Adult spawning and early larval development of the endangered bivalve *Pinna nobilis*.

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ABSTRACT

The development of aquaculture activities has posed an alternative solution for the preservation of some overexploited shellfish fisheries worldwide. In the same way, endemic Mediterranean bivalves such as *Pinna nobilis*, highly threatened by habitat loss and coastal pollution, could found in aquaculture a solution for preserving the continuity of the species. Given the endangered status of the species, the biological and ecological processes regulating natural populations have been well studied, but there are still important knowledge gaps preventing the development of viable artificial cultures. This study describes for the first time the larval development of *P. nobilis* (from fertilization until pediveliger larval stages) in captivity conditions. Moreover, different rearing tanks of 5, 16 and 80L, larvae density from 1 to 600 larvae mL^{-1}, light conditions, food doses, were tested in order to establish the bases for the optimal rearing of the species and provide a source of individuals for restoring field populations. Results showed that 16L tanks with a concentration of 2 larvae mL^{-1}, constant temperature of 21˚C, 12/12h photoperiod and fed with an "*optimal*" mixture of 25 cells per µL of *Chaetoceros calcitrans* + 33.3 cells per µL of *Pavlova lutheri* + 100 cells per µL of *Isochrysis galbana*" appear to be the best conditions to rear larvae of *P. nobilis*. Different captivity conditions such as lower or higher tank volume, larvae density, or food doses; light privation did not report better results for larval development.

Keywords: *Pinna nobilis*, conservation, larvae development, rearing, captivity.

Introduction

In the last decades the rearing of endangered bivalve species for both commercial and ecological purposes has received considerable attention (Ellis, 2000; Knop, 2009). In spite of the economic and scientific interest, the rearing of bivalve species has been proved to be rather difficult due to the great larval mortalities of the group (Rumrill, 1990). Field studies show that larval mortality in natural conditions ranges from 25 to 80% during first days of life (Talmage and Glober, 2009; Gazeau *et al.*, 2010). Both natural and/or anthropogenic-driven mortality in bivalve larvae –i.e.., from egg to postlarval recruit- are often difficult to measure and are considered as a "constant" (Philippart *et al.*, 2003). Possible causes may include failure of fertilization, which is often related to sea acidification and may produce shape abnormalities in embryos or avoid development in over 50% of the larvae (Kurihara *et al.*, 2007); coastal development (Gómez *et al.*, 2000; Guest *et al.*, 2008); shortage of food resources (Rico-Villa *et al.*, 2006), absence of a suitable substrate for benthic settlement (Su *et al.*, 2007); combined effects of short planktonic durations and local patterns of marine circulation (Shanks and Brink, 2005); and/or lethal environmental temperatures (Philippart *et al.*, 2003). Any of these scenarios usually leads to a reduction of recruitment rates and consequently, to the disappearance of populations.

Historically, mortality problems have been observed in the marine bivalve industry for decades (Samain and McCombie, 2008; Barton *et al.*, 2015). The main goal in hatchery production is to improve larval and post-larval survival by achieving larval growth and metamorphosis success. In this term, the proper formulation of larval diets has been considered as the most critical aspect in hatchery operations focusing on the type of microalgae used as feed (Brown *et al.*, 1998; Knuckey *et al.*, 2002; Ponis *et al.*, 2006; Rico-Villa *et al.*, 2006; Pettersen *et al.*, 2010; Ragg *et al.*, 2010; Gui *et al.*, 2016) or even in artificial substitutes to phytoplankton diet (Couteau and Sorgeloos, 1992; Knauer and Southgate 1999; Nevejan *et al.*, 2007; Gui *et al.*, 2016).

In other cases, recurrent mortality episodes are described as a result of virus or vibrio-like bacteria infecting bivalve hatcheries and nurseries (Renault and Arzul, 2001; Dubert *et al*., 2015; Rojas *et al*., 2015) and reducing commercial production by approximately 40% as reported for French farming facilities of Pacific oyster (*Crassostrea gigas*) since 2008 (Pernet *et al*., 2014).

From an ecological perspective, the rearing of endangered bivalves in captivity conditions may pose a potential solution for the rehabilitation of seriously damaged populations (Ronquillo and Mckinley, 2006; Vinvie, 2008; Thomas and De Leaniz, 2010; Theodorou *et al.,* 2015) and face the same early stages' bottlenecks than those reported for bivalve commercial farming (Arnold, 2008). One of these endangered species is the Mediterranean fan mussel, *Pinna nobilis*. The populations of this bivalve have significantly declined during the last decades (De Gaulejac and Vicente, 1990; Vicente and Moreteau, 1991; Garcia-March, 2005; Basso *et al.,* 2015; Rouanet *et al.,* 2015) as a result of different anthropogenic stressors such as coastal development, fishing pressure, and/or accidental harvesting by trawling and shell breakage by anchoring (Katsanevakis, 2005; Acarli *et al.,* 2011; Hendriks *et al.,* 2011). For these reasons, the European Union included it as an endangered species in the ANNEX IV of the Council Directive 92/43/EEC (EC Habitats Directive), and strictly forbids any kind of culling. Although these measures have helped certain recovery of populations in some Mediterranean regions (Pérez-Vallazza *et al.,* 2008; Theodorou *et al.,* 2017), the species is still at risk by unknown factors such as those causing mass mortality event along the Spanish Mediterranean coast and the Balearic Islands during the summer of 2016 (Darriba, 2017; Vázquez-Luis *et al.,* 2017). Temperature effects due to climate change, coastal pollution, and pathogenic mechanisms are amongst potential explanatory factors, all of them mediated by human influence (Rodrigues *et al.,* 2015). In this scenario, the rearing of *P. nobilis* in captivity appears as an alternative solution to obtain healthy stocks of recruits that could be reintroduced in suitable areas with declining population densities (Trigos, 2017). Yet, the duration of the different larval stages from fertilized eggs, as well as the main factors influencing survival rates are still largely

unknown, although they are proposed to follow similar patterns to other bivalves. Following this hypothesis, Vicente (1986) described the larval stages of trochophore, veliger, and pediveliger followed by metamorphosis, and juvenile development of *P nobilis*. De Gaulejac (1989), observed larvae shells with electronic microscope and pointed the possibility of a time gap of 5 to 10 days between gametes expulsion and substrate settlement. Peharda and Vilibic (2008) suggested that veliger fan mussel larvae could have a negative phototactism that allow vertical migration to deeper waters during the day to return to upper bathymetric ranges at night, as also indicated for other bivalves (Gosling, 2003), presumably to avoid UV light or predator activity (Manuel *et al.,* 1996). However, there is still no available information on the behavioural responses of *P. nobilis* larvae to different environmental factors under captivity conditions that could provide a solid basis for designing and implementing a viable larval hatchery. In addition, factors determining patterns of benthic recruitment are virtually unknown, although in other species is often linked to the availability of preferential substrates for settlement (Prado *et al.*, 2012), which highlights the importance of simulating optimal settlement conditions to rear *P. nobilis* in captivity. Other biological and physical aspects such as the possible correlation between adult size and numbers of oocytes expelled also require further investigation.

In this context, the main objective of this work was to describe, for the first time, the larval development of the ecologically important Mediterranean fan mussel (*P. nobilis*) with the aim of developing cultivation techniques and providing a practical guideline for problem identification during larval rearing in this species. More specifically we conducted experiments aimed (1) at different larval densities (2) with different tank volumes, (3) fed with three doses of microalgae mixture and (4) reared in light/dark conditions.

Materials and methods

Field sampling

A total of 40 individuals of *P. nobilis* were collected around the Embiez archipelago, South East of France from April 2012 to September 2014. The collected specimens were carefully transported to laboratory facilities within portable tanks and their total height (Ht) registered and labelled. Shells were brushed to remove epibiontic organisms, including other bivalves (Rabaoui *et al.*, 2015) that could affect the fertilization process. All individuals were reintroduced to the field at the end of the experiments.

Gamete release and fertilization

Small groups of 4-6 individuals were placed in two 120 L tanks within controlled temperature facilities (20-21°C) with filtered (1µm) and treated (UV) seawater for a maximum of 24h. A ≈10°C gradient is necessary to induce a thermal shock causing gametes release in bivalves (Helm *et al.*, 2006) which takes place at various temperature thresholds, depending on the species (Drent, 2004). Herein, in order to keep this temperature gradient two induction tanks were prepared; one tank was maintained at 15 ºC while a second tank was heated to 25°C thus respecting the conformability range of temperatures described for *P. nobilis* (Trigos *et al.*, 2015).

Individuals were transferred to the induction tanks and individuals were translocated between both of them every 50 minutes for a maximum of 6 times. If no response was achieved, individuals were introduced in 2,600L tanks with constant aeration and water renovation, and the process repeated on the following days. When only males expelled gametes, small volumes of sperm were poured in the tanks on the following thermal shocks, with the aim to stimulate the females (if any) while the thermal shock was occurring. When male and female spawning occurred, individuals were introduced individually in 60L tanks in order to avoid possible polyspermia. Both oocytes and spermatozoids were recovered from individual tanks using a sterilized 60 mL micropipette and then filtered through a 30µm sieve in order to remove any

possible fecal waste that could be attached (mainly to oocytes). Volumes of 4 mL of a homogenized sample of oocytes were counted (N= 3) in order to determine the possible correlation between adult size and numbers of oocytes expelled. After that, gametes were introduced together in a 15L tank favouring the mixture, thus enhancing fertilization.

Larval cultures

Preliminary essays were conducted to determine the optimal rearing conditions according to survival time observed. To this aim and after fertilization, larvae were maintained in the same tank with constant aeration and temperature (21°C) until they reached the trochophore stage. Then, they were transferred to different small rearing tanks of 5, 16 and 80L (N=3) in order to better control the larvae development and cultures were also adjusted at different larval concentrations of 1, 2 and 600 larvae mL^{-1} respectively, according to Helm *et al.* (2006) with water renewal fluxes (600 $mL \cdot h^{-1}$) through a 35µm strainer and constant aeration. In addition to exhaustive filtration of seawater circuits periodic, bacterial cultures were carried out to monitor the presence of pathogens within tanks. For each concentration and rearing tank, survival time was estimated daily by sampling (N=1) of 4 mL and counting of living larvae.

Larvae diet was established using a mixture of three microalgae (*Isochrysis galbana*, *Pavlova lutheri* and *Chaetoceros calcitrans*), as suggested in the literature (Pernet *et al.,* 2005; Milke *et al.,* 2004, 2006). The final concentration mix of the three phytoplankton species was adjusted according to three different doses named as "*low*", "*optimal*" and "*high*" using the formula proposed by Helm *et al.* (2006) for bivalves feeding in breeding facilities:

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

Where:

V_{dose} = supplied dose in liters.

Cell density needed [μL] = cell concentration according to low, optimal or high dose.

$V_{tank}(L)$ = tank volume.

Cell density available [μL] = cell concentration in laboratory cultures.

For each concentration mix, the equation included the cell density suggested by the author:"15 *Chaetoceros* cells per µL + 25 *Pavlova* cells per µL + 50 cells per µL of *Isochrysis*" for the "*low*" dose, "25 *Chaetoceros* cells per µL + 33.3 *Pavlova* cells per µL + 100 cells per µL of *Isochrysis*" for the "*optimal*" dose, and "30 *Chaetoceros* cells per µL + 50 *Pavlova* cells per µL + 150 cells per µL of *Isochrysis*" for the "*high*" dose.

Effect of rearing conditions on larval development and settlement

Once previous essays allowed determination of the maximum survival time of larvae under the different parameters established, cultures were also used for testing the influence of light on larvae development due to negative phototactism, as observed for certain bivalve larvae (Raby *et al.,* 1994). To this end, six 16L tanks were set with open circulation at densities of 2 larvae·mL^{-1}. Three of those tanks were kept at 12/12h photoperiod whereas the other three tanks were covered with opaque plastic (darkness conditions). Microalgae doses were identically established as "*low*", "*optimal*" and "*high*" to elucidate if the use of any of them could affect directly the larvae growth. The latter was determined by registering larval length with a "Leica DM2500" microscope. A total of 60 fan mussel larvae were daily placed on a dig dish, and their length measured for average estimations (±SD). The process was carried out by triplicate using larvae coming from different spawns.

For all tanks, abiotic parameters were kept as stable as possible. Temperature: 19-21ºC; salinity: 32-37; pH: 7.7-8.4; and dissolved oxygen: 5.9-7.0 mg O_2. Larvae concentrations were daily monitored (N= 1 per tank) to determine the conditions with lower mortality rates. Moreover, an artificial substrate made of 250µm PVC mesh was deployed in all tanks in order

to enhance larvae settlement. The substrate was autoclaved (120°C. 30 minutes) to prevent the introduction of pathogens within rearing tanks.

Statistical analyses

All statistical analyses were conducted with SPSS® Statistics 21 program. The possible association between the size of broodstocks and the number of oocytes expelled was studied using the Pearson correlation factor.

The best survival rate was determined studying the effect of the tank volume (fixed factor, three levels) and the larvae density (fixed factor, three levels) and calculated by a two-way factorial ANOVA.

The effect of photoperiod (fixed factor, two levels), food dose (fixed factor, three levels) and rearing day on larvae survival, was investigated using a three-way factorial ANOVA followed by a *post-hoc* analysis (DHS-Tukey) to establish significant groupings. All data were tested for ANOVA assumptions of normality (Levene's test) and homogeneity of variances (Cochran's test).

Results

Gamete release and fertilization

A total of 31 individuals (47.10 ± 10.61 cm Ht), of the 40 subjected to thermal shock, released gametes for periods of 40 minutes and, in some instances for up to 3h. Among individuals that responded positively, 5 of them (16.1%) were strictly males and 14 (45.2%) expelled only oocytes. The remaining 12 specimens (38.7%) released almost simultaneously male and female gametes. The release of female gametes in *P. nobilis* was estimated in $1.9 \cdot 10^6$ oocytes $\cdot L^{-1}$ (averaged for the 26 individuals releasing female gametes) with a mean Ht of 51.8 ± 9.98 cm (**Fig 1**).

There was a significant a positive association between the size of individuals and the number of expelled oocytes ($F = 0.765$. $p < 0.01$) as reported for other bivalve species (Helm *et al*. 2006). The smallest spawning female was 37.7 cm Ht, and 34.6 cm Ht in males. In those instances in which there was only male spawning, sperm was stored in a temperature controlled room at 4°C and visual observations revealed that sperm remained alive for a maximum of 3 days.

Larval cultures

Viable oocytes were spherical with an average diameter of Ø = 55 ± 1µm whereas spermatocytes hardly exceed 1µm length. Embryonic development of *P. nobilis* started with the rapid fertilization of oocytes by surrounding spermatocytes at a temperature of 21ºC (**Table 2**). After 15-30 minutes the appearance of the first polar body and the formation of a periviteline membrane confirmed successful fertilization (**Fig. 2**). The first zygote inclusions were observed after 40 minutes and gradually increase the number of blastomeres in a successive formation of inclusions until they attained a ciliated blastula stage 5 h later. Herein, the phase in which the motility of larvae begins (**Fig. 3**) and last for 24 h until the trocophore stage at an average size of 65 ± 5µm. This was followed by a period of frenetic activity where larvae can reach speeds of 0.5 to 1 cm·second^{-1} and displayed a helical swimming pattern as observed in other bivalves

239 (Troost *et al.* 2008). This speed was considerably reduced after the first 48 h when the larvae
240 started to generate their own shell (Prodissoconch I) and become a D-larva or early veliger stage
241 (85 ± 3µm;). Here, the appearance of a ciliary structure or "*vellum*" produced an incessant
242 movement that generates a current of attraction that allows the capture of phytoplankton cells.
243 Later, the carbonate shell that protects the visceral cavity of the larvae and the food become
244 more important, reducing cilia movement to an intermittent rotation. Progressively, the larvae
245 secrete more calcium carbonate causing the thickening of shell layers and the development of
246 the first growth rings (Prodissoconch II). Herein, the characteristic straight hinge that gives
247 name to the "D" larva tends to bend thus reaching the umbonate phase. From this moment the
248 larvae stopped swimming completely but few developed the foot that allows benthic settlement.
249
250 From the spawning to pediveliger stage the average growth of larvae was estimated at 8.57
251 $\mu m \cdot day^{-1}$. However, at some point of the experiments (day 6 in 80L tanks and day 7 in 16L
252 tanks) all growth was stopped and no more larval development was observed regardless of the
253 rearing conditions and coinciding with the period when larvae stop swimming (red line) (**Fig.**
254 **4**). Thus, larvae remained alive for a maximum of 22 days and statistical analysis showed that
255 there is a significant difference between survival of larvae and the parameters selected for the
256 rearing activity occurring best survival results in 16L tanks with an initial larvae density of 2
257 $larvae \cdot mL^{-1}$ ($F = 13.542$, $p < 0.05$).
258
259 **Effect of rearing conditions on larval development and settlement**
260 Results from ANOVA showed higher survival of larvae due to the presence of light in the tanks
261 ($F = 4.597$, $p < 0.05$) and evidenced important differences according to the dose of food
262 provided ($F = 3.434$, $p < 0.05$) while interaction between "Tank + Dose" was not significant ($F =$
263 2.910, $p = 0.58$). Post-hoc analysis showed that the "*optimal*" dose significantly improved (p
264 < 0.05) larval survival compared to "*low*" and "*high*" doses where more than 80% of larvae had
265 died after 48 h and 96 h, respectively.

The relative daily mortality registered in tanks with photoperiod 12/12 showed mean values of 54.2% when the dose was "*low*", 18.3% when the dose was "*optimal*" and 34.4% when the dose was "*high*". In addition, the highest values of mortality were also recorded during mainly the first three days, during the trochophore and veliger phases (**Fig. 5**).

Pathologies observed

In most cases typical symptoms of bacterial infection (**Fig. 6**) are suspected to have caused the observed mortalities over 80% during the first 2-9 days. Herein, the loss of the larval "*vellum*" was observed during the veliger phase. Despite larvae continue alive, the absence of the ciliary structure prevents them to feed properly and after some days die. Another disease observed in dead larvae is easily identifiable by the continuous movement of the bacteria around and inside larvae shells referred as "*swarming*". In some cases, bacterial infection seems to affect larval motility, generating large clusters of larvae referred as "*spotting*" at the bottom of the tanks due to the secretion of mucous filaments.

Discussion

This study reports for the first time the successful spawning and larval rearing to pediveliger stage in captivity conditions of the endangered bivalve *P. nobilis*. This is also the first daily graphic documentation of the early development on this species.

Recent episodes of mass mortality occurred in southwestern Mediterranean coasts for *P. nobilis* accentuates the population regression registered in the last decades and consequently points captivity cultures as a potential solution to restore damaged populations. As described in Trigos (2017) the maintenance of large number of adults in captivity conditions for prolonged periods of time is necessary to obtain enough gametes of both sexes in a species in which the absence of external sexual dimorphism difficult the development of a hatchery protocol.

This study shows how 12 specimens (38.7%) from the total studied, released almost simultaneously male and female gametes, an event so far unknown in this species, which is described as a successive hermaphroditism, as a mechanism to prevent self-fertilization (De Gaulejac *et al*. 1995a. b). Interestingly, some expelled oocytes were still in a division process, suggesting that internal fertilization may occur as described for other bivalves such as *Ostrea edulis* (Peteiro *et al*. 2007). This observation coupled with the fact that oocytes present higher density than seawater and tended to sink after being released to the media could be indicate of internal fertilization as a common mechanism enhancing the survival of larvae, but further research is needed to confirm this hypothesis.

Spawned oocytes stayed at the tank bottom until the ciliated blastula stage (5h post-spawning) when they became motile and swimming activity was observed. Life stages from late embryos to the veliger phase are considered as planktonic and potentially disperse by currents. Once the pediveliger stage was reached, larvae ceased to be planktonic and searched for a suitable substrate to start benthic development. Our study observed up to 22 days to achieve the pediveliger stage then to acquire the capability to get attached. In this context, the hypothesis

312 proposed by De Gaulejac (1989) with a period between 5 to 10 days to settlement phase,
313 contrast with our longer period which might be affected by captivity conditions. This could
314 indicate that artificial conditions can be improved in order to obtain better results.
315
316 The massive mortality rates observed (up to 100% at day 4 and day 22, respectively in dark and
317 light cultures), prevented observing more development phases for *P. nobilis* but as indicated by
318 Hernández-Hernández (2000) and Robles-Mungaray (2004) for other species of Pinnidae,
319 simultaneously to foot development, the progresses of the pediveliger phase (110 ± 10 µm size)
320 is characterized by a gradual loss of the "*vellum*" that gives way to the formation of gills.
321 Subsequently the process of metamorphosis begins with the secretion of new shell from the
322 edge of the Prodissoconch II. Based on the type of growth of other specimens of the same
323 family,the Prodissoconch II should continue its growth in a transverse direction to that
324 previously recorded (Robles-Mungaray, 2004). The appearance of this new structure called
325 dissoconch represents the turning point at which individuals reach the juvenile phase and
326 acquire all adult characteristics such as the typical "pen" shape of the Pinnidae.
327
328 In our case, the mortality rates observed at this pediveliger phase were possibly associated to
329 bacterial pathologies which are widespread in hatcheries of commercial species, as well as in
330 regular experimental activities in the laboratory (Andersen *et al.*, 2000; Prado *et al*. 2016). The
331 infection of larval cultures may be caused by external or horizontal factors such as bacteria
332 escaping the mechanisms of water filtration or other broodstock individuals (Fontanez and
333 Cavanaugh, 2014). The other type of transmission can be vertical when bacterial contamination
334 occurs in gonads and intestinal tracts of broodstock and passes to offspring (Beninger *et al.*
335 2003; Prado *et al.* 2013). These pathologies are thought to mainly affect larvae because they are
336 much more susceptible to bacterial infections than adults (Lambert and Nicolas 1998). In
337 particular, *Vibrio* species are regarded as central pathogens in larval bivalve cultures (Gómez-
338 León *et al*. 2005; Elston *et al*. 2008; Kesarcodi-Watson. 2009) with new species described in the

339 last years (Prado *et al*. 2005; Dubert *et al*. 2015). The most common problem arising from
340 *Vibrio* action is the necrosis of soft tissues and ciliary structures (Sugumar *et al*. 1998; Neo *et*
341 *al*. 2011), thus preventing filtration and feeding mechanisms that cause the death of the larvae
342 (Dubert *et al*. 2016). This disease is easily identifiable by the continuous movement of the
343 bacteria around and inside larvae shells referred as "*swarming*" (Beaz-Hidalgo *et al.*, 2010). The
344 loss of the larval "*vellum*" during the veliger phase was a typical symptom of bacterial infection.
345 Hence, *Vibrio* is suspected to have caused the observed mortalities over 80% during the first 2-9
346 days, depending on light conditions and food dose. A low dose of phytoplankton could weaken
347 the larvae being consequently more susceptible to infection. By the contrary, the addition of
348 high doses of the microalgae mixture could be responsible of an excess of non-profited food in
349 the tanks, thus enhancing the proliferation of bacteria. The tanks exposed to the darkness
350 registered mortalities higher than 80% on the second day and survival tended to improve with
351 the "*high*" dose. Therefore, the photoperiod is presented as a limiting factor for the development
352 of *P. nobilis* larvae and the high mortality observed could be explained by the absence of light
353 which move the larvae away from natural conditions. This fact is supposed to stress
354 considerably the larvae being consequently more susceptible to infection. According to our
355 results, *P. nobilis* needs light to complete its larvae cycle in contrast with described by Peharda
356 and Vilibic (2008) who suggested that *P. nobilis* veliger larvae have a negative phototactism
357 and migrates vertically to deeper waters during daylight and returns to superficial areas at night,
358 as also indicated for other bivalves (Gosling. 2003).
359
360 The loss of vellum tissue also affected larval motility, generating large clusters of larvae
361 referred as "*spotting*" at the bottom of the tanks due to the secretion of mucous filaments by the
362 larval foot that may measure up to one meter in length (Gérard *et al.,* 1989; Bachelet *et al.,*
363 1992; Rojas *et al.,* 2009). Further studies need be conducted to determine whether these larvae
364 clusters following the secretion of mucous filaments are a side effect of the veil loss or a

mechanism to improve larval buoyancy and facilitate dispersal as proposed by Beninger *et al.* (2003) thus, discerning if it is a natural process or a negative consequence of bacterial activity.

In other Pinnidae such as *Atrina maura*, success in larvae rearing has not been achieved despite its commercial interest has prompted the study of aquaculture conditions for more than a decade. Coupled with pathologic problems, the larvae of *A. maura* appear to show a high hydrophobicity which causes the larvae adhesion to the water surface and consequently the death from desiccation and/ or starvation (Maeda-Martínez, 2008). According to González-Corona (2003) and Robles-Mungaray (2004) there are still some biological and technical aspects such as the adjustment of larval density or cleaning protocol, that need be optimized in order to reduce mortality and allow the sustainable commercial production of this bivalve. Therefore, there is a lack of empirical knowledge regarding the mechanisms that trigger disease transmission (Arechavala-Lopez *et al.*, 2013), thus, the optimisation in the hatchery process is accordingly necessary and involves a better understanding of bivalve physiological requirements.

To conclude, this work presents the first detailed information on the biological cycle of *Pinna nobilis* and provides information concerning important variables determining larval mortality and settlement success, such as light conditions or food dose, establishing the bases for the rearing of the endangered fan mussel *P. nobilis* in captivity. Yet, the closure of its biological cycle in captivity appears to be rather difficult since large mortality rates are observed during first days of life (4 to 22 depending on light treatment). Given that our results are conclusive on the suitability of light conditions and "optimal" food doses, the experimental activity should be intended from a pathological approach, considering bacterial infection as one of the main bottlenecks in the rearing of *P. nobilis* larvae and preventing the development of this species at pediveliger stages.

392

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646 **Table 1.** Different established doses depending on volume and cell density.

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	LOW DOSE (L)	OPTIMAL DOSE (L)	HIGH DOSE (L)
TANK 5L			
Chaetoceros calcitrans	-	*0.04*	-
Isochrysis galbana	-	*0.14*	-
Pavlova lutheri	-	*0.05*	-
TOTAL (L)	-	***0.23***	-
TANK 16L			
Chaetoceros calcitrans	*0.07*	*0.11*	*0.14*
Isochrysis galbana	*0.23*	*0.46*	*0.69*
Pavlova lutheri	*0.11*	*0.15*	*0.23*
TOTAL (L)	***0.41***	***0.72***	***1.05***
TANK 80L			
Chaetoceros calcitrans	-	*0.57*	-
Isochrysis galbana	-	*2.29*	-
Pavlova lutheri	-	*0.76*	-
TOTAL (L)	-	***3.62***	-

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654 **Table 2.** Time of larvae development in *Pinna nobilis* at 21˚C. pH 8.50. O_2 6.5 $mgO_2 \cdot L^{-1}$ and
655 salinity 38.0psu.

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Stage	**Cumulated time (h:min)**	**Size (µm)**
Spawning	*0:00*	*50*
Sperm attachment	*0:00*	*50*
1st polar body	*0:15*	*50*
Double membrane	*0:30*	*50*
1st inclusion	*0:40*	*55*
Blastule	*5:00*	*55*
Gastrule	*8:00*	*55*
Early trocophore	*22:00*	*65*
Late trocophore	*30:00*	*70*
Early veliger	*48:00*	*85*
Late veliger	*72:00*	*90*
Early umbonade	*144:00*	*100*
Pediveliger	*168:00*	*110*

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Fig. 1 Correlation between broodstock size (Ht) and number of oocytes expelled per L. N = 26 adults.

Fig 2. Early developmental stages of *Pinna nobilis*: (**A**) first polar body (00:15); (**B**) fertilized oocytes with double membrane (00:30); (**C**) first inclusion (00:40); (**D**) first complete division (01:00); (**E**) 3th and 4th division (03:00); (**F**) end of cell division phase (04:30). cp. polar body; ep. periviteline membrane; ma. macromere; mi. micromere.

Fig 3. Early larval stages in *Pinna nobilis*: (**A**) early trocophore (22:00); (**B**) late trocophore (30: 00); (**C** and **D**) isometric view of late veliger (72:00); (**E** and **F**) early umbonate (144:00); (**G**) pediveliger (168:00) (**H**) lateral view of attached larva. ci. cili; fa. Apical flagella; v. vellum; ma. posterior adductor muscle; gd. digestive glandule; u. umbo; p. foot; pI. Prodissoconch I; pII. Prodissoconch II.

Fig 4. Larval growth at the different experimental volumes (5, 16, and 80L). The red line indicates the moment when larvae stop swimming, concurring with the end of growth within 16 and 80 L tanks (arrows).

Fig 5. Daily evolution of larval survival according to photoperiod and phytoplankton dose.

Fig 6. Common diseases observed in *Pinna nobilis*: (**A**) loss of vellum structure; (**B**) bacterial movement inside and around the larvae "*swarming*"; (**C**) larvae clusters attached by mucus "*spotting*".