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**Can we save a marine species affected by a highly infective, highly lethal, waterborne disease from extinction?**

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# Can we save a marine species affected by a highly infective, highly lethal, waterborne disease from extinction?

## Abstract

Anthropogenic drivers and global warming are altering the occurrence of infectious marine diseases, some of which produce mass mortalities with considerable ecosystemic and economic costs. The Mediterranean Sea is considered a laboratory to examine global processes, and the fan mussel *Pinna nobilis* a sentinel species within it. Since September 2016, fan mussels suffer a die-off, very likely provoked by the protozoan *Haplosporidium pinnae*. Population dynamic surveys, rescue programmes, larvae collector installation and protection of infected adults from predators, have increased knowledge about the factors conditioning the spread of the die-off; previous model simulations indicate that water temperature and salinity seem to be related to the manifestation of the disease, which at the end are strongly influenced by climate change and anthropogenic actions. The absence of natural recruitment implies that fan mussel populations are not recovering, but the survival of populations living in paralic environments provides an opportunity to study the disease and its conditioning factors. The present situation is proposed as an example of what is to come in the global context of climate change and poses several questions: are we the witnesses of the potential extinction of a sentinel species? Can we avoid the potential extinction of this species by applying active measures, and which measures will be more effective? How many other more overlooked species might experience a massive and unnoticed die-off before it is too late to implement any preservation action? For the extinction of community structure species will provoke unpredicted ecological cascade effects with global implications.

**Key words:** *Pinna nobilis*; *Haplosporidium pinnae*; Mediterranean endemism; mass mortality; protozoan; parasite

## 1. Introduction

Anthropogenic drivers and global warming are altering the occurrence of infectious marine diseases, in opposing directions depending on taxon and the nature of the impact Tracy et al., (2019). Ectothermic hosts with parasites with environmental transmission stages that can persist outside the host are among the most vulnerable species to global warming (Altizer et al, 2013). Reduction in biodiversity, translocation of species and alteration of natural habitat and community composition due to human action and climate change, facilitate the occurrence of

pandemic infectious marine diseases, with considerable economic and ecosystemic costs (Lafferty, et al., 2015; Harvell et al., 2019). However, both, evaluating whether disease is increasing or decreasing in the ocean, and quantifying anthropogenic drivers of infectious marine disease, is challenging owing to the lack of baseline data and of long-term records (Tracy et al., 2019). On this regard, sentinel species such as the Mediterranean endemic fan mussel *Pinna nobilis* (Basso et al, 2015) could provide information on what is to come to global ecology in the context of the present environment overexploitation. Globally, several authors have pointed out that the Mediterranean Sea could be used as a natural laboratory to study geodynamic and paleoclimatic processes on different scales and as a miniature model of the world's oceans (Krijgsman, 2002; Lejeusne et al, 2010).

Fan mussel populations have been recently devastated by a die-off that is very likely to be associated with the protozoan *Haplosporidium pinnae* (Catanese et al., 2018). The first reports of the die-off occurred in September 2016 in southeastern Spain (Vázquez-Luis et al., 2017), and it has since spread throughout all Spanish Mediterranean coasts, reaching France, Italy, Greece, Cyprus and other Mediterranean countries, in less than two years (IUCN 2018a). To date, data indicate that the protozoan species is specific to *P. nobilis*, leaving other invertebrates unaffected, including the congeneric species *P. rudis* (Catanese et al., 2018). Prevalence and subsequent mortality virtually reach 100% in infected populations. Previously reported deaths caused by protozoa that affect commercial bivalve species such as *Crassostrea virginica* or *Ostrea edulis* can be massive but do not usually reach 100% (Culloty and Mulcahy, 2007; Bower, 2014). Although commercial exploitation of these species has collapsed in some areas, their extinction was never considered a possibility. After many decades, the selection of cohorts that are resistant to parasites (naturally and artificially) has improved the aquaculture of these bivalves (Dégremont et al., 2015). Growing them in low-salinity deltaic water has also been effective for attaining commercially profitable production, although this strategy is compromised during dry years (Soniati et al., 2008). Likewise, previous reported bivalve die-offs in the Mediterranean Sea affecting *Spondylus gaederopus* (Meinesz, 1983; Kersting et al., 2006) and *Arca noae* (Botari et al., 2017) showed reduced mortality percentages and/or extensions compared with observed die-offs of fan mussels. Although dramatic, these die-offs did not suppose a short-term risk of extinction for the affected species.

When in early 2017 a Haplosporidian protozoan was found to be the most likely causal agent of the fan mussel die-off, a group of experts following up the event predicted a high risk that the disease would be spread by marine currents throughout the Spanish Mediterranean coasts and beyond its borders in the following months and years, potentially unimpeded due to the absence of marine barriers (Vázquez-Luis et al., 2017). This observation, together with the characteristic

biology of fan mussels, could, eventually, cause the ecological, if not total, extinction of the species, which would represent an unprecedented process in the Mediterranean Sea. As a consequence, Spanish authorities changed the status of *P. nobilis* from "endangered" to "endangered with extinction" along the Spanish coasts (Orden TEC/596/2019, de 8 de abril). The International Union for Conservation of Nature (IUCN) also raised the attention of neighbouring countries regarding the imminent arrival of the parasite to their coasts, which could be considered for each country along the Mediterranean (IUCN, 2018b). Recently, a compilation of recommended actions has been released by IUCN (IUCN, 2019). A follow-up of as many as possible of the remaining Spanish populations was initiated, while a rescue programme to maintain individuals in captivity before die-off caused by parasite arrival, was proposed by the Spanish experts and started as early as 2017. The follow-up has helped to better understand the spread of the disease, with surface currents being the main factor influencing local dispersion, whereas disease expression seems to be closely related to temperatures above 13.5°C and to a salinity range between 36.5–39.7 psu (Cabanellas-Revoredó et al., 2019). Rescued individuals have provided valuable advances in the study of the Haplosporidian parasite (Catanese et al., 2018), helping to prepare new tools for the quick identification of its presence in samples (qPCR) (López-Sanmartín et al. 2019), to conduct breeding studies, and in general, to try to better understand the aetiology of disease and its global implications. Additionally, a few individuals in affected populations have shown some resilience, remaining sick for weeks, or even months in indoor facilities before dying, some of which were found being eaten alive by opportunistic predators. It was hypothesized that these individuals could potentially resist the parasite, but the weakness caused by prolonged disease made them extremely vulnerable to predators, which killed them before they could recover from the infection. Consequently, an attempt to protect some sick fan mussels from predators was also undertaken.

In the present study, compiled field and ecological data regarding the spread of the die-off in Spain and neighbouring countries between October 2016 and September 2018 are presented. Additional results on the rescue initiative and predator exclusion actions are shown and recommendations provided to improve results for future actions, based on all the acquired knowledge. This information will serve as a baseline for the implementation of protection programmes and to increase the success of active initiatives to save the species from extinction. It will also help to raise awareness and prepare scientists and managers for the future predicted increment of marine invertebrate die-offs provoked by emerging waterborne diseases (Harvell et al., 2002; Fey et al., 2015).

The fan mussel already endured some depletions during the last decades of the twentieth century due to the overexploitation and destruction of its main habitat (*Posidonia oceanica*

meadows), pollution and indiscriminate boat anchoring (Basso et al., 2015, Vázquez-Luis et al. 2015). The species has long been collected for its decorative value (the shell can reach more than one-metre-long), food and bait (Butler et al., 1993). Considerable pressure still exists upon some populations of fan mussels in the Eastern Mediterranean, as its flesh is considered a delicacy (Katsanevakis et al., 2011, 2008). The byssus threads were considered sea-silk or sea-gold by the antic Roman and Greek civilizations due to their soft consistency and iridescent colouration, which were highly appreciated and impossible to imitate in ancient times (Maeder, 2017). The creation of marine protected areas and Natura 2000 sites, as well as the implementation of European laws and Directives (Annex II of the Barcelona Convention (SPA/BD Protocol 1995) and Annex IV of the EU Habitats Directive (EU Habitats Directive 2007)), contributed to a notable recovery of the population in the Mediterranean Sea until late 2016.

## 2. MATERIALS AND METHODS

### 2.1 Follow-up of the die-off

After the first report released by Vázquez-Luis et al. (2017), new stations and areas were added to control the spread of mortality. In total, the evolution of mortality in 58 sites along the Spanish and French Mediterranean coasts is reported in the present manuscript (Fig. 1, Table A1). For all locations, the sampling effort was distributed using control areas of different extensions (minimum 1 unit of 100 m<sup>2</sup>) as the epicentre of the surveys, which usually corresponded to stations that had been periodically surveyed before the onset of the die-off. Adjacent areas were surveyed using extensive explorative sampling (García-March and Vicente, 2006) for comparison to control areas. Citizen scientific data (local diving clubs, professional and amateur divers) was added to increase the number of observations (besides the 58 stations) and the extension of the surveyed areas. These data, mainly focused on the presence of alive/dead fan mussels, were always controlled by experts, either directly, by visiting the area, or indirectly, by using digital imaging, before being scientifically validated (Cabanellas-Reboredo et al., 2019). Given the variability of data sources and sampling periods, data were provided as monthly percentages of living individuals, including all individuals who were alive on the date of the observation, despite some showing symptoms of sickness. Monitoring of the mortality spread included only populations that still had living individuals in October 2016 (i.e., many populations from Andalucía and most of Murcia, except Mar Menor Lagoon, were excluded). Monthly water temperatures for monitored locations were obtained from different sources, including data collected in situ monthly or bimonthly with a multiparametric probe, every hour with in situ data



loggers, averages extracted from daily satellite readings provided by the NOAA (www.seatemperature.org) and local meteorological stations.

## 2.2 Relationship between water temperature and survivorship

To evaluate the relationship between water temperature and mortality in the presence of *H. pinnae*, data from Delta del Ebro (used as the control group) and Port Lligat (used as the experimental group), both from open waters and tanks, were analysed. These data were selected from the whole dataset (Table A1), because 1) the presence/absence of *H. pinnae* had been confirmed by PCR (numerous positives in Port Lligat individuals, and no positive in samples from Delta del Ebro individuals until July 2018) and, 2) both temperature and survivorship had been recorded monthly between November 2017 and September 2018.

## 2.3 Recruitment and larvae collectors

To monitor recruitment and spat survival of fan mussels after the die-off, juvenile collectors were installed between June 2017 and November 2017 in 11 stations along Spanish Mediterranean coasts (Fig. 1). Collectors were based on the designs of García-March and Vicente (2006), Cabanellas-Reboredo et al. (2009) and Kersting and García-March (2017), using plastic mesh-bags with either fishing rod or two plastic mesh-bags inside. The lines of collectors were placed at 1 or 1.5-m intervals between depths of 0.5 m and 20 m, depending on the area: in Delta del Ebro, collectors were installed only at a depth of 0.5 m, and in the remaining locations, the shallowest collector was deployed at a depth of 3 m.

Between October and November 2017, the collectors were extracted and thoroughly examined, and all pinnids that were visible to the naked eye were collected and stored in acclimated and aerated water. All individuals were transported alive to the Institute of Environment and Marine Science Research (IMEDMAR-UCV) facilities within the day of collection. Once at the laboratory, the juveniles were placed in closed circuit water systems and fed the same phytoplankton gel as the adults (see section 2.3.4). Less than 1 ml/day of each species of phytoplankton gel was necessary to feed all juveniles. Water was filtered to 1  $\mu$  and exposed to  $>100 \text{ mWs}^{-1}\text{cm}^{-1}$  ultraviolet radiation.

Additional censuses of recruitment in situ were carried out each time an adult survival station was monitored in the present study.

## 2.4 Rescue of individuals

#### 2.4.1 Extraction of individuals

In February 2017, 16 adult fan mussels were collected from the “Marina Real de Valencia” in the Port of Valencia. Eight individuals were hosted at the IMEDMAR-UCV and the other 8 at the Oceanogràfic Aquarium of Valencia. Given the fast spread of the protozoan along Spanish coasts during spring-summer 2017, another 100 and 115 individuals were collected from the Alfacs Bay (Ebro Delta, Tarragona) and Port Lligat (Cap de Creus, Girona), respectively, in November 2017 and transported to indoor facilities. The 100 individuals from Delta del Ebro were hosted at the Institute of Agrifood Research and Technology (IRTA), and the 115 individuals from Port Lligat were separated into 4 groups: 50 were hosted at the Institute for Agricultural and Fisheries Research and Training (IFAPA) Agua del Pino, 50 at the Spanish Oceanographic Institute (IEO) - Murcia, 5 at the Oceanogràfic Aquarium of Valencia and 10 at the marine station of IMEDMAR-UCV in Calpe. The institutions were selected by their experience in the maintenance of marine bivalves and/or *P. nobilis* in indoor facilities, and by their availability to host the fan mussels. The number of individuals per institution depended on the space available to host them under the appropriate conditions. To ensure that all collected fan mussels were adults with reproductive capacity, only specimens with a shell length larger than 40 cm were extracted. For the extraction of individuals from La Marina Real de Valencia and Port Lligat, a hole was dug in the sediment close to the ventral part of the shell to liberate the byssus. For the extraction of individuals from the Alfacs Bay, an electric water pump was used on selected pen shells dwelling on unvegetated patches at a low water depth (0.5-0.7 m). Pressurized water was pumped into the ventral part of the individuals to remove the mud and sand covering the byssus. Each individual was maintained with water until later transportation and only exposed to air for a few seconds for placement within transport tanks.

#### 2.4.2 Transport

A truck was equipped with 3 tanks each filled with 300 l of seawater. Constant water aeration was achieved with a portable air compressor. The water temperature was maintained at 14°C, due to a similar ambient temperature during winter or late autumn, when the collection of individuals was performed. During transport, fan mussels were placed within individual mesh containers (5 to 10 individuals per tank) with the antero-posterior axis resting horizontally and either the dorsal or ventral part facing upwards. Although distances from the collection site to

the hosting institution ranged from 4 km to 1270 km, all trips were conducted within a single day, switching drivers when necessary.

#### 2.4.4 Maintenance in indoor facilities

The fan mussels were placed in 400 to 5.000-l tanks in groups of 4 to 12 individuals. Closed circuits were used for the small groups hosted at the Oceanogràfic Aquarium and IMEDMAR-UCV (8 + 8 individuals from la Marina Real and 5 + 10 individuals from Port Lligat, respectively, for each institution). Semi-closed circuits were used for the 50 individuals hosted in the IEO-Murcia (water was renewed twice a week) and IFAPA (water was renewed every two days). Open circuits were used for the 100 fan mussels hosted at the IRTA. In all systems, water was passed through 1- $\mu$ m filters and exposed to UV radiation ( $>100 \text{ mWs}^{-1}\text{cm}^{-1}$ ) before passing through the system with the individuals, following the recommendations for *Haplosporidium nelsoni* and *Perkinsus marinus* by Ford et al. (2001). For the reception of individuals at hosting institutions, the water temperature within the tanks was targeted to be above the minimum comfort limit of 16°C (Trigos et al., 2015). However, due to the large volume of water used in the open circuits, water temperature could only be raised up to 13.9 and 15°C at IRTA and IFAPA, respectively. In smaller closed and semi-closed circuits at the IEO-Murcia, Oceanogràfic and IMEDMAR-UCV, water temperature during reception was maintained at 17°C. All individuals were laid vertically with the anterior end of the shell pointing downwards, but unburied and without sediment. Various supports, such as concrete blocks where the individuals were inserted in holes, or PVC tubes where the individuals leaned on one side, were used to maintain the individuals in the vertical position. Individuals were also arranged to avoid the proximity of inhalant and exhalant chambers between contiguous specimens.

To feed fan mussels, an initial daily mixture of phytoplankton gel (easyreefs®), autoclaved muddy detritus with high organic matter (OM) content and supplements (lyophilized easy SPS®) was supplied in the following concentrations per average individual of 55 cm:

2.4-7.2 ml of *Isochrysis galbana* (0.0120-0.036 g OM/ml)

2.4-7.2 ml of *Phaeodactylum tricornutum* (0.0121-0.0363 g OM/ml)

1.5-4.5 ml of *Tetraselmis chuii* (0.0172-0.0516 g OM/ml)

0.3 g of muddy detritus (10% OM content)

0.1 g of easy SPS (83.88% OM content)

Modifications of the quantity of the various components of the diet were also implemented a posteriori, according to the specific settings of the tanks at each institution, food acceptance and pseudofaeces production of fan mussels. The food was provided diluted in water as follows: continuously (IFAPA), in a single dose (Oceanogràfic Aquarium), two doses (IRTA and IEO-MURCIA), or four doses (IMEDMAR-UCV) daily.

Epibionts living on the shells were cleaned one month after reception of the individuals to minimize the stress of captivity. Shells were bruised maintaining the fan mussels within trays filled with seawater and then returned to the tanks.

## 2.5 Predator exclusion cages

A total of 35 individuals from the Port lligat (Cap de Creus) population were caged in May (30 cages) and June (5 more cages) 2018 -one and two months after detecting the local die-off. Four areas of circa 500 m<sup>2</sup> were selected, with 8 or 9 individuals in each protected by cages (Figure A1). Monthly surveys were conducted until the last of the individuals in the cages died. Every month, the cages were opened and cleaned of epibionts and possible predators that could have entered as juveniles and grown inside the cage.

The cages consisted of a cylinder of 5 mm mesh, 30 cm in diameter and 50 cm in length, with closure of the upper part with a 2-mm mesh. The lower part of the flexible mesh cylinder was fitted with a draw cord closure surrounding the shell of the fan mussel, at the point of protrusion from the sediment, to avoid the entry of predators. The cage was opened and closed at the top with another draw cord closure (Figure A2). Three steel pegs with a length of 1 m were used to attach the cage to the sea floor and provide better adjustment of the structure to the flat shape of the fan mussels.

## 3. Results

### 3.1 Follow-up of the die-off

Table A1 shows the living individuals found in the monitored stations from October 2016 to September 2018. The die-off spread northwards and eastwards with time from southeastern Spain, showing mortality peaks in the hotter months. It is noteworthy that the sampling periodicity was variable among locations and that sometimes surveys were not performed during the hottest months. Several critical points were observed, such as the spread to northeastern Spain (Castellón and Cataluña) in late 2017, the start of mortality in France in mid-

2018 and the onset of mortality in Delta del Ebro in the summer of 2018, but constrained to the outermost stations closer to the open sea. In the Columbretes Islands and Balearic Islands, surveys were also performed of deep populations down to a depth of 45 m. In all stations surveyed, where adult specimens had previously been observed, only empty shells of dead individuals were found (equivalent to 0% survival). Unfortunately, no information was available on the status of deeper populations, the fate of which is unknown to date. By September 2018, only 6 fan mussels were found alive in open waters of Spanish coasts. Excluding the populations from the inner sites of the Delta del Ebro and Mar Menor lagoon, as well as 2 individuals from the Columbretes Islands and 4 individuals from the Balearic Islands, all surveys resulted in the identification of 0% live individuals.

### 3.2 Relationship between water temperature and survivorship

Fig. 2 shows survivorship and temperature data for the stations used in the experiment. In Port Lligat, mortality was first observed in April 2018, when the temperature rose to 14°C from the winter minimum of 12.3°C observed in March 2018 (see Table A1). From that moment, the survivorship curve displayed a highly negative slope, with a total mortality after 5 months from the last observation without mortality. Regarding the tanks in Oceanogràfic, where temperature in the tanks remained constant at 17°C, mortality followed a similar trend to Port Lligat (highly negative slope with total mortality after 5 months from the last observation without mortality). In contrast, at IFAPA, IEO and IMEDMAR, where the water temperature was reduced below 14°C, there was a change in slope of the survivorship curve after reducing the water temperature, maintaining live individuals in the three locations by September 2018. Furthermore, survivorship was lower in the stations where water temperature remained above 15°C for a longer time (i.e., IEO - IMEDMAR - IFAPA in increasing order of survivorship and decreasing order of temperature); at IFAPA, where water temperature was maintained at or below 15°C most of the time, survivorship was the highest.

### 3.3 Recruitment and larvae collectors

Only the collectors located in Castellón and Alicante hosted fan mussel juveniles in October 2017. Additionally, 51 juveniles were found among hundreds of *P. rudis* juveniles recruited in the ropes of the Vilajoiosa fish farm (Alicante) -these juveniles were found thanks to citizen scientific collaborations. Considering *P. nobilis* growth rates (Kersting & García-March 2017) and that by February 2018, those juveniles had a size >20 mm, the spat were most likely recruited

between July and December 2017. Fifty-two juveniles were also found in a shallow bank at a depth of <0.2 m in Alfacs Bay in Delta del Ebro in January 2018. In September 2018, another 30 fan mussel juveniles were recently found settled in Calpe (Alicante) attached to an exclusion cage structure suspended at a depth of 15 m, designed to protect from predators *P. rudis* juveniles recruited the previous season. The juveniles from the Columbretes Islands and Calpe, collected in October 2017, and from Vilajoiosa fish farm, which were transported to aquaria, only grew a few millimetres in the tanks and died. In fact, the longest life span in the aquarium did not exceed 6 months. The 30 individuals from Calpe collected in September 2018 were placed in situ in the same cages where *P. rudis* juveniles were hosted (29 individuals), and one individual was maintained in the tanks. In November 2018, all *P. nobilis* placed in situ were dead (but no *P. rudis* in the same cage, which all remained alive), and the individuals maintained in the tanks was still alive but showed no growth.

Thirty samples of recently deceased *P. nobilis* juveniles from the tanks were analysed for the presence of *H. pinnae* using specific PCR (Catanese et al., 2018). Twenty-five of them were positive, and the DNA quality of the remaining 5 individuals was poor and, thus, provided unreliable results.

Juvenile fan mussels have not been found anywhere else apart from the collectors, the ropes of the fish farm and the predator exclusion cages, in any of the surveys carried out to search for live *P. nobilis*.

### 3.4 Rescue of individuals

#### 3.4.1 Individuals from la Marina Real of Valencia

The 16 individuals collected from la Marina real of Valencia survived during the transport. Temperature was maintained between 17 and 20°C. Of the 8 individuals maintained at the IMEDMAR-UCV, one individual died in May 2017, another two in July 2017 and the remaining 5 in August 2017, seven months after collection. All individuals who died showed symptoms of being affected by the parasite: mantle retraction, lack of response to stimuli and no growth. Samples of the dead individuals were positive to the presence of *H. pinnae* (note that the species was yet unknown by then). Of the 8 individuals maintained at the Oceanogràfic Aquarium, one individual died in April 2017, another two in May 2017 and another one in August 2017. Samples of the dead individuals were positive for the presence of *H. pinnae*. The four remaining individuals, all living in the same tank, showed some symptoms (slight mantle retraction) in August 2017, but they subsequently recovered, extended their mantle again and survived for 18

months until August 2018, at which time all four died. One of the individuals closed the gap in July 2018 and remained in that position until August 2018, at which time it opened the gap, retracted its mantle and died. The other three individuals also retracted their mantle, opened the gap and died in few days. Samples collected after death were positive for infection by the parasite.

#### 3.4.2 Individuals from Port Lligat and Alfacs Bay

All individuals survived the transport. Only one specimen from Portlligat, transported to IFAPA -Agua del Pino (the longest trip of 1270 km), showed signs of stress upon arrival. In two days, this fan mussel had retracted its mantle and opened the ventral part of the gap. When no response to mechanical stress was observed, it was sacrificed. Analysis of the tissues confirmed that it was infected by the parasite. This was the first evidence that the donor population from Port Lligat was already infected when the rescue was undertaken in November 2017. In contrast, 25 random individuals among the 100 collected in Alfacs Bay and stabled at IRTA facilities were subjected to mantle biopsies, all of which were negative for the parasite, demonstrating that the population was free of *H. pinnae* at the time of rescue.

During the November 2017 - January 2018 period, water temperature was maintained at 11.8°C at IRTA, 15°C at IFAPA, 16°C at IEO-Murcia, 17°C at Oceanogràfic Aquarium and 18°C at IMEDMAR-UCV. In February-March, water temperature was raised to 18°C at IEO-Murcia, reduced to 12°C at IMEDMAR-UCV and maintained at 11.8°C at IRTA and 15.5°C at IFAPA. Beginning in April, temperatures were maintained below 14°C at all sites except IRTA and the Oceanogràfic Aquarium (16°C and 17°C respectively). Temperatures of the tanks in the IRTA installations increased the following months similarly to the Delta del Ebro water temperatures, reaching a maximum of 26.2°C in August 2018 (Table A1, Fig. 2).

In January 2018, two months after collecting the individuals, another 11 individuals from Port Lligat died. In April 2018, 63 individuals from Port Lligat were dead. In July 2018, 81 individuals from Port Lligat and 6 from Delta del Ebro had died, and in September 2018, 12 individuals from Port Lligat and 62 from Delta del Ebro remained alive (Table A1). The tests of dead individuals from Port Lligat were positive for the presence of the parasite, but they were negative for the dead individuals from Delta del Ebro. In contrast, the dead individuals in Delta del Ebro were found to be positive for bacterial infection by *Vibrio mediterranei* (Prado et al., 2020).

#### 3.5 Predator exclusion cages

In June 2018, 10 individuals protected by the cages were found dead. Five cages were reused and installed again with the only 5 individuals remaining alive close to the sites where the cages were installed the previous month. In July 2018, only 7 individuals remained alive in the cages, and no additional individuals were observed alive around the monitoring stations. In August 2018, only dead individuals were found inside or outside the cages, leading to the conclusion that the disease affected all individuals irrespective of the effect of predators. Further surveys in subsequent months confirmed the absence of live individuals in Port Lligat.

#### 4. Discussion

The data gathered during the two years following the start of the die-off showed that it is causing the ecological extinction of *Pinna nobilis*. The pathogen is most likely waterborne and spreads with currents (Vázquez-Luis et al., 2017; Cabanellas-Reboredo et al., 2019). Direct observations have shown the fast spread in only two years of the die-off from southeastern Spain to eastern and northeastern Spain and to other countries such as France, Italy, Malta, Greece and Cyprus (IUCN 2018a; Katsanevakis et al., 2019; Panarese et al., 2019). The presence of uni- and multinucleate cells capable of direct infection, together with the occurrence of resistant spores, confer *H. pinnae* a strong infective and spreading capacity and a strong resilience to changes in environmental conditions (Catanese et al., 2018). Thus, the parasite could remain alive in reservoirs such as unknown hosts or sediment and infect new settlers similarly to *Bonamia ostreae* (Culloty and Mulcahy, 2007), thus constraining the repopulation of habitats after local extinction. To date, juvenile settlement has been missing in the affected areas, despite recruits found in seed collectors in the 2017 and 2018 recruitment seasons. In the Spanish Mediterranean, the species has been cornered in the Mar Menor coastal lagoon and the Ebro Delta (Alfacs and Fangar Bays), although the disease had already arrived at Alfacs Bay. Moreover, these locations are heavily anthropogenic habitats, subjected to a number of impacts that make them highly vulnerable and prone to sudden environmental changes (Nasi et al., 2018; Vidovic et al., 2016). These impacts could contribute to compromising the survival of local communities (e.g., Mar Menor lagoon; García-Ayllon, 2018; Quintana et al., 2018) and lead to the near total population extinction of fan mussels throughout the Spanish Mediterranean Sea. It should be noted that, similarly, populations in French lagoons (along the Occitanie coast) have not been impacted to date, also suggesting that, in a few years, lagoons may well be the only areas throughout the Mediterranean Sea where fan mussels will remain. The factors explaining why fan mussels can survive the parasitosis longer in these areas are unknown, but a change in these



hypothetical factors would terminate the only reservoir refuge known to date for fan mussels, with fatal and catastrophic consequences for the species. Natural recolonization of fan mussels in these environments, when conditions again become optimal, will be impracticable due to the barrier of open waters with the presence of the parasite. The few living individuals in open waters may be expected to be unable to reproduce due to their low numbers and isolation, making them dismissible as source of seeds to repopulate decimated populations. Consequently, it is reasonable to assume that *P. nobilis* could be facing total extinction over the medium term unless manipulative actions are undertaken, e.g., artificial repopulation of habitats free of parasite using individuals of surviving populations and repopulation with resistant individuals. This action would be favoured by the features of *P. nobilis* of low inter-population differentiation due to high connectivity and low isolation of populations. Therefore, the hypothesis of small isolated populations is inconsistent for this species (Wesselman et al., 2018). Records of marine invertebrate extinctions are extremely rare (Carlton, 1993; Carlton et al., 1999; CIESM, 2013) and have not been previously documented in a consistent manner. Furthermore, recent die-off events have been mostly related to positive thermal anomalies and prolonged exposure to warmer summer conditions, coupled with water column stability and reduced food resources (Coma et al., 2009; Rivetti et al., 2014). Die-offs due to disease are almost never included among the causes of extinction, with a few exceptions, such as Dulvy et al. (2003). In this regard, the eelgrass Limpet *Lottia alveus* went extinct in the 1930s due to the catastrophic decline of its main habitat, the eelgrass *Zostera marina* from Labrador to New York in the western Atlantic Ocean. In this case, the limpet was unable to adapt to the eelgrass brackish water refuge and became extinct (Carlton et al., 1991). The spread and virulence of the infection by *H. pinnae* and the geographical range of fan mussels, which are endemic to the Mediterranean Sea, provide similarly pessimistic predictions for fan mussels. The capacity to live in paralic environments currently seems to be the only ecological opportunity left to fan mussels to avoid the same fate as the eelgrass limpet. Two questions arise, however. Are we the witnesses of the potential extinction of a sentinel species? Can we avoid the potential extinction of this species by applying active measures, and which measures will be more effective?

#### 4.1 Relationship between water temperature and survivorship

A null hypothesis was postulated according to the observations of mortality after the development of the rescue programme, which seemed to be related to the increased water temperature in the tanks with respect to the water temperature in situ. The (null) hypothesis

was that water temperature would have little if any effect on the parasite, and the die-off would start in Port Lligat independently of the water temperature. Consequently, reducing the water temperature in the tanks would have little if any effect on reducing fan mussel mortality. Alternatively, water temperature and fan mussel mortality would be correlated, and if water temperature was the determinant of infection and the dispersion capacity of the parasite, the die-off would start in Port Lligat as soon as (and only when) the water temperature rose from the winter minimum, and the mortality in the tanks would be reduced if the water temperature was kept at temperatures similar to the winter minimum in the Mediterranean Sea (between 12°C and 14°C). Most remarkable was the observation of the start of mortality in Port Lligat when temperatures rose from 12.3°C to 14°C (Fig. 2), indicating the existence of a threshold temperature for activation of the parasite situated in between these two temperatures. This observation coincides with the model calculations by Cabanellas et al. (2019) and suggests that refuge temperatures for *P. nobilis* are indeed below 14°C. The importance of temperature in the survivorship of fan mussels was reinforced by the higher survivorship in the tanks where a low water temperature was maintained for a longer time. In IFAPA, where the temperature was maintained between 14.1°C and 15.5°C in the December 2017 - March 2018 period, mortality exhibited a lower trend than in the other stations and spiked in April, immediately after the increase to 15.5°C in March. In Oceanogràfic, total mortality of the individuals occurred after maintaining a constant water temperature at 17°C. This result is interpreted as the existence of a range of temperatures between circa 13.5°C and 15°C, at which *H. pinnae* seems to be activated with moderated virulence, but maximum virulence capacity is observed at higher temperatures >15°C. It should be noted that in the tanks, once the temperature was reduced below 14°C, sick individuals could remain alive for several months (in contrast to what occurred when a high water temperature was maintained as in Oceanogràfic). Therefore, mortality of sick individuals who were infected during periods of warm temperatures could occur when the water temperature was cold (<14°C). The Haplosporidian needed a longer time to kill fan mussels at low temperatures, a trend that was clearly observed by the gentle change in slopes of survivorship of IEO, IMEDMAR and IFAPA after reducing the water temperatures. Mortality of sick individuals still occurred for several months at low temperatures before stopping because the reduction of water temperatures did not automatically halt mortality (Fig. 2).

Infections of *Ostrea edulis* with the protozoan *Perkinsus marinus* are also seasonal, peaking in summer and fall and decreasing in winter and spring. Parasite latency could occur during the colder winter months and reactivate with the rising temperatures in spring (McCollough et al., 2009). Likewise, infections of *Haplosporidium nelsoni* acquired in late summer by *Crassostrea*

*virginica* may persist over the winter and cause mortality in April/May of the following year (Ford and Haskin, 1982), as observed with *H. pinnae* in the Port Lligat population.

Regarding the individuals rescued from Alfacs Bay, which were unaffected by *H. pinnae*, the 13.5-14°C threshold affecting their survival was not observed. The mortality sustained by these individuals was, however, due to a bacterial (*V. mediterranei*) infection related to warm summer temperatures when water in the tanks rose above 22°C and led to a spike of mortality above 25°C. Rodriguez et al. (2017, 2018) isolated *V. mediterranei* strains in two sick fan mussels collected in Almería (Spain) in October 2016 and observed 100% mortality in experimental infections of turbot (*Scophthalmus maximus*) and manila clam (*Ruditapes philippinarum*) at 25°C and 24°C, respectively, using these strains. These temperature values are very similar to those observed during the peak mortalities at IRTA. Considering that *V. mediterranei* is a cosmopolitan species (Tarazona et al., 2014) and that its virulence is dependent on hot summer temperatures  $\geq 24^{\circ}\text{C}$ , the bacteria seem to be opportunistic, affecting some weak individuals, but not responsible for the die-offs in open waters. A mycobacterium bacteria has recently been associated with the die-offs observed in the Tyrrhenian Sea (Carella et al., 2019), which suggests that the importance of bacterial infections and the synergistic effects among different pathogens increasing the virulence of the disease should be studied in future research. Conversely, despite the arrival of *H. pinnae* in Alfacs Bay in July 2018, it killed mostly individuals in the external part of the bay, closer to its mouth, suggesting the presence of some ecological barrier constraining its capacity for dispersion or survival in this environment.

#### 4.2 Recruitment and larvae collectors

The presence of juveniles from the summer 2017 reproductive cycle in the larval collections from Castellón and Alicante, the fish farm in Villajoyosa and in Alfacs Bay, suggest that 2017 was a good year for fan mussel reproduction. The origin of these larvae is unknown. Apparently, four main sources were available in summer 2017: Columbretes Islands, Delta del Ebro, continental France and Corsica (Wesselmann et al., 2018). Juveniles and larvae of *Ostrea edulis* may be infected by *Bonamia ostreae*, but mortalities mainly affect oysters older than 2 years (Arzul et al., 2011). *Crassostrea virginica* juvenile oysters exposed to *Perkinsus marinus*, however, are rapidly infected and die (McCollough et al., 2009). The presence of fan mussel juveniles in collectors and the fish farm indicates that at least they can survive for a few months in open waters. However, if fan mussel juveniles could survive for up to two years, like those of *O. edulis* infected by *B. ostreae*, at least some of the spat that settled in summer 2016, the size of which should be approximately 20 cm by summer 2018 (García-March et al., 2007; García-March et al.,

2011; Kersting & García-March 2017), should have been spotted already. Data gathered to date, however, show that the affected areas are not being repopulated naturally by *P. nobilis*. The confirmed infection of the *Pinna nobilis* juveniles maintained in tanks in the present study, as well as the lack of observations of new juveniles in situ to date, indicate that the juveniles are also being infected by the protozoan and that the response is more similar to that of *C. virginica* infected by *Perkinsus marinus*. This observation differs from the dynamics of infectious diseases of other marine invertebrates, such as the sea urchin *Diadema antillarum* in the Caribbean. Shortly after the onset of the die-off suffered by this species in 1983-1984, natural recruitment was observed, probably from larvae that were already present in the plankton upon arrival of the disease. During the subsequent 30 years, episodic low-level recruitment events of individuals were observed. These new recruits, however, subsequently succumbed to mortality, which was interpreted as the consequence of an unknown factor of post-settlement mortality. The populations of *D. antillarum* have recovered to 12% of those before the die-off because recruitment in populations after the post-mortality period was persistently limited for decades, despite a potentially slowly increasing larval supply (Lessios, 2016). In comparison to the absence of natural recruitment observed in fan mussel populations after mortality, a similar outcome after 30 years seems to be optimistic scenario. Considering the extra constraints encountered by fan mussels to repopulate affected areas, we may be the witnesses of its potential extinction. The recovery of fan mussel populations, if possible, will, in any case, be a long-term process lapsing for many decades. Previously reported die-off events of Mediterranean invertebrates were usually caused by warming seawater temperatures and anomalous low circulation patterns during summer, both in deeper (Garrabou et al., 2009, 2001) and shallow habitats (Kersting et al. 2013), and have not affected *P. nobilis*. These events, however, were usually punctual and did not kill the entire populations, so densities could be recovered through recruitment after a few years (Cerrano et al., 2005). The present die-off experienced by fan mussels is more similar to those observed for *O. edulis* and *C. gigas* and the eelgrass Limpet *Lottia alveus* (Carlton et al., 1991). Ultimately, natural populations of *Pinna nobilis* in the Mediterranean Sea are likely to disappear in the next few years, with the exception, perhaps, of lagoonal population. Such a pandemic is very unusual in marine organisms and raises questions concerning actions that should be undertaken in the very near future, before all open sea population go extinct. These actions should be planned considering the emergency, because the disease has eliminated fan mussel populations from Spanish coasts and spread to most of the Mediterranean Sea in just a couple of years.

#### 4.3 Rescue of individuals

The rescue programmes have shown that fan mussels are resilient to extraction and transport, even for long distances, as described in the present study.

#### 4.3.1 Individuals from la Marina Real of Valencia

Maintenance temperatures (above 14°C), probably accelerated the rapid death of the fan mussels collected from la Marina Real of Valencia, especially at the IMEDMAR-UCV installations. These fan mussels were going to be used for in situ reproduction experimentation, and warm temperatures are necessary to facilitate gonad maturation. The first exploratory survey carried out on this population in December 2016 showed no mortality. Two months later, however, when the collection of individuals was undertaken, 40% mortality was already observed. It is worth noting that by then, the pathogen was still unidentified and that the relationship between water temperature and disease virulence was unsuspected. In contrast, it was hypothesized that maintaining individuals within their comfort temperature range (between 16°C and 24°C) (Trigos et al., 2015) could help improve their capacity to fight the disease under captivity conditions. The higher water temperature maintained at the IMEDMAR-UCV facilities probably accelerated the mortality of the fan mussels, which were all dead 7 months after collection. The four individuals who survived in good condition for 18 months in the Oceanogràfic Aquarium were maintained at a constant temperature of 17°C. These individuals should have died if infected because that temperature was sufficiently high to cause the rapid death of infected individuals. It is possible that these fan mussels had some kind of resistance and were maintaining the protozoan in a chronic condition and that an uncontrolled weakening of their immune system could have led to their final death. Alternatively, the individuals were “luckily” free of the protozoan when collected, and remained uninfected until July 2018, when the tank could have been unintentionally exposed to the protozoan, provoking rapid death of the fan mussels. Regardless of the causes, as the individuals came from an affected population and survived for 18 months, prolonging the life of fan mussels rescued from affected areas is possible.

#### 4.3.2 Individuals from Port Lligat and Alfacs Bay

In September 2018, 12 individuals (11%) among those rescued from Port Lligat were still alive, when the donor population had already disappeared and no individuals could be found in situ since one month prior. The main factor constraining the mortality in tanks was the declining

water temperature below 14°C. Again, rescuing individuals prolonged their life, confirming that it is possible to save fan mussels originating from infected populations. It is worth noting a priori that only those individuals who have not been infected are expected to survive, which means that survivorship will depend on the prevalence of the infection at the time of collection. The survival was much higher for individuals from Alfacs Bay, which was expected because the disease had not arrived in this region when the individuals were collected. However, the mortality observed throughout the stabling period was produced by the bacterium (*V. mediterranei*), which is also known to infect other species of bivalves (Tarazona et al., 2014). With the new knowledge of the occurrence of *V. mediterranei* in *P. nobilis* maintained in captivity, samples analysed for the presence of *H. pinnae* should be re-checked for bacteria to evaluate the possibility of cohabitation of other pathogens in fan mussels.

#### 4.4 Predator exclusion cages

All fan mussels used in the experiment died in August 2018. Higher numbers of cages could favour the survival of some individuals –the strongest–, however, installing and maintaining one or two orders of magnitude more cages in situ would be highly demanding for the low level of expected success (only a few individuals would be expected to survive). Considering these results, it seems more reasonable and affordable to rescue individuals than encage them. Comparatively, however, the most positive impact of encaging vs. rescuing individuals is that, because of the cage, a survivor would have stronger resistance to *H. pinnae*, which would make it much more capable of fighting the die-off.

#### 4.5 Potential actions and recommendations

Based on the information gathered over the previous two years, a series of actions and recommendations for improvement of the survival of fan mussels can be undertaken. These actions will allow extra time and hence a greater probability of survival to *P. nobilis* as a species, but whether their implementation will avoid fan mussel potential extinction or not is currently unpredictable, because extinction depends on many uncontrolled (and some yet unknown) variables. One imminent action should be the protection of the remaining lagoon populations by eliminating or minimizing activities that could create a threat to fan mussels living within. Protecting individuals in cages seems to be inefficient unless large numbers of specimens are protected, and even so, the success of using more cages would need to be re-evaluated. Alternatively, rescuing individuals seems to be a good option even if they originate from an

affected population. Another important issue is to understand why individuals living in paralic environments and coastal lagoons are not affected by the die-off (e.g., Mar Menor Lagoon) or are less severely affected (Alfacs Bay). It is not clear if it is a question of temperature and/or salinity, or of some chemical condition occurring in these environments, preventing direct (by killing it) or indirect (by killing its potential vector) entry of the parasite. Although both Mar Menor lagoon and Ebro Delta do not show extreme salinity variations compared with those occurring in the Caloosahatchee estuary in Florida (La Peyre et al., 2003), salinities are hiperhaline and hipohaline, respectively, compared with the Mediterranean Sea. *H. pinnae* could be unadapted to these variations in water temperature and/or salinity (Cabanellas et al., 2019). Alternatively, fan mussels surviving in these environments could possess some kind of resistance. Studying the environmental conditions of the lagoons and the genetic differences between individuals living inside and outside coastal lagoons and deltas could help to better understand this issue. Additionally, gathering more information about other fan mussel populations surviving in paralic environments in affected areas would improve understanding of the factor(s) conditioning their survival, by comparison of coincident environmental features among them. Studies of reproduction in vitro are fundamental to close the life cycle of *P. nobilis*. Once reproduction in vitro is mastered, it could be applied to resistant individuals to grow resistant spat to repopulate devastated fan mussel populations. Finally, extensive searches for resistant individuals in already affected populations is of paramount importance, since these individuals could provide the seeds for future open water repopulations with artificially produced juveniles.

Regarding the specific recommended action of the rescue of individuals, it is advisable to extract fan mussels prior to the arrival of the parasite. Otherwise, small groups of no more than 5 to 10 individuals should be collected to enable the selection of only those that seem healthy, i.e., total mantle extension and a quick closing response of the valves in response to external stimuli. After placing them in cold water, preferably 12.5°C, individuals should be checked daily for mantle extension during the following weeks to retain only those that show total mantle extension. It is recommended to place no more than 5-6 individuals per tank in independent systems with acclimatizers. If any individual is infected, it may infect others in the same tank, particularly if the water temperature rises above 14°C. Hence, it is very important to maintain low temperatures and quickly isolate any individual showing any symptom of the disease. It is advisable to obtain mantle samples of all individuals, conserve them in absolute ethanol, and analyse them for the presence of the parasite by PCR (Catanese et al., 2018; López-Sanmartín et al. 2019). *Perkinsus marinus* and *H. nelsoni*, infecting *Crassostrea virginica*, undergo reduced transmission and prevalence at winter temperatures from 10-15°C -although *P. marinus* can

survive at temperatures as low as 4°C (Soniat et al., 2008). Repeated freshets can also reduce *P. marinus* infections in *C. virginica* to low levels, resulting in an overall low prevalence, low oyster mortality and good growth (La Peyre et al., 2003). *Pinna nobilis* can survive in paralic environments subjected to important seasonal salinity and temperature oscillations. Although there is a knowledge gap regarding its tolerance limits, the habitat distribution suggests it is a euryhaline, eurytherm species. Considering that the extreme salinity of Delta del Ebro and Mar Menor is one of the possible environmental variables preventing infection of fan mussels by the parasite, it would be worth determining whether fan mussels can survive low-temperature (<8°C) and extreme salinity (<15 psu or >45 psu) treatments. Based on the data published by Cabanellas-Reboredo et al. (2019), it is reasonable to suspect that *H. pinnae* transmission and replication may be constrained at least by low temperatures and probably also by extreme salinities, as observed for other similar protozoans. A combined treatment with low temperatures and extreme salinities could be a good option to maintain individuals during the initial weeks of quarantine. This strategy could reduce transmission among individuals, while healthy and sick individuals are separate. Furthermore, it would be worth examining whether the prevalence of the parasite in infected individuals could be reduced with these kinds of treatments.

In conclusion, one additional global view is provided. The Mediterranean Sea has been considered a laboratory to examine global processes (Krijgsman, 2002; Lejeusne et al., 2010) using *P. nobilis* as a model species (Basso et al., 2015). When the fan mussel die-off started, many research teams were already studying the species because of its ecological and social interest. As previously suggested by Dulvy et al. (2003), marine extinctions may be underestimated because of low detection power and a long-term lapse (on average 53 years) between the last sighting of an organism and the reporting of the extinction. If a species of interest that is closely monitored, such as *P. nobilis*, has been shown to experience such a sudden and fatal die-off event, practically eliminating its populations in two years, How many other more overlooked species might experience a massive and unnoticed die-off before it is too late to implement any preservation action? In the present context of anthropogenic impacts and climate change, the case of fan mussels shows that the virulence and speed of mass mortality events may escalate in the following years. Quick responses are going to be necessary, and researchers and stakeholders will have to be ready to act collaboratively in order to respond to the new threats. Furthermore, the present pandemic shows that the effects of infectious diseases have important implications in the future redistribution of species and biodiversity, which would justify their consideration in prediction models (e.g. Jones and Cheung, 2014), because their effects are global and occur in very short time periods, compared even to the



speed of climate change, and may have considerable structural effects in the biological community (Harvell et al, 2019).

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## **7. Compliance with Ethical Standards**

- The submitted work results from all original research conducted by the authors.

- All authors agree with the contents of the manuscript and its submission to the journal.
- No part of the research has been published in any form elsewhere, unless fully acknowledged in the manuscript.
- The manuscript is not being considered for publication elsewhere while it is being considered for publication in Biological Conservation.
- All research in the paper not carried out by the authors is fully acknowledged in the manuscript.
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- All appropriate ethics, permissions to manipulate animals and other approvals were obtained for the submitted research.

## 8. Competing interests statement

There are no competing interests.

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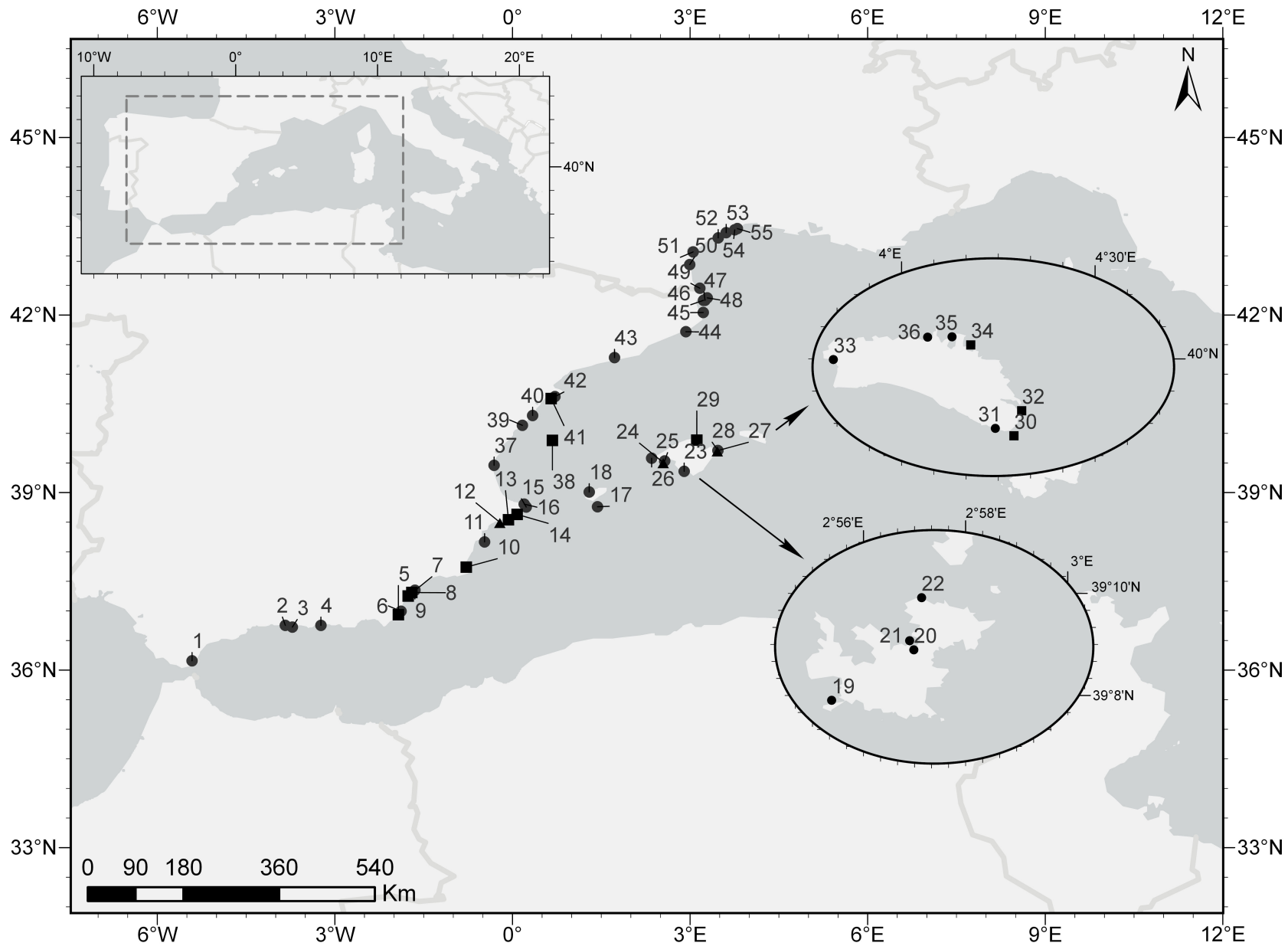
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Fig. 1. Distribution map of the stations monitored for population dynamics and installation of larvae collectors. Circles indicate population dynamic stations, triangles indicate stations of larvae collectors and squares indicate stations with both population dynamic and larvae collector stations. 1, Algeciras. 2, Caleta Maro. 3, Marina del Este. 4, Melicena. 5, Agua Amarga. 6, Isla de San Andrés. 7, Loza del Payo. 8, El Calón. 9, Terreros. 10, Mar Menor. 11, Illa de Tabarca. 12, Villajoyosa. 13, Serra Gelada. 14, Calpe. 15, Illa Portixol. 16, Cap de Sant Antoni. 17, Illetes. 18, Cala Gelada. 19, Rates (20 m). 20, Santa María (10 m). 21, Santa María (20 m). 22, Morobuti (30 m). 23, Cap Pinar. 24, Magaluf. 25, Portals. 26, Sant Elm. 27, Cala Ratjada. 28, Cala Gat. 29, Pollença. 30, Illa de l'aire. 31, Benibecar. 32, La Mola. 33, Sa Farola. 34, Son Saura. 35, Fornells-Cabra Salada. 36, Cala Mica. 37, Marina Reial. 38, Illes Columbretes. 39, Prat de Cabanes. 40, Serra d'Irta. 41, Bahía dels Alfacs. 42, El Fangar. 43, Canyelles. 44, Tossa de Mar. 45, Illes Medes. 46, Montjoi. 47, Joncols. 48, Port Lligat. 49, Peyrefite. 50, Etang de Leucate. 51, Etang de Ayrolles. 52, Agde. 53, Etang de Thau. 54, Frontignan. 55, Les Aresquiers.

Fig. 2. Survivorship curves and water temperature in Ebro Delta and IRTA (DeltaIrta) (top panel), Port Lligat (medium panel) and the tanks in IEO, IFAPA, Oceanogràfic and IMEDMAR-UCV (bottom panel). When two lines of a similar type occur in a panel, thick lines represent the water temperature and thin lines the survivorship for the same tanks. In the top panel, AlfacsZ1C1 (Alfacs circle 1, zone 1) and AlfacsC1C2 (Alfacs circle 1, zone 2) share the same water temperature (AlfacsT). Note that water temperature in Port-Lligat (medium panel) corresponds to the closest monitoring station located in open waters of Startit (one mile off the Medas Islands). Port-Lligat is an enclosed small bay with in situ recorded water temperature during the rescue programme conducted in November 2017 below 13°C. Therefore, the registered water temperature at Startit was higher than the directly observed water temperature in Port Lligat in November 2017.



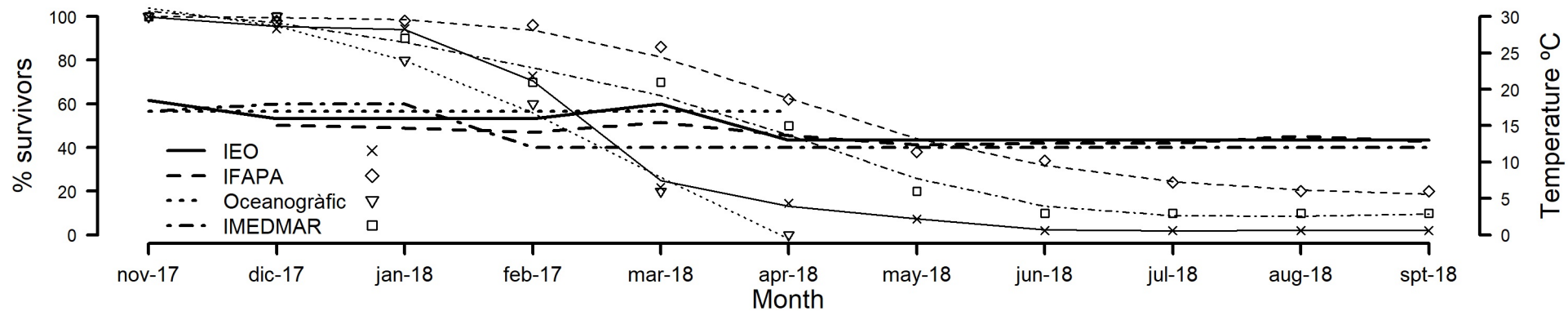
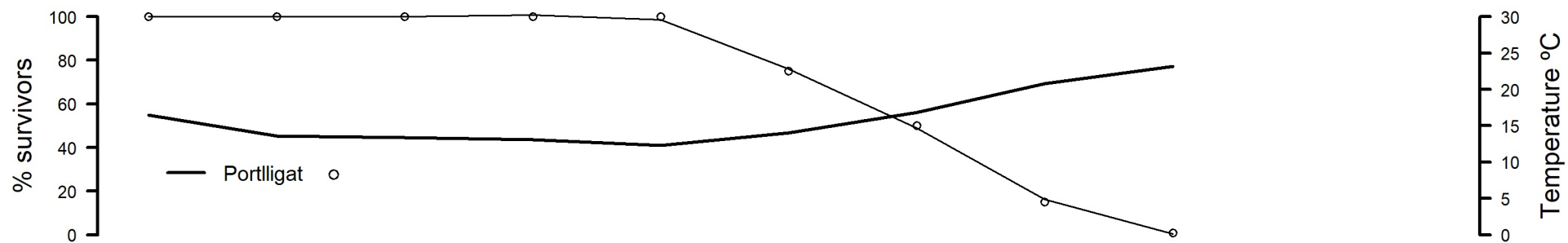
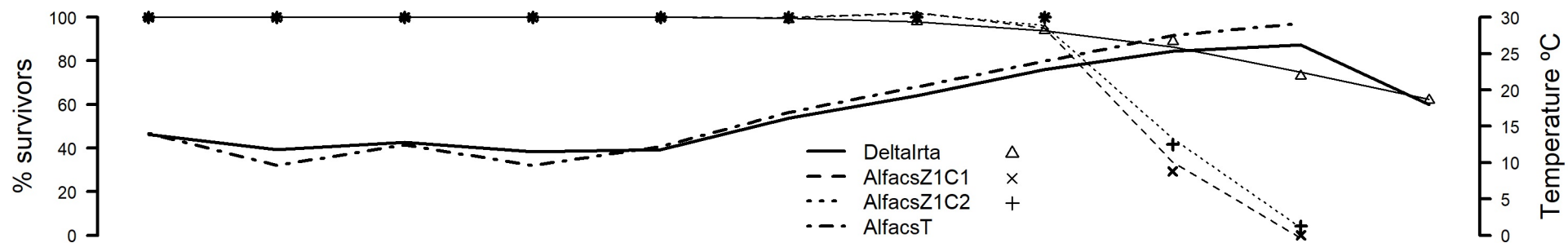




Fig. A1. Location of the stations where the fan mussels were protected with predator exclusion cages. Numbers in brackets indicate how many fan mussels were caged at each site.

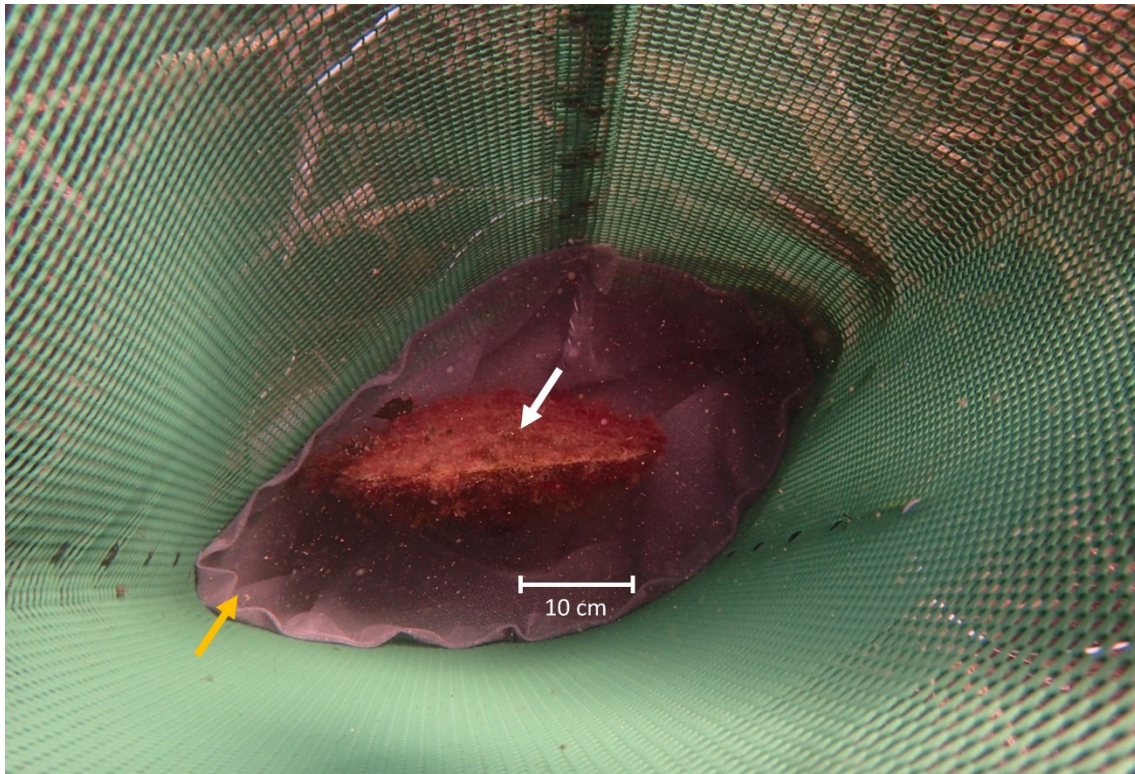


Fig. A2. Example of the interior of a predator exclusion cage used in Port Lligat (zenith view). White arrow shows the fan mussel, whereas the yellow arrow points the bottom cage closure that is wrapped around the shell to avoid the entry of predators through the base of the cage



### **Conflict of interest**

- The submitted work results from all original research conducted by the authors.
- All authors agree with the contents of the manuscript and its submission to the journal.
- No part of the research has been published in any form elsewhere, unless fully acknowledged in the manuscript.
- The manuscript is not being considered for publication elsewhere while it is being considered for publication in Biological Conservation.
- All research in the paper not carried out by the authors is fully acknowledged in the manuscript.
- All sources of funding are acknowledged in the manuscript, and the authors declare no direct financial benefits that could result from publication.
- All appropriate ethics, permissions to manipulate animals and other approvals were obtained for the submitted research.

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