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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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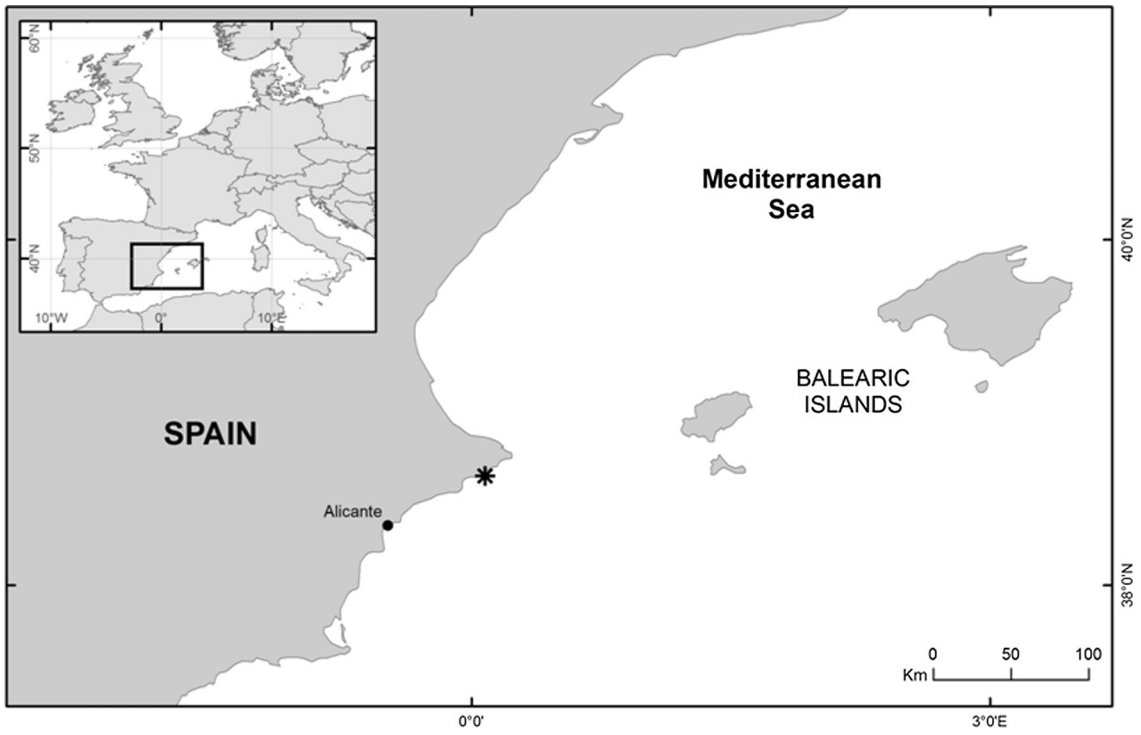

Fig. 1. Map of the Western Mediterranean showing the sampling point (asterisk) in Calpe (Alicante).

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

shell weight between 355 and 510 g.

They showed a wide range of epibionts attached to the periostracum: tube-building polychaetes, bryozoans, red coralline algae, sponges and one *Arca noae* bivalve in one case.

During the macroscopical observation realized in the laboratory, the four specimens had the valves closed, no byssus thread and the mantle retracted. Specimens 1 and 3 had a pair of shrimps *Pontonia pinnophylax* alive within the valves whereas specimen 2 released foul-smelling and seemed to have remains of a dead shrimp.

Mantle folds were black and white radial muscle fibres were clearly observed in the inner side. Specimen 4 had a little white nacred spherical pearl (15 mm of diameter) attached to the mantle. Digestive gland and kidney were so dark too. All the individuals had the kidney full of rounded brilliant black granules. That granules were observed with a Differential interference contrast (DIC) optical microscopy (Nomarski) showing an orange-red colour, a length ranging 490–700 µm, inorganic nature and concentric layers, resembling nephroliths described by Ghiretti et al. (1972) for *P. nobilis*.

One out of the four specimens – number 3 – had faeces inside the branchial cavity, near the anus, and it was the only one in which the crystalline style (7 cm of length) was present.

3.2. Histopathological study

Light microscopic observation of stained sections showed that three out of the four *P. nobilis* processed were infected by an haplosporidan-like parasite in different cell stages.

In the infected individuals, large numbers of uni-nucleate stages (2.8 ± 1.4 µm of length; mean ± SD; n = 50), with central or slightly eccentric dense nucleus, were disseminated in the connective tissue throughout all over the analysed organs and in some haemocyte vessels (Fig. 2). Early plasmodial stages (2–4 nuclei) were observed too, but at lower abundance. The nuclei of the plasmodia have ring shape with a dense perimeter and a light space inside (Fig. 2C). Host haemocyte reaction appeared to be little or absence, indeed few haemocytes were observed in the vicinity of the parasite cells. Moreover, sporocysts were observed in the digestive tubules (Fig. 3). Spores (3.27 ± 0.39 µm of length; mean ± SD; n = 52) enclosing an eosinophilic sporoplasm and a lateral nucleus were observed, longitudinal section revealed that they were ovoid with an overhanging lid (operculum) at one end. Clusters of retractile spores were also seen in some cells.

The sporulation stages collapsed epithelial cells leading the disintegration of digestive diverticula and free spores appeared in the lumen of many digestive tubules.

No prokaryotes or other eukaryote parasites were observed in histological sections of *P. nobilis*, unless some ciliates in gills and a copepod-like in one case. In the foul-smelling specimen – number 2 – free bacteria were observed in necrosed tissues.

Although the fixative used was suboptimal for electron microscopy, it was possible to observe some free uni-nucleate stages in the connective tissue and early plasmodia with two and more nuclei and rounded haplosporosomes-like (around 120 nm of diameter) in the cytoplasm. Abundant sporocysts containing spores in different stages of development were also seen (Fig. 4), with up to 25 spores per sporocyst. The epispore cytoplasm was observed surrounding some spores with a unidentified structure resembling a nucleus in contact with the wall. Spores were pleomorphic, but usually elongated ovoid (up to 4 µm) with a wall composed by two electro-dense layers (outer and inner layers – around 15 nm thick) and an electro-light middle layer (around 10 nm thick), round to elongated haplosporosomes-like in the cytoplasma (around 220 nm length) and an external lid (operculum) covering the apical orifice. The operculum was situated in the apical zone of the wall, composed of 2 plates and the nucleus tends to be eccentric in the basal zone. Spore ornamentation was not observed.

3.3. DNA isolation and PCR amplification

The PCR reactions with the degenerated primers HAPF1-HAPR3 produced consistently positive results in three of the four specimen analysed (Fig. 5), confirming that the parasite observed in histolopathological study is a haplosporidan parasite.

Nevertheless, PCR reactions with primers BO-BOAS and MSXA'-MSXB produced negative results –no amplification- discarding the presence of *Bonamia* spp. or *H. nelson*i.

4. Discussion

The histopathological study revealed that three specimens were infected with a haplosporidan parasite causing a systemic infection – with uni-nucleate stages and early plasmodia – in the connective tissue and spores in the digestive tubules. The sporulation of the parasite in the digestive gland caused the collapse of the digestive cells infected, precluding the digestion process. Assuming that uni and binucleated forms precede the multinucleate stages, early infection occurred in the connective tissue spreading throughout the entire animal in the bloodstream, given that uni-nucleate stages were observed in the hemolymph vessels. When the infection achieves the digestive tubules sporulation process takes place.

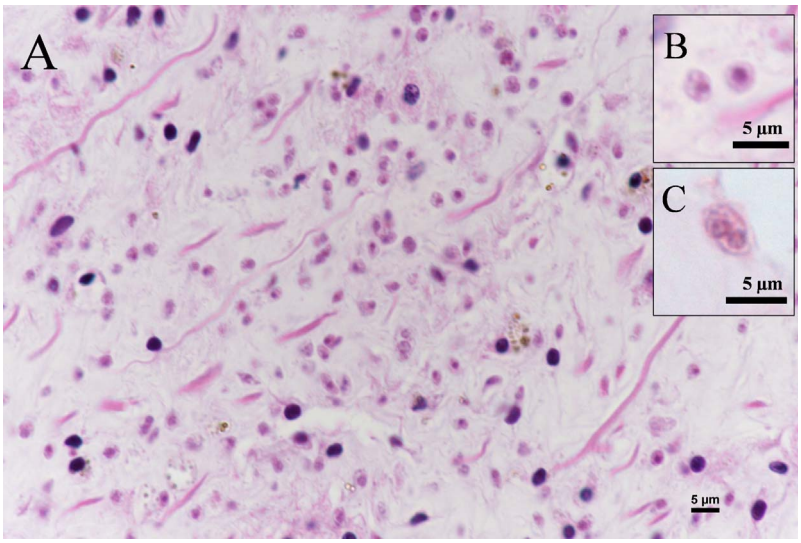

Fig. 2. A: Connective tissue of *Pinna nobilis* infected with uni-nucleate stages and early plasmodia. B: Two uni-nucleate cells. C: Early plasmodia with 2 nuclei.

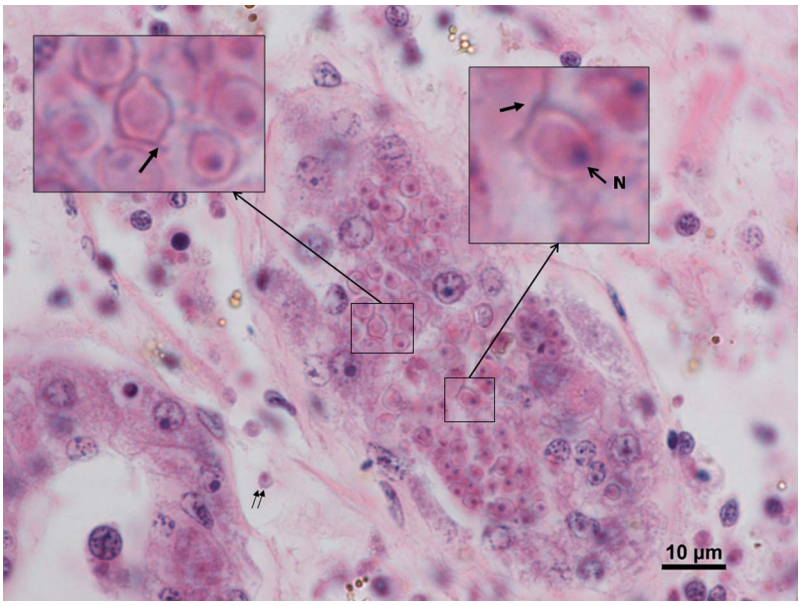

Fig. 3. Digestive gland of *Pinna nobilis* infected with free uni-nucleate cells (double arrow) and spores of an haplosporidan parasite. Magnifications show spores where the lid (arrow) and an eccentric nucleus (N) can be observed.

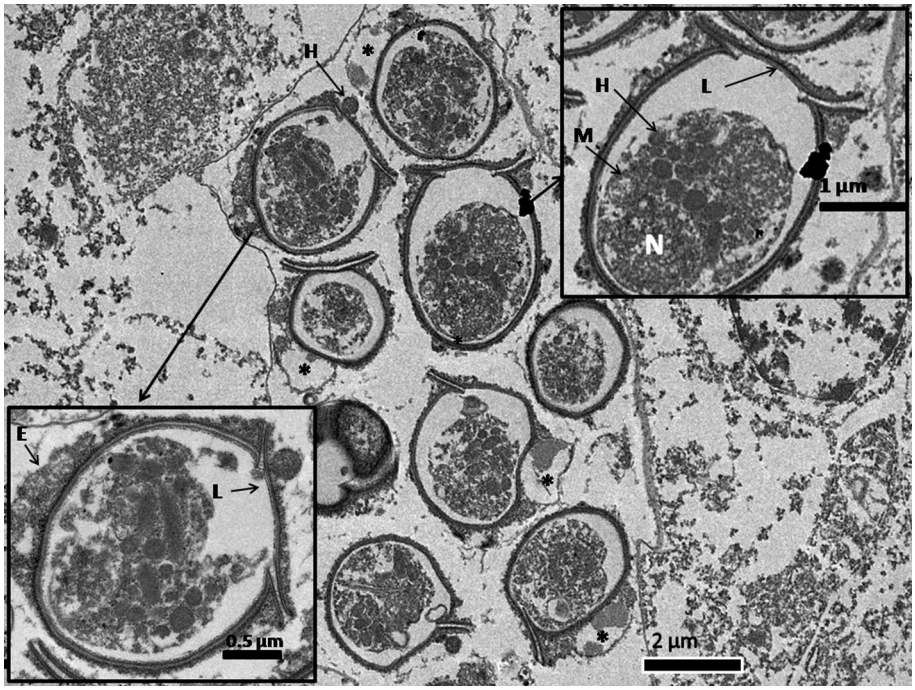

Fig. 4. Transmission electron micrographs (TEM) of sporocysts with spores in different stages of development infecting digestive tubules of *Pinna nobilis*. N: nucleus, H: haplosporosomes-like, L: superior lid of the operculum; M: mitochondria; E: epispore cytoplasm and asterisks: unidentified structures in the epispore cytoplasm.

No other parasite and/or pathological alteration that might account for the mortality event were observed. Regarding to the uninfected specimen, among the information obtained during the macro and microscopic study, it seemed to be healthy.

Perkins (2000) described the Haplosporidia as parasitic protists that form ovoid, walled spores with an orifice in one pole, covered either externally by a hinged lid or internally by a flap or wall material. The ultrastructural study confirmed that the spores observed in *P. nobilis* can be included in the classical definition of the group. Moreover, the presence of an external hinged lid suggests that the parasite belongs to either *Minchinia*, *Haplosporidium* or *Bonamia*. Currently, the most common method to separate the genera *Haplosporidum* and *Bonamia* from the *Minchinia* was based on the origin of the spore ornamentation, from either the spore wall in *Haplosporidium* and *Bonamia* or from the epispore cytoplasm in the *Minchinia* (Bearham et al., 2008). In our case, spore ornamentation was not observed but the lack of wall projections must be verified with further studies.

We observed in several cases an unidentified structure resembling a nucleus in the epispore cytoplasm in contact with the wall spore, but there are no references in the literature about that. Moreover, we observed presumed mitochondria in the epispore cytoplasm, but the quality of the images made difficult a clear identification.

Comparison of data observations with other haplosporidan studies in the literature is difficult because, as Hine and Thorne (2002) stated, there are inconsistencies in terminology and because spore dimensions vary between fresh smears, sections in light and electron microscopy

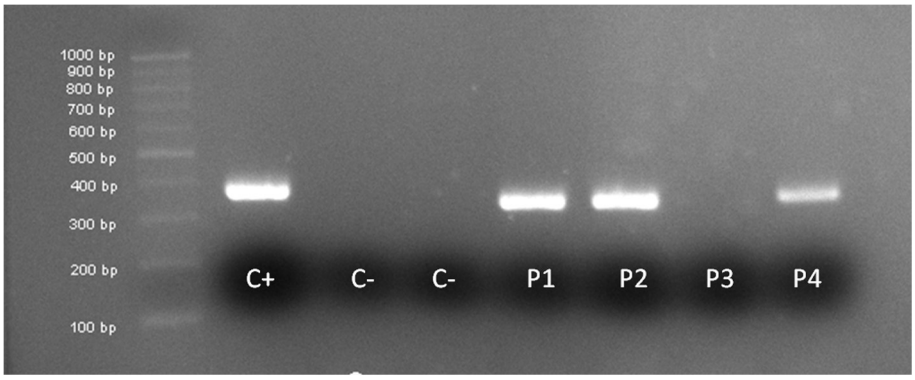

Fig. 5. Agarose gel electroforesis of the PCR products amplified in 3 out of 4 specimens of *Pinna nobilis* using the generic HAPF1-HAPR3 primers (Renault et al., 2000). C+: positive control C− negative controls, P1 to P4: specimens 1–4.

and re-embedding of tissues for TEM from paraffin blocks.

Results of the DNA assays supported that the parasite belongs to the haplosporidan group as the histopathological observations suggested. Moreover *H. nelsoni* and *Bonamia spp.* are discarded, given that samples of DNA isolated from infected tissues were not amplifiable with the primer pairs MSXA'-MSXB or BO-BOAS.

Haplosporidan parasites have been detected infecting species of bivalves, gastropods, crustacean, worms, ascidians and even hyperparasiting trematode larvae (reviewed by Burreson and Ford (2004) and Arzul and Carnegie (2015)), but they were never reported in a member of Pinnoidea Superfamily.

They are widely distributed around the world in both marine and freshwater environments, but the prevalence of infection is often extremely low and they do not appear to be important pathogen because of this low prevalence. However, some species have been associated with epizootic mortalities of commercially important molluscs. The most well-studied members of the group are *Haplosporidium nesoni*, which causes MSX disease in *C. virginica* on the east coast of North America, and *Bonamia ostreae* which caused the destruction of *O. edulis* populations in France before spreading through other European countries (reviewed by Burreson and Ford (2004) and Arzul and Carnegie (2015)). The haplosporidan detected infecting *P. nobilis* seems to be as relevant as these two species, especially if it is confirmed as the cause of mortalities of this endangered and protected bivalve in other locations along the Mediterranean waters.

Several parasite stages were described in bivalves: uni-nucleate cells, early plasmodia (two or few nucleus), big multi-nucleate plasmodia, sporocysts with spore walls forming around each nucleus and mature spores. However, spores are often not present in infected hosts. In the genus *Bonamia*, spores were only observed in *B. prespora* (Carnegie et al., 2006). *Bonamia* spp. are mostly intracellular, infecting haemocytes whereas *Haplosporidium* spp. are typically extracellular invading connective tissues or epithelium. Sporulation, when observed in *H. nelsoni* generally occurs in the epithelium of the digestive diverticula in advanced infections and in connective tissues in other *Haplosporidium* spp. (Arzul and Carnegie, 2015). The haplosporidan parasite infecting *P. nobilis* is extracellular in the initial stages (uni-nucleate cells and early plasmodia) and the sporulation process takes place in digestive diverticula, as only was detected in *H. nelsoni*.

Dynamics of haplosporidans in their hosts is seasonal and depends on environmental parameters. *H. nelsoni* is highly sensitive to temperature and salinity whereas bonamiosis has no a clear correlation with between the disease and environmental parameters (Arzul and Carnegie, 2015). In the case of *P. nobilis*, it is impossible to asseverate if this parasite is new infecting this bivalve species or if the parasite is an old simbiont that has changed their relation with the host due to recent changes in environmental or inner conditions of the host leading a mortality outbreak. In any case, pathogens must be included as a new threat for this species.

In conclusion, this is the first report of a haplosporidan parasite infecting in the Superfamily Pinnoidea. Further studies are required to identify the species and to evaluate the association with the mass mortality that is spreading along the Mediterranean waters.

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References

Arzul, I., Carnegie, R.B., 2015. New perspective on the haplosporidian parasites of molluscs. J. Invertebr. Pathol. 131, 32–42. http://dx.doi.org/10.1016/j.jip.2015.07.014.

Basso, L., Vázquez-Luis, M., Gracía-March, J.R., Deudero, S., Álvarez, E., Vicente, N., Duarte, C:M., Hendriks, I.E., 2015. Chapter three – the Pen Shell, Pinna nobilis: a review of population status and recommended research priorities in the Mediterranean Sea. Adv. Mar. Biol. 71, 109–160. http://dx.doi.org/10.1016/bs.amb.2015.06.002.

Bearham, D., Spiers, Z., Raidal, S., Jones, J.B., Burreson, E.M., Nicholls, P.K., 2008. Spore ornamentation of *Haplosporidium hinei* n. sp. (Haplosporidia) in pearl oysters *Pinctada maxima* (Jameson, 1901). Parasitology 135, 521–527. http://dx.doi.org/10.1017/S0031182008004149.

Burreson, E.M., Ford, S.E., 2004. A review of recent information on the Haplosporidia, with special reference to *Haplosporidium nelsoni* (MSX disease). Aquat. Living Resour. 17, 499–517. http://dx.doi.org/10.1051/alr:2004056.

Carnegie, R.B., Burreson, E.M., Hine, P.M., Stokes, N.A., Audemard, C., Bishop, M.J., Peterson, C.H., 2006. *Bonamia perspora* n. sp. (Haplosporidia), a parasite of the oyster *Ostreola equestris*, is the first *Bonamia* species known to produce spores. J. Eukaryot. Microbiol. 53, 232–245. http://dx.doi.org/10.1111/j.1550-7408.2006.00100.x.

Cochennec, N., Le Roux, F., Berthe, F., Gerard, A., 2000. Detection of *Bonamia ostreae* based on small subunit ribosomal probe. J. Invertebr. Pathol. 76, 26–32. http://dx.doi.org/10.1006/jipa.2000.4939.

EEC., 1992. Council directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora. Off. J. Eur. Commun. Brussels, No L 206. 35, 7–50.

Fernández-Tajes, J., Longa, A., García-Gil, J., Chiu, Y.W., Huang, Y.S., Méndez, J., Lee, R.S., 2011. Alternative PCR-RFLP methods for mussel *Mytilus* species identification. Eur. Food. Res. Technol. 233, 791–796. http://dx.doi.org/10.1007/s00217-011-1574-x.

Ghiretti, F., Salvato, B., Carlucci, S., De Pieri, R., 1972. Manganese in *Pinna nobilis*. Specialia 28 (2), 232–233.

Hine, P.M., Thorne, T., 2002. *Haplosporidium* sp. (Alveolata: Haplosporidia) associated with mortalities among rock oysters *Saccostrea cuccullata* in north Western Australia. Dis. Aquat. Org. 51, 123–133.

Linnaeus, C., 1758. Tomus I. Syst. nat., ed. 10. Holmiae, Laurentii Salvii: [1–4], 1–824.

Perkins, F.O., 1899. Phylum Haplosporidia Caullery & Mesnil, 1899. In: Lee, J.J., Leedale, G.F., Bradbury, P. (Eds.), The Illustrated Guide to the Protozoa. Society of Protozoologists, Lawrence, KS, pp. 1329–1341.

Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Burreson, E.M., 2000. Haplosporidiosis in the Pacific oyster, *Crassostrea gigas*, from the French Atlantic

coast. Dis. Aquat. Org. 42, 207–214.
Shaw, B.L., Battle, H.I., 1957. The gross and microscopic anatomy of the digestive tract of the oyster, *Crassostrea virginica* (Gmelin). Can. J. Zool. 35, 325–347.
Vázquez-Luis, M., Álvarez, E., Barrajón, A., García-March, J.R., Grau, A., Hendriks, I.E., Jiménez, S., Kersting, D., Moreno, D., Pérez, M., Ruiz, J.M., Sánchez, J., Villalba, A., Deudero, S.S.O.S., submitted for publication. *Pinna nobilis*: a mass mortality event in western Mediterranean Sea. Front. Mar. Sci.