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First haplosporidan parasite reported infecting a member of the Superfamily Pinnoidea (*Pinna nobilis*) during a mortality event in Alicante (Spain, Western Mediterranean)

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ABSTRACT

Several stages of a haplosporidan parasite, including spores, were detected infecting three out of four specimens of the Pen Shell *Pinna nobilis* from the coast of Alicante (Western Mediterranean). A mortality event initiated few weeks before the sampling. The infection was systemic in the connective tissue, with free uni-nucleate stages and early plasmodia, whereas sporulation process took place in the digestive tubules disrupting them. Morphological details, by light and transmission electron microscopy, and PCR amplification confirmed that the parasite belongs to the haplosporidan group. Spores were pleomorphic, usually elongated ovoid, with round to elongated haplosporosomes-like in the sporoplasma. The operculum was situated in the apical zone of the wall, with an external lid, and the nucleus tended to be eccentric in the basal zone. Spore ornamentation was not observed. The single uninfected specimen appeared to be healthy. This is the first report of a haplosporidan parasite infecting a member of the Superfamily Pinnoidea and this is the first histopathological study of a mortality event in the endangered and protected *P. nobilis*.

1. Introduction

The Pen Shell *Pinna nobilis* (Linnaeus, 1758) ranks among the largest bivalves in the world and is the largest bivalve of the Mediterranean Sea, where it is endemic. It is long-lived with a maximum reported age of 27 years reaching a size of up to 120 cm. It occurs at depths between 0.5 and 60 m, mostly on soft-botton areas overgrown by *Posidonia oceanic* meadows. The dense network of robust rhizomes and roots formed by seagrass meadows provide a substrate, where *P. nobilis* can anchor themselves through attachment of their byssus threads. They also become anchored to the substrate via compression of the basal part of the shells, embedded within the seagrass matte, as the individuals grow (Basso et al., 2015).

The populations of *P. nobilis* have been greatly reduced due to anthropogenic and environmental threats and it has been listed as an endangered and protected species under the European Council Directive 92/43/EEC (EEC, 1992). Therefore, it is under strict protection, in fact all forms of deliberate capture or killing are prohibited as the deliberate disturbance, destruction or taking of eggs and the deterioration or destruction of breeding sites or resting places.

Among the threats identified in the review of Basso et al. (2015) for the already endangered populations of *P. nobilis*, they included contaminants, invasive species, the climate change and mainly the degradation of *Posidonia* meadows. Nevertheless, parasites were never considered as a threat for the Pen Shell populations.

However, one of the main threats identified for commercial bivalve molluscs, around the world, have been parasites and one of the major pathogens concerning for aquatic animal health managers and shellfish industries were haplosporidan parasites. They have been responsible for some of the most significant and consequential marine disease epizootics on record, such as the *Haplosporidium nelsoni* outbreaks devastating oyster populations (*Crassostrea virginica*) along the Mid-Atlantic coast of the USA and the microcell haplosporidan *Bonamia ostreae* infecting flat oyster *Ostrea edulis* populations in Europe (Burreson and Ford, 2004; Arzul and Carnegie, 2015).

In early Autumn 2016, a mass mortality event in *P. nobilis* populations was detected in the Western Mediterranean coast, affecting specimens of all sizes reaching up to 100% in the centre and southernmost coasts of the Iberian Peninsula Populations whereas the northern coasts of the Spanish Mediterranean Sea seemed to be unaffected (Vázquez-Luis et al., submitted for publication). The aim of the present study was to investigate the event detected from a histopathology point of view to evaluate if a pathogen could be involve in the mortality event of the Pen Shell in Alicante (Mediterranean coast of Spain).

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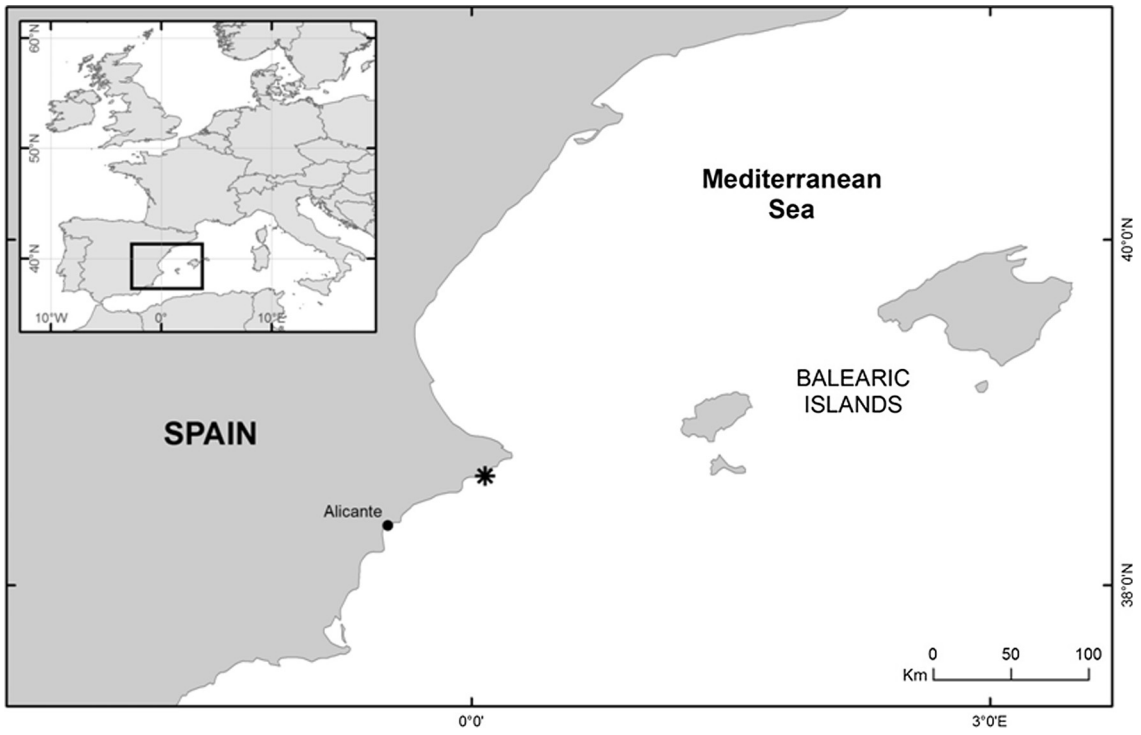

Fig. 1. Map of the Western Mediterranean showing the sampling point (asterisk) in Calpe (Alicante).

2. Material and methods

2.1. Sample description

Four specimens of *Pinna nobilis* were sampled diving from the subtidal coast of Calpe –8 m depth–, one of the first locations where the mortality event was detected (Alicante-Southeast Spain) (Fig. 1). Samples were transported to the laboratory under cool conditions in 24 h. During the macroscopical evaluation of the individuals – at the laboratory – a description of the specimens condition were registered as well as epibionts and biometric parameters.

2.2. Histopathological study

Samples from adductor and retractor muscles, mantle, labial palps, gills, digestive gland, gonad and byssus gland were fixed in Davidson's solution (Shaw and Battle, 1957) for histopathological study and also preserved in 96% ETOH for DNA extraction. Paraffin blocks were sectioned at 5-µm with a rotary microtome. Tissue sections were deparaffinised, stained with Harris' haematoxylin and eosin and examined by light microscopy for parasites and pathological conditions.

Some portions of digestive gland infected with a protozoan parasite were taken out from the paraffin blocks and processed for transmission electron microscopy (TEM). 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 in a JEOL JEM 1010 transmission electron microscope at 80 kV.

2.3. DNA isolation and PCR amplification

Small pieces of digestive gland preserved in ethanol were chopped with sterile scissors and washed twice (15 min) with sterile distilled water (800 µl) and three times (15 min) with lysis buffer (1 M Tris, 0.2 M EDTA, 2.5% N-Laurylsarcosine). The pellet was mixed with 800 µl of lysis buffer and 10 µl of proteinase K (20 mg/ml) and incubated at 50 °C overnight. DNA was extracted by precipitation with isopropanol 44% (v/v) and ammonium acetate 500 mM after digestion of RNA by RNase (10 µg/mL) and the elimination of proteins by extraction with phenol/chloroform/isoamylalcohol (25:24:1) solution (Fernández-Tajes et al., 2011).

The DNA isolated from each individual sampled was subjected to PCR amplification using generic haplosporidan primers (HAPF1-HAPR3), *Haplosporidium nelsoni* specific primers (MSXA'-MSXB) (Renault et al., 2000) and generic primers for *Bonamia* spp. (BO-BOAS; Cochennec et al., 2000) given that histopathological study revealed the presence of an haplosporidan-like parasite with unicellular stages resembling *Bonamia* spp. and sporulating in digestive tubules, as *Haplosporidium nelsoni*.

The PCR was carried out in 25 µl of reaction volume containing 1 µl of genomic DNA, 12.5 µL of RealStart™ DNA Polymerase premix (Yeastern Biotech) at 1x concentration, 6.5 µl of water and 2.5 µl of each primer (10 µM) for HAPF1-HAPR3 and MSXA'-MSXB primers and 9.5 µl of water and 1 µl of each primer for BO-BOAS. For PCR reactions with BO-BOAS and HAPF1-HAPR3 pairs of primers, two negatives controls (no DNA and DNA of uninfected flat oyster *O. edulis*) and one positive control (DNA of *O. edulis* co-infected with *Bonamia ostreae* and *Bonamia exitiosa*) were used in each reaction. For PCR reactions with MSXA'-MSXB primers, two negative controls (no DNA and DNA of *O. edulis* co-infected with *B. ostreae* and *B. exitiosa*) were used.

Reaction mixtures were cycled in a GeneAmp PCR System 9700 thermal cycler (Applied Byosistems) according the specifications indicated by Renault et al. (2000) and Cochennec et al. (2000).

PCR products were electrophoresed on 2% agarose gels in 1x TAE buffer (Tris 40 mM, acetic acid 1 mM, EDTA 20 mM), stained with RealSafe (Durviz) and scanned in a EBox-VX2/20 M Photo documentation system (VilberLourmat).

3. Results

3.1. Sample description

The length of the four specimens of *P. nobilis* processed ranged between 40 and 50 cm, they weighted between 389 and 599 g, with a