21st INTERNATIONAL EAFP CONFERENCE 2023 ABERDEEN
DISEASES IN FISH AND SHELLFISH 11-14 September 2023

21st International Conference on Diseases of Fish and Shellfish
11 - 14 September 2023
Aberdeen, UK

LIFE20-NAT/ES/001265

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Phagocytosis based assay for an *in vitro* assessment of immunocompetence of the penshell *Pinna nobilis* during *Mass Mortality Events*

What happened: Mass Mortality Events of the mediterranean penshell *P. nobilis* since 2016

What we found: Animal immunosuppression

Next steps: Repeat the experiment on a bigger number of animals; TEM observation of immune cells;

INTRODUCTION

Mass Mortality Events (MMEs) close to 100% of the populations affecting the noble pen shell *P. nobilis* have been reported since 2016. Currently, residual populations are present only in enclosed bays and lagoons in few countries and conservation efforts have been made to maintain individuals in indoor facilities. The mortality events are presumably due to the cooperation of a parasitic and bacterial pathogens, along with possible additional opportunistic pathogens, suggesting an underlying reduced capability to actively respond to pathogenic stimuli.

AIM OF THE STUDY

Use an *in vitro* test for study phagocytosis, coupled with cytology, to evaluate animal immunocompetence.

METHOD

The study was performed in July 2021 and May 2022 on two natural population from the Ebro Delta (Catalonia, Spain) and animals maintained in captivity at IMEDMAR-UCV (Catholic University of Valencia) system and Murcia Aquarium (September 22) (Murcia). Hemolymph was collected from animals in the field and in captivity as a non-destructive sampling. After the procedure, the animals were immediately relocated into the water bottom/in the tanks and checked the next days for health state. (**Figure A**). From each bivalve, a hemolymph aliquot was used to define the cell types and counted to obtain the Total Haemocyte Count (THC). The phagocytic activity was measured as follow: PH rodo green BioParticles (Invitrogen) conjugated with *Staphylococcus aureus* and *Zymosan* A were incubated with *P. nobilis* hemocytes at 25°C at two different timing (30 min and 3 h). After the stimulation, the cells were read at a FACScalibus Flow Cytometer (**Figure B**). The number of positive cells was measured as the percentage of cells showing MFI (Mean Fluorescence Intensity) higher than the negative control and evaluted as Mean value among individuals. The phago-lysosome fusion efficiency was also evaluated.

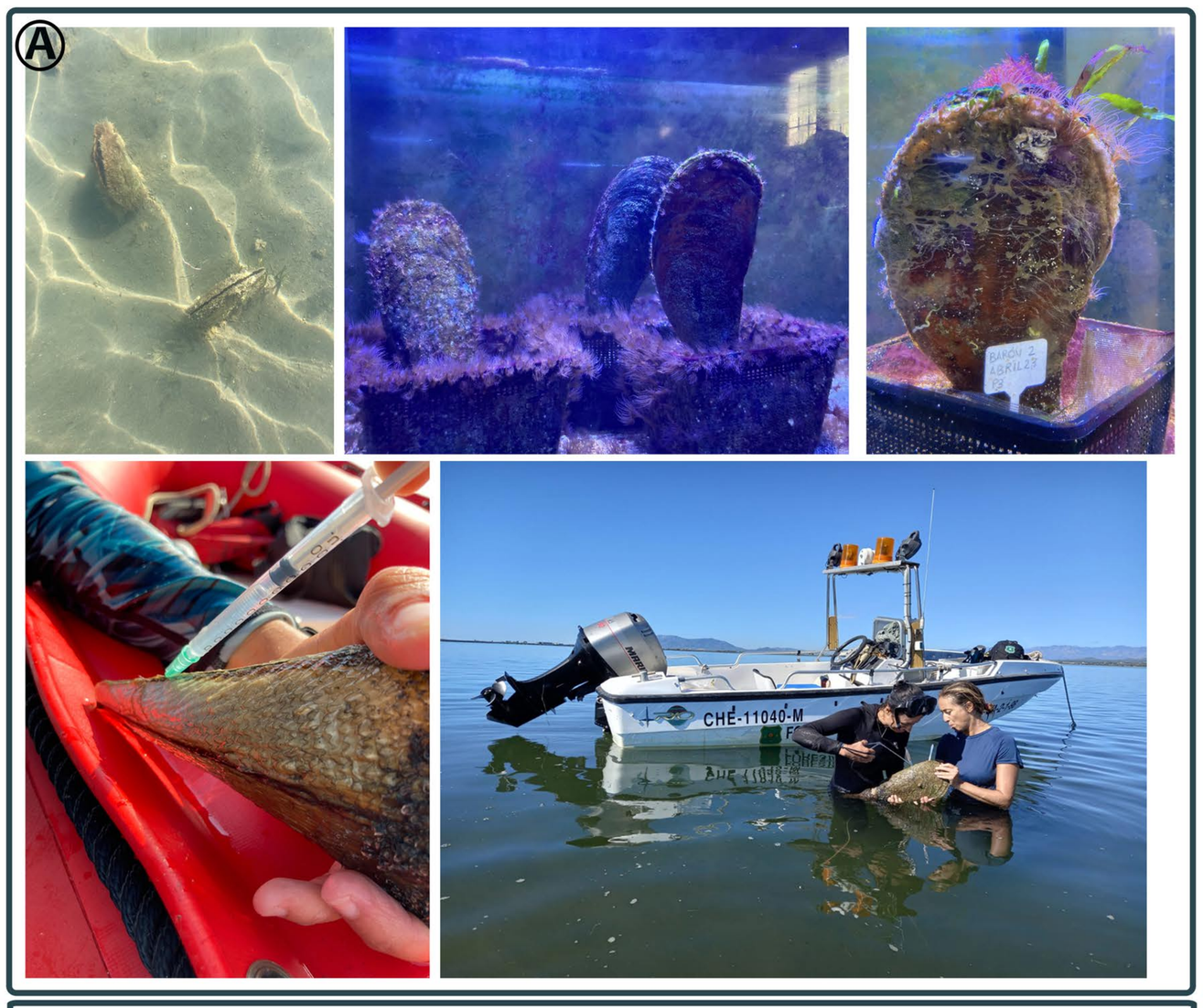
RESULTS

Flow cytometry showed the presence of 3 types of heamocytes constituted by a Type 1, rich in granules (68-70%)(white arrow), a Type 2 smaller agranular cells (13-35%) (blue arrow) and a less represented Type 3, bigger and with granules (3-5%) (black arrow) (**Figure C**). Animals in captivity at IMEDMAR-UCV and Murcia Aquarium had a significantly lower THCs compared with natural population of Alfacs Bay (mean number of 7-9 x 10^4 vs 2-5 x 10^5 cells/mL, respectively) and also varied markedly among individuals (**Figure D**). In the two natural population of Alfacs Bay in July 2021, the number of phagocyic cells MFI reached the 70% after 3h only in one individual, while the other displayed a MFI value between 30-40%. In animals in captivity, in both the seasons, scarce or absent ability to phagocyte the two stimuli was present. In May 2022, at 30 minutes of incubation, MFI of natural population ranged between 10-30% with both the stimuli and increased to 30-50% after three hours. The capability of phagolysosome fusion, represented by a second peak, was observed only in few individulas, after 3h, depending on the stimuli and visible with cytology (**Figura E-F**). *Pearson* correlation of THC values was positive and strong (p-value = 0.7) to the MFI of the 3h experimental conditions.

CONCLUSIONS

-This represents the first in vitro study of *P. nobilis* immunity during MMEs

-The study showed strong immunodepression of captive animals and a scarce capacity of natural population to respond to pathogenic stimuli.



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Funding:LIFE PINNARCA grant number LIFE20 NAT/ES/001265)