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## Abnormal mortality of triploid adult Pacific oysters: Is there a correlation with high gametogenesis in Normandy, France?

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27 **ABSTRACT**

28 Summer mortalities of the adult Pacific oysters *Crassostrea gigas* are an important economic  
29 concern. In 2015 and 2016, the mortality of hatchery origin spat comprising 4 diploid batches  
30 and 9 triploid batches as well as 12 batches of wild caught diploids was followed at 3 sites in  
31 Normandy. Abnormal mortalities (> 20%) were observed at one site only and were  
32 significantly higher in triploid animals ( $P < 0.05$ ). Triploid oysters are believed to be partially  
33 sterile but a high level of gametogenesis was observed in all monitored triploid batches. In  
34 2015, preliminary results revealed that triploid oysters underwent strong gametogenesis with  
35 mature gametes (stage 3) and 50% of them were non-perturbed (i.e. named alpha). In 2016,  
36 42% of triploid oysters were alpha. This study revealed that triploid oyster maturation  
37 occurred in September, which corresponded to the time of high mortalities in Normandy.

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40 **Keywords:** *Crassostrea gigas*, triploid, mortality, gametogenesis

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## 52 INTRODUCTION

53 Mass mortalities of Pacific oysters *Crassostrea gigas* have occurred since the 1950s in  
54 different countries including Japan, the United States and France (Glude 1975, Perdue et al.  
55 1981, Costil et al. 2005). The causes remain unclear, but a multifactorial etiology is suspected.  
56 Since 2012, an upsurge of mortality of adult oysters on the French coast has been observed.  
57 The most important extrinsic factor seems to be the presence of the pathogen *Vibrio*  
58 *aestuarianus* (François et al. 2013, François et al. 2014, François 2015, Garnier et al. 2007,  
59 Vezulli et al. 2015, Goudenège et al. 2015) but intrinsic factors such as gametogenesis and  
60 spawning may be important. The influence of reproduction on oyster mortality has been  
61 reported by several authors who inferred metabolic and physiological disturbances, sometimes  
62 associated with gonadal maturation (Tamate et al. 1965, Glude 1975, Maurer and Comps  
63 1986, Huvet et al. 2010).

64 Triploid animals have partial or complete gonadal sterility (Benfey, 1999). The reduction of  
65 gametogenesis in triploid oysters enhances their marketability during the reproductive season  
66 and improves meat quality (Nell, 2002). These reproductive cells present some perturbations  
67 in their development. In 2010, Jouaux et al. established a classification to determine the  
68 gametes disorders of triploid oysters.

69 Until 2008, several methods were available to produce triploid Pacific oysters, including  
70 artificial induction that involves chemical treatment or physical stress (Allen and Downing  
71 1986, Yamamoto et al. 1988, Gerard et al.1999). Another method, which was patented in the  
72 USA with licenses for the EU (Guo and Allen, 1988), consists of crossing gametes from  
73 induced tetraploids and normal diploids (Guo and Allen 1994, Guo et al.1996, Eudeline et al.  
74 2000). The tetraploids were produced first by crossing triploid females with diploid males,  
75 and blocking the first phase of meiosis.

76 The induction method usually has a success rate of 80-90% (Guo and Allen 1994, Guo et al.  
77 1996) whereas the tetraploid technique can **theoretically** produce 100% triploid embryos.  
78 Unfortunately, the induction method and tetraploid technique (Guo and Allen 1988) could  
79 also produce fertile triploids in 2% and 13.4%, respectively (Benabdelmouna and Ledu 2007,  
80 Benabdelmouna et al. 2007). In order to avoid this risk, two new methods to produce  
81 tetraploids **were** patented in 2007 (Benabdelmouna and Ledu 2007, Benabdelmouna et al.  
82 2007). The authors observed a mixture of diploid, triploid and tetraploid **larvae after the**  
83 **classical first induction on diploids**. Tetraploid larvae were smaller than other larvae and  
84 usually died in the mixture **but when** separated by sieving **and** reared alone could survive to  
85 **become** new adult tetraploids.

86 **In 2012, oyster farmers throughout France reported higher mortality among adult triploid**  
87 **populations of *C. gigas* in comparison to diploid stocks**. (Pers. communic.). **This was in**  
88 **contrast with the results of a multidisciplinary study conducted between 2001 and 2006 in**  
89 **which triploid oysters suffered low mortalities due to greatly reduced gametogenesis and**  
90 **higher potential defense capacities than diploid animals (Samain and Mc Combie, 2008).**

91 In **this** study, the gametogenesis of 9 triploid batches in Normandy **was** analyzed and  
92 monitored for 2 years with a control of ploidy for each animal studied. Experiments **were**  
93 conducted between March 2015 and November 2016 with the following objectives:

94 (1) To study the effect of ploidy (diploid vs triploid) on survival during the **oyster breeding**  
95 cycle.

96 (2) To study gametogenesis stage of triploids during mortality monitoring.

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## 99 **MATERIALS AND METHODS**

### 100 **Animals**

101 Twenty five batches of oysters of different origin and ploidy (Table 1) were studied: 12  
102 diploid batches from natural capture in the Marennes-Oléron bassin (Charente-Maritime,  
103 France) and in the Arcachon bassin (Gironde, France), 4 diploid batches from 3 hatcheries (A,  
104 B, C) and 9 triploid batches from 5 hatcheries (A, B, C, D and E). These batches were  
105 received between 9<sup>th</sup> January and 12<sup>th</sup> May 2014 and were placed at 3 sites in Normandy  
106 (Meuvaines, St Vaast-La-Hougue, and Géfosse-Fontenay) (figure1). Upon receipt,  
107 approximately 30,000 units per batch were dispatched to each of three sites in 2014 (3 bags  
108 per batch and per site). These oysters were reared off-bottom in culture bags on iron tables, in  
109 exactly the same manner as commercially-grown oysters (duplication, screening...) and  
110 remained on each site for the duration of the study. In 2015 and 2016, an average of 2,500 and  
111 600 per batch per site were kept, respectively.

112

### 113 **Characterization of batches**

114 Upon receipt, the ploidy of oysters was identified by analysis of 10 individuals per batch by  
115 flow cytometry (Gallios Beckman Coulter, Flow cytometric technical platform, SF ICORE,  
116 Caen). Part of the gill was sampled and stored in ethanol solution (70%) at -20°C until the  
117 next step. One square millimeter of gill in ethanol solution (70%) was homogenized (Dounce  
118 all glass tissue Grinders) and filtered (80 µm) to eliminate coarse particles. Cells were stored  
119 at -20°C. Subsequently, samples were centrifuged at 2000 g for 1 minute and the pellets were  
120 suspended in 1mL sterile artificial seawater (per 1L of reverse osmosis purified water: 23g  
121 sodium chloride; 1.49g potassium chloride; 0.3g calcium chloride; 1.23g Magnesium sulfate  
122 heptahydrate) for 5 minutes at room temperature. Samples were again centrifuged at 2000 g  
123 for 1 minute, pellets were suspended in 500µL of propidium iodide solution (propidium  
124 iodide (0.05mg/mL); RNase (0.02mg/mL); PBS 1X) and stored for 15 minutes in the dark at  
125 room temperature. 10 000 single cells per sample were analyzed by flow cytometry with

126 fluorescence detected at 610 nm (FL3). Ploidy of the individual cell was determined from the  
127 histogram of single cell number on a linear scale. During the second and third year of the  
128 study, the same process was used to determine the ploidy of each sample for gametogenesis  
129 analysis. Animals with unexpected ploidy were reported as “unexpected”. Between 10 and 80  
130 individuals per batch were analyzed per year from 2014 to 2016 (table 2).

131

### 132 **Dynamics of mortality**

133 A mortality count on each batch per site was conducted each month from March to  
134 November. For this purpose, each year in February, from among all individuals in a batch on  
135 the foreshore, 100 individuals were randomly selected and placed in an oyster bag. Mortality  
136 rates were determined by counting dead and moribund vs healthy animals in the bag each  
137 month. The dead and moribund individuals were removed from the oyster bag each month. In  
138 the case of abnormal mortality (>20%), samples (5 individuals analyzed in 1 pool) were  
139 systematically sampled from the 3 sites at the time of the monthly count to detect the presence  
140 or absence of *V. aestuarianus*.

141 In 2016, environmental monitoring was conducted monthly by analyzing samples of seawater  
142 collected from the 3 sites.

143 DNA extraction was performed with QIAmp DNA mini kit® (Qiagen, Courtaboeuf, France)  
144 according to the manufacturer's instructions using 50 milligrams of fresh oyster tissues for  
145 each pool of the molluscs and for 100 µL of the pellet obtained by centrifuging 500 mL of  
146 seawater at 10 000 g for 30 min. Elution was performed in 60 µL of buffer AE provided with  
147 the kit. An extraction process control was included to evaluate contamination between  
148 samples. A real-time PCR assay, based on TaqMan® technology (Applied Biosystems) was  
149 carried out for the detection and quantification of the amount of *V. aestuarianus* DNA copies  
150 in the extract. This was based on amplification of part of the *dnaJ* gene, encoding heat shock

151 protein 40 (Saulnier et al. 2009). The primer pair dnaJ- F (5'  
152 GTATGAAATTTTAACTGACCCACAA 3') and dnaJ-R (5'  
153 CAATTTCTTTCGAACAACCAC 3') with the dnaJ-probe (5'  
154 TGGTAGCGCAGACTTCGGCGAC 3') was used. The reaction volume of 25 µL contained  
155 12.5 µL of Takara Premix Ex Taq™ 2X (Takara Bio Inc., Shiga, Japan), 0.5 µL of each  
156 primer (20 µM), 0.5 µL of probe (10 µM), 9 µL of DNA/nuclease-free water, and 2 µL of  
157 extracted DNA. The thermal cycling profile consisted of 95°C for 10 s followed by 40 cycles  
158 of 95°C for 5 s and 62°C for 30 s and Real-Time PCR was performed with a Smart Cycler®  
159 (Cepheid, USA).

160

#### 161 **Characterization of gametogenesis by histology**

162 **Histological assessment** included observation of **gender and** the stage of **gameteogenesis**. This  
163 **was done** on all triploid and diploid batches from hatcheries and on 1 **wild caught** diploid  
164 batch (control) **from** Meuvaines and Géfosse in 2015 and 2016, respectively. **Ten individual**  
165 **oysters per date and batch were randomly sampled every two months between March and**  
166 **November**. Transverse sections (5 mm) cut in the vicinity of gill-palp junction were fixed in  
167 Davidson's solution (48h; 10% glycerol, 20% formaldehyde, 30% ethanol (95%), 30% sterile  
168 sea water), dehydrated and embedded in paraffin wax. Sections of 5 µm were then prepared  
169 for subsequent treatments. One slide per sample was stained by the Prenant-Gabe trichrome  
170 method (Gabe, 1968). Stage was determined according to Heude Berthelin et al. 2001. A  
171 triploid classification (Jouaux et al. 2010) was also used to identify perturbed, beta, and non-  
172 perturbed, alpha, reproductive cells.

173

#### 174 **Statistical analysis**



175 ANOVA was performed to determine significant differences between sites in 2015 after log  
176 transformation of adult mortality rates. In 2016, raw data did not follow normal distribution  
177 and a Kruskal Wallis test therefore was employed. Student's t test after log transformation  
178 was performed to compare survival rates between triploid and diploid batches in 2015  
179 whereas a Wilcoxon Mann Whitney test was employed for data from 2016. A Wilcoxon  
180 Mann Whitney test was used to compare gametogenesis data.

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## 196 **RESULTS**

### 197 **Characterization of oyster batches**

198 In 2014, there was homogenous ploidy within a batch, except for batch 21, which was  
199 composed of diploid and triploid individuals in equal numbers.

200 During the second year, only 7 batches presented ploidy results in line with what was  
201 expected (5 triploid batches, 1 diploid hatchery and 1 diploid from **wild caught**). Among the  
202 heterogeneous batches, batch 21 **presented a majority of unexpected animals (i.e. 55% of**  
203 **diploids) similar to** the first year (Table 2).

204 In the third year, 3 batches out of 14 **had** the expected ploidy (2 triploid batches from hatchery  
205 A and 1 triploid batch from hatchery D) and for the first time a triploid individual was found  
206 in **the** control **wild caught** batch (batch 8).

207 In view of these results, all **wild caught** batches from Meuvaines and St Vaast-La-Hougue  
208 sites were analyzed by flow cytometry in December 2016 and March 2017, respectively.  
209 **Among** these 736 samples, 10 triploid individuals were found, including 6 individuals in  
210 batch 14 and 1 individual in **each of** batches 7, 8 and 24 from Arcachon and batches 14 and 17  
211 from Charente.

212 Overall in 2014 and 2016, **there were** abnormal results **for** 12.8% **of** 749 samples **of triploid**  
213 **hatchery stock**, 3.8% **of** 522 samples **of diploid hatchery stock** and 1.4% **of** 736 samples **of**  
214 wild stock. The proportion of inconsistencies was significantly different between triploid and  
215 diploid batches ( $p < 0.05$ ).

216 The hatcheries **did not** have the same **level of** inconsistency. Hatchery D **had** 100%  
217 homogeneity (130/130). Hatchery E was inconsistent, with 62.4% (93/149) of deviant  
218 animals.

219 Hatchery A produced 2 batches with the triploidy rate reaching 100.0%, as was expected  
220 (batch 1 **had** 170/170; **batch** 2 **had** 138/138) whereas batch 19 was inconsistent for 6/**139**  
221 individuals. This hatchery also provided 2 diploid batches (batches 3 and 18) that were  
222 heterogeneous, with 7.0% (9/129) and 4.7% (6/127) of abnormal results, respectively.

223 Hatchery B provided 2 batches of triploid animals (batches 4 and 25) with a rate of  
224 inconsistency of 2.3% (3/128) and 20.7% (31/150), respectively. The diploid batch contained  
225 3.1% (4/127) of **unexpected** animals.

226 Hatchery C provided 2 batches of triploid animals (batches 13 and 20) with a rate of  
227 inconsistency of 4.3% (6/139) and 1.2% (2/139), respectively. The diploid batch (12)  
228 contained 0.7% (1/139) of abnormal results.

229

230 Batches that contained **unexpected** animals were removed from the gametogenesis monitoring  
231 in order to have a true evaluation of gametogenesis according to ploidy. In the same way,  
232 batches 21 and 25 were removed from the mortality rate evaluation.

233

#### 234 **Mortality dynamics**

235 Mortality dynamics were monitored for all batches on the 3 sites (Figure 2) except **at**  
236 Meuvaines in 2016 **when some** bags of **oysters or identification tags** were lost **due to** weather  
237 conditions.

238 **From September to November** 2015 and **in August** 2016, abnormal mortalities (higher than  
239 20.0%) were observed **in some batches but only at the** Géfosse site. The mortality rates ranged  
240 from **15.0% to 55.0% in 2015 and from 18.0% to 54.0%** in 2016.

241 In 2015, triploid oysters presented a level of mortality **that was significantly** higher than  
242 diploids ( $P < 0.05$ ). In November, the mortality reached 43.0% for triploid oysters and 27.0%,  
243 for diploid oysters (Table 3).

244 In 2016, a similar significant difference was observed at the onset of **the** mortalities in August,  
245 **being 25.0% for** triploid batches **and only 12.0%** for the diploid batches ( **$P < 0.05$** ). In  
246 September, the recorded mortalities were on average 32.0% for triploids and 23.0% for  
247 diploids.

248 In case of mortalities, *V. aestuarianus* was systematically detected.  
249 Monthly seawater analysis showed that *V. aestuarianus* was present on sites from May, when  
250 mortality rates were less than 10.0% at all three sites. Pathogen loads reached  $2.3 \times 10^4$  UG/500  
251 mL in July on the sites of Meuvaines and G fosse **but** without triggering mortality **at** the  
252 Meuvaines site.

253

#### 254 **Histological monitoring of gametogenesis**

255 In 2015, gametogenesis was monitored on the Meuvaines site. As no mortality was recorded  
256 on this site, gametogenesis monitoring was conducted on the G fosse site in 2016.

257 In 2015 and 2016, in March, our control (batch 8) was mainly in stage 1 (90.0%) and  
258 therefore began its gametogenesis cycle (Figure 3). It should be noted that in 2015 the  
259 hatchery batches were delayed compared to the control, as only 55.0% of the diploid batches  
260 and 51.4 % of triploid batches were at this stage (Figure 3). This delay was not observed in  
261 2016; 87.5% of the hatchery diploid batches and 87.8% of triploid batches were at stage 1  
262 (Figure 3).

263 In May the control batch was in stage 2 (80.0% in 2015 and 77.8% in 2016). Unlike 2016, in  
264 2015, hatchery batches were slightly behind with only 65.0% in 2015 *vs* 84.2% in 2016 for  
265 diploid batches and 49.9% in 2015 *vs* 68.6 in 2016 for triploid batches **being** at this stage  
266 (Figure 3).

267 In July **in** both years, stage 3 was present in 100.0% of batch 8 animals (control). For diploid  
268 hatchery batches, this stage was present in 88.9% in 2015 and in 100.0% in 2016. A delay in  
269 the evolution of triploid gametogenesis when compared with diploid animals was also noticed  
270 in July 2015 (only 8.9% reached stage 3). This delay was less in 2016 (53.0% were in stage  
271 3). For both years, triploids were significantly less advanced than diploid animals (stage 3

272 rate,  $p < 0.01$ ). We observed that at this stage only 22.0% of the triploid animals were alpha in  
273 2016 when compared with 2015 (29.0%).

274 In September 2015, 50.0% of the control animals were at a resorption stage (stage 4), and the  
275 hatchery batches were late when compared with the control (batch 8) because only 36.9% of  
276 diploid batches and 11.4% of triploid batches were at this stage. In September 2016, all  
277 batches showed delayed gametogenesis: 100.0% of the control batch, 97.5% of hatchery  
278 diploid oysters and 52.4% of triploid oysters were in stage 3.

279 In November, the control batch was mainly in quiescence with 50.0% of the individuals at this  
280 stage in 2015 and 67.0% in 2016. In 2015, the majority of hatchery diploid and triploid  
281 individuals were at stage 0 with 62.5% and 58.6%, respectively, and like the control batch,  
282 began a new cycle, stage 1 (Figure 3). In 2016, the majority of diploid hatchery batches  
283 (68.7%) were also at stage 0. Only 42.5% of the triploid individuals were at this stage, but  
284 some individuals continued their cycle with a delay since individuals at stages 2, 3 and 4 were  
285 also observed.

286 Of all the triploid individuals whose cell blocking was identified, alpha individual ratio was  
287 significantly higher when compared with beta individuals with respectively 64.0% vs 36.0%  
288 in 2015 and 52.3% vs 47.6% in 2016. Moreover, the percentage of triploid  $\alpha$  animals in stage  
289 3 was 50.0% in 2015 and 42.0% in 2016.

290 In July and September 2016, the same batches were sampled from Meuvaines to assess the  
291 site impact on gametogenesis. For the control batch and diploid hatchery batches, a similar  
292 profile was observed at both sites on both sampling dates. In July, on Meuvaines, triploid  
293 batches had significantly more advanced gametogenesis than on G fosse ( $P < 0.05$ ). In  
294 September, the progress of gametogenesis was identical at both sites.

295 The sex ratio kinetics was monitored on two sites, Meuvaines in 2015 and Géfosse in 2016.  
296 Both diploid and triploid oysters showed high rates of sexuate individuals from May to  
297 September (Figure 4).

298 The percentage of sexually differentiated individuals was significantly higher in diploid  
299 oysters than in triploids, 63.5% vs 53.0% in 2015 ( $P < 0.05$ ) and 75.2% vs 52.0% in 2016  
300 ( $P < 0.005$ ) (Figure 4). In the two groups considered, males were predominant in 2015 (44.3%  
301 males vs 22.2% females in the diploid group and 43.5% males vs 9.6% females in the triploid  
302 group ( $P < 0.05$ )) (Figure 4). In 2016, little difference was observed (39.1% males vs 36.1%  
303 females in the diploids and 25.7% males vs 26.3% females in the triploids) (Figure 4).

304 The percentage of hermaphrodites was significantly higher in 2016 for triploids ( $P < 0.05$ )  
305 (1.3% (2/238) in the diploids vs 7.1 % (22/311) in the triploids) whereas in 2015 no difference  
306 was observed between diploids and triploids (0.8% (2/248) vs 2.9% (10/345), respectively).

307

308 In July and September 2016, the same batches were taken from Meuvaines to assess the site  
309 impact in sexually differentiated individuals. For the control and hatchery batches, the same  
310 profile was observed at both sampling dates.

311

## 312 **DISCUSSION**

313

### 314 **Characterization of batches**

315 In this study, some discrepancies between the expected and the observed ploidy for some  
316 batches were revealed. In 2014, these discrepancies were confined to batch 21. This low  
317 frequency of discrepancies measured in 2014 could be linked to the number of analyzes  
318 performed per batch (only 10 individuals), which may have been insufficient. In the second  
319 year of the study, more individuals per batch were studied and the discrepancies were brought

320 to light. The variation in the proportions of inconsistencies from one year to the next was the  
321 same, irrespective of the specified ploidy of the batch. The incoherence rates of the triploid,  
322 diploid hatchery and wild batches were 12.8% (749 samples), 3.8% (522 samples) and 1.4%  
323 (736 samples), respectively, with a more pronounced discrepancy in hatchery oysters  
324 ( $P < 0.05$ ).

325 Concerning triploid batches, these discrepancies could be explained either by a hatchery  
326 traceability problem, or by their manufacturing process. Numerous studies have shown that  
327 depending on the process employed, the success rate for triploid production could be random  
328 and not give 100.0% triploid production (Allen and Downing 1986, Yamamoto et al. 1988,  
329 Guo et al. 1992, Gerard et al. 1999). These discrepancies could be explained by a reversion  
330 mechanism where triploids revert to a diploid stage by a loss of chromosomes (Allen et al.  
331 1996, Allen et al. 1999).

332 For the hatchery diploids as with triploid batches, traceability could be a concern. A delay in  
333 hatcheries fertilization may also increase the risk of triploid. In this case, the polar body may  
334 not be expelled, which would induce a high triploid production rate (Glover et al. 2015).

335 On the wild oysters, 736 oysters were analyzed and 10 were incoherent, (1.4%). Although this  
336 number is surprising, American scientists reported a 1.3% risk of obtaining triploid animals  
337 after diploid *C. gigas* oyster crossing (Guo et al. 1992). This observation was also observed in  
338 fish farming due to an extended oocyte fertilization time. Surprisingly, the "biovigilance"  
339 network (French network providing quantifiable information on the presence of "triploid or  
340 tetraploid" polyploid oysters in areas where "natural" spat recruitment occurs) did not observe  
341 polyploid animals in the environment before 2014, when a triploid spat was first detected by  
342 this network (oral communication Benabdelmouna, 2014). Our observation and the 2014  
343 "biovigilance" campaign showed that triploid animals could occur naturally in the  
344 environment.

## 345 **Mortalities dynamics**

346 **Of** the three studied sites, only the G fosse site **had** abnormal mortality rates. Mass mortalities  
347 have been recorded **at** this site since 1994 (Goyard, 1996), which is described as a high  
348 carrying capacity ecosystem. **A**dult oyster losses **at** this site reached 35.0% in 1997 and 51%  
349 in 1998 (Fleury et al 1999). In our study, mortality was observed, **but** especially in adult  
350 triploids (55.0% in 2015 and 54.0% in 2016 for some batches).

351 Field studies on *C. gigas* summer mortality have been conducted in France for **many** years  
352 (Soletchnik et al. 1999, Royer et al. 2007) but few studies have compared the mortality  
353 between triploid and diploid oysters. **The** effect of ploidy on the mortality of adult oysters **has**  
354 **been** studied **only through** experimental infections and involved **only** small numbers **of**  
355 individuals (D gremont et al. 2014, Az ma et al. 2016).

356

357 G fosse is an estuarine site with regular rainfall. Studies have shown that triploid oysters were  
358 **underperforming** at low salinity sites when compared with all diploid lines. Their performance  
359 was similar to selected diploid lines at moderate salinity sites (Callam 2013). Low salinity  
360 may also influence the resistance of triploid oysters to mortalities. However, this would  
361 indicate that these triploids have adaptation problems when compared with diploids.

362

363 In the Morest project (Samain and Mc Combie 2008), abnormal mortalities were also  
364 observed at the G fosse site, where triploid oysters were more resistant than diploid oysters.  
365 The difference in mortality reported in this study was explained by reduced reproductive  
366 development that conferred resistance to summer mortalities. However, in our current study,  
367 certain batches of triploids reached an advanced **stage** of reproduction and some suffered  
368 mortalities in a similar way to diploids. Mortality outbreaks appeared when oysters were in  
369 gonadal maturation (Berthelin et al. 2000, Gagnaire et al. 2006) and increased mortality



370 occurred when the gonad was most extensively developed. The peak of mortality coincided  
371 with the spawning and post spawning periods when the gonadal volume began to decrease  
372 (Royer et al. 2007).

373 Gagnaire et al. 2006, showed that triploids presented the highest values for several hemocyte  
374 parameters and the lowest mortality rates, with differences between triploids and diploids at  
375 the highest during the ripe gamete period. In this study, we observed a converse situation,  
376 where triploid mortality rates were higher than diploid mortality rates ( $P < 0.05$ ), especially  
377 with animals at stage 3 of gonadal maturation.

378

379 The relationship between summer mortality and potential infection by pathogens has been  
380 previously established (Lipp et al. 1976, Garnier et al. 2007, Saulnier et al. 2010, Travers et  
381 al. 2017). *V. aestuarianus* has been detected in mortality outbreaks (Labreuche et al. 2006)  
382 but these bacteria have been more frequently identified since 2012 (François et al. 2013,  
383 François et al. 2014, François 2015). Recently, it has been reported that susceptibility to *V.*  
384 *aestuarianus* infection was low for oysters at the spat stage but increased with later life stages  
385 (Azéma et al. 2017).

386 In the current study, mortality was always associated with the detection of *V. aestuarianus* but  
387 the presence of the bacterium alone does not systematically cause mortality. It would appear  
388 that adult oyster infection by *V. aestuarianus* is not directly related to its presence in the  
389 environment but could also involve the animals' physiological state, especially during the  
390 final stage of gametogenesis.

391 Wadsworth (2018) has recently shown in the US that triploid oysters presented significantly  
392 higher mortality rate (site dependent) when compared with diploid oysters, highlighting the  
393 need to improve our knowledge about factors that trigger summer mortality in triploids.

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### 399 **Characterization of gametogenesis by histology**

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401 In comparison to diploid animals, gametogenesis in triploids was delayed but not absent  
402 (Allen and Downing 1990, Normand et al. 2008). We confirm this observation in our study. A  
403 delay in gametogenesis **in triploids** when compared with diploids was observed, with a  
404 significant difference in stage 3 levels of diploid and triploid batches ( $p < 0.01$ ).

405

406 Jouaux et al. (2010) observed that triploid oysters showed normal gametogenesis at early  
407 stages but not at later ones, resulting in an incomplete maturation process. Two types of  
408 gametogenic pattern were identified, named either alpha, for pacific oysters unblocked during  
409 their gametogenesis and able to produce a significant number of gametes or beta for  
410 individuals with blocked gametogenesis and producing few or no gametes. In our study, we  
411 observed an increase in **the proportion of** stage 3 alpha triploid oysters (50.0% in 2015 and  
412 42.0% in 2016) when compared with 25% in previous studies (Jouaux et al. 2010). Moreover  
413 we observed **in** 2015 a higher mortality differential between triploids and diploids than in  
414 2016. We believe that the alpha rate **may** have **had** an impact on mortality rate, because as  
415 indicated in the Morest project (Samain and Mc Combie 2008), diploid animals (with  
416 reproduction) are more sensitive to mortality contrary to triploids. All these data suggest that  
417 alpha animals suffered greater mortality when compared with none alpha animals, which are  
418 Beta animals.

419 This study showed that these triploid oysters had a hermaphroditism rate of less than 8.0%,  
420 which is low when compared with the rate previously reported by Jouaux et al, where 30% of

421 triploid animals were hermaphrodites (Jouaux et al. 2010). In *C. hongkongensis*, a low rate of  
422 hermaphroditism < 5% was also observed (Zhang et al 2016). The diploid batches always  
423 showed low rates of hermaphroditism (Jouaux et al. 2010). Hermaphrodites did not occur  
424 uniformly throughout the sampling season, with a higher rate when maturity reached its peak  
425 (Allen and Downing 1990) and our results confirm this observation.

426

427 In conclusion, we observed abnormal mortalities at one of three sites, and these were  
428 significantly higher for triploid animals (during the whole first year of study and only during  
429 the summer of the second year). Also, we noticed a high level of advanced gametogenesis in  
430 all triploid batches monitored in this study. Triploid oyster maturation occurred in September,  
431 which corresponded to high mortalities in Normandy.

432 In addition, we observed inconsistencies in relation to the expected ploidy in both hatchery  
433 and wild animals.

434 Our study seems to show that the new generation of triploid oysters (obtained with tetraploids  
435 patented in 2007) has a gametogenesis more and more similar to those of diploid oysters.

436

437

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FIGURE 1: *Three study sites in Normandy: Saint-Vaast-la-Hougue, Gêfosse-Fontenay, Meuvaines.*

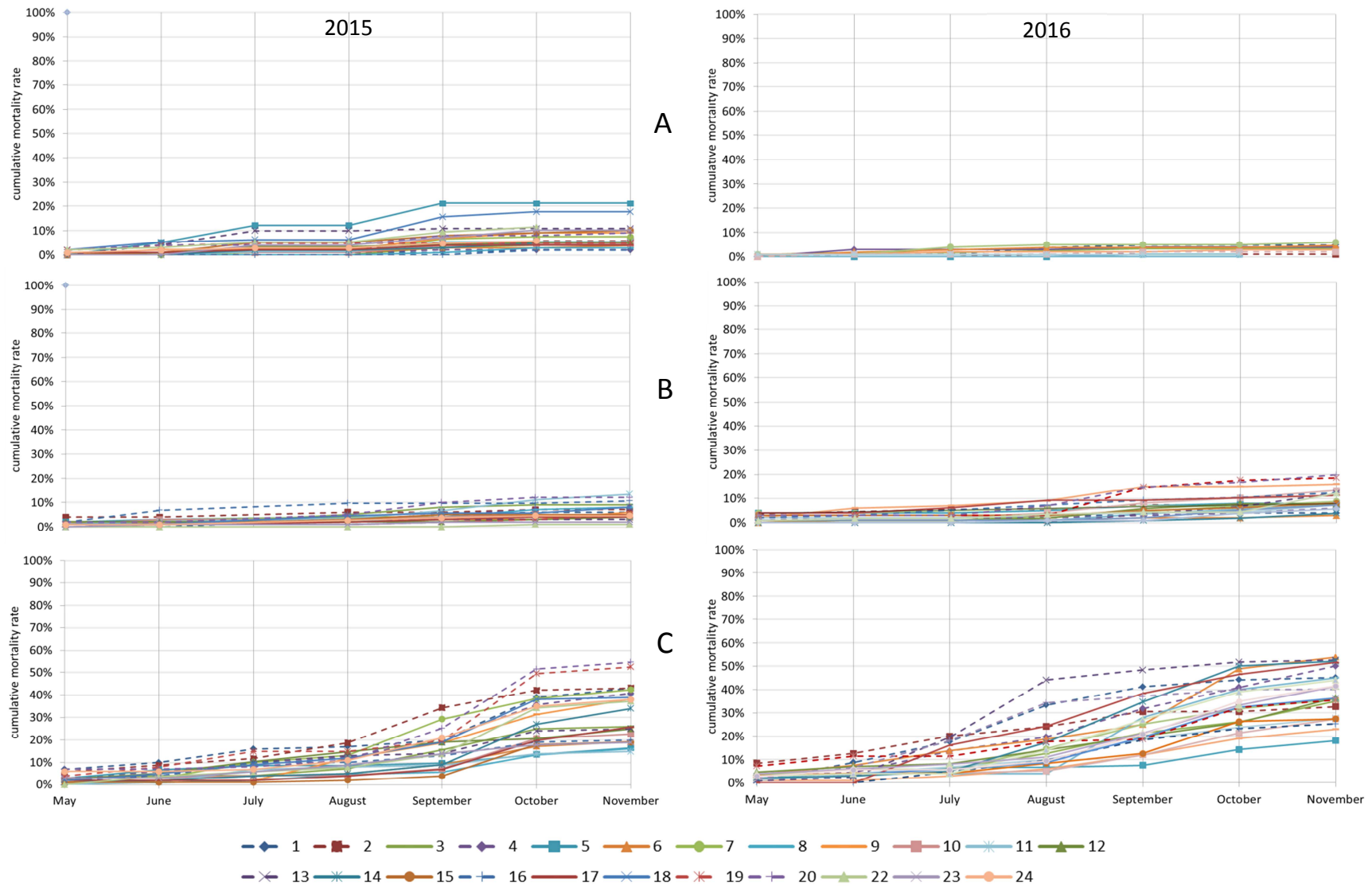


FIGURE 2: Trend of cumulative mortality of 23 batches in 2015 (left) and 2016 (right) on three sites: Meuvaines (A), Saint-Vaast-la-Hougue (B), G fosse (C). Triploid and diploid batches are respectively in dotted and solid line.

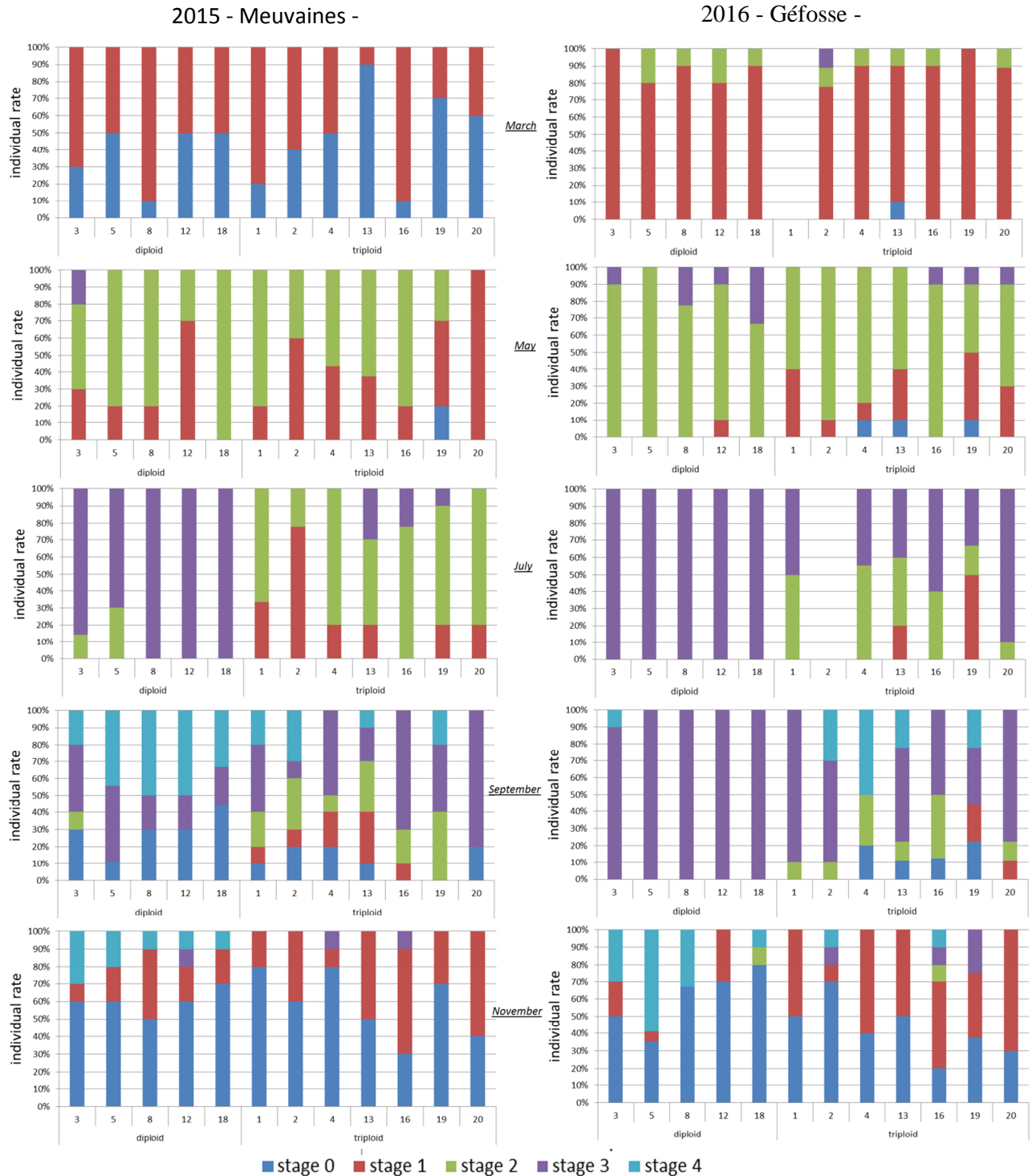


FIGURE 3: Histogram of gametogenesis stage of diploid and triploid batches in accordance with months in Meuvaines in 2015 (left column) and in Géfosse in 2016 (right column) on March, May, July, September, November. The X-axis corresponds to batches and the Y-axis to individual rates in stage 0 (quiescent stage), 1 (start of gametogenesis), 2 (differentiation of gamete), 3(rip stage), 4 (resorption stage).

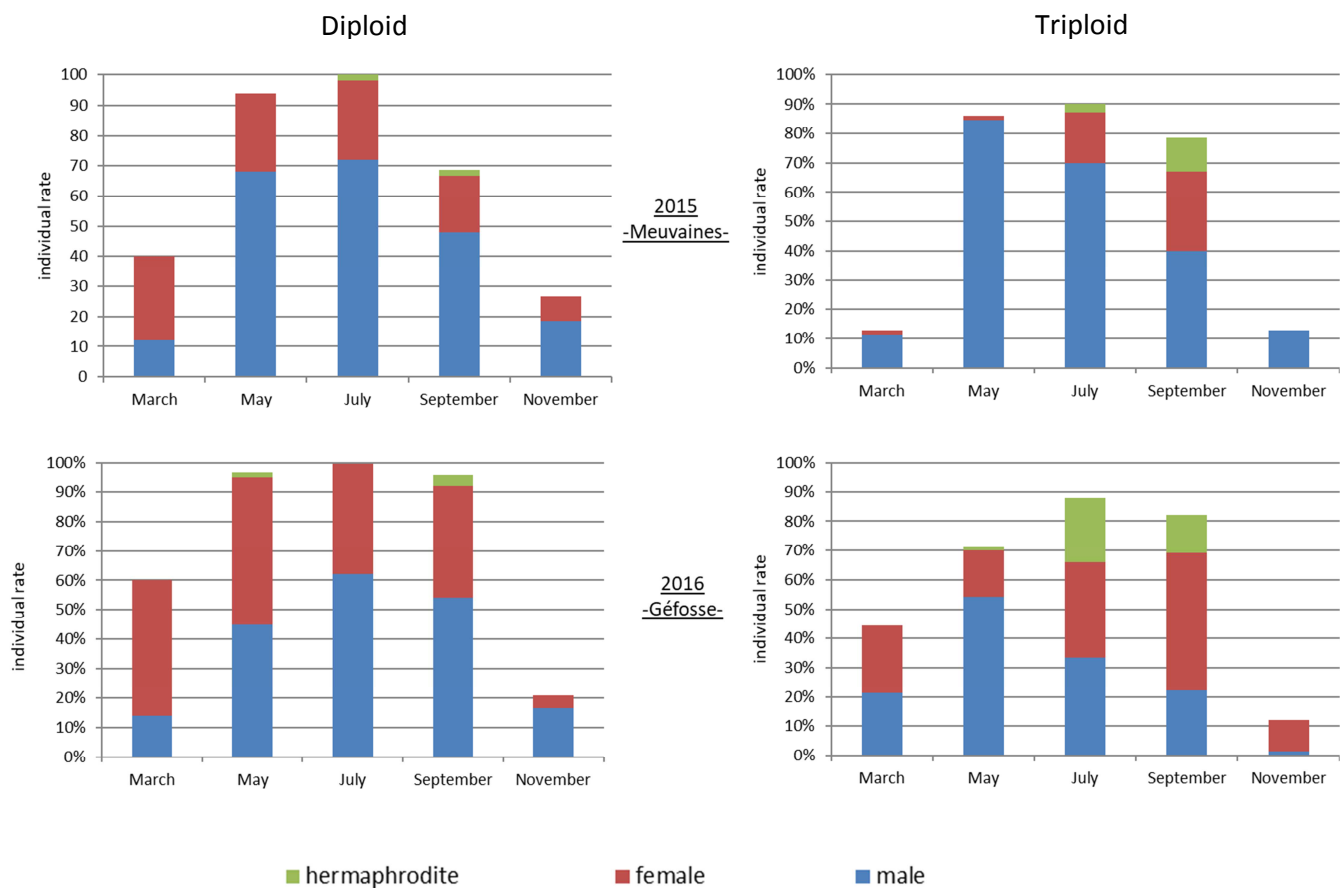


FIGURE 4: Percentage of males, females and hermaphrodites individuals in 2015 and 2016 in the diploid (left) and triploid (right) batches ( $n= 50$  individuals/diploid group/sampling point) and ( $n=70$  individuals/triploid group/sampling point).

TABLE 1: Characterization of 25 batches. (The gray lines correspond to the 14 batches chosen for the gametogenesis study)

Batches	Date of receipt	Ploidy	Origin	Date of laying
1	28 <sup>th</sup> January 2014	3N	Hatchery A	end of April 2013
2	28 <sup>th</sup> January 2014	3N	Hatchery A	end of July 2013
3	28 <sup>th</sup> January 2014	2N	Hatchery A	end of July 2013
4	29 <sup>th</sup> January 2014	3N	Hatchery B	No specified
5	29 <sup>th</sup> January 2014	2N	Hatchery B	26 <sup>th</sup> July 2013
6	10 <sup>th</sup> February 2014	2N	natural recruitment Arcachon (Mapouchet)	11 <sup>th</sup> August 2013
7	11 <sup>th</sup> February 2014	2N	natural recruitment Arcachon (Piraillan)	between 25 <sup>th</sup> July and 11 <sup>th</sup> August 2013
8	13 <sup>th</sup> March 2014	2N	natural recruitment Arcachon (Les Arrouillats)	between 25 <sup>th</sup> July and 11 <sup>th</sup> August 2013
9	20 <sup>th</sup> March 2014	2N	natural recruitment Cap Ferret (L'herbe)	between 25 <sup>th</sup> July and 11 <sup>th</sup> August 2013
10	20 <sup>th</sup> March 2014	2N	natural recruitment Arcachon (Arams)	between 25 <sup>th</sup> July and 11 <sup>th</sup> August 2013
11	20 <sup>th</sup> March 2014	2N	natural recruitment Cap Ferret (Belisaire)	between 25 <sup>th</sup> July and 11 <sup>th</sup> August 2013
12	9 <sup>th</sup> April 2014	2N	Hatchery C	15 <sup>th</sup> October 2013
13	9 <sup>th</sup> April 2014	3N	Hatchery C	10 <sup>th</sup> September 2013
14	14 <sup>th</sup> April 2014	2N	natural recruitment Charente Ile d'Aix	6 <sup>th</sup> August 2013
15	15 <sup>th</sup> April 2014	2N	natural recruitment Charente Les Longées	6 <sup>th</sup> August 2013
16	14 <sup>th</sup> April 2014	3N	Hatchery D	6 <sup>th</sup> August 2013
17	16 <sup>th</sup> April 2014	2N	natural recruitment Charente La Moulière	6 <sup>th</sup> August 2013
18	16 <sup>th</sup> April 2014	2N	Hatchery A	29 <sup>th</sup> August 2013
19	16 <sup>th</sup> April 2014	3N	Hatchery A	13 <sup>th</sup> September 2013
20	16 <sup>th</sup> April 2014	3N	Hatchery C	August 2013

21	16 <sup>th</sup> April 2014	3N	Hatchery E	No specified
22	16 <sup>th</sup> April 2014	2N	natural recruitment Charente	6 <sup>th</sup> August 2013
23	17 <sup>th</sup> April 2014	2N	natural recruitment Charente Fouras	6 <sup>th</sup> August 2013
24	18 <sup>th</sup> April 2014	2N	natural recruitment Cap Ferret	between 25 <sup>th</sup> July and 11 <sup>th</sup> August 2013
25	5 <sup>th</sup> December 2014	3N	Hatchery B	1 <sup>th</sup> January 2014



TABLE 2: *Percentage of incoherent individuals of batches monitored for the gametogenesis (all sites included).*

Batch	Ploidy	Hatchery	Percentage of incoherent individuals		
			2014	2015	2016
1	3N	A	x (0/10)	x (0/80)	x (0/80)
2	3N	A	x (0/10)	x (0/70)	x (0/58)
3	2N	A	x (0/10)	8% (4/50)	7.2% (5/69)
4	3N	B	x (0/10)	2% (1/50)	2.9% (2/68)
5	2N	B	x (0/10)	2% (1/50)	4.5% (3/67)
8	2N	recruitment	x (0/10)	x (0/48)	1.4% (1/69)
12	2N	C	x (0/10)	x (0/59)	1.4% (1/70)
13	3N	C	x (0/10)	6.6% (4/60)	2.9% (2/69)
16	3N	D	x (0/10)	x (0/50)	x (0/70)
18	2N	A	x (0/10)	2% (1/49)	7.3% (5/68)
19	3N	A	x (0/10)	x (0/70)	10.2% (6/59)
20	3N	C	x (0/10)	x (0/60)	2.9% (2/69)
21	3N	E	50% (5/10)	55% (33/60)	69.6% (55/79)
25	3N	B	x (0/10)	10% (6/60)	31.2% (25/80)

TABLE 3: Mean mortality rate,  $m$ , and standart deviation associated,  $SD$ , observed in Géfosse for triploid and diploid adults.

	Years	Triploids	Diploids	Significant difference
		m +/- SD	m +/- SD	p-value
May	2015	5 +/- 2%	2 +/- 1 %	0.009
	2016	3 +/- 3%	3 +/- 1 %	>0.05
June	2015	7 +/- 2%	3 +/- 2 %	0.003
	2016	4 +/- 2%	7 +/- 5 %	>0.05
July	2015	11 +/-3%	5 +/- 3 %	0.002
	2016	15 +/- 5%	7 +/- 4 %	0.005
August	2015	14 +/- 3%	8 +/- 4%	0.003
	2016	25 +/- 12%	12 +/- 5%	0.003
September	2015	21 +/- 7%	13 +/- 7%	0.025
	2016	32 +/- 11%	23 +/- 8%	>0.05
October	2015	37 +/- 12%	24 +/- 9%	0.015
	2016	34 +/- 9%	34 +/- 11%	>0.05
November	2015	43 +/- 14%	27 +/- 9%	0.003
	2016	39 +/- 10%	36 +/- 11%	>0.05