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ORIGINAL ARTICLE

Crassostrea gigas (Thunberg 1793) cultivation in southern Adriatic Sea (Italy): A one-year monitoring study of the oyster health

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Abstract

Pacific oyster (*Crassostrea gigas*) farming has developed in recent years along the Italian coasts of the Adriatic sea. This is particularly true in sites located in the northern area and only recently becoming more common in the southern area, which is different particularly in regards to temperature and trophic conditions. There is a lack of studies on the status of the oysters farmed in this area; therefore, the present research aimed to monitor the health conditions of adult triploid oysters that were kept in offshore longline culture system over one year. Environmental parameters were daily estimated from satellite. Biometric, immunological and oxidative stress parameters were measured monthly, looking also for the presence of pathogens, such as OsHV-1, *Vibrio aestuarianus* and *Vibrio splendidus*. During summer months, high temperatures and low food availability had a detrimental impact on the growth and immunological parameters which showed a progressive recovery during the autumn and winter months. Overall, no abnormal mortality was observed, and no histological alterations were evident. The presence of pathogens was negligible, and the oxidative stress parameters were poorly detected, except for the digestive glands during the autumn and winter seasons. On this basis, the data herein reported suggest that the area could be suitable for the development of triploid Pacific oyster farming, although animals would require a longer time to reach commercial size.

KEYWORDS
environment, immunity, oxidative stress, oyster, pathogens

1 | INTRODUCTION

In the European Union, marine bivalve molluscs account for 46.6% of the overall aquaculture production and for 23.7% of the value, with mussels and Pacific cupped oysters playing the predominant role for volume and for economic value respectively (Eurostat, 2018). The Japanese oyster *Crassostrea gigas* (Thunberg, 1793) adapts to different environmental conditions, showing good growth performances also in presence of wide temperature and turbidity fluctuations. These features have contributed to its worldwide diffusion (Grizel & Héral, 1991; Miossec et al., 2009) and to its introduction in Europe for aquaculture purposes in the late 60 s. In Italy, the annual production of shellfish is derived mainly from mussel (*Mytilus galloprovincialis*) and clam (*Ruditapes philippinarum*) cultivation in coastal areas and the lagoons of the Adriatic sea. Oyster production is currently limited to small-scale farming despite the rise of national consumer

demand. According to several field and modelling studies (Brigolin et al., 2017; Roncarati et al., 2010), the trophic level and temperature of the Adriatic sea are suitable for growing Pacific oyster (Tamburini et al., 2019), although the region is characterized by oligotrophic condition (Cerino et al., 2012) and by temperatures close to the critical upper thermal limit of the species (Bougrier et al., 1995; Pastor et al., 2018).

The present study aimed to investigate a set of indicators concerning the health conditions of adult triploid oysters (*C. gigas*), towards the end of the grow-out phase, in a longline farm located offshore in southern coast of the Adriatic sea. The set of indicators included biometric, immunological and oxidative stress parameters, which were monitored for one year with a monthly frequency, in association with the detection of bacterial (*Vibrio* spp.) and viral (Oyster Herpesvirus type-1) pathogens. In addition, environmental parameters were estimated from satellite data.

2 | MATERIALS AND METHODS

2.1 | Sampling site

The study was conducted in a longline farm in which Pacific oysters and Mediterranean mussels are co-farmed. The site is located in open sea at about 1.5 miles off the coast of the Puglia region in Italy, along the southern coast of the Adriatic sea (41°56.469′ North, 15°41.539′ East) (Figure 1). Seed (size T4-T6) of triploid oysters (*Crassostrea gigas*) was imported from France in December 2016. Oysters were cultured at an average depth of 6 m inside stacked baskets, made up of 4 polyethylene copolymer (model MP650) trays (65 cm × 40 cm × 10 cm). In the present study, specimens were sampled from 4 trays and each tray contained 216 oysters at the beginning of the study, for a total population of 864 animals. The same number of animals (n = 15) was collected monthly from each tray for a total of 60 oysters which were then pooled. Oysters were placed on ice and transferred within four hours to the laboratory of the Faculty of Veterinary Medicine of Teramo. Twenty oysters were used both for biometric and haemocyte parameters, while the remaining 40 oysters were shipped on ice to the laboratory of IZS Venezie of Legnaro (Padova). Here, only the oysters in good state of conservation were processed. Seven oysters were considered for histology analysis and 5 of them were also investigated for oxidative parameters, while the ones left were frozen for further molecular detection of the pathogens. The field survey started in March 2018 and ended in February 2019; however, due to adverse weather conditions, it was not possible to collect samples in November 2018. The mortality rate was estimated by direct counting of all oysters in four trays before each monthly sampling and was expressed as a percentage of dead oysters out of the whole live oyster population.

Daily time series of Sea Surface Temperature (SST) and chlorophyll-*a* concentration (Chl-*a*) were estimated at the sampling site by means of Satellite Remote Sensing (Porporato et al., 2020), both parameters being taken at 1 km^2 spatial resolution and retrieved from the Copernicus Marine Environment Monitoring Service (CMEMS; https://marine.copernicus.eu) on a daily basis. For SST, Level 4 was used (i.e. continuous spatio-temporal data resulting from model outputs) while for Chl-*a*, Level 3 was used (i.e. data mapped on uniform

FIGURE 1 Area investigated [Colour figure can be viewed at wileyonlinelibrary.com]

spatio-temporal grid but with gaps due to clouds presence). Daily data were aggregated as monthly means, in order to match the other indicator sampling frequencies.

2.2 | Haemocytes parameters

Haemolymph was sampled from 20 oysters, which were partitioned into two groups, in order to obtain 2 haemolymph pools. The fluid was collected from the pericardial sinus of living oysters by removing the upper shell after cutting the adductor muscle at its attachment to the shell. Total haemocyte count (THC) and haemocyte viability (HV) were calculated for each pool by a Cell Viability Analyzer (Vi-Cell, Beckman Coulter) that consists of a video imaging system associated with an automated analysis of suspended cells. HV was measured by the trypan blue dye exclusion method, and the results were reported as percentage of viable cells. THC was expressed as the number of haemocytes × 10^6 haemolymph ml^{-1}, including both viable and nonviable cells. After THC and HV determination, the Cell Viability Analyzer (Vi-Cell, Beckman Coulter) was used to measure the haemocyte spreading, expressed as a morphological parameter that was indicative of the cell shape changes following phagocytosis in vitro stimulation (Mosca et al., 2013). The haemolymph concentration of both pools was adjusted to 5 × 10^5 of viable haemocytes ml^{-1} and, for phagocytosis stimulation, an insoluble preparation of cell wall from the yeast *Saccharomyces cerevisiae* (Zymosan A, Sigma Aldrich) was incubated for 15 min with haemolymph, using a Zymosan-to-haemocytes ratio of 80:1. Haemolymph not incubated with Zymosan was analysed to measure the basal level of the parameter. In parallel, the haemocyte's ability to generate reactive oxygen species (ROS) following *in vitro* stimulation was monitored by a chemiluminescence assay (Lacoste, Malham, et al., 2001). Briefly, both haemolymph pools were adjusted to 5 × 10^5 of viable haemocytes ml^{-1} and then placed in 96-well microplate. Luminol (Sigma Aldrich) was added at 1 mM, and the Zymosan A was used as described above. The chemiluminescence was monitored by a multimode plate reader (Sinergy H1, Bio-Tek) for 60 min with 1 min time interval between consecutive readings of the same well. The intensity of the chemiluminescence was calculated by integrating the area under curve (AUC). All the haemocyte parameters were reported as a monthly mean that was obtained from the 2 haemolymph pools, each analysed in duplicate.

2.3 | Biometric parameters

Length, total weight and meat wet weight were determined monthly from the 20 oysters which were also used for the haemolymph collection.

The meat yield was expressed as the percentage ratio between meat wet weight and total weight (Cruz-Romero et al., 2007). The parameters were reported as a mean value for each month.

2.4 | Molecular detection of pathogens

Three pathogens, namely OsHV1, *Vibrio splendidus* and *Vibrio aestuarianus*, were monitored over one year on a total of 193 oysters. The variability of the number of the oysters analysed in each month was due to the fact that only the animals in good state of conservation were considered at their arrival to the reference laboratory. The gills and mantles from each animal were homogenized and stored at −20°C. DNA extraction was performed with the QIAamp® DNA Mini Kit (QIAGEN) following the manufacturer's protocol, and the purity was determined with a spectrophotometer. The samples were then diluted to a final concentration of 5 ng/µl and stored at −20°C.

2.4.1 | Molecular detection of ostreid Herpesvirus type 1 (OsHV-1)

OsHV-1 presence was revealed by a real-time PCR protocol, specifically designed to amplify the conserved region of OsHV-1 genome located in the ORF 100 gene with the primers HVDP-F (5′ ATTGATGATGTGGATAATCTGTG 3′) and HVDP-R (5′GGTAAATACCATTGGTCTTGTTCC 3′), according to the guidelines of the European Union Reference Laboratory for Molluscs Diseases (IFREMER, 2011a). The expected amplicon size was of 197 bp. The amplification was performed in a 25 µl reaction volume, comprising 5 µl DNA, 1× QuantiFast SYBR Green Master Mix (QIAGEN) and 0.5 µM of each primer. Cycling conditions included an initial activation step at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 60 s, 72°C for 45 s. The specificity of the amplification reaction was assessed by melting temperature curve analysis, in the temperature range of 65–95°C, with a step of 0.5°C and a hold of 10 s. The expected amplicon melting temperature was 79.5 ± 0.5°C. The entire analysis was carried out with a CFX96 Touch Real Time PCR System (Bio-Rad) and the software MANAGER 2.1.

2.4.2 | Molecular detection of *Vibrio aestuarianus* and *Vibrio splendidus*

Detection and quantification were carried out with distinct real-time PCR protocols targeting a specific region of *V. splendidus* 16S rDNA gene and *V. aestuarianus* dnaJ gene respectively (IFREMER, 2013). The primers and probe used for *V. splendidus* corresponded to the 16S SpF2 (5′ ATCATGGCTCAGATTGAACG 3′), 16S SpR2 (5′ CAATGGTTATCCCCCACATC 3′) and 16S probe (5′ FAM-CCCATTAACGCACCCGAAGGATTG-BHQ1 3′). The primers and probe used for *V. aestuarianus* corresponded to dnaJ-F (5′ GTATGAAATTTTAACTGACCCACAA 3′), dnaJ-R (5′ CAATTTCTTTCGAACAACCAC 3′) and dnaJ-probe (5′ Texas Red TGGTAGCGCAGACTTCGGCGAC-BHQ2 3′). The qPCR reactions for *V. splendidus* were carried out in 20 µl of reaction volume containing 5 µl of DNA, 1× Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies), 0.8 µM of each primer and 0.2 µM of probe. The PCR was performed

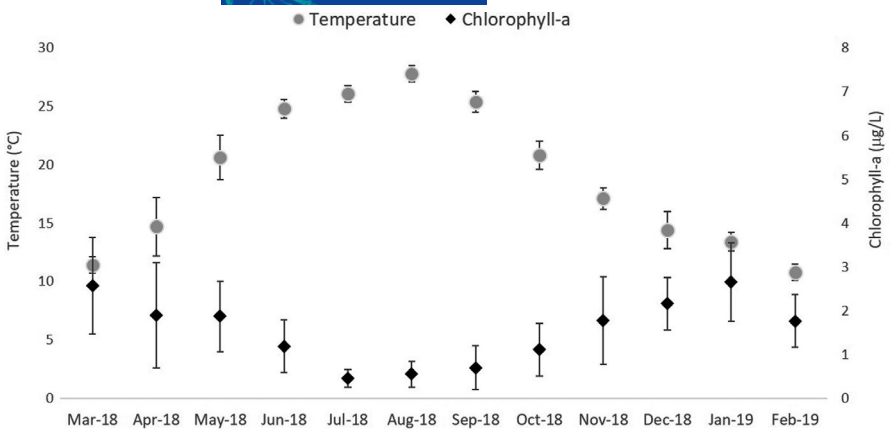

FIGURE 2 Monthly variations of seawater temperature and chlorophyll-*a* concentration. Mean values were calculated from daily data obtained from satellite

under the following thermal conditions: initial activation step at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 90 s.

The qPCR for *V. aestuarianus* was performed in 20 µl of reaction volume containing 5 µl of DNA, 1× Brilliant III Ultra-Fast QPCR Master Mix, 0.3 µM of each primer and 0.2 µM of probe. The PCR was performed under the following thermal conditions: initial activation step at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 20 s. The entire analysis was carried out with a CFX96 Touch Real Time PCR System (Bio-Rad) and the software MANAGER 2.1. In order to quantify the samples under analysis, in each run standard samples consisting of quantified plasmidic DNA of Vibrio of the species of interest were amplified and analysed. Such standards covered the range 10^6–10^2 plasmidic copies/µl by 5 log10 dilution points for *V. splendidus* and the range 10^5–10^1 plasmidic copies/µl by 5 log10 dilution points for *V. aestuarianus*, each represented by independent triplicates. A regression analysis was then carried out, relating the observed standard Cycle Threshold values to their log-transformed concentration. For *V. aestuarianus* method, the limit of quantification was 10 copies/µl. For *V. splendidus* method, the limit of quantification was 100 copies/µl.

2.5 | Evaluation of Malondialdehyde (MDA) and Nitrotyrosine (NT) by immunohistochemistry

Malondialdehyde (MDA) and Nitrotyrosine (NT) were determined monthly on 5 specimens by immunohistochemistry, according to previous methods (Boscolo Papo et al., 2014; Fiocchi et al., 2020). Both parameters were measured in the digestive glands, visceral connective tissue, gills and mantle. Transverse sections (about 5 mm) were fixed in 4% paraformaldehyde at 4°C. After 48 h, soft tissues were washed in distilled water, dehydrated and embedded in paraffin. Fine sections (3 µm) were cut using a microtome, deparaffinized in xylene, rehydrated in graded ethanol and washed in distilled water. After mounted in adhesion microscope slides (Marienfeld, DE), serial sections were incubated in Cell Conditioning Solution (Roche, SA) at 97°C for 15 min and in EnVision FLEX Peroxidase-Blocking Reagent (Dako, USA) at RT for 10 min. Slides were incubated overnight at 4°C with rabbit polyclonal MDA antiserum, dilution 1:1500 (Abcam, UK) and mouse monoclonal NT antiserum, dilution 1:1000 (Genetek, USA). Negative control was performed by incubating sections with PBS instead of the specific antibody. The incubation of sections was then performed with the detection system EnVision FLEX/HRP (Dako, USA) and the chromogen EnVision FLEX Substrate Buffer DAB (Dako, USA). Finally, slides were counterstained with the EnVision FLEX Haematoxylin (Dako, USA).

2.6 | Histological evaluation

Histological examination of 7 oysters was carried out monthly, according to Standard Operative Procedures (IFREMER, 2011b). Transverse sections containing mantle, gills, stomach, intestine and digestive diverticula were fixed for 48 h in Carson's solution. The tissues were dehydrated by immersion in a graded series of ethanol and embedded in paraffin; 3 µm sections were obtained and stained with Harris' Haematoxylin and Eosin-Phloxine. Microscope slides were examined using 100×, 250×, 400× and 1000× magnification to detect the presence of any parasites and pathological alterations.

2.7 | Statistical analysis

One-way analysis of variance (ANOVA) was applied to verify overall significant differences between sampling dates for the haemocyte parameters and meat yield. The level for accepted statistical significance was $p < 0.05$. Pearson's correlation analysis was used to find relationships between environmental and haemocyte parameters or meat yield, with a level for accepted statistical significance of $p < 0.05$.

3 | RESULTS

3.1 | Environmental parameters and mortality rate

The mean monthly SST ranged from 10.8°C in February to 27.8°C in August (Figure 2). Chlorophyll-*a* concentration was lower than 1 µg/L from July to September, while in the other months, the mean values were below 2 µg/L, except for March (2.57 µg/L), January (2.66 µg/L) and December (2.16 µg/L) (Figure 2). The oyster survival

rate was optimal from February to July, while a low rate of mortality was detected in August (3.6%), followed by intense episode in September (13.3%). At the end of the study period, the cumulative mortality reached 19.5%.

3.2 | Haemocytes parameters

Mean values of the HV showed significant seasonal variations between sampling dates ($p < 0.05$), ranging from a minimum of 74.3% in July and a maximum of 94.8% in December (Figure 3). Significant changes were also observed for the THC, which ranged from 0.62×10^6 haemolymph ml^{-1} in May to 1.66×10^6 haemolymph ml^{-1} in April ($p < 0.01$) (Figure 3). However, no correlation was found between HV or THC and temperature or chlorophyll-*a* concentration (p = n.s.).

The haemocyte's ability to respond to phagocytosis in vitro stimulation showed significant variations between sampling dates, both in terms of spreading activity and of ROS generation ($p < 0.05$). In particular, the best morphological activity was detected in December and January, while the lowest response was seen in May, July and October (Figure 4). The generation of ROS, as monitored by chemiluminescence assay, revealed the best response from December to February, while the lowest value was recorded in July (Figure 5).

The haemocyte spreading was negatively correlated to temperature ($\rho_s = -0.662$, $p < 0.05$) and positively correlated to chlorophyll-*a* concentration ($\rho_s = 0.664$, $p < 0.05$). Also, the luminescence activity was negatively correlated to temperature ($\rho_s = -0.736$, $p < 0.05$) and positively associated with chlorophyll-*a* concentration ($\rho_s = 0.602$; $p < 0.05$). Finally, a strong positive correlation was noted between the haemocyte spreading and the haemocyte luminescence ($\rho_s = 0.859$, $p < 0.05$).

3.3 | Biometric parameters

A significant variation of the meat yield was observed between sampling dates ($p < 0.05$) with higher values in April, May, January and February, and lower values in the summertime (Figure 6). The meat yield was negatively correlated to SST ($\rho_s = -0.801$, $p < 0.05$) and positively to chlorophyll-*a* concentration ($\rho_s = 0.808$, $p < 0.05$). Oysters reached a final mean length of 87 mm in February 2019, starting from 62 mm in March 2018. The mean meat wet weight reached 14.5 g at the end of the study, starting from 3.1 g in March 2018. The greatest increase in both parameters was observed from December to February, while a constant trend was revealed from March to October (Figure 7).

3.4 | Molecular detection of pathogens

Only 3 oysters (3/193) resulted as positive for OsHV1 in different months and only 1 animal (1/193) was found positive for *V. aestuarianus*, thus resulting in a prevalence of 1.5% and 0.5% respectively. *V. splendidus* was found in most of the oysters (174/193), showing the highest prevalence (90.1%). The bacterial DNA load in oysters positive to *V. splendidus* was highly variable between animals with mean values in the order of $10^2/10^3$ DNA copies. Moreover, some animals (27/174, 15.5%) resulted as positive, but with a non-quantifiable bacterial load. All data are reported in Table 1.

3.5 | Evaluation of Malondialdehyde (MDA) and Nitrotyrosine (NT) by immunohistochemistry

As emerged through the qualitative evaluation of red-dark colour precipitates, NT immunoreactivity was observed with the greatest intensity in the digestive glands and in periglandular connective tissue of the oysters which were sampled during the autumn and winter seasons (Figure 8). MDA immunoreactivity was weaker than NT and was mainly observed in the periglandular connective tissue (Figure 9). As also described for NT, the highest number of MDA precipitates was detected in autumn and winter samples.

3.6 | Histological evaluation

The examination of histological sections revealed overall good health conditions; pathological alterations were not detected.

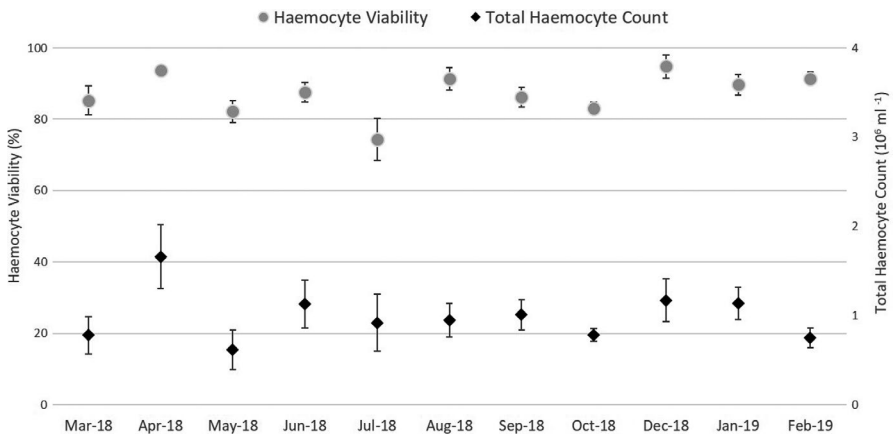

FIGURE 3 Monthly variations of total haemocyte count (THC) and haemocyte viability (HV). Mean values were calculated from 2 haemolymph pools, each composed of 10 oysters and analysed in duplicate

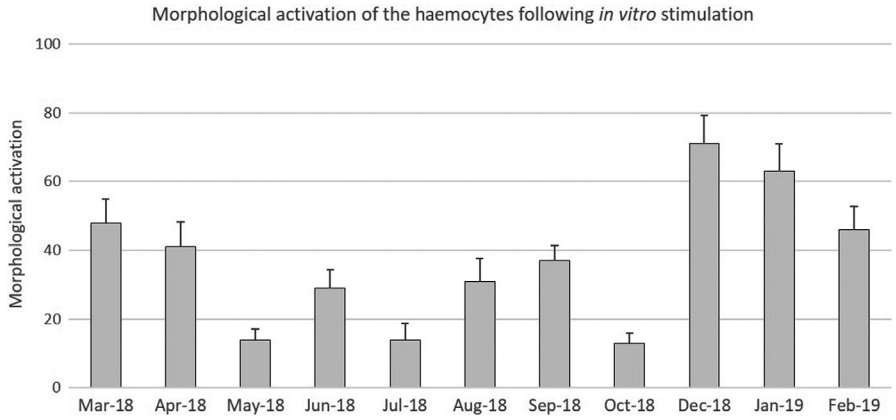

FIGURE 4 Monthly variations of the morphological activity of the haemocytes following phagocytosis *in vitro* stimulation. Mean values were calculated from 2 haemolymph pools, each composed of 10 oysters and analysed in duplicate. Morphological activity was reported as an arbitrary parameter of the haemocyte circularity variations

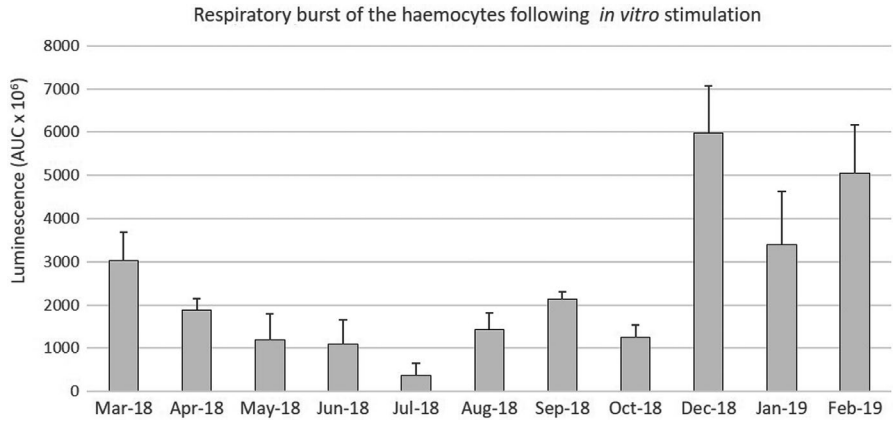

FIGURE 5 Monthly variations of the respiratory burst of the haemocytes following phagocytosis in vitro stimulation. Mean values were calculated from 2 haemolymph pools, each composed of 10 oysters and analysed in duplicate. Respiratory burst was reported as luminescence Area Under Curve (AUC)

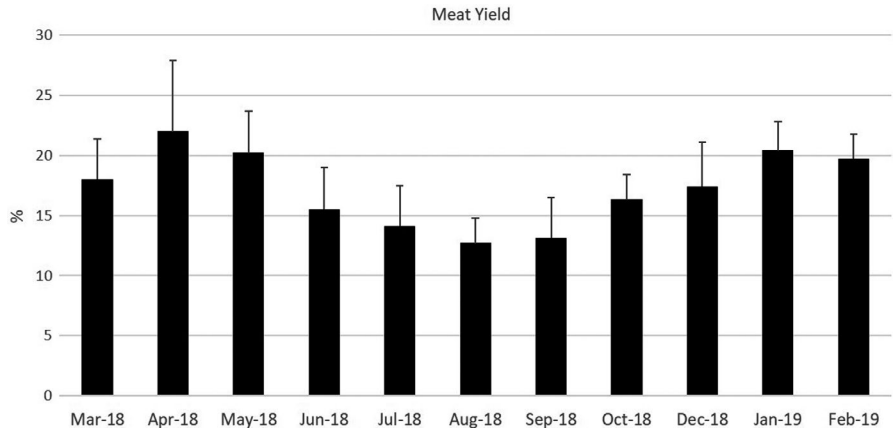

FIGURE 6 Monthly variations of meat yield, reported as mean values from 20 oysters

4 | DISCUSSION

Haemocytes represent the pivotal cells for the immunity of marine bivalve molluscs (MBM) (Zannella et al., 2017). Their morpho-functional characteristics have been widely investigated as parameters for monitoring the health conditions of MBM in field and laboratory studies (Bocchetti et al., 2008; Farcy et al., 2013; Gorbi et al., 2008; Hannam et al., 2009).

In the present study, the total haemocyte count (THC) showed significant variations between months, as previously described (Delaporte et al., 2006, 2007). However, no correlations were found with the environmental parameters, including the temperature, differently from what was described by some authors in *C. gigas* (Rahman et al., 2019).

Regarding this discrepancy, we could speculate on the site of haemolymph collection, conducted in this study from the pericardial cavity and, as described by previous authors, the origin of haemolymph may influence the level of haemocyte parameters (Gagnaire et al., 2008).

A relatively mild haemocyte response to phagocytosis in vitro stimulation was observed from May to October, with a negative peak in July, both in terms of morphological activity and generation of the respiratory burst. The haemocyte activity was negatively

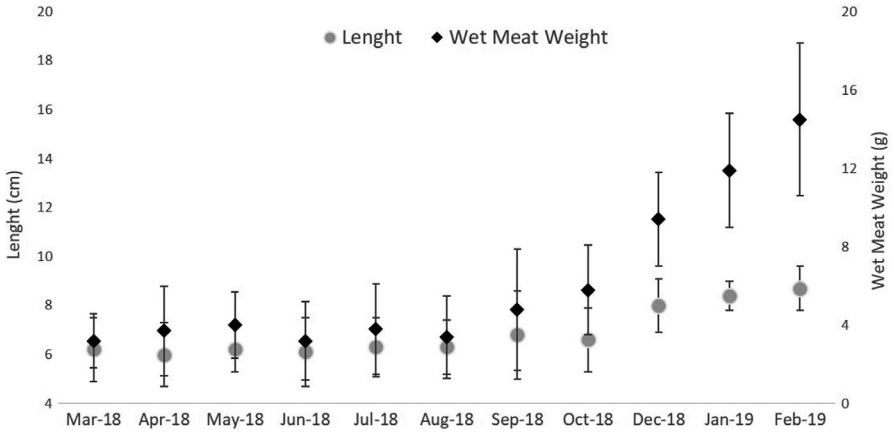

FIGURE 7 Monthly variations of lenght and meat wet weight, reported as mean values from 20 oysters

TABLE 1 Monthly prevalence of OsHV-1, *Vibrio splendidus* and *Vibrio aestuarianus* and DNA bacterial load in the positive oysters

	OsHV-1 (number of positive oysters on total)	*Vibrio aestuarianus* (number of positive oysters on total and copies of DNA μl^{-1})	*Vibrio splendidus* (number of positive oysters on total and copies of DNA μl^{-1})
March 2018	1/15	0/15	10/15 Positive with mean copies of 296 (100 - 692) 5/15 Positive not quantifiable
April 2018	0/15	0/15	14/15 Positive with mean copies of 1357 (111 - 6958) 1/15 Positive not quantifiable
May 2018	0/16	0/16	15/16 Positive with mean copies of 492 (117 - 2067) 1/16 Positive not quantifiable
June 2018	0/20	0/20	20/20 Positive with mean copies of 2384 (200 - 7019)
July 2018	1/30	0/30	29/30 Positive with mean copies of 2085 (111 - 22359) 1/30 Positive not quantifiable
August 2018	0/19	0/19	0/19
September 2018	0/22	0/22	20/22 Positive with mean copies of 1378 (171 - 6123) 2/22 Positive not quantifiable
October 2018	0/15	0/15	11/15 Positive with mean copies of 769 (143 - 3594) 4/15 Positive not quantifiable
December 2018	1/15	0/15	14/15 Positive with mean copies of 756 (126 - 2313) 1/15 Positive not quantifiable
January 2019	0/10	1/10 Positive (362)	10/10 Positive with mean copies of 9235 (2322 - 29313)
February 2019	0/16	0/16	4/16 Positive with mean copies of 172 (136 - 271) 12/16 Positive not quantifiable

correlated with SST, which was characterized by mean values higher than 25°C from June to September, and between 20°C and 25°C in May and October. The effects of temperature on the oyster haemocyte have been widely investigated, mainly in laboratory experimental condition. Previous studies reported that haemocytes from *C. gigas* kept at 21°C for 4 weeks showed a reduced ability to ingest *Vibrio anguillarum* when compared with oysters maintained at 12°C (Malham et al., 2009). However, other authors reported that haemocytes from *C. gigas* exposed to 35°C for 4 h preserved their ability to ingest fluorescent beads (Gagnaire, Frouin, et al., 2006). Additionally, *C. gigas* exposed to a temperature of 25°C for 2 weeks showed a greater haemocyte activity to ingest yeasts, when compared with oysters kept at

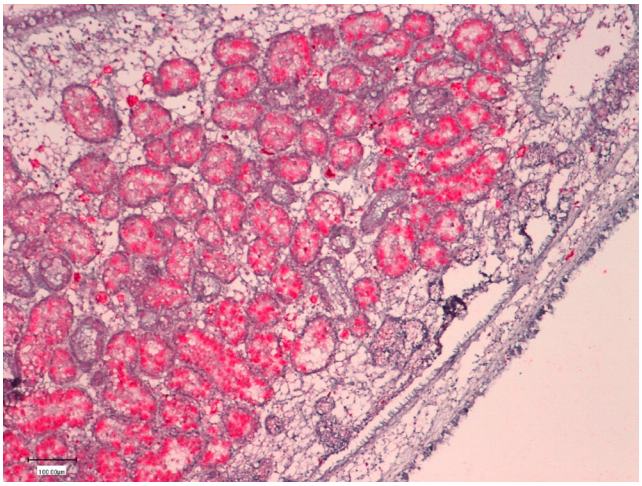

FIGURE 8 Nitrotyrosine (NT) immunoreactivity in the digestive glands and relative connective tissue in a representative winter sample (100×) [Colour figure can be viewed at wileyonlinelibrary.com]

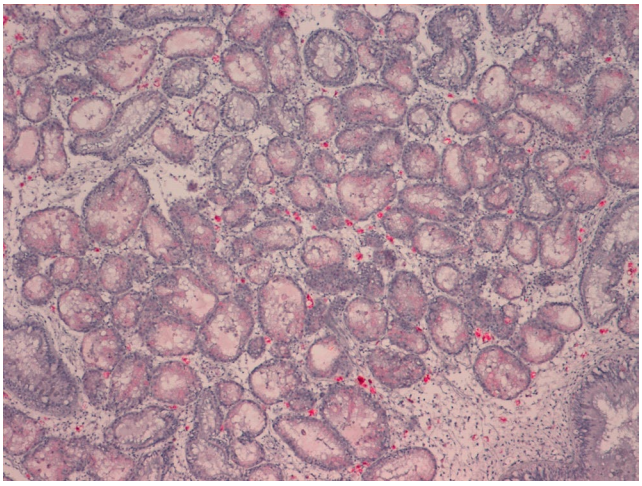

FIGURE 9 Malondialdehyde (MDA) immunoreactivity in the periglandular connective tissue in a representative winter sample (100×) [Colour figure can be viewed at wileyonlinelibrary.com]

15°C (Rahman et al., 2019). The haemocytes of *C. virginica* exposed at 28°C for 1 week showed a reduced ability to engulf fluorescent beads and a non-significant increase in the respiratory burst after zymosan A stimulation (Hegaret et al., 2003), whereas a temperature of 25°C impaired the haemocyte adhesion in *Ostrea edulis* (Fisher et al., 1987). Laboratory and field data are difficult to compare because multiple environmental stressors can occur in field condition at the same time, acting in a synergistic way and for a longer time (Soletchnik et al., 2007). In this sense, salinity variations (Guo et al., 2015), hypoxia (Boyd & Burnett, 1999), hypercapnia (Ivanina et al., 2014) and pollutants (Gagnaire, Thomas-Guyon, et al., 2006) all represent stressors that can impair the immune competence of the oyster haemocytes. In the present study, we did not have the possibility to conduct a more exhaustive monitoring of environmental parameters. In addition, we cannot exclude that seasonal variations of the total haemocyte response in *C. gigas* could depend not only on the direct effect of single or multiple stressors, but also on seasonal distribution patterns of the various haemocyte types in the haemolymph (Delaporte et al., 2007; Malham et al., 2009), primarily hyalinocytes and granulocytes, the latter having the most active role in the phagocytosis (Wang et al., 2017).

The present study also revealed a positive correlation between the haemocyte response and the chl-*a* concentration, which is an expression of phytoplankton abundance. Low levels of chl-*a*, particularly during the summer season, corresponded with a weaker haemocyte activity. Therefore, we could suppose a negative impact of low nutrients availability on the energy reserves, such as to determine a general weakness of the oyster physiology, including the immune response. The relationship between a reduced haemocyte activity and a low energy status was investigated in *C. gigas* oysters during the active gametogenesis (Delaporte et al., 2006). The reproductive cycle demands high levels of energy consumption; thus, oyster may have insufficient energy for immune defence and could become more susceptible to environmental stress (Samain et al., 2007) and pathogens (Li et al., 2009). In the present study, triploid oysters were used and they are generally considered as sterile or partially sterile animals (Jouaux et al., 2010). For this reason, they are characterized by a lower mortality rate and by a higher defence ability compared to diploid oysters (Gagnaire, Soletchnik, et al., 2006). Therefore, the impairment of the haemocyte response could be due to an insufficient energy input associated with high temperatures and low values of phytoplanktonic density, rather than to a shift of the energy allocation among different physiological processes. However, considering that gametogenesis in some lineages of triploid oysters is delayed but not absent, some batches can reach an advanced stage of reproduction and can suffer homeostasis disruption leading to mortality events comparable or even more intense than diploid oysters (Houssin et al., 2019).

In the present study, the growth of the oysters was negatively correlated with temperature and positively associated with chl-*a* concentration, both parameters being widely recognized as the main environmental driving forces for oyster growth (Cassis et al., 2011; Kang et al., 2010; King et al., 2006). The concentration of chl-*a* has been commonly used to investigate the oyster growth dynamics; however, other parameters should be considered as food quantifiers, such as composition and size of phytoplankton (Barraza-Guardado et al., 2008), bacteria (Rosa et al., 2018) and particulate organic matter (Nathalie et al., 2008). Our study lacks these additional trophic parameters that could aid further understanding of the slow growth rates observed. Indeed, our results suggest that oysters would require longer time to reach a commercial size in comparison with other productive sites in the Adriatic sea (Bordignon et al., 2020) and in the Mediterranean sea (Graham et al., 2020). Although we have no detailed data about economic outcomes of the farm, we presume low competition in terms of oyster productivity. However, the commercial distribution of the product in a regional context with strong tourist vocation and the proper management of this short supply chain would seem to guarantee a certain degree of profitability for farmers. Moreover,

the area investigated is classified as 'zone A' by European Union and National regulations; therefore, the oysters satisfy microbiological requirements and can be submitted directly to market with further shortening of supply chain.

On the basis of environmental, biometric and immunological parameters, a poor health condition of the oysters was hypothesized during summer months, while a recovery of the homeostatic condition was observed during autumn and winter months, although the oxidative stress parameters (MDA and NT) were particularly detected from November to February in digestive glands and periglandular connective tissue. Malondialdehyde (MDA) is a toxic aldehyde that is derived from the degradation of polyunsaturated fatty acids (PUFAs) and its presence at high levels is indicative of lipid peroxidation (Zapata-Vivenes & Nusetti, 2007). Nitrothyrosine (NT) is a relatively stable stress marker for the production of peroxynitrite that represents a powerful oxidant molecule (Fiocchi et al., 2020). The increase in MDA and NT that we observed in digestive glands and relative connective tissue during colder months could probably be ascribed to the greater food availability and to the subsequent increase in the energy metabolism that is strictly related to the production of oxidant molecules (Chaini et al., 2016; Quijano et al., 2016).

The discrepancy between the high expression of NT, as an indicator of the oxidant peroxynitrite generation, and the low detection of MDA, as an indicator of the lipid peroxidation, may rely on the protective effect of the antioxidant systems that prevents cell oxidative damage (Canesi, 2015; Ferreira et al., 2019; Liu & Wang, 2016). In this sense, the limited efficiency of the antioxidant defences and the increased level of lipid peroxidation may have been associated with oyster mortality events (Gènard et al., 2011).

In the present study, a mortality event occurred in September (13.3%), while low rates of mortality were registered in August (3.6%) and October (2.3%). As indicated by the French network monitoring programs ('Repamo' and 'Remora'), oyster mortality is considered abnormal if it affects more than 15% of cultivated stock over an interval of 15 days, while summer mortality reach or exceed 30% on a regular basis (Ropert et al., 2008). Therefore, the mortality peak that we found in September could be considered as a physiological event. The event was not related to sudden and intense variations of temperature or chl-*a* and, to the best of our knowledge, was not due to other environmental stressors that occurred in that specific period. Moreover, the presence of the pathogens that we investigated cannot account for this mortality. Oyster Herpesvirus type-1 (OsHV-1) and its microvariants, particularly µVar and related genotypes, have been associated worldwide with oyster mortality outbreaks, mainly involving juveniles (Solomieu et al., 2015 and references inside). In the present study, only 3 oysters resulted as positive for OsHV-1, thus revealing a very low prevalence of all the animals that were analysed for the virus. Previous molecular study showed a high prevalence of OsHV-1 in healthy adult oysters, suggesting that OsHV-1 can infect adult animals in the absence of symptoms and that it can also persist in hosts following primary infection (Arzul et al., 2002). Nevertheless, the determination of viral load thresholds for OsHV-1 is fundamental to discriminating the different degrees of infection from the mechanical carriage (Odeon et al., 2011; Pèpin et al., 2008). At least nine OsHV-1 genotypes, mostly related to the µVar cluster, were found in healthy adult and juveniles oysters from natural beds in Italy and this epidemiological study also revealed a low viral load and a prevalence ranging from 0% to 26.7%, depending on the sites of collection along Italian coasts (Burioli et al., 2016). OsHV-1 and the pathogenic µVar were previously described at low concentration in juveniles oysters that were reared off-coast of the middle Adriatic sea, but abnormal mortality, impairment of growth and histological lesions were not observed (Dundon et al., 2011). Nevertheless, the role of OsHV-1 as an opportunistic pathogen was described in the Sacca of Goro lagoon in the northern Adriatic sea, where the summer mortality was correlated with the critical environmental factors (high temperature, hypoxia and eutrophication) and to the co-existence of OsHV-1 with other pathogens for oysters, such as *Vibrio splendidus* and *Vibrio aestuarianus* (Domeneghetti et al., 2014). *V. splendidus* polyphyletic group and *V. aestuarianus* have been widely investigated as bacterial causes for mortality outbreaks of *Cr. gigas*, both in adults and juveniles animals (Azèma et al., 2016; Garnier et al., 2007; Gay et al., 2004; Lacoste, Jalabert, et al., 2001). Both *Vibrio* species represent common environmental microorganisms that colonize marine niches, but *V. splendidus* clade is constantly found in abiotic and biotic matrices while *V. aestuarianus* is poorly detected (Lopez-Joven et al., 2018; Vezzulli et al., 2015). Indeed, in the present study *V. aestuarianus* was detected only in 1 oyster while a high prevalence was found for *V. splendidus* groups. Although no further investigation was conducted to reveal the presence of pathogenic strains, the bacterial load that we detected by the molecular method and the ubiquitous nature of the *V. splendidus* group represent two elements that may be indicative of a natural level of contamination. Moreover, some characteristics of the monitored area, such as the low concentration of plankton and temperatures higher than 25 °C during summer months, did not represent ideal conditions for the diffusion and viability of both *Vibrio* species (Pernet et al., 2012; Vezzulli et al., 2015).

5 | CONCLUSIONS

The present study revealed critical environmental conditions during the summer season, particularly related to a low trophic level, which may have inhibited oyster growth and weakened their immune system, thus making the organisms potentially more susceptible to infectious agents. However, the negligible presence of pathogens, the triploid nature of the animals lacking reproductive stress, the absence of eutrophication phenomena, the reduced levels of oxidative stress and the absence of pathological signs at histological evaluation, all represent positive features that may support the development of the offshore longline culture of *C. gigas* oysters in the investigated area.

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AUTHOR CONTRIBUTIONS

F.M. involved in acquisition and interpretation of data, and writing of the manuscript. P.G.T. involved in design of the study and revision of the manuscript. J.H., A.M.D. and D.D. involved in acquisition of data. G.A., C.B. and R.P. involved in acquisition and interpretation of data, and revision of the manuscript. A.V. involved in acquisition and interpretation of data. All the authors approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL APPROVAL

The present study did not require an ethical approval.

DATA AVAILABILITY STATEMENT

The authors elect to not share data.

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