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First detection of *Pinna nobilis* infection by *Haplosporidium pinnae* in the sanctuary area of Thau lagoon, France

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Abstract

The noble pen shell *Pinna nobilis* is an endemic and emblematic giant bivalve whose populations have been recently affected by a mass mortality event (MME), primarily due to the spread of the pathogen *Haplosporidium pinnae*. Since the beginning of the MME in Spain in 2016, nearly one hundred percent of monitored open sea water populations have been decimated around the Mediterranean Sea. The only refuge areas with living *P. nobilis* populations were found in coastal lagoons. Today, the Thau lagoon in France is home to a vitally important population of *P. nobilis* and was thought to be safe from the parasite. Here, we report the first molecular detection of *H. pinnae* (isolate PN1) in tissue samples of moribund individuals from Thau lagoon.

Keywords: Bivalvia; Pen shell; *Pinna nobilis*; *Haplosporidium pinnae*; Coastal lagoon; Conservation; IUCN Red list.

Introduction

Since autumn 2016, the Mediterranean endemic fan mussel *Pinna nobilis* is experiencing recurring episodes of mass mortality events (MME) (Vázquez-Luis *et al.*, 2017; Cabanellas-Reboredo *et al.*, 2019), most of them due to the specific parasite *Haplosporidium pinnae* (Catanese *et al.*, 2018; Grau *et al.* 2022). The extent and virulence of the phenomenon have resulted in the classification of *P. nobilis* as a critically endangered species on the IUCN global red list of threatened species (Kersting *et al.*, 2019).

On the French Western Mediterranean coast (Occitanie, France), the first mortality cases were reported in 2018 in the marine reserve of Cerbère-Banyuls (Cadene *et al.*, 2018). By the end of 2019, the populations located in the Agathoise Coast Marine Protected Area were, in turn, also affected by the epidemic (City of Agde, pers. comm.). By 2020, except for Thau lagoon, all known populations on the Occitan coast, including those from a few lagoons, seemed to be infected by the parasite (Peyran *et al.*, 2022, Grau *et al.*, 2022). Before 2021, populations living in a few coastal lagoons appeared to have been spared by the epidemic, as could be observed in the Diana and Urbinu lagoons in Corsica (Simide *et al.*, 2019; Foulquié *et al.*, 2022), or Thau lagoon (Foulquié *et al.*, 2020).

Hence, these lagoon populations have been considered in France as sanctuaries for *P. nobilis*.

However, following a field survey with tissue sample collection during summer and fall 2020, molecular infection with *H. pinnae* could be detected for the first time in individuals from Thau lagoon.

Materials and Methods

P. nobilis monitoring

Five sites presenting contrasted population densities were surveyed between May and December 2020 : Barrou (43.4226500° N, 3.6779500° E), Mèze plagette (43.4232833° N, 3.6098000° E), Lafarge (43.4310000° N, 3.7035167° E), Ponton (43.4245167° N, 3.7003833° E), and Sète (43.4231667° N, 3.7034333° E) (Fig. 1).

For the Barrou and Mèze plagette stations, random transects of 50 m (100 m²) were carried out respectively in May 2020, between 0.5 and 2.5 m depth (Foulquié *et al.*, 2020). Survey dives carried out at these two sites in October 2020 revealed a mortality rate of around 55 % at the Barrou station (Foulquié pers. obs.). Regarding the Lafarge station, apart from the impact study carried out by Seaneo in June 2017 (Fabre & Dalías, 2017), du-

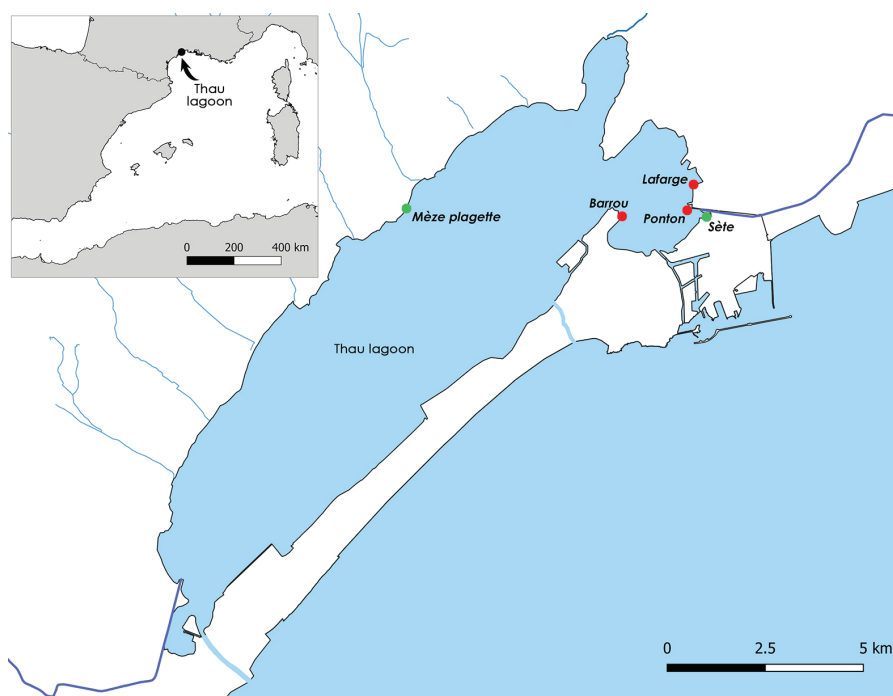


Fig. 1: Map of sites monitored for *P. nobilis* in Thau lagoon, by the end of 2020 (red dots: mortality observed, green dots: no mortality).

ring which the presence of *P. nobilis* was not reported, no specific monitoring had been undertaken before the surveys carried out between July and October 2020. Two random dives were carried out in July and August 2020 to check the presence of *P. nobilis*. Thirty-four living individuals together with four dead individuals were observed. In October 2020, a third dive enabled observation of an increase in the mortality rate reaching more than 50% (Foulquié pers. obs.).

The information and data concerning the Ponton site comes from surveys carried out by diving clubs, as part of citizen science. During the last census carried out in December 2020, divers were able to observe a mortality rate of around 85 % [Pascal Girard (FFESSM), unpublished data].

The Sète station was surveyed in November 30, 2020 along a random 200 m transect where 34 living individuals were observed, with no signs of mortality (Foulquié pers. obs.).

P. nobilis tissue sampling and nucleic acid preparation

Tissue biopsies using a non-lethal method described elsewhere (Bunet *et al.*, 2021) were conducted on 6 *P. nobilis* (Table 1): 4 healthy individuals from Mèze plage and two moribund individuals from the Lafarge site exhibiting symptoms of the disease during summer 2020 (Fig. 2).

Individuals of *P. nobilis* were sampled by scuba diving using a non-lethal sampling method specifically developed to avoid manipulation of *P. nobilis*. A small piece (6 mm², ~15 mg) of the mantle tissue was quickly excised using biopsy forceps before the fan mussel closed its valves. The tissue was transferred in a tube filled with 95% ethanol and stored at -80°C until use. Total DNA

was isolated using the Nucleospin® tissue kit from Macherey-Nagel. DNA quantity was measured using the Qubit™ dsDNA HS Assay kit (Invitrogen), and quality was checked on a standard 0.8% agarose gel.

Haplosporidium pinnae detection

The presence of the parasite *H. pinnae* within the animals was assessed using quantitative PCR, as previously described by López-Sanmartín *et al.* (2019). *H. pinnae* was also detected using NGS data from whole genome sequencing (Illumina 2x150 bp paired-end sequencing, GENEWIZ Germany GmbH, Leipzig). High-quality paired-reads (Q>40) with a read length higher than 150 nucleotides were aligned against the small subunit ribosomal RNA partial gene available in the NCBI (LC338065). The presence of specific reads mapping the reference sequence was assessed visually using IGV (Thorvaldsdóttir *et al.*, 2013) and subsequently assembled in the case of specific read alignments. The obtained sequences were then used as queries in BLASTn to identify the isolates.

Results and Discussion

We monitored *P. nobilis* populations from Thau lagoon between May and December 2020. Five sites presenting contrasted population densities were more closely surveyed: Mèze plage, Lafarge, Ponton, Barrou and Sète (Fig. 1). Except from the sites Mèze plage and Sète where no mortality was observed, we noticed mortality rates ranging from 55 to 100 % at the other studied sites.

Monitoring dives carried out in October 2020 at the Barrou station revealed a mortality rate of around 55 %. Regarding the Lafarge station, by the end of August 2020,

Table 1: Summary of sampling and diagnosis information.

Location	Geographical coordinates of sampling sites	Sampling date	Habitat	Depth (m)	Water temperature at sampling sites	P. nobilis sample id.	Shell height to sediment (cm)	Shell width to sediment (cm)	Shell max width (cm)	Calculated total shell length (cm)	Status (asymptomatic/sick)	H. pinnae PCR test	Sequencing result
Méze plagette	43.4232833° N, 3.6098000° E	30/08/2020	Zostera noltei	2	25°C	Mez1	24	10	14	42,4	Asym	Negative	Negative
Méze plagette	43.4232833° N, 3.6098000° E	30/08/2020	Zostera noltei	2	25°C	Mez2	28	10	18	46,4	Asym	Negative	Negative
Méze plagette	43.4232833° N, 3.6098000° E	30/08/2020	Zostera noltei	2	25°C	Mez3	22	10	15	40,4	Asym	Negative	Negative
Méze plagette	43.4232833° N, 3.6098000° E	30/08/2020	Zostera noltei	2	25°C	Mez4	28	9	17	44,6	Asym	Negative	Negative
Lafarge	43.4310000° N, 3.7035167° E	11/10/2020	Mud sand	2	17°C	Laf1	≈ 20	NA	NA	NA	Sick	Positive	isolate H. pinnae PN1
Lafarge	43.4310000° N, 3.7035167° E	11/10/2020	Mud sand	2	17°C	Laf2	≈ 20	NA	NA	NA	Sick	Positive	isolate H. pinnae PN1



Fig. 2: Moribund *Pinna nobilis* at the Lafarge site showing half-open valves and retracted body (© Mathieu Foulquié).

thirty-four living individuals together with four dead individuals were observed. By the end of October 2020, the mortality rate had reached 100 %.

For the Ponton site, during the last census carried out in December 2020, divers were able to observe a mortality rate of around 85 % [Pascal Girard (FFESSM), unpublished data].

During the survey carried out at the Sète station, by the end of November 2020, thirty-four living individuals were observed, with no signs of mortality.

The molecular diagnosis of the presence/absence of *H. pinnae* in the tissues of the biopsied *P. nobilis* individuals revealed consistent results between quantitative PCR and NGS data (Table 1). DNA signals from *H. pinnae* could be detected only in the two moribund individuals from Lafarge and parasite sequences obtained from these two specimens were found to be homologous (i.e., highest alignment score; query cover >89%; percent identity > 99.75%) to the *H. pinnae* isolate PN1 (MN104247).

A previous larger and geographically more wide-ranging study that investigated the presence of *H. pinnae* by PCR analysis, including 53 samples of mantle biopsies from asymptomatic adults fan mussels from Thau lagoon between June 2019 and January 2020, did not reveal the presence of the parasite (Grau *et al.*, 2022).

Hence, these are the first reported cases of symptomatic infection by *H. pinnae* in Thau lagoon that established the presence of the parasite within this sanctuary area during summer of 2020. The rapid propagation of the disease within the Lafarge site population (100 % mortality in three months), and to the other nearby sites (Barrou and Ponton) by the end of 2020 [Mathieu Foulquié and Pascal Girard (FFESSM), unpublished data], indicates that the parasite would seem to be able to cope with the unusual environmental parameters in the lagoons, such as salinity and temperature (Garcia-March *et al.*, 2020;

Prado *et al.*, 2021; Nebot *et al.*, 2021; Grau *et al.*, 2022). However, these sites are all located in the eastern part of the lagoon (see Fig. 1). The detection of *H. pinnae* at the Lafarge station and the mortality episodes that occurred at the same time at the Ponton and Barrou stations suggest that the parasite probably arrived and spread via the canals of the city of Sète, which directly communicate with the contaminated open sea waters.

It is therefore crucial to monitor on a regular basis the possible expansion of the epizootic disease in these neighbouring populations of *P. nobilis*, which currently represent one of the last significant reservoirs of genitors for this highly threatened species.

Acknowledgements

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Ethics Statement: No experimentation involving removal, dislocation, or killing of *Pinna nobilis* individuals was performed. The authorization to sample tissue of *P. nobilis* in Thau lagoon was obtained from the DREAL (Direction Régionale de l’Environnement, de l’Aménagement et du Logement) of the Occitania Region. **Competing interests:** The authors declare that they have no competing interests. **Authors’ contributions:** MF, SC, NV and RB designed and supervised the study. MF conducted the sampling and field work, SC the laboratory work. MF, RB and SC analyzed the data and wrote the note. All authors read and approved the final manuscript.

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