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

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ABSTRACT

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

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

INTRODUCTION

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Animals

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

Characterization of batches

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

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

Dynamics of mortality

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

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

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

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

Characterization of gametogenesis by histology

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

Statistical analysis

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

RESULTS

Characterization of oyster batches

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

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

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

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

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

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

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

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

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

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

Mortality dynamics

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

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

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

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

In case of mortalities, *V. aestuarianus* was systematically detected.

Monthly seawater analysis showed that *V. aestuarianus* was present on sites from May, when mortality rates were less than 10.0% at all three sites. Pathogen loads reached $2.3x10^4$ UG/500 mL in July on the sites of Meuvaines and Géfosse but without triggering mortality at the Meuvaines site.

Histological monitoring of gametogenesis

In 2015, gametogenesis was monitored on the Meuvaines site. As no mortality was recorded on this site, gametogenesis monitoring was conducted on the Géfosse site in 2016.

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

Characterization of batches

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

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

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

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

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

Mortalities dynamics

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

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

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

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

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

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

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

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

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

Characterization of gametogenesis by histology

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

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

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

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

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

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

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

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REFERENCES

Allen, S.K. Jr, Downing, S.L., 1986. Performance of triploid Pacific oysters, *Crassostrea gigas* (Thunberg). I. survival, growth, glycogen content, and sexual maturation in yearlings. J. Exp. Mar. Biol. Ecol. 102 : 197-208 DOI:10.1016/0022-0981(86)90176-0

Allen, S.K. Jr, Downing, S.L., 1990. Performance of triploid oysters, *Crassostrea gigas*: Gametogenesis. Can. J. Fish. Aquat. Sci., Vol 47, 1213-1222

Allen, S.K.Jr., Guo, X., Burreson, G., Mann, R., 1996. Heteroploid mosaics and reversion among triploid oysters, *Crassostrea gigas*. Fact or artifact. Journal of Shellfish Research, 15(2), 514 (abstract).

Allen, S.K.Jr., Howe, A., Gallivan, T., Guo, X., Debrosse, G., 1999. Genotype and environmental variation in reversion of triploid *Crassostrea gigas* to the heteroploid mosaic state. Journal of Shellfish Research, 18(1), 293 (abstract).

Azéma, P., Lamy, JB., Boudry, P, Renault , T., Travers, M.A., Dégremont, L., 2017. Genetic parameters of resistance to *Vibrio aestuarianus*, and OsHV-1 infections in the Pacific oyster, *Crassostrea gigas*, at three different life stages. Genet Sel Evol. Feb 15;49(1):23. DOI: 10.1186/s12711-017-0297-2.

Benabdelmouna A., 2014. Résultats 2014 du réseau de suivi de l'aneuploïdie du naissain de captage . Journées "Observatoire Conchylicole, 17-18 décembre 2014 Nantes (Ifremer et Ecole Centrale).

Benabdelmouna, A., Ledu, C. 2007. Obtention de mollusques bivalves tétraploïdes à partir de géniteurs diploïdes. FR patent # 2 913 982 –A1

469 Benabdelmouna, A., Ledu, C., Gerard, A., 2007. Obtention de mollusques bivalves
470 tétraploïdes à partir du croisement de femelles diploïdes et de males tétraploïdes. FR
471 patent #2 913 983-A1
472 Benfey, T.J., 1999. The physiology and behaviour of triploid fishes. Rev. Fish. Sci. 7: 39-67.
473 DOI: 10.1080/10641269991319162
474 Berthelin, C., Kellner, K., Mathieu, M., 2000. Storage metabolism in the Pacific oyster
475 (*Crassostrea gigas*) in relation to summer mortalities and reproductive cycle (West
476 Coast of France). Comp Biochem Physiol B Biochem Mol Biol.;125:359–69. DOI :
477 10.1016/S0305-0491(99)00187-X
478 Callam, B.R., 2013. Genetic and environmental influence on triploid *Crassostrea virginica*
479 grown in Chesapeake Bay: Growth. Thesis. The College of William and Mary. 258 pp.
480 Retrieved from: http://www.vims.edu/library/Theses/Callam13.pdf
481 Costil, K., Royer, J., Ropert, M., Soletchnik, P., Mathieu, M. 2005. Spatio temporal variations
482 in biological performances and summer mortality of the Pacific oyster *Crassostrea*
483 *gigas* in Normandy (France). Helgol. Mar. Res. 59, 286-300. DOI : 10.1007/s10152-005-
484 0004-5
485 Dégremont, L., Ledu, C., Maurouard, E., Nourry, M., Benabdelmouna, A. 2014. Effect of
486 ploidy on the mortality of *Crassostrea gigas* spat caused by OsHV-1 in France using
487 unselected and selected OsHV-1 resistant oysters Aquaculture Research 47(3) DOI:
488 10.1111/are.12536
489 Eudeline, B., Allen, S.K. Jr., Guo, X., 2000. Optimization of tetraploid induction in Pacific
490 oysters, *Crassostrea gigas*, using first polar body as a natural indicator. Aquaculture.
491 187, 73–84 Manque DOI

Fleury, P.G, Ruelle, F., Claude, S., Palvadeau, H., Robert, S., d'Amico, F., Vercelli, C., Chabiran, J.M., 1999. Résultats des stations nationales REMORA (suivi de la croissance de l'huître creuse sur les côtes françaises), année 1998. IFREMER

François, C., Joly, J.P., Garcia, C., Lupo, C., Travers, M.A., Pépin, J.F., et al., 2013. Bilan 2012 du réseau REPAMO - Réseau national de surveillance de la santé des mollusques marins. http://archimer.ifremer.fr/doc/00143/25470/23625. pdf.

François, C., Joly, J.P., Garcia, C., Lupo, C., Travers, M.A., Tourbiez, D., et al., 2014. Bilan 2013 du réseau Repamo—Réseau national de surveillance de la santé des mollusques marins. http://archimer.ifremer.fr/ doc/00197/30798/29167.pdf.

François, C., 2015. Bilan 2014 du réseau Repamo—Réseau national de surveillance de la santé des mollusques marins. http://archimer.ifremer.fr/ doc/00256/36691/35303.pdf.

Gabe, M. 1968. Techniques histologiques. Masson et Cie, Paris VI.

Gagnaire, B., Soletchnik, P., Madec, P., Geairon, P., Le Moine, O., Renault, T., 2006. Diploid and triploid Pacific oysters, *Crassostrea gigas* (Thunberg), reared at two heights above sediment in Marennes-Oleron Basin, France: Difference in mortality, sexual maturation and hemocytes parameters, Aquaculture, 254: 606-616. DOI: 10.1016/j.aquaculture.2005.10.008

Garnier, M., Labreuche, Y., Garcia, C., Robert, M., Nicolas, J.L., 2007. Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. Microb Ecol 53 (2): 187-196. DOI: 10.1007/s00248-006-9061-9

Gérard, A., Ledu, C., Phelipot, R., and Naciri-Graven, Y., 1999. The induction of MI and MII triploids in the Pacific oysters *Crassostrea gigas* with 6-DMAP or CB. Aquaculture. 174. 229-242.

Glover, K.A., Madhun, A.S., Dahle, G., Sørvik, A.G.E., Wennevik, V., Skaala, Ø., Craig Morton, H., Hansen, T.J., Fjelldal, P.G., 2015.The frequency of spontaneous triploidy

517 in farmed Atlantic salmon produced in Norway during the period 2007–2014. BMC
518 Genetics 16:37 DOI: 10.1186/s12863-015-0193-0
519 Glude, J.B., 1975. A summary report of Pacific coast oyster mortality investigations (1965-
520 1972). In: The 3rd Us Japan meeting on Aquaculture, Tokyo, Japan, October 15 16,
521 1974, 29-34.
522 Goudenège, D., Travers, M.A., Lemire, A., Petton, B., Haffner, P., Labreuche, Y., Tourbiez,
523 D., Mangenot, S., Calteau, A., Mazel, D., Nicolas, J.L., Jacq, A., Le roux, F., 2015. A
524 single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogenicity in oysters.
525 Environ Microbiol. 2015 Nov; 17(11):4189-99. DOI: 10.1111/1462-2920.12699.
526 Goyard, E., 1996. Bilan national de croissance de l'huître creuse de 1993 à 1995. Rapport du
527 réseau REMORA. IFREMER La Trinité-sur-mer
528 Guo, X., Allen, S.K.Jr., 1988. Tetraploid shellfish. USA patent #5824841 (20/10/1998)
529
530 Guo, X., Cooper, K., Hershberger, W. K., Chew, K.K., 1992. Genetic Consequences of
531 Blocking Polar Body I with Cytochalasin B in Fertilized Eggs of the Pacific Oyster,
532 Crassostrea gigas: I. Ploidy of Resultant Embryos Biol. Bull. 183, 381-386. doi:
533 10.2307/1542013
534 Guo, X., Allen, S.K Jr., 1994. Viable tetraploids in the Pacific oyster (*Crassostrea gigas*
535 Thunberg) produced by inhibiting polar body I in eggs from triploids. Mol. Mar. Biol.
536 Biotechnol. 3 (1): 42-50
537 Guo, X., De Brosse, G.A., Allen, S.K Jr., 1996. All-triploid Pacific oyster (*Crassostrea gigas*
538 Thunberg) produced by mating tetraploids and diploids. Aquaculture. 142, 149-161
539 Heude Berthelin, C., Laisney, J., Espinosa, J., Martin, O., Hermandez, G., Mathieu, M.,
540 Kellner, K., 2001. Storage and reproductive strategy in *Crassostrea gigas* from two
541 different growing areas (Normandy and the Atlantic coast, France). Invertebrate
542 reproduction and development 40(1) :79-86 DOI: 10.1080/07924259.2001.9652500

543 Huvet, A., Normand, J., Fleury, E., Quillien, V., Fabioux, C., Boudry P., 2010. Reproductive
544 effort of Pacific oysters: A trait associated with susceptibility to summer mortality.
545 Aquaculture 304, 1-4, 95-99. DOI :10.1016/j.aquaculture.2010.03.022
546 Jouaux, A., Heude-Berthelin, C., Sourdaine, P., Mathieu, M., Kellner, K., 2010.
547 Gametogenetic stages in triploid oysters *Crassostrea gigas* : irregular locking of gonial
548 proliferation and subsequent reproductive effort. Journal of Experimental Marine
549 Biology and Ecology, 395 :162-170. DOI : 10.1016/j.jembe.2010.08.030
550 Labreuche, Y., Lambert, C., Soudant, P., Boulo, V., Huvet, A., Nicolas, JL., 2006. Cellular
551 and molecular hemocyte responses of the Pacific oyster, *Crassostrea gigas*, following
552 bacterial infection with *Vibrio aestuarianus* strain 01/32. Microbes Infect. 8: 2715–24.
553 DOI:10.1016/j.micinf.2006.07.020
554 Lipp, P.R., Brown, B., Liston, J., Chew, K., 1976. Recent findings on the summer diseases of
555 pacific oysters. Proc Nat Shellfish Assopc 65 : 9-10
556 Maurer, D., Comps, M. 1986. Mortalités estivales de l'huître *Crassostrea gigas* dans le bassin
557 d'Arcachon : facteurs du milieu, aspects biochimiques et histologiques. In : Pathology
558 in Marine Aquaculture, Vivarès, C.P., Bonami, J.R. and Jaspers, E. (Ed). European
559 Aquaculture Society, Special Publication N° 9, Bredene, Belgium, 29-41.
560 Nell, J.A., 2002. Farming triploid oysters. Aquaculture. 201, 69-90. DOI : 10.1016/S0044-
561 8486(01)00861-4
562 Normand, J., Le Pennec, M., Boudry, P. 2008. Comparative histological study of
563 gametogenesis in diploid and triploid pacific oysters (*Crassostrea gigas*) reared in an
564 estuarine farming site in France during the 2003 heatwave. Aquaculture 282 (1-4): 124-
565 129 DOI : 10.1016/j.aquaculture.2008.06.026

566 Perdue, J.A., Beattie, J.H., Chew, K.K., 1981. Some relationships between gametogenic cycle
567 and summer mortality phenomenon in the Pacific oyster (*Crassostrea gigas*) in
568 Washington State. J. Shellfish Res. 1, 9-16.
569 Royer, J., Ropert, M., Costil, K., 2007. Spatio-temporal in mortality, growth and condition of
570 the pacific oyster, *Crassostrea gigas*, in normandy (France) Journal of shellfish research
571 Vol 26, 4, 973-984
572 Samain J.F., Mc Combie H., 2008. Summer mortality of Pacific oyster *Crassostrea gigas*.
573 Collection Synthèse. Editions QUAE Cemagref, Cirad, Ifremer, Inra. 400p. ISSN :
574 1777-4624 Ref. : 02038
575 Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., Garcia, C., (2010). A large-
576 scale epidemiological study to identify bacteria pathogenic to Pacific Oyster
577 *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity.
578 Microb Ecol 59:787–798 DOI:10.1007/s00248-009-9620-y
579 Soletchnik, P., Le Moine, O., Faury, N., Razet, D., Geairon, P., Goulletquer, P., 1999.
580 Summer mortality of the oyster in the Bay Marennes-Oleron: Spatial variability of
581 environment and biology using a geographical information system (GIS). Aquatic
582 Living Resources Vol. 12 , 2 , 131-143. DOI : 10.1016/S0990-7440(99)80022-9
583 Tamate, H., Numachi, K.I., Mori, K., Itikawa, O., Imai, T., 1965. Studies on the mass
584 mortality of the oyster in Matsushima Bay VI. Pathological studies. Bull. Tohoku Reg.
585 Fish. Res. Lab. 25, 89-104.
586 Travers, M-A., Tourbiez, D., Parizadeh, L., Haffner, P., Kozic Djellouli, A., Aboubaker, M.,
587 Koken, M., Dégremont, L., Lupo, C., 2017. Several strains, one disease:experimental
588 investigation of *Vibrio aestuarianus* infection parameters in the Pacific oyster,
589 *Crassostrea gigas*. Vet. Res. (2017) 48:32 DOI 10.1186/s13567-017-0438-1

590 Vezzulli, L., Pezzati, E., Stauder, M., Stagnaro, L., Venier ,P., Pruzzo, C., 2015.Aquatic
591 ecology of the oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus*. Environ
592 Microbiol. 17(4):1065-80. DOI :10.1111/1462-2920.12484
593 Wadsworth, P.C., 2018. Comparing triploid and diploid growth and mortality in farmed
594 oysters, *Crassostrea virginica*, in the northern gulf of mexico. A thesis for the Degree of
595 Master of Science. Auburn University, 87 p http://hdl.handle.net/10415/6074
596 Yamamoto, S., Sugawara, Y., Nomaru, T., and Oshino, A., 1988. Induced triploidy in the
597 Pacific oyster, *Crassostrea gigas*, and performance of triploid larvae. Tohoku J. Agr.
598 Res. 39 (1): 47-59
599 Zhang, Y., Li, J., Qin, Y., Zhou, Y., Zhang, Y., Yu, Z. 2016. A comparative study of the
600 survival, growth and gonad development of the diploid and triploid Hong Kong oyster,
601 Crassostrea hongkongensis (Lam & Morton 2003)[J]. Aquaculture Research, 1-10 doi:
602 10.111/are.13081.
603
604
605
606
607
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FIGURE 1: *Three study sites in Normandy: Saint-Vaast-la-Hougue, Géfosse-Fontenay, Meuvaines.*

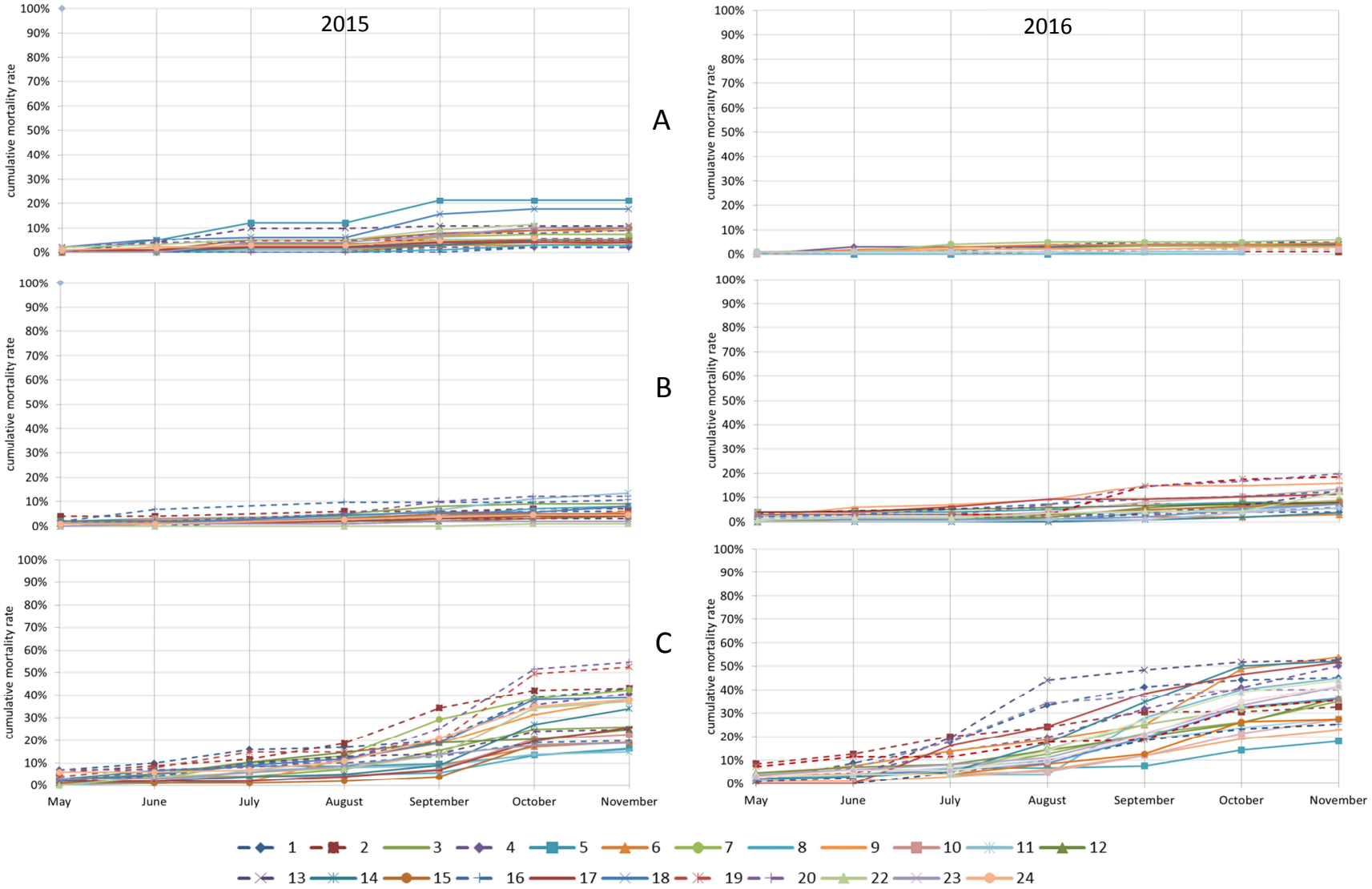

FIGURE 2: *Trend of cumulative mortality of 23 batches in 2015 (left) and 2016 (right) on three sites: Meuvaines (A), Saint-Vaast-la-Hougue (B), Géfosse (C). Triploid and diploid batches are respectively in dotted and solid line.*

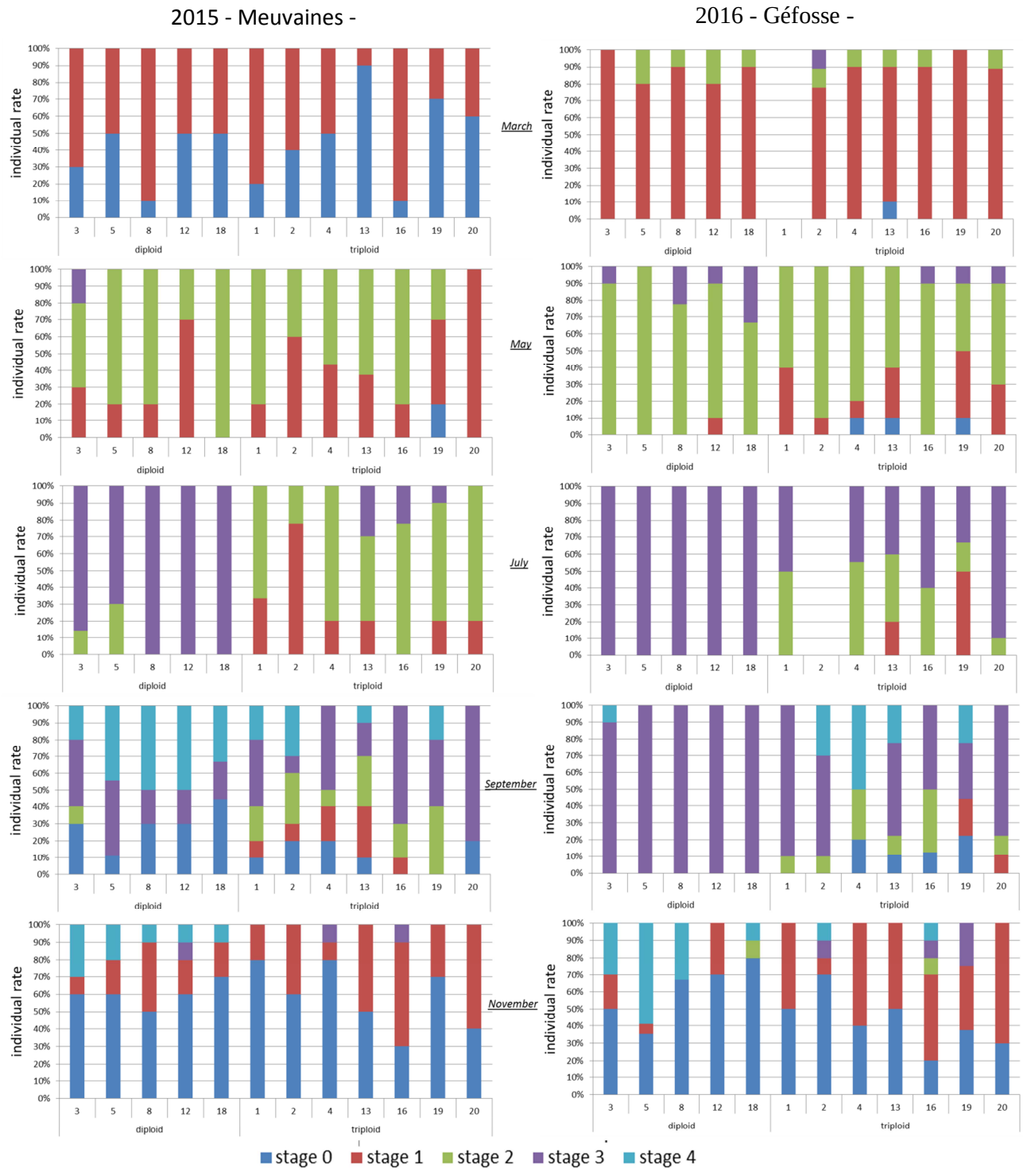

FIGURE 3: *Histogram of gametogenesis stage of diploid and triploid batches in accordance with months in Meuvaines in 2015 (left column) and in Géfosse in 2016 (right column) on March, May, July, September, November. The X-axis corresponds to batches and the Y-axis to individual rates in stage 0 (quiescent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

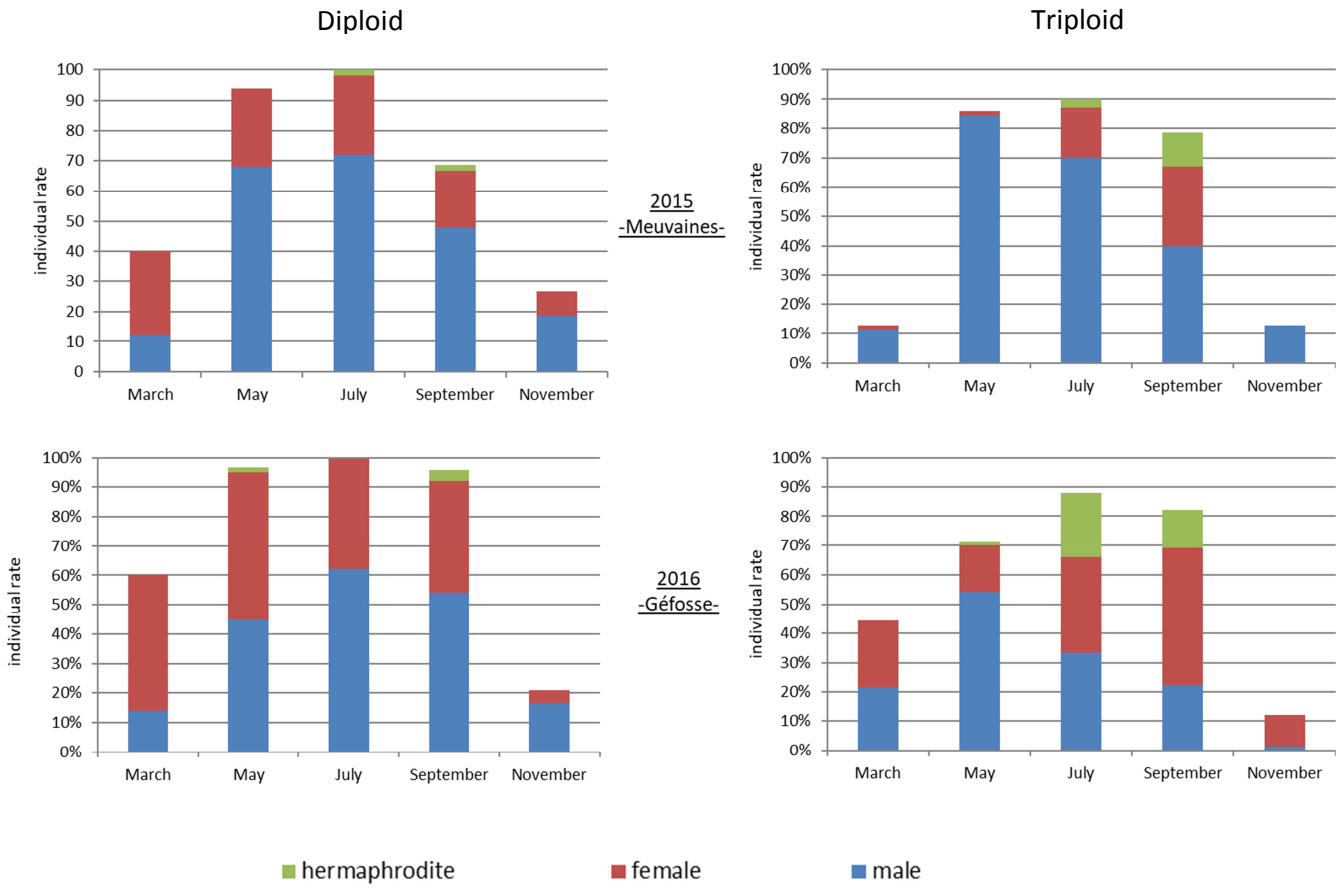

FIGURE 4: *Percentage of males, females and hermaphrodites individuals in 2015 and 2016 in the diploid (left) and triploid (right) batches (n= 50 individuals/diploid group/sampling point) and (n=70 individuals/triploid group/sampling point).*

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

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

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

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

			Percentage of incoherent individuals		
Batch	Ploidy	Hatchery	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