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Sex determination in the oyster *Crassostrea gigas* - a large longitudinal study of population sex ratios and individual sex changes

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Running title: Sex-ratio in *Crassostrea gigas*

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Abstract

Understanding sex determination in the Pacific oyster *Crassostrea gigas,* a sequential hermaphrodite, can provide prospective on the evolution of sex-determining systems for comparative reproduction from an evolutionary perspective. Surprisingly, this mechanism is still poorly understood. To date, sex ratio and sex change have never been studied at the individual level for a large size group and long-term monitoring. To this purpose, we performed an ambitious individual long-term follow-up (6 years) on a large population (cohort 1: 7488 oysters) produced from wild oysters, as well as for a second population produced from the cohort 1 (cohort 2: 4320 oysters). All oysters were individually sexed from 2014 to 2019. For the cohort 1, our results showed a significantly female-biased sex ratio each year, ranging from 61 to 73% for the cohort 1. The proportion of oysters exhibiting sex change between the first two breeding seasons was 34% and decreased each year, ending at 9% between years 5 and 6. From the initial population, 1386 oysters were sexed six years in a row. Among them, 58% were sequential hermaphrodites, within which 32% changed sex once (19% protandric and 13% protogynic), 19% twice, 5% three times, 1% four times and 0.1% five times. In contrast, 42% never exhibited a sex change, within which 34% were potentially true females and 8% potentially true males. However, a logistic regression model indicates that those oysters could experience one sex reversal in subsequent years resulting that all oysters of our population of *C. gigas* would be sequential hermaphrodites. Similar results were observed for the cohort 2, although the proportion of sequential hermaphrodite was higher than the one observed for cohort 1. It is supposed that a genetic basis exist for sex change in *C. gigas*. Our work participates to unravel the barriers existing about the sequential hermaphroditism, the protandry and the sexual system in *C. gigas,* still currently debated.

Keywords: sex-ratio; sex change; oysters; hermaphroditism; *Crassostrea gigas*

1. Introduction

As sex determination is of major importance to sexual reproduction, it is the subject of many studies across the animal kingdom. Although the mechanisms of sex determination are remarkably diverse among organisms, they can be grouped into three main modes: (i) genotypic sex determination where sex is established by the genotype (gonosomes or autosomes), (ii) environmental sex determination where sex is influenced by environmental cues, and (iii) a mix of genotypic and environmental sex determination (Bachtrog et al., 2014).

The sex-determining mechanisms observed across the tree of life are very diverse because they can evolve rapidly (Bachtrog et al., 2014). A striking example of diversity in sex determination is freshwater crustaceans in the family Limnadiidae (Weeks et al., 2006). Consequently, different modes of sex determination are found among closely related species or populations of the same species and in contrast, the same mode may evolve independently in distant clades (Bachtrog et al., 2014). This diversity of sex-determining mechanisms is associated with two modes of sexual reproduction in animals: gonochorism (only one distinct sex in any individual organism) and hermaphroditism (simultaneous when individuals function as male and female at the same time; sequential when individuals first function as one sex and then switch to the other sex). Gonochorism appears more widespread than hermaphroditism, which is only observed in approximately 5% of all animal species (Bachtrog et al., 2014).

Sex determination defines individual sex and is therefore closely related to the sex ratio of a population and its variation. Fisher (1930) theorized that the sex ratio within a population

should be balanced (1:1) under the hypothesis that producing males or females requires an equal cost. This balance is the "evolutionarily stable strategy" and is maintained by natural selection, which promotes the rarer sex. However, in the animal kingdom, biased sex ratios are commonly observed. This may be induced by differential mortality related to sex (Arendt et al., 2014), inbreeding and local competition for mates and food (Hamilton, 1967), endocrine-disrupting environmental pollutants (Mills and Chichester, 2005), or adaptive maternal effects that result in differential investment in male or female offspring (Trivers and Willard, 1973).

Mollusca, the phylum to which oysters belong, provides a rich source of material to better understand the evolution of sex and sex determination (Breton et al., 2017). Indeed this phylum (i) is the second largest in the animal kingdom, (ii) belongs to Lophotrochozoa, a clade poorly understood in terms of reproduction, (iii) provides a richness of species with highly diverse modes of sexual reproduction ranging from functional hermaphroditism (simultaneous and sequential) to gonochorism, suggesting diverse underlying sex-determining mechanisms, and (iv) includes species of economic and nutritional importance, which makes knowledge of their sex determination highly necessary to provide useful tools for the control of their sex in aquaculture. Within molluscs, gonochorism appears as the most common sexual system, occurring in seven of the eight extant classes (Collin, 2013), while approximately 40% of the 5,600 genera are classified as hermaphrodites (Heller, 1993). Among bivalves, only approximately 4% of the 10,000 extant species have been determined to not be strictly gonochoric (Coe, 1943); indeed, hermaphroditism has been identified in 13 out of the 117 families (Heller, 1993). However, this number of hermaphroditic bivalve species is probably an underestimation because of (i) its determination based on a limited number of individuals and groups that sometimes lack sexual dimorphism, (ii) the

misidentification of sex in simultaneous hermaphrodites based on the study of gonad fragments, and (iii) the misidentification of sex change in sequential hermaphrodites observed at a population scale and not at an individual scale (Yusa, 2007).

Concerning the Pacific oyster *Crassostrea gigas*, its sex-determination system has not been established and there are two longstanding paradigms concerning hermaphroditism in this species:

i) Oysters are sequential hermaphrodites (encountering sex changes at some point in their lifespan). However, few studies provide direct observations of individual sex changes, and these observations have been limited to two years of life in *C. gigas* (Amemiya, 1929; Lango Reynoso, 1999; Lannan, 1971; Park et al., 2012; Yasuoka and Yusa, 2016).

ii) Oysters are protandrous hermaphrodites (born male and change sex to a female) with a striking example provided by Guo et al. (1998), suggesting a higher proportion of males in younger oysters. Nevertheless, five independent studies reported primary sex ratios that were biased in favor of females or were well-balanced (1:1) (Amemiya, 1929; Fabioux et al., 2005; Lango Reynoso, 1999; Park et al., 2012; Santerre et al., 2013).

None of the above studies has investigated the mode of reproduction in individual *C. gigas* for more than two years and/or used a large number of oysters, leading to a lack of consensus among them. Direct observations are crucial and are a mandatory component of experimental design for better understanding of sex determination in *C. gigas.*

In this study, we aimed to assess the temporal variation of the sex ratio for a *C. gigas* population (cohort 1) over the six first-years to identify potentially true males and potentially true females, as well as sequential hermaphrodites. Thus, 7488 oysters were tagged and then sexed from 2014 to 2019 to clarify the sex determination in this major species used in

120 aquaculture. In addition, the sex ratio and the sex change from 2015 to 2019 was also
121 recorded for a second cohort using 4,320 *C. gigas*.

122 ## 2. Materials and methods

123 ### 2.1. First cohort using wild oysters

124 #### *2.1.1. Biological material*

125 Twenty half-sib families, each consisting of two full-sib families of *C. gigas,* were produced
126 at the Ifremer hatchery in La Tremblade (France) on 27 March 2013 from a wild oyster
127 population sampled from the Marennes-Oléron Bay (France). The parents were opened to
128 determine their sex as well as their level of maturity by microscopic observation of gonad
129 samples spread on a slide (presence of spermatozoa or oocytes). Twenty males and forty
130 females were kept for mating, each male being crossed with two females. The gametes were
131 collected from each parent by stripping the gonad. After fertilization, the larvae were raised
132 in 30-L tanks at 25°C in UV-treated, filtered, and aerated seawater. All families were raised
133 separately. The water was changed three times per week. Larvae were fed daily with
134 *Isochrysis galbana* (30,000 cells/mL) until they reached 140 µm, after which the diet was
135 supplemented with *Skeletonema costatum* (30,000 cells/mL). Two weeks after fertilization,
136 larvae were placed on cultch in flow-through raceways at 20°C supplied with UV-treated
137 seawater enriched with *S. costatum*. Oyster spat were reared under standard hatchery
138 conditions until they reached a size of 2 mm. In May 2013, 5000 oysters per family were
139 transferred to the Ifremer nursery in Bouin (France) (Baud and Bacher, 1990). Density was
140 reduced during the nursery period as some oysters were used in studies to determine the
141 genetic basis for resistance to pathogens (Azéma et al., 2017a; Azéma et al., 2017b).
142 Meanwhile, each family was kept in one sieve at high density to reduce the growth until
143 November 2013 and they were protected within the facility under biosecurity control to avoid
144 contamination with major oyster pathogens such as OsHV-1 and *Vibrio aestuarianus*. Further
145 details on these families are provided elsewhere (Azéma et al., 2017a; Azéma et al., 2017b).

146

147 *2.1.2. Field study*

148 The field study lasted from November 2013 to July 2019. In November 2013, 38 families
149 were transferred to the field study site at La Floride in the Marennes-Oléron Bay, which is the
150 main area for shellfish culture in Europe (Goulletquer and Le Moine, 2002). This site is
151 located in the intertidal area and the mean immersion time is around 50%, which is low in
152 comparison to growing leases. This choice was based to avoid a second gametogenesis within
153 a year. Each family was grown separately throughout the study. Approximately 14,000
154 oysters were deployed (Table 1) (average individual weight 8.0g); the mean number of
155 oysters per family was 367 and ranged from 150 to 964 among families. Each family was
156 placed into a single labeled sealed oyster bag, except for eight families for which two bags
157 were needed because of the high number of oysters. All bags were randomly attached to
158 racks. Every month, bags were checked to make sure that they were well-attached to the
159 racks and that they were free of defects that would cause loss. The seawater temperature was
160 recorded every hour throughout the study using two probes (ThermoTrack; supplementary
161 data 1). For this study, data are presented without distinguishing the families to have a broad
162 view of sex ratio and sex change for the studied population of *C. gigas*.

163

164 *2.1.3. Sex observation*

165 All oysters were checked annually at the time of sexual maturity in June from 2014 to 2019.
166 At the beginning of June, oysters were transferred from the field to the laboratory and held in
167 a flow-through system. Seawater was chilled to 15°C until sex was determined to avoid
168 unintentional spawning events. The number of oysters sexed each year is indicated in Table
169 1; it decreased throughout the study mainly because of mortality and to a much lesser extent,
170 sampling for molecular analyses (data not shown). After the first sexing (June 2014), male

171 and female oysters were separated into two labelled oyster bags until individual labelling. In
172 April 2015, all live oysters were individually marked with a plastic-laminated number glued
173 with epoxy resin (Sader©) on the upper valve. After labelling, males and females were mixed
174 in one oyster bag per family. Two non-destructive methods were used to determine oyster
175 sex: induced spawning and gonad biopsy. For years 1, 2, 3 and 5, gonad biopsy concerned
176 less than 5% of the oyster population, while it was 37% at year 4 in 2017 and 100% at year 6
177 in 2019 due to technical reasons (12,000 additional oysters sexed in 2017, data not shown,
178 and hatchery closed in 2019). The biopsy method did not induce higher mortality than oysters
179 that spawned (data not shown). To visualize the emission of the gametes during induced
180 spawning, oysters were placed in a single layer with sufficient distance from each other in a
181 black-bottomed 200-L tank filled with seawater. Thermal shocks in the form of alternating
182 ambient (20°C) and warm (30°C) water were used to trigger spawning. Oyster gametes were
183 also added to the tank as a stimulant. Males emit their spermatozoa as a long, opaque white
184 mesh. Females are identifiable by the emission of their oocytes in the form of repeated,
185 dense, and granular clouds.

186 After spawning commenced, each oyster was placed in a transparent 300-mL beaker
187 containing seawater at 25°C to ensure that the observed gametes were from the suspected
188 oyster and to confirm the nature of the gametes. When massive spawns occurred, seawater
189 was removed, and all oysters were individually placed into beakers.

190 The thermal shock cycle was repeated up to 10 times, but some oysters did not respond to
191 induction. For non-responding individuals, a biopsy of the gonad was performed and sex was
192 determined by microscopic observation of gonad smears. Oysters were placed in a 5-L tray
193 with a muscle relaxant solution consisting of seawater (3/5), freshwater (2/5), and magnesium
194 chloride (50 g/L). As soon as the shells opened, a smear of gonad was taken using a needle
195 (0.9 × 38 mm; Terumo©) and a 1-mL syringe (Terumo©). Mature gametes were visualized

microscopically (40×) to determine the sex. Oysters with oocytes were identified as females and those with spermatozoa were classified as males. Oysters with both mature oocytes and spermatozoa were identified as simultaneous hermaphrodites (represented less than 1% per year). For some oysters, sex could not be determined, and they were categorized as "empty". These two categories were excluded from the results presented below. After spawning and biopsies, male and female oysters were placed in separate trays until all data was recorded. Males and females from the same family were placed into culture bags and the bags were returned to the study site.

2.2. Second cohort

The first cohort was produced in March 2013, kept in high density until November 2013, and then sexed for the first time in June 2014. There is a chance that the primary sex ratio could have been missed. Consequently, a second cohort was produced in June, then deployed in the field in November and sexed for the first time in June of the following year. This protocol is also close to the life cycle of oysters in the Marennes-Oléron region, with spawning occurring in June/July. The second cohort was produced on June 16th 2014 using four families of the cohort 1 that were selected for their higher resistance to OsHV-1 and *Vibrio aestuarianus*. Thus, 14 females and seven males, all sibling of the cohort 1 (i.e. those oysters were not followed in the longitudinal study), were used producing 15 full-sib families and 5 half-sib families. The same hatchery and nursery protocols used for the cohort 1 were applied for the cohort 2. Spat were transferred on the same site used for the cohort 1 in November 2014 (mean individual weight 2.6 g, one bag of 1 kg per family, i.e. around 406 oysters per family and 6,090 oysters deployed). Each family was grown in separate bag until individual tagging (Pit-tag, Biolog-ID, BERNAY France) in April 2016. Then, oysters were mixed and grown

using standard field on-growing method. Sex was recorded as described above, in June of each year from 2015 to 2019 (Table 1). As for cohort 1, data are presented without distinguishing the families to have a broad view of sex ratio and sex change for a second cohort of *C. gigas*.

2.3. Statistical analyses

All statistical analyses were conducted using R® (version x64 3.4.1, RCore Team) with significance set at $\alpha = 0.05$. No data required transformation before analysis.

Sex ratio was calculated each year from year 1 to year 6 as the number of females divided by the number of females and males sexed at year Y. The standard error (SE) was calculated such SE=$\sqrt{(p * q)/n}$ where p is the proportion of females, q=1-p the proportion of males, and n the sample size. The sex ratio of each cohort for years 1 to 6 was compared to the suggested "ideal" ratio of 1:1 using χ^2 tests. Sex ratio was compared among years by logistic regression and a logit transformation, and pairwise comparisons among years were conducted using least-squares means.

Sex was recorded from year 1 to year 6, leading to five sets of data recording the sex change between two consecutive years, defined as sets Y1/2, Y2/3, Y3/4, Y4/5, and Y5/6 for the cohort 1. Similarly, four sets were available for the cohort 2 defined as sets Y1/2, Y2/3, Y3/4, and Y4/5. For each set, the percentage of sex change was calculated from the ratio of the number of oysters that exhibited sex change between year Y and year Y+1 and the total number of oysters sexed in year Y+1. Sex change was compared among sets by logistic regression and a logit transformation.

Finally, the estimated regression equations were obtained for the cohort 1, as well as for males and females sexed at year 1 to compute the predicted cumulative percentage of sequential hermaphrodites at the desired age (in years) from year 1 to year 30.

3. Results

3.1. First cohort

3.1.1. Sex ratio

The sex ratio of the population every year is shown in Fig 1. The mean percentage of females among years was 67% ranging from 61% in year 2 to 73% in year 4. The sex ratio was significantly different from 1:1 every year ($P < 0.0001$). Similarly, the sex ratio was significantly different among years ($P < 0.0001$). All pairwise comparisons were significant ($P < 0.01$) except between year 1 and year 5, between year 1 and year 6, and between year 5 and 6.

3.1.2. Sex change between two consecutive years

For set Y1/2, 66% of the population did not change sex (Fig. 2). This proportion significantly increased for the subsequent sets ($P < 0.0001$) with 84% for Y2/3, 82% for Y3/4, 89% for Y4/5 and 91% for Y5/6.

3.1.3. Percentage of sequential hermaphrodites at year 6

For oysters sexed each year from year 1 to year 6 (n = 1386), 42% never exhibited any sex change (Fig. 3). Among them, 34% were potentially true females and 8% were potentially true males. The percentages of oysters undergoing sex changes were 32%, 19%, 5%, 1% and 0.1% for 1, 2, 3, 4 and 5 sex changes, respectively (Fig. 3).

266 *3.1.4. Prediction of the percentage of sequential hermaphrodites during the lifespan*
267 *in C. gigas*

268 The regression equations to predict the cumulative percentage of sequential hermaphrodites
269 according to the age of the oysters are given in Table 2. Although 42% of the oysters were
270 identified as potentially true females and potentially true males at year 6 (Fig. 3), Fig. 4
271 predicts that almost all oysters should experience at least one sex change during their
272 lifetime, occurring at any time, even if the probability for sex change decreased in older
273 oysters. Thus, 95% of the population are predicted to exhibit at least one sex change between
274 year 1 and year 19. It may occur significantly earlier for the males (in the first 11 years)
275 compared to the females (in the first 27 years) (Fig.4). The percentages of new sequential
276 hermaphrodites each year are given in supplementary data 2.

277

278 *3.2. Second cohort*

279 *3.2.1. Sex ratio*

280 The sex ratio of the population every year is shown in Fig 5. The mean percentage of females
281 among years was 59% ranging from 54% in year 1 to 67% in year 3. The sex ratio was
282 significantly different from 1:1 every year ($P < 0.01$). Similarly, the sex ratio was
283 significantly different among years ($P < 0.0001$). Pairwise comparisons were significant ($P <$
284 0.01) between year 1 and year 2, and between year 3 and the other years.

285 *3.2.2. Sex change between two consecutive years*

286

287

288 For set Y1/2, 53% of the population did not change sex (Fig. 6). This proportion significantly
289 increased for the subsequent sets ($P < 0.0001$) with 62% for Y2/3, 86% for Y3/4, and 89%
290 for Y4/5.

291 *3.2.3. Percentage of sequential hermaphrodites at year 5*

292 For oysters sexed each year from year 1 to year 5 (n = 333), 24% never exhibited any sex
293 change. Among them, 17% were potentially true females and 7% were potentially true males.
294 The percentages of oysters undergoing sex changes were 44%, 26%, 5% and 1% for 1, 2, 3,
295 and 4 sex changes, respectively.

296

297 **4. Discussion**

298

299 This study aimed to investigate, for the first time, the time-course of the sex ratio and the
300 ability to change sex during the first six years of the life of *C. gigas*. It allows us to estimate
301 the proportion of sequential hermaphrodites in the population, and to clarify three milestones
302 that are still debated regarding sex determination in this species: sequential hermaphroditism,
303 protandry, and the sexual system. As a consequence, this study will also provide useful
304 information for comparative reproductive biology as it concerns (i) a representative of
305 Lophotrochozoa, which is poorly documented in this aspect of its biology, (ii) an organism
306 with a very plastic reproductive system, and (iii) an invertebrate with sex-determining genes,
307 something more in common with vertebrates than with other invertebrates (Santerre et al.,
308 2012; Santerre et al., 2014; Zhang et al., 2014).

309

310

311 *4.1. Sequential hermaphroditism in C. gigas*

312

313 Simultaneous hermaphroditism was observed during our study, with an annual frequency of
314 less than 1% (data not shown). This small proportion is similar to that previously reported in
315 *C. gigas* (Amemiya, 1929; Guo et al., 1998; Normand et al., 2009; Steele and Mulcahy, 1999;
316 Yasuoka and Yusa, 2016). In contrast, sequential hermaphroditism describes animals that
317 first function as one sex and then switch to the other sex. From the handful of studies on sex
318 determination in oysters, sequential hermaphroditism has been poorly characterized at the
319 individual scale and the distinction between individuals that undergo a sex change and those
320 that do not is rarely achieved. For this reason, our study showed an accurate identification of
321 sequential hermaphrodites based on the number of sex changes observed during the five or
322 six years recorded and the evolution of sex change by age.
323 Thus, 66% of oysters of the cohort 1 did not change sex during the two first years (Fig. 2)
324 which is in agreement with the results reported in *C. gigas* after two breeding seasons by
325 Amemiya (1929) (66%) and Park et al. (2012) (60%). For the cohort 2, a lower proportion
326 was observed with 53% of the oysters that did not change sex during the two first years (Fig.
327 6) matching with the results reported by Lango Reynoso (1999) (45-52%). Although
328 environment might play a role, this could be explained by the parents of the cohort 2. Indeed,
329 one the four families used to produce the cohort 2 exhibited the highest tendency for sex
330 change among the 38 families of the cohort 1. This family contributed to 9 of the 15 families
331 of the cohort 2 suggesting that genetic factors might be involved for sex change in *C. gigas*.
332 From the 1386 oysters sexed six years in a row for the cohort 1, 42% did not change sex
333 which is within the range for similar studies in *C. virginica* (33-57%) (Haley, 1979; Needler,
334 1942). Within the 42% of oysters that did not experience sex change, 34% were potentially
335 true females and only 8% were potentially true males. This contrasts with the two studies in
336 *C. virginica* that found 45% true males and 12% true females (Haley, 1979) and 30% males

337 and 4% females (Needler, 1942). Although Coe (1932) introduced the idea of true males and
338 Hedrick and Hedgecock (2010) added true females, this is the first time that the proportions
339 of these groups are reported in *C. gigas*. Again, the lower proportion of true females (17%)
340 and true males (7%)(Fig 7) for the cohort 2 than those reported in cohort 1 could be explained
341 due to the inherence of genetic factors through the families used to produce the cohort 2.
342 Consequently, 58% of the oyster population for the cohort 1 were sequential hermaphrodites
343 after six breeding seasons. The percentages of oysters encountering 1, 2, 3, 4 or 5 sex changes
344 in our study were 32, 19, 5, 1 and 0.1%, respectively for the cohort 1 (Fig. 3). Our study
345 demonstrates that most of the sex-changing oysters exhibit only one or two sex changes
346 (51%), while only 6% of the population had at least three sex changes. Similar results were
347 found for the cohort 2 (Fig. 7). This is also in agreement with the results observed in *C.*
348 *virginica* after five years with 59% and 7%, respectively, although this study was only based
349 on 57 oysters (Needler, 1942). Also, 25% of the oyster population experienced bidirectional
350 changes and that true alternating sexuality, with a sex change encountered each year, only
351 involved a very limited proportion of the population (0.1% at year 6 for the cohort 1 and
352 0.9% at year 5 for the cohort 2).
353 Among the sequential hermaphrodites, older animals exhibited less frequent sex change, even
354 if sex change was observed over the whole study. Thus, 34% of the oysters (n = 4850)
355 changed sex between the two first breeding seasons, while it decreased to 9% between the
356 fifth and sixth breeding season (n = 1386) (Fig. 2). Similar tendency was observed for the
357 cohort 2 from 47% of the oysters (n = 2465) that changed sex between the two first breeding
358 seasons to 11% between the fourth and fifth breeding season (n = 339) (Fig. 7). There is a
359 lack of information on sex change at the individual level in the literature for *C. gigas,* as
360 previous studies have only recorded the sex ratio for two years (Amemiya, 1929; Lango
361 Reynoso, 1999; Park et al., 2012). In *C. virginica*, no distinct pattern was apparent in the

362 rhythm of changes from younger to older oysters with 12, 15, 18, 18 and 6%, respectively
363 (Galtsoff, 1937; 1964), and with 39, 12, 28 and 35%, respectively (Needler, 1942). The
364 variability in the rates of sex change with oyster age cannot be explained, as the cues that
365 control sex change in oysters remain poorly understood. Meanwhile, several factors might
366 control sex change as demonstrated for hermaphroditic fishes with environmental cues
367 (temperature, pH, hypoxia), density, social structure, or attainment of a critical age or size
368 (reviewed in Todd et al. (2016)). Thus, it could be assumed that younger oysters could be
369 more sensitive to the factors triggering a sex change in our *C. gigas* populations.
370 Even if our collected data showed the existence of potentially true males and potentially true
371 females after six years of follow-up, the predicted cumulative percentage of sequential
372 hermaphrodites was up to 95% over their life period. It suggests that all oysters of our
373 population of *Crassostrea gigas* could be potentially sequential hermaphrodites.
374 Nevertheless, results obtained in the first six years could be useful for aquaculture and
375 research purposes, to control the conditioning in hatchery by optimizing the number of adults
376 (Helm et al., 2004), to produce inbreed lines (Lannan, 1971; Yang et al., 2015) or to improve
377 sex-specific growth (Baghurst and Mitchell, 2002).
378
379 *4.2. Protandry in C. gigas*
380 Previous studies considered *Crassostrea* oysters as protandrous hermaphrodite (Coe, 1934;
381 Galtsoff, 1964; Guo et al., 1998). Protandrous animals are defined here as those (i) exhibiting
382 a primary sex ratio within the population distorted toward males (first-maturing sex in sex-
383 changing animals as suggested by Charnov (1982)) that evolves toward females, and also (ii)
384 exhibiting one sex change.
385 Our populations of *C. gigas* exhibited a primary sex ratio significantly biased toward females
386 with 69% for the cohort 1 (n = 7409) (Fig.1) and in a lesser extent, 54% for the cohort 2

387 (n=4320)(Fig.5). However, previous studies did not reach a consensus concerning the
388 primary sex ratio in *C. gigas.* The sex ratio was biased in favour of females in some studies
389 (Amemiya, 1929; Lango Reynoso, 1999; Santerre et al., 2013), while some observed 1:1
390 primary sex ratios (Fabioux et al., 2005; Park et al., 2012), and others observed sex ratios
391 biased toward males (Enriquez-Diaz et al., 2009; Guo et al., 1998; Yasuoka and Yusa, 2016).
392 In other oyster species, male-biased sex ratios have been reported in *C. virginica* (Coe, 1936;
393 Galtsoff, 1937; Haley, 1979; Kennedy, 1983; Powell et al., 2013) and *Saccostrea cucullata*
394 (Morton, 1991), while there is a large predominance of females in *C. rhizophorae*
395 (Littlewood and Gordon, 1988) and no significant dominance of either sex in *C. madrasensis*
396 (Mohan Joseph and Madhyastha, 1984) and *C. gasar* (Ramos et al., 2013). In bivalves,
397 Morton (1991) proposed that a pronounced female bias could optimize the reproductive
398 success, by maximizing resource allocation into the more energy-demanding process of
399 oogenesis. However, this diversity of primary biased sex ratio falls in line with the high
400 phenotypic plasticity of the oyster due to complex genotype-environment interactions. In this
401 respect, many biological mechanisms are proposed to affect the primary sex ratio of
402 organisms, which is expected to be 1:1 under heterogamety, including genes and cytoplasmic
403 factors, the sexual system, and the mode of sex determination (Yusa, 2007). Cross-
404 generational plasticity may also induce bias. Thus, the ecological conditions experienced by
405 the mother could influence life-history trade-offs in offspring and result in the production of
406 more of the sex that provides greater fitness returns (Wade et al., 2003). Sex ratio may also be
407 distorted to survive in heterogeneous environments (Ghiselin, 1969), especially for organisms
408 with low mobility such as the oyster. However, according to Yusa (2007), several other
409 factors may explain the existence of bias in sex ratios, such as the misidentification of sex,
410 sampling size bias, sex-related differences in mortality, and age differences at the time of
411 sexual maturity. The design of our study limited such bias as follows: (i) hatchery-produced

412 oysters were the same age and were individually monitored on our experimental oyster farm;
413 (ii) a large number of oysters (7,409 and 4,320 individuals for the cohorts 1 and 2,
414 respectively) at the beginning of the survey make the results robust; (iii) mortalities were not
415 significantly correlated with sex (results not shown); and, (iv) the time of sexual maturity is
416 well-known for both sexes (Berthelin et al., 2000) and was accurately checked annually by
417 spawning and/or gonad biopsy.
418 The significant bias in the primary sex ratio toward females in the first year was maintained
419 over the following five years (Fig. 1 and Fig. 5). This tendency is explained by the (i) higher
420 proportion of true females (34% and 17%) than the proportion of true males (8% and 7%)
421 that did not change sex during the five/six years of the study, (ii) high percentage of females
422 among the oysters showing two sex reversals (74% and 63% for cohorts 1 and 2, respectively
423 Fig.3 and Fig.7), and (iii) protandrous males (19% and 23%) (Fig.3 and Fig.7). Female-
424 biased sex ratios that were maintained over the second year have also been previously
425 reported in *C. gigas* (Amemiya, 1929; Lango Reynoso, 1999), while reports of primary male-
426 biased sex ratios showed an increase over time of the proportion of females in *C. gigas* (Guo
427 et al., 1998) and *C. virginica* (Haley, 1979).
428
429 During our study, 19% of the oyster population underwent protandrous sex change while
430 13% underwent protogynous sex change for the cohort 1 (Fig.3), while it was 23% and 21%
431 for the cohort 2 (Fig. 7). Although many previous studies suggested that protandry is the
432 typical form of sexuality in oysters (Coe, 1934; Galtsoff, 1964; Guo et al., 1998; Parker et al.,
433 2018; Powell et al., 2011), protogynous sex changes have also been observed in *C. gigas* for
434 70% of the animals changing their sex only once (calculated from Park et al. (2012)) and in
435 *C. virginica* (Haley, 1979).

436 Our results strongly encourage the scientific community to consider the oyster as a very
437 flexible sex-changer, undoubtedly experiencing both protandrous and protogynous sex
438 changes, as well as multiple sex changes (Fig.3 and Fig.7).
439
440 *4.3. Hypotheses for the sexual system of C. gigas*
441 As our study involved long-term monitoring of a large population of oysters that were
442 individually sexed each year for six years, it allowed us to gather a large amount of reliable
443 data related to the sex ratio and the ability to change sex in *C. gigas*. These data highlight the
444 plasticity of reproduction in *C. gigas*, as previously mentioned for instance by Guo et al.
445 (1998) who discussed "protandric sex change, dioecy and hermaphroditism" and Hedrick and
446 Hedgecock (2010) who discussed "dioecious, sequential hermaphrodites and some rare
447 simultaneous hermaphrodites". From our data, the mode of reproduction of *C. gigas* could
448 only involve sequential hermaphrodites and some rare simultaneous hermaphrodites. Our
449 work highlights a plasticity for the mode of reproduction at the individual level in *C. gigas* by
450 proposing robust percentages of potentially true males and true females and sequential
451 hermaphrodites and the temporal variation for sex change among the hermaphrodites after six
452 years as well as the simulated percentage of hermaphrodites during the lifespan of one *C.*
453 *gigas* population.
454 Based on these results, we propose a hypothesis involving changes in the mode of
455 reproduction in *C. gigas* in France. When environmental conditions change or when a species
456 occupies a new habitat, selection may favor a transition from hermaphroditism to
457 gonochorism or vice versa (Weeks et al., 2006). In France, the production of *C. angulata*
458 collapsed, and to sustain the oyster production, *C. gigas* was massively introduced during the
459 1970s from Japan and British Columbia (Grizel and Héral, 1991). Although there was no
460 genetic differentiation or decrease in diversity between the population of *C. gigas* from Japan

461 (the origin of European populations) and those from France (Rohfritsch et al., 2013), the
462 latter may have experienced selection for the mode of sex determination to adapt to its new
463 habitat along the French coast. Similarly, global warming has increased seawater
464 temperature, a parameter known to be involved in environmental sex determination, as well
465 as ocean acidity. Recently, it was found that ocean acidification altered sex determination in
466 *Saccostrea glomerata* leading to a significant change in the population sex ratio by increasing
467 the proportion of females (Parker et al., 2018). A similar trend was also observed in *C.*
468 *hongkongensis* concerning trace metal pollution (Weng and Wang, 2015). Thus, this
469 phenotypic plasticity could be an adaptive response to spatially heterogeneous and/or
470 temporally varying environments (Ernande et al., 2004), and such variation may switch the
471 mode of reproduction of *C. gigas* from hermaphroditism to gonochorism or vice versa. This
472 modulation could involve three transitionary sexual systems (i) trioecy (mix of males,
473 females and simultaneous hermaphrodites), (ii) androdioecy (mix of males and simultaneous
474 hermaphrodites), and (iii) gynodioecy (mix of females and simultaneous hermaphrodites)
475 (Charlesworth and Charlesworth, 1978; Charnov, 1982). However, these modes of
476 reproduction do not take into account the sequential hermaphroditism that was very evident
477 in *C. gigas* in this study. This particularity can be an intermediate strategy developed by the
478 oyster to quickly cope with environmental variations.
479
480 In conclusion, the results of the present study showed a sex ratio distorted in favor of females
481 each year for the two cohorts for five and six years. Among the oysters sexed six years in a
482 row, 42% didn't change sex, while changing sex more than two times was scarce (7%).
483 Similar trends were observed for the cohort 2, although sex reversal was higher. This could
484 be explained by a genetic basis for sex change, as one of the family used as parents showed
485 the highest proportion of sex changer oysters (i.e. sequential hermaphrodites). For the first

time in *C. gigas*, we found that sex changes decreased with the age of the oyster. Finally, it appears that the entire population of oysters should be sequential hermaphrodites. Our study provides valuable information for designing future studies to (i) better understand genetic control of sex-determining mechanisms in *C. gigas*, (ii) manage production in hatcheries (control sex ratios and implement breeding programs) and assist in fisheries management, (iii) study comparative reproductive biology as very little information is available regarding this topic in molluscs, Lophotrochozoa, and other species exhibiting hermaphroditism (a well-conserved mode of reproduction in the animal kingdom), and (iv) advance evolutionary perspectives on the sexual system.

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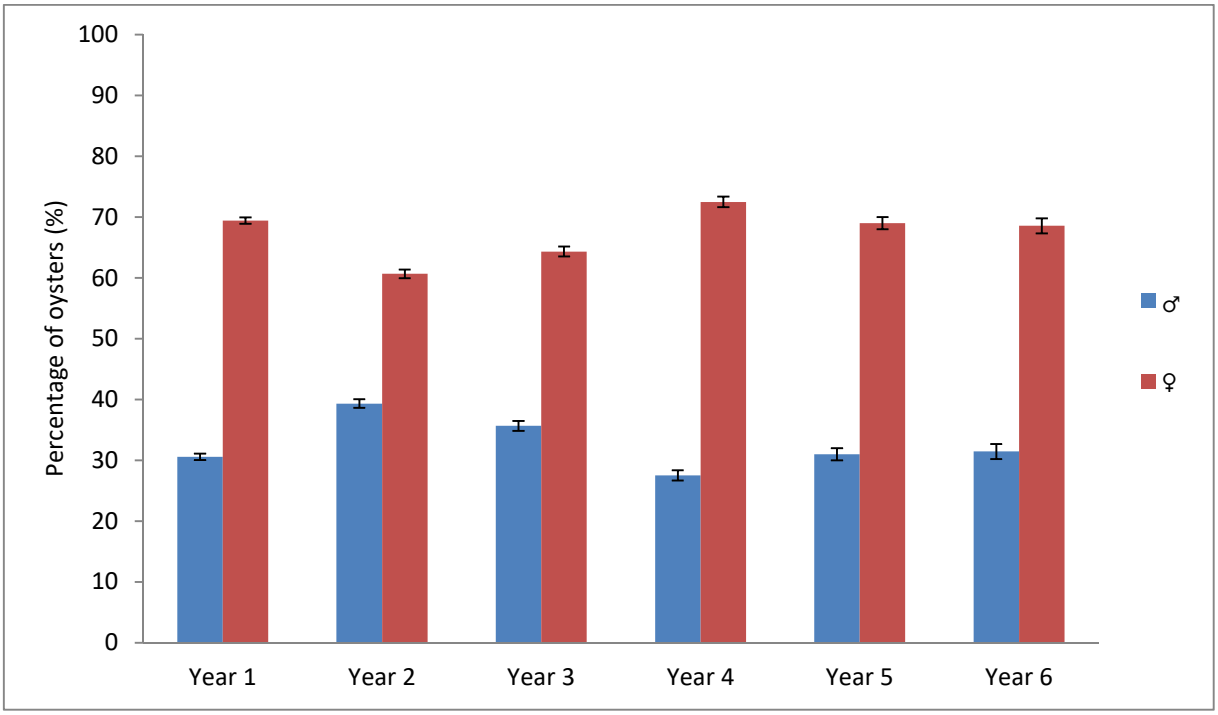

Fig. 1 Sex ratio (±SE) for the cohort 1 from year 1 to year 6. The number of oyster sexed each year is reported in Table 1.

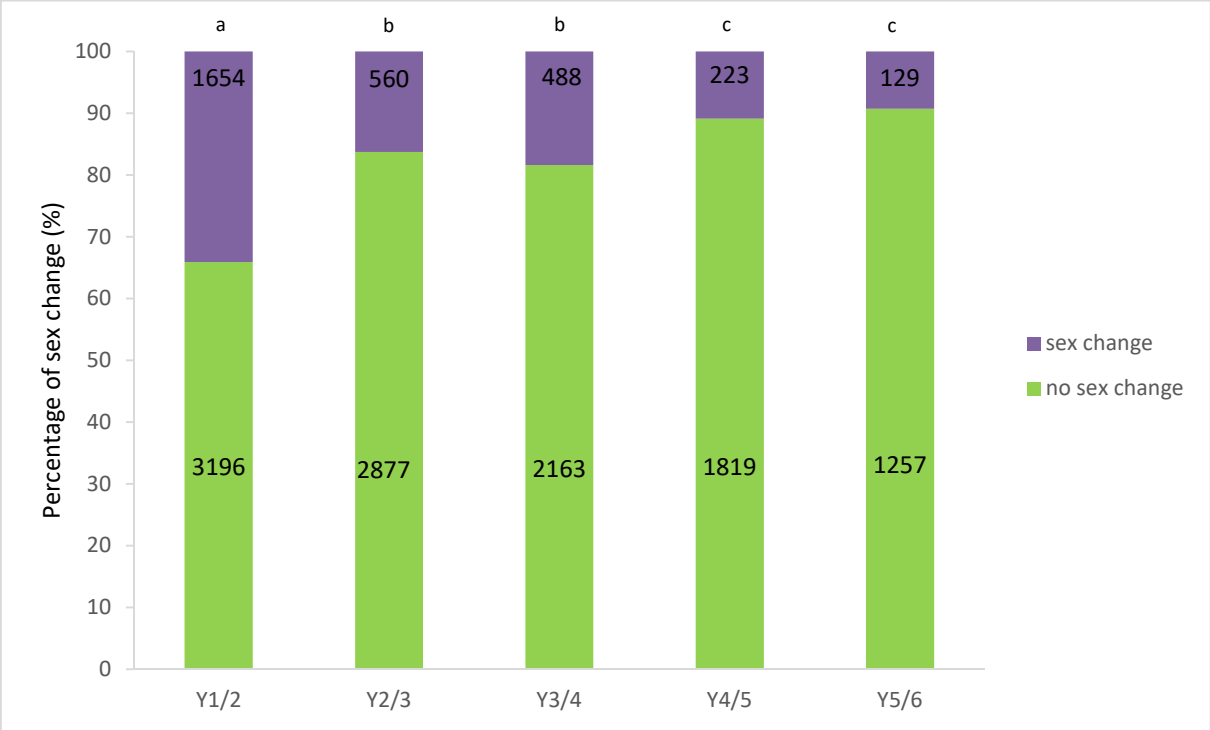

Fig. 2 Percentage of the oyster population for the cohort 1 experiencing or not a sex change between two consecutive years for each set (Y1/2 to Y5/6, Y being the year). The numbers of oysters that experienced or not a sex change are reported inside the bar. Oysters without any observable gametes and simultaneous hermaphrodites at year Y were excluded. The letters a, b, and c indicate significant differences among sets ($P < 0.0001$).

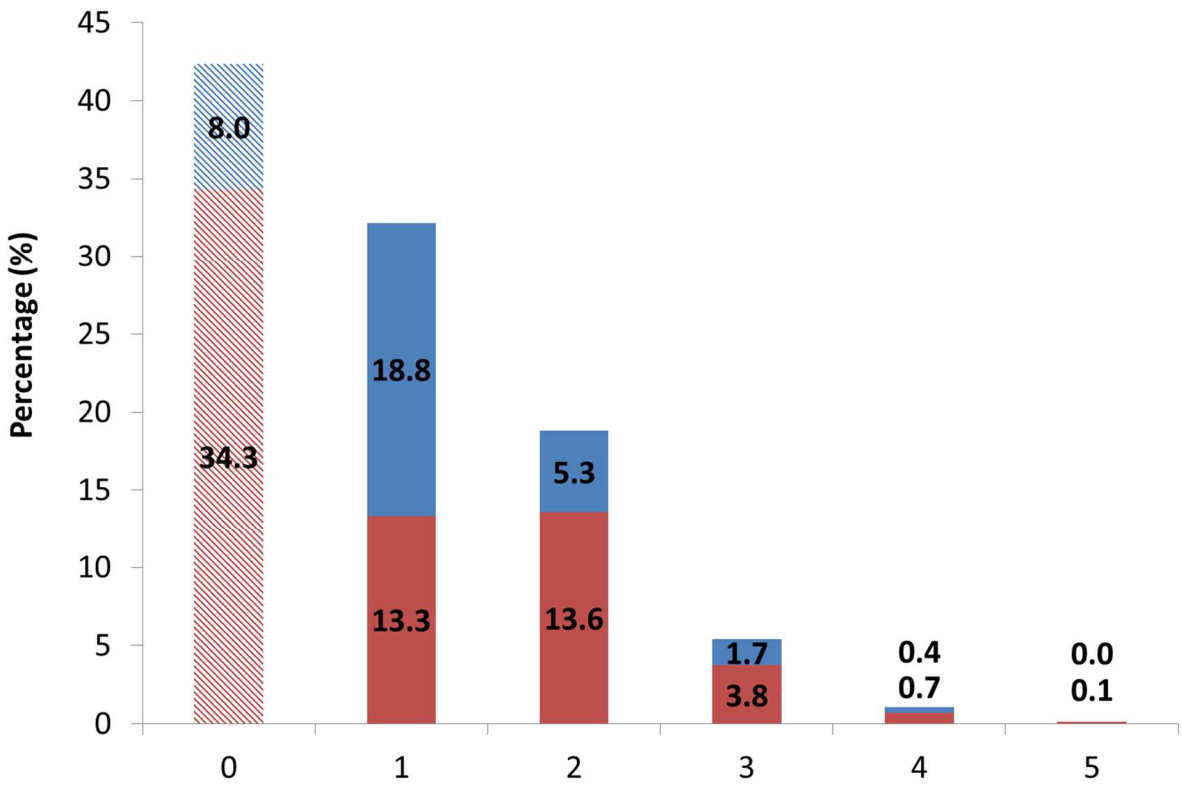

Fig. 3: Percentage of females and males that never experienced a sex change throughout the study (hatched red and blue, respectively) and that underwent one to five sex changes using their primary sex observed in year 1 (red and blue, respectively) for the cohort 1. Only oysters sexed every year from year 1 to year 6 are included (n = 1386). Oysters that changed only once are the protandric (18.8%) and protogynic (13.3%) oysters.

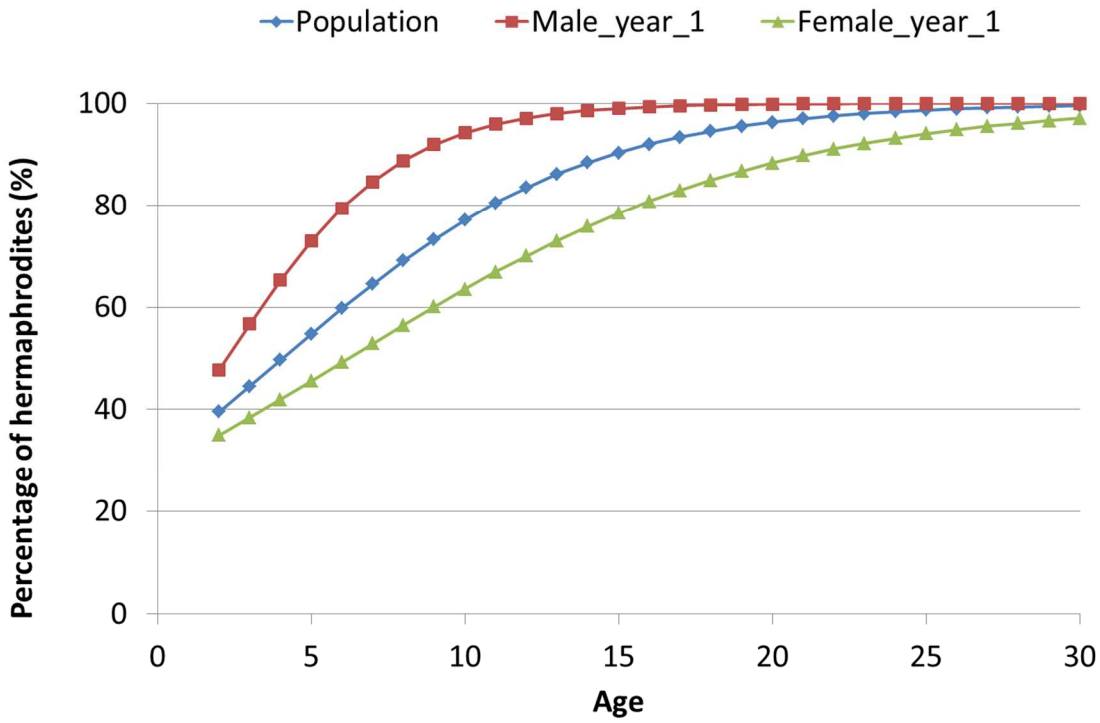

Fig. 4: Predicted cumulative percentage of sequential hermaphrodites in our population of *Crassostrea gigas* according to their age (in years), as well as for oysters sexed either male or female at year 1.

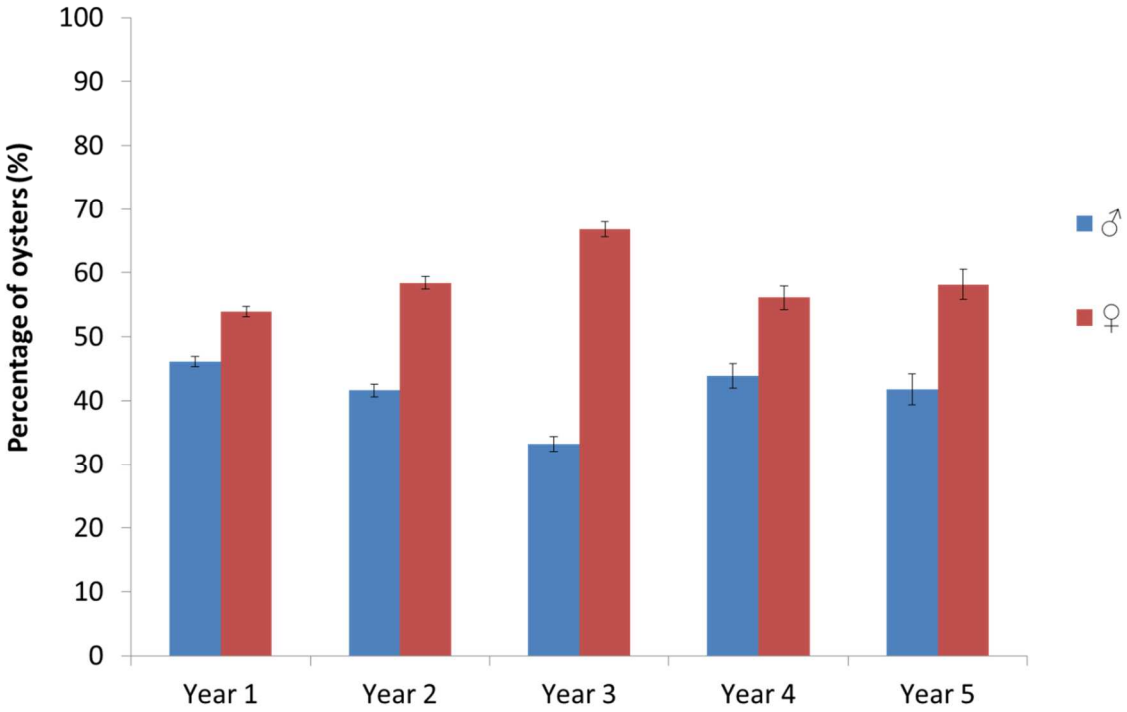

Fig. 5 Sex ratio (±SE) for the cohort 2 from year 1 to year 5. The number of oyster sexed each year is reported in Table 1.

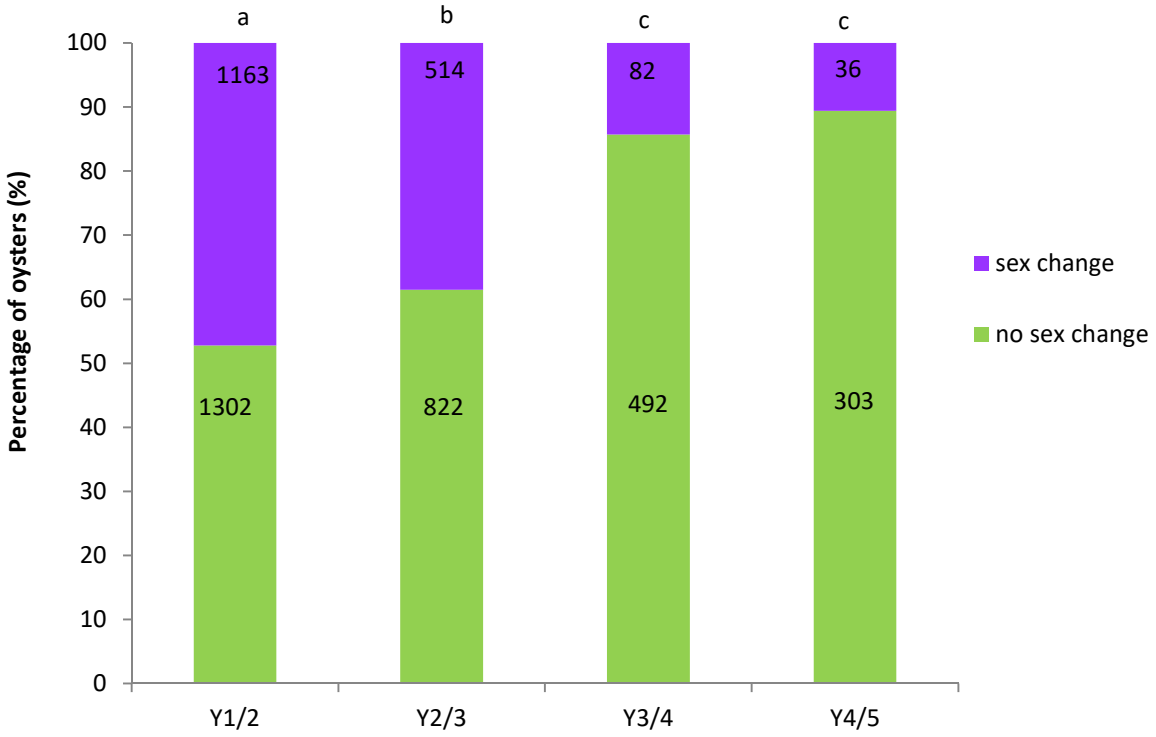

Fig. 6 Percentage of the oyster population for the cohort 2 experiencing or not a sex change between two consecutive years for each set (Y1/2 to Y4/5, Y being the year). The numbers of oysters that experienced or not a sex change are reported inside the bar. Oysters without any observable gametes and simultaneous hermaphrodites at year Y were excluded. The letters a, b, and c indicate significant differences among sets ($P < 0.0001$).

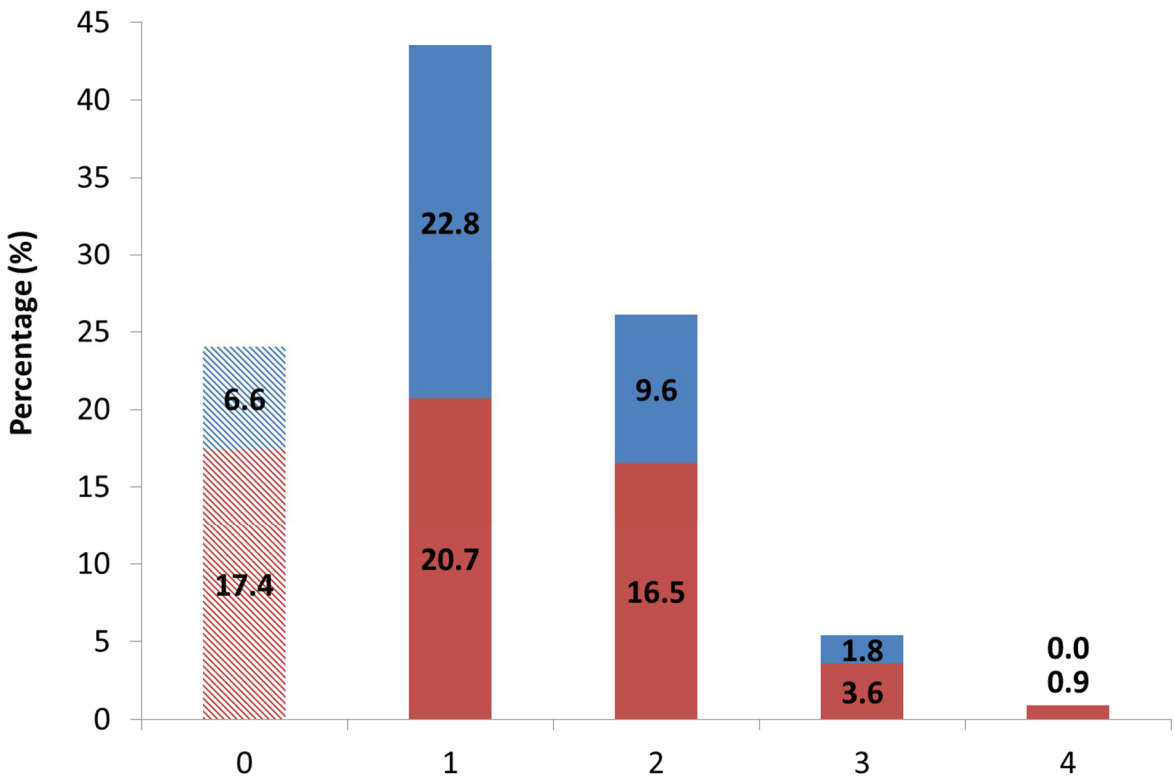

Fig. 7: Percentage of females and males that never experienced a sex change throughout the study (hatched red and blue, respectively) and that underwent one to four sex changes using their primary sex observed in year 1 (red and blue, respectively) for the cohort 2. Only oysters sexed every year from year 1 to year 5 are included (n = 333). Oysters that changed only once are the protandric (22.8%) and protogynic (20.7%) oysters.

Table 1 Number of oysters deployed in the field in year 0 for cohorts 1 and 2, and then sexed male or female each year

Year	2013	2014	2015	2016	2017	2018	2019
Cohort 1[1]	Y0	Y1	Y2	Y3	Y4	Y5	Y6
	13946	7488	4851	3440	2699	2093	1426
Cohort 2[1]		Y0	Y1	Y2	Y3	Y4	Y5
		6090	4320	2519	1541	685	421

[1] Y for year. Some oysters (<1%) were not sexed for a particular year (any gametes observed by biopsy/spawn). So they did not appear for that year while they did for the others. For example, an oyster of the cohort 1 could have been sexed in Years Y1, Y2, Y3, Y5 and Y6, but not in Y4.

Table 2 Regressions equations and inverse link given the cumulative percentage (CP) of the sequential hermaphrodites according to the age of the oysters in years for the cohort 1

Year	Regression equations	Inverse link
Population	Y= -0.8345+0.2047 x age	CP = Exp (Y)/(1+exp(Y))
Male at year 1	Y= -0.8097+0.3598 x age	CP = Exp (Y)/(1+exp(Y))
Female at year 1	Y= -0.9162+0.1471 x age	CP = Exp (Y)/(1+exp(Y))