LIFE20 NAT/IT/001122

LIFE PINNA

Conservation and re-stocking of the Pinna nobilis in the western Mediterranean and Adriatic sea

PROTOCOLS FOR COLLECTION AND TRANSPORT OF ALL THE ORGANISM AND SMALL FRAGMENTS TISSUE

DELIVERABLE C 5.1

Life Pinna

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Conservation and re-stocking of the *Pinna nobilis* in the western Mediterranean and Adriatic sea

AC/Task responsible: ARPAL

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0. Summary

Deliverable C 5.1 brings together all the technical protocols developed as part of the LIFE PINNA project to support the monitoring, conservation and controlled reproduction of Pinna nobilis. The documents provide a standardised description of the procedures adopted by the partners to ensure quality, reproducibility and methodological consistency in field and laboratory activities.

The protocols cover the entire operational cycle of Action C5, including:

- **Collection and management of sentinel organisms** used to monitor the presence of environmental pathogens.
- **Non-lethal sampling methods** (mantle tissue and mucus swabs) for genetic and diagnostic analysis.
- **Procedures for the transport and storage of biological samples**, with particular attention to the cold chain.
- **Transport, acclimatisation and tank maintenance of Pinna nobilis adults**, including feeding strategies and environmental parameter management.
- **Controlled reproduction protocols**, including gametogenesis induction, spawning stimulation, fertilisation and larval rearing.

The deliverable is an essential technical reference for the management of the species, consolidating the best practices developed during the project and making them available for future conservation initiatives.

1. Introduction

This **Deliverable C 5.1** is the official compendium of technical protocols developed, optimised and applied within the **LIFE PINNA** project, dedicated to the conservation, monitoring and controlled reproduction of the species Pinna nobilis, now classified as critically endangered. The definition of standardised procedures is one of the key objectives of the project, as it ensures methodological uniformity among partners, reliability of the data collected and transferability of knowledge to management bodies, research institutes and future conservation programmes.

LIFE PINNA has operated in a complex context, characterised by the need to integrate field activities, genetic and pathological analyses, captive management and reproductive experiments. To meet these needs, the consortium developed a series of operational protocols covering the entire cycle of activities envisaged by Action C5, from the collection of sentinel organisms to the management of larvae produced in the laboratory. The documents collected in this deliverable therefore represent a complete methodological corpus, the result of joint work between scientific, technical and institutional partners, and based on experimental evidence gained during the project.

The protocols developed concern in particular:

• **Collection and management of sentinel organisms** used to monitor the presence of environmental pathogens potentially involved in the mortality of *P. nobilis*. The procedures describe both the collection of wild individuals and the use of farmed and subsequently acclimatised organisms, with particular attention to maintaining the cold chain and preventing contamination.

• **Non-lethal sampling methods, including the collection of coat fragments and mucus swabs**, which are essential for obtaining genetic material and for diagnostic analyses without compromising the survival of individuals. These protocols are based on scientifically validated methodologies that have been further adapted to the needs of the project.

• **Procedures for the transport and storage of biological samples**, which ensure the correct management of materials intended for genetic and pathological analysis, guaranteeing integrity, traceability and high quality standards.

• **Protocols for the transport, acclimatisation and maintenance in captivity of *Pinna nobilis* adults**, developed on the basis of previous experience and refined during the course of the project. These include the preparation of transport containers, the management of environmental parameters, aeration systems, methods for positioning individuals in tanks and feeding strategies adopted during the various years of experimentation.

- **Controlled reproduction protocols**, which describe in detail the techniques for inducing gametogenesis, methods for stimulating spawning, procedures for spontaneous and controlled fertilisation, and methods for rearing larvae. These documents represent one of the most innovative results of the project, as they bring together for the first time a structured set of experimental practices successfully applied to the species.

Taken together, the protocols presented here constitute a valuable technical and scientific reference, both for the continuation of the project's activities and for future application by organisations and institutions involved in the protection of Pinna nobilis. Their standardisation allows for the consolidation of a shared and replicable approach, promoting data comparability and the dissemination of best practices across the Mediterranean.

Deliverable C 5.1 therefore represents a strategic result of the LIFE PINNA project: a comprehensive, up-to-date and validated methodological archive that contributes substantially to the construction of a solid operational basis for the long-term conservation of the species.

The following paragraph contains all the outlines of the protocols developed:

1) Collection of various types of biological samples: sentinel organisms, mantle tissue fragments, and DNA samples collected via swabs (sections 2.1.1, 2.1.2, 2.1.3).

2) Transport of various types of biological samples: sentinel organisms, mantle tissue samples (sections 2.2.1, 2.2.2).

3) Transport and preservation of adult *Pinna nobilis* specimens (section 2.3).

4) Reproduction of *P. nobilis* specimens in the laboratory (section 2.4).

2. List of Protocols

2.1 Collection of various types of biological samples

2.1.1 Sentinel Organism Collection Protocol

Organism Sampling Methods

In the context of the LIFE PINNA project, sentinel species such as Mytilus galloprovincialis and other filter-feeding bivalves were used to assess the presence of pathogens in marine environments. Unlike non-lethal sampling approaches (e.g., for Pinna nobilis), this protocol involves lethal sampling, allowing for comprehensive tissue analysis. These species were selected due to their filtration capacity and widespread distribution, which make them effective bioindicators of environmental contamination, including pathogens. Importantly, these filter-feeding organisms inhabit the same ecosystems as Pinna nobilis, often sharing similar environmental conditions. As such, they are likely to accumulate the same waterborne pathogens that have been implicated in the mass mortality events affecting Pinna nobilis, including protozoans and bacteria. Their use as sentinel species provides an indirect but valuable means of monitoring the presence and potential spread of these threats in the marine environment.

To ensure the reliability and scientific validity of the results, it is essential that the sentinel organisms used for pathogen detection have been continuously exposed to the local marine environment for a sufficient period of time. Specifically, individuals must have filtered the local seawater for at least three consecutive months prior to sampling. This exposure period is considered critical, as it allows the organisms to accumulate any pathogens or contaminants present in the environment, thereby making them effective indicators of potential threats to Pinna nobilis.

This condition can be satisfied in two ways:

- by introducing farmed individuals into the monitoring site at least three months prior to the scheduled sampling;
- by directly collecting wild individuals that are naturally present in the area.

In the case of farmed organisms, individuals must first undergo a stabulation process—a controlled acclimation phase carried out in clean, monitored conditions. This step is crucial to reduce any pre-existing microbial load or stress-related conditions acquired during farming. Only after this stabulation period are the mussels placed in the selected monitoring sites, where they are left to filter the local seawater for a minimum of three months.

Both strategies are equally valid, provided that there is reasonable certainty that the organisms have remained within the same water body for the required time period without relocation. The selection of wild individuals is often advantageous, as they are already well-adapted to the local habitat and may offer a more representative snapshot of the environmental microbial load.

Live individuals of the selected sentinel species were collected once a month from predefined monitoring sites as part of the environmental surveillance activities. During collection, particular care was taken to minimise external contamination by using clean equipment and handling specimens with gloves or sterilised tools. Immediately after collection, each individual was placed into a sterile container to preserve sample integrity. The number of organisms collected at each site varied depending on local abundance but typically ranged from 10 to 30 individuals per location.

Sample Storage and Transport

Following collection, specimens were stored in thermally insulated containers with freezer packs to maintain low temperatures (~4 °C) and prevent tissue degradation. This step was essential to preserve the biological integrity of the samples prior to laboratory analysis. The organisms were then sent to the laboratories of the University of Sassari, with strict adherence to cold chain protocols throughout the entire journey.

2.1.2 Mantle Fragments Collection Protocol

Mantle Tissue Sampling Methods

For tissue collection from live individuals, we employed a specialised non-lethal sampling method, carried out by scuba divers, which was developed by Sanna et al. (2013, 2014) and shown not to cause significant harm to the shells or soft tissues of *P. nobilis*.

The following steps should be followed for tissue collection:

1. Valve Opening: the valves are held open with a wooden stick (diameter of about 0.5 cm), put in proximity of the hinge ligament (4-5 cm).

2. Sample Collection: a 20–50 mg sample of mantle tissue is carefully excised using Hartmann Alligator Forceps or a similar instrument (see Image). The procedure is as follows:

- Stabilise the Tissue: using a straight Hartmann haemostatic forceps (Image A), gently grasp the flap of mantle tissue to be sampled. The forceps should be placed just below the site where the excision will be made to stabilise the tissue and minimise movement during sampling;
- Perform the Excision: with a curved Hartmann auricular forceps (Image B), carefully excise the tissue sample. The curved design allows precise cuȷ̂ing and helps avoid damage to surrounding tissue. Excise approximately 20–50 mg of tissue.

3. Sample Size and Handling: handle the sample carefully to prevent contamination or loss. Place the tissue immediately into a pre-labelled Eppendorf-type tube (minimum 1.5 ml) containing ethanol at 90% to preserve it for DNA extraction.

4. Finalising the Sampling Procedure: once the sample is secured, gently remove the wooden stick used to hold the valves open, ensuring the valves close naturally without causing stress or damage to the individual.

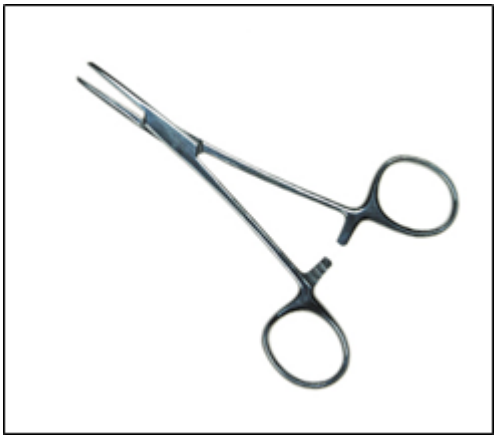

Immagine A

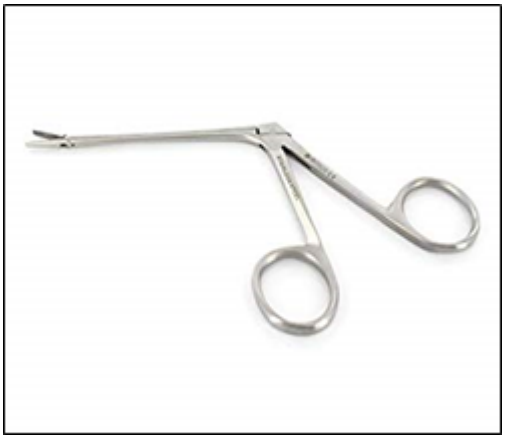

Immagine B

Figure 1. Instruments for mantle tissue sampling. (A) Straight Hartmann haemostatic forceps, used to stabilise the tissue flap. (B) Curved Hartmann auricular forceps, used to excise the tissue precisely without damaging surrounding tissue.

Method Validation

The effectiveness of this method was first verified in 2011 through survival tests conducted in multiple areas within Zone C of the Capo Caccia–Isola Piana Marine Protected Area. Specimens were monitored for over two months, comparing treated and untreated individuals (15 per site).

In both sites, mortality averaged below 4% for both treated and untreated individuals, indicating that mortality was related to physiological factors associated with population dynamics rather than experimental manipulation. These results demonstrate that sampled individuals are unaffected by the treatment, which can therefore be considered non-lethal.

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2.1.3 DNA samping swab collection

Dna Sampling Swab Protocol

Following the authorization for non-lethal sampling received from the Ministry (MASE) to sample Pinna nobilis tissues for genetic analysis and pathogen research, and in order to standardize an alternative sampling method, this method is described below.

Basically, sample collection follows the phases already authorized by ISPRA:

1) The valves are held open with a wooden stick (diameter of about 0.5 cm), put in proximity of the hinge ligament (4-5 cm);

2) A 20-50 mg sample of mantle tissue is excised using Hartman Alligator Forceps, or similar;

3) The stick is then removed, and the tissue sample is stored in a 1.5 ml tube, and then preserved in 75% ethanol.

Nonetheless, point (2) will be supported/replaced, after a preliminary experimentation, by the following experimental application:

A coĵon or a sampling brush swab is gently rubbed to collect mucus samples from the soft tissues of Pinna nobilis. To collect as much material as possible, it is advisable to take samples from different parts of the upper edge of the mantle and, eventually, from the gills (when the size of the specimen allow to easily access to this organ with that swab) of Pinna nobilis specimen, also in relation to the size of the individual.

After that, the soft head of the swab is cut off, preserved in proper tubes filled with 90% ethanol and then stored at -20° C.

This swab-based method represents an implementation of the experimental procedure first tested by Sarafidou et al. (2023) and has been adapted for the specific requirements of Pinna nobilis sampling under the LIFEPINNA project, focusing on improving DNA yield and minimizing specimen stress.

It should be noted that the main purpose of the experimental application of this protocol, which does not fully replace the one already in use but rather implements it during the preliminary step of standardization, is to verify whether data comparable to those coming from tissue collection can be obtained from the sample collection of mucus, in terms of (1) quality and quantity of Pinna nobilis DNA and (2) possibility of collecting data on the pathogens searched for the LIFEPINNA project, primarily Haplosporidium pinnae.

It should also be noted that the collection of DNA from epithelial mucus has already been used by UNISS with great effectiveness and very low level of invasiveness for the specimen in other aquatic organisms (see e.g. Casu et al. 2019).

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2.2 Transport of various types of biological samples

2.2.1 Transport of Sentinel Organisms Protocol

The collection of wild sentinel organisms (i.e., filter-feeding bivalves, not necessarily belonging to the species *Mytilus galloprovincialis*) must take place in the areas where the *Pinna nobilis* individuals are planned to be implanted or as close as possible to the installation areas. The minimum number of individuals to be collected must be 30 individuals per site.

The choice of the collection site is constrained by the presence of wild animals, suitable to be utilized as sentinel organisms on the seabed of the translocation site and along the rocky coastline. A careful survey of the translocation site and the surrounding areas aimed to identify suitable sites for sentinel collection should be realized. Sampling should be conducted via snorkeling and/or SCUBA diving.

The organisms were collected manually or with the aid of a diving knife, held in net bags with canvas bags inside. Once out of the water, the organisms should be wrapped in cloths soaked in seawater and transported to the laboratory, where they were labeled and maintained at a temperature of -20°C until shipment.

Shipment Procedure

For shipping, a polystyrene box of opportune dimension was used, containing ice packs previously stored in a freezer at -20°C. The courier service ensure that the organisms were delivered with cold express transport, with delivery within 48 hours.

2.2.2 Transport of Mantle Tissue Samples Protocol

Mantle samples are collected by scuba divers on live individuals; the excised tissue, approximately 20-50 mg, is placed into an Eppendorf-type tube (minimum 1.5 ml) and then stored at a temperature of -20°C until shipment.

Shipment Procedure

For shipping, a polystyrene box of opportune dimension was used, containing ice packs previously stored in a freezer at -20°C. The courier service ensure that the samples were delivered with cold express transport, with delivery within 48 hours.

2.3 Transport and maintenance Pinna Protocol

Based on the initial experience of transporting and maintaining adult specimens of *Atrina fragilis* in tanks at the Camogli laboratory, it was possible to apply the same transport and maintenance methodology to specimens of *Pinna nobilis*. The first transport of eleven specimens of *Pinna nobilis* was carried out on 19 June 2023, departing from the Venetian lagoon and arriving at the Camogli laboratory. The transport lasted six hours. The same protocol was used for transport in subsequent years: 6 specimens in 2024 and 17 specimens in 2025 from the Venetian lagoon. Consequently, this document summarises the stages of transport and maintenance in the laboratory.

Preparation of containers for transport:

1) The container chosen for transport is made of food-grade plastic (30/40 L), easily found in any agricultural or hardware store (it is usually used for olive harvesting). The cover is equipped with a seal and has an opening diameter of 30 cm (Fig. 1). In addition, the cover has been perforated to allow the insertion of small tubes to ensure ventilation for the *P. nobilis* individuals. Depending on the size of the individuals, a container can contain a maximum of 3 individuals with a shell height of between 20 and 30 cm.

Fig. 1: Transport container.

2) Inside, a thick layer of soft material (15/20 cm) consisting of rock wool, frequently used for mechanical filters in aquariums, is placed at the bottom (Fig. 2). Alternatives for this material could be: jute sacks, potato sacks, artificial foam that does not float, or a layer of natural bath sponges. For subsequent transport, jute sacks and blankets were used to house the *Pinna nobilis* individuals.

Fig. 2: Rock wool.

3) Of the two systems tested with *A. fragilis* (vertical and horizontal), the vertical system was preferred. In the vertical system, the structure used to keep the individuals in place consists of a circular plastic tube to which a rubber net is attached, available from fishing shops (the type of net used in trawling, Fig. 3). This system was chosen because we were looking for a material that would keep the individuals immobile without blocking the opening of their shells.

Fig. 3: Vertical system - Base for individuals.

4) The aeration system is assembled using a 1L plastic jar with the bottom cut off, inside which a small tube with a porous stone is inserted. A wad of rock wool is placed at the top to prevent too many bubbles from circulating in the water, which could end up inside the organism and damage it (Fig. 4).

Fig. 4: The aeration system.

5) Finally, the container is filled with sea water and the organisms are placed inside without exposing them to air. To transfer the organisms, the container was immersed directly in sea water to prevent the specimens from drying out. Once inside the container, the specimens are secured and wrapped in soft material (rags, cotton wool, jute, etc.) between the nets of the container. This operation is carried out directly on the boat (Fig. 5). Finally, the cover is screwed on, after first passing the aeration tube through one of the two holes made in the cover, and then connecting it to the bubbler device (AMTRA, 5V, 360 L/h).

Fig. 5: Moving specimens into transport containers.

6) During transport (lasting a total of 7 hours), the condition of the individuals, the containers and the efficiency of the bubblers were checked approximately every 2 hours.

Placing the specimens in the tanks

As soon as they arrive at the laboratory, the water parameters of the transport containers and the water in the laboratory tanks in which the specimens will be placed are measured. The parameters are measured with a HANNA HI98194 multiparameter probe; specifically, data on temperature, salinity, pH and dissolved oxygen are collected. The acclimatization phase consists of gradually transferring water from the laboratory tanks to the transport containers in order to equalize the parameters of the water in the tanks with those in the containers (Fig. 6). The procedure takes approximately 1 hour, with a change of 3 liters of water every 10-15 minutes.

Fig. 6: Acclimatisation of *Pinna nobilis* specimens.

In 2023, the eleven specimens of *P. nobilis* were placed in three tanks:

- two tanks of 240 litres each (three specimens per tank)

- one tank of 545 litres (five specimens)

Inside the tanks, the specimens were placed in a horizontal position on plastic boxes (Fig. 7) to facilitate subsequent handling/management of the specimens during spawning induction tests

Fig. 7: Placing specimens of *Pinna nobilis* in the tank.

In 2024, the six specimens collected (shell height between 31.2 cm and 60 cm) were placed in tanks in an oblique position inside plastic containers to facilitate subsequent handling/management of the specimens during spawning induction tests (Fig. 8). The six specimens were divided into two tanks, each with a capacity of 540 L: four specimens (average length 38.3 cm) were placed in one tank, while the other two specimens (length 44 and 60 cm) were placed in the other tank. We decided to place only two specimens in the latter tank so that the 60 cm specimen could have both a double dose of food and a greater volume of water available.

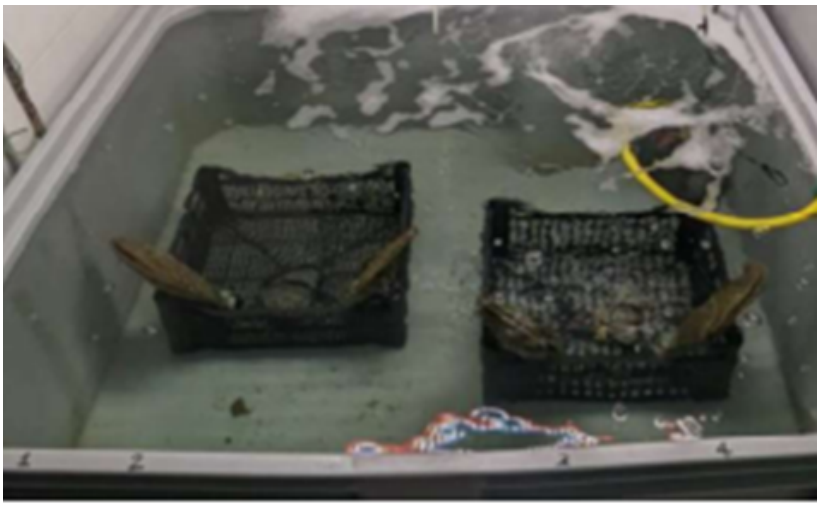

Fig. 8: Positioning of *Pinna nobilis* specimens inside the tank (2024).

In 2025, 17 specimens (shell height between 23.5 cm and 29 cm) were collected and placed in approximately 540 L tanks in a vertical position, thanks to the support of plastic boxes (Fig. 9). Thirteen specimens were kept at the University of Genoa laboratory and four at the Shoreline Soc. Coop laboratory.

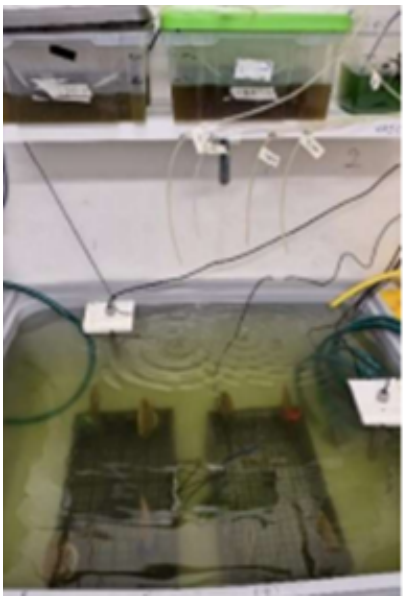

Fig. 9: Positioning of specimens in the tank: University of Genoa (left) and Shoreline Soc. coop. (right).

Maintenance of specimens in tanks

The tanks are cleaned three times a week by siphoning the bottom of the tank to remove pseudofeces and any food residue. The volume of water removed is subsequently replaced with clean water. The water change is partial (1/3 of the total volume) to avoid leaving the specimens dry. The water used to fill the tanks is filtered and sterilized: it filters through a sand filter, then a biological filter, two 10- and 1-micron cartridge filters, and finally through three 36W UV lamps. All tanks contain porous stones connected to a compressor for aeration. The photoperiod is regulated using an LED lamp, set to increase the hours of light according to the natural photoperiod.

In 2023, the specimens were fed three times a week with a mix of live microalgae (Isochrysis galbana: at an average concentration of 3.25×10^6 cells/ml; *Chaetoceros calcitrans*: at an average concentration of 3.75×10^6 cells/ml; *Diacronema lutheri*: at an average concentration of 2.33×10^6 cells/ml) and an aliquot of Easy Reef artificial feed (Najdek et al., 2013; Trigos et al., 2015; Prado et al., 2020; Hernandis et al., 2022). The specimens (average height 37.85 cm) were fed 350 ml of each microalgae species per day (for a total of 1050 ml of microalgae mix per Pinna specimen). While the live microalgae feed ration was provided per individual pinniped (regardless of tank size), the artificial feed was provided according to tank size (240 L tanks: 4 ml/day; 540 L tanks: 9 mL/day), following the product instructions (4 mL/day for every 250 L of tank water).Approximately three and a half months after being transported to the laboratory, six specimens died and were analysed by colleagues at the University of Sassari to determine whether or not they were infected with the protozoan *Haplosporidium pinnae*. The dead specimens all tested positive for *H. pinnae*, while the five survivors tested negative. Furthermore, during the summer, given the high temperatures reached in the laboratory, the temperature of the tanks containing the positive specimens was lowered using a cooler to improve the conditions for keeping the specimens. The temperature was maintained between 21 and 23°C.

In 2024, based on the size of the specimens and the density of the algae culture, the specimens were fed daily with a mixture of two live microalgae (Isochrysis galbana: culture density 8 *10^6 cells/ml, *Chaetoceros calcitrans*: culture density 7 *10^6 cells/ml), an aliquot of Easy Reef® artificial feed (8 ml/day/tank, following the product instructions) and a small amount of decapsulated *Artemia salina* cysts (Gold Pearls®, the dose was gradually increased from 0.25 g to 2.5 g/tank), to increase the protein intake in the diet. The microalgae dose was administered to the specimens using peristaltic pumps (Jebao Doser 3.4®) every hour for 19 hours a day, while routine tank cleaning and water changes (3 times a week) were carried out during the remaining 5 hours, during which no feed was administered. In this way, it was possible to administer approximately 79 ml/h/specimen of I. galbana and 53 ml/h/specimen of C. calcitrans. As a result, each specimen was fed a total of 2.5 L/day of microalgae mix (*I. galbana*: 1.5 L and *C. calcitrans*: 1 L per day).

The different strains of microalgae are maintained and grown in the laboratory in 40-litre bioreactors in order to produce microalgae in large quantities (Fig. 9). I. galbana, D. lutheri and T. suecica were maintained at a salinity between 31.5 and 32 practical salinity units (PSU) and C. calcitrans at 25 PSU (Helm et al., 2006). The density of the cultivated algal cells was calculated using a Bürker haemocytometer. The water used for microalgae was filtered and sterilized in the same way as that used for adult P. nobilis, but was also chemically sterilized (0.4 ml/L sodium hypochlorite with aeration for 24 hours and then 0.024 g/L sodium thiosulphate to remove excess chlorine). The culture medium used for microalgae growth is Guillard F/2 (Cell-Hi-F2P; Varicon aqua solution®).

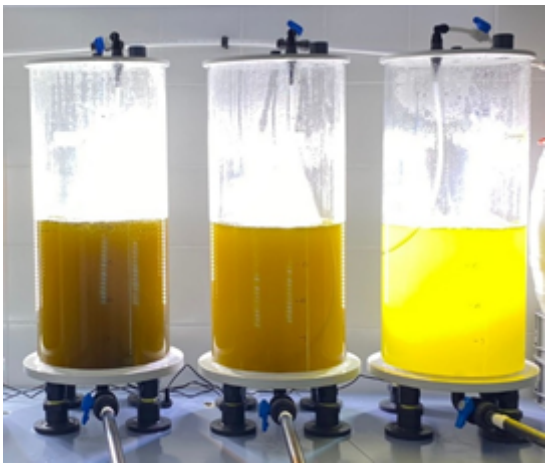

Fig. 9: 40 L bioreactors for microalgae production in the laboratory.

The specimens of *P. nobilis* from 2025, both for the University of Genoa and for Shoreline Soc. Coop., were collected in January and subjected to gonadal maturation simultaneously in the two laboratories, one in Genoa and one in Trieste. Consequently, the temperature of the tanks was regulated using heaters and coolers, as the temperature was increased from 15° to 20°C between January and April. The photoperiod was regulated using an LED lamp, increasing from 10 to 18 hours of light during the months of the experiment.

The specimens were fed daily with a diet consisting of a mix of three algal strains (between $1.05*10^{10}$ and $2.1*10^{10}$ cells/sample), depending on the water temperature: 60% Isochrysis galbana; 30% *Chaetoceros calcitrans/Phaeodactylum tricornutum*; 10% *Tetraselmis suecica/T. chuii*. In addition, zooplankton was administered in vivo (copepods, *Artemia salina* nauplii and rotifers) or as artificial feed (Bea zoo plus®; Gold pearl®). In this case too, the different strains of microalgae used for feeding were maintained and grown in the two laboratories in 40-litre bioreactors, in order to produce microalgae in large quantities.

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2.4 Breeding of Pinna

Spawning induction and fertilization *Pinna nobilis* specimens reacted to various spawning induction stimuli:

a) stress from collection and transport

b) thermal shock

c) gradual increase in the temperature of the water

On 20/06/2023, following stress caused by detachment and transport (occurred on 19/06/2023), some of the 11 specimens of *Pinna nobilis* began to release gametes into the water of tank (Fig. 1).

Fig. 1: Spawning of already fertilized eggs

Between June and July 2023, there were five spontaneous gamete releases, but only three of these produced fertilization. The released eggs, which were already fertilized, were collected, counted and monitored, tracking their rapid development until the formation of larvae.

A total of 14,623,800 eggs were released, with high fertilization rates of between 95.31% and 99% (Table 1).

Date	Spawning (n° specimens: Female and Male)	ID specimens	ID Fecondaz.	N° eggs release	N° fertilized eggs	Fertilization rate	Trocophore rate
20/06/2023	8 ? (3F; 2/5M?)	F: 4; 5; 6 (Positives)	**Pn_F1**	11.310.000	10.780.000	95.31 %	99.6 %
21/06/2023	8 (3F; 2 M)	F: 4; 5; 6 M: 7; 9 (Positives and negatives)	-	-	-	-	-
22/06/2023	2 M	M: 7; 10 (Negatives)	-	-	-	-	-
28/06/2023	8 (3F)	F: 4; 5; 6 (Positives)	**Pn_F2**	3.313.800	3.280.200	99 %	94.5 %
05/07/2023	5 ?? (F)	7; 8;9; 10; 11 (Positives and negatives)	Pn_F3	eggs not quantified			
TOT.				**14.623.800**	**14.060.200**		

Tab. 1: Data relating to spontaneous spawning events of *Pinna nobilis*.

In this first attempt at controlled reproduction, it was therefore not necessary to induce adult specimens using thermal shock (Trigos et al., 2018; Hernandis et al., 2023). Using transport stress is a practice also used by other authors (Hernandis et al., 2023) precisely to avoid subjecting breeders to further stress.

In 2024, between May and July, there were seven spawning induction events: two induced by transport stress and five by thermal shock (Table 2). The first two spawning events, as in the previous year, were induced by the stress of collection and transport of the individuals, but only on one of the two occasions did the release of already fertilised eggs (fertilisation Pn_F4) occur, with relative embryonic and larval development. In June and July, five stimulations were carried out using thermal shock, but not all of them led to the release of gametes. Three stimulations led only to the release of male gametes, while in one case female gametes were also released, making it possible to carry out controlled fertilisation (Pn_F5) using gametes from different individuals.

Tab. 2: Data relating to *Pinna nobilis* spawning events in 2023 and 2024 (*specimens tested positive for *Haplosporidium pinnae*; M: male; F: female/already fertilised eggs; f: female/unfertilised eggs; I: indeterminate; E: hermaphrodite; $^{\Delta}$ specimens subjected to thermal shock; ^{O} self-fertilisation; $^{\square}$ controlled fertilisation).

Date	Spawning (n° specimens: Female, Male, hermaphrodite and Indeterminate)	ID specimens	ID fertilization	N° eggs release	N° fertilized eggs	Fertilization rate	Trocophore rate
23/05/2024	1 M	M: 6	-	-	-	-	-
31/05/2024	2 (2F)	F: 5; 6	**Pn_F4^{O}**	Uova non contate (stima: 118.800.000)	-	-	-
18/06/2024	0$^{\Delta}$	I: 2; 4	-	-	-	-	-
19/06/2024	3$^{\Delta}$ (2f; 1 E)	f: 1; 3 E: 6	**Pn_F5$^{\square}$**	44.280.000	31.560.000	72,5%	37,2%
08/07/2024	2$^{\Delta}$ M	M: 1; 4	-	-	-	-	-
15/07/2024	2$^{\Delta}$ M	M: 4; 6	-	-	-	-	-
17/07/2024	3$^{\Delta}$ M	M: 1; 3; 4	-	-	-	-	-
TOT. 2024				**44.280.000**	**31.560.000**		

The specimens subjected to thermal shock were repeatedly moved every 30-50 minutes from the hot water tank to the cold water tank and vice versa (Fig. 2A, B). The temperature difference between the two tanks was 10 °C (± 5 °C compared to the water temperature in the adult maintenance tank). The temperatures were maintained using heaters (Tetra HT 100 W) and a chiller (Teco TK500). The entire stimulation process lasted between 2 and 4 hours, depending on the response observed in the specimens.

Once the stimulation operations were complete, and after the release of gametes, the specimens were returned to their maintenance tanks.

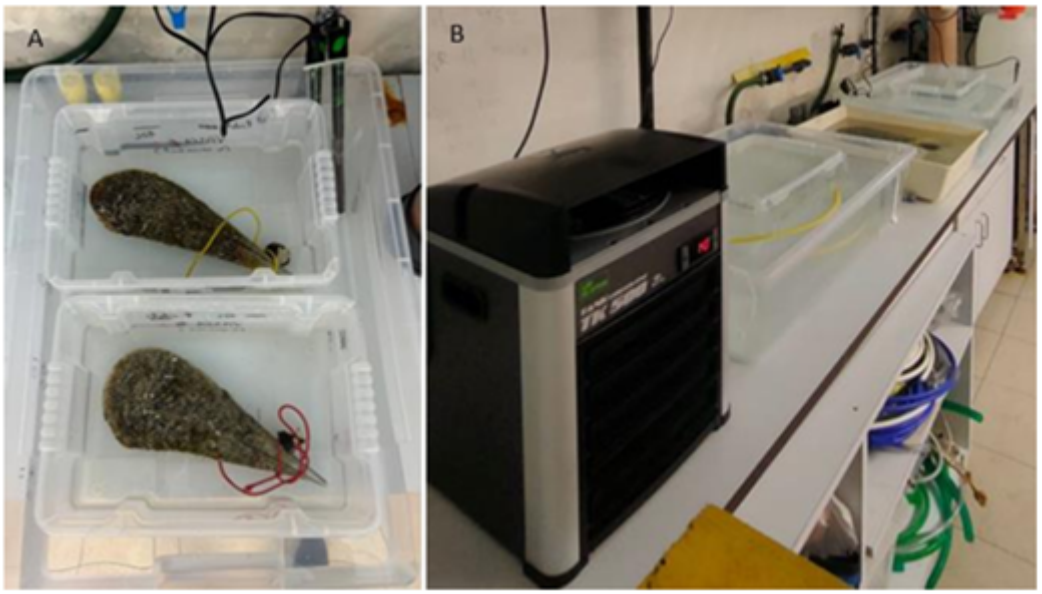


Fig. 2: Specimens of *P. nobilis* subjected to thermal shock: A, tank with heaters; B, tank with cooler.

In Pn_F5 fertilization, the eggs (Fig. 3) were collected in a beaker and counted using a Sedgewick-Rafter counting chamber. This time, it was possible to perform controlled fertilization using gametes from different specimens. The embryos were then placed in tanks of different volumes, and their development was observed until the development of larvae.

Fig. 3: Spawning of eggs after thermal shock (the arrow indicates the eggs).

All specimens, except for one (I: indeterminate), released gametes at least once. One specimen was identified as having released only male gametes (M: male), one specimen released only fertilized eggs (F: female/fertilized eggs), while three specimens alternated between releasing

unfertilized eggs (f: female/unfertilized eggs) and male gametes during different spawning events. One of them was seen to release, in the same reproductive event, alternately and at intervals, first female gametes and then male gametes (E: hermaphrodite) (Table 2). In Pn_F4 fertilization (self-fertilization), the fertilized eggs were not counted in their entirety, but it was decided to use only those present in a volume of 11L (2,420,000 eggs), whose embryonic and larval development was then monitored (Table 2; Fig. 4). For controlled fertilization Pn_F5, the released eggs were filtered, collected and then counted before proceeding with fertilization. A total of 44,280,000 eggs were counted, with a fertilization rate of 72.5% and a trochophore rate of 37.5%, resulting in 11,835,000 trochophores (Table 2).

Larval maintenance and feeding

In 2023, the larvae were reared at a temperature of approximately 25 °C (room temperature) and a salinity of 37.5 PSU. The larvae were placed in two 240-litre tanks and two aquariums (60 litres and 83 litres), plus additional 30-litre tanks as needed. Initially, the concentration of larvae/ml was very high (7-50 larvae/ml), but it was gradually reduced to approximately 1 or 3 larvae/ml. The water used to fill the tanks has been filtered and sterilized: it passes through a sand filter, a biological filter, two 10- and 1-micron cartridge filters, and finally through three 36W UV lamps. Each tank has been equipped with aeration blown through a rigid tube, creating a chain of bubbles (approximately 1 bubble/s). The photoperiod is regulated using an LED lamp (12 hours of light and 12 hours of darkness).

Every day, after gently homogenizing the contents of each tank, a known volume was taken from which a 1 ml sub-sample was observed using a Sedgwick Rafter counting chamber to estimate the number of larvae present and monitor their development. The water in the larval cultures was changed three times a week. For this purpose, all the water in the tanks was filtered through a sieve with a mesh size of Ø = 45 µm, partially submerged in water, to prevent the larvae from drying out on the filter. Once the tanks had been refilled with fresh water, the larvae were returned and fed.

The larvae were fed three times a week with a mix of live microalgae: *Isochrysis galbana, Chaetoceros calcitrans, Diacronema lutheri* (Trigos et al., 2018; Prado et al., 2020).

Tab. 2: feeding Pinna nobilis larvae

Tank (L)	Larvae/ml	ml/algae/day
Tank 250 l	1	*250ml (Isocrysis galbana)* *250ml (Diacronema lutheri)* *250ml (Chaetoceros calcitrans)*
Tank 60 l	1	*80ml (Isocrysis galbana)* *80ml (Diacronema lutheri)* *80ml (Chaetoceros calcitrans)*

Given the rapid larval development due to the high temperature, after a few days, settlement substrates were placed in the various tanks. These substrates consist of plastic nets and conical structures used for oyster farming (Fig. 4). Unfortunately, the larvae were unable to develop beyond the initial veliger stage, so it was not possible to observe the settlement phase.

Fig. 4: Installation of settlement substrates inside the tanks containing P. nobilis larvae

In 2024, the larvae obtained were reared differently from the previous year, with changes made to the management of the tanks, temperature and feeding. The larval cultures were maintained at a temperature of 21°C, with the aid of an air conditioner in the room, which allowed for better development than in the previous year. The salinity of the water was 37.5 PSU and the water used was filtered and sterilized in the same way as that used for adult specimens. The larvae were placed in tanks of different volumes (15 L and 31 L), with a density of 2.5 larvae/ml and 5 larvae/ml respectively. The water in the larval tanks was changed every two days using a 45 µm filter on which the larvae were collected, which was kept submerged in the tank so that the larvae were not left to dry on the filter. The larvae retained on this filter were then transferred to a known volume of water, counted in a Sedgwick Rafter counting chamber (1 ml of subsample/3 replicates) and then reintroduced into the tanks filled with fresh water and fed, trying to maintain concentrations of approximately 1-2 larvae/ml after initial mortality. At the same time, the developmental stage of the larvae was monitored.

The larvae were fed daily with a mixture of *I. galbana* and *C. calcitrans*, but the dose fed was based on the formula reported by Trigos et al. (2018), with some modifications. I. galbana is a species characterized by nutritional profile rich in docosahexaenoic acid (DHA) (Helm et al., 2004; Martino et al., 2023), while *C. calcitrans* has nutritional profile rich in eicosapentaenoic acid (EPA). Essential fatty acids (EFAs), particularly omega-3 fatty acids, EPA and DHA, are important for larval growth and development because they are the main components of cell membranes and play a key role in modulating membrane functions (Marshall et al., 2010).

Starting from what Trigos et al. (2018) indicated as the optimal dose (33.3 cells/µl of D. lutheri + 25 cells/µl of *C. calcitrans* + 100 cells/µl of I. galbana), the diet was modified by replacing the D. lutheri cells with the equivalent amount of I. galbana cells (Helm et al., 2004), obtaining and administering a total of 133.3 cells/µl of *I. galbana*. Meanwhile, the amount of *C. calcitrans* was

increased compared to that indicated by Trigos et al. (2018) to make the diet richer in EPA and test for any differences in larval survival. This is because, although the polyunsaturated fatty acid content depends heavily on the microalgae cultivation conditions (Fernandes et al., 2016), an equivalent biomass of *C. calcitrans* and *I. galbana* provides an EPA/DHA ratio close to 2/1 (2.25 cells/µl of *C. calcitrans* = 1 cell/µl of I. galbana; Helm et al., 2004). To approach an EPA/DHA ratio of 1:1, a quantity of *C. calcitrans* biomass equivalent to half the biomass of *I. galbana* was measured, and consequently 150 cells/µl of *C. calcitrans* were administered (Table 1). The following formula was used to calculate the quantity of microalgae to be administered:

$$V_{dose}(L) = \frac{Desired\ cell\ density\ [\mu L] \cdot V_{tank}}{Available\ cell\ density\ [\mu L]}$$

Where:

V_{dose} = dose in litres to be administered.

Desired cell density [µL] = optimal dietary cell concentration.

Available cell density [µL] = cell concentration of laboratory cultures.

Furthermore, once the larvae reached a size greater than 120 µm, a third microalga, Tetraselmis suecica, was added to the diet. This microalga is known to improve the survival of bivalve mollusc larvae compared to a diet based on only two microalgae (Helm et al., 2004). Consequently, microalgal concentrations were recalculated by increasing the dose administered. 88.86 cells/µL of I. galbana and the equivalent amount of T. suecica (8.89 cells/µL; knowing that 1 cell of I. galbana = 0.1 cells of T. suecica), while *C. calcitrans* was dosed to achieve a biomass equivalent to that of I. galbana (200 cells/µL; knowing that 1 cell of *I. galbana* = 2.25 cells of), thus further enriching the diet in EPA (Table 4).

Tab. 4: Microalgal concentrations (cells/µL) used in the diet supplied to Pn_F5 larvae (2024), compared with Trigos et al. (2018).

Ceppi di microalghe	**Trigos et al., 2018** (dose per tutto il ciclo larvale)	**Questo studio** (dose fino alla taglia larvale di 120 µm)	**Questo studio** (dose dopo la taglia larvale di 120 µm)
Isochrysis galbana	100 cell./µL	133,3 cell./µL	88,86 cell./µL
Diacronema lutheri	33,3 cell./µL	-	-
Chaetoceros calcitrans	25 cell./µL	150 cell./µL	200 cell./µL
Tetraselmis suecica	-	-	8,89 cell./µL

Before feeding the necessary amount of food, the cell density of the microalgal cultures was counted daily using a Bürker chamber haemocytometer. In this way, using the formula reported by Trigos et al. (2018), it was possible to calculate the amount of each algae to be fed to the larvae.

Larval development

In 2023, the different stages of zygote development, up to the first larval stage of trochophore and the subsequent veliger stage, were observed during the larval rearing period. Larval development occurred very rapidly, with the initial veliger stage being observed 22 and 26 hours after fertilization (hpf) for Pn_F1 and Pn_F2, respectively (Fig. 5f; Table 5). The rapid development observed was probably due to the high water temperature of the larval cultures (~25 °C). It was not possible to maintain the larval cultures under controlled temperature conditions. Due to the rapid development, it was not possible to document all the timings of the different stages of embryonic and larval development. The initial trochophore stage was observed at approximately 5-6 hpf, while Trigos et al. (2018) observed it at approximately 22 hpf at a constant temperature of 21 °C. The same difference in development speed was observed at the advanced trochophore and early veliger stages (Fig. 5e, 5f; Table 5).

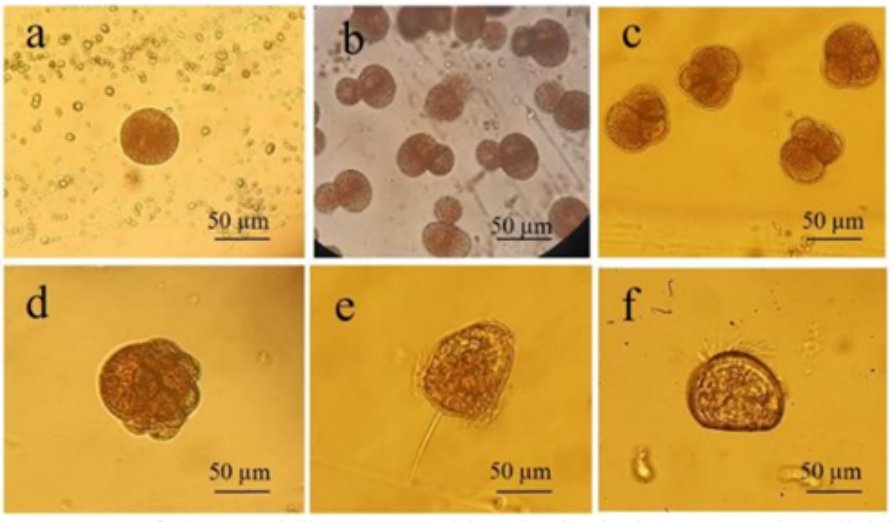


Fig. 5: Larval development stages of Pinna nobilis: a, eggs with polar body; b, 1st complete division; c, 3rd and 4th divisions; d, end of cell division phase (>8 cells); e, late trochophore; f, early veliger (Scale bar: 50 µm).

Although it was not possible to measure the size of the larvae at each stage of development, the measurements taken show no significant differences from those reported by Trigos et al. (2018) (Table 5). Between the eighth and ninth day after fertilization, almost all of the larvae were distributed at the bottom of the tanks, moving slowly, indicating low vitality. After 9 dpf, only empty shells were observed at the bottom of the tanks, marking the end of larval rearing for both fertilization events (Fig. 6A).

Tab. 5: Timing and size of *Pinna nobilis* larval development (2023: at approximately 25°C and 2024: at 21°C), compared with Trigos et al., 2018 (21°C). NM: not measured.

Development stage	Cumulative time (h:min)					Size (µm)		
	This study				Trigos *et al.*, 2018	This study		Trigos *et al.*, 2018
	2023		2024			2023	2024	
	Pn_F1	Pn_F2	Pn_F4	Pn_F5				
Spawning/Fertilization	00:00	00:00	00:00	00:00	00:00	50	50	50
Egg with polar body	NM	-	-	-	00:15	50	50	50
Double membrane	NM	-	-	-	00:30	50	50	50
1ª inclusion	NM	-	-	-	00:40	-	-	55
1ª complete division	00:25	00:22	01:00	00:29	01:00	-	-	-
3ª and 4ª division	01:15	00:30	01:00	00:43	03:00	53	53	-
End of the cell division phase (>8 cells)	01:50	02:10	01:00	01:43	04:30	-	-	-
Blastula	-	-	-	-	05:00	-	-	55
Gastrula	-	-	-	-	08:00	-	-	55
Early trochophore	05:55	04:41	01:00	21:17	22:00	-	60	65
Late trochophore	20:50	-	25:20	-	30:00	65	65	70
Early veliger	22:36	26:30	121:04	45:17	48:00	75-80	80	85
Late veliger	-	-	-	-	72:00	-	110-120	90
Early umbonate	-	-	-	383:17	144:00	-	145	100
Pediveliger	-	-	-	-	168:00	-	-	110

In 2024, embryonic and larval development was monitored for both fertilizations, observing the transition to trochophore and subsequently to veliger. For Pn_F4, larval development stopped at the veliger stage 10 days after fertilization (Fig. 5f; Fig. 6B), while the larvae obtained from Pn_F5 developed further, surviving up to 21 dpf (Fig. 6B) and reaching the umbonate larva stage (Fig. 7). Unlike in 2023, the temperature of the larval development tanks was maintained at 21 °C. This allowed the larval development times, up to the veliger stage, to be in line with Trigos et al. (2018) for both fertilisation events, while, for Pn_F5 only, the umbonate larva stage was reached at 384 hpf, unlike the result reported by Trigos et al. (2018), who observed this stage at 144 hpf.

Furthermore, the size reached by the umbonate larvae also differs, with a size of 150 µm in the present study and 100 µm in the study by Trigos et al. (2018) (Table 5). Nine and 21 days after fertilization, for Pn_F4 and Pn_F5 respectively, only empty shells were found at the bottom of the tanks.

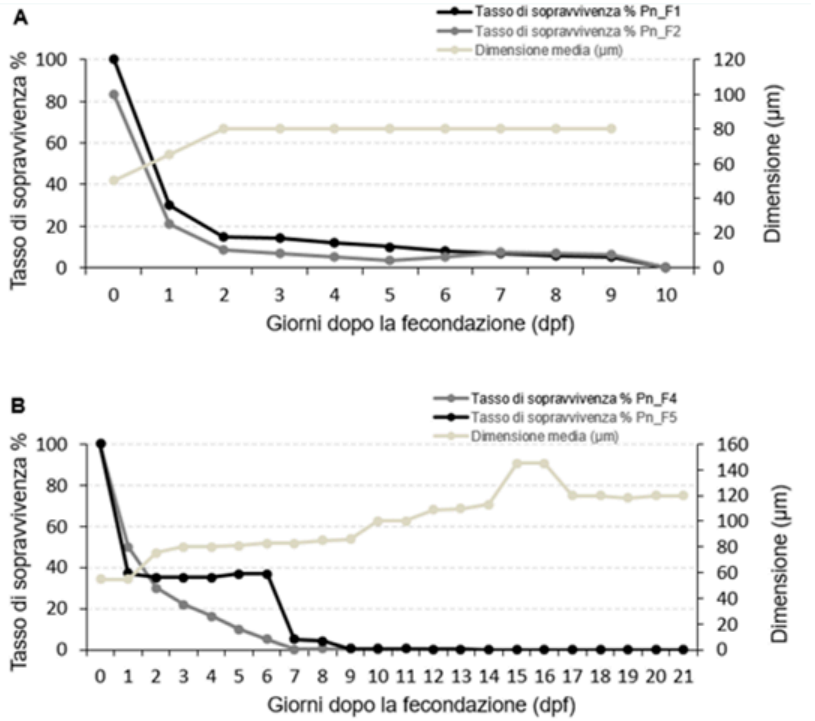


Fig. 6: Survival and size trends of *Pinna nobilis* larvae; A, 2023; B, 2024.

Fig. 7: Umbonate larva of *Pinna nobilis* (Scale bar 50 µm).

The data collected during 2022 and 2023 were used to write a scientific article, published in the journal Ecology and Evolution:

Ferranti M.P., Azzena I., Batistini E., Caracciolo D., Casu M., Chiantore M., Ciriaco S., Firpo V., Intini L., Locci C., Montefalcone M., Oprandi A., Sanna D., Scarpa F., Segarich M., 2024. Handling of the bivalve Pinna nobilis, endangered and pathogen affected species, for controlled reproduction: precautions taken. Ecology and Evolution, 14: e70565. https://doi.org/10.1002/ece3.70565.

With regard to the data collected during the 2024 breeding season, another scientific article was drafted and submitted to the journal Aquaculture Reports in August 2025, pending evaluation:

Ferranti M.P., Azzena I., Batistini E., Casu M., Ciriaco S., Di Napoli C., Farris S., Intini L., Locci C., Oprandi A., Prandoni L., Sanna D., Scarpa F., SegarichM., Chiantore M., submitted. Controlled reproduction of the noble pen shell Pinna nobilis: comparison of larval development from 1 self-fertilization and controlled fertilization. Aquacuture Reports.

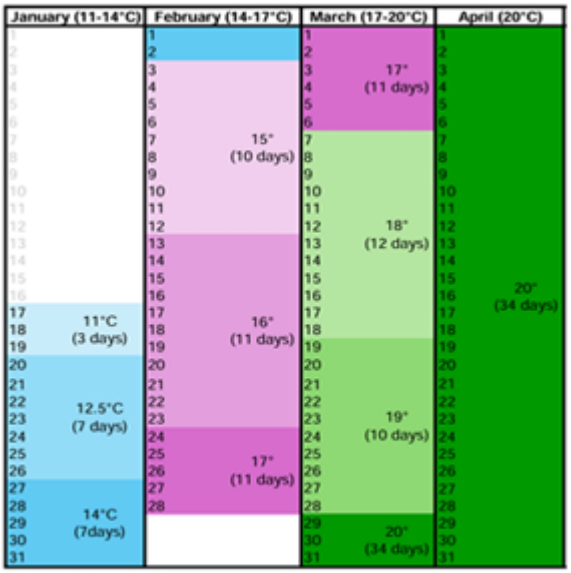
Gonadal maturation induction

Maintenance of adult specimens

In 2025, from mid-January to April, a gonadal maturation induction test was carried out in parallel in three laboratories: University of Genoa (at the Camogli-CNR-IBF laboratory; Genoa - Liguria), Pula Aquarium (Croatia) and Shoreline Soc. Coop. (Trieste). The aim of this experiment was to induce gonadal maturation in some adult specimens of P. nobilis in about four months, in order to bring forward the reproductive period and the release of gametes. To this end, four specimens were collected and kept in each laboratory, with the exception of the University of Genoa (eight specimens in tanks).

Only the data relating to the University of Genoa and Shoreline soc.coop., as partners in the Life Pinna project, will be reported below.

The specimens were placed in a vertical position in tanks of approximately 500 litres, and the seawater used was subjected to a filtration process involving a sand filter, two cartridge filters (10 µm and 1 µm), and was subsequently sterilised using a series of three UV lamps. The water temperature was gradually increased from 15° to 20°C, using heaters and chillers to maintain the set temperature. The photoperiod was adjusted using an LED lamp, increasing from 10 h to 18 h of light. (Fig. 8).



Mese¤	Fotoperiodo¤
Gennaio¤	14h·luce/10h·buio¤
Febbraio¤	14h·luce/10h·buio¤
Marzo¤	16h·luce/8h·buio¤
Aprile¤	18h·luce/6h·buio¤

Fig. 8: Set-up temperature trend (left) and photoperiod (right).

The specimens were fed a diet composed of a mixture of three algal strains (between $1.05*10^{10}$ and $2.1*10^{10}$ cells/sample), depending on the water temperature:

- 60% *Isochrysis galbana*

- 30% *Chaetoceros calcitrans/Phaeodactylum tricornutum*

- 10% *Tetraselmis suecica/T. chuii*

In addition, live zooplankton (copepods, *Artemia salina* nauplii and rotifers) or artificial feed (Bea zoo plus®; Gold pearl®) was supplied.

Spawning induction and fertilization

In April 2025, when the temperature reached 20°C in both Trieste and Genoa, we observed a total of four spontaneous spawnings, which led to three fertilizations and respective larval cycles. Of the three fertilizations, one occurred spontaneously in the tank and the other two were controlled fertilizations.

Fertilization rates were very low (8.9-17.5%), with a total of approximately 7,802,600 eggs collected (Table 6).

Tab. 6: Data relating to spawning and fertilization following induced gonadal maturation of Pinna nobilis.

Date	Spawning (n° specimens: Female and Male)	ID specimens	ID Fecondaz.	N° eggs collected	Fertilization rate
15/04/2025 (Shoreline)	1M (Spontaneous spawning, 20°C)	M: 1	-	-	-
16/04/2025 (Shoreline)	2 (1F; 1 M) (Spontaneous spawning, 20°C)	M: 1 F: 2	**Pn_F1** (Controlled fertilization)	eggs not quantified	-
25/04/2025 (Unige)	2 (1F; 1 M) (Spontaneous spawning, 20°C)	M: 5 F: 4	**Pn_F2** (Spontaneous fertilization)	3.693.600 (eggs collected on the bottom of the tank)	8.9 %
25/04/2025 (Unige)	2 (1F; 1 M) (Spontaneous spawning, 20°C)	M: 5 F: 4	**Pn_F3** (Controlled fertilization)	4.109.000	17.5 %
12/05/2025 (Unige)	1M (Thermal shock)	M: 5	-	—	-
12/05/2025 (Shoreline)	0 (Thermal shock)	-	-	-	-
14/05/2025 (Unige)	0 (Thermal shock)	-	-	-	-
21/05/2025 (Shoreline)	0 (Thermal shock)	-	-	-	-

Unfortunately, larval development stopped at the first trochophore stage, which was observed up to 8– 10 days after fertilisation, without ever developing into the subsequent larval stages.

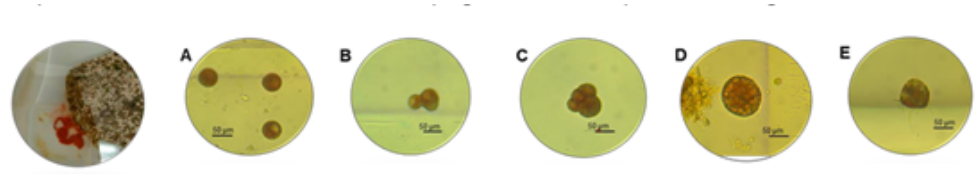


Fig. 9: Egg spawning (left); Larval development of *Pinna nobilis*: A: fertilized eggs with polar body; B: first division; C: 3rd and 4th divisions; D: early trochophore; E: late trochophore.

In May 2025, four thermal shock tests were carried out to induce the release of more gametes, but unfortunately only one specimen released male gametes, resulting in the inability to proceed with controlled fertilisation.

Unfortunately, gonadal maturation led to gamete release, but with a reduced number of eggs released, low fertilisation rates and larval development interrupted in the early stages.

Furthermore, given the poor results obtained in May, on 16 July 2025, another five specimens (size range 26.5-42 cm) were transported to the laboratory to undergo thermal shock. The specimens were kept under the same conditions as described above. On 23 and 30 July, two thermal shock tests were carried out, but no gametes were released. These specimens were probably already in a state of sexual dormancy, perhaps due to the high sea temperatures reached in July at the donor site.