, and once for 10 min in 0.4x SSC at 42 °C. The detection step was performed according to the manufacturer's instructions (Dig Nucleic Acid Detection Kit; Boehringer-Mannheim, Mannheim, Germany). Counter staining was carried out with Bismarck brown yellow and rinsing in ethanol, followed by application of cover slips using an aqueous mounting medium. Heavily infected mussel tissues were used as positive controls in each batch, while uninfected mussel tissues were used as negative controls.

*Marteilia refringens* was observed by histology in the 12 samples of 60 animals collected monthly with prevalences ranging from 3.34% (2/60) in January to 26.67% (16/60) in July. Plasmodial early stages could be detected throughout the year, mostly in association with more mature stages of the parasite. These early stages were observed in the epithelia of the mantle, stomach and primary digestive

tubules. Mature stages of the parasite were observed in the epithelium of the secondary digestive tubules.

*In situ* hybridization showed strongly labeled stages of different morphology in the digestive gland of most individuals. However, in one sample collected in July, numerous labelled cells of the parasite were also observed in the epithelium of the gills (Fig. 1) and the mantle (Fig. 2), as well as in the connective tissue of the mantle and digestive gland (Fig. 3). Parasites located in the epithelia of the gills and mantle epithelium appeared in clusters along the basal membrane of the epithelium. Parasites located in the connective tissues usually appeared isolated. Different stages of the parasite were also observed in the digestive tissues of the same individual (Fig. 4). No haemocytic host response was observed around the parasites. Sequential paraffin sections of the same parasitized individual were stained by H&E for histological observations and although some parasites could be observed in the connective tissue, they were difficult to detect. Some of the examined mussels (11) showed very faint labelling, even if the positive control and the remainder of the slides from the same batch showed strong labelling. Initial stages of paramyxean development are not easily detectable by routine histological observation, especially when there is no host reaction or tissue disruption around the parasite. For this reason, ISH may be very useful to elucidate the parasite cycle within its host. *Marteilia sydneyi* had never been detected in palps and gills of *Saccostrea glomerata* until ISH was used (Kleeman et al. 2002). The presence of early developmental stages of *Marteilioides chugmuensis* was also described in *Crassostrea gigas* gills, palps and mantle epithelia after ISH and electron microscopy (Itoh, Komiyama, Ueki & Ogawa 2004). In the present ISH study, only one mussel showed parasites in the epithelium of the gills and mantle, and in the connective tissues of the mantle and digestive gland. Nevertheless, such occurrences are probably underestimated because the number of animals tested by ISH was low (36) and some individuals showed only very faint labelling (11), which did not allow detailed analysis of the material. The mussel with parasites in the gill epithelium was collected in July, which is during the transmission and infection period of the parasite in *Mytilus galloprovincialis* (Robledo & Figueras 1995; N. Carrasco, unpublished data). For this reason, and considering the similarities with the results of Kleeman et al. (2002), the epithelium of the gills may be considered as a potential route of entry for *M. refringens*. However, *M. refringens* appears to penetrate its host through most epithelia, including gills, mantle and also stomach and primary digestive tubules. Parasites located in gill and mantle epithelia could move, through systemic circulation and/or by migration through tissues (supported here by the occurrence of labeled parasites in the connective tissues), to the digestive tissues to complete their development by sporogenesis. Detection of the initial infective stages of *M. refringens* in the epithelia of the gills and mantle epithelia of *M. galloprovincialis* suggests that infection results from a "free-floating" parasite stage, acquired through the bivalve filter-feeding process, which comes into contact with gills and palps, as proposed by Kleeman et al. (2002). This hypothesis implies that the parasite is released from the intermediate host to its surrounding environment as the free-floating form. Additional studies of the initial stages of development of *M. refringens* using electron microscopy are thus required to further understand the parasite life cycle.

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## Figures

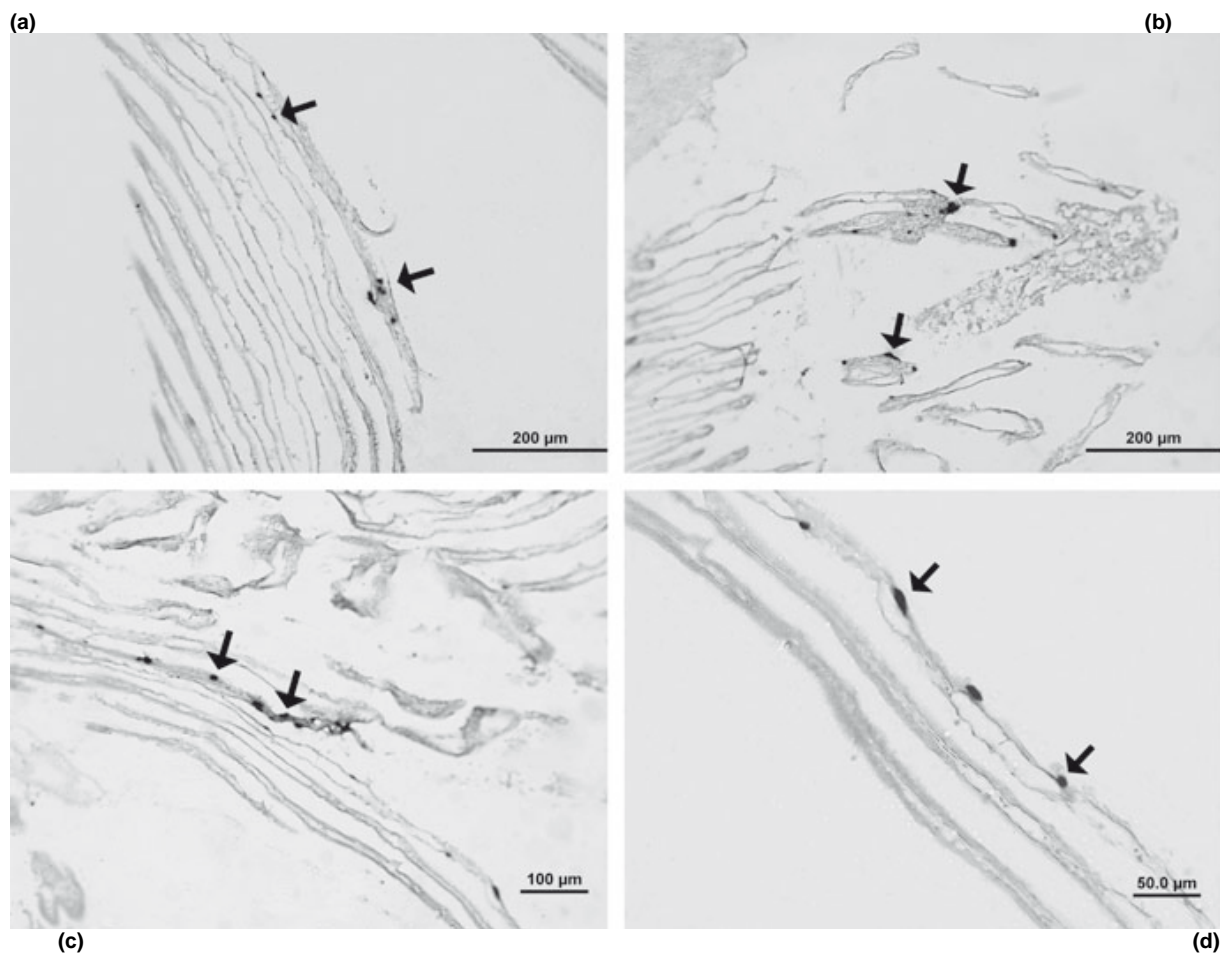


Figure 1 (a–d) Detection of early stages of *Marteilia refringens* (arrows) in gill epithelium of *Mytilus galloprovincialis* by in situ hybridization using the digoxigenin-labelled Smart2 DNA probe.

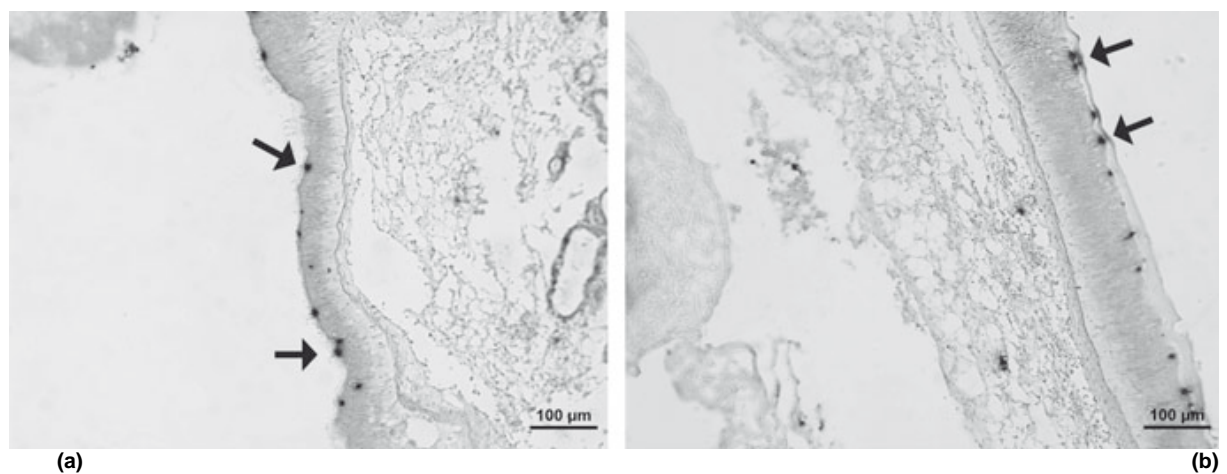


Figure 2 (a, b) Detection of early stages of *Marteilia refringens* (arrows) in mantle epithelium of *Mytilus galloprovincialis* by in situ hybridization using the digoxigenin-labelled Smart2 DNA probe.

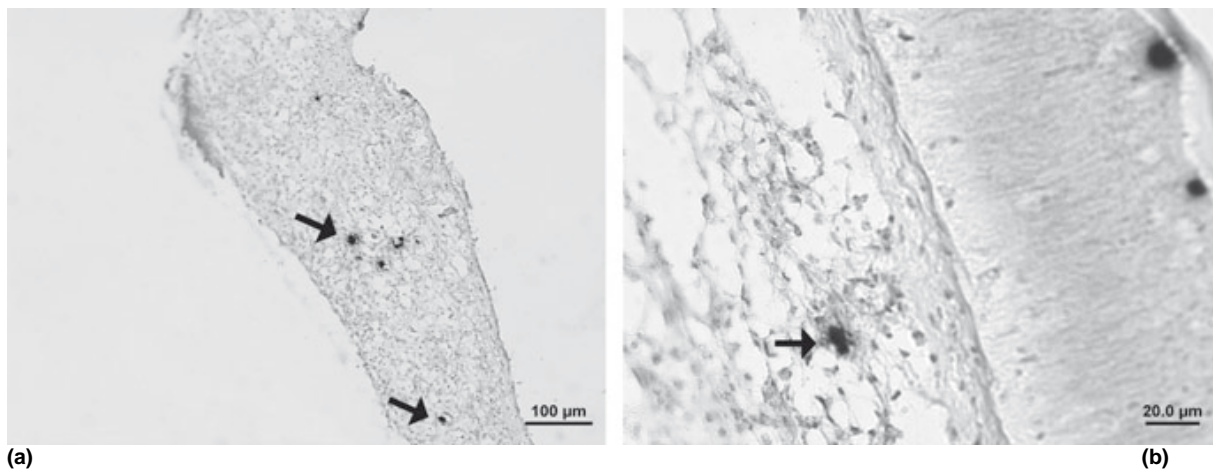


Figure 3 (a, b) Detection of early stages of *Marteilia refringens* (arrows) in mantle connective tissue of *Mytilus galloprovincialis* by in situ hybridization using the digoxigenin-labelled Smart2 DNA probe.

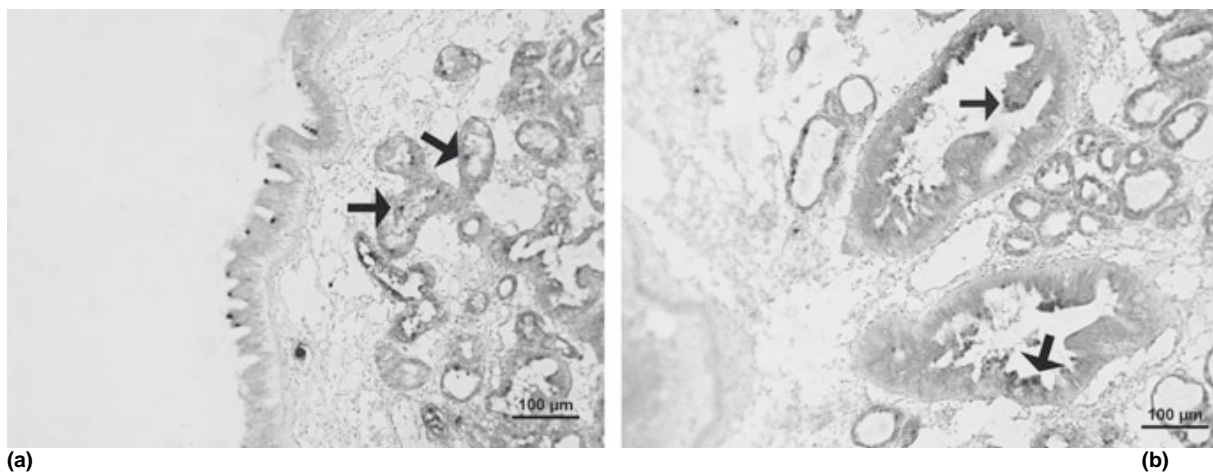


Figure 4 Detection of different stages of *Marteilia refringens* in digestive tissues of *Mytilus galloprovincialis* by in situ hybridization using the digoxigenin-labelled Smart2 DNA probe. (a) Labelled *M. refringens* cells in secondary digestive tubules (arrows), the parasite can also be observed in epithelium and connective tissue; (b) labelled *M. refringens* cells in primary digestive tubules (arrows).