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1 **Breeding, planktonic and settlement factors shape recruitment patterns of one of**
2 **the last remaining major population of *Pinna nobilis* within Spanish waters**

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28 **Abstract** The pen shell, *Pinna nobilis* L. is a critically endangered bivalve threatened by mass
29 mortality events throughout the Mediterranean, but the Alfacs Bay (Ebro Delta) still hosts
30 many healthy individuals. Herein, we study the main factors controlling recruitment patterns in
31 this locality, including gonadal development and abundance of critical life-stages, as well as the
32 effect of environmental factors. Growth records from empty shells suggested a single major
33 peak of recruitment during a period of 11 years, although many juveniles were found in two
34 very shallow sand bars possibly acting as a barrier for water circulation and as a trap for larvae.
35 Collectors deployed outside these sand bar areas showed zero settlers, and the availability of
36 planktonic larvae was very low. Gonadal examination evidenced breeding throughout the
37 summer period with successive hermaphroditism, but 20% of individuals were simultaneous
38 hermaphrodites, a condition that has been associated with environmental stress and that
39 could lead to in-breeding depression and potentially reduced fertility. Yet, given the large size
40 of the population and the wide breeding period observed, planktonic processes causing larval
41 mortality such as freshwater discharges from rice locally important rice agriculture are also
42 proposed as possible impacts accounting for patterns of low larval availability.

43

44 **Keywords:** larval availability; anomalous simultaneous hermaphroditism; agricultural pollution;
45 salinity; habitat features; parasites; Western Mediterranean

46

47 **Introduction**

48 The pen shell *Pinna nobilis* L. is an endemic Mediterranean bivalve strictly protected by the
49 European Union (Habitats Directive Annex IV EEC, 1992), the Barcelona convention (Protocol
50 ASPIM Annex 2), and recently listed as “critically endangered” by the Spanish government
51 (BOE 251-14181, 2018). Recent mortalities of 90-100% of the populations have been reported
52 along the entire Mediterranean Spanish coast and other Mediterranean countries such as
53 France, Italy, Greece, Tunisia, Cyprus, and Turkey (Cabanellas-Reboredo et al., 2019) have been
54 associated with the presence of the parasite *Haplosporidium pinnae* (Catanese et al., 2018).
55 Additionally, the virulence of other pathogens such as *Mycobacteria* spp. has also been
56 proposed as a mortality agent (Carella et al., 2019) and the possible association with
57 *Haplosporidium* outbreaks is currently being investigated. At present, only two Spanish
58 populations located in Fangar Bay (North Ebro Delta, containing hundreds of individuals) and
59 the Mar Menor (also hundreds of individuals by recent census) remain uninfected. A third
60 population located in Alfacs Bay (South Ebro Delta) was infected in July 2018 in an area
61 adjacent to the open sea, but the rest of the bay remains healthy (García-March et al., in
62 review). Although the reasons driving these patterns are still uncertain, lower salinities
63 associated to the rice cultivation period (Cerralbo et al., 2019) have been proposed to hamper
64 the spread of the parasite (Cabanellas-Reboredo et al., 2019).

65 In healthy populations, natural mortality in *P. nobilis* has been indicated to be size-
66 dependent, with young stages suffering much higher mortality rates than adults (García-March
67 et al., 2007; Katsanevakis, 2007), which coupled with long life-expectancy (over 50 yrs age, and
68 120 cm length, Butler et al. 1993; Rouanet et al. 2015), typically leads to the dominance of
69 large-sized individuals. Population sizes may vary from thousands to tens of individuals in
70 densities that do not usually exceed one individual per 100 m², although aggregated patterns
71 with tens of individuals per 100 m² are common in some places (review by Prado et al., 2014).
72 Ultimately, the population structure is the result of a multiplicity of biotic and abiotic factors

73 influencing spawning, larval production, pelagic dispersal and survival, settlement and post-
74 settlement success, and recruitment at different spatial and temporal scales (Pineda et al.
75 2010).

76 The breeding cycle of *P. nobilis* has been described in detail (De Gaulejac, 1995), although
77 little is known about mechanisms controlling sex determination, mass-spawning events and
78 fertilization rates. Individuals are not strictly gonocoric but feature a particular type of
79 asynchronous and successive hermaphroditism, in which different degrees of sexual maturity
80 are observed between male and female dominant phases (De Gaulejac 1995; Deudero et al.,
81 2017). Hence, each type of sexual gamete is sequentially released from June to September (De
82 Gaulejac, 1995) at temperatures above 20 °C (Deudero et al., 2017). Yet, many other
83 environmental factors including temperature, phytoplankton blooms, tides, moonlight,
84 chemicals, storms or high wave action may also have a role in mass-spawning events, as
85 evidenced in many other marine invertebrates (Giese, 1959; Himmelman, 1975). Under some
86 unclear factors captive individuals of *P. nobilis* may also exhibit simultaneous release of male
87 and female gametes (38.7 % of the individuals) leading to self-fertilization and full mortality of
88 trocophora larvae (Trigos et al., 2017). Oocytes released to the water column (De Gaulejac et
89 al., 1995a) appear to have a low buoyancy and a tendency to sink (Trigos et al. 2017), which
90 may make dispersal difficult. Fertilization of oocytes occurs rapidly at 21 °C and can be
91 confirmed after 15-30 min by the appearance of the first polar body and the formation of a
92 perivitelline membrane (Trigos et al. 2017). The exact duration of the larvae is not known, but
93 incomplete studies of the life cycle until the pediveliger stage suggest a minimum of 10 days
94 (Trigos et al., 2018; P. Prado pers. observ.) during which they may be exposed to large-scale
95 factors such as currents, nutrient concentrations, temperature, and salinity that can strongly
96 influence larval availability (Pineda et al., 2010). According to indirect genetic evidence from
97 natural populations, larvae may occasionally travel distances in the order of hundreds of km,
98 although self-recruitment seems to predominate (Wesselmann et al., 2018; Nebot et al.,

99 2019). Given the strong similarity in larval bivalve morphology, identification to species level is
100 very challenging (Malchus & Sartori, 2013), and no studies have been conducted to assess
101 natural variability in the availability of *P. nobilis* larvae. Yet, recent methodological approaches
102 using q-PCR detection are now available to allow the estimation of larval abundances “per se”
103 (Andree et al., 2018) and might provide a better understanding of early planktonic processes.
104 Otherwise, the use of collector devices for *P. nobilis* settlers has been widely used across many
105 populations as a proxy for larval supply (e.g., Cabanellas-Reboredo et al., 2009; Theodorou et
106 al., 2015; Kersting & García-March 2017), although they may be affected by differential factors
107 such as preferential substrates for settlement, chemical cues, predation, and competition,
108 among others (Pineda et al., 2010). Once in the benthos, reported differences in the age
109 structure of populations (Richardson et al., 2004; Katsanevakis 2005), as well as data obtained
110 *in situ*, suggest that recruitment peaks in the Columbretes Islands (W Mediteranean) may
111 occur every 3-4 years possibly associated to sea water temperature at the beginning of the
112 reproductive period (Kersting & García-March 2017).

113 The population of *P. nobilis* within Alfacs Bay (Ebro Delta, Catalonia) was estimated in the
114 summer of 2012, previous to the infection by *Haplosporidium pinnae* at over 90,000
115 individuals, with peak densities of up to 20 individuals per 100 m², and mean densities of 1.61
116 individuals per 100 m² (Prado et al., 2014). The population features an extremely shallow
117 distribution of individuals (from 20 cm to 130 cm depth) growing over extensive seagrass
118 meadows of *Cymodocea nodosa* (Prado et al., 2014), and is dominated by large adults of 45-55
119 cm length (Prado et al., 2014) although smaller individuals are also observed. The distribution
120 of individuals in the bay is highly irregular but is inversely related to the influence of
121 agricultural discharges (Cerralbo et al., 2019), with large unpopulated areas in the northern
122 shore, low-density areas in the inner part of the southern shore, and high-density areas in the
123 region closer to mouth of the bay (Prado et al., 2014).

124 In the context of extreme risk for the conservation of the species, the aim of this study was
125 to identify the main factors controlling recruitment patterns and the age structure of the
126 Alfacs Bay population, including gonadal development, larval availability, and juvenile densities
127 in the benthos. To this end we conducted: (1) long-term (>10 years) and mid-term (3 years,
128 2016 to 2018) assessment of recruitment events based on age determination from shells and
129 on the abundance of juveniles (field densities) and settlers (artificial collectors), respectively.
130 Further, we investigated the possible relationship between these data and long-term series of
131 seawater temperature, salinity and phytoplankton availability; (2) evaluations of larval
132 availability in the water column using qPCR analysis (Andree et al., 2018), and (3) histological
133 examination of the gonads during the fertility period were also conducted in 2016. Both larval
134 availability and early benthic stages were investigated in three areas of the bay with distinctive
135 adult densities in order to establish a potential relationship between the number of breeding
136 individuals and reproductive outputs.

137

138 **Materials and methods**

139 **Study Area**

140 Alfacs Bay is a semi-enclosed estuarine water body (ca. 49 km² and an average depth of
141 3.13 m) located at the south of the Ebro River delta (40°36'39" N, 0°39'25" E; Catalonia, NW
142 Mediterranean), which has a great economic importance due to the presence of extensive
143 shellfish aquaculture, particularly of oysters and mussels. The northern shore is bordered by
144 rice fields and receives seasonal agricultural discharges (ca. 275 · 10⁶ m³ yr⁻¹) high in nutrients
145 and organic matter that causes a salinity gradient (from below 30 to 37; Camp & Delgado,
146 1987; Llebot et al., 2011; Cerralbo et al., 2019) and favors the development of epiphytes and
147 fast growing macroalgae (Prado, 2018). Hence, the shallow submerged platform along the
148 inner shore (<2 m) is mostly composed of silty sediments and dominated by the seagrass
149 *Cymodocea nodosa* along with patches of *Zostera noltii* in areas closer to the freshwater

150 discharge (Prado 2018). The southern shore (the Banya Sandspit) was included in the Ebro
151 Delta Natural Park in 1986 and is also part of the Natura 2000 network of the European Union
152 because of its importance for both *C. nodosa* and *P. nobilis* (Prado et al., 2014). Nevertheless,
153 nearly half of the Banya Sandspit is occupied by extensive salt pans used for traditional salt
154 extraction activities that are not subjected to the same regulations as the rest of the area. The
155 Banya Sandspit stretches along a shallow platform of 18 km² (ca. 700 m wide), which gently
156 slopes from 0 to 1.5 m, with an average depth of 0.64 m. At a distance of ca. 500-600 m from
157 the coast, the platform forms a large sand bar with several parts (two major areas of 13.79 and
158 12.56 Ha and several isolated areas) that emerge at low tides, at least during the winter
159 period, when particularly low tides occur (see Fig. 1). By following the density of *P. nobilis*
160 within these areas along the summer salinity gradient (Cerralbo et al., 2019), the Alfacs Bay
161 can be divided into three zones with high (half outer region of the Banya Sandspit),
162 intermediate (half inner region of the Banya Sandspit), and low abundance of individuals (inner
163 northern shore; Prado et al., 2014). The high abundance zone was located in the outer half of
164 the Banya Sandspit, adjacent to the mouth of the bay (ca. 2.5 km wide) and experienced major
165 mortality by *H. pinnae* in July 2018; the intermediate abundance zone in the inner half of the
166 bay (currently free of the parasite); and the low abundance zone (also free of the parasite) in
167 the Northeast corner of the bay, facing a recovered salt marsh area (Fig. 1).

168 Environmental variables including temperature, salinity, and phytoplankton availability (Chl
169 *a* concentration) are measured weekly in a central point of the Alfacs Bay (40° 36.54'N, 0°
170 39.36' E) as a part of a monitoring program for shellfish safety of the Catalanian Government.
171 Values from late May to October (potential reproductive period) were requested for study
172 purposes and examined for possible associations with recruitment patterns of the population
173 using multiple correlation analysis.

174

175 **Gonadal development**

176 Given the protected status of the species only 10 individuals were collected from the area
177 with high adult densities (2 in June and 8 in August 2016) in order to minimize the impact on
178 the population. Individuals were placed within iceboxes and transported to the lab for
179 anatomical examination and description, measurement of valve length to the nearest mm and
180 dissection of organs for tissue sampling. From each animal, different transverse sections of
181 each tissue, including gonad, digestive gland, kidney, mantle and gills were fixed in Davidson's
182 solution and preserved for at least 48 h at room temperature. Subsequently, tissues were
183 dehydrated in ascendant alcohol series and embedded in paraffin blocks, 3 μm thick sections
184 were cut and stained with haematoxylin and eosin (H&E). The slides were examined under a
185 light microscope (Nikon DS-Fi1 video camera mounted on a Nikon 50i microscope) for possible
186 presence of pathogens and diseases. Estimation of gonadal status was evaluated following De
187 Gaulejac (1995). In three selected individuals showing gonadal degeneration, the percentage
188 of degenerated follicles over a total of 500 was estimated using a magnification of 10X.
189 Captured images were examined using the *cell counter* plugin of *ImageJ* software (v1.50 h9).

190

191 **Estimates of Larval Abundances by qPCR**

192 Field sampling for *P. nobilis* larvae was conducted every 15 days between July and October
193 2016 in the three sites with high, intermediate and very low density of individuals (Fig. 1) with
194 a plankton net of 0.2 m diameter and 30 μm size mesh. Samples were collected (n= 3
195 replicates per site) by walking ca. 30 m over the seagrass bed of *Cymodocea nodosa*,
196 equivalent to a filtration volume of 3.77 m^3 . All samples were preserved in 70% ethanol and
197 transported to the lab for further processing. All bivalve larvae within each sample were
198 carefully separated from the rest of the plankton under a dissecting microscope using a
199 zooplankton micro-spoon with a loop of 100 μm in diameter.

200 Full methodological details of DNA extraction, qPCR amplification, calibration curves and
201 assay optimization can be obtained from Andree et al. (2018). Briefly, specific primers

202 designed for the amplification of a small fragment (less than 200 bp) of the ribosomal DNA of
203 *P. nobilis* were designed for assessing specific identity and abundance of target sequences in
204 water samples, or for species identification among other bivalve larvae. Due to the
205 methodology of qPCR a higher abundance of specific target is inversely correlated to Ct values;
206 that is, a low Ct value correlates to high abundance (Andree et al., 2018).

207 Significant differences in qPCR Ct values for each Zone and Date were investigated with a 2-
208 way factorial ANOVA (Zone and Date fixed factors with two and six levels). For all analyses, the
209 critical level of significance was fixed at $p < 0.05$. SNK post hoc comparisons were used to
210 identify significant differences between zones at each sampling date.

211

212 **Abundance of Settlers**

213 Collector devices were constructed for observing new bivalve settling stages. Each collector
214 consisted of one plastic mesh bag (ca. 5 mm opening) filled with nylon fishing thread or pieces
215 of fishing net and a buoy, then each was anchored to a mooring on the seafloor with a rope.
216 The efficiency of this type of collector for capturing *P. nobilis* settlers has been previously
217 established by García-March and Kersting (2006) in the Columbretes Islands Marine Reserve,
218 located ca. 100 km south from Alfacs Bay. The devices remained immersed during 4 months,
219 after which they were recovered and taken into the lab for separation and identification of
220 juvenile bivalve species under the dissecting microscope.

221 These collectors were deployed between the first of June and the first of October of 2016
222 and 2017 at the same three sites used to estimate settlement rates, whereas in 2018 they
223 were only installed along the main sand bar of 1100 m² during the same period. In each site,
224 five devices were randomly distributed within seagrass beds of *C. nodosa* (ten at the single site
225 of 2018), close to living adults and at least 5 m apart from each other. Five additional devices
226 were deployed beyond the seagrass limit of the site with high abundance of individuals at a
227 depth of ca. 1.5 to 2 m, in order to assess possible habitat preferences.

228 Variability in the abundance of bivalve assemblages among study zones and between years
229 was investigated with a 2-way Permanova available within the PRIMER v6 software package
230 (Clarke & Gorley, 2006).

231

232 **Short-term recruitment patterns**

233 Juveniles from each summer recruitment period in 2016, 2017, and 2018 were sampled by
234 snorkeling along 20 m long by 1 m wide transects (N= 20 per site and year). The three sites of
235 Alfacs Bay with distinctive abundance of individuals were sampled at a depth of ca. 60-80 cm,
236 at which adult densities are highest (Prado et al., 2014). Two additional areas of ca. 1100 m²
237 and 380 m², that were exposed or with very shallow depth at low tide and within which were
238 observed high numbers of juveniles in early 2018 (from 2017 recruitment), were also sampled
239 that year and again in early 2019 to obtain further information of interannual patterns at the
240 bay scale.

241

242 **Long-term patterns of recruitment**

243 Twenty shells between 42.8 and 62.1 cm long from Alfacs Bay –these being the most
244 common sizes observed– were used to evaluate the age range of the population and to
245 determine the availability of different cohorts indicative of successful long-term recruitment.
246 Ten of them included the individuals used for histological analysis (i.e., the exact moment of
247 death was known; see Table 1), and the other ten were found empty during the fieldwork in
248 summer 2016. These consisted of recently dead individuals (i.e., in the order of a few days),
249 which could be identified from the absence of encrusting epifauna and macroalgae in the inner
250 surface of the valves, so its pearly sheen was still intact. A sample size of N= 20 has been
251 shown as sufficient for accurate age estimation in population studies (García-March et al.,
252 2011; García-March & Márquez-Aliaga 2007). One valve was processed to study the record of
253 the posterior adductor muscle scar (PAMS) from the interior of the shell according to Garcia-

254 March et al. (2011). The valve was radially cut through the PAMS and ca. 8 cm dorso-ventral
255 sections of one side were mounted in slides. A thick section (ca. 200 μ) of the portion glued to
256 the slide was cut using a low-speed Buehler Isomet saw. The free surface of the slide
257 preparation was polished to improve observation of the growth record. From each polished
258 section, the growth record was counted. Missing records were calculated using the width of
259 the calcite layer in the three oldest records of all specimens (García-March et al., 2011).

260 The possible association between available environmental variables (temperature, salinity,
261 and phytoplankton availability) and long-term recruitment patterns from the age study was
262 investigated with multiple correlation analysis using the Statistica v. 12 software.

263

264 **Results**

265 **Environmental variables**

266 Seasonal patterns of water temperature in the Alfacs Bay showed lowest values in May and
267 October (20.4 ± 0.2 °C), intermediate in June and September (24.4 ± 0.2 °C), and peaks in July-
268 August (27 ± 0.2 °C). Interannual differences (2001 to 2018) in temperature during the
269 potential period of reproduction were small (Fig. 2a). For salinities, values fluctuated from 31.7
270 in May to 37 in October, depending on agricultural discharges but without any clear monthly
271 trend (Fig. 2b). In contrast, chlorophyll-*a* levels tended to increase through the summer period
272 but were highly variable among study years (May-June: 1.8 to 6.6 μ g/L; Jul-Aug: 2.1 to 8.7
273 μ g/L; and Sep-Oct: 2.6 to 9.6 μ g/L) (Fig. 2c).

274

275 **Gonadal Development**

276 Six developmental stages were identified for the 10 sacrificed individuals (2 in June and 8 in
277 August) and indicated in Table 1 and Fig. 3. There was no apparent relationship between
278 gonad development stage and valve length.

279 Microscopic examination of gonads showed that some individuals had overlapping stages of
280 male and female development within individual follicles (ie., successive and asynchronous
281 hermaphroditism; De Gaulejac 1995; Deudero et al., 2017). There was one spawning male
282 releasing spermatozoa from the central part of the follicle; one male in a degenerating state
283 with spermatozoa in the lumen of the acini and oocytes developing on the wall of the follicle;
284 and one active female with mature oocytes in the center of the follicle (see Fig. 3, Table 1). It
285 was possible to determine that gamete production is continuous during the summer period
286 whereas spawning is partial, particularly in females, with remains of residual oocytes in most
287 examined individuals. Two of the individuals collected in August already presented a
288 disorganized gonad structure with no evidence of any type of male or female germ lines,
289 suggesting the end of the reproductive period. However, in two of the examined individuals
290 (one from June and one from August), the development of both types of sexual gametes
291 occurred simultaneously from the germinal epithelium of the same follicle (Fig. 3, Table 1), a
292 gonadal stage not described in previous works.

293 Further, individuals 1 and 7 collected in August showed 100% degenerating gonad, whereas
294 individual 8 presented some residual final oocytes in some follicles (10%) (Fig. 3). Such
295 significant degeneration coincided with the presence of multiple nematode eggs (gonad of
296 individual number 7) and with the occurrence of oocysts of a possible coccidian and of some
297 unknown crustacean (individuals 1, 7 and 8) (Fig. 4A-C). Extensive damage represented by
298 necrosis of digestive tissue was also observed in individual 1 of June with no evident
299 aetiological agent (Fig. 4D). Nevertheless, microscopic evaluation of the different tissues
300 (gonad, digestive gland, kidney, mantle and gills) showed no evidence of haplosporidian
301 infections.

302

303 **Larval Abundances**

304 q-PCR results for the three study zones and six study dates (from 7-15-2016 to 1-10-2016)
305 only detected 15 positives for the presence of *P. nobilis* DNA (total N= 54 samples). In addition,
306 Ct values were rather high (between 31 and 39, with a global average of 36.4), which indicates
307 very low larval densities ranging from 0.2 to 32 larvae per sample (see Fig. 5) equivalent to
308 0.05 to 8.5 larvae per m³. In addition, q-PCR for water samples was in agreement with these
309 patterns, since no positives were detected, suggesting low availability of gametes and/ or
310 tissue remains in the water column.

311 The high Ct values observed in Zone 2 and 1 (31.9 and 32.1, respectively), analyzed by
312 ANOVA showed no differences among zones (df=2, MS= 166.7, p= 0.433). In contrast, there
313 were significant effects related to time of sample collection (df= 5, MS= 517.9, p= 0.038), and a
314 Zone x Date interaction (df=10, MS= 509.69, p= 0.0166). Further SNK post hoc analyses
315 indicated that significant effects were only due to differences between two dates: the first of
316 September 2016 (positive samples recorded in the three zones, but particularly at Zone 2 with
317 an estimate of ca. 47 individuals) and the first of August 2016 (no positive samples recorded at
318 any site).

319 Fluctuations in the abundance of *P. nobilis* larvae from qPCR were found to be unrelated to
320 overall abundances for all bivalve larvae, which tended to be higher in Zone 3 (df= 2, F= 2.038,
321 p= 0.1449), particularly on 9-15-2016 with a peak of over 5,000 bivalve larvae per sample (Fig.
322 5), although neither temporal effects (df= 5, F= 1.235, p= 0.3129) nor the Zone x Date
323 interaction were significant (df= 10, F= 1.2247, p= 0.3088).

324

325 **Abundance of Settlers**

326 No *P. nobilis* settlers were observed within collectors deployed from 2016 to 2018 at
327 different bay locations (in 2018 only adjacent to the main sand bar). Yet, collectors deployed in
328 2016 and 2017 showed the presence of ten different bivalve species, thus demonstrating
329 trapping efficiency. Community results from PERMANOVA showed significantly higher settling

330 rates in 2016 than in 2017 (MS= 10968; Pseudo-F= 20.04; $p= 0.001$), particularly in zones 1 and
331 2; whereas zone 3 showed similar numbers between years (MS= 6842; Pseudo-F= 12.5; $p=$
332 0.001). Additionally, differences among zones were also significant (MS= 14351; Pseudo-F=
333 26.2; $p= 0.001$, see Fig. 5), with zone 1 (closer to the open sea) displaying ca. 2 and 15 times
334 higher number of settlers than zone 2 and 3, respectively. The most abundant species in
335 collectors from all zones were juveniles of *Parvicardium* spp., especially in Zones 1 and 2
336 during 2016 (Fig. 6). *Limaria hians* was also locally abundant, especially at the deeper site
337 (Zone 1), as well as *Ruditapes* spp. and *Mytilus galloprovincialis* in Zone 1. Other species
338 present in low numbers in the collectors were: *Anomia ephippium*, *Arca noae*, *Barbatia*
339 *barbata*, *Mimachlamys varia*, *Modiolula phaseolina*, and *Musculus subpictus*.

340

341 **Short-term recruitment patterns**

342 Only one juvenile was observed within transects in 2016, two in 2017 (N= 20 transects per
343 site, each of 20 m²), and none in 2018 in the study area of high adult densities and close to the
344 collectors deployed for evaluation of benthic settlement outside the main sand area. This
345 resulted in densities ranging from 0 (Zones 2 and 3) and 0.25 to 0.5 ind./100 m² (Zone 1).
346 Nevertheless, in two of the emerged areas along the main bay sand bar (9271.8 m²) and (434.5
347 m²), densities of 2.5 and 20.7 indiv./100 m² from the 2017 recruitment were observed,
348 respectively, many of them fully emerged at low tide and with compromised survival (Fig. 7).
349 Considering only the dimensions of the largest sandbar area but with lowest density, the
350 estimated abundance of juveniles is over 3,000 individuals. In contrast, juvenile densities from
351 2016 and 2018 recruitments was comparably low (0.1 to 2 and 0 to 0.01 indiv./100 m²,
352 respectively). The higher recruitment observed in 2017, could not be explained by the
353 environmental variables measured (see Fig. 2a-c).

354

355 **Long-term patterns of recruitment**

356 The analysis of the growth record (posterior adductor muscle scars) of the empty shells
357 showed a population dominated by the 8 ± 1 year old class (Fig. 8). Nine of the 20 shells cut
358 had this age when they died. The remaining shells were 5, 6, 7, 9 and 15 ± 1 years of age (two
359 shells for each size class except for the 7-year-old age class, which occurred for three shells).
360 Counting back from the year of collection for empty shells (shells N= 10) and living adults
361 (sacrificed N= 10), approximate years of recruitment could be estimated, ranging from 2001 to
362 2011, with a recruitment peak in 2008 and very little or no recruitment between 2002 and
363 2006.

364 Multiple correlation analysis between available shell records of recruitment and
365 environmental factors (temperature, salinity and chlorophyll-*a* levels) measured in the middle
366 point of the bay, showed no evident association with values recorded at any summer month
367 (May to October) during the 2001 to 2011 period ($R= 0.618$; $F_{5,5}= 37.304$; $p= 0.5884$). The 2008
368 peak (± 1 year methodological error) alone could also not be explained by the presence of any
369 maximum or minimum in the environmental variables (Fig. 2a-c).

370

371 **Discussion**

372 Populations of *P. nobilis* in the Ebro Delta (Alfacs and Fangar Bays) constitute a critical
373 genetic resource for the long-term conservation of the species, currently threatened by
374 massive large-scale mortalities (Cabanellas-Reboredo et al., 2019). Examined individuals from
375 the Alfacs Bay showed no evidence of abnormal gametes at any gonadal development stage.
376 Eight of them were considered to be within some stage of the asynchronous and successive
377 hermaphroditic sexual cycle described for *P. nobilis* (De Gaulejac 1990, 1995a, b; Deudero et
378 al., 2017). However, the other two presented simultaneous sexual maturation which has not
379 been described in previous studies (De Gaulejac, 1995; Deudero et al., 2017), and may result in
380 self-fertilization and low larval viability (Trigos et al., 2018). In other bivalves, the incidence of
381 simultaneous hermaphrodites has been reported to range from ca. 0.4 to 2.5% (Syasina et al.,

1996). However, unfavorable ecological situations (including pollution) may have the potential to trigger the development of simultaneous hermaphrodites in bivalve populations (Pekkarinen 1991). For instance, Syasina et al., (1996) found that up to 6% of scallops (*Mizuhopecten yessoensis*) in a bay polluted with hydrocarbons were simultaneous hermaphrodites, along with reduced percentage of fertilized eggs and early veligers. Although rates observed in our study (20%) must be interpreted with care (low N of 10 individuals), this stage has not been observed in other pristine locations from the Cabrera National Park (Balearic Islands, Spain) where a total of 120 individuals were examined during a seasonal study previous to the *Haplosporidium* outbreaks (Deudero et al., 2017). The application of pesticides for rice cultivation is suspected to be associated to recurrent episodes of shellfish mortality in the Ebro Delta (Köck-Schulmeyer et al., 2010). In fact, up to 26 endocrine disrupting chemicals (EDCs) (pesticides, phthalates, alkylphenols, and natural and synthetic hormones) have been identified in water samples from the Ebro River, irrigation canals, and bays (Brossa et al., 2005). The possible stress induced by agricultural pollution is also consistent with the low age of adult individuals in the Alfacs Bay (7 to 9 years old) compared with the theoretical life expectancy of the species (> 50 years; Rouanet et al., 2015). Among other abnormal reproductive conditions, the presence of a degenerating gonad in the presence of parasites (a possible coccidian and an unknown crustacean) in three individuals collected in August could also reduce the reproductive output. Although identification of observed parasites to species level was not attempted, copepods have been shown to account for intense haemocytic reactions and metaplasia of infected epithelia (Carballal et al., 2001). Furthermore, coccidian parasites are commonly reported across different tissues of marine bivalves, including gonads, with responses varying from no apparent effect to light to moderate lesions and hypertrophy of infected tissues (Whyte et al., 1994; Carballal et al., 2001).

407 In spite of these deviations in gonadal development, the availability of breeding individuals
408 (ripe female and female in developing stages) throughout the summer period, and the large
409 number of adult pen shells in the bay (> 90,000 individuals; Prado et al., 2014) suggest that
410 there are factors occurring later in the water column that interfere with fertilization success
411 and/or cause larval mortality. Given the great environmental gradients occurring within the
412 Alfacs Bay (Llebot et al., 2011; Cerralbo et al., 2019) and the potential transport of larvae
413 across different water masses, a single central monitoring point is insufficient to capture all the
414 variability to which they might be exposed. Elevated temperatures and inadequate food supply
415 have been associated with the mortality of bivalve larvae (Rumrill, 1990), but interannual
416 differences in these variables at the monitoring point were small or uninformative. In contrast,
417 the salinity gradient associated to agricultural discharges (along with agrochemicals and/or
418 siltation) could be structuring the species distribution within the bay (see Prado et al., 2014;
419 Cerralbo et al., 2019) and variable inputs and exposure times might be affecting the viability of
420 planktonic stages. Although the effects of salinity have not been yet investigated for *P. nobilis*
421 embryos and larvae, evidence in other species such as the marine snail *Ilyanassa obsoleta*
422 suggest that short-term fluctuations in salinity could influence planktonic growth rates, size,
423 and the overall larval viability with direct ecological effects on recruitment success (Richmond
424 & Woodin, 1996). Similarly, Lough & Gonor (1971) found that low salinities retards the
425 development of the boring bivalve *Adula californiensis*, and it becomes abnormal below
426 26.3‰, which is comparable to lowest salinities occurring in the northern shore of the Alfacs
427 Bay during the summer period (Camps & Delgado, 1987; Llebot et al., 2011). In this scenario,
428 the dominant local winds and summer currents acting along the NW-SW axis of the Alfacs Bay
429 (Llebot et al., 2014; Cerralbo et al., 2019) may also contribute to the random transport of
430 larvae from the Banya Sandspit to the North coast of the bay with lower salinity and vice versa.

431 Although the plankton survey was only conducted in 2016, our results for that particular
432 year evidenced very low larval availability (only 15 positive samples for *P. nobilis* DNA, ca. 0.05

433 to 8.5 larvae per m⁻³), despite that gonadal examination showed the presence of breeding
434 individuals throughout most of the summer (June to August). To our knowledge, this is the first
435 time that larval stages of *P. nobilis* are quantified using qPCR analysis, so no previous
436 information of natural larval supply is available. Nevertheless, compared to other bivalve
437 species such as the brown mussel *Perna perna* or the clam *Ruditapes decussatus* showing
438 larval peaks of hundreds to thousands of larvae per m³ and high rates of recruitment (Chícharo
439 & Chícharo, 2001; Porri et al., 2008), numbers of *P. nobilis* larvae identified by qPCR were too
440 low. In fact, the number of total bivalve larvae that were visually counted ranged from 0 to a
441 peak of 15,272 individuals per sample (ca. 4,051 per m⁻³), most of which were other species, as
442 also occurred with settlers found in collectors, including oysters, mussels, and/or clams, some
443 of which are cultivated in the bay. Zone 3 recorded the highest overall numbers of bivalve
444 larvae (ca. 4 and 8-fold those of Zone 1 and 2, respectively) but those peaks did not include *P.*
445 *nobilis* larvae, which was more frequent in Zone 1 with high abundance of adults. Temporally,
446 the highest number of positive samples (n= 9) for *P. nobilis* were detected in late summer
447 when bay temperatures range from ca. 20 to 24 °C (similar to early summer), and therefore
448 must have been originated in late August or early September, when breeding individuals were
449 still present. In contrast, deeper populations in the Balearic Islands (>5 m) have only been
450 observed to spawn from May to July, at water temperatures ranging from approx. 20 to 26°C
451 (Deudero et al., 2017). During the study, larval availability was not estimated in May-June 2016
452 since the qPCR technique was still under development (Andree et al., 2018), and therefore the
453 presence of an earlier peak could have been overlooked.

454 Unfortunately, collector devices deployed from beginning of June to the end of September
455 (2016-2018) in different zones outside the sand bar area showed no evidence of *P. nobilis*
456 settlers. They were, however, effective for other common Mediterranean bivalves, particularly
457 *Parvicardium* spp. that reached values of from 15 to ca. 500 individuals per collector
458 depending on zone and year. Settlers of *P. nobilis* are commonly observed in other

459 Mediterranean locations, although with great variability. For instance, an average of 29 and 46
460 settlers were found in two different types of collectors deployed in the Marine Protected Area
461 of Palma Bay, Balearic Islands (Cabanelas-Reboredo et al., 2009). In the nearby location of
462 Columbretes Islands, settlers have been detected during nine consecutive years, with
463 interannual differences ranging from zero to >50 individuals per collector (Kersting & García-
464 March, 2017). In particular, a peak of 200 settlers was also found in the collectors deployed at
465 that locality in 2017, even though most adult individuals had presumably died due to infection
466 by *H. pinnae* (Diego Kurt, pers. comm.). Given that the Columbretes Islands are located only
467 100 km south from the Ebro Delta in the direction of the main North to South current (Millot &
468 Taupier-Letage, 2005) the Alfacs Bay population could have been a possible source, as also
469 proposed for the Balearic Islands (Wesselman et al., 2018). However, this long-distance
470 connectivity is possibly occasional, since oocytes appear to have a tendency to sink and the
471 greatest swimming activity occurs during the trochophore stage lasting only 24 h of the overall
472 planktonic period (a minimum of 10 days; Trigos et al., 2017; Prado pers. observ.). Recent
473 research conducted on the family structure of *P. nobilis* in the Cabrera National Park (Balearic
474 Islands), featuring a comparable size to the Alfacs Bay, demonstrated that individuals (N= 771)
475 formed a single panmictic population, and that the number of connections with other
476 individuals from the same exact location can reach values close to 60% (Nebot et al., 2019).
477 Although no similar research has been conducted in the Alfacs Bay, the more restricted
478 patterns of water circulation compared to open sea sites suggest that self-recruitment could
479 be even higher.

480 According to the age distribution obtained from the growth record of empty shells, the
481 Alfacs Bay population seems to undergo major recruitment events followed by periods of slow
482 population growth. A first recruitment event would have occurred around 2001, with
483 subsequent slow or no population growth between 2002 and 2006, a moderate recruitment
484 around 2007, and a major recruitment event in 2008. The time lapse between 2002 and 2006,

485 when little or no recruitment was observed, cannot be solely explained by the time necessary
486 for the 2001 recruits to attain maturity (ca. 2 years, Richardson et al., 1999; PPrado pers.
487 observ.) and points to some ongoing unfavorable local factors. This is coherent with patterns
488 observed in Fangar Bay (North Ebro Delta), where the population (not affected by mass
489 mortality) is entirely comprised by individuals of ca. 37-38 cm total length (Prado, pers.
490 observ.), suggesting that they were originated during a short window period of favorable local
491 conditions by larvae arriving from elsewhere. In fact, the salinity regime of the Fangar Bay is
492 considerably lower than that in the Alfacs Bay (below 20‰; Camps & Delgado, 1987; Llebot et
493 al., 2011), which may also account for the lack of reproductive success. In the particular case of
494 the Alfacs Bay, given that the population is located south from the Ebro River acting as a main
495 geographical barrier from possible larvae transported by the main North to South circulation
496 along the coast (Millot & Taupier-Letage, 2005), recruitment patterns seem to be due to
497 factors affecting individuals already dwelling within the bay.

498 Large numbers of juveniles (2.5 to 20.7 indiv. · 100 m⁻²) from the 2017 recruitment were
499 found in two very shallow areas of a large sand bar running parallel to the Banyà Sandspit,
500 suggesting that the geomorphology of the site may act as a physical barrier for local tidal and
501 wind currents possibly involved in the dispersal of larvae. Recruitment in the largest sand bar
502 area (ca. 13 Ha), resulted on juveniles' exposure to desiccation at low tide, which can shatter
503 survival rates, and account for enhanced abundance of individuals at greater depths (60 to 80
504 cm of water; Prado et al., 2014). In contrast, in Lake Vouliagmeni (Greece), a location also
505 featuring restricted patterns of water circulation and a large population size of ca. 8,500
506 individuals, the abundance of juvenile sizes has been reported to be equivalent to that of
507 adults. Interestingly, a clear preference for shallower areas has been indicated, although at
508 greater depth (< 7 m vs. > 7 m, respectively; Katsanevakis 2005) than in Ebro Delta sites. Yet,
509 the presence of large numbers of adult individuals at greater depths demonstrates that
510 recruitment also occurs outside the sand bar, possibly during exceptional major peaks.

511 To conclude, temporal recruitment patterns in the *P. nobilis* population of Alfacs Bay
512 appear to be the result of factors acting on the fecundity of the adult stock and during the
513 phases in the water column. At the individual level, although the total number of samples
514 examined was low, the numerous reproductive anomalies presented, collectively give a
515 negative overall view for the population, which also features reduced life expectancy (Rouanet
516 et al., 2015). Additionally, simultaneous hermaphroditism is a condition associated to
517 environmental stress (Pekkarinen 1991; Syasina et al., 1996) which might result from local
518 agricultural pollution (Brossa et al., 2005). Nevertheless, given the large population of the bay
519 (Prado et al., 2014) and the extended breeding period observed, detrimental effects of low
520 salinities on larval stages (Lough & Gonor, 1971; Richmond & Woodin, 1996) are proposed as a
521 the most plausible factor accounting for patterns of low larval availability. Since agricultural
522 discharges are extremely variable depending on management by the farmers, this might cause
523 stochastic temporal effects on the survival of larval pools and later recruitment. The remaining
524 larvae may be entrapped and forced to settle in the shallow sand bar areas, where juveniles
525 and adults have a limited chance of survival. These constrictions contrast with predatory
526 bottlenecks reported for benthic stages in other nearby areas such as the Columbretes Islands
527 (Kersting & García-March, 2017) suggesting factors controlling recruitment patterns may also
528 be dependent on local habitat conditions.

529

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656

657 **Fig. 1** Map of the Alfacs Bay showing the location of sampling sites according to adult densities
658 (Z1 = zone of high abundance also sampled at the deep limit of the seagrass bed (Z1-deep); Z2
659 = zone of intermediate abundance; Z3 = zone of low abundance). The emerged parts of the
660 Sand Spit sand bar are indicated in black. Juveniles from the 2017 recruitment were observed in
661 the main area of 13.79 Ha adjacent to Z2, and in other minor area of ca. 434 m² closer to Z1.

662

663 **Fig. 2** Variation in **(a)** temperature (°C), **(b)** salinity, and **(c)** chlorophyll-*a* content (µg · L⁻¹)
664 during the reproductive period of *P. nobilis* from 2007 to 2018. In the X axis, years of larger
665 recruitment (2008 and 2017) are indicated in bold, and years corresponding to the ± 1 year
666 error in age estimations (i.e., 2007 and 2009) in dark grey.

667

668 **Fig. 3** Histological feature of gonadal developmental stages of *P. nobilis* during the summer
669 season. **(a). Simultaneous maturation:** presence of visible male germinal epithelium
670 represented by spermatogonia (Sg) and female previtellogenic oocytes (PVO) and residual
671 spermatozoa (Spz); an early male germ line is visible in a few follicles (insert); **(b). Mature**
672 **female:** female gonad with vitellogenic (VO) and mature oocytes (MO); phagocytes in the
673 lumen (*); male early germ line are present in some follicle (insert); **(c). Male degeneration-**
674 **female formation:** previtellogenic oocytes (PVO) border the follicle and spermatozoa (Spz) are
675 visible in the lumen; **(d). Female/male line degeneration:** regressive phenomena at both male
676 and female germ cells with apoptotic oocytes (AO) and residual male germ line in the lumen
677 **(e). Spent:** empty follicles with visible phagocytes (*) and residual germ cells; **(f). Male**
678 **Spawning:** spermatozoa (Spz) fill the follicles with phagocytes in the center (*). Few early
679 developing oocytes against the follicle wall are also visible.

680

681 **Fig. 4** Pathogens and disease observed in individuals of *Pinna nobilis* during the summer
682 season. **a-c. Pathogens detected in the gonad:** Nematode eggs (arrowheads) **(a)** in a

683 degenerated gonad, oocyst of a possible coccidian (arrowheads) **(b)** and a crustacean
684 (arrowheads) **(c)**; **(d)**. **Extended colliquative necrosis (*) of digestive tubules (DT)** of the
685 digestive gland.

686

687 **Fig. 5** Abundance of bivalve larvae (all species) within plankton samples from July to October
688 2016. Estimate numbers of *P. nobilis* larvae within positive samples extrapolated from the
689 calibration curve (Andree et al., 2018) are indicated per zone below each sampling date.

690

691 **Fig. 6** Abundance of bivalve species within collector devices deployed at each study zone and
692 at the deep site in zone 1 (2016 only, those at the deep site were lost in 2017). Other species
693 present in very low abundances and not visible in bars are indicated in the results section. **(a)**
694 Summer 2016; and **(b)** summer 2017.

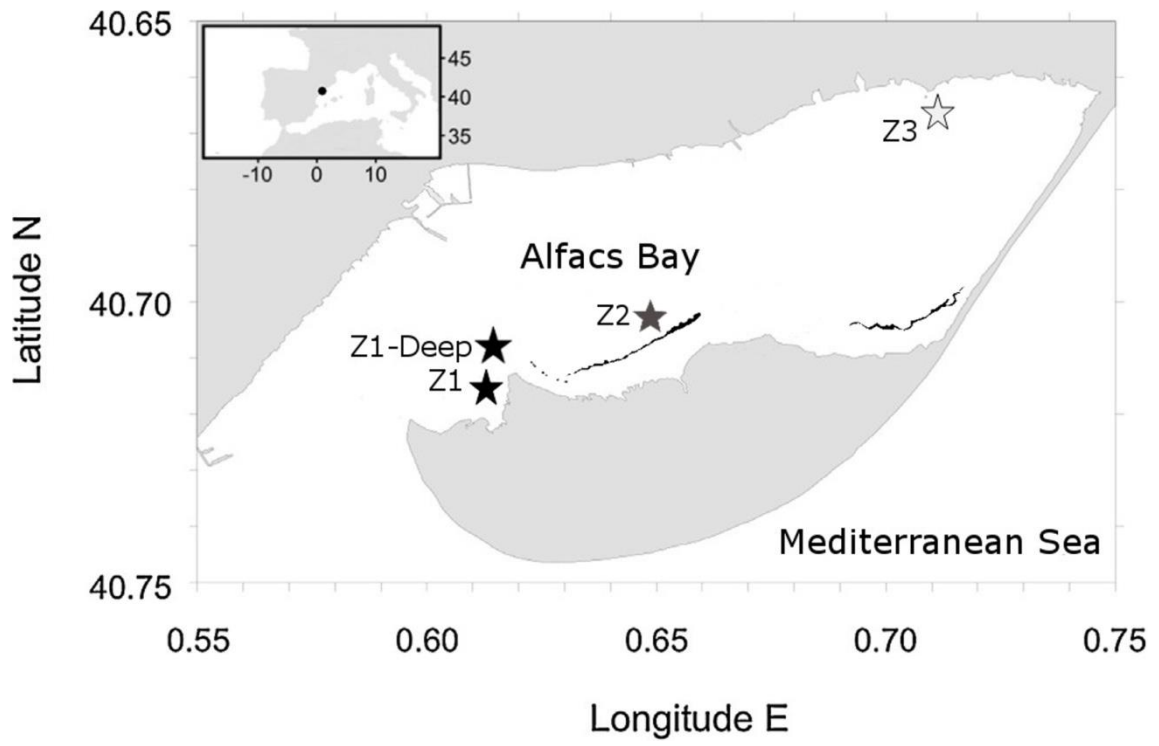
695

696 **Fig. 7 (a)** Close-up of an individual exposed to air in the sand bar at low tide, and **(b)** general
697 view of the sand bar with juveniles from the 2017 recruitment.

698

699 **Fig. 8** Age structure and year of recruitment of Alfacs Bay population from shells (N= 20)
700 collected in 2016.

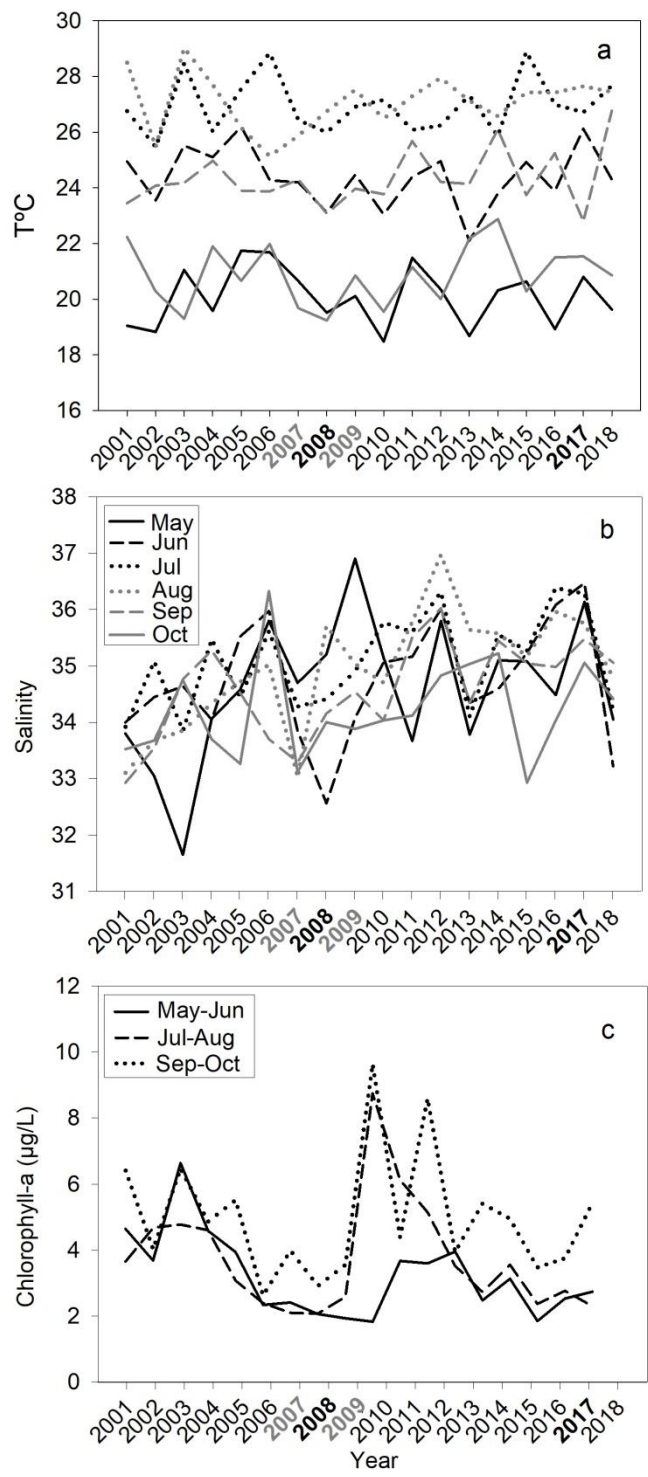
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702

703 Fig. 1

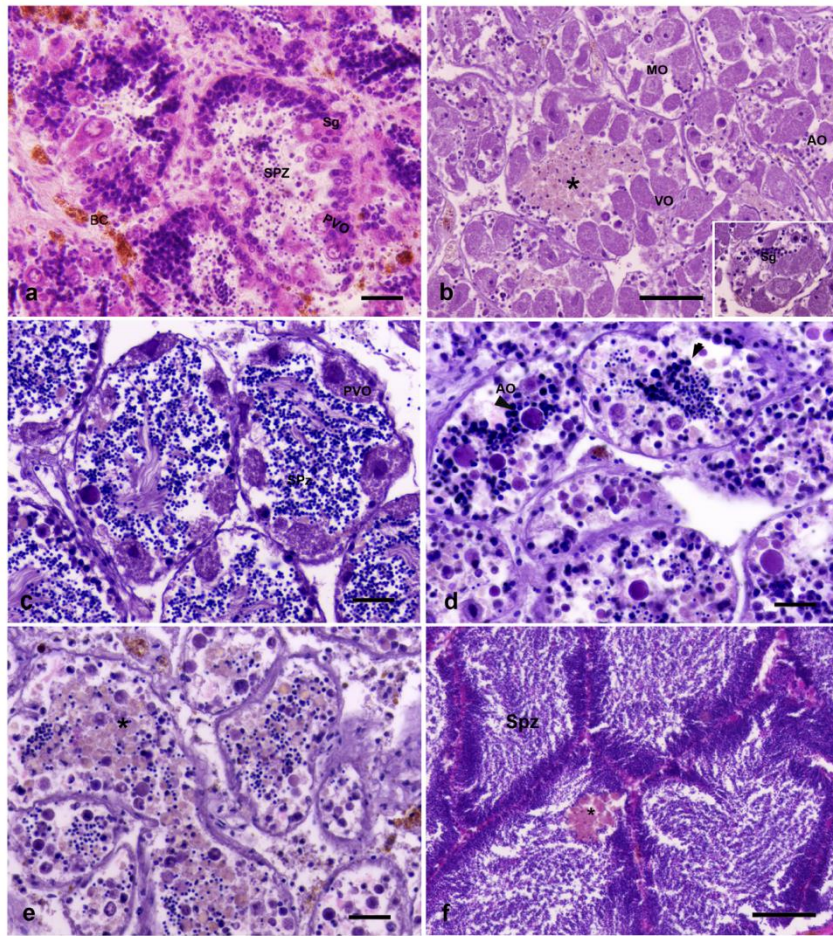
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706 Fig. 2

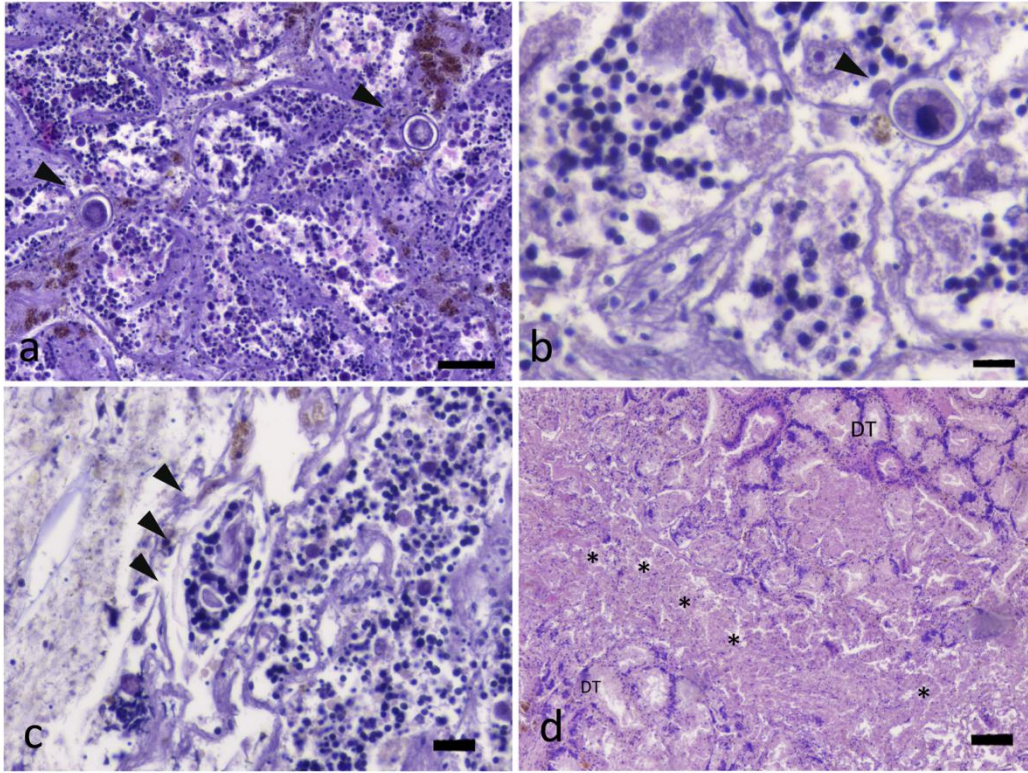
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708

709 Fig. 3

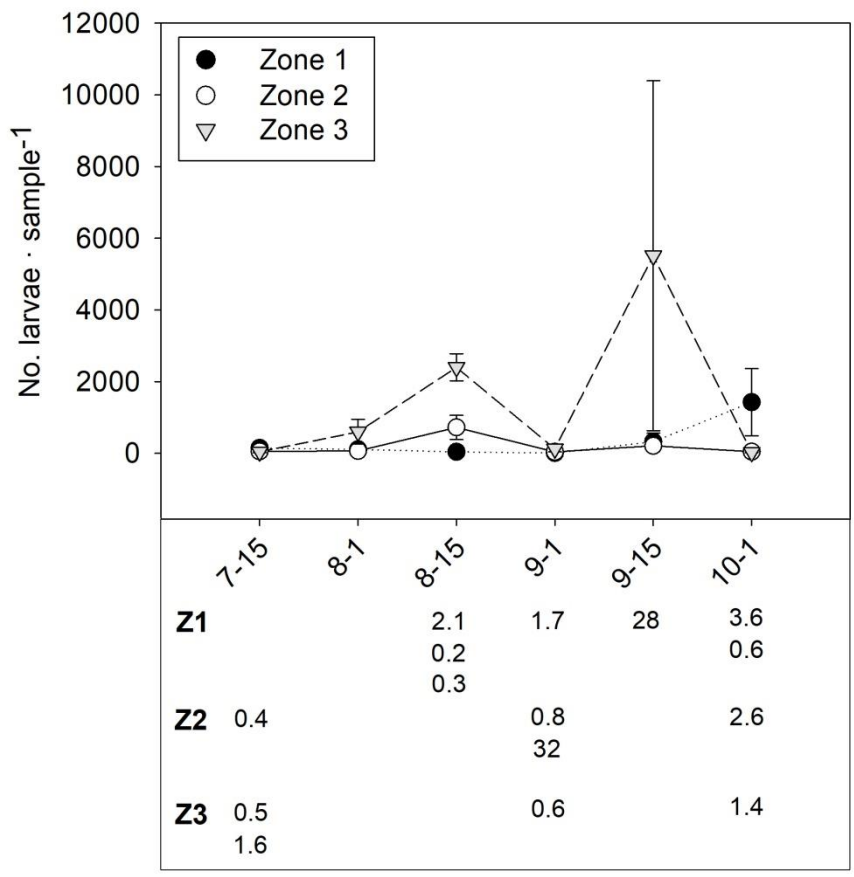
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711

712 Fig. 4

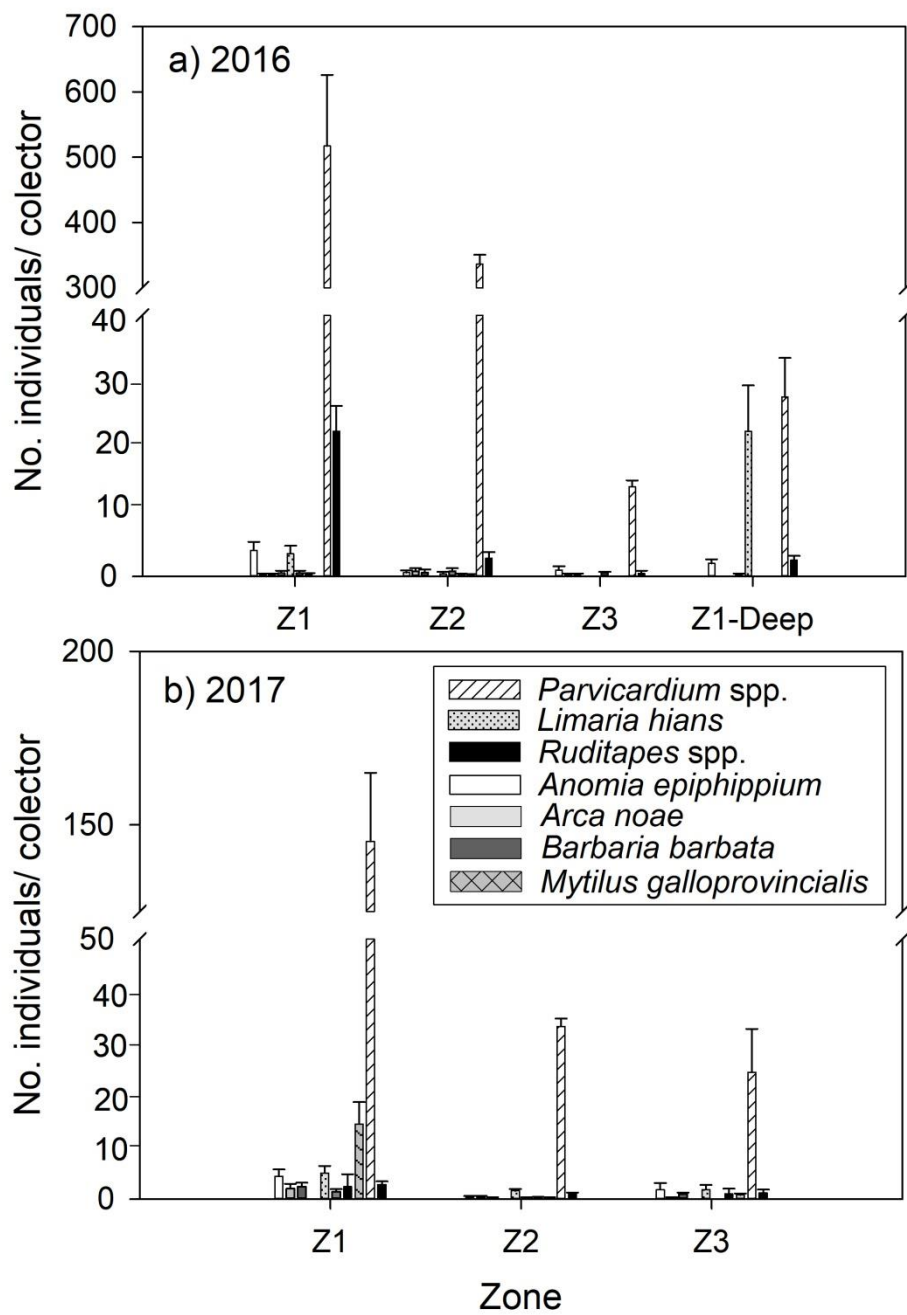
713



714

715 Fig. 5

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718 Fig. 6

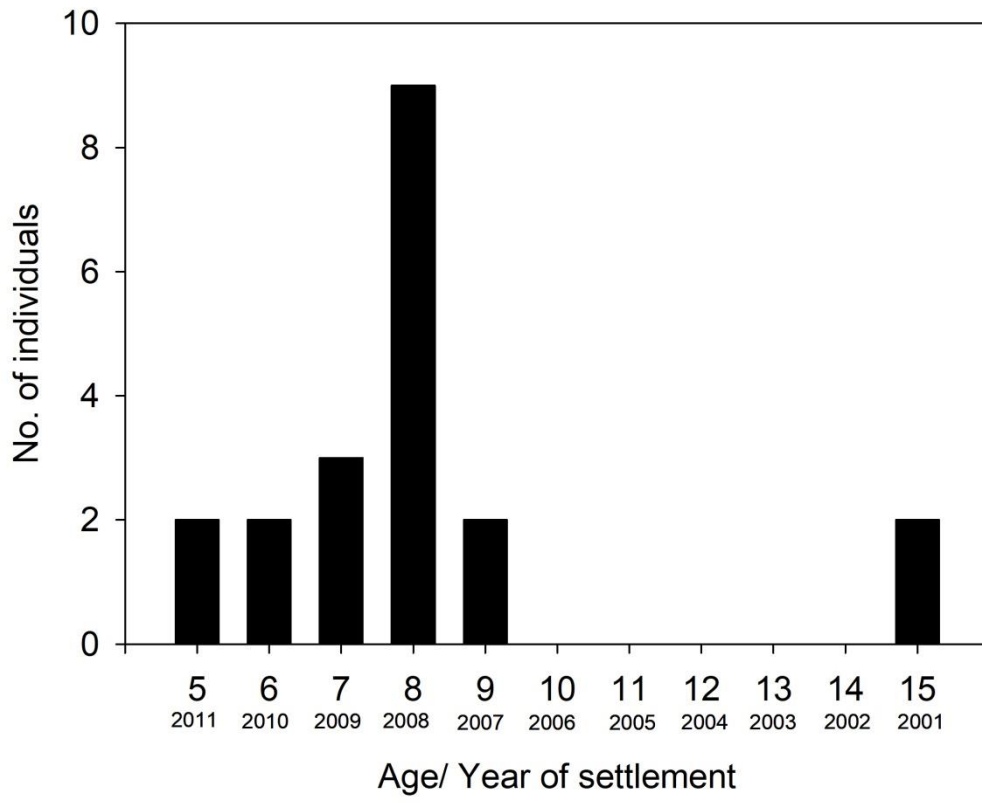
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720

721 Fig. 7

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724 Fig. 9

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726 **Table 1** Description of gonadal development stages in *P. nobilis* individuals from the Alfacs Bay
 727 collected in June and August 2016. The total length of the valves is also indicated between
 728 brackets. For the female/ male line degeneration stage DF indicates the % of degenerated
 729 follicles.
 730

Gonad stage	Description	Individual (shell size)
Simultaneous maturation	Both sexual lines develop on the wall of the follicle from the germinal epithelium. In some instances, some residual spermatozoa can be observed in the lumen. Not described by De Gaulejac.	Ind. 1 June (47 cm)
		Ind. 1 Aug (61.5 cm)
Mature female	Vitellogenic and mature oocytes with presence of empty phagocytes in the center of the follicle are seen. Atretic oocytes are also visible. In some follicle, early male germinal epithelium is also present.	Ind. 4 Aug (60 cm)
Male degeneration-Female formation	According to De Gaulejac (1995): Spermatozoa in the lumen of the acini. The wall of the follicle is covered by developing oocytes.	Ind. 5 Aug (63.5 cm)
Female/male line degeneration	Follicles concurrently containing both degenerating sex lines are present: regressive phenomena at level of male and female germ cells are visible. Apoptotic and vacuolated vitellogenic oocytes are scattered in the follicles and residual spermatogones/ spermatocytes are also present in the lumen. In this phase, presence of nematode eggs, oocysts of protozoan and some unknown crustaceans are visible	Ind. 2 Aug (62 cm) DF:100%
		Ind. 7 Aug (47 cm) DF:100%
		Ind. 8 Aug (43.5 cm) DF: 90% (450/500); few follicles presented final residual oocytes
Spent	Empty acini in sexual pause. There is only conjunctive tissue with few haemocytes phagocyte cellular debris.	Ind. 3 Aug (58 cm)
		Ind. 6 Aug (58 cm)
Spawning male	The follicles are filled of spermatozoa oriented toward the central part. Only few, developing oocytes are present in some areas against the follicle wall.	Ind. 2 Jun (54 cm)

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