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Sexual Maturation of Fan Mussel *Pinna nobilis* (Linnaeus, 1758) (Mollusca: Bivalvia) in Experimental Cages in the Mali Ston Bay (South Adriatic Sea)

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Abstract: The research included 120 specimens of *Pinna nobilis* cultivated at a commercial park for oyster (*Ostrea edulis*) and black mussel (*Mytilus galloprovincialis*) cultivation. Research was carried out from 2012 to 2016, prior to the start of the mass extinction of fan mussel in the Mediterranean During the study period, the average sea temperature at a depth of 3 m was 14.87 ± 4.22 °C, ranging from 7.83 to 24.90 °C. The age of the specimens at the beginning of sexual maturity was approximately three years. The average length of the specimens throughout the study was 293.01 ± 16.58 mm. Gonad status was monitored monthly by sampling. The gender ratio in the study was 46 females (38.4%), 50 males (41.6%), and 24 undetermined individuals (20%). In our study, the results showed that males mature slightly earlier than females. The main spawning season occurred during the warmer part of the year when seawater exceeded 18 °C, starting in May and lasting until November. During the study, the GSI varied as expected and peaked in June (12.1), increasing from May to September.

Keywords: marine ecology; aquaculture; conservation; *Pinna nobilis*; Adriatic Sea

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1. Introduction

The fan mussel *Pinna nobilis* is endemic to the Mediterranean Sea and is one of the largest bivalves that reach a size of up to 86 cm [1]. However, according to data from the Adriatic Sea, the maximum length is up to 120 cm [2]. It is a long-living bivalve, up to 20 years according to [3] Butler et al. (1993). On the other hand, an age of 27 years has been reported in the Thermaikos Gulf (Greece) [4]. Recruitment [3] is highly variable, occurring at depths between 0.5 and 60 m, mostly on soft bottom areas overgrown by meadows of the seagrass *Posidonia oceanica*, *Cymodocea nodosa*, *Zostera marina* or *Zostera noltii* [2], but also on bare sandy bottoms [5,6].

The state of fan mussel populations can be discussed by referring to two periods, one before 2016 and the other after. Up until 2016, the population of *Pinna nobilis* was endangered as a result of recreational and commercial fishing for food, using its shell for decorative purposes, and incidental killing by trawling and anchoring. In the European Union, it has been listed as an endangered species and was placed under strict protection according to the European Council Directive 92/43/EEC. In some areas, dense populations of 15–16 ind. per ha^{-1} were reduced to 1 ind. per ha^{-1} in a single year [7]. Destruction of eggs, larvae and adults by chemical pollutants and regression of its common habitat, the seagrass beds of *Posidonia oceanica* due to anthropogenic activities, have also contributed to the acceleration of the decline of this formerly abundant species [8].

This situation persisted until 2016, when all the aforementioned problems faded into the background, as this largest shellfish of our sea almost no longer graced the seabed. After 2016, and over the following 3–4 years, fan mussel populations in the Mediterranean almost disappeared due to a massive die-off caused by the protozoan *Haplosporidium pinnae* [9]. The die-off reached such proportions in subsequent years that the European authorities

decided to classify the fan mussel as a critically endangered species. on the IUCN global red list of threatened species [10].

In 2019, the first cases of mortality were recorded in our southern Adriatic aquatorium. In the Bay of Mali Ston (where we conducted research and maintained fan mussel breeding stocks), during the summer months of the same year, the natural population of pen shells perished, as well as all specimens we had in the breeding stock cultivated since 2005 [11]. Prior to 2016, knowledge of the biology and ecology of the species was fragmentary, and several aspects needed further investigation [3,12]. Investigations focused on the age and growth rate of the *Pinna nobilis* in natural conditions [6,13–16]. Biofouling was also investigated [17], and its distribution and habitat [18], organisms that are symbiotically related, such as the shrimp, *Pontonia pinnophylax* and the crab, *Nepinnotheres pinnotheres* (Decapoda) associated with this bivalve [18]. In the Western and Central Mediterranean, research involved sexual development and maturation [19], the influence of hydrodynamic forces on population structure [12], and monitoring of the populations [20].

Research was also conducted on growth under natural conditions, mainly on the seafloor [5,7,12–15,21–23]. Gametogenesis was also investigated, with findings indicating it is generally similar to other shellfish species [24]. The potential for suspension farming was explored in the eastern Mediterranean [25] alongside growth possibilities in commercial production conditions for oyster and mussel parks in the Adriatic [11].

Mass mortality of populations and critical endangerment now require more intensive research on potential laboratory cultivation and natural repopulation in order to save the remaining specimens and stimulate repopulation. Since 2016, intensive research has been conducted on mass die-offs and the causative agents of die-offs, as well as the biology of the causative agent of mass mortality, *Haplosporidium pinnae* [26–34], as well as other possible causative agents found on deceased fan mussel specimens such as *Mycobacterium* sp., *Vibrio* sp. and *Perkinsus* sp. [35–37]. In order to save the species, it is necessary to understand sexual development and controlled spawning methods, which are also under investigation [38,39]. As there are not enough surviving individuals, research is also focused on close relatives as potential victims of future die-offs, such as *Pinna rudis* [40].

In order to achieve quality protection for *Pinna nobilis*, it is necessary to obtain better knowledge of its biology. Equally, studies on controlled breeding are very important. Solving the problem of controlled breeding could protect its natural populations. The preliminary results of sexual maturation in the cage rearing of fan mussel in the first years of life were presented. This research was carried out just prior to the huge extinction of the fan mussel throughout the entire Mediterranean, which is not possible in today's conditions where the species is on the verge of extinction.

2. Materials and Methods

2.1. Study Area

We cultivated the fan mussels in the Bay of Mali Ston (42°52′12″ N, 17°42′07″ E) (Figure 1). Mali Ston Bay is a protected marine park located on the eastern side of the Adriatic Sea. Special ecological conditions, due to the elongated shape of the bay, are significantly influenced by the land compared to the open sea [41]. Such conditions have enabled high productivity and survival of numerous shellfish species, with 96 species of shellfish recorded in 35 families [42]. Besides being an area with the highest shellfish production on the eastern coast of the Adriatic, the history of cultivation is very long, especially concerning the European flat oyster (*Ostrea edulis*) and black mussel (*Mytilus galloprovincialis*). Additionally, due to the low human population density of the surrounding area, the bay has not been significantly exposed to anthropogenic eutrophication [43]. Throughout the bay, especially in its inner and shallower parts, until 2019, the population of fan mussel was well developed. Natural populations of fan mussel in the bay are mainly located within the meadows of the seagrass *Posidonia oceanica* or on bare sandy bottoms.

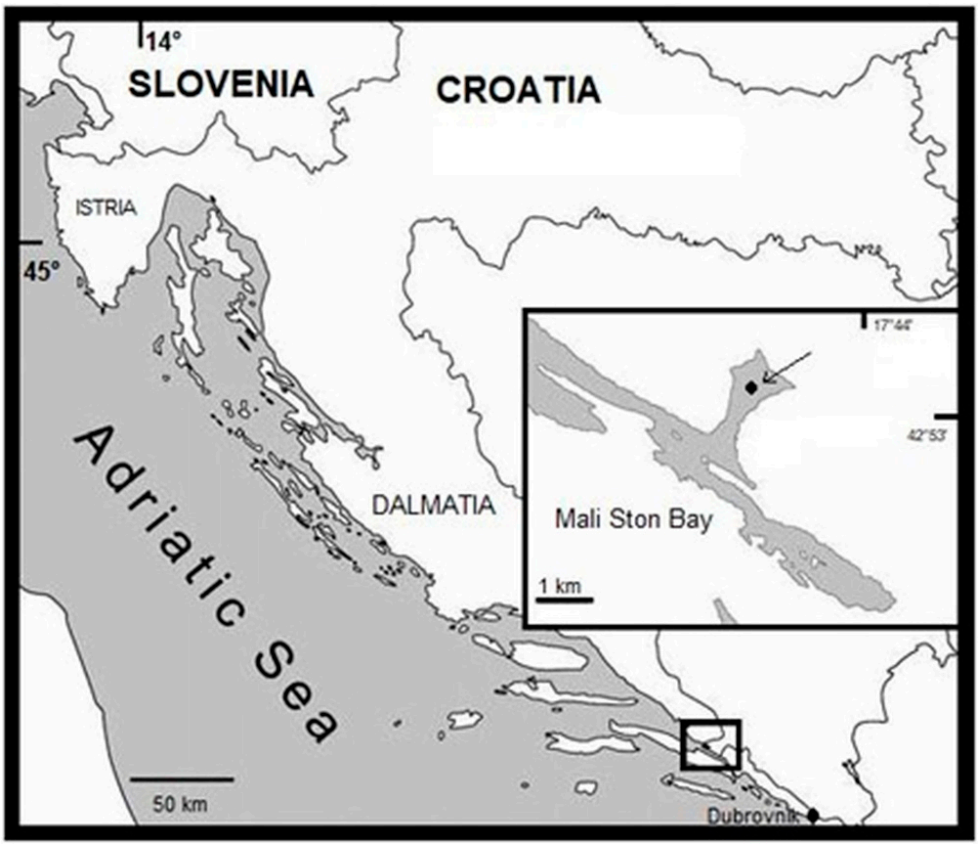


Figure 1. Map of the study area. Mark indicates location of the cages with fan mussel.

2.2. The Sampling of Juvenile Stages

All specimens were collected at the commercial collectors for the flat oyster and black mussel. Several lengths of "Christmas tree" type collectors were placed in the central part of the bay along 500 m of floating line. The collector is a solid core polyurethane (plastic) rope with a diameter of 12 mm and plastic threads around it that increase the total diameter of the rope to 34–36 mm [41]. Juvenile fan mussel individuals from the collector were collected in November 2012. A total of 163 individuals (18.1 to 94.3 mm SL, mean 35.2 ± 14.3 mm) were collected and transferred to specially prepared cages for cultivation at commercial mussel and oyster farms in the bay (Figure 2).

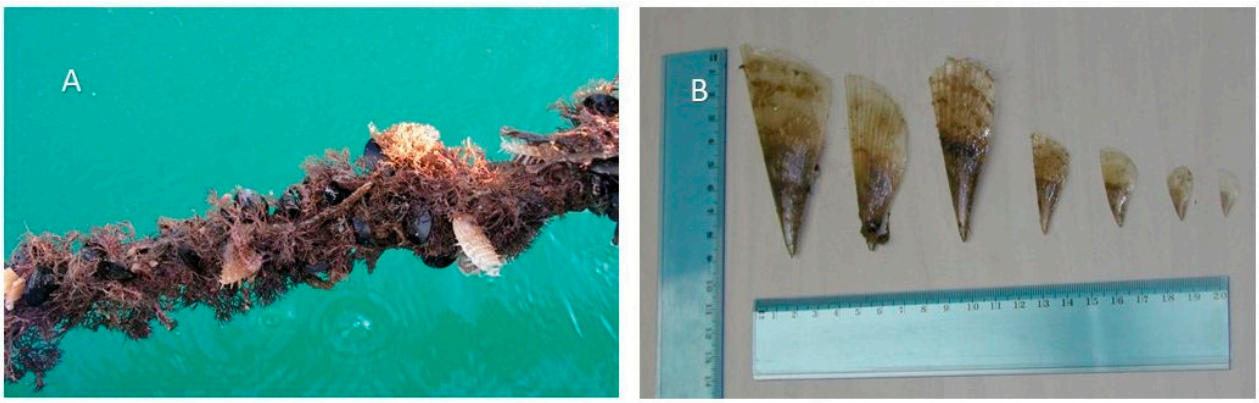


Figure 2. *Cont.*



Figure 2. (**A**) Fan mussel *P. nobilis* spat on the "Christmas tree" collector, (**B**) Measuring of juvenile *P. nobilis* individuals, (**C**) styrofoam with holes in which individuals were placed, (**D**) cages with postlarvae with fishing net and plastic net on the bottom, (**E**) Juvenile specimens of *P. nobilis* in experimental cages. (**F**) Picture of 1-year-old *P. nobilis* specimens in experimental cages.

2.3. Growth to Sexual Maturity

Specially designed cages were constructed (30 cm in height and 15 cm in diameter) (Figure 2). The cages were made of wire netting covered with fishing net (mesh size 4 mm). The wire frame cages were insulated with plastic. The cage format was cylindrical, and within it, at the middle and bottom, there were two circular pieces of styrofoam (3 cm thickness) with holes (1 cm deep) in which specimens were placed. The holes in the styrofoam were made so that the lower third part of the shells was positioned in the hole in the styrofoam. During the growth to sexual maturity, the cages were adjusted according to the growth of individuals to ensure optimal growth [11]. The cages (20 individuals/cage) were placed at a depth of 3 m, and the bottom depth at the cultivation site was 6 m.

2.4. Sexual Maturation in Cage Cultivation

After 24 months of cultivation, we began investigations into the sexual maturation of individuals. Ten individuals were sacrificed monthly to monitor the annual cycle of gonad development and other measurable parameters. Ten individuals were transported to the laboratory in two separate tanks of seawater enriched with oxygen using battery-operated air pumps. Transportation lasted 40 min, and in warm months, the temperature was maintained at natural values at the time of collecting organisms from the cages. Biometrics and gonad sampling were conducted within two hours of sample extraction from the sea. We measured the length of the individual (SL), width of the individual (SH),

individual weight (TW), soft tissue weight (STW), shell weight (SW), and gonad weight (GW). Dimensions were measured to the nearest 0.1 mm using Vernier calipers. Sample weight was measured with an analytical balance with a precision of 0.1 g. Gonadosomatic index and condition index values were calculated after measuring the parameters of mollusks in the laboratory.

We measured the gonadosomatic index (GSI):

$$\text{GSI} = \text{gonad weight/soft tissue weight} \times 100$$

We measured the condition index (CI):

$$\text{CI} = \text{fresh weight/total weight} \times 100$$

Temperature and salinity were measured at the location of cultivated fan mussels. Biometric data and gonadal tissue sampling was conducted in 2015.

2.5. Histological Analysis

The sex of individuals and the developmental stages of male and female gametes were determined by examining biopsy samples. Gonad samples preserved in 10% formaldehyde were further processed with formaldehyde, and alcohol-fixed in paraffin. Embedded samples in paraffin were sectioned with a microtome at a thickness of 4–5 µm and stained with hematoxylin-eosin and gentian-violet stains. The degree of gonad development was determined according to [44] Deudero et al., who presented five developmental stages in the gonads of both sexes: 1. Undifferentiated, 2. Early developing, 3. Late developing, 4. Spawning, and 5. Regressing. Microscopic examination of histological samples was examined, and developmental stages were determined using a Leica binocular microscope DM 750 at a magnification of 400×. A series of micrographs for each monthly sample were made using Toupcam L3CMOS camera attached to the microscope. Micrographs were further analyzed in FIJI (1.54f) software, where a diameter of 100–150 oocytes per sample was measured.

2.6. Statistical Analysis

Statistica 8.0 software package (Statsoft Inc., 2300 E 14th St., Tulsa, OK, USA) was used for statistical analysis of the raw data (oocyte diameter). Monthly data groups (N = 100) were tested for normality using Shapiro–Wilk W test ($p < 0.001$), and subsequently, upon confirmation of normality, one-way ANOVA and Fisher LSD post-hoc were used to assess different stages in oocyte size and development. For each month, a frequency distribution histogram of oocyte size in samples was created.

3. Results

3.1. Hydrological Parameters

During the investigated period, the average sea temperature at a depth of 3 m was 14.87 ± 4.22 °C, ranging from 7.83 to 24.90 °C. The salinity at the same depth during the research period was 35.2 ± 2.68 psi.

3.2. Growth to Sexual Maturity

The age of individuals at the beginning of the study was 2+ years, approximately three years. The average length of individuals during sampling for sexual development research was 293.01 ± 16.58 mm, while the width was 117.31 ± 6.09 mm. The average weight of individuals without seawater was 257.04 ± 43.35 g, while with seawater in the shellfish, it was 400.75 ± 68.91 g during annual sampling (Table 1).

Table 1. Monthly and annual average values of biometrics of *P. nobilis* individuals in cage culture.

Month	Weight Shell (g)	Length Shell (mm)	Width Shell (mm)
March	290.48 ± 64.28	306.6 ± 23.37	123.4 ± 2.07
Apilr	220.52 ± 37.47	292.2 ± 14.02	110.2 ± 8.76
May	291.22 ± 59.56	326 ± 31.02	122.6 ± 8.41
June	221.44 ± 45.88	291.8 ± 22.04	117 ± 3.39
July	221.1 ± 34.13	285.6 ± 30.39	112.8 ± 7.66
August	240.54 ± 54.28	285.2 ± 30.69	119.8 ± 7.95
September	203.34 ± 25.98	269.6 ± 20.94	107.2 ± 3.83
October	338.28 ± 76.75	311.4 ± 24.32	126.4 ± 9.84
November	259.84 ± 25.98	288.4 ± 22.83	111.8 ± 11.73
December	232.42 ± 76.75	275.4 ± 13.18	114.2 ± 4.49
January	245.14 ± 73.39	276.4 ± 33.00	118.8 ± 11.54
February	320.2 ± 134.59	307.6 ± 54.97	123.6 ± 18.45
AVG/year	257.04 ± 43.35	293.01 ± 16.78	117.31 ± 6.09

3.3. Histological Analysis

The sex ratio of 120 individuals in the study was balanced: 46 females (38.4%), 50 males (41.6%), and 24 (20%) undetermined individuals. The frequencies of oocyte diameter throughout the year are shown in Figure 3. By measuring the diameter of oocytes, we determined that the largest recorded diameter was in September, at 98.17 µm, while the average largest diameter of oocytes was in July, at 17.12 ± 27.08 µm. The smallest average values were recorded in March, at 3.92 ± 0.94 µm. One-way ANOVA showed differences in oocyte size through months ($F = 8.7001$, $p < 0.001$), and subsequent Fisher LSD post-hoc analysis revealed several phases in oocyte development throughout the yearly cycle. The first statistically different oocyte size was noted during March and April, indicating intensive early gametogenesis and oocyte growth, while the second phase, noted during July and August, is probably the peak of development and the oocyte maturation phase. The third phase, as revealed by post-hoc analysis, is the degenerative oocyte stage at the end of the year (November through December).

We initiated research on gonad development in March, when the sample consisted of eight individuals whose sex could not be determined, and only two females in early developmental stages (Figure 4A). Primary oocytes predominate in the ovaries. April is the month when our gonad sampling results show progress in sexual development, with two females in an advanced stage of sexual development with vitellogenic oocytes (Figure 4C), four in the initial stage (Figure 4A), and two males in an early developmental stage (Figure 4B). The sex could not be identified in two individuals. May is characterized by further progress in individual sexual maturation, with no individuals whose sex could not be determined. In May, the analysis of qualitative stages of gonad maturation showed, as depicted in Figure 5, that females were in an advanced stage (65%), and males were ready for spawning (50%). Males are ready for spawning from May to March (10 months), but this number and percentage decrease as the spawning season progresses and sea temperature decreases (Figure 5). In June, four females (65%) and four males (100%) were recorded in the spawning stage, and there were no individuals whose sex could not be determined (Figures 4E,F and 5). July, August, and September are months in which we record males in the spawning stage and females in the spawning stage or high maturity stage. In these months, two individuals could not be determined by sex or all were defined as is the case in September. In October, along with individuals in a high stage of maturity and in the spawning stage, we first recorded two males in the post-spawning stage. In November, we first found a female in the post-spawning stage and more males in this stage (20%). Other than females in the spawning stage, December is characterized by a smaller number of males in spawning, only two, and two individuals whose sex could not be determined. January and February are characterized by a larger number of individuals in lower degrees of sexual maturity or in a resting stage, as well as a larger number of individuals whose sex

cannot be determined. Throughout the research period, we did not record mortality in the groups of individuals sampled for biopsy.

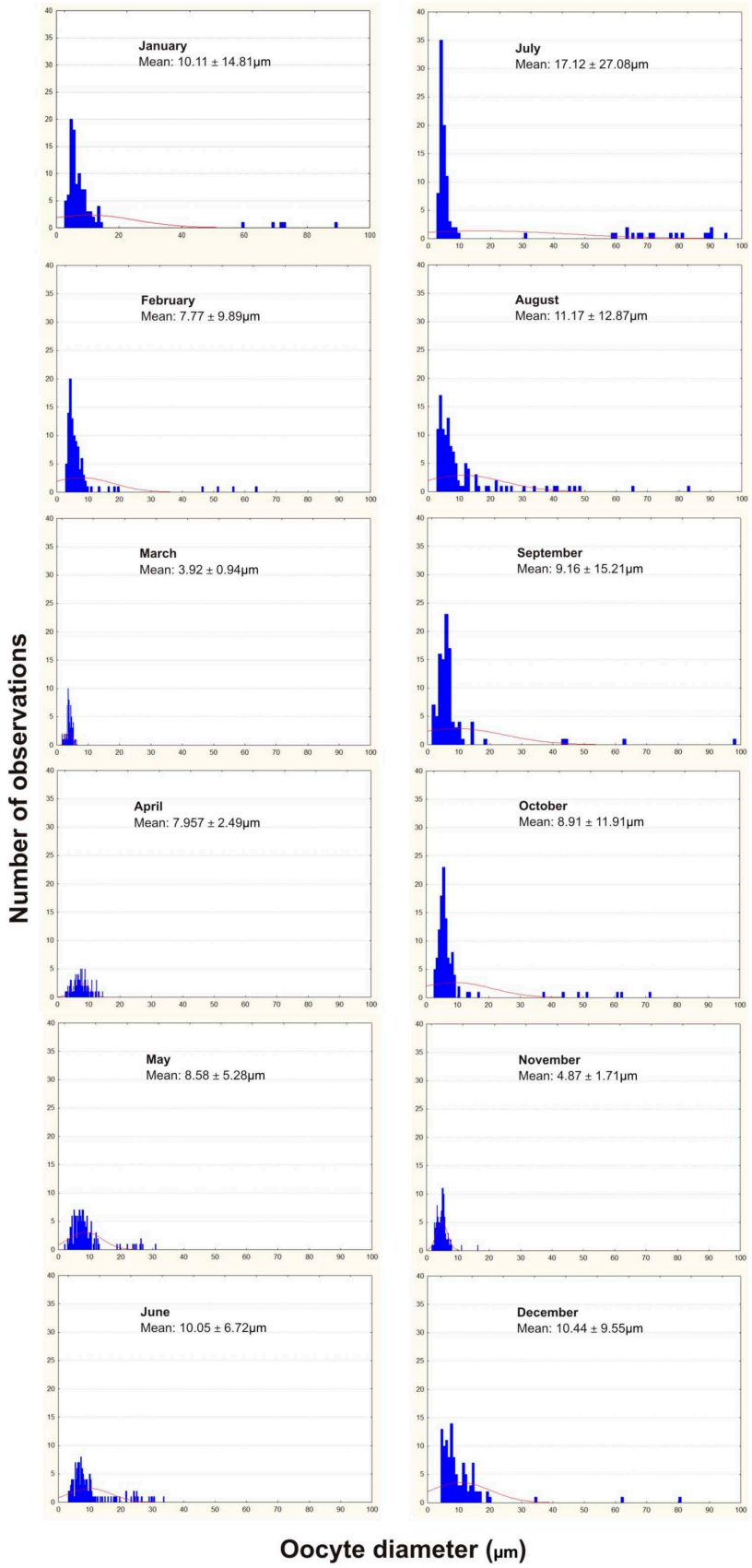


Figure 3. Monthly frequency histograms of oocyte size measured for *P. nobilis* in cage rearing.

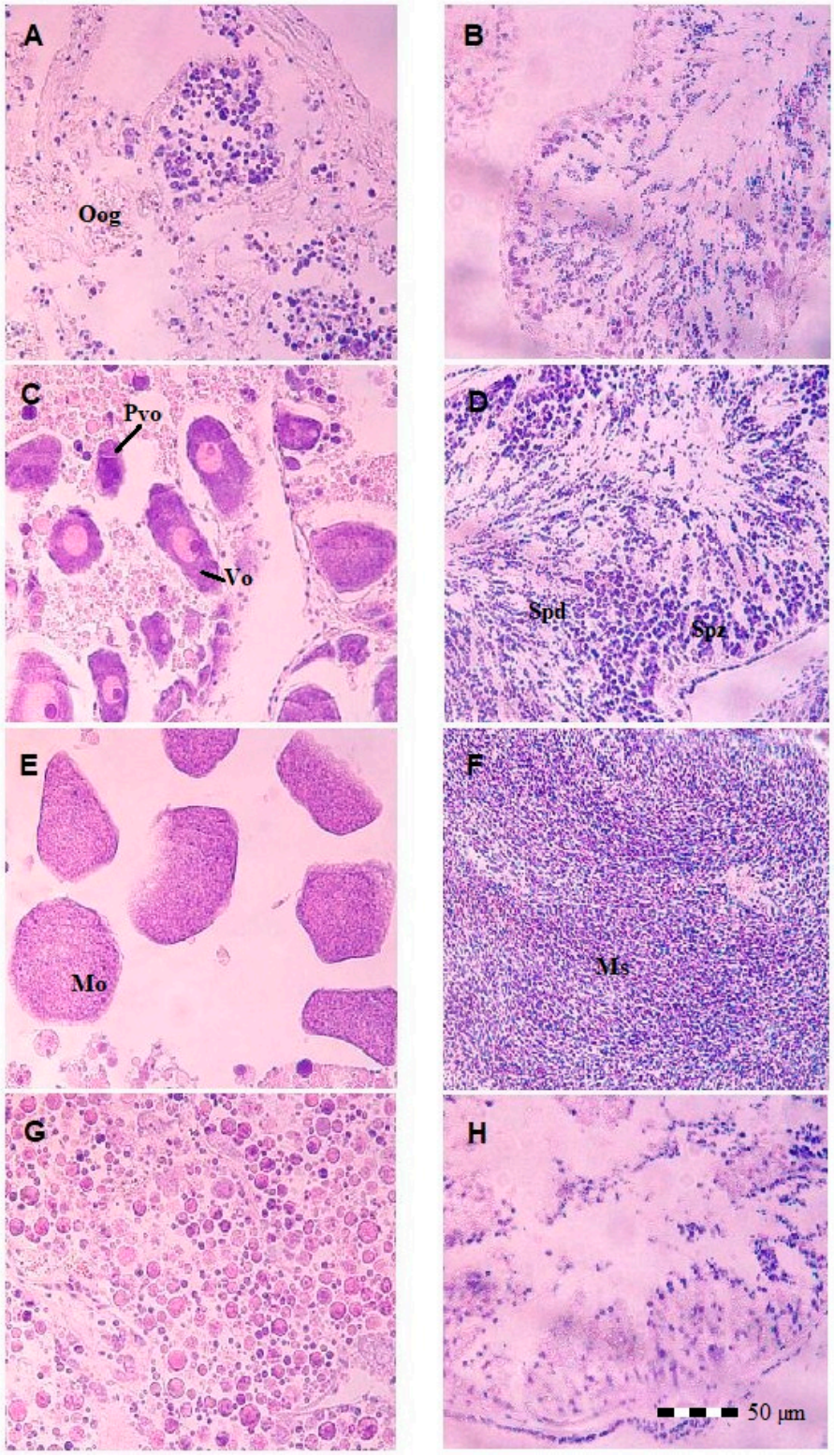
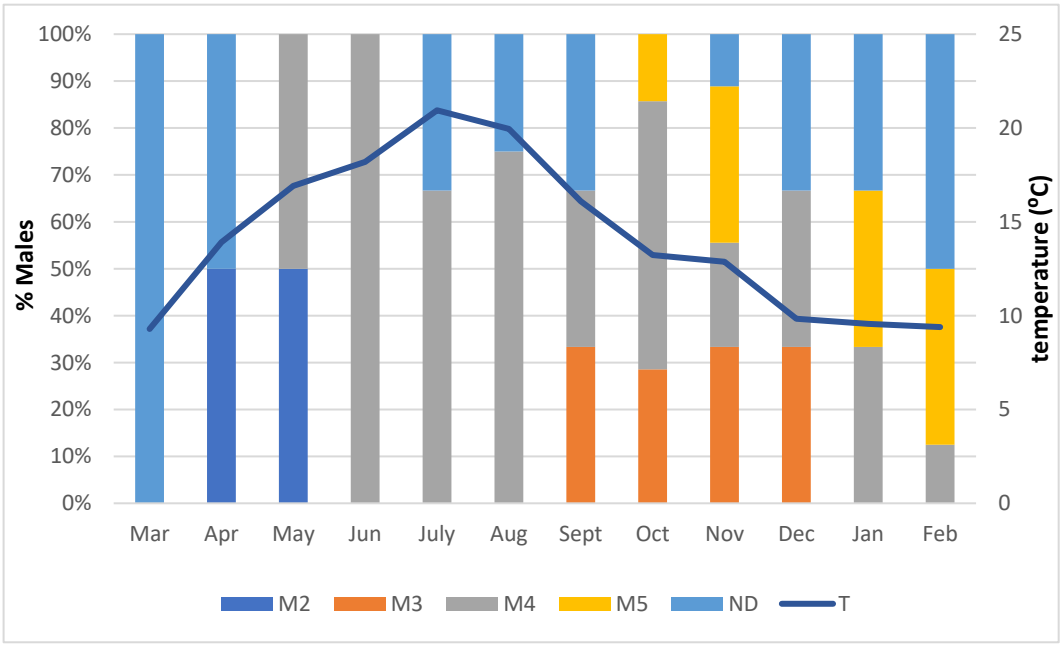
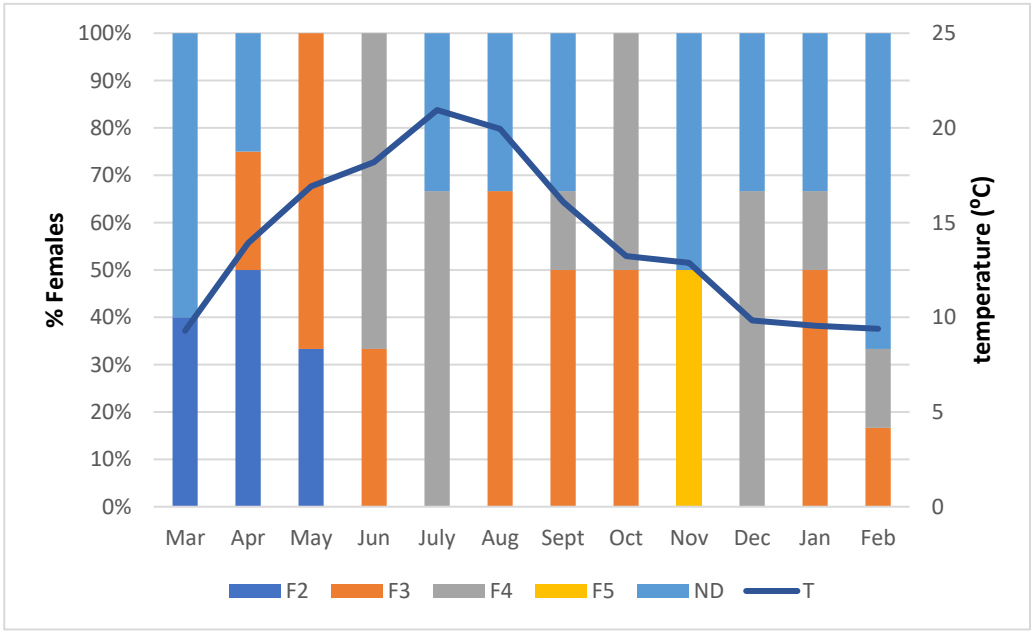


Figure 4. Photos of the different histological gonad development stages of *P. nobilis*: (**A**) early developing female, (**B**) early developing male, (**C**) late developing female, (**D**) late developing male, (**E**) spawning female, (**F**) spawning male, (**G**) regressing, and (**H**) resting, (Mo, mature oocyte; Ms, mature spermatozoa; Oog, oogonia; Spz, spermatocytes; Spd, spermatids; Vo, vitelogenic oocyte; Pvo, previtelogenic oocyte).



(**A**)



(**B**)

Figure 5. Gonad maturation *P. nobilis* for (**A**) males and (**B**) females with 1. (light blue) non-differentiated, 2. (blue) early developing, 3. (orange) late developing, 4. (grey) spawning, 5. (yellow) resting stage. The line is temperature (°C) at the 3 m deep during the sampling period.

GSI varies during the study period as expected, being highest in June (12.1 ± 6.88) and increasing from May to August, when it drops during September to 3.5 ± 0.94, which is also the lowest value in the study period. The condition index was highest in February, at 26.44 ± 2.05, and lowest in August, at 16.93 ± 1.85 (Figure 6).

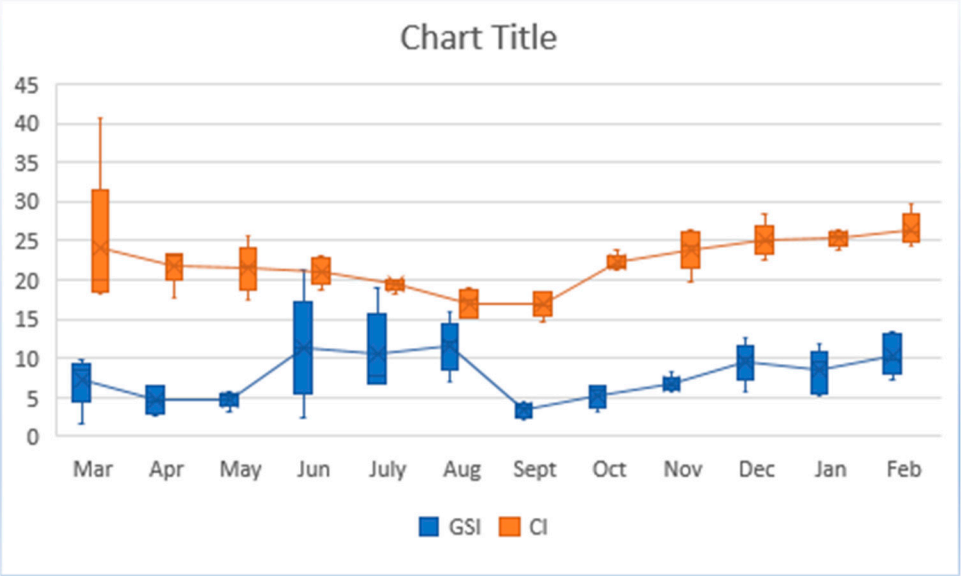


Figure 6. *P. nobilis* gonad index values, condition index and corresponding standard deviation.

4. Discussion

Favorable conditions for the sustainability of mollusk populations in Mali Ston Bay have been confirmed by the number of cultured species and the long tradition of commercial cultivation in the bay, 96 mollusk species from 35 families have been identified [42,45]. Due to the threat to the fan mussel even before the great mortality that began in 2016, this species attracted our attention with the desire to save and maintain the population in our waters. The research was carried out from 2012 to 2016, just before the mass extinction of the fan mussel in the Mediterranean. As the opportunity arose to collect sufficient juveniles with "Christmas tree" collectors [41], research focused on the possibility of cultivation in commercial mollusk farms and the formation of broodstocks. This provides new insights into the species that can be used for area repopulation. After the great mortality, such a method of collection and strategic positioning in the aquatorium becomes even more important for the repopulation of the almost lost species.

4.1. Hydrological Parameters

The bay is naturally moderately eutrophic, which is very favorable for natural and cultivated mollusk populations [46,47]. The temperature of 7.83–24.90 °C and salinity of 30.8–37.8 psi during the research period did not show significant deviations from those usually present in the bay. To avoid significant deviations of water parameters, which are sometimes pronounced in the surface layer, the cages were located at three meters' depth, which, according to previous experience, is the best for the growth of fan mussels at the same density [11]. Additionally, we did not record mortality in the cages with fan a mussel, which is consistent with low mortality in our previous research [11], but also in research from Turkish waters [26].

4.2. Sexual Maturity

This research has confirmed previous findings on the sexual development of the fan mussel [44] and has shown that fan mussel individuals can successfully develop, grow, and sexually mature in conditions similar to commercial cultivation. The conditions in Mali Ston Bay, as previously mentioned, are very characteristic due to the bay's position, depth, and the influence of the coastal zone, so spawning may have slightly different timing than in the surrounding waters. Based on the recorded temperature and salinity measurements, our individuals entered the spawning phase in May and June when the temperature values at a depth of three meters where the cages were located were 17–18 °C and salinity was

34–36 ppt. At the surface during the same period, the temperature was 17–20 °C, while at one meter above the bottom (five meters' depth), it was 11–15 °C. This is the period of temperature increase in the cultivation area, which rises from 9°C in March to 18°C in June. Deudero et al. [44] mention the beginning of spawning at 20 °C, which is slightly higher than in our research. In our research, the average temperature at a depth of 3 m in July and August was 19–20 °C, which is the peak of the spawning season, after which both the temperature and spawning intensity decrease.

4.3. Histological Analysis

All sampled individuals were sexually mature after the second year of life. Throughout the season, we recorded individuals in the spawning stage from May to February. A one-year period of sexual maturity in *Pinna nobilis* in cages for cultivation can be divided into three parts: the colder part of the year (March and April), when we record dormancy and no individuals in spawning; the spawning period from May to October; and the remaining months from November to February where some individuals in higher maturity stages before spawning and during spawning are recorded. The gonadosomatic index in this research is most pronounced from May to September, although we also recorded individuals in high maturity stages before spawning and during spawning later, but the number of these individuals was not such as to maintain total higher GSI values as in these months. Also, the condition index follows the gonadosomatic index except during the period of intensive spawning.

Histological analysis revealed a slightly higher ratio of males to females, with 20% of individuals being undetermined, primarily recorded at the beginning of the study in the winter–spring months before the spawning season. Deudero et al. [44] recorded similar but slightly lower numbers for males and females and a slightly higher number of undetermined sexes. Histological analysis of gonad samples showed that the spawning of fan mussels in our conditions is somewhat longer compared to previous research [44]. Histology showed that males in our study mature slightly earlier in the season compared to females. The first female individuals in the spawning stage were found in June, but some specimens were in a high stage of sexual maturity until February. Male samples showed continuity of high maturity from May to February, while in females, we have individuals in a late maturation stage in August and September but not yet in spawning.

The number of spawning events and the duration of the spawning period can vary greatly depending on the species, geographical area, and environmental conditions [48]. In our conditions, we recorded a long spawning period, which can be indirectly confirmed by the relatively large oscillation in the dimensions of collected juveniles with collectors, where lengths range from 18.1 to 94.3 mm, with mean values of 35.2 $\pm$ 14.3 mm, similar to the size values in previous cultivation experiments [41]. According to some authors, the planktonic phase of larvae lasts 5–10 days [49] and we can conclude that acceptance on our collectors occurred over 5–6 months because such a growth rate from the minimum to maximum size of collected individuals was confirmed in previous post-larval growth experiments [11,25]. The length of spawning and multiple spawning events can characterize the species in a particular aquatorium. In contrast to our research, Cabanellas-Reboredo et al. [50] and Deuredo et al. [44] mention shorter acceptance periods and spawning. Of course, these differences are likely determined by the ecological conditions of the respective research areas. In the research of Deudero et al. [44], an extended spawning season and other peaks in larval acceptance may be determined by the depth at which individuals from shallower and deeper waters spawn at different times of the year or spawning season. Such a situation must be excluded from our research because it concerns individuals from the same location and the same depth cultured for almost three years at a high density in cages.

5. Conclusions

All insights into the ecology of a species that is critically endangered represent a significant contribution to attempts to save its existence. Mastering closed-system cultivation

or suspension on commercial mollusk farms is the next and most important step toward species conservation [51–53].

This research shows that fan mussel individuals can be successfully cultivated on commercial mollusk farms along with other cultivated species. In cage culture conditions with higher density than in natural conditions, *Pinna nobilis* undoubtedly achieves sexual maturity in the third year of cultivation. The sex ratio in cage breeding is equal, and the first seasonal development of oocytes is recorded in March and April. In our breeding conditions, we recorded an extended spawning season with a peak in August. Mortality was not recorded in this study. If a population begins to regenerate in an area or if laboratory cultivation to the post-larval phase is successfully resolved, cage cultivation in a targeted area can likely accelerate repopulation.

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Conflicts of Interest: The authors declare no conflict of interest.

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