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Bonamia-like parasite found in the Suminoe oyster *Crassostrea rivularis* reared in France

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ABSTRACT: Considering the economic importance of the Pacific oyster *Crassostrea gigas* to the French shellfish industry, the appearance of major diseases in this species could cause dramatic decreases in production. Suminoe oysters, of the non-indigenous species *Crassostrea rivularis* (Gould), were introduced into France to test their ability to adapt to local conditions. These oysters were imported after careful examination, and were maintained in laboratory quarantine. Some mortalities occurred 7 mo after importation. Histological and electron microscope examinations of 9 dead specimens revealed a parasite presumed to be a *Bonamia*-like protozoan. This is the first report concerning a parasite of the genus *Bonamia* in a species belonging to the genus *Crassostrea*. Thus, *C. rivularis* is not considered to be a suitable substitute for *C. gigas* in France.

KEY WORDS: *Crassostrea rivularis* · Suminoe oyster · Bonamiosis · *Bonamia*-like parasite

INTRODUCTION

The Pacific oyster *Crassostrea gigas* was introduced into France from Japan in the late 1960s after the disappearance of the Portuguese oyster *C. angulata* due to iridoviral infections (Comps & Duthoit 1976, Comps et al. 1976, Grizel & Héral 1991). Considering the economic importance of the Pacific oyster to the French shellfish industry, the appearance of major diseases in this species could be catastrophic. In this context, the search for species that could replace *C. gigas* was initiated by IFREMER at La Tremblade (Charente-Maritime, France). Non-indigenous species, such as *C. virginica*, *C. sikamea* and *C. rivularis*, have been imported following careful examination according to ICES (International Council for the Exploration of the Sea) rules, and maintained in quarantine facilities. Their ability to adapt to local rearing conditions was tested with experiments on comparative growth and susceptibility to known local parasites.

As part of this research, *Crassostrea rivularis* was imported from the USA in 1994. Before the importation of live oysters, histological examinations were performed on 30 fixed specimens to check for the presence of lesions or pathogens. In the absence of any recognised pathogens, 100 adult *C. rivularis* oysters were imported from New Jersey to the La Tremblade laboratory. These oysters were maintained in strict quarantine.

Seven months after introduction, some mortalities occurred in quarantine. Histological examinations were performed and revealed the presence of an intracellular protozoan parasite in connective tissues of 9 dead specimens. In order to investigate further, the parasite was purified and examined using an electron microscope. Ultrastructure analysis suggested that the protozoan might belong to the genus *Bonamia*. This paper is the first report of the presence of a *Bonamia*-like parasite in *C. rivularis* introduced to and reared in France. Furthermore, it is the first report of a *Bonamia*-like parasite in any *Crassostrea* species.

MATERIALS AND METHODS

Oysters. 30 fixed and 100 live adult *Crassostrea rivularis* (Gould), from the same stock, were provided by Haskin Shellfish Research Laboratory (Rutgers University, New Jersey, USA). The fixed specimens were

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received in January 1994 and were examined histologically at the IFREMER laboratory following ICES rules. Live oysters were received in July 1994 and were kept in quarantine. The quarantine system uses chlorine treatment for outlet water, but no treatment is applied to inlet water. Inlet water comes from the Marennes Oleron basin, where bonamiosis is present on the flat oyster *Oshea edulis*.

Histological examination. After the oysters were removed from their shells, they were sectioned sagittally. Half of each was placed in Davidson's fluid, and the other half in buffered formalin, according to Carson et al. (1973). The samples fixed in Davidson's fluid were dehydrated through an ascending ethanol series, cleared in xylene and embedded in paraffin wax. Blocks were sectioned at 3 to 4 µm thickness, stained with hematoxylin and eosin (H/E) and then examined for lesions and pathogens. The samples in Carson's fixative were examined by electron microscopy (see below). Heart smears were also performed on the dead oysters. These were stained with a Hemacolor kit (Merck) and were then examined by microscope for the presence of parasites.

Electron microscopy. Pieces of gill and digestive gland were collected from the oysters stored in Carson's fixative. They were rinsed in cacodylate buffer and fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer at a pH of 7.2, then post-fixed in 1% osmium tetroxide in the same buffer. The specimens were then cleared in propylene oxide and embedded in Epon resin. Sections of 1 µm were taken for light microscopy and stained in 2.5% toluidine blue in 1% aqueous sodium borate solution. Ultra-thin sections were made using copper grids and double stained with uranyl acetate and lead citrate. These were then examined in a Jeol JEM 1200 EX transmission electron microscope.

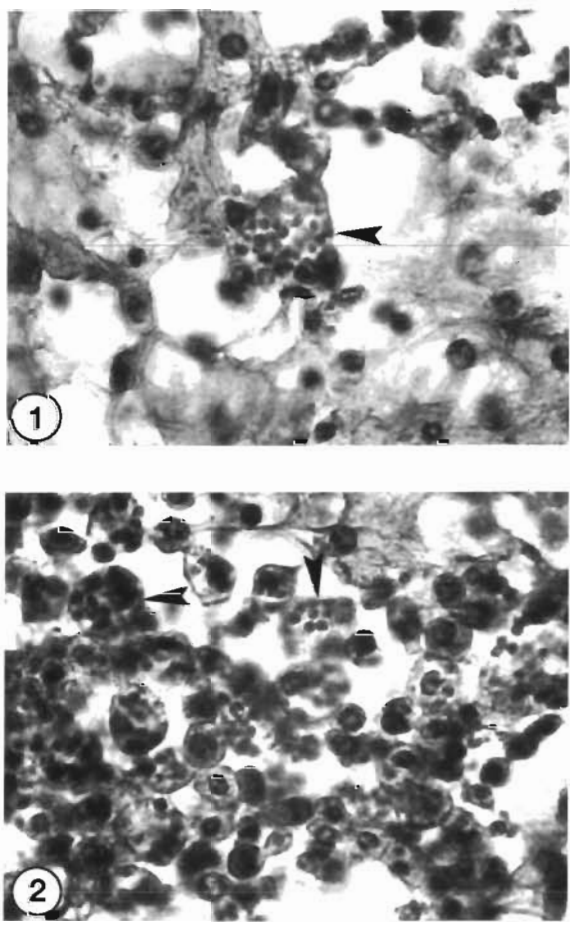
Pellets of purified parasites were fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer and post-fixed with 1% osmium tetroxide in the same buffer. The pellets were placed in low melting point agarose, dehydrated in an ethanol series and then treated following classical techniques for electron microscopy.

Purification. Heart smears of dead oysters were prepared and examined by microscope to determine infection level. Three strongly infected oysters were selected for purification. Parasites were purified according to the protocol of Mialhe et al. (1988). After homogenization of all organs except the adductor muscle, the parasites were concentrated by differential centrifugation on sucrose gradients and then separated by isopyknic centrifugation on a Percoll gradient. Finally, the purified parasites were resuspended in filtered sea water (0.22 µm) and counted using a Malassez-cell haemocytometer.

RESULTS

Histological examination

Parasitic infections were found in connective tissues of 9 dead *Crassostrea rivularis* which had been reared in the quarantine facilities of the IFREMER laboratory at La Tremblade. Histological sections revealed the presence of numerous spherical basophilic organisms, 2 to 3 µm in diameter, with a characteristic non-central large nucleus. The parasites were located in the hemocyte cytoplasm (Fig. 1). The infected cells typically accumulated in the vascular sinuses around the stomach, intestine and digestive gland. However, infected hemocytes were observed in all connective tissues. A characteristic histological feature of the infected oysters was the disassociated appearance of connective tissues which were infiltrated by groups of infected hemocytes (Fig. 2).



Figs. 1 & 2. *Crassostrea rivularis* with *Bonamia*-like infection. Hematoxylin/eosin stain. Light microscopy (×1000). Fig. 1. Numerous parasites located in hemocyte cytoplasm (arrow). Fig. 2. Connective tissue infiltrated by groups of infected hemocytes (arrows)

Electron microscope examination

Organisms were found in hemocytes, in gills and within the digestive gland. Infected hemocytes generally contained several parasitic cells. These were sometimes enclosed within a large vacuole. Occasionally, parasitic cells were visible in the cytoplasm; these were not delimited by a vacuolar membrane (Figs. 3 & 4). The structure of some hemocytes was only slightly affected, while others were more profoundly altered and displayed vacuolization (Fig. 4).

After purification, the parasite was visible as a spherical or ovoid cell of about 3 to 5 µm in diameter (Fig. 5). It was bound by a unit membrane as well as a plasmalemma of similar thickness to that of the host cell (Fig. 6). A distinct peripherally located nucleolus was sometimes present. The nuclear envelope consisted of an inner and an outer membrane, with some nuclear pores. The cytoplasm was moderately dense and contained 1 or 2 mitochondria. These mitochondria were variable in size (0.5 to 1.5 µm in diameter) and displayed short cristae pointing toward the matrix, which was electron-lucent (Fig. 6). Haplosporosomes were characteristically present, scattered throughout the cytoplasm. The haplosporosomes displayed 2 parallel layers of membranous appearance, one external and the other internal, embedded in a very electron-dense material (Fig. 7). Occasionally a dense body of about 0.8 µm was present. Numerous free ribosomes could be observed in the cytoplasm, as well as a few small citernae of the endoplasmic reticulum.

DISCUSSION

This study reports the presence of a protozoan infection in the connective tissues of *Crassostrea rivularis* found at the IFREMER laboratory in La Tremblade (Charente Maritime, France). The infected oysters were maintained in quarantine facilities where chlorine treatment prevents the escape of pathogens. However, without inlet water treatment, the hypothesis that *Bonamia* was transmitted to the experimental oysters from neighboring waters which are endemic for bonamiosis is strongly supported.

The structure and the histological location of the detected parasite are characteristic of parasites belonging to the genus *Bonamia*. Comparison between infected tissues of *Crassostrea rivularis* and the European flat oyster *Ostrea edulis* parasitized by *Bonamia ostreae* reveal no morphologic differences at the light or electron microscope level. In order to confirm that the parasite is the same, it was first necessary to isolate and purify it. The use of the *B. ostreae* purification protocol enabled the parasites found in *C. rivularis* to be purified, further highlighting their similarity. These purified cells displayed no difference from *B. ostreae* at the ultrastructural level either.

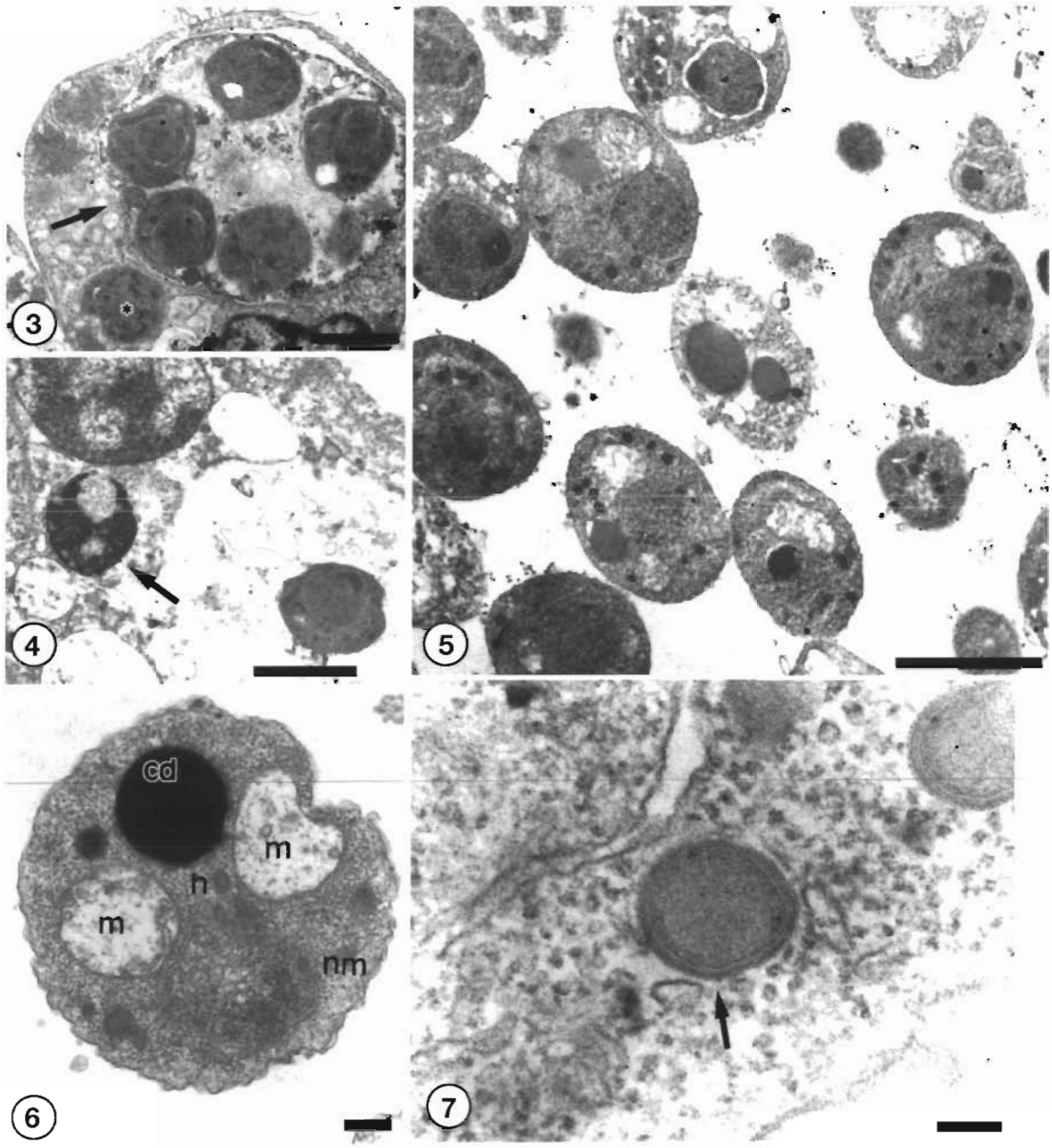
Morphological features of the parasite found in *Crassostrea rivularis* are in agreement with those described by Pichot et al. (1980) and Comps et al. (1980) for the species *Bonamia ostreae*. They also agree with those of *Bonamia* sp. in *Tiostrea lutaria* (Dinamini et al. 1987). The only differences found between these 2 presumed species of *Bonamia* are ultrastructural differences: number of haplosporosomes and large globules, morphology of mitochondria and nuclear/cytoplasmic ratio.

It is also of note that location and morphological structure of the parasite found here in *Crassostrea rivularis* are not in agreement with those previously described by Farley et al. (1988) for 2 species of disease-causing crassostreid parasites, which they assigned to the genus *Mikrocytos*: *M. mackini* in *Crassostrea gigas* from British Columbia (Canada) and *M. roughleyi* in *Saccostrea commercialis* from Australia. Histopathologically, *M. mackini* is characterized by acute inflammatory abscesses which remain focal until the oyster dies. Electron microscopy has demonstrated that organelles resembling haplosporosomes only occur at one stage in *Mikrocytos*. The haplosporosome-like organelles often tend to be elongated and contain layers of membranes. The internal structure of these organelles is not nearly as dense as that seen in *Bonamia* or other haplosporidans. In contrast with *Bonamia*, no clear demonstration of mitochondria has been achieved in *Mikrocytos*. *Mikrocytos* is always associated with focal abscesses and occurs in crassostreid oysters whereas *Bonamia* is always associated with generalized infections and has only been reported in ostried oysters up until now (Farley et al. 1988).

All the characteristics of the parasite detected indicate an infection by the parasite *Bonamia ostreae* in an endemic area. Control oysters which were examined before importation, following ICES rules, did not contain detectable parasites and the mortalities appeared only after 7 mo in quarantine.

Previous controlled introductions of healthy stocks of *Ostrea chilensis* from Chile and *Ostrea angasi* from Australia were followed by *Bonamia ostreae* infection in locations along the French coasts where *B. ostreae* is endemic and naturally infects the local *O. edulis* (Grizel et al. 1983, Bougrier et al. 1986). In this study we report the presence of a parasite clearly related to the genus *Bonamia* but in an oyster species belonging to the genus *Crassostrea*.

Crassostrea rivularis (Gould, 1861) is synonymous with *C. ariakensis* (Wakiya) (Torigoe 1981). This species occurs naturally in Japan (Torigoe 1981), Pakistan and India (Ahmed 1971), and China (Zhuang 1992). It



Figs. 3 to 7. *Crassostrea rivularis* with *Bonamia*-like infection. Electron microscopy. Fig. 3. Naturally infected oyster hemocyte showing numerous parasites enclosed within a large vacuole (arrow) and one (asterisk), in the cytoplasm, not delimited by a vacuole membrane. Fig. 4. Infected hemocyte showing altered parasite (arrow) in the cytoplasm. Fig. 5. Ultrathin section of purified parasites. Scale bar = 2 µm. Fig. 6. Parasite showing nuclear membrane (nm), haplosporosome (h), mitochondria (m), and dense body (cd). Scale bar = 500 nm. Fig. 7. Detail of haplosporosome-like body (arrow). Scale bar = 200 nm

is of commercial importance in China (Yongjia et al. 1995) and has also been introduced from Japan to the USA, where it is cultivated on the west coast (Langdon & Robinson 1991). Its gametogenic cycle makes it a good candidate for a 'summer' oyster (Perdue & Erickson 1984). Although *'Ostrea rivularis'* is mentioned in a recent paper on oysters from southern China (Yongjia et al. 1995), the placement of the oysters we studied in the genus *Crassostrea* is certain. DNA studies based on partial 28S rRNA gene and on mitochondrial 16S rRNA gene sequences confirm the close relationship between *C. rivularis* and other members of the genus *Crassostrea* (Littlewood 1994, O'Foighil et al. 1995). These are all non-incubatory oysters and hybridiza-

tions may occur within the genus as has been shown with *C. gigas* and *C. rivularis* (Allen & Gaffney 1993). Such hybrids have been produced recently in our laboratory at La Tremblade.

Crassostrea gigas has been recognized by the European Commission as a non-carrier species for the parasites *Bonamia ostreae* and *Marteilia refringens* (93-169-CEE). Moreover, Renault et al. (1995) obtained no proliferation of *B. ostreae* in *C. gigas* experimentally challenged by the parasite, confirming the previous observations obtained by epidemiological observation. A comparative study of the resistance of *C. gigas*, *C. rivularis* and their inter-specific hybrids to parasites of the genus *Bonamia* could be of great interest for the understanding of host-parasite relationships in bivalves.

Our report demonstrates the possible susceptibility of *Crassostrea rivularis* to a *Bonamia*-like parasite. Thus, *C. rivularis* is no longer considered as a potential substitute for *C. gigas* in France. This study also underlines the potential danger of introducing non-indigenous species of bivalves for rearing without sufficient control protocols.

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