

1 **Inheritance and temporal plasticity of simultaneous hermaphroditism in the Pacific**
2 **oyster *Crassostrea gigas***

3

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13 **Running title: Sex-ratio in *Crassostrea gigas***

14

15 **Abstract**

16 The Pacific oyster, *Crassostrea gigas*, is commonly described a protandrous alternative
17 hermaphroditic species in which simultaneous hermaphrodites are rarely observed. However,
18 its sex determinism is still debated and remains unclear. Genetic sex determinism has been
19 investigated in bi-parental progenies, i.e. offspring obtained by crossing one male and one
20 female oyster. This makes difficult to interpret the results, as it is not possible to distinguish
21 whether the parents are genetically male and female, or sequential hermaphrodites. This study
22 reports the sex ratio at 14 and 15-month-old in the progeny of a simultaneous hermaphrodite
23 oyster. SNP genotyping confirmed that they were produced by selfing. In addition, sex ratio
24 was also recorded in bi-parental control progenies, produced by mating two parents (i.e. one
25 female and one male). When 14-month old, oysters produced by selfing were mainly males
26 (98.8%) while sex ratio of control oysters was close to 1:1. Most interestingly, when 15-month
27 old, 70% of the oysters produced by selfing had or were undergoing a sex change between May
28 and June, and 68.8% of the oysters were simultaneous hermaphrodites suggesting inheritance
29 of this reproductive trait, but also its temporal plasticity. In contrast, control progenies showed
30 less than 1% of simultaneous hermaphrodites, as usually reported in *C. gigas*. To our
31 knowledge, this is the first report of such high proportion of simultaneous hermaphrodites in
32 *C. gigas* and of the inheritance of this trait. Our results open new perspectives in the production
33 of inbred lines of Pacific oysters by self-fertilization through successive generations of selfing.

34

35 *Keywords:* sex-ratio; self-fertilization; inheritance; hermaphroditism; Pacific oyster;

36 *Crassostrea gigas*

37

38 1. Introduction

39

40 The Pacific oyster, *Crassostrea gigas*, is a species major aquaculture interest worldwide. To
41 date, selective breeding mainly focused on disease resistance (Azéma et al., 2017; Camara et al.,
42 2017; Divilov et al., 2019), growth (Gomes et al., 2018; Wang et al., 2012) and triploids (Ibarra et
43 al., 2017; Wang et al., 2002), shell coloration (Ge et al., 2015; Xu et al., 2017) and yield (Dégremont
44 et al., 2005; Langdon et al., 2003). Significant genetic improvement can also be gained through
45 the production of triploid seed (Dégremont et al., 2012; Hand et al., 2004). Production of
46 hatchery seed relies on the control of successive steps of reproduction: gametogenesis of
47 broodstock, fertilization, larval rearing, settlement and early growth of juveniles. The first step
48 for breeding relies on sex ratio in the conditioned broodstock. Surprisingly, sex of parental
49 individuals is still uncontrolled in *C. gigas* hatcheries.

50 To date, sex determinism in *C. gigas* is supposed to be controlled genetically and influenced
51 by environmental factors (Guo et al., 1998). Meanwhile, few studies have investigated sex-ratio
52 and sex change over time in this species. In contrast to *C. virginica* where protandry is obvious
53 (Haley, 1977; Needler, 1942), Normand et al. (2009) reported female individuals in 5-month-old
54 oysters (i.e. first gametogenesis). Broquard et al. (2020) also reported that (1) most studied
55 hatchery-produced oysters of one large *C. gigas* population were not protandric, (2) sex-ratio
56 was biased toward females each year, (3) sex change mainly occurs once or twice during the
57 first six years, (4) sex change decreases in older oysters, and (5) that 42% of the individuals
58 did not change sex after six years. Furthermore, Broquard et al. (2020) also suggest that all
59 oysters can become sequential hermaphrodites throughout their life. Beside sequential
60 hermaphrodites, simultaneous hermaphrodites are also observed, but those are scarce, usually
61 less than 2% in diploid *C. gigas* (Broquard et al., 2020; Guo et al., 1998; Yasuoka and Yusa, 2016).

62 Simultaneous hermaphrodites are however more frequently observed in triploid Pacific oysters
63 (Allen and Downing, 1990; Guo and Allen, 1994; Ibarra et al., 2017; Jeung et al., 2016;
64 Normand et al., 2009).

65 Simultaneous hermaphroditism is potential interest to study the genetic determinism of sex
66 ratio in *C. gigas*. To date, genetic determinism of sex ratio has been investigated in biparental-
67 families (Guo et al., 1998). The fact that one or both of the parents might be successive
68 hermaphrodites complexifies these studies. Thus, the analysis of female/male ratios based on
69 a single-locus model for sex determination in the Pacific oysters with two (Guo et al., 1998) or
70 three genotypes (Hedrick and Hedgecock, 2010) could be more difficult to interpret as done by
71 Baghurst and Mitchell (2002). Lately, we reported the possibility to produce oyster families by
72 self-fertilization in *C. gigas* over two successive generations (Dégremont et al., 2022). Animals
73 were viable beyond the larval and spat stages, as reported by Hedgecock et al. (1995), while
74 previous studies based on cryopreserved sperms were unsuccessful in *C. gigas* (Lannan, 1971)
75 and in *C. virginica* (Yang et al., 2015). Sex ratio in the progeny of self-fertilized oysters remained
76 to be further investigated, following the report of 3 hermaphroditic oysters out of 6 individuals
77 (Dégremont et al., 2022).

78 In this study, we report the sex ratio in one family of *C. gigas* produced by self-fertilization.
79 As a control, sex ratio was also recorded in bi-parental. The aim of our study is to determine if
80 oysters produced by selfing of a synchronous hermaphrodite could present a higher frequency
81 of simultaneous hermaphrodites, suggesting a significant inheritance of this reproductive trait
82 in *C. gigas*.

83 2. Materials and methods

84 2.1. Oysters produced by self-fertilization

85 The oysters produced by self-fertilization originated from one female and one grand-parental
86 sampled in 2012 from a wild population located in the Marennes-Oléron Bay, France (Fig. 1).
87 This family, named H4, was produced in March 2013. To produce a new generation, this family
88 was maintained in Ifremer's facilities located in La Tremblade and Bouin. Full-sib oysters of
89 this H4 family were placed in 240 L conditioning tank in La Tremblade in January 2016, and
90 seawater temperature was gradually increased to 21 °C during 1 week. Seawater flow was 400
91 L/h, and a cultured phytoplankton diet (*Isochrysis galbana*, *Tetraselmis suecica*, and
92 *Skeletonema costatum*) was provided ad libitum (50,000 cells/mL). A mass spawning event
93 induced by heat shock was performed on March 2016 using 17 full-sib oysters. The sex ratio
94 of broodstock was not recorded (Fig.1), meaning that at least one female and one male
95 spawned, but simultaneous hermaphrodites cannot be excluded. The progenies were again
96 always kept in Ifremer facilities in La Tremblade and in Bouin. In order to produce the new
97 generation, ten oysters were conditioned for reproduction from January to March 2018. Four
98 oysters were opened in March 2018, and sexed by microscopic observation of gonad samples
99 spread on a slide. Among them, one was found to be a simultaneous hermaphrodite (named
100 SH1). Gametes were collected by stripping the gonad, and then diluted in 1L beaker for 5 min.
101 The gametes were sieved on 100- μ m and then 20- μ m mesh screens to remove large and small
102 tissue debris, respectively, including the sperm. Unfertilized and fertilized eggs retained on the
103 20- μ m mesh screen were transferred in a 30 L tank at 25°C using UV-treated (40 mj/cm²) and
104 filtered seawater (5 μ m). Larval rearing was performed in Ifremer hatchery in La Tremblade,
105 and settlement lasted from day 14 post-fertilization to day 23. Around 1000 spat (5 mm) were
106 transferred to Ifremer nursery in Bouin on May 2018 until June 2019, and then the number of

107 oysters was reduced to 500 oysters in September 2018. This family produced by fertilization is
108 named hereafter H4-self.

109

110 *2.2. Control oysters produced by crossing one female by one male*

111 From the same broodstock described above, one full-sib family (named H4fm) was produced
112 by mating one female with one male in March 2018. In addition, six other groups (named A to
113 F) were produced; two of them were full-sib families, while the others were a mix of three
114 independent full-sib families each (i.e. three females and three males used, each female mated
115 with one male). All oyster groups had the same life-history than H4-self, from fertilization to
116 sampling for sex determination in May 2019. Finally, six other groups of hatchery-produced
117 oysters from mass spawning events from 2015 to 2018 (named I to N), were maintained at the
118 Ifremer facilities in Bouin for at least one year, and were sampled for sex determination in June
119 2019.

120

121 *2.3. Sex determination*

122 *2.3.1. Sex determination by biopsy*

123 In May 2019, sex determination was controlled by opening 10 to 42 oysters for H4mf, H4-self,
124 and groups A to F (Table 1). For each oyster, gonad tissue was spread on a slide and check by
125 microscopic observation (x40) (Table 1). In addition, 214 H4-self oysters were sexed by biopsy
126 as described in Broquard et al. (2020). Briefly, oysters were placed in a 5-L tray with a muscle
127 relaxant solution consisting of seawater (3/5), freshwater (2/5), and magnesium chloride (50
128 g/L). As soon as the shells opened, a smear of gonad was taken using a needle (0.9×38 mm;
129 Terumo©) and a 1-mL syringe (Terumo©). Gametes were visualized microscopically, and
130 oysters with oocytes were identified as females and those with spermatozoa were classified as

131 males. Then, all H4-self biopsied oysters were individually tagged (Pit-tag, Biolog-ID, Bernay,
132 France) in order to follow their sex the following month. The mean individual total weight of
133 the H4-self biopsied oysters was 19.5 ± 5.6 g in May 2019.

134

135 2.3.2. Sex determination by histology

136 In June 2019, all H4-self oysters had to be sacrificed and stored in ethanol for DNA analysis,
137 and in Davidson's fluid for sex determination by histology, as oysters were fully riped at this
138 period of the year. They were shorted by sex recorded by biopsy one month before. All females,
139 (i.e. only 2 individuals), and 18 males were individually sampled for DNA analyses and then
140 fixed individually in Davidson's fluid. The other males were sampled for DNA analyses, and
141 fixed in Davidson's fluid, but it cannot be possible to individually connect a DNA analyses to
142 a sex determined subsequently by histology. Two transverse cross-sections were done for 189
143 oysters to determine if gametes and their distribution were similar across the gonad. Only one
144 transverse cross-section was done for the remaining oysters due to their smaller size. All cross-
145 sections were dehydrated and embedded in paraffin for histology according to standard
146 procedures. Sections of 2–3 μm thickness were stained by hematoxylin and eosin. Slides were
147 then observed with a microscope, and one to three pictures of each slide were recorded. Oysters
148 with oogonies/oocytes were identified as females, those with spermatogonies/spermatozoa
149 were as males, and oysters with both as simultaneous hermaphrodites. In addition, sex
150 determination was also performed by histology for 111 oysters from groups B, C, I, K, K, M
151 and N (Table 1). Finally, for 130 of the simultaneous hermaphrodites of H4-self, the percentage
152 of area showing either undetermined, male and female gametes where obtain from a crop of
153 each picture to maximise the gonad area using Fiji (Schindelin et al., 2012).

154

155 *2.4. Genetic confirmation of self-fertilized oysters*

156 We used a SNP-genotyping array (Illumina Infinium) developed by Lapègue et al. (2014) and
157 226 markers were successfully genotyped. The list of markers is provided in supplementary
158 Table 1. DNA extraction and genotyping were performed from a piece of gill by Labogena
159 (Jouy-en-Josas, France). For the parent of the H4-self oysters, DNA extraction and SNP-
160 genotyping were done in duplicates. Among the H4-self oysters, 44 were genotyped including
161 20 sampled for both DNA analyses and histology. Besides, 434 oysters (325 progenies and 109
162 parents) from other germplasms were also genotyped and included for the parental assignment.
163 Parentage assignment were determined using Cervus 3.0.7, as well as summary statistics for
164 the H4-self oysters (Kalinowski et al., 2007). The frequencies of genotypes were examined using
165 the Proc Allele in SAS genetics (SAS Institute Inc., 2013).

166

167 **3. Results**

168 *3.1. Parentage analyses*

169 The genotypes of SH1 and the 44 H4-self oysters are provided in supplementary Table 2.
170 Among the 226 SNP, 6 failed, 174 were monomorphic and 46 were polymorphic
171 (Supplementary Table 3). The mean number of alleles per locus was 1.177, the mean expected
172 heterozygosity was 0.101 and the mean polymorphic information content was 0.075. For the
173 polymorphic SNP, the mean expected heterozygosity should be 0.5 while the mean observed
174 heterozygosity was 0.817 (Supplementary Table 3). All H4-self oysters were unambiguously
175 assigned to SH1 as parent, confirming that they were produced by selfing of this oyster
176 (Supplementary Table 4).

177

178 3.2. *Sex checked by biopsy or sacrifice*

179 In May 2019, only three females were observed among the H4-self oysters, while there were
180 241 males. Thus, sex ratio of H4-self oysters was strongly skewed toward males (98.8%) with
181 98.6% and 100% when checked by biopsy or sacrificed, respectively (Table 1). In a lesser
182 extent, H4fm oysters, produced from two parents, showed also a higher proportion of males
183 with 61.9% (Table1). For the other groups A to F, sex ratio was close to 1:1, and ranged from
184 40.0 to 53.3% of females (Table 1).

185

186 3.3. *Sex observed by histology*

187 H4-self oysters sexed by biopsy in May 2019 were all sacrificed in June. Their sex was checked
188 by histology, except for one male and one female that died in between. For all the 189 H4-self
189 individuals that were checked using two transverse cross-sections, sex was identical between
190 the two sections (Supplementary Table 5). Among the 212 H4-self oysters, only one did not
191 show gametes while sex was identified for 211 oysters (Table 1). The two oysters that were
192 females in May still showed the same sex in June, and four males in May undergo a sex change
193 and were females in June. Nevertheless, the overall percentage of females in H4-self remained
194 low in June (2.8%). In contrast, the percentage of males decreased from 98.6 in May to 28.9%
195 in June. This is because 68.2% of the oysters became simultaneous hermaphrodites (Table 1).
196 In total, 148 H4-self oysters had or were undergoing a sex change between May and June,
197 representing 70% of this progeny. While it was not possible to obtain data on eventual sex
198 change over time for oyster produced from two parents, sex in groups B to N was 40.5% of
199 females, 58.6% of males and only 0.9% of simultaneous hermaphrodites (Table 1). High
200 variation for sex was observed among stocks from 8.3 to 83.3% of females, and only the stock
201 C had one simultaneous hermaphrodite (Table 1).

202

203 *3.4. Gonad of simultaneous hermaphrodite*

204 In June 2019, the simultaneous hermaphrodites were functional hermaphrodites, i.e. showing
205 mature gametes of both sexes. However, they showed variable levels of hermaphroditism,
206 ranging from a very low to a high percentage of gonad area showing mature oocytes (Fig.2
207 panel A to panel F, respectively). The percentages of indeterminate, male and female gamete
208 areas of each simultaneous hermaphrodite are shown in Supplementary Table 6. In summary,
209 considering the percentage of surface area presenting male and female gametes, 5% of
210 simultaneous hermaphrodites presented less than 25% of gonadal surface area with male
211 gametes, 12% presented 25-50%, 42% presented 50-75%, and 41% presented more than 75%
212 (Table 2).

213

214 **4. Discussion**

215

216 This study aimed to investigate, for the first time to our knowledge, the sex ratio in a progeny
217 of Pacific oysters produced by self-fertilization. This is the third report on the production of
218 oysters by selfing in *C. gigas*. Indeed, such oysters are less common and have been bred in
219 only two previous studies in *C. gigas*, the first by Hedgecock et al. (1995), confirming the
220 pedigree using allozyme markers, and more recently by Dégremont et al. (2022), checking the
221 pedigree using SNP markers, and who managed to produce two successive generations by
222 selfing. However, neither of these two studies examined in details sex ratio and sex change in
223 the selfed progeny. Hedgecock et al. (1995) produced a new generation of bi-parental inbred
224 families and reported females and males, but no simultaneous hermaphrodites. Meanwhile,
225 Dégremont et al. (2022) reported three simultaneous hermaphrodites among six siblings

226 produced by selfing, suggesting inheritance of this reproductive trait, although the number of
227 studied oysters was very low. It was therefore worth investigating sex determination in oysters
228 produced by selfing. In our study, the oysters were 14 months old when sex was determined
229 and were in the nursery system for one year. Sex ratio and sex change were recorded for 244
230 oysters produced by selfing, which gives more reliable data.

231 Genetic models for sex determination first proposed in *C. gigas* a single major gene: a 2-
232 genotype model, with a dominant male allele (M), and a protandric female allele (F) and two
233 genotypes, FM oysters being true males, and FF oysters being protandric females capable of
234 sex change (Guo et al., 1998). A second single-gene model proposed 3-genotypes : FF and MM
235 oysters being true females and males, respectively, whereas FM are protandric oysters (Hedrick
236 and Hedgecock, 2010). These models did not include simultaneous hermaphrodites. By
237 recording the sex of oysters produced by selfing, only one parent was used and its genotype
238 could be assumed as FF for the 2-genotype model or FM for the 3-genotype model. While for
239 the former all progenies should be FF, as so potentially hermaphrodites (sequential and/or
240 simultaneous), the latter questioned about the FF genotypes which should be 25% of true
241 females in the oysters produced by selfing, while it was only 1.8% in May. In addition, the 3-
242 genotype model impose 50% of FM, that is to say hermaphrodite during the entire lifespan of
243 *C. gigas*, whereas in our study, 70% of the H4-self oysters were already identified as sequential
244 or simultaneous hermaphrodite when 15 month-old. Unfortunately, it was not possible to
245 follow the H4-self oysters during the subsequent years, which would have provided valuable
246 data on further sex changes. Nevertheless, this indicates either most of FF and MM oysters
247 died during the early stage due to deleterious genes, or that 3-genotype model is not valid for
248 genetic sex determinism in *C. gigas*.

249

250 Our second finding was a very high proportion of males (98.8%) in May 2019 for the oyster
251 produced by selfing (H4-self) in comparison to H4fm oysters produced from two parents using
252 the same germplasm (61.9%) or to other groups (A to F, ranging from 46.7% to 60%) (Table
253 1). Caution should be taken when comparing the sex ratio among groups due to the relative
254 number of oysters sexed for groups A to F (10-30). Meanwhile, the family of oysters produced
255 by selfing first showed a very high and infrequent sex ratio skewed toward males. Surprisingly,
256 comparison of sex ratio between families are scarce in *C. gigas* as many studies focused on
257 wild populations or hatchery-produced oysters (Baghurst and Mitchell, 2002; Buroker, 1983;
258 Fabioux et al., 2005; Ibarra et al., 2017; Lango-Reynoso et al., 1999; Park et al., 2012; Santerre
259 et al., 2013; Yasuoka and Yusa, 2016). To our knowledge, sex-ratio of families in *C. gigas*
260 have only been investigated in two studies, which showed high variation underlying the
261 evidence for genetic determination (Guo et al., 1998; Hedgecock, 2023). Among the families
262 studied by Guo et al. (1998), the highest frequency of one-year-old males was 90%, but relies
263 on relative low numbers of oysters sexed in this family (n=20), leading to limited precision.
264 This result is the closest reported in the literature for male-skewed sex ratio to ours (close to
265 99% in 244 oysters) in *C. gigas* which could be in agreement with protandric dioecy as reported
266 by Guo et al. (1998). Nevertheless, protandry is called into question by data obtained from
267 crosses using two parents, with a balanced sex ratio, as recently demonstrated by Broquard et
268 al. (2020).

269

270 The third main results concern the frequency of simultaneous hermaphrodite observed just one
271 month later in oysters produced by selfing, reaching 68.2%, while it was less than 1% for
272 control oysters produced by crossing males and females (Table 1). To our knowledge, this is
273 highest value ever recorded and by far, as simultaneous hermaphroditism in diploid Pacific
274 oyster is generally considered a rare occurrence, with environmental and genetic factors

275 influencing its expression. Indeed, studies usually report less than 2% of simultaneous
276 hermaphrodites in diploid *C. gigas* (Amemiya, 1929; Broquard et al., 2020; Guo et al., 1998;
277 Normand et al., 2009; Steele and Mulcahy, 1999; Yasuoka and Yusa, 2016), and one study
278 reports around 10% of simultaneous hermaphrodites in diploid *C. gigas* (Ibarra et al., 2017). It
279 is likely that oysters recorded in these studies were produced from two parents, and not by
280 selfing, even if this possibility could not be excluded. In triploid *C. gigas*, all produced from
281 two parents, higher proportion of simultaneous hermaphrodites, are usually reported with
282 values ranging from 10-35% (Allen and Downing, 1990; Guo and Allen, 1994; Ibarra et al.,
283 2017; Normand et al., 2009). This high value of simultaneous hermaphrodites is the result from
284 genetic abnormalities due to the third set of chromosomes as described by Guo et al. (1998),
285 but remains twice as low than in our study. Consequently, oysters produced by selfing are
286 more prompt to present simultaneously both gamete males and females during a part of their
287 life, suggesting an inheritance for this reproductive characteristic, which is in agreement with
288 the finding observed in only six oysters by Dégremont et al. (2022).

289
290 The speed of gametogenesis in *Crassostrea gigas* appears to be accelerated in oysters produced
291 by self-fertilization compared to those generated through crossing males and females as 68.2%
292 of the H4-self oysters were undergoing a sex change in only one month, with the development
293 of oocytes between May and June. *C. gigas* is usually described as a sequential hermaphrodite
294 meaning that sex change is observed between two consecutive reproductive periods (Broquard
295 et al., 2020; Park et al., 2012). This rapid change of gametogenic tissues could result from
296 compensatory mechanisms in response to the genetic challenges imposed by selfing. The high
297 proportion of simultaneous hermaphrodites observed in H4-self oysters suggests a potential
298 adaptive response or consequence of inbreeding, and may indicate a disruption in the sex-
299 determining pathways, possibly due to increased homozygosity resulting from selfing. Even if

300 the mean observed heterozygosity was higher than expected (0.817 vs 0.5) for the informative
301 SNPs (Supplementary Table 3), homozygosity increased faster by selfing than crossing sibling,
302 which could affect the expression of genes involved in sex determination at the certain level of
303 inbreeding, leading to the simultaneous presence of both male and female reproductive tissues.
304 We hypothesize that the increase of simultaneous hermaphrodite in selfed oysters might reflect
305 an adaptive strategy to overcome the potential fitness costs associated with selfing, such as
306 reduced viability or lower growth rates, mainly observed during the larval stage due to the
307 genetic load in *C. gigas* (Launey and Hedgecock, 2001; Plough, 2018). By accelerating the
308 reproductive cycle, oysters produced by selfing could potentially increase the probability of
309 successful fertilization events, mainly with neighbouring individuals that are not simultaneous
310 hermaphrodites, which are much more common in oyster populations, thus compensating for
311 any reduction in offspring fitness, and explaining why simultaneous hermaphrodite remains
312 scarce. Further investigation into the molecular and environmental factors influencing
313 gametogenesis in selfed *C. gigas* could provide valuable insights into the evolutionary
314 strategies employed by these organisms under inbreeding conditions.

315

316 Finally, all simultaneous hermaphrodites observed for the H4-self oysters in June 2019 began
317 gametogenesis as males, as observed one month earlier, and had rapid gametogenesis to
318 produce mature oocytes. Most of the simultaneous hermaphrodites still showed a higher
319 tendency toward males (Table 2), as reported by Dégremont et al. (2022). We observed around
320 5% of simultaneous hermaphrodites with a higher tendency towards females (>75% of the
321 gonad area). It is likely that this proportion would have increased in subsequent weeks, and in
322 the same way, would have increased the percentage of females and thus decreased the
323 percentage of simultaneous hermaphrodite for the H4-self family. According to our experience,
324 it might have been possible to produce a new generation of oysters by selfing using the H4-self

325 oysters in June 2019, with a higher fecundity for those showing a tendency towards females.
326 Such strategy would be useful to produce standardized highly inbred lines that are of
327 importance for genetic studies, such as mapping mutations (Allen et al., 2013; Doitsidou et al.,
328 2016; Smith et al., 2016), experiments and epigenetic studies based on using isogenic lines (see
329 for example in rainbow trout: (Lallias et al., 2021; Pouil et al., 2023)).

330

331

332 In conclusion, our study of sex ratio in *C. gigas* produced by selfing revealed a strikingly high
333 proportion of males, with 99% of the population exhibiting male phenotypes. More
334 importantly, a significant portion of these oysters (68%), were identified as simultaneous
335 hermaphrodites one month later, revealing temporal plasticity and suggesting a potential
336 genetic basis for this trait. These findings underscore the complexity of sex determination in
337 *C. gigas* and point to a genetic mechanism that may govern the development of sexual
338 phenotypes in this species. However, the precise genetic factors and their interactions remain
339 poorly understood. Therefore, further studies are essential to unravel the genetic determinants
340 of sex in *C. gigas*, which could have significant implications for aquaculture practices and the
341 conservation of oyster populations.

342

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348

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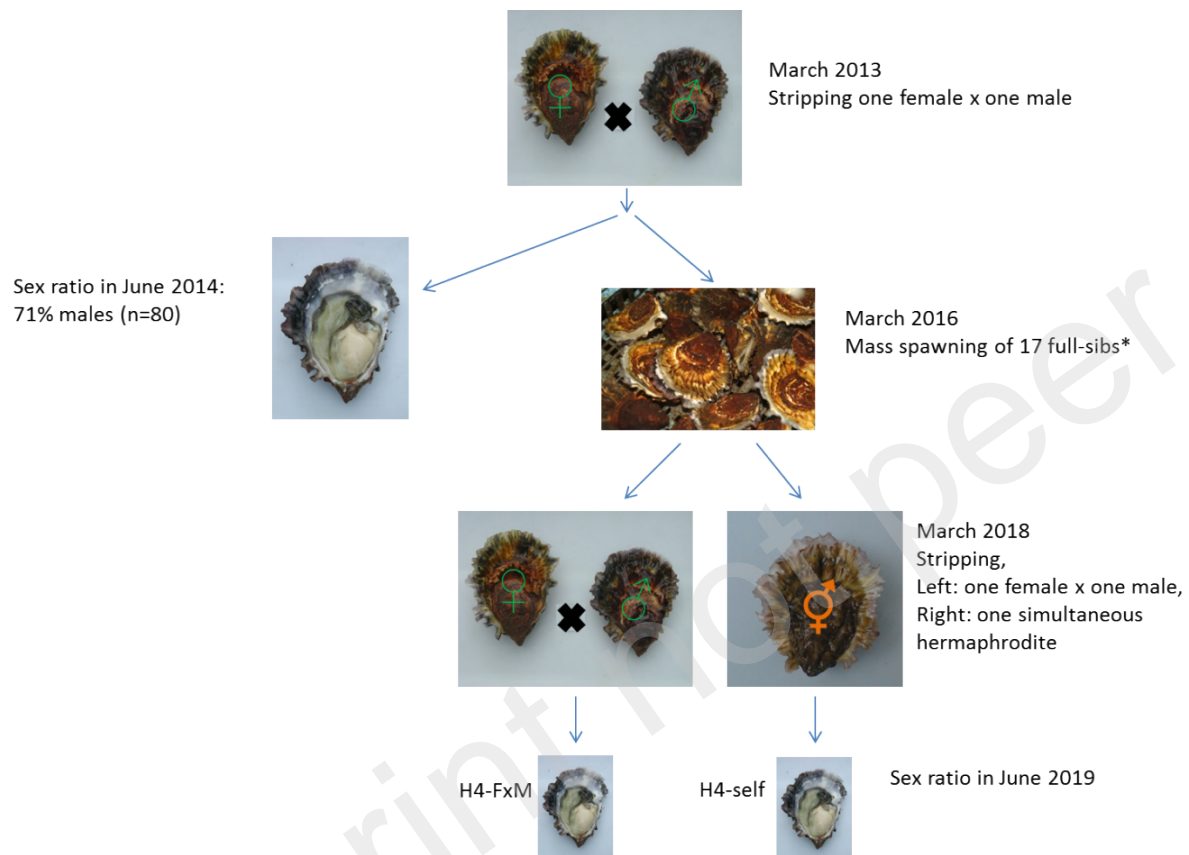


Figure 1 Pedigree of the family H4-self produced by self-fertilization in March 2018 (* no data on sex ratio recorded)

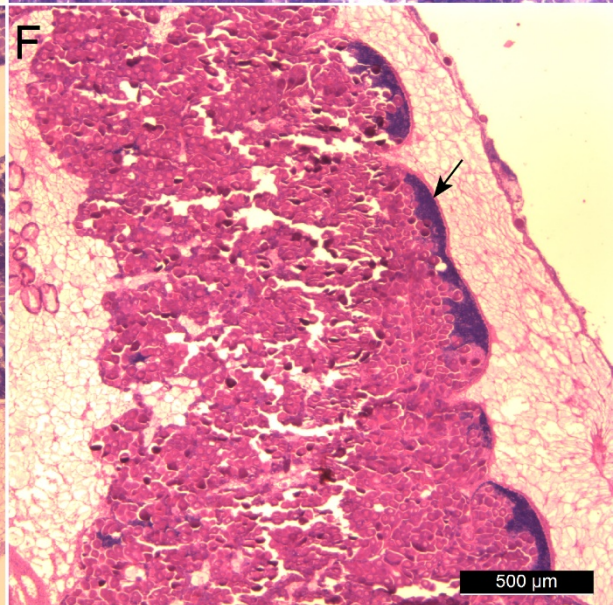
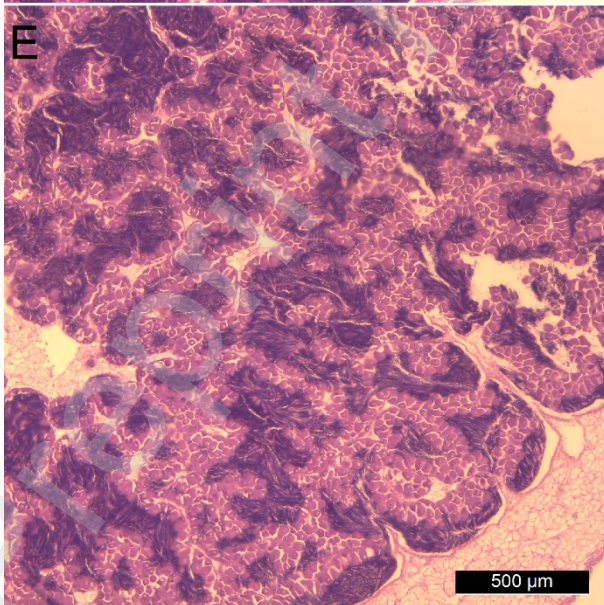
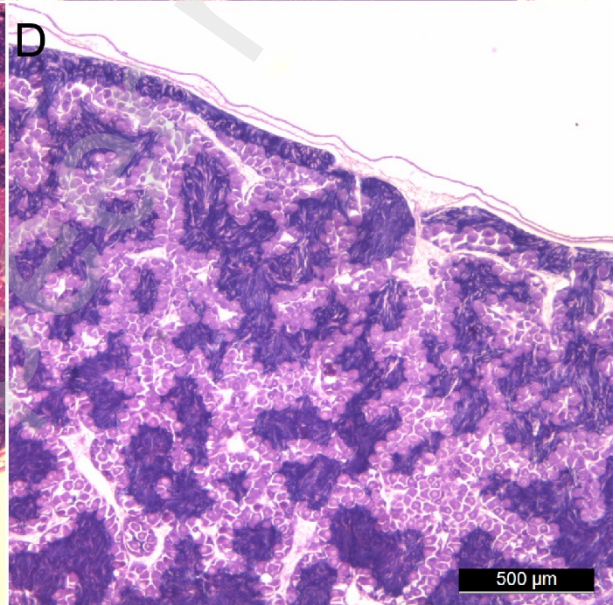
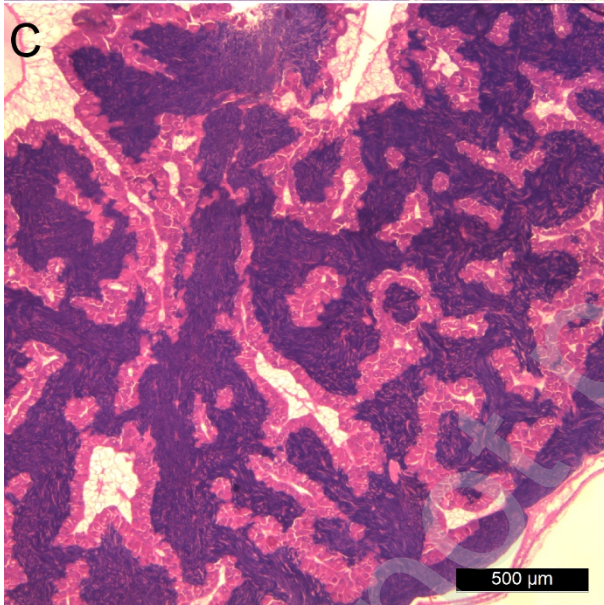
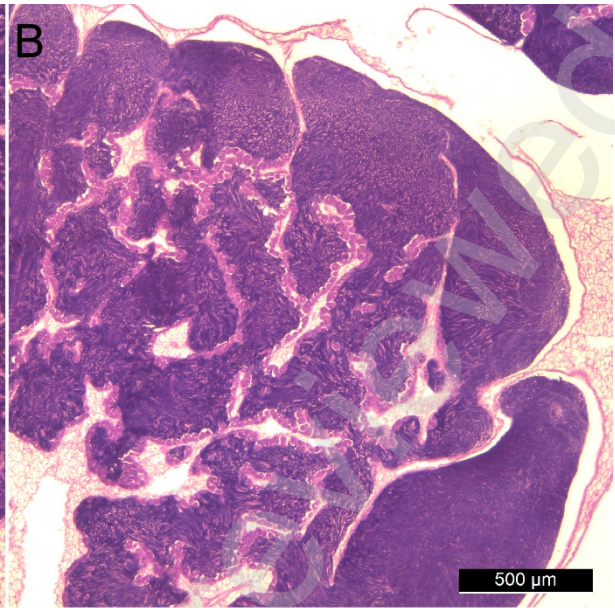
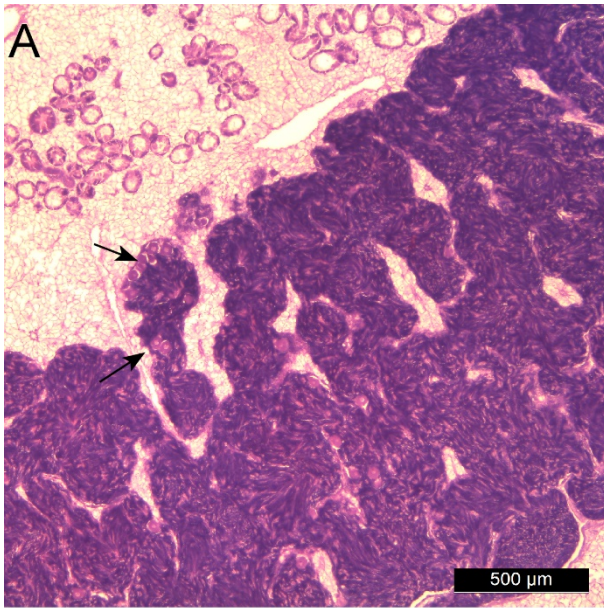


Figure 2: Histological description of simultaneous hermaphrodites of *C. gigas*. From A to F, female tendency increase. Arrows in panel A indicate mature oocytes, while it indicates sperm in panel F

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Table 1: Sex of oysters produced by selfing or by mating two different parents (i.e. « No selfing ») recorded at 14 and 15 month-old in May and June 2019, respectively.

Date	Reproduction	Stock	Method	Number sexed	Female	Male	Herma-phrodite	% Female	% Male	% Herma-phrodite
May	Selfing	H4-self	biopsy	214	3	211	0	1.4	98.6	0
	Selfing	H4-self	sacrificed	30	0	30	0	0	100	0
	Selfing	H4-self	All	244	3	241	0	1.2	98.8	0
	No selfing	H4fm	sacrificed	42	16	26	0	38.1	61.9	0
	No selfing	A	sacrificed	10	4	6	0	40.0	60.0	0
	No selfing	B	sacrificed	20	10	10	0	50.0	50.0	0
	No selfing	C	sacrificed	22	11	11	0	50.0	50.0	0
	No selfing	D	sacrificed	11	5	6	0	45.5	54.5	0
	No selfing	E	sacrificed	10	5	5	0	50.0	50.0	0
	No selfing	F	sacrificed	30	16	14	0	53.3	46.7	0
No selfing	Total A-F	sacrificed	103	51	52	0	49.5	50.5	0	
June	Selfing	H4-self	histology	211*	6	61	144	2.8	28.9	68.2
	No selfing	B	histology	10	8	2	0	80.0	20.0	0
	No selfing	C	histology	12	1	10	1	8.3	83.3	8.3
	No selfing	I	histology	10	3	7	0	30.0	70.0	0
	No selfing	J	histology	30	7	23	0	23.3	76.7	0
	No selfing	K	histology	16	7	9	0	43.8	56.3	0
	No selfing	M	histology	12	10	2	0	83.3	16.7	0
	No selfing	N	histology	9	6	3	0	66.7	33.3	0
	No selfing	Total B-N		111	45	65	1	40.5	58.6	0.9

* 212 were sexed by histology but one was undetermined (no gametes observed), and one female and one male biopsied one month earlier died.

TABLE 2 Percentage of the gonad area showing male, female or undetermined gametes for the simultaneous hermaphrodite of H4-self in June 2019

Gonad area	All			Without undetermined	
	% male	% female	% undetermined	% male	% female
]0 - 25%[14	58	76	5	41
[25 - 50%[23	37	14	12	42
[50 - 75%[51	5	9	42	12
[75 -100%[12	1	1	41	5