1 **Beneficial effects of carpet shell clam (*Ruditapes decussatus*) depuration during short periods of**

2 **conditioning in shellfish hatchery: role of the temperature and phytoplankton on reduction and diversity**

3 **of vibrios.**

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Abstract

Broodstock conditioning in hatcheries is the step previous to spawning and its optimization may be a key to the success of larval cultures. Cleaning and brushing of the broodstock and the utilization of antibiotics to reduce their vibrios load are used regularly as routine prophylactic measures prior to spawning induction. The development of protocols to reduce these bacteria using cheap and harmless techniques is of utmost importance for commercial bivalve production in hatcheries. With this aim, we have evaluated initially different conditionings (A-D) during short periods (a total of four weeks): first two weeks under gradient temperature (increasing +0.3ºC day^{-1} from 14.5ºC to 20ºC), without (A) or with phytoplankton (B), and constant temperature (20ºC) without (C) or with phytoplankton (D). Afterwards, all conditionings were kept at 20ºC and fed for two more weeks. Furthermore, broodstock optimal feeding time was re-evaluated during a second trial series. In all conditionings, bacterial loads were determined in terms of marine heterotrophic bacteria (MHB) and presumptive vibrios (PV). Broodstock under the optimal short period of conditioning (conditioning C) obtained the best gonadal development and a significant reduction in PV load at a lower expense. A total of 61 PV were isolated from all conditionings and identified by sequencing the 16S rRNA gene. Splendidus clade was dominant in the samples coming from natural beds. Diversity of vibrios changed throughout the conditionings in the hatchery favoured by exogenous factors whose effect was mainly observed at the end of the trials: Splendidus clade was also dominant in the broodstock conditioned at gradient temperature and Mediterranei and Harveyi clades were prevalent at constant temperature. Moreover, the percentage of transformation to D-larvae was estimated and the vertical transmission of vibrios from broodstock to eggs and D-larvae was suggested. Implementation of conditioning C reduces considerably the *Vibrio* load of clams without using antibiotics, and thus it represents a novel, cheap, environmental friendly and harmless methodology that can be easily transferred to commercial hatchery.

Keywords: Shellfish hatchery; *Ruditapes decussatus*; clam; broodstock; conditioning; *Vibrio*.

53 **1. Introduction**

54

55 Carpet shell clam (*Ruditapes decussatus*) constituted one of the most important bivalve species in
56 European shellfish aquaculture (4,128.73 T and 45 million €) (FishStatJ, FAO). Bivalve spat from hatchery is
57 currently the only sustainable alternative for the support of aquaculture activities (da Costa et al., 2013; Ojea et
58 al., 2008). The first step in hatchery culture is broodstock conditioning, previously collected from wild beds, in
59 tanks where maturation is induced by artificial means, i.e. manipulating the seawater temperature and supplying
60 adequate feed. Therefore, the production season can be extended and the gamete development of broodstock
61 undergoing gametogenesis accelerated (Helm and Bourne, 2004). Different studies, reviewed by da Costa et al.
62 (2013), demonstrated that the conditioning of *R. decussatus* broodstock at a high constant temperature (20ºC) or
63 by a gradual increase in temperature from natural environment to the conditioning temperature (20ºC) constitutes
64 two good alternatives to obtain sexually mature adults.
65 Monitoring bacteria entry routes should be the first step in the prevention of bacterial proliferation in
66 bivalve cultures and the subsequent detrimental effects (Dubert et al., 2015a). Broodstock constitutes one of the
67 most important bacterial sources for larval cultures due to the vertical transmission of bacteria from adults to
68 larvae (Sandaa et al., 2008; Schulze et al., 2006). Microbiota may include opportunistic pathogens harmless to
69 broodstock but harmful to larvae. Indeed, members of the genus *Vibrio*, including known larval pathogens, have
70 been isolated in shellfish hatcheries from the gonad of broodstock (Lodeiros et al., 1987; Prado et al., 2014b;
71 Riquelme et al., 1995; Sainz-Hernandez and Maeda Martinez, 2005).
72 Routine prophylactic measures for bivalve broodstock arriving hatchery facilities include cleaning and
73 brushing broodstock prior to spawning induction (Helm and Bourne, 2004). In addition to that, the use of
74 antibiotics to reduce bacterial load of bivalve broodstock is very frequent in experimental and commercial
75 broodstock conditioning (González-Araya et al., 2012; Holbach et al., 2015). This causes additional expense and
76 efficiency of antibiotic treatments for ubiquitous pathogens is seriously questioned (Berthe, 2005). The use of
77 antibiotics in shellfish hatcheries without an effluent treatment system is highly unadvisable, since these
78 hatcheries constitute a potential source of antibiotic residues and resistant bacteria to the surroundings (Dubert et
79 al., 2015b). Consequently, bivalve hatcheries have to develop protocols to reduce bacterial load in broodstock
80 using cheap and harmless techniques.
81 Periods of conditioning will be directly related to reproductive activity of the broodstock, i.e. the
82 conditioning periods will be shorter if the broodstock from the natural environment are ripe. In that case, vibrios

83 load associated to broodstock must be reduced rapidly as step previous to spawning induction to minimize the
84 potential risk of vertical transmission to larval cultures. This is the first study until now, in which we have
85 evaluated the bacterial load of *R. decussatus* broodstock during short periods of conditioning previous to the
86 spawning induction, modifying exogenous factors (temperature and phytoplankton). Cultivable bacteria,
87 including *Vibrio* spp., associated to the gonad of these broodstock were also quantified. Vibrios were isolated,
88 characterized and identified to evaluate the direct influence of exogenous factors on these bacterial populations.
89 The optimal conditioning was based on the best reduction in vibrios load and gonadal developmental stage
90 (GDS) of the broodstock. Moreover, in certain cases, D-larvae transformation rate was determined and vertical
91 transmission suggested.
92
93 **2. Materials and methods**
94
95 *2.1. Design of short periods of conditioning and sampling of broodstock*
96
97 Two series of trials (Fig. 1) were designed in consecutive years to optimize the short periods of
98 conditioning of *R. decussatus* broodstock in a shellfish hatchery for a total of 4 weeks. Ripe gametes could be
99 released by the natural populations of carpet shell clam in Ría de Arousa (NW Spain) between April and August
100 and major spawning efficiency occurs in August-September (Rodriguez-Moscoso and Arnaiz, 1998). Hence,
101 adult specimens were collected in May by rake from a natural bed located in Illa de Arousa (Ría de Arousa,
102 Galicia, NW Spain) in two consecutive years depending of the trial. Clams were kept at 10°C, transferred to the
103 hatchery of the Centro de Investigacións Mariñas (CIMA) (Ribadeo, Galicia, NW Spain) and conditioned under
104 different exogenous factors (temperature and phytoplankton) for a total of 4 weeks. Broodstock were maintained
105 in 150 L rectangular tanks with an open circuit of sand-filtered (1 µm) and UV-sterilized seawater at ambient
106 salinity of 32-33 ppt. Tanks were kept with aeration under a photoperiod regime of 12:12 h with a continuous
107 renewal of seawater in a ratio of 20 L/h/kg. Gametogenic scale proposed by Wilson and Seed (1974) was used
108 with slight variations to adapt it for *R. decussatus* to assign the sex and gonadal developmental stage (GDS) of
109 each individual after the observation by microscope (20x) of the histological preparations corresponding to the
110 gonad: stage 0 (rest stage), 1 (start of gametogenesis), 2 (advanced gametogenesis), 3 (ripe), 4 (spawning) and 5
111 (restoration).
112

113 *2.1.1. First trial*

114

115 In the first trial series, the bacteriological optimization of the short periods of conditioning was studied
116 modifying the temperature of the tanks and supplying phytoplankton or kept the broodstock in a brief depuration
117 without phytoplankton. Four challenges were performed in duplicate and a total of 560 clams (mean shell length:
118 43.5±3.3 mm) were distributed in eight tanks (70 individuals per tank; 1.3 kg/150 L) and conditioned for a total
119 four weeks. During the first two weeks (Fig. 1A), clams were conditioned varying combinations of temperature
120 and food (conditionings A-D) following the conditions described by da Costa et al. (2013): gradual increase of
121 temperature (increasing +0.3ºC day^{-1} from 14.5ºC to 20ºC), without (A) or with phytoplankton (B), or at high
122 constant temperature (20ºC) without (C) or with phytoplankton (D). Broodstock corresponding to conditionings
123 B and D were continuously fed with a mixture of phytoplankton based on *Isochrysis galbana*, *Diacronema*
124 *lutheri*, *Tetraselmis suecica*, *Chaetoceros* sp. and *Skeletonema marinoï* in equal cell concentration proportions
125 (1:1:1:1:1), representing a ratio of 6% dry meat by weight (Aranda-Burgos et al., 2014). After the second week
126 of conditioning, all batches were kept at 20ºC and fed as described above until the end of the challenges (Fig.
127 1A). Ten clams were taken in a ratio 1:1 (male:female) from each tank and each gonad sampled to assign the
128 sex, pertinent GDS and for microbiological analyses: at arrival to the hatchery (Sampling 0), two weeks later
129 (Sampling 1) and finally at the end of the experiments (Sampling 2) (Fig. 1A).

130

131 *2.1.2. Second trial*

132

133 In the second trial series, the depuration time and thus the period appropriate to feed the broodstock was
134 re-evaluated on basis of the best short period of conditioning determined in the first trial. Three challenges were
135 performed in duplicate and a total of 390 clams (mean shell length: 49.1±3.5 mm) were divided into six tanks
136 (65 individuals per tank; 1.7 kg/150 L) and conditioned for a total of four weeks. All broodstock were
137 conditioned at constant temperature according to the best bacteriological results obtained previously (Fig. 1B).
138 The same mix of phytoplankton used in the first challenge was supplied at the beginning of the trial in
139 conditioning C0 (=conditioning D) (Fig. 1A and B). Broodstock was kept without phytoplankton for one week in
140 the conditioning C1 and two weeks during the conditioning C2 (=conditioning C) (Fig. 1B), so they were
141 depurated during that time. After two weeks, parameters were unified following the conditions described for the
142 first trial until the end of the challenge (Fig. 1B). Sex, GDS and microbiological analyses were performed

143 sampling six clams (ratio male:female, 1:1) from each tank at the beginning of the trial (Sampling 0) and after of
144 the first (Sampling 1), second (Sampling 2) and fourth week (Sampling 3) (Fig. 1B).
145
146 *2.2. Spawning induction and D-larvae transformation*
147
148 Broodstock conditioned under conditionings C0 and C2, corresponding to the second trial, were selected
149 for further studies. For the spawning induction, clams were cleaned by brushing and kept dry at 4°C for 12 h.
150 Then, broodstock were placed in a tray with UV-sterilized seawater. Specimens were subjected to thermal shock,
151 with temperatures up to 25ºC for 2 h, decreasing to 14ºC for 30 min. A total of 2 cycles were performed.
152 Additional stimuli were provided by adding gametes stripped from one of the conditioned bivalves and mixture
153 of phytoplankton. Each spawning was quickly transferred into individual receptacles for the release of sperm or
154 eggs, thus avoiding self-fertilization. Once spawning was completed, sperm from several males was pooled and
155 added to the container, at 20°C, with oocytes to obtain synchronous fertilization. After fertilization, the eggs
156 were sieved through a 45-µm mesh screen to eliminate the excess of sperm. Total number of fertilised eggs was
157 determined and the embryos were transferred to 500 L larval culture tanks with aerated and filtered UV-
158 irradiated seawater at a temperature of 20°C and the density was adjusted to 30 embryos mL^{-1}. No food was
159 supplied during embryo incubation. In the post-fertilization, the percentage of transformation to D-larvae was
160 estimated by the total number of viable D-larvae collected on 60-µm mesh screen per mL.
161
162 *2.3. Microbiological samples*
163
164 Fragments of gonad (0,08-0,1 g) were aseptically homogenized *in situ*, diluted 1:10 (v/v) in sterile seawater
165 and 100 µL corresponding to appropriate dilutions in seawater were spread on Marine Agar (MA; Difco, USA)
166 for detection of marine heterotrophic bacteria (MHB), and on Thiosulphate-Citrate-Bile-Sucrose (TCBS; Oxoid,
167 UK) for detection of presumptive vibrios (PV). TCBS and MA plates were incubated under aerobic conditions at
168 20ºC for 24-48 h and 1 week, respectively. Bacterial were counted as colony forming units per gramme of gonad
169 (CFU g^{-1}) and expressed in mean values of males, females and the average of both sexes. Eggs from the
170 spawning and D-larvae were streaked *in situ* directly on the plates (MA and TCBS) plates with an inoculating
171 loop (1 µL) to detect the presence/absence of bacteria obtaining a semiquantitative estimates of bacterial
172 numbers. In all cases, different types of colony on MA and TCBS plates were identified in basis of

173 morphological and growth characteristics an one colony of each predominant type was isolated, purified and
174 maintained frozen for further studies at -80ºC in Marine Broth (MB; Difco, USA) supplemented with 20%
175 glycerol (v/v). All isolates were analysed for their basic phenotypic properties using a set of tests described by
176 Prado et al. (2014b), and only those that shared the main phenotypic features of the genus *Vibrio* (Noguerola and
177 Blanch, 2008) were selected for further studies. Genomic DNA of these isolates was extracted with the
178 InstaGene™ Matrix (Bio-Rad, USA) and their 16S rRNA gene was amplified and sequenced using specific
179 bacterial primers (27F, 1100R and 1510R) to obtain an almost complete sequence (approx. 1400 bp) and
180 bioinformatic analyses were performed as described Dubert et al. (2015a). Sequences were deposited in the
181 DDBJ/EMBL/GenBank database under accession numbers LN867527 to LN867600 (Supplemental Fig. S1 and
182 S2; Table S1).

183 Finally, inlet seawater tank and mixture of phytoplankton used as food were sampled throughout the
184 conditionings as described Dubert et al. (2015a), processed *in situ* and spread on TCBS to discard them as
185 potential source of vibrios. PV counts were expressed as colony forming units per millilitre (CFU mL^{-1}).

186

187 *2.4. Statistical analysis*

188

189 Data normality was first evaluated using the Shapiro-Wilk test and then one-way analysis of variance
190 (ANOVA) for significant differences was performed using STATISTICA software version 12 (Stat Soft, Inc.,
191 USA). Homogeneity of variances was checked by means of the Barlett test. Also, a three-way analysis of
192 variance (ANOVA) with phytoplankton, temperature and sex as factors was performed using STATISTICA
193 software version 12 (Stat Soft, Inc., USA). MHB and PV data were log transformed to meet homogeneity of
194 variances criteria for ANOVA analysis. When necessary, *post hoc* analyses with the LSD test were applied.
195 Differences were considered statistically significant if $p \leq 0.05$.

196

197 **3. Results**

198

199 *3.1. Bacterial load of broodstock during the optimization of the short periods of conditioning*

200

201 *3.1.1. First trial*

202

203 Bacterial load (MHB and PV) expressed in mean values demonstrated that PV loads corresponding to the
204 broodstock coming from natural beds (Sampling 0) (Fig. 1A; Table 1) were similar and not significant (p>0.05)
205 regardless of sex (≈10^4 CFU g^{-1}), whereas MHB counts were one logarithm higher for females although not
206 significant (p >0.05). Quantitative analysis revealed that values of standard deviation exceed the mean in part of
207 samples due to differences found in the bacterial loads (see maximum and minimum values in Table S2) and
208 gonadal development of the clams collected from the natural environment (Table S2). This fact was observed
209 throughout the first trial (Table 1). Hence, GDS values were above of the stage 2 (minimum: 2; maximum: 3)
210 (Table S2 and 1). Ratio PV:MHB was 1:1 for males and 1:10 for females.

211 Quantitative analysis revealed that during the first two weeks of conditioning (Sampling 1) (Fig. 1A;
212 Table 1) the PV and MHB loads of males and females decreased simultaneously in conditioning B and more
213 clearly in conditioning C, in which a significant reduction of two logarithms was observed in the PV load
214 compared to Sampling 0 (Table 1, p <0.05). During the first two weeks of conditioning (Sampling 1), a
215 significant reduction of one logarithm was observed in the MHB load for males in treatment C (Table 1, p
216 <0.05), and for females also a significant reduction of one logarithm was observed in treatment B and C (Table
217 1, p <0.05). For males, PV:MHB ratio was 1:10 in conditionings A, B and C and 1:100 for conditionings D. For
218 females, this ratio was 1:1 under conditionings A, 1:10 in B and 1:100 in conditionings C and D at constant
219 temperature.

220 In the Sampling 2 (Fig. 1A; Table 1), PV load decreased significantly in males and females conditioned
221 under conditionings C (p <0.05) compared to the initial samples, whereas in conditioning D only the males
222 decreased their PV load although it was not significant (p >0.05). In both cases, the PV:MHB ratio was 1:10 for
223 males and 1:100 and females. In addition, in all conditioning the MHB load was similar to Sampling 0 (≈10^4-10^5
224 CFU g^{-1} for both sexes) and it was not significant (p >0.05) (Table 1).

225 Finally, GDS increased significantly throughout all conditionings in relation to the initial samples
226 (GDS=2.3 and 2.5 for females and males, respectively; Table 1). Highest value was reached at the end of trial in
227 broodstock under conditioning C (GDS=4.0 and 3.5 for females and males, respectively; Table 1).

228 As an additional step, the effect of temperature and phytoplankton on the bacterial load of broodstock was
229 analysed separately.

230 Influence of the constant temperature (conditioning C and D) promoted a significant reduction at the end
231 of trials in PV load (p <0.05) (Sampling 2), in comparison to gradient temperature (conditionings A and B)

232 (Table 1). MHB loads were similar in Sampling 2 regardless of temperature (≈10^4-10^5 CFU g^{-1}), although
233 slightly lower and not significant (p >0.05) for constant temperature conditionings (Table 1).
234 Influence of phytoplankton was only evaluated in broodstock corresponding to conditionings A and C,
235 since they were fed for the first time after two weeks of depuration (Fig. 1A; Table 1). Comparison of
236 conditionings A and C let to determine an increase not significant (p >0.05) of the PV and MHB loads of the
237 most of males and females, excluding only the females corresponding to conditioning A, when they were fed for
238 the first time, i.e. after second week (Sampling 2)
239 Interestingly, PV load of inlet seawater and the mixture of phytoplankton used as food in the first trial
240 were below the detection limits in all cases (<10^1 CFU mL^{-1}).
241 In summary, the greatest reduction in PV and MHB was detected in the broodstock under constant
242 temperature (C and D), and particularly under conditionings C, which provided the best microbiological results
243 and the highest GDS.
244
245 *3.1.2. Second trial*
246
247 In the second set of trials, the optimal feeding time, and thus the depuration period, was re-evaluated in
248 broodstock conditioned under constant temperature (Fig. 1B; Table 2). Bacterial loads of broodstock coming
249 from natural beds (Sampling 0) (Fig. 1B; Table 2) were slightly different (not significant; p > 0.05) from the first
250 trial (Table 1, 2 and S2): PV loads were lower for both sexes, whereas MHB load was higher only for females.
251 Indeed, ratio PV:MHB was 1:10 for males and 1:100 for females. Similarity to the first trial was observed for
252 values of standard deviation and GDS (Table 2 and S2).
253 After the first week (Sampling 1) (Fig. 1B; Table 2), PV load decreased significantly (p <0.05) in all
254 cases with respect to the initial samples. Hence, PV and MHB loads were significantly lower under depuration
255 conditionings C1 and C2 (Table 2, p <0.05) and these values were similar in both batches. PV loads were below
256 the detection limits (<10^2 CFU g^{-1}) in conditioning C2. Broodstock corresponding to non-depurated batches
257 (conditioning C0), which were fed from the beginning of the trial, showed the highest PV and MHB values and
258 the PV:MHB ratio was 1:1000 for males and 1:100 for females.
259 As expected, phytoplankton had a similar effect on the bacterial load as observed during the first trial.
260 Hence, males and females corresponding to conditioning C1 (Fig. 1B; Table 2) increased significantly their PV
261 and MHB loads (p <0.05) when they fed for the first time, i.e. after one week of depuration (Sampling 2). In

262 contrast, PV load was kept below detection limits (<10^2 CFU g^{-1}) in broodstock did not feed until that moment
263 (conditioning C2). In all samples, MHB loads remained at 10^4-10^5 CFU g^{-1} regardless of conditioning treatment
264 (Table 2).

265 In the final sampling (Sampling 3) (Fig. 1B; Table 2), PV load was lower than initial samples in all cases,
266 although results were not significant (p >0.05). Interestingly, PV values corresponding to conditionings C1 and
267 C2 were below the detection limits (<10^2 CFU g^{-1}). MHB values were similar (≈10^3-10^4 CFU g^{-1}) and not
268 significant (p >0.05) in all conditionings (Table 2). Interestingly, influence of the phytoplankton was only
269 observed for PV load of the males not fed until now (conditioning C2) but not in females. As observed in the
270 first trial, PV load of inlet seawater and the mixture of phytoplankton used as food were below the detection
271 limits in all cases (<10^1 CFU mL^{-1}).

272 Moreover, GDS increased throughout the study, reaching the highest values for females of conditioning
273 C2 and males in batches C0, C1 and C2 (Table 2).

274 In summary, results demonstrated that broodstock conditioned under conditioning C (=C2), that is at 20ºC
275 for a total of four weeks, with an initial depuration of two weeks and fed only for the remaining two weeks
276 previous to spawning induction obtained the best GDS, a considerable reduction in PV and MHB loads and at a
277 lower economic cost.

278

279 *3.2. Changes in the Vibrio spp. of conditioned broodstock*

280

281 Qualitative analysis of the predominant *Vibrio* spp. was done during all conditionings to evaluate the
282 potential vertical transmission to larval cultures. A total of 164 strains were isolated in the samples from MA and
283 TCBS plates: 68 in the first trial and 96 in the second trial. Phenotypic analyses showed that 61 isolates shared
284 the main phenotypic features of the genus *Vibrio* (33 and 28 isolates were isolated from the first and second
285 trials, respectively): Gram-negative rods, facultative anaerobic, growth on TCBS, reduced nitrates to nitrites,
286 positive for catalase, oxidase and indole production and growth at 4-25ºC and 3-6% NaCl (data not shown).
287 They were identified by sequencing the 16S rRNA gene (Table 3), and analysis of their similarities confirmed
288 that most (56/61 isolates) belonged to the genus *Vibrio*. Only five strains pertained to other taxonomic groups,
289 such as *Photobacterium* (n=3) and *Pseudovibrio* (n=2).

290

291 *3.2.1. First trial*

292

293 *Vibrio* species were very homogeneous in the different conditionings (Table 3A). Species belonged to
294 Splendidus clade (Supplemental Fig. S1; Table 3A) as *V. atlanticus*/*V. tasmaniensis*, *V. hemicentroti* and mainly
295 *V. gigantis*/*V. celticus* were the most common in the gonad of the broodstock directly collected from natural
296 beds, being the main types of colony in the plates corresponding to Sampling 0. Interestingly, other species as *V.*
297 *jasicida*, related to Harveyi clade, was punctually detected in this sampling.

298 Strains related to the bivalve larval pathogen *V. tubiashii* subsp. *tubiashii* (Supplemental Fig. S2; Table
299 3A) were isolated in all samples after 2 weeks of conditioning (Sampling 1) regardless of conditioning.
300 However, only one isolate was obtained in conditioning C where was not the dominant type of colony. Other
301 genera, such as *Photobacterium* or *Pseudovibrio*, were only detected in samples corresponding to unfed batches
302 (A and C).

303 The effect of the short periods of conditionings on the *Vibrio* spp. was mainly observed at the end of the
304 trial (Sampling 2). Hence, certain *Vibrio* clades were favoured by either constant or gradient temperature, seeing
305 as all batches in the last samples were fed for two weeks until the end of the challenges. Isolates mainly
306 belonging to Splendidus clade (Supplemental Fig. S1), identified as *V. gigantis*/*V. celticus*, were related to
307 conditionings with gradient temperature (A and B) (Table 3A). In contrast, Harveyi clade (it includes *V.*
308 *neocaledonicus*) and particularly Mediterranei clade (it includes *V. thalassae* and *V. meditarranei*)
309 (Supplemental Fig. S2) were regularly isolated from broodstock kept at constant temperature (C and D) (Table
310 3A).

311

312 *3.2.2. Second trial*

313

314 A similar qualitative evolution of the *Vibrio* spp. was observed in the second trial, where conditionings
315 C0 and C2 were equivalent to batches D and C in the first trial, respectively. Splendidus clade (Supplemental
316 Fig. S1; Table 3B) was again the predominant *Vibrio* group in clams directly collected from natural beds
317 (Sampling 0). As expected in the conditionings at constant temperature, the presence of this clade reduced
318 throughout the conditioning (Table 3B). However, Mediterranei clade (it includes *V. thalassae* and *V.*
319 *meditarranei*) and Harveyi clade (it includes *V. neocaledonicus, V. owensii* and *V. jasicida*) (Supplemental Fig.
320 S2; Table 3B) gained relevance at the end of conditionings (Sampling 3). Interestingly, one isolate from

321 conditioning C0 (Sampling 3) was close to the larval pathogen *V. tubiashii* subsp. *europaeus* (Supplemental Fig.
322 S2; Table 3B). In addition, some strains (n=10) seemed to be non-recognizable species (<98.8%).
323
324 *3.3. Spawning induction and D-larvae transformation*
325
326 Spawning was induced in the broodstock corresponding to batches C0 and C2 (Table 3C) to establish the
327 relationship between conditionings at constant temperature and the transformation rates. In conditionings C2
328 (=conditioning C) the 33.7% of the eggs were transformed to D-larvae in relation to 10.5% corresponding to the
329 batch C0.
330 The influence of *Vibrio* populations associated to broodstock gonad on eggs and D-larvae after spawning
331 was suggested by means of the vertical transmission. Indeed, similar strains related to the Harveyi clade, namely
332 isolates identified as *V. neocaledonicus*, were isolated previously at the end of the second trial (strain 3683)
333 (Table 3B, Sampling 3) and detected subsequently in eggs and D-larvae (strain 3693) (Table 3C) (Supplemental
334 Fig. S2). Isolate 3614 close to larval pathogen *V. tubiashii* subsp. *europaeus* was isolated in the eggs released
335 from the broodstock of conditioning C0, in which it was also isolated (strain 3610) (Table 3; Supplemental Fig.
336 S2).
337
338 **4. Discussion**
339
340 Knowledge of vibrios associated to broodstock during conditioning in shellfish hatcheries is essential to
341 better overall management and reducing risk of vibriosis. Broodstock are a direct source of bacteria specific to
342 each larval batch due to the close relationship by means of the vertical transmission of bacteria (Prado et al.,
343 2014b; Riquelme et al., 1995; Sainz-Hernandez and Maeda Martinez, 2005; Sandaa et al., 2008; Schulze et al.,
344 2006). Results have demonstrated that short periods of conditioning constitute a suitable procedure to acclimatise
345 broodstock in advanced gametogenesis and to reach the sexual maturity decreasing considerably the vibrios load
346 in relation to the natural environment. In line with other authors, the high quantity of nutritional reserves in the
347 gonad promotes the bacterial accumulation and supports the detection of vibrios in the gonad of the bivalve
348 broodstock (Lodeiros et al., 1987; Prado et al., 2014b; Riquelme et al., 1995; Sainz-Hernandez and Maeda
349 Martinez, 2005; Sugumar et al., 1998). Interestingly, we have observed variability in the bacterial loads of the
350 clams coming from the natural environment due to different microbiological/physiological state of each

351 specimen. This fact could explain the variability in the bacterial loads of the broodstock found throughout the
352 study within the same conditioning treatment, suggesting that the process of conditioning is not uniform.
353 Moreover, clams collected in natural environment in May were in advanced gametogenesis and ripe stage,
354 according to Rodriguez-Moscoso and Arnaiz (1998). Besides, analysis of the samples showed some differences
355 among sexes but it did not let to establish a direct relationship between sex and the bacterial loads. In contrast,
356 other authors detected higher bacterial loads in females than males since the nutritional reserves in the female
357 gonad promoted the bacterial proliferation (Riquelme et al., 1995; Sainz-Hernandez and Maeda-Martínez, 2005;
358 Sugumar et al., 1998).

359 Exogenous factors, mainly temperature and food, are determinant during the conditioning of broodstock
360 due to their impact on reproductive activity. In fact, some authors have reported the beneficial effects of food and
361 the temperature on gametogenic development during this process, e.g. temperature 20ºC, gradient of temperature
362 or mixture of phytoplankton based on *Isochrysis* sp. clone T-iso + *Pavlova lutheri* + *S. costatum* + *Chaetoceros*
363 *calcitrans* + *T. suecica* (da Costa et al. 2013; Matias et al. 2009; Ojea et al., 2008). In other studies, Riquelme et
364 al. (1995) demonstrated that adequate conditioning of *Argopecten purpuratus* broodstock may be an effective
365 procedure for reducing the risk of *Vibrio* vertical transmission. Subsequently, Holbach et al. (2015) obtained a
366 strong reduction of *Vibrio* loads in oocytes and D-larvae when *Pecten maximus* broodstock were conditioned
367 without sandy-bottom for 2 months and treated 6 days with erythromycin. Our results demonstrate the useful and
368 effectiveness of the short periods of conditioning in carpet shell clam broodstock from a microbiological,
369 physiological and economic standpoint. Interestingly, significant results in the reduction of PV load were
370 obtained for optimal conditioning C, i.e. at 20ºC for a total of four weeks, with an initial depuration of two weeks
371 and feeding the clams only for the remaining two weeks previous to spawning induction.

372 Routine prophylactic measures for bivalve broodstock arriving hatchery facilities include its
373 cleaning/brushing and the very frequent use of antibiotics (e.g. erythromycin, florfenicol, oxytetracycline or even
374 chloramphenicol despite of its prohibition) to reduce its bacterial load, particularly vibrios, as step previous to
375 spawning induction (Dubert et al., 2015b; Holbach et al. 2015; Miranda et al., 2013; Uriarte et al., 2001). The
376 conditioning protocol proposed is a good alternative to the use of antibiotics whose administration causes
377 additional expenses, do not guarantee culture success and promotes the rapid development of resistant vibrios in
378 larval tanks. Recently, Dubert et al. (2016) reported the rapid colonization of the bivalve larvae by the
379 pathogenic *Vibrio* spp. and demonstrated that once the pathogen is inside the bivalve larvae the infection process
380 cannot be stopped. Hence, preventive treatments are particularly the key to successful larval cultures decreasing

381 the economic losses for the shellfish hatcheries. The appearance of bacteria resistances is favoured by the routine
382 of use of antibiotics in hatcheries as preventive treatment, with a concomitant rise and persistence of diseases
383 that cannot be efficiently treated (McPhearson et al., 1991).

384 Analysis of the exogenous factors separately let to evaluate the effect of temperature and phytoplankton
385 on the bacterial load of broodstock. Quantitative results demonstrated a significant reduction in PV load
386 promoted by constant temperature. Changes in seawater temperature have been identified as the most important
387 variable regulating *Vibrio* abundances in marine environments when culture-dependent methods have been
388 applied (Huq et al., 2005; Louis et al., 2003; Romalde et al., 2014; Turner et al., 2009). Different *Vibrio* species
389 are favoured by environmental conditions, i.e., temperature, salinity and nutrient availability (Armada et al.,
390 2003; Dubert et al. 2015a; Eiler et al., 2006; Takemura et al., 2014). We have demonstrated in our study that
391 gradient temperature promoted at the end of the trials the proliferation of isolates belonging to the Splendidus
392 clade and constant temperature favoured the presence of the Mediterranei and Harveyi clades. In addition, a
393 number of studies (Arias et al., 1999; Pujalte et al., 1999) have shown the influence of temperature on these
394 clades in bivalve molluscs coming from natural beds. These authors established the predominance and stability
395 of *V. splendidus* at temperatures below 20°C and *V. harveyi* and *V. mediterranei* at ≥ 20ºC.

396 Most of depurated broodstock (conditionings A, C, C1 and C2) increased their bacterial loads when they
397 were fed for the first time after the depuration despite the PV load of the mixture of phytoplankton was below
398 the detection limits. Different authors have determined the presence of high and constant MHB levels associated
399 to the phytoplankton cultures (≈10^6 CFU mL^{-1}) (Dubert et al., 2015a; Makridis et al., 2012; Nicolas et al., 2004;
400 Salvensen et al., 2000). Phytoplankton harbours a wide spectrum of bacterial populations that utilize metabolites
401 and organic substances released by living or dead algal cells (Bruckner et al., 2011; Eiler et al., 2007; Riquelme
402 et al., 1987). Dubert et al. (2015a) demonstrated that vibrios were never the main component of the population of
403 cultivable bacteria in a mixture of phytoplankton composed by species *Isochrysis* sp., *Diacronema* sp.,
404 *Chaetoceros* sp. and *Tetraselmis* sp. However, this vibrios detection in the microalgal cultures should be taken in
405 mind because the phytoplankton is supplied directly to bivalves. This fact could support the increase of the PV
406 load after the broodstock were fed since bivalves due to their filter-feeding habit accumulate large numbers of
407 bacteria, representing an important ecological niche for vibrios which are regular components of their microbiota
408 (Pruzzo et al., 2005).

409 The 16S rRNA gene has a rather low interspecies resolution and is not useful for species differentiation
410 but may provide an useful information at clade level (Dubert et al. 2015a). Qualitative analysis of the *Vibrio*

411 population in basis of 16S rDNA sequencing let to demonstrate that most vibrios in broodstock coming from
412 natural-bed belonged to Splendidus clade, which includes the dominant *Vibrio* species in coastal marine
413 sediments, seawater and bivalves, including *R. decussatus* (Beaz-Hidalgo et al. 2008; Romalde et al., 2014).
414 Detection of Splendidus and Harveyi clades throughout the conditioning should be taken into account because
415 they include aquaculture pathogens such as *V. splendidus*, *V. harveyi*, *V. alginolyticus* or *V. owensii* (Austin et
416 al., 2010; Cano-Gomez et al., 2010; Rojas et al., 2015). Interestingly, isolates close to *V. tubiashii* was detected
417 during conditionings. This species was reported as larval and juvenile pathogen for several bivalve species but
418 not in adult specimens (Elston et al., 2008; Hada et al., 1984; Prado et al., 2014a; Travers et al., 2014). Hence,
419 the *Vibrio* broodstock populations must be checked seeing as they may include *Vibrio* spp. that are harmless to
420 broodstock but harmful to larvae. Indeed, our results suggested a vertical transmission of *Vibrio* species,
421 including isolates close to the larval pathogen *V. tubiashii* subsp. *europaeus*, from broodstock to eggs and D-
422 larvae after spawning. Moreover, *Vibrio* species detected in the eggs could not be transferred through the
423 phytoplankton since during this stage the cultures are not fed.

424 In summary, our findings support that vibrios loads of broodstock could be reduced in a short time with
425 the use of an adequate conditioning protocol. Indeed, clams conditioned at 20ºC for a total of four weeks, with
426 an initial depuration of two weeks and fed only for the remaining two weeks obtained the best gonadal
427 development and an important reduction of vibrios load with at lower cost. Implementation of this conditioning
428 in hatcheries would improve the management of *R. decussatus* broodstock since did not imply the use of
429 antibiotics, and thus it represents a novel, cheap, environmental friendly and harmless (i.e. prevents from
430 releasing resistant bacteria to the environment) methodology that can be easily transferred to commercial
431 hatchery production of clams.

432

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434

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437

438 **Appendix A. Supplementary data**

439

440 Supplementary Figure S1, S2 and Table S1 and S2 can be found online.

441

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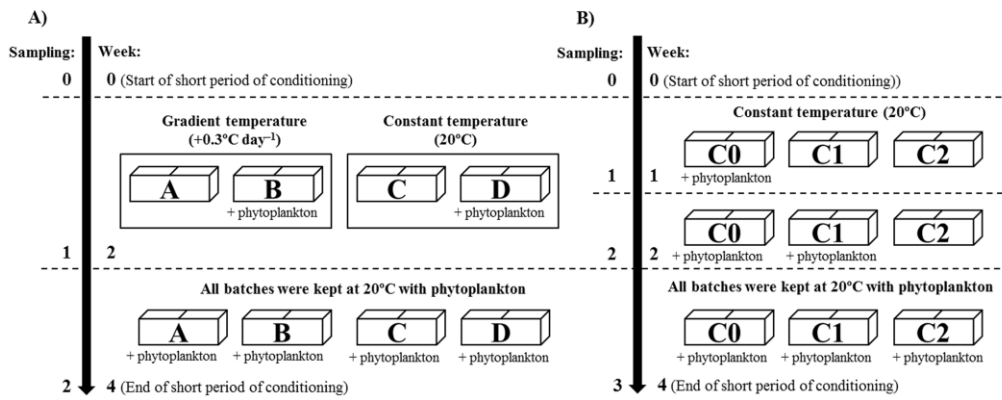
557 **Figure legends**



558

559 **Fig. 1.** Series of trials designed to optimize the short periods of conditioning modifying only temperature and

560 supplying or not phytoplankton: first (A) and second (B) trial. Trials were performed in duplicate.