



# Life Pinna

LIFE20 NAT/IT/001122

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

### **BEST PRACTICE PROTOCOL FOR *Pinna nobilis* REPRODUCTION IN CONTROLLED CONDITIONS**





## BEST PRACTICE PROTOCOL FOR *Pinna nobilis* REPRODUCTION IN CONTROLLED CONDITIONS

# Deliverable\_C4

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

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## SUMMARY

Action C4 refers to the adaptation, breeding and reproduction of adults transferred to the Camogli laboratory (controlled environment).

The present Deliverable reports the best results regarding the *Pinna nobilis* reproduction events in controlled conditions from 2023 to 2025.

We have standardized the method of acclimation specimens to seawater parameters in maintenance tanks upon their arrival at the laboratory.

The first spawning occurred in June 2023. As it was not possible to collect adult specimens of *Pinna nobilis* in Trieste in the summer of 2022, due to the severe depletion of adult stocks, it was consequently not possible to obtain and maintain the larvae in the laboratory. The second spawning occurred in May 2024, the third in April/May 2025 and the fourth in July 2025 (only male).

In 2023, we obtained five spawning events, induced by transport stress. In 2024, we carried out seven induction tests (two induced by transport stress and five by thermal shock), observing six spawning events. Whereas, in 2025, induced gonadal maturation was carried out, performing 8 spawning induction tests and obtaining 5 spawning events (4 spontaneous spawning and 1 induced by thermal shock).

In all years, we obtained fertilization and observed larval development. In 2024, the umbonate larval stage was reached 16 days after fertilization. The shell size was 150  $\mu\text{m}$ , which is a size that has never been described before for this larval stage of *P. nobilis*. Unfortunately, the settlement and metamorphosis phases have not been achieved.

In order to encompass also the results from the 2025 spawning inductions, the present document was delivered later than the expected date (June 2025).

## 1. Adaptation, breeding and reproduction for active restocking

Following the collection and transfer of adult specimens (Action C5), four consecutive phases are foreseen in the framework of the present action:

1. Acclimatization and sexual maturation
2. Spawning induction and eggs fertilization
3. Larval rearing and metamorphosis
4. Juveniles rearing

### 1.1 Acclimatation and sexual maturation

In the laboratory, the specimens were acclimatized, and the procedure lasted around 1 h, with a water change rate of 3 L of water changed every 10 min inside the 30 L boxes used for the transport.

The adult *P. nobilis* specimens can be placed in different positions inside the tank: horizontal, oblique or vertical using plastic boxes (Fig. 1), to facilitate their handling for spawning induction trials in order to achieve controlled reproduction. The horizontal positioning is also in agreement with the study carried out by Hernandis et al. (2022) who did not observe significant differences in the physiology of Pinnids between the laying down and standing positions. However, we recommend the vertical position, as in nature. Trying to maintain a specimen of average size 38 cm in at least 100L each specimen.



Fig. 1: specimens of *Pinna nobilis* placed in different positions within the tank: A: horizontal, B: oblique, C: vertical

As it is a semi-closed system, the tank was cleaned three times a week to remove feces and any food residual, and partial water change (i.e., 2/3 of the total volume, three times a week) was performed to avoid air exposure of the specimens. The sea water used for the tanks was filtered through a sand filter to remove large particles; then, the finest fraction was removed with a microfiltration system composed by two cartridge filters (10  $\mu\text{m}$  and 1  $\mu\text{m}$ ), and, at the end of the process, water was sterilized through three UV lamps in series (Amtra Scudo Inox UVC- 36w; Ford and Borrero 2001;



Prado, Cabanes et al. 2020; Hernandis, 2021). Air was provided in the tanks through air stones connected to a compressor, and the photoperiod was regulated using an LED lamp (12 h light/12 h dark or in according to natural photoperiod).

During the summer season, the specimens were maintained in tanks at about 21 °C, using air-conditioned room temperature or chillers. If the specimens need to be maintained in tanks during the winter months (e.g. for gonadal maturation trials), the temperature can be regulated using a combination of heaters and coolers to maintain a constant temperature.

For *P. nobilis* specimens testing positive for *Haplosporidium pinnae*, management of the water change was more complex as the wastewater was stored in separate tanks and chemically sterilized (0.4 mL/L sodium hypochlorite with aeration for 24 h and then 0.024 g/L sodium thiosulfate to eliminate excess chlorine) to avoid discharging potentially pathogen- contaminated water into the sea.

On the basis of the size of the specimens and the algal culture density, the specimens were fed daily, with a mix of two live microalgae (*Isochrysis galbana*: culture density  $8 \cdot 10^6$  cell/mL, *Chaetoceros calcitrans*: culture density  $7 \cdot 10^6$  cell/mL), an aliquot of Easy Reef artificial feed® (8 mL/day/tank, following the instructions of the product) and a small quantity of decapsulated *Artemia salina* cysts (Gold Pearls®, the dose was gradually increased from 0.25g to 2.5g/tank) to increase protein intake in the diet. This diet is founded on the study by Ferranti et al. (2024), with some changes in order to improve the diet for adult individuals (microalgal mixture and supply – continuous instead of discontinuous). The choice of a feed based on two microalgal species, in addition to easy culture handling, followed the choice made by Hernandis et al. (2022), supported by the fact that the polyunsaturated fatty acid content of *I. galbana* and *Diacronema lutheri* is on average similar, making one species a substitute for the other (Helm et al., 2006). The dose of microalgae was supplied to the specimens with peristaltic pumps (Jebao Doser 3.4®) every hour for 19 hours/day and routine tank cleaning and water changes (3 times a week) were carried out during the remaining 5 hours, during which the feed was not supplied. In this way it was possible to feed around 79 mL/h/specimen of *I. galbana* and 53 mL/h/specimen of *C. calcitrans*. In this way, each specimen was fed with a total of 2.5 L/day of microalgae mix (*I. galbana*: 1.5 L and *C. calcitrans*: 1 L per day).

An alternative daily diet for adult specimens could consist of a mix of three live microalgae: 60% *Isochrysis galbana*, 30% *Chaetoceros calcitrans*/*Phaeodactylum tricornutum* and 10% *Tetraselmis suecica*/*T. chuii* (between  $1.05 \cdot 10^{10}$  –  $2.1 \cdot 10^{10}$  cells/specimen). In addition, zooplankton was also



given (copepods, *Artemia salina* nauplii and rotifers) in vivo or artificial feed (2 ml of Bea zoo plus®; 2.5 g/tank of Gold pearls®). This type of diet was provided to adult *P. nobilis* during a trial of gonadal maturation induction, with doses to be adjusted according to need and size of specimens.

The microalgae were grown in the laboratory inside 4 bioreactors (40 L/each), *I. galbana* at a salinity between 31.5 - 32 practical salinity units (PSU) and *C. calcitrans* at 25 PSU (Helm et al., 2006). The density of cultured algal cells was calculated using the Bürker hemocytometer. The water used for microalgae was filtered and sterilized in the same way as for the adults of *P. nobilis*, but was additionally chemically sterilized (0.4 mL/L sodium hypochlorite with aeration for 24 h and then 0.024 g/L sodium thiosulfate to eliminate excess chlorine). The culture medium used for microalgae growth is Guillard F/2 (Cell-Hi-F2P; Varicon aqua solution®).

## 1.2 Spawning induction and egg fertilization

Spawning events can occur because of the stress from collection and transport, following stimulation with thermal shock or a gradual increase in the temperature of the water in the specimen maintenance tank.

In order to induce thermal shock, the adult specimens were maintained at around 21°C and moved repeatedly (about every 30-50 minutes) from cold to warmer water tanks and *viceversa*, with a thermal gap of 10°C ( $\pm 5$  °C from the temperature of the adult specimen tanks). Temperatures were maintained through the use of two heaters (Tetra HT® 100 w) and a chiller (Teco TK500®). The stimulation process lasted 2-4 hours (Trigos et al., 2018; Hernandis et al., 2023; Ferranti et al., 2024), depending on the response observed in the specimens. At the end of the induction trials and after the possible release of gametes, the specimens were returned to their tanks.

The gradual increase in temperature was applied to induce gonadal maturation in some specimens of *P. nobilis*, collected in winter and matured in a controlled environment, bringing forward the release of gametes compared to the natural spawning period.

In 2023, 2024 and 2025, we carried out a total of 20 spawning induction trials, obtaining 16 spawning events (7 induced by transport stress, 5 by thermal shock and 4 by gradual temperature increase, and 8 fertilizations (5 self-fertilization and 3 controlled fertilization) (Table 1).

Tab. 1: Data relating to spawning events of *Pinna nobilis* in 2023, 2024 and 2025 (\*specimens tested positive for *Haplosporidium pinnae*; M: male; F: female/already fertilized eggs; f: female/unfertilized eggs; I: indeterminate. Stimuli: <sup>T</sup>transport stress, <sup>A</sup>thermal shock, <sup>G</sup>gradual temperature increase. <sup>O</sup>Self-fertilization; <sup>□</sup>Controlled fertilization).

| Data                   | Spawning (n° specimens: Female, Male and Indeterminate) | ID specimens                | ID fertilization                  | N° released/collected eggs                           | N° fertilized eggs | Fertilization rate | Trochophorae rate |
|------------------------|---|-----------------------------|-----------------------------------|--|--------------------|--------------------|-------------------|
| 20/06/2023             | 5 <sup>T</sup> (3F; 2M)                                 | F: 4*; 5*; 6*<br>M: 7; 9*   | Pn_F1 <sup>O</sup>                | 11.310.000   | 10.780.000         | 95.31 %            | 99.6 %            |
| 21/06/2023             | 5 <sup>T</sup> (3F; 2M)                                 | F: 4*; 5*; 6*<br>M: 7; 9*   | -                                 | eggs not counted                                     | -                  | -                  | -                 |
| 22/06/2023             | 2M <sup>T</sup>   | M: 7; 10*                   | -                                 | -  | -                  | -                  | -                 |
| 28/06/2023             | 3F <sup>T</sup>   | F: 4*; 5*; 6*               | Pn_F2 <sup>O</sup>                | 3.313.800  | 3.280.200          | 99 %               | 94.5 %            |
| 05/07/2023             | 5 <sup>T</sup> (3M; 2I)                                 | M: 7; 9*; 10*<br>I: 8*; 11* | Pn_F3 <sup>O</sup>                | eggs not counted                                     | -                  | -                  | -                 |
| 23/05/2024             | 1 <sup>T</sup> (1M)                                     | M: 6                        | -                                 | -  | -                  | -                  | -                 |
| 31/05/2024             | 2 <sup>T</sup> (2F)                                     | F: 5; 6                     | Pn_F4 <sup>O</sup>                | not quantified (in 11 L: 2.420.000 larvae)           | -                  | -                  | -                 |
| 18/06/2024             | 0 <sup>A</sup>  | I: 2; 4                     | -                                 | -  | -                  | -                  | -                 |
| 19/06/2024             | 3 <sup>A</sup> (2f; 1f/M)                               | f: 1; 3; 6<br>M: 6          | Pn_F5 <sup>□</sup>                | 44.280.000 (in 11 L)                                 | 31.560.000         | 72.5%              | 37.2%             |
| 08/07/2024             | 2 <sup>A</sup> M  | M: 1; 4                     | -                                 | -  | -                  | -                  | -                 |
| 15/07/2024             | 2 <sup>A</sup> M  | M: 4; 6                     | -                                 | -  | -                  | -                  | -                 |
| 17/07/2024             | 3 <sup>A</sup> M  | M: 1; 3; 4                  | -                                 | -  | -                  | -                  | -                 |
| 15/04/2025 (Shoreline) | 1M <sup>+</sup>   | M: 1                        |                                   |  |                    |                    |                   |
| 16/04/2025 (Shoreline) | 2 <sup>+</sup> (1F; 1 M)                                | M: 1<br>F: 2                | Pn_F6 <sup>□</sup>                | eggs not quantified                                  |                    |                    |                   |
| 25/04/2025 (Unige)     | 2 <sup>+</sup> (1F; 1 M)                                | M: 5<br>F: 4                | Pn_F7 (Spontaneous fertilization) | 3.693.600 (eggs collected on the bottom of the tank) |                    | 8.9 %              |                   |
| 25/04/2025 (Unige)     | 2 <sup>+</sup> (1F; 1 M)                                | M: 5<br>F: 4                | Pn_F8 <sup>□</sup>                | 4.109.000  |                    | 17.5 %             |                   |
| 12/05/2025 (Unige)     | 1M <sup>A</sup>   | M: 5                        |                                   |  |                    |                    |                   |
| 12/05/2025 (Shoreline) | 0 <sup>A</sup>  | -                           |                                   |  |                    |                    |                   |
| 14/05/2025 (Unige)     | 0 <sup>A</sup>  | -                           |                                   |  |                    |                    |                   |
| 21/05/2025 (Shoreline) | 0 <sup>A</sup>  | -                           |                                   |  |                    |                    |                   |

Following spawning (Fig. 2), the eggs released (already fertilised or not) were collected, filtered on two filters: 125  $\mu\text{m}$  (or less, depending on the availability of filters) to retain faeces or aggregates of decomposing organic matter, and 45  $\mu\text{m}$  on which the eggs were retained (egg diameter 55  $\mu\text{m}$ ). During this process, the eggs were kept submerged so as not to dry out on the filter. Then, the eggs were placed in a known volume, counted and measured. The number of eggs was counted by homogenizing the sample, taking a 1 mL subsample (at least 3 replicates), and counting in a Sedgwick Rafter Counting Chamber. Embryo development was monitored during egg release counts to determine fertilization rate. If unfertilized eggs were released, fertilization was achieved by adding a few mL of sperm. The eggs were then gently stirred several times to facilitate contact between the gametes, and after about half an hour the development of the embryos was observed. As the eggs settled to the bottom, it was important to fertilise them in a container (beaker or tank) with sufficient surface area to ensure the formation of a monolayer on the bottom. In this way, the eggs do not overlap and are more likely to be fertilised.



Fig. 2: Eggs spawning of *Pinna nobilis* specimen

The zygotes were then placed in several tanks of different volume and equipped in order to insufflate air through a small rigid tube, creating a chain of bubbles (about 1 bubble/second). The development of the embryo was monitored until the formation of the larvae (Fig. 3).

### 1.3 Larval rearing

The larvae were reared at a water temperature of about 21°C (room temperature), according to Trigos et al. (2018), to avoid the rapid development due to the high temperature observed in Ferranti et al. (2024). The water used was filtered and sterilized in the same way as for adult specimens. The larvae were placed in tanks with different volumes, at the density of 2.5 larvae/mL and 5 larvae/mL.



The larvae were fed daily with a mix of 2 microalgae (*I. galbana*, *C. calcitrans*): the first day a full dose was provided, the second day only the amount consumed was added. *I. galbana* is a species characterised by a nutritional profile rich in docosahexaenoic acid (DHA) (Helm et al., 2006; Martino et al., 2023), whereas *C. calcitrans* has a nutritional profile rich in eicosapentaenoic acid (EPA). Essential fatty acids (EFAs), particularly the 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). Consequently, the larvae were fed a total of 133.3 cells/ $\mu\text{L}$  of *I. galbana* and 150 cells/ $\mu\text{L}$  of *C. calcitrans*. This is because, although the polyunsaturated fatty acid content is highly dependent on the culture conditions of the microalgae (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/ $\mu\text{L}$  of *C. calcitrans*: 1 cell/ $\mu\text{L}$  of *I. galbana*; Helm et al., 2006). However, we preferred to test an EPA/DHA ratio of 1/1 and dosed an amount of *C. calcitrans* biomass equivalent to half the biomass of *I. galbana* (Tab. 1). In addition, once the larvae reached a size above 120  $\mu\text{m}$ , the diet was supplemented with another microalga, *Tetraselmis suecica* ( $4.4 \cdot 10^6$  cell/mL), which is known to improve the survival of bivalve mollusc larvae, compared to a diet based on only two microalgae (Helm et al., 2006). Therefore, the microalgal concentrations were recalculated, supplying 88.86 cells/ $\mu\text{L}$  of *I. galbana*, 8.89 cells/ $\mu\text{L}$  of *T. suecica* (knowing that 1 cell of *I. galbana* = 0.1 cells of *T. suecica*) and 200 cells/ $\mu\text{L}$  of *C. calcitrans* (knowing that 1 cell of *I. galbana* = 2.25 cells of *C. calcitrans*), thus further enriching the diet in EPA.

No microalgae were fed to the larvae until they reached the D-larvae stage, as they only start feeding at this developmental stage. (Helm et al., 2006).

The water in the larval tanks was changed every two days using a 45  $\mu\text{m}$  filter on which the larvae were collected, which was kept submerged inside 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 subsample/3 replicates) and then were put back into the tanks filled with renewed water and fed, trying to maintain concentrations of approximately 1-2 larvae/mL, after the initial mortality. At the same time, larval survival and development was monitored.

In both 2023 and 2024, we observed that larvae produced by self-fertilization stopped their cycle at 9 days post fertilization (dpf), at the D-larva stage, with an average shell size of 86  $\mu\text{m}$ , resulting in less viable larvae (Fig. 3Af). Whereas the larvae obtained from controlled fertilization developed better, surviving until 21 dpf (Fig. 3B), of these, 40% reached the umbonate larval stage at 16 dpf

with a shell size of 150  $\mu\text{m}$  (Fig. 3Ah), in accordance with Hoyos-Chairez & Chavez-Villalba (2023), who report that *Atrina maura* (Family Pinnidae) reaches this larval stage at the same size and after about 15 dpf. This larval size was never achieved before for *P. nobilis*

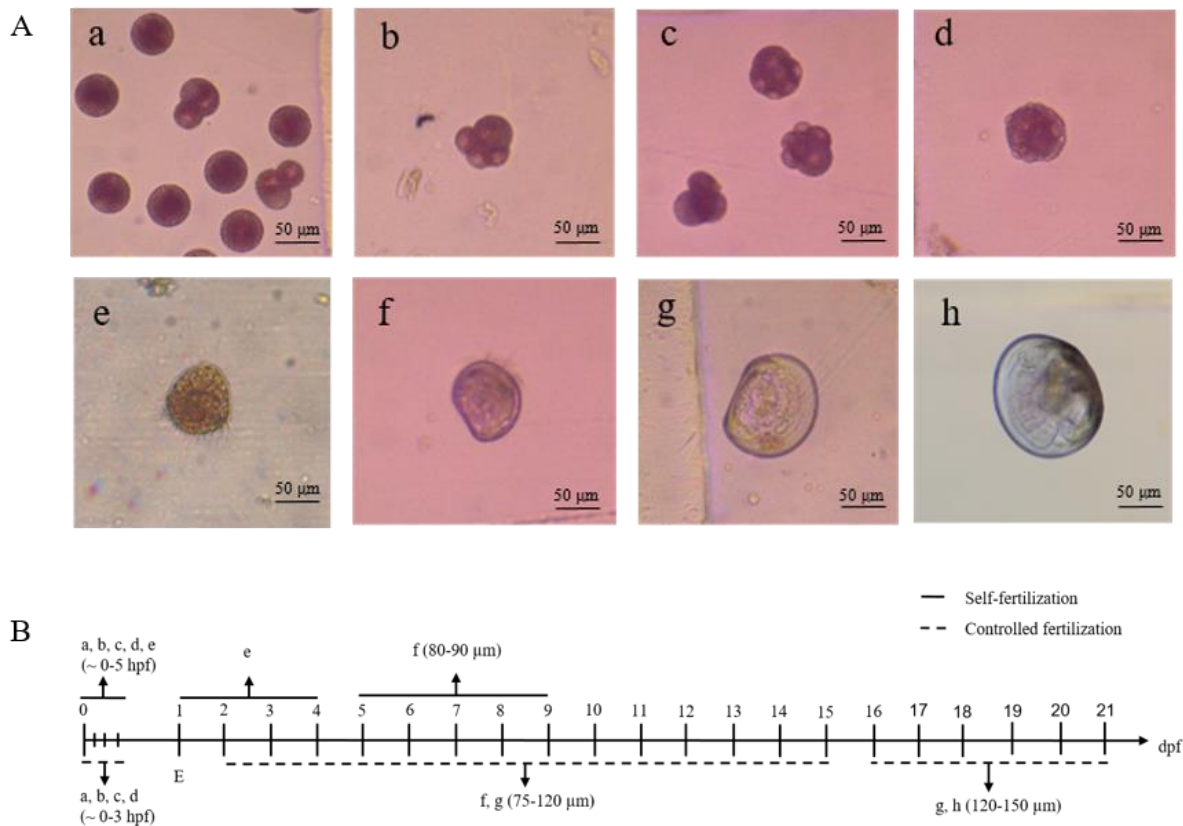


Fig. 3: A) Larval developmental stages of *Pinna nobilis*: a: Egg and 1<sup>st</sup> complete division; b: 4<sup>th</sup> division; c: End of cell division phase (>8 cells); d: Morula; e: Early trochophore; f: Early D-larvae (~80  $\mu\text{m}$ ); g: D-larvae (~120  $\mu\text{m}$ ); h: Umbonate larvae stage (~150  $\mu\text{m}$ ). Line bar: 50  $\mu\text{m}$ . B) Timing of *Pinna nobilis* larvae development, comparison between self-fertilization (continuous line) and controlled fertilization (dotted line).

Figure 4 shows the trend in larval survival for both types of fertilizations: for self-fertilization, the first count was only at 5 dpf, with a survival rate of 16% (D-larva stage). On the other hand, in controlled fertilization, the survival rate reached 37.2% at 1 dpf (trochophore stage) and remained around this percentage until 5 dpf with the development of the D-larva. At 7 dpf there was a further marked decrease with a survival rate of 5%.

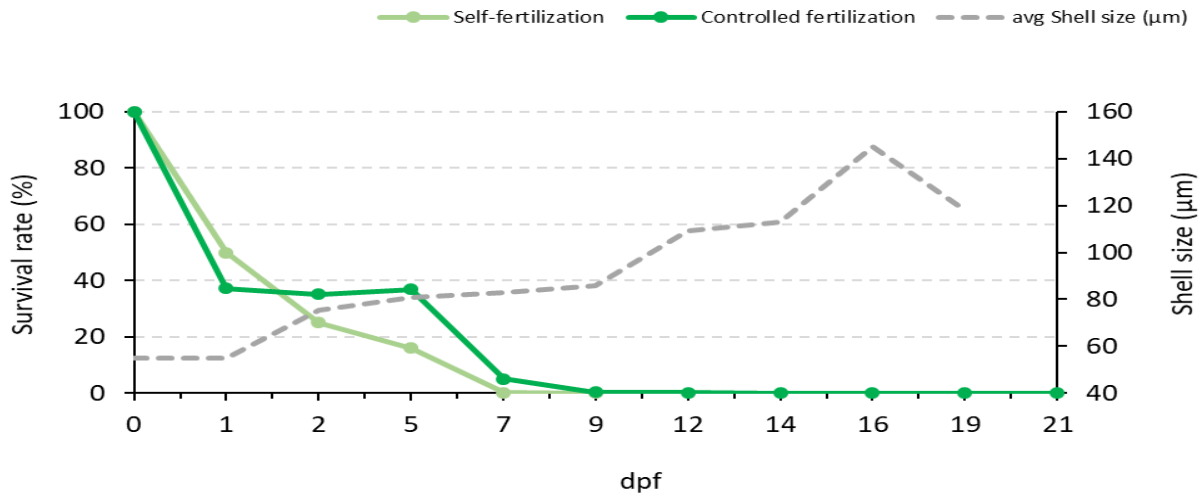


Fig. 4: Larval survival rate (self-fertilization and controlled fertilization) and average shell size of larvae of *Pinna nobilis*

Unfortunately, the pediveliger stage and settlement were not reached. However, it was hypothesised that these would be achieved within a few days, based on information reported for other species of Pinnidae. In fact, Hoyos-Chairez & Chavez-Villalba (2023) report that settlement starts at 29 dpf for *A. maura*; Hashimoto et al. (2023) at 24 dpf for *A. lischkeana*. Consequently, the previous assumption that the stage of settlement of *P. nobilis* larvae is between 5 and 10 days (De Gaulejac, 1989; Basso et al., 2015), contrasts with the information reported in the recent literature and with what was observed in the present study. Although the diet used resulted in good larval development and survival up to 21 dpf, it is hypothesized that in order to achieve settlement it will be necessary to further develop techniques for larval management of *P. nobilis*, focusing efforts on improving the nutritional profile of the diet provided and also on water quality management. In this regard, it could be useful to work with larger volume rearing systems, which can guarantee a greater stability of water parameters (i.e. nutrients) for a longer period of time, as proposed by Hashimoto et al. (2023), facilitating cleaning and water changes without significant manipulation of the larvae.

#### 1.4 Juveniles rearing

As reported above, since the settlement was not reached, this did not allow for the obtaining of juveniles.

## 2. Gonadal maturation induction trials

In 2025, from mid-January to April, a gonadal maturation induction trial 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).

The experimental set-up was designed based on the experience gained by the team at the Pula Aquarium (Garcia, 2023: personal communication; Rodriguez-Goenaga et al., 2024 \_not published) and information found in the literature (Hernandis et al., 2023).

Only 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 L, and the sea water used was filtered and sterilized as described above. The water temperature was gradually increased from 15°C to 20°C, using heaters and coolers to stabilize the set temperature. The photoperiod was adjusted using an LED lamp, increasing from 10 hours to 18 hours of light (Fig. 5).

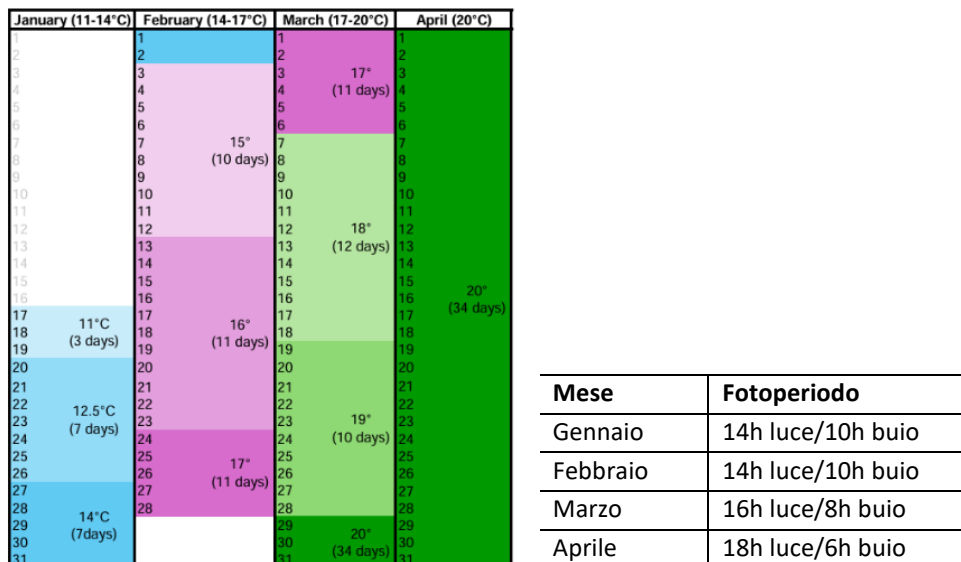


Fig. 5: Set-up temperature trend (left) and photoperiod (right)



The specimens were fed a daily diet consisting of a mix of three live microalgae: 60% *Isochrysis galbana*, 30% *Chaetoceros calcitrans/Phaeodactylum tricornutum* and 10% *Tetraselmis suecica/T. chunii* (between  $1.05 \cdot 10^{10}$  –  $2.1 \cdot 10^{10}$  cells/specimen). In addition, zooplankton was also given (copepods, *Artemia salina* nauplii and rotifers) in vivo or artificial feed (2 ml of Bea zoo plus®; 2.5 g/tank of Gold pearls®).

In April 2025, when the temperature reached 20°C in both Trieste and Genoa, we observed a total of 4 spontaneous spawning, which led to 3 fertilizations and respective larval cycles.

Of the 3 fertilizations, one occurred spontaneously in the tank and the other 2 were controlled fertilizations. Fertilization rates were very low (8.9-17.5%), with a total of about 7.802.600 eggs collected (Table 1, see data 2025).

Unfortunately, larval development stopped at the trochophore stage (Fig. 3Ae), which was observed up to 8–10 days after fertilization, without ever progressing to the subsequent larval stages.

In May 2025, four thermal shock trials were carried out to induce the release of additional gametes, but unfortunately, only one specimen released male gametes, making it impossible to proceed with controlled fertilization. Unfortunately, gonadal maturation led to the release of gametes, but with a reduced number of eggs, low fertilization rates and larval development interrupted in the early stages.

### 3. Paper published

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 reproductive 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., Segarich M., Chiantore M., submitted. Controlled reproduction of the noble pen shell *Pinna nobilis*: comparison of larval development from 1 self-fertilization and controlled fertilization. *Aquaculture Reports*.

Finally, data relating to gonadal maturation trials in 2025 are currently being processed and a paper is being drafted.



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