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20 **ABSTRACT**

21 The development of aquaculture activities has posed an alternative solution for the preservation
22 of some overexploited shellfish fisheries worldwide. In the same way, endemic Mediterranean
23 bivalves such as *Pinna nobilis*, highly threatened by habitat loss and coastal pollution, could
24 found in aquaculture a solution for preserving the continuity of the species. Given the
25 endangered status of the species, the biological and ecological processes regulating natural
26 populations have been well studied, but there are still important knowledge gaps preventing the
27 development of viable artificial cultures. This study describes for the first time the larval
28 development of *P. nobilis* (from fertilization until pediveliger larval stages) in captivity
29 conditions. Moreover, different rearing tanks of 5, 16 and 80L, larvae density from 1 to 600
30 larvae mL⁻¹, light conditions, food doses, were tested in order to establish the bases for the
31 optimal rearing of the species and provide a source of individuals for restoring field populations.
32 Results showed that 16L tanks with a concentration of 2 larvae mL⁻¹, constant temperature of
33 21°C, 12/12h photoperiod and fed with an “optimal” mixture of 25 cells per µL of *Chaetoceros*
34 *calcitrans* + 33.3 cells per µL of *Pavlova lutheri* + 100 cells per µL of *Isochrysis galbana*
35 appear to be the best conditions to rear larvae of *P. nobilis*. Different captivity conditions such
36 as lower or higher tank volume, larvae density, or food doses; light privation did not report
37 better results for larval development.

38

39

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

41

42 **Introduction**

43 In the last decades the rearing of endangered bivalve species for both commercial and ecological
44 purposes has received considerable attention (Ellis, 2000; Knop, 2009). In spite of the economic
45 and scientific interest, the rearing of bivalve species has been proved to be rather difficult due to
46 the great larval mortalities of the group (Rumrill, 1990). Field studies show that larval mortality
47 in natural conditions ranges from 25 to 80% during first days of life (Talmage and Glober,
48 2009; Gazeau *et al.*, 2010). Both natural and/or anthropogenic-driven mortality in bivalve
49 larvae –i.e., from egg to postlarval recruit- are often difficult to measure and are considered as a
50 “constant” (Philippart *et al.*, 2003). Possible causes may include failure of fertilization, which
51 is often related to sea acidification and may produce shape abnormalities in embryos or avoid
52 development in over 50% of the larvae (Kurihara *et al.*, 2007); coastal development (Gómez *et*
53 *al.*, 2000; Guest *et al.*, 2008); shortage of food resources (Rico-Villa *et al.*, 2006), absence of a
54 suitable substrate for benthic settlement (Su *et al.*, 2007); combined effects of short planktonic
55 durations and local patterns of marine circulation (Shanks and Brink, 2005); and/or lethal
56 environmental temperatures (Philippart *et al.*, 2003). Any of these scenarios usually leads to a
57 reduction of recruitment rates and consequently, to the disappearance of populations.

58

59 Historically, mortality problems have been observed in the marine bivalve industry for decades
60 (Samain and McCombie, 2008; Barton *et al.*, 2015). The main goal in hatchery production is to
61 improve larval and post-larval survival by achieving larval growth and metamorphosis success.
62 In this term, the proper formulation of larval diets has been considered as the most critical
63 aspect in hatchery operations focusing on the type of microalgae used as feed (Brown *et al.*,
64 1998; Knuckey *et al.*, 2002; Ponis *et al.*, 2006; Rico-Villa *et al.*, 2006; Pettersen *et al.*, 2010;
65 Ragg *et al.*, 2010; Gui *et al.*, 2016) or even in artificial substitutes to phytoplankton diet
66 (Couteau and Sorgeloos, 1992; Knauer and Southgate 1999; Nevejan *et al.*, 2007; Gui *et al.*,
67 2016).

68 In other cases, recurrent mortality episodes are described as a result of virus or vibrio-like
69 bacteria infecting bivalve hatcheries and nurseries (Renault and Arzul, 2001; Dubert *et al.*,
70 2015; Rojas *et al.*, 2015) and reducing commercial production by approximately 40% as
71 reported for French farming facilities of Pacific oyster (*Crassostrea gigas*) since 2008 (Pernet
72 *et al.*, 2014).

73 From an ecological perspective, the rearing of endangered bivalves in captivity conditions may
74 pose a potential solution for the rehabilitation of seriously damaged populations (Ronquillo and
75 Mckinley, 2006; Vinvie, 2008; Thomas and De Leaniz, 2010; Theodorou *et al.*, 2015) and face
76 the same early stages' bottlenecks than those reported for bivalve commercial farming (Arnold,
77 2008). One of these endangered species is the Mediterranean fan mussel, *Pinna nobilis*. The
78 populations of this bivalve have significantly declined during the last decades (De Gaulejac and
79 Vicente, 1990; Vicente and Moreteau, 1991; Garcia-March, 2005; Basso *et al.*, 2015; Rouanet
80 *et al.*, 2015) as a result of different anthropogenic stressors such as coastal development, fishing
81 pressure, and/or accidental harvesting by trawling and shell breakage by anchoring
82 (Katsanevakis, 2005; Acarli *et al.*, 2011; Hendriks *et al.*, 2011). For these reasons, the European
83 Union included it as an endangered species in the ANNEX IV of the Council Directive
84 92/43/EEC (EC Habitats Directive), and strictly forbids any kind of culling. Although these
85 measures have helped certain recovery of populations in some Mediterranean regions (Pérez-
86 Vallazza *et al.*, 2008; Theodorou *et al.*, 2017), the species is still at risk by unknown factors
87 such as those causing mass mortality event along the Spanish Mediterranean coast and the
88 Balearic Islands during the summer of 2016 (Darriba, 2017; Vázquez-Luis *et al.*, 2017).
89 Temperature effects due to climate change, coastal pollution, and pathogenic mechanisms are
90 amongst potential explanatory factors, all of them mediated by human influence (Rodrigues *et*
91 *al.*, 2015). In this scenario, the rearing of *P. nobilis* in captivity appears as an alternative
92 solution to obtain healthy stocks of recruits that could be reintroduced in suitable areas with
93 declining population densities (Trigos, 2017). Yet, the duration of the different larval stages
94 from fertilized eggs, as well as the main factors influencing survival rates are still largely

95 unknown, although they are proposed to follow similar patterns to other bivalves. Following this
96 hypothesis, Vicente (1986) described the larval stages of trochophore, veliger, and pediveliger
97 followed by metamorphosis, and juvenile development of *P. nobilis*. De Gaulejac (1989),
98 observed larvae shells with electronic microscope and pointed the possibility of a time gap of 5
99 to 10 days between gametes expulsion and substrate settlement. Peharda and Vilibic (2008)
100 suggested that veliger fan mussel larvae could have a negative phototactism that allow vertical
101 migration to deeper waters during the day to return to upper bathymetric ranges at night, as also
102 indicated for other bivalves (Gosling, 2003), presumably to avoid UV light or predator activity
103 (Manuel *et al.*, 1996). However, there is still no available information on the behavioural
104 responses of *P. nobilis* larvae to different environmental factors under captivity conditions that
105 could provide a solid basis for designing and implementing a viable larval hatchery. In addition,
106 factors determining patterns of benthic recruitment are virtually unknown, although in other
107 species is often linked to the availability of preferential substrates for settlement (Prado *et al.*,
108 2012), which highlights the importance of simulating optimal settlement conditions to rear *P.*
109 *nobilis* in captivity. Other biological and physical aspects such as the possible correlation
110 between adult size and numbers of oocytes expelled also require further investigation.

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

117

118

119 **Materials and methods**

120 **Field sampling**

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

127

128 **Gamete release and fertilization**

129 Small groups of 4-6 individuals were placed in two 120 L tanks within controlled
130 temperature facilities (20-21°C) with filtered (1µm) and treated (UV) seawater for a maximum
131 of 24h. A $\approx 10^\circ\text{C}$ gradient is necessary to induce a thermal shock causing gametes release in
132 bivalves (Helm *et al.*, 2006) which takes place at various temperature thresholds, depending on
133 the species (Drent, 2004). Herein, in order to keep this temperature gradient two induction tanks
134 were prepared; one tank was maintained at 15 °C while a second tank was heated to 25°C thus
135 respecting the conformability range of temperatures described for *P. nobilis* (Trigos *et al.*,
136 2015).

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

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

150

151 **Larval cultures**

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

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

167

$$168 \quad V_{dose} (L) = \frac{\text{cell density needed } [\mu L] \cdot V_{tank}}{\text{cell density available } [\mu L]}$$

169

Where:

V_{dose} = supplied dose in liters.

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

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

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

170

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

176

177 **Effect of rearing conditions on larval development and settlement**

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

189 For all tanks, abiotic parameters were kept as stable as possible. Temperature: 19-21°C;
190 salinity: 32-37; pH: 7.7-8.4; and dissolved oxygen: 5.9-7.0 mg O₂. Larvae concentrations were
191 daily monitored (N= 1 per tank) to determine the conditions with lower mortality rates.
192 Moreover, an artificial substrate made of 250 μm PVC mesh was deployed in all tanks in order

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

195

196

197

198 **Statistical analyses**

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

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

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

210

211

212 **Results**

213 **Gamete release and fertilization**

214 A total of 31 individuals (47.10 ± 10.61 cm Ht), of the 40 subjected to thermal shock,
215 released gametes for periods of 40 minutes and, in some instances for up to 3h. Among
216 individuals that responded positively, 5 of them (16.1%) were strictly males and 14 (45.2%)
217 expelled only oocytes. The remaining 12 specimens (38.7%) released almost simultaneously
218 male and female gametes. The release of female gametes in *P. nobilis* was estimated in $1.9 \cdot 10^6$
219 oocytes $\cdot L^{-1}$ (averaged for the 26 individuals releasing female gametes) with a mean Ht of 51.8
220 ± 9.98 cm (**Fig 1**).

221 There was a significant a positive association between the size of individuals and the number
222 of expelled oocytes ($F = 0.765$. $p < 0.01$) as reported for other bivalve species (Helm *et al.*
223 2006). The smallest spawning female was 37.7 cm Ht, and 34.6 cm Ht in males. In those
224 instances in which there was only male spawning, sperm was stored in a temperature controlled
225 room at 4°C and visual observations revealed that sperm remained alive for a maximum of 3
226 days.

227

228 **Larval cultures**

229 Viable oocytes were spherical with an average diameter of $\emptyset = 55 \pm 1\mu\text{m}$ whereas
230 spermatocytes hardly exceed $1\mu\text{m}$ length. Embryonic development of *P. nobilis* started with the
231 rapid fertilization of oocytes by surrounding spermatocytes at a temperature of 21°C (**Table 2**).
232 After 15-30 minutes the appearance of the first polar body and the formation of a perivitelline
233 membrane confirmed successful fertilization (**Fig. 2**). The first zygote inclusions were observed
234 after 40 minutes and gradually increase the number of blastomeres in a successive formation of
235 inclusions until they attained a ciliated blastula stage 5 h later. Herein, the phase in which the
236 motility of larvae begins (**Fig. 3**) and last for 24 h until the trocophore stage at an average size
237 of $65 \pm 5\mu\text{m}$. This was followed by a period of frenetic activity where larvae can reach speeds
238 of 0.5 to $1 \text{ cm} \cdot \text{second}^{-1}$ 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 ($85 \pm 3\mu\text{m}$). 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 $\mu\text{m}\cdot\text{day}^{-1}$. 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·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.

266 The relative daily mortality registered in tanks with photoperiod 12/12 showed mean values of
267 54.2% when the dose was "low", 18.3% when the dose was "optimal" and 34.4% when the dose
268 was "high". In addition, the highest values of mortality were also recorded during mainly the
269 first three days, during the trochophore and veliger phases (**Fig. 5**).

270

271 **Pathologies observed**

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

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281

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284

285 **Discussion**

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

289

290 Recent episodes of mass mortality occurred in southwestern Mediterranean coasts for *P. nobilis*
291 accentuates the population regression registered in the last decades and consequently points
292 captivity cultures as a potential solution to restore damaged populations. As described in Trigos
293 (2017) the maintenance of large number of adults in captivity conditions for prolonged periods
294 of time is necessary to obtain enough gametes of both sexes in a species in which the absence of
295 external sexual dimorphism difficult the development of a hatchery protocol.

296

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

306 Spawned oocytes stayed at the tank bottom until the ciliated blastula stage (5h post-
307 spawning) when they became motile and swimming activity was observed. Life stages from late
308 embryos to the veliger phase are considered as planktonic and potentially disperse by currents.
309 Once the pediveliger stage was reached, larvae ceased to be planktonic and searched for a
310 suitable substrate to start benthic development. Our study observed up to 22 days to achieve the
311 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 ($110 \pm 10 \mu\text{m}$ 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-profit 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

365 mechanism to improve larval buoyancy and facilitate dispersal as proposed by Beninger *et al.*
366 (2003) thus, discerning if it is a natural process or a negative consequence of bacterial activity.

367

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

380

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

391

392

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398

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

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

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654 **Table 2.** Time of larvae development in *Pinna nobilis* at 21°C. pH 8.50. O₂ 6.5 mgO₂·L⁻¹ and
655 salinity 38.0psu.

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

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

667 **Fig 2.** Early developmental stages of *Pinna nobilis*: **(A)** first polar body (00:15); **(B)** fertilized
668 oocytes with double membrane (00:30); **(C)** first inclusion (00:40); **(D)** first complete division
669 (01:00); **(E)** 3th and 4th division (03:00); **(F)** end of cell division phase (04:30). cp. polar body;
670 ep. periviteline membrane; ma. macromere; mi. micromere.

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

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

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

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