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## Low pH reduced survival of the oyster *Crassostrea gigas* exposed to the Ostreid herpesvirus 1 by altering the metabolic response of the host

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### Abstract :

Environmental change in the marine realm has been accompanied by emerging diseases as new pathogens evolve to take advantage of hosts weakened by environmental stress. Here we investigated how an exposure to reduced seawater pH influenced the response of the oyster *Crassostrea gigas* to an infection by the Ostreid herpesvirus type I (OsHV-1). Oysters were acclimated at pH 8.1 or pH 7.8 and then exposed to OsHV-1. Their survival was monitored and oyster tissues were sampled for biochemical analyses. The survival of oysters exposed to OsHV-1 at pH 7.8 was lower (33.5%) than that of their counterparts at pH 8.1 (44.8%) whereas levels of OsHV-1 DNA were similar. Energetic reserves, fatty acid composition and prostaglandin levels in oyster did not vary consistently with pH, infection or their interactions. However, there was a reduction in the activities of superoxide dismutase (SOD) and nitric oxide synthase (iNOS) in oysters at low pH, which is associated with the observed difference in survival.

### Highlights

► The susceptibility of *Crassostrea gigas* to OsHV-1 increased at pH 7.8 in comparison to pH 8.1 ► The amount of OsHV-1 in oyster tissues was the same at both pH, suggesting the role of host metabolic response in differential survival ► A lower activity of SOD and a basal activity of iNOS at pH 7.8, in comparison to pH 8.1, may have impaired the defence of oysters to OsHV-1 explaining the lower survival

**Keywords :** Acidification, Bivalve, Disease, Environment, Metabolism, Mortality risk

## 1. Introduction

Oysters are grown in estuaries and bays where they are exposed to short-term fluctuations of environmental parameters. Among them, pH can vary daily and seasonally of 0.2-0.3 pH units due to freshwater and nutrient inputs of the watershed, and photosynthetic and respiration activities of living organisms (Duarte et al., 2013; Wootton et al., 2008). In addition to these short-term fluctuations, pH in the ocean is decreasing over the long-term due to increasing amount of dissolved carbon dioxide (CO<sub>2</sub>) in the seawater as a consequence of increasing anthropogenic CO<sub>2</sub> emissions (Doney et al., 2009). The pH in the ocean is expected to decrease by 0.4 units by 2100 (Caldeira and Wickett, 2003), and acidification is likely to affect all areas from the deep sea to coastal areas and estuaries (Feely et al., 2009; Orr et al., 2005).

Calcifying animals and more specifically bivalves are among the most susceptible species to pH reduction (Kroeker et al., 2013). They allocate energy to the maintenance of acid–base balance at the expense of shell and somatic growth, protein synthesis, behaviour, reproduction and immune response (Gazeau et al., 2013; Parker et al., 2013). Reduced pH decreases immune and antioxidant responses of molluscs (Beesley et al., 2008; Bibby et al., 2008; Ellis et al., 2015; Li et al., 2015; Malagoli and Ottaviani, 2005; Matozzo et al., 2012; Range et al., 2014; Tomanek et al., 2011; Wang et al., 2016) and also changes virulence, and community of pathogens (Asplund et al., 2014; Mackenzie et al., 2014; Zha et al., 2017). The pH can modulate host-pathogen interactions (Burge et al., 2014; Hernroth and Baden, 2018) and acidified conditions might render animals more vulnerable to pathogens. The few studies that have investigated the immune response of marine bivalves to pathogen challenges under acidified conditions mainly showed that in this condition host immune suppression was beneficial for vibrio infection (Asplund et al., 2014; Cao et al., 2018b; Castillo et al., 2017; Ellis et al., 2015; Hernroth et al., 2016; Zha et al., 2017). Furthermore, among these studies

only Ellis et al. (2015) looked at the in vivo progression of the infection for more than 72h and examined host survival.

Here we investigated the influence of an exposure to reduced seawater pH on the survival of the oyster *Crassostrea gigas* infected by the Ostreid herpesvirus type I (OsHV-1) and the response of host metabolism. In 2008, massive mortality events of young Pacific oysters were reported in France, and since then, in other European countries and Oceania (Barbosa Solomieu et al., 2015; Pernet et al., 2016). These mortality events are caused by a polymicrobial disease which first involves the infection of oysters with a newly described genotype of OsHV-1  $\mu$ var (de Lorgeril et al., 2018; Segarra et al., 2010). For instance, OsHV-1 creates an immune-compromised state of oysters evolving towards subsequent bacteraemia by opportunistic bacterial pathogens leading to oyster death (de Lorgeril et al., 2018). This disease poses a major challenge for Pacific oyster production around the world. Oysters were monitored for OsHV-1 DNA quantification and for analyses of energetic reserves, key enzyme activities, fatty acid composition and prostaglandin level. These parameters may change at low pH (Freitas et al., 2017; Velez et al., 2016) and therefore modulate the host response to pathogens. For instance, energy reserves are used to sustain immune, antioxidant and cytoprotection processes and modulate disease outcome (Fuhrmann et al., 2018; Genard et al., 2013; Pernet et al., 2010; Tamayo et al., 2014). Antioxidant and immune related enzymes protect the host against oxidative stress resulting from accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are toxic to invaders (Donaghy et al., 2015; Hermes-Lima, 2004; Lesser, 2006; Sies, 1993). Then, membrane fatty acids provide information about the degree of oxidative stress and damage at the cellular level (Hulbert, 2003). Some of them are precursors of eicosanoids, a group of highly biologically active hormones such as prostaglandins, associated with stressful or energetically expensive situations such as stimulation of immune function (Stanley and Howard, 1998). To our

knowledge, this study is the first to investigate how pH influences the survival of a bivalve to a viral infection and the role of host metabolism.

## 2. Material and method

### 2.1. Animals

The specific-pathogen-free oysters (SPF) came from a single cohort produced in 2015 according to Le Roux et al. (Le Roux et al., 2016; 2015a). In March 2015, adult oysters were held at 17°C in 500 l flow-through tanks for conditioning. Fertilization was achieved on 28 April 2015 by stripping the gonads. The embryos developed in 150 l tanks at 21°C for 48 h, and D-larvae were transferred to flow-through rearing systems at 25°C. After 15 days, competent larvae were collected and allowed to settle on cultch in downwellers. The entire rearing cycle was conducted in UV-sterilized, 1- $\mu\text{m}$ -filtered seawater enriched with living phytoplankton at the Ifremer Marine Station (Argenton, France, 48° 48' 24.49" N, 3° 0' 22.84" W).

At the onset of the experiment (10 August) oysters were 4 months old,  $0.6 \pm 0.2$  g wet weight and  $18.8 \pm 2.9$  mm shell length (mean  $\pm$  SD) and OsHV-1 DNA was not detected upon PCR testing of representative samples. They were maintained at  $21.1 \pm 0.17^\circ\text{C}$ , 35.2‰ salinity, and fed with *Chaetoceros muelleri* (CCAP 1010/3) and *Tisochrysis lutea* (CCAP 927/14) (1:1 in dry weight). Phytoplankton concentration was maintained at  $800 \mu\text{m}^3 \mu\text{l}^{-1}$  algae. These environmental parameters were checked daily. A light aeration was provided in each tank to maintain the oxygen level between 85 and 100% of saturation.

### 2.2. Experimental design

From 10 to 17 August 2015, a subsample of the oyster cohort was transferred to a farming area of the Bay of Brest (Brittany, France, 48° 20' 06.19" N, 4° 19' 06.37" W) during a disease outbreak. After 7 days of field exposure, few individuals died. The remaining living oysters were moved back to the laboratory. These field infected oysters were then used as pathogen donors (Petton et al., 2015b). This ecologically-realistic method of infection retains

the whole complexity of the disease and reproduces the natural route of infection (de Lorgeril et al., 2018). The level of OsHV-1 DNA in donors was  $2.94 \times 10^9 \pm 1.56 \times 10^9$  OsHV-1 DNA copies  $\text{mg}^{-1}$  wet weight (mean  $\pm$  SD, n=3 pools of 10 oysters).

In the meantime, oysters kept in the laboratory were acclimated for 6 days at two pH levels: 8.1 ( $8.1 \pm 0.1$  Figure S1) which is the current pH of ocean (control) or 7.8 ( $7.8 \pm 0.1$ , Figure S1) which corresponds to the predicted value at the end of the 21<sup>st</sup> century (acidified seawater). Seawater was acidified by injection of pure CO<sub>2</sub> and pH was regulated with a pH-computer (JBL ProFlora m1003, Germany). Acidified and control seawater was then distributed to six tanks per treatment at 6-8 l h<sup>-1</sup> (Figure S2). Each tank was 47 l and contained *ca* 150 oysters for a total biomass of 85.5 g. This combination of oyster density and water flow allowed limiting the acidification of seawater which naturally occurs as a consequence of animal respiration. The pH<sub>NBS</sub> was monitored daily with a pH 3310 WTW probe (WTW, Alles, France), calibrated once per week. These oysters were then used as pathogen recipients or controls.

On 17 August 2015 (day 0), pathogen donors were placed in a 60 l flow-through tank filled with seawater at pH 8.1. The seawater surrounding the donors was used as the source of infection (SI). The SI was left at pH 8.1 or acidified by injection of CO<sub>2</sub> to reach pH 7.8. Then the SI was pumped to tanks where pathogen recipients were held (n=3 tanks per pH  $\times$  infection combination). Control tanks were not connected to the SI but supplemented with Filtered Sea Water at a pH 8.1 or 7.8, as previously described. At day 4, when recipients began to die, the donors were removed but the seawater distribution remained unchanged.

Survival of recipients and controls was followed every day for 15 days. Dead oysters were removed from tanks. Alive oysters were sampled at days 0, 2 and 4 for each treatment combination, before the onset of mortality. Whole tissues from 10 oysters per tank were removed from the shells, pooled together, flash frozen and stored in liquid nitrogen.

### 2.3. Laboratory analyses

The oyster tissues were ground in liquid nitrogen with a MM400 homogenizer (Retsch, Eragny, France). The resulting oyster powders were stored at  $-80^{\circ}\text{C}$  until further analyses. Pathogen and biochemical analyses were conducted on oyster powder subsample as fully described in Supplementary File S1.

### 2.3.1. *OsHV-1*

Total DNA from oyster tissues was then extracted with a QIAamp tissue mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extract was stored at  $-20^{\circ}\text{C}$  before detection and quantification according to a real-time PCR protocol based on SYBR® Green chemistry (Pépin et al., 2008). Results were expressed as OsHV-1 DNA copy number per milligram of wet tissue. The detection of the  $\mu\text{var}$  strain of OsVH-1 was done using specific DPFor/DPRev primers targeted the region of the OsHV-1 genome predicted to encode a DNA polymerase catalytic sub-unit (Webb et al., 2007).

### 2.3.2 *Energetic reserves and membrane fatty acids*

Carbohydrate concentration was determined according to Dubois (1956). Neutral lipid classes were analysed by high performance thin layer chromatography. Identified compounds were triacylglycerols (TAG  $\mu\text{g mg}^{-1}$ ) and sterols (ST  $\mu\text{g.mg}^{-1}$ ). Since TAG are mainly reserve lipids and ST are structural constituents of cell membranes, we used the TAG-ST ratio as a proxy for the relative contribution of reserve to structure. Polar lipids were separated from neutral lipids using a silica gel micro column and their fatty acid were analysed by gas chromatography (Marty et al., 1992). Results are expressed as % of polar lipids.

### 2.3.3 *Proteins, antioxidant and immune related enzymes and prostaglandins*

Proteins were quantified following Bradford (1976) and expressed as mg of proteins per g of dry weight. Total activity of superoxide dismutase (SOD; EC 1.15.1.1) was assayed using a SOD assay kit (Sigma-Aldrich, Saint-Louis, Missouri, USA) according to a modified procedure (Le Bris et al., 2015). Catalase activity (CAT; EC 1.11.1.6) was measured

according to Aebi et al. (1984). Total Glutathione Peroxidase activity (t-GPx, EC 1.11.1.9) and Glutathione-S-Transferase activity (GST, EC 2.5.1.18) were assayed according to McFarland et al. (1999). Glutathione Reductase activity (GR, EC 1.pH 8.1.7) was assessed following Cribb et al. (1989). The inducible Nitric Oxide Synthase activity (iNOS, EC 1.14.13.39) was measured according to the non-radioactive method developed by Richard et al. (2016). For SOD and CAT, results are expressed in U mg<sup>-1</sup> protein whereas for the other enzymes, results are expressed in mU.mg<sup>-1</sup> protein. Assays were done in triplicate using 96-well micro-plates (Greiner Bio One, Les Ulis, France), a POLARstar Omega micro-plate reader and MARS data analysis software (BMG Labtech, Champigny-sur-Marne, France). Catalase activity was measured in quartz Hellma cuve 110 (Dutscher, Brumath, France) using a Libra S12 spectrophotometer (Biochrom, Cambourn, UK).

*In vivo*, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is converted to Prostaglandin E<sub>2</sub> Metabolite (PGEM) assayed using the PG screening EIA Kit (Cayman Chemical No. 514531, Ann Arbor, MI, USA) according to a modified procedure from Segueineau et al. (2011).

## 2.4. Statistical analyses

### 2.4.1. Survival analysis

Nonparametric estimates of the survivor function were computed according to Kaplan and Meier (1958). Survival time was measured as days from the onset of the experiment (day 0) when recipient oysters were exposed to the SI. The data were read as the number of dead animals within each tank on each day. Survival curves of recipient oysters were plotted and compared among the pH treatments.

The survival curves of recipient oysters were compared using the Cox regression model (Cox, 1972). The survival of control oysters (not exposed to the SI) was not included in the statistical models because it was always 100%. The proportionality of hazards (PH) was checked and validated with Schoenfeld residuals.

### 2.4.2. Multivariate analyses



Time series of membrane fatty acids for each pH, infection status and time were associated using a hierarchical clustering method based on the Euclidian distance and Ward's linkage methods (Ward, 1963). The clustering and related time series were represented using heat map plots of the raw dataset (Eisen et al., 1998). Only fatty acids >1% of total fatty acids in at least one treatment combination were included in this analysis.

#### 2.4.3. ANOVAs

Split plot ANOVAs were conducted to determine differences in all biochemical variables, according to pH, infection and sampling time and their interactions. The experimental unit was the tank in which pH and infection were applied. Main plots were pH and infection levels, subplots were sampling times. Significant differences are considered at  $\alpha = 0.05$ . Where significant differences were measured a Tukey test was computed to characterize which means were different.

All statistical analyses were conducted using R Software ([www.R-project.org/](http://www.R-project.org/)). Only significant effects are shown.

### 3. Results

#### 3.1. Survival

The survival of controls was 100% at the end of the experiment. In recipients, the onset of mortality began at days 2 and 3. Their final survival at pH 7.8 was lower (33.5%) than that of animals at pH 8.1 (44.8%, Fig. 1). Overall, the risk of mortality of recipients at pH 7.8 increased by 37% compared to that of oysters held at pH 8.1 (Supplementary Table S1).

#### 3.2. OsHV-1

The levels of OsHV-1 DNA were not influenced by pH in any experimental treatments (Supplementary table S2). However, the levels of OsHV-1 DNA in recipient oysters were 4 to 5 orders of magnitude higher than in controls (Fig. 2a, Supplementary Tables S2, S3). Low levels of OsHV-1 DNA were occasionally detected in controls ( $<10^3$  cp mg<sup>-1</sup> wet tissue).

Although controls were no longer considered SPF, absence of mortality suggest that they were healthy. Overall, levels of OsHV-1 DNA increased 1.4 order of magnitude between day 2 and 4 (Fig. 2b, Supplementary Table S2, S3).

### 3.3. Proximate composition

Carbohydrate content of oysters at pH 7.8 was 26% higher than at pH 8.1 (Fig. 3a) but the level of TAG (relative to ST) and protein were similar between pH treatments (Supplementary Table S4, S5). The level of protein in recipient oysters was 15% lower than in controls (Fig. 3b).

### 3.4. Antioxidant and immune related enzymes

Among the six tested enzymes, only SOD and iNOS activities were modulated by pH. Indeed, the activity of SOD in both recipients and controls at pH 7.8 was 19% lower than at pH 8.1 (Fig. 4a, Supplementary Table S6, S7). For iNOS, the effect of pH interacted with infection and time (Fig. 4b, Supplementary Tables S6, S7). The activity of iNOS in recipients at pH 8.1 was on average double than in other factor combinations.

Activities of SOD, GST or GR varied as a function of the interaction of infection and time (Supplementary Table S6, S7). Activities of SOD and GST were respectively 69% and 141% higher in recipients at day 4 than in other factor combinations (Fig 4a, c) whereas no consistent pattern was found for GR.

### 3.5. Membrane fatty acids

The major fatty acids found in oyster tissues were 22:6n-3, 16:0, 18:0 dimethylacetal (DMA), 20:5n-3 and 20:4n-6, which together accounted for >50% of the total fatty acids (Table S8). Hierarchical clustering identified four distinct groups of fatty acids (Fig. 5). Groups 1 and 2 included plasmalogen-related fatty acids, such as non-methylene-interrupted (20:2 and 22:2), 20:1n-11 and DMA (18:0 and 20:1) (Kraffe et al., 2004) and 20:4n-6, a precursor of eicosanoids (Smith 2003). Group 3 contained the short chain fatty acids (14:0, 16:0, 16:1n-7), and branched fatty acids characteristic of bacteria. The group 4 included all

the major polyunsaturated fatty acids: 18:2n-7, 18:3n-3, 18:4n-3, 20:5n-3, 22:5n-6, and 22:6n-3. Overall, there was no consistent clustering of pH, infection status or time on these fatty acids (Fig. 5, Table S8, S9). The pH had a significant effect on the levels of 14:0, 18:1n-9, 18:3n-3, 20:4n-6, 22:5n-3, 22:6n-3 and the unsaturation index of polar lipids (Table S9).

### 3.6. Prostaglandins

The level of Prostaglandin E<sub>2</sub> Metabolite (PGEM) was influenced by the interaction of pH, infection and time (Supplementary Table S10, S11). The level of PGEM in controls at pH 8.1 (4.1 pg mg<sup>-1</sup> wet weight) was on average double than in other factor combinations (2.0-2.4 pg mg<sup>-1</sup> wet weight), except for recipients at pH 8.1 day 2 and pH 7.8 day 4 where values were similar (2.8-3.1 pg mg<sup>-1</sup> wet weight).

## 4. Discussion

This study shows that pH modulates oyster immunity and the outcome of a disease induced by a virus in a marine bivalve. The survival of oysters exposed to OsHV-1 at pH 7.8 was lower (33.5%) than that of their counterparts at pH 8.1 (44.8%). Mussels *M. edulis* acclimated for 90 days at 5 different pH (from 6.5 to 8.05) show mortality at pH 6.5 and 7.3 and a reduction in antibacterial activity at low pH. However, no additional mortality was reported after a challenge with the bacteria *Vibrio tubiashii* because mussels were able to restore antibacterial activity when infected (Ellis et al., 2015). Mussels were able to cope with an infection event under stressful conditions which was not the case of oysters. Our results are in line with previous studies reporting that low pH influences activities of haemocytes and antioxidant enzymes, thus leading to immunosuppression and increased disease susceptibility (Burge et al., 2014; Cao et al., 2018b; Hernroth and Baden, 2018; Matozzo et al., 2012; Wang et al., 2016).

Levels of OsHV-1 DNA in infected oysters were similar at the two pH, suggesting that pH had no effect on virus infectivity. The effects of pH on marine eukaryotic viruses are barely known (Mojica and Brussaard, 2014). However, studies conducted on marine

bacteriophages show that they can be inactivated when pH is lower than 7 (Mojica and Brussaard, 2014). Overall, it is generally suggested that the most important changes are due to the effects of pH on the host organisms on which the viruses rely (Danovaro et al., 2011). Here the difference in oyster survival at pH 7.8 and 8.1 may reflect changes in the metabolic status of the host.

The carbohydrate content of oysters in the acidified condition was 26 % higher than in control pH. Similarly, carbohydrate, but also lipid and protein of mussels exposed at pH 7.3 for 28 d were higher than in control condition, suggesting that exposure to low pH result in a lower metabolic activity and energy sparing (Freitas et al., 2017; Velez et al., 2016). Interestingly, carbohydrate sparing was already observed in oysters exposed to low salinities (Fuhrmann et al., 2018), so that it may be viewed as an overall stress response. In contrast, oysters maintain their carbohydrate and lipid reserves after 29 days at low pH (7.3, 7.6, 7.7 in comparison to pH 8.0), by restructuring their proteome (Timmins-Schiffman et al., 2014). Maintaining homeostasis is essential to acclimate and tolerate stress, but this can put strains on mechanisms involved in energy acquisition, conversion and conservation (Sokolova et al., 2012). Here the fact that oysters held under acidified condition showed higher energetic reserves did not necessarily mean there was no energetic cost to pH acclimation, but that there was a metabolic response of the host.

The unsaturation index (UI) of membrane lipids, which is an index of basal metabolic rate in animal species (Hulbert and Else, 1999) including bivalves (Pernet et al., 2007; Pernet et al., 2008), increased marginally (less than 2%) at low pH. These variations in UI are minor compared to those that can occur in response to a change of temperature in bivalves (~20% when  $\Delta T^{\circ}\text{C}=20^{\circ}\text{C}$ ; Pernet et al. 2007).

The activity of SOD in oysters increased at day 4 in response to OshV-1 and was lower at pH 7.8 than at pH 8.1, regardless of infection status. This may reflect a lower production of reactive oxygen species (ROS), and consequently, a lower capacity to neutralize

pathogens at pH 7.8 (Freitas et al., 2017; Hu et al., 2015; Velez et al., 2016). Although some studies report increased activities of SOD at low pH reflecting a stress response (Hu et al., 2015; Velez et al., 2016), others show no difference (Cao et al., 2018a; Freitas et al., 2017; Velez et al., 2016). Discrepancies among studies may reflect differences in duration of acclimation to pH, species and tissues sampled. This pinpoints the needs for not only investigating proxies for overall immunity, but to concomitantly assess the functional capacity of the immune system by measuring disease susceptibility of the host and the mortality caused by a pathogen during or after exposure to pH conditions (Ellis et al., 2015).

Infected oysters at pH 8.1 showed an early increased in iNOS activity, but this was not observed in infected oysters at pH 7.8. Given that increasing iNOS activity generally coincides with increasing RNS production to neutralize pathogens (Fang, 1997; Nathan, 1997), maintenance of iNOS activity at a basal level at pH 7.8 may reflect absence of this type of immune response. The effects of pH on SOD and iNOS activities in oyster tissues converged toward the idea that low pH exposure did not result in activation of the defence against pathogens while it was the case at control pH.

Activities of SOD and GST in oysters exposed to OsHV-1 increased during the time course of infection, as previously reported for other bivalve species exposed to pathogens (Canesi, 2015; De Zoysa et al., 2011; Genard et al., 2013), suggesting that oysters responded to infection by producing ROS.

These ROS can however peroxidise membrane lipids (Hermes-Lima, 2004; Manduzio et al., 2005). In our study, the UI of membrane lipids, which is also viewed as a peroxidation index (Hulbert, 2003), remained similar in oysters regardless of pH and infection. This suggests that oysters were able to cope with low pH and pathogens to maintain membrane lipids composition. Accordingly, clams *R. decussatus* have the same level of lipid peroxidation at pH 7.3 in comparison to pH 7.8, likely reflecting the maintenance of the integrity of lipid membranes (Velez et al., 2016).

Although eicosanoids which are precursors of prostaglandin, leukotriene and thromboxane are produced from 20:4n-6 and control inflammation or immunity during pathogen exposure in mammals and invertebrates (Smith and Murphy, 2003; Stanley and Howard, 1998), there was no consistent effect of pH or infection on prostaglandin E2 metabolite and 20:4n-6 in oysters. These variables were influenced by pH x infection x time so that there was clear relation with pH or infection. It is likely that the level of stress induced by pH and infection in our experiment was not sufficient to enhance prostaglandin production in oysters.

Here we show that low pH increases the susceptibility of oysters to OsHV-1 and this coincides with lower activity of SOD and basal activity of iNOS at pH 7.8, two enzymes that are involved in the host immune response, whereas these activities were enhanced at pH 8.1. Our study leads to the conclusion that experimental acute acidification of seawater is likely to induce modulation of immune response in oyster infected by OsHV-1. Further developments should consider long-term acclimation and multi-generational effects, and examine other species of bivalves with other factors such as temperature, to fully explore the impacts of ocean acidification on bivalves (Riebesell and Gattuso, 2015).

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## References

- Aebi, H.** (1984). Catalase in vitro. *Methods Enzymol* **105**, 121-6.
- Asplund, M. E., Baden, S. P., Russ, S., Ellis, R. P., Gong, N. P. and Hernroth, B. E.** (2014). Ocean acidification and host-pathogen interactions: blue mussels, *Mytilus edulis*, encountering *Vibrio tubiashii*. *Environ. Microbiol.* **16**, 1029-1039.
- Barbosa Solomieu, V., Renault, T. and Travers, M.-A.** (2015). Mass mortality in bivalves and the intricate case of the Pacific oyster, *Crassostrea gigas*. *J. Invertebr. Pathol.* **131**, 2-10.
- Beesley, A., Lowe, D. M., Pascoe, C. K. and Widdicombe, S.** (2008). Effects of CO<sub>2</sub>-induced seawater acidification on the health of *Mytilus edulis*. *Clim. Res.* **37**, 215-225.
- Bibby, R., Widdicombe, S., Parry, H., Spicer, J. and Pipe, R.** (2008). Effects of ocean acidification on the immune response of the blue mussel *Mytilus edulis*. *Aquatic Biol.* **2**, 67-74.
- Bradford, M. M.** (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
- Burge, C. A., Eakin, C. M., Friedman, C. S., Froelich, B., Hershberger, P. K., Hofmann, E. E., Petes, L. E., Prager, K. C., Weil, E., Willis, B. L. et al.** (2014). Climate Change Influences on Marine Infectious Diseases: Implications for Management and Society. *Ann. Rev. Mar. Sci.* **6**, 249-277.
- Caldeira, K. and Wickett, M. E.** (2003). Anthropogenic carbon and ocean pH. *Nature* **425**, 365-365.
- Canesi, L.** (2015). Pro-oxidant and antioxidant processes in aquatic invertebrates. *Anna N Y Acad Sci* **1340**, 1-7.
- Cao, R., Liu, Y., Wang, Q., Yang, D., Liu, H., Ran, W., Qu, Y. and Zhao, J.** (2018a). Seawater Acidification Reduced the Resistance of *Crassostrea gigas* to *Vibrio splendidus* Challenge: An Energy Metabolism Perspective. *Front Physiol* **9**, 880.
- Cao, R., Wang, Q., Yang, D., Liu, Y., Ran, W., Qu, Y., Wu, H., Cong, M., Li, F., Ji, C. et al.** (2018b). CO<sub>2</sub> induced ocean acidification impairs the immune function of the Pacific oyster against *Vibrio splendidus* challenge: An integrated study from a cellular and proteomic perspective. *Sci Total Environ* **625**, 1574-1583.
- Castillo, N., Saavedra, L. M., Vargas, C. A., Gallardo-Escarate, C. and Detree, C.** (2017). Ocean acidification and pathogen exposure modulate the immune response of the edible mussel *Mytilus chilensis*. *Fish Shellfish Immunol* **70**, 149-155.
- Cox, D. R.** (1972). Regression Models and Life Tables. *J R Stat Soc Series B Stat Methodol* **20**, 187-220.
- Cribb, A. E., Leeder, J. S. and Spielberg, S. P.** (1989). Use of a microplate reader in an assay of glutathione reductase using 5,5'-dithiobis(2-nitrobenzoic acid). *Anal Biochem* **183**, 195-6.
- Danovaro, R., Corinaldesi, C., Dell'anno, A., Fuhrman, J. A., Middelburg, J. J., Noble, R. T. and Suttle, C. A.** (2011). Marine viruses and global climate change. *FEMS Microbiol Rev* **35**, 993-1034.
- de Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., Vidal-Dupiol, J., Chaparro, C., Galinier, R., Escoubas, J.-M. et al.** (2018). Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. *Nat Commun* **9**, 4215.
- De Zoysa, M., Whang, I., Nikapitiya, C., Oh, C., Choi, C. Y. and Lee, J.** (2011). Transcriptional analysis of disk abalone (*Haliotis discus discus*) antioxidant enzymes against marine bacteria and virus challenge. *Fish Shellfish Immunol* **31**, 155-60.
- Donaghy, L., Hong, H.-K., Jauzein, C. and Choi, K.-S.** (2015). The known and unknown sources of reactive oxygen and nitrogen species in haemocytes of marine bivalve molluscs. *Fish. Shellfish Immunol.* **42**, 91-97.
- Doney, S. C., Fabry, V. J., Feely, R. A. and Kleypas, J. A.** (2009). Ocean acidification: The other CO<sub>2</sub> problem. *Ann. Rev. Mar. Sci.* **1**, 169-192.
- Duarte, C. M., Hendriks, I. E., Moore, T. S., Olsen, Y. S., Steckbauer, A., Ramajo, L., Carstensen, J., Trotter, J. A. and McCulloch, M.** (2013). Is Ocean Acidification an Open-Ocean Syndrome? Understanding Anthropogenic Impacts on Seawater pH. *Estuaries Coasts* **36**, 221-236.
- DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F.** (1956). Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**, 350-356.

- Eisen, M. B., Spellman, P. T., Brown, P. O. and Botstein, D.** (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* **95**, 14863-8.
- Ellis, R. P., Widdicombe, S., Parry, H., Hutchinson, T. H. and Spicer, J. I.** (2015). Pathogenic challenge reveals immune trade-off in mussels exposed to reduced seawater pH and increased temperature. *J. Exp. Mar. Biol. Ecol.* **462**, 83-89.
- Fang, F. C.** (1997). Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest* **99**, 2818-2825.
- Feely, R. A., Doney, S. C. and Cooley, S. R.** (2009). Ocean Acidification Present Conditions and Future Changes in a High-CO<sub>2</sub> World. *Oceanography* **22**, 36-47.
- Freitas, R., De Marchi, L., Bastos, M., Moreira, A., Velez, C., Chiesa, S., Wrona, F. J., Figueira, E. and Soares, A. M. V. M.** (2017). Effects of seawater acidification and salinity alterations on metabolic, osmoregulation and oxidative stress markers in *Mytilus galloprovincialis*. *Ecol Indic* **79**, 54-62.
- Fuhrmann, M., Delisle, L., Petton, B., Corporeau, C. and Pernet, F.** (2018). Metabolism of the Pacific oyster, *Crassostrea gigas*, is influenced by salinity and modulates survival to the Ostreid herpesvirus OsHV-1. *Biol Open* **7**.
- Gazeau, F., Parker, L. M., Comeau, S., Gattuso, J. P., O'Connor, W. A., Martin, S., Portner, H. O. and Ross, P. M.** (2013). Impacts of ocean acidification on marine shelled molluscs. *Mar. Biol.* **160**, 2207-2245.
- Genard, B., Miner, P., Nicolas, J. L., Moraga, D., Boudry, P., Pernet, F. and Tremblay, R.** (2013). Integrative study of physiological changes associated with bacterial infection in Pacific oyster larvae. *Plos One* **8**.
- Hermes-Lima, M.** (2004). Oxygen in Biology and Biochemistry: Role of Free Radicals. In *Functional Metabolism*, pp. 319-368: John Wiley & Sons, Inc.
- Hernroth, B., Baden, S., Tassidis, H., Hörnaeus, K., Guillemant, J., Bergström Lind, S. and Bergquist, J.** (2016). Impact of ocean acidification on antimicrobial activity in gills of the blue mussel (*Mytilus edulis*). *Fish Shellfish Immunol* **55**, 452-459.
- Hernroth, B. E. and Baden, S. P.** (2018). Alteration of host-pathogen interactions in the wake of climate change – Increasing risk for shellfish associated infections? *Environ Res* **161**, 425-438.
- Hu, M., Li, L., Sui, Y., Li, J., Wang, Y., Lu, W. and Dupont, S.** (2015). Effect of pH and temperature on antioxidant responses of the thick shell mussel *Mytilus coruscus*. *Fish Shellfish Immunol* **46**, 573-583.
- Hulbert, A. J.** (2003). Life, death and membrane bilayers. *J. Exp. Biol.* **206**, 2303-11.
- Hulbert, A. J. and Else, P. L.** (1999). Membranes as possible pacemakers of metabolism. *J Theor Biol* **199**, 257-74.
- Kaplan, E. L. and Meier, P.** (1958). Nonparametric Estimation from Incomplete Observations. *J. Am. Stat. Assoc.* **53**, 457-481.
- Kraffe, E., Soudant, P. and Marty, Y.** (2004). Fatty acids of serine, ethanolamine, and choline plasmalogens in some marine bivalves. *Lipids* **39**, 59-66.
- Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, C. M. and Gattuso, J.-P.** (2013). Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. *Glob. Change Biol.* **19**, 1884-1896.
- Le Bris, C., Richard, G., Paillard, C., Lambert, C., Seguineau, C., Gauthier, O., Pernet, F. and Guerard, F.** (2015). Immune responses of phenoloxidase and superoxide dismutase in the manila clam *Venerupis philippinarum* challenged with *Vibrio tapetis*-Part I: Spatio-temporal evolution of enzymes' activities post-infection. *Fish Shellfish Immunol* **42**, 16-24.
- Le Roux, F., Wegner, K. M. and Polz, M. F.** (2016). Oysters and Vibrios as a Model for Disease Dynamics in Wild Animals. *Trends Microbiol* **24**, 568-80.
- Lesser, M. P.** (2006). Oxidative stress in marine environments: biochemistry and physiological ecology. *Annu. Rev. Physiol.* **68**, 253-78.
- Li, S. G., Liu, Y. J., Liu, C., Huang, J. L., Zheng, G. L., Xie, L. P. and Zhang, R. Q.** (2015). Morphology and classification of hemocytes in *Pinctada fucata* and their responses to ocean acidification and warming. *Fish Shellfish Immunol.* **45**, 194-202.
- Mackenzie, C. L., Lynch, S. A., Culloty, S. C. and Malham, S. K.** (2014). Future oceanic warming and acidification alter immune response and disease status in a commercial shellfish species, *Mytilus edulis* L. *Plos One* **9**.



- Malagoli, D. and Ottaviani, E.** (2005). Cytotoxicity as a marker of mussel health status. *J. Mar. Biol. Assoc. UK* **85**.
- Manduzio, H., Rocher, B., Durand, F., Galap, C. and Le Boulenger, F.** (2005). The point about oxidative stress in molluscs. *ISJ* **2**, 91-104.
- Marty, Y., Delaunay, F., Moal, J. and Samain, J. F.** (1992). Changes in the fatty acid composition of *Pecten maximus* (L.) during larval development. *J. Exp. Mar. Biol. Ecol.* **163**, 221-234.
- Matozzo, V., Chinellato, A., Munari, M., Finos, L., Bressan, M. and Marin, M. G.** (2012). First evidence of immunomodulation in bivalves under seawater acidification and increased temperature. *Plos One* **7**.
- McFarland, V. A., Inouye, L. S., Lutz, C. H., Jarvis, A. S., Clarke, J. U. and McCant, D. D.** (1999). Biomarkers of oxidative stress and genotoxicity in livers of field-collected brown bullhead, *Ameiurus nebulosus*. *Arch Environ Contam Toxicol* **37**, 236-41.
- Mojica, K. D. A. and Brussaard, C. P. D.** (2014). Factors affecting virus dynamics and microbial host-virus interactions in marine environments. *Fems Microbiology Ecology* **89**, 495-515.
- Nathan, C.** (1997). Inducible nitric oxide synthase: what difference does it make? *J Clin Invest* **100**, 2417-2423.
- Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F. et al.** (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437**, 681-686.
- Parker, L. M., Ross, P. M., O'Connor, W. A., Portner, H. O., Scanes, E. and Wright, J. M.** (2013). Predicting the response of molluscs to the impact of ocean acidification. *Biology* **2**, 651-92.
- Pernet, F., Barret, J., Marty, C., Moal, J., Le Gall, P. and Boudry, P.** (2010). Environmental anomalies, energetic reserves and fatty acid modifications in oysters coincide with an exceptional mortality event. *Mar. Ecol. Prog. Ser.* **401**, 129-146.
- Pernet, F., Lupo, C., Bacher, C. and Whittington, R. J.** (2016). Infectious diseases in oyster aquaculture require a new integrated approach. *Phil. Trans. R. Soc. Lon. B Biol. Sci.*
- Pernet, F., Tremblay, R., Comeau, L. and Guderley, H.** (2007). Temperature adaptation in two bivalve species from different thermal habitats: energetics and remodelling of membrane lipids. *J. Exp. Biol.* **210**, 2999.
- Pernet, F., Tremblay, R., Redjah, I., Sévigny, J.-M. and Gionet, C.** (2008). Physiological and biochemical traits correlate with differences in growth rate and temperature adaptation among groups of the eastern oyster *Crassostrea virginica*. *J Exp Biol* **211**, 969.
- Petton, B., Boudry, P., Alunno-Bruscia, M. and Pernet, F.** (2015a). Factors influencing disease-induced mortality of Pacific oysters *Crassostrea gigas*. *Aquac Environ Interact.* **6**, 205-222.
- Petton, B., Bruto, M., James, A., Labreuche, Y., Alunno-Bruscia, M. and Le Roux, F.** (2015b). *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease. *Front. Microbiol.* **6**.
- Pépin, J. F., Riou, A. and Renault, T.** (2008). Rapid and sensitive detection of ostreid herpesvirus 1 in oyster samples by real-time PCR. *J Virol Methods* **149**.
- Range, P., Chicharo, M. A., Ben-Hamadou, R., Pilo, D., Fernandez-Reiriz, M. J., Labarta, U., Marin, M. G., Bressan, M., Matozzo, V., Chinellato, A. et al.** (2014). Impacts of CO<sub>2</sub>-induced seawater acidification on coastal Mediterranean bivalves and interactions with other climatic stressors. *Reg. Environ. Change* **14**, S19-S30.
- Richard, G., Guerard, F., Corporeau, C., Lambert, C., Paillard, C. and Pernet, F.** (2016). Metabolic responses of clam *Ruditapes philippinarum* exposed to its pathogen *Vibrio tapetis* in relation to diet. *Dev Comp Immunol* **60**, 96-107.
- Riebesell, U. and Gattuso, J.-P.** (2015). Lessons learned from ocean acidification research. *Nature Clim. Change* **5**, 12-14.
- Segarra, A., Pepin, J. F., Arzul, I., Morga, B., Faury, N. and Renault, T.** (2010). Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus Res.* **153**, 92-99.
- Seguineau, C., Racotta, I. S., Palacios, E., Delaporte, M., Moal, J. and Soudant, P.** (2011). The influence of dietary supplementation of arachidonic acid on prostaglandin production and oxidative stress in the Pacific oyster *Crassostrea gigas*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **160**, 87-93.
- Sies, H.** (1993). Strategies of antioxidant defense. *Eur J Biochem* **215**, 213-9.

**Smith, W. L. and Murphy, R. C.** (2003). The eicosanoids: cyclooxygenase, lipoxygenase, and epoxygenase pathways. In *Biochemistry of lipids, lipoproteins and membranes*, vol. 36 eds. D. E. Vance and J. E. Vance), pp. 341-371. Amsterdam: Elsevier Science.

**Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G. and Sukhotin, A. A.** (2012). Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Mar Environ Res* **79**, 1-15.

**Stanley, D. W. and Howard, R. W.** (1998). The biology of prostaglandins and related eicosanoids in invertebrates: Cellular, organismal and ecological actions. *Amer. Zool.* **38**, 369.

**Tamayo, D., Corporeau, C., Petton, B., Quere, C. and Pernet, F.** (2014). Physiological changes in Pacific oyster *Crassostrea gigas* exposed to the herpesvirus OsHV-1  $\mu$ Var. *Aquaculture* **432**, 304-310.

**Timmins-Schiffman, E., Coffey, W. D., Hua, W., Nunn, B. L., Dickinson, G. H. and Roberts, S. B.** (2014). Shotgun proteomics reveals physiological response to ocean acidification in *Crassostrea gigas*. *BMC Genomics* **15**, 1-18.

**Tomanek, L., Zuzow, M. J., Ivanina, A. V., Beniash, E. and Sokolova, I. M.** (2011). Proteomic response to elevated P CO<sub>2</sub> level in eastern oysters, *Crassostrea virginica*: evidence for oxidative stress. *J Exp Biol* **214**, 1836-44.

**Velez, C., Figueira, E., Soares, A. M. V. M. and Freitas, R.** (2016). Native and introduced clams biochemical responses to salinity and pH changes. *Sci Tot Environ* **566-567**, 260-268.

**Wang, Q., Cao, R. W., Ning, X. X., You, L. P., Mu, C. K., Wang, C. L., Wei, L., Cong, M., Wu, H. F. and Zhao, J. M.** (2016). Effects of ocean acidification on immune responses of the Pacific oyster *Crassostrea gigas*. *Fish Shellfish Immunol.* **49**, 24-33.

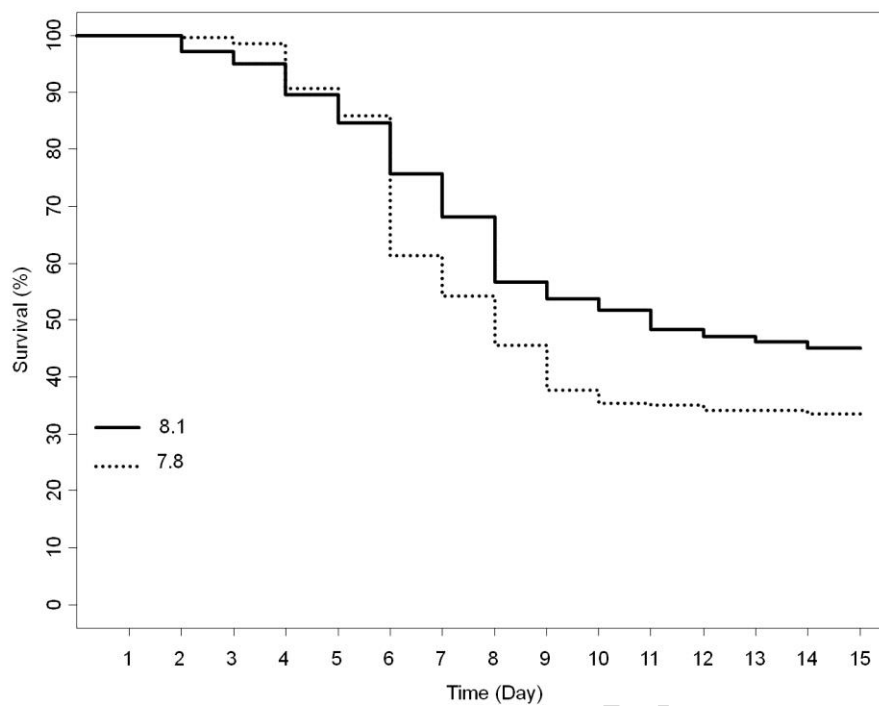
**Ward, J. H.** (1963). Hierarchical Grouping to Optimize an Objective Function. *J Am Stat Assoc* **58**, 236-244.

**Webb, S. C., Fidler, A. and Renault, T.** (2007). Primers for PCR-based detection of ostreid herpes virus-1 (OsHV-1): Application in a survey of New Zealand molluscs. *Aquaculture* **272**, 126-139.

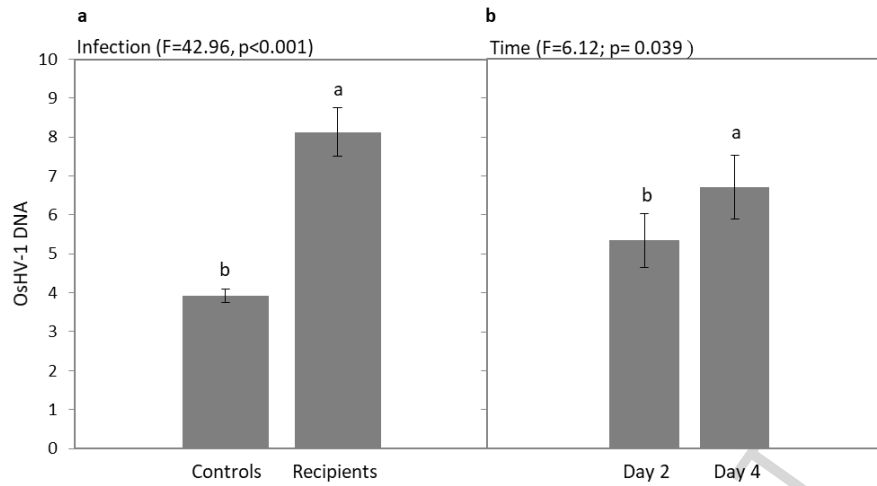
**Wootton, J. T., Pfister, C. A. and Forester, J. D.** (2008). Dynamic patterns and ecological impacts of declining ocean pH in a high-resolution multi-year dataset. *PNAS* **105**, 18848-18853.

**Zha, S., Liu, S., Su, W., Shi, W., Xiao, G., Yan, M. and Liu, G.** (2017). Laboratory simulation reveals significant impacts of ocean acidification on microbial community composition and host-pathogen interactions between the blood clam and *Vibrio harveyi*. *Fish Shellfish Immunol* **71**, 393-398.

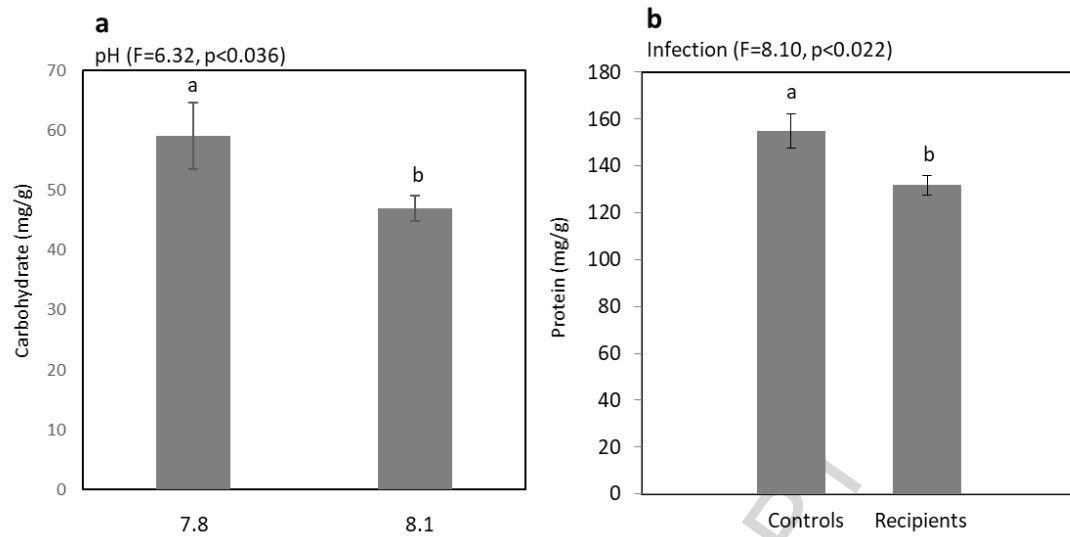
Figures and Tables



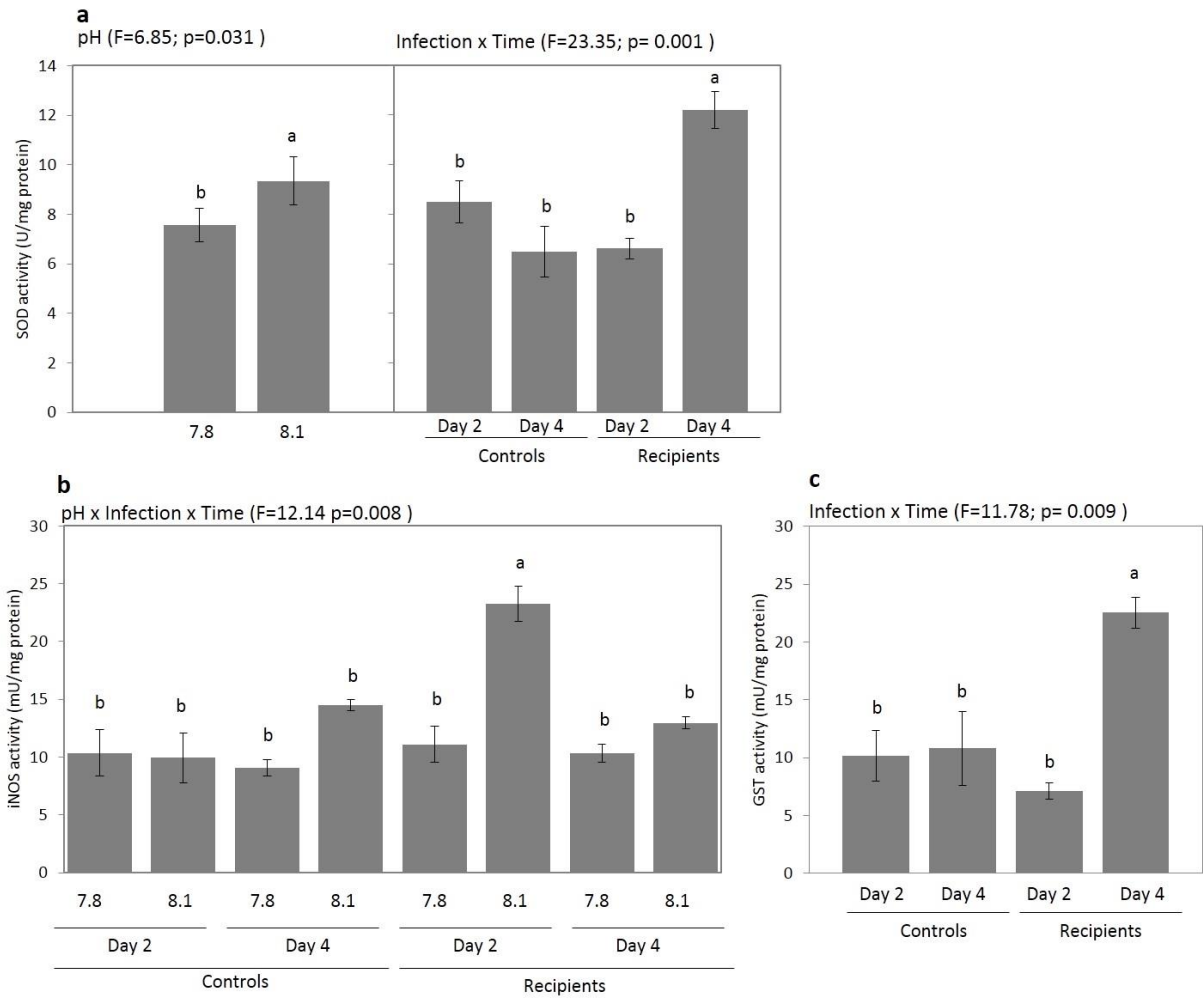
**Fig. 1:** Survival of oysters challenged at pH 8.1 (control) and 7.8 (acidified) and with a source of infection containing Ostreid Herpesvirus 1 (OsHV-1). Survival time was measured as days from the onset of exposure to the source of infection.



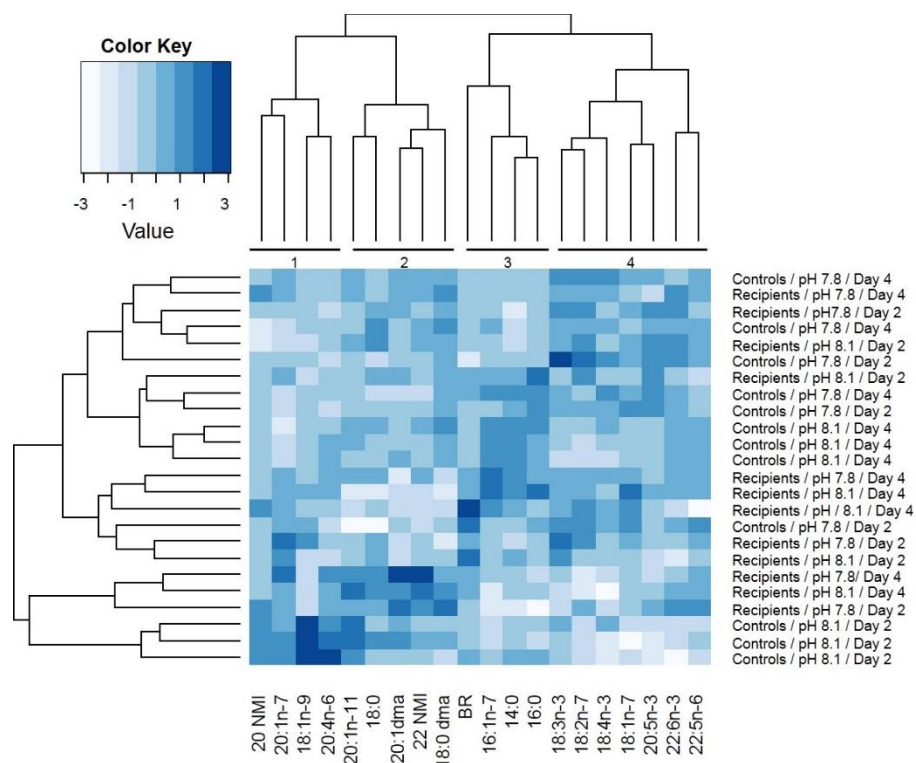
**Fig. 2:** Quantification of the OsHV-1 DNA as a function of exposure to the source of (a) infection and (b) time, results are expressed as DNA copy number per mg of fresh weight and were  $\log(x+1)$  transformed. Data are means  $\pm$  SE between replicates tanks (n=12). Letters indicate significant differences.



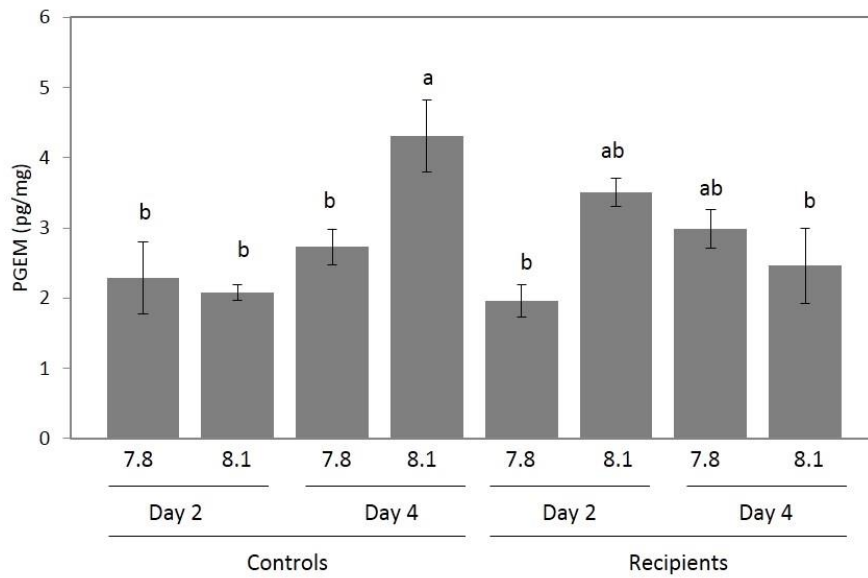
**Fig. 3:** Proximate composition of oyster tissues giving the content of (a) carbohydrate as a function, of pH and (b) protein as a function of exposure to the source of infection. Data are means  $\pm$  SE between replicates ( $n=12$ ) and are expressed in  $\text{mg g}^{-1}$  of dry weight. Letters indicate significant differences.



**Fig. 4:** (a) SOD activity as a function of pH and infection x time separately, (b) iNOS activity as a function of pH x infection x time, (c) GST activities as a function of infection x time. Data are means  $\pm$  SE between replicates depending on combination of treatments (for triple interaction  $n=3$ ; for double interaction  $n=6$ ; for single effect  $n=12$ ). Letters indicate significant differences. Abbreviations: SOD, total superoxide dismutase; iNOS, inducible nitric oxide synthase; GST, glutathione-S-transferase.



**Fig. 5:** Heat map showing lipids contributing  $>1$  % of total fatty acids. Fatty acids were reordered according to the hierarchical clustering result given by the dendrogram at the top. The dendrogram on the left shows how the lipid profiles cluster by individuals. Abbreviations: BR, branched; 20 and 22 NMI, total of  $C_{20}$  and  $C_{22}$  non-methylene interrupted; DMA dimethylacetal fatty acids.



**Fig.6:** Prostaglandin E2 as a function of pH x infection x time. Data are means  $\pm$  SE between replicates ( $n=3$ ) and are expressed in  $\text{pg mg}^{-1}$  of wet weight. Letters indicate significant differences.



**Highlights**

- The susceptibility of *Crassostrea gigas* to OsHV-1 increased at pH 7.8 in comparison to pH 8.1
- The amount of OsHV-1 in oyster tissues was the same at both pH, suggesting the role of host metabolic response in differential survival
- A lower activity of SOD and a basal activity of iNOS at pH 7.8, in comparison to pH 8.1, may have impaired the defence of oysters to OsHV-1 explaining the lower survival

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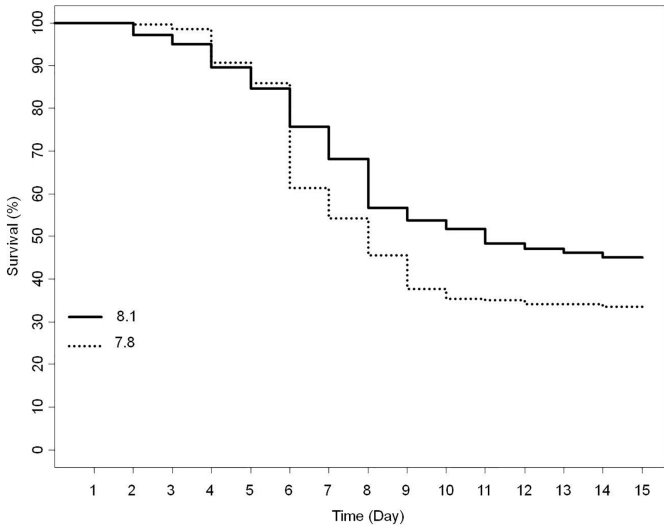


Figure 1

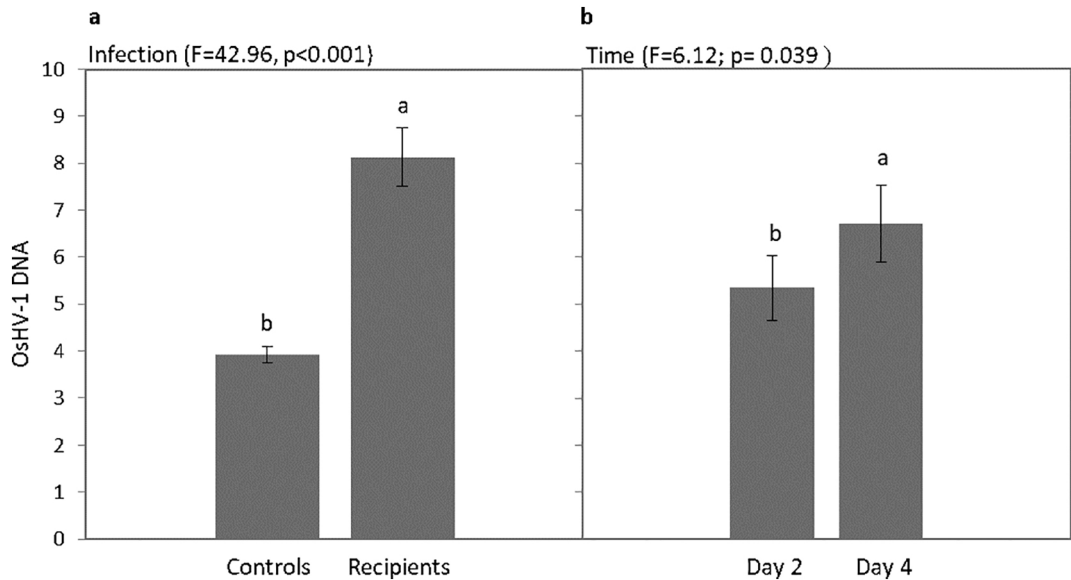


Figure 2

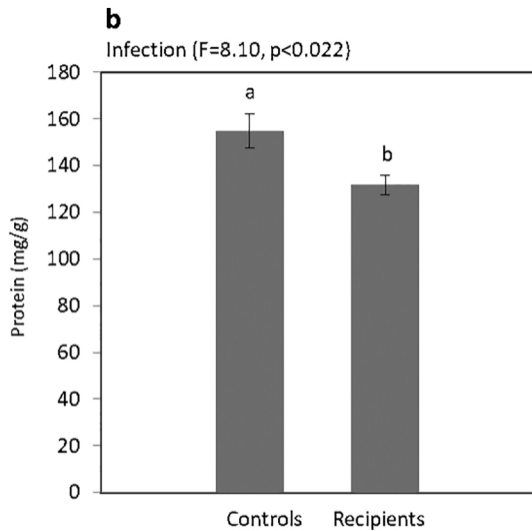
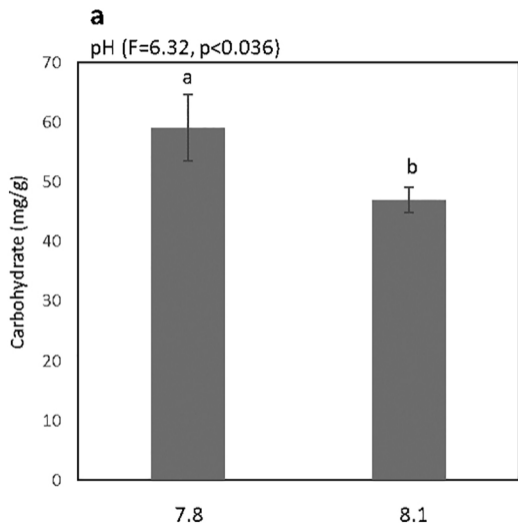


Figure 3

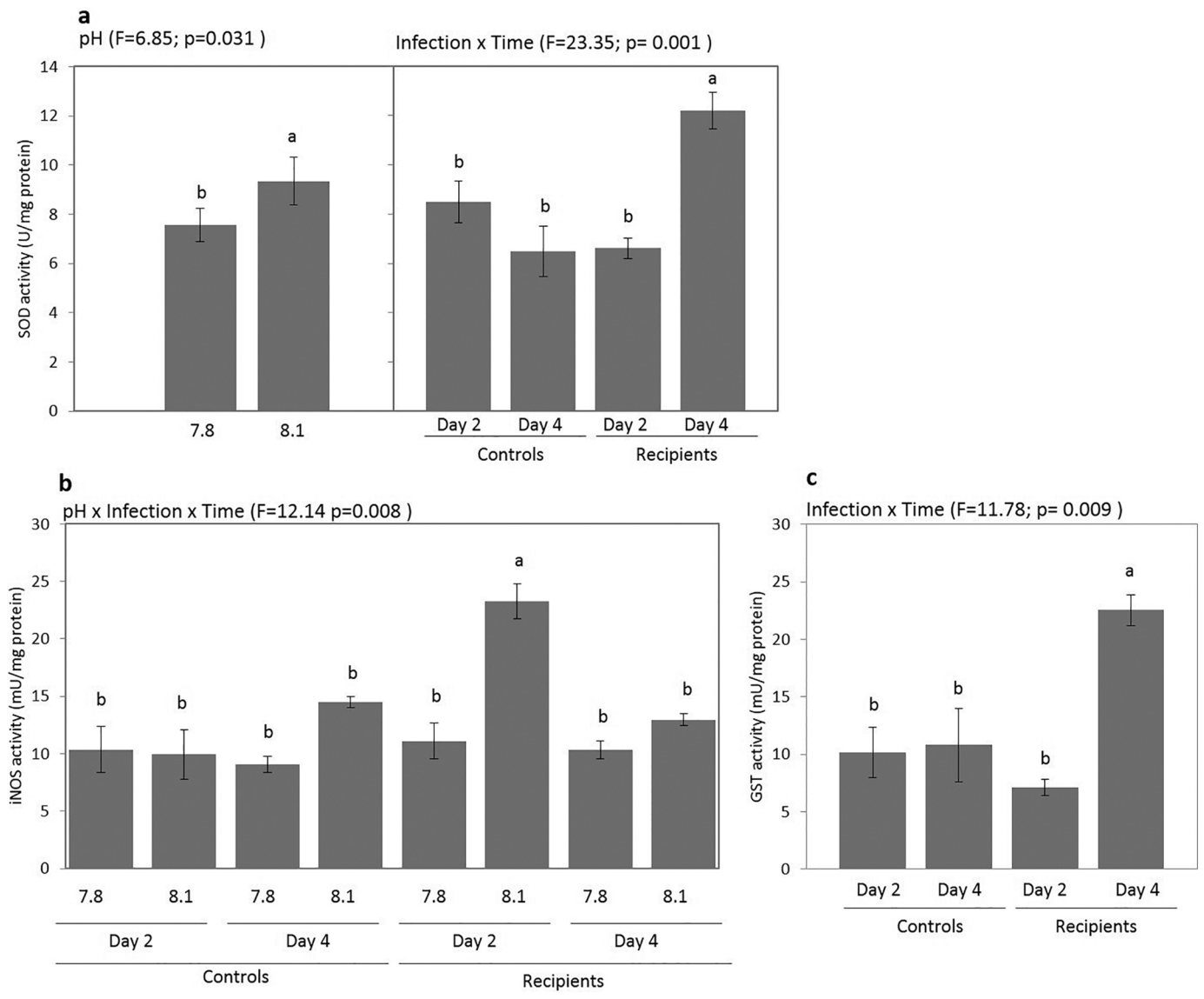


Figure 4

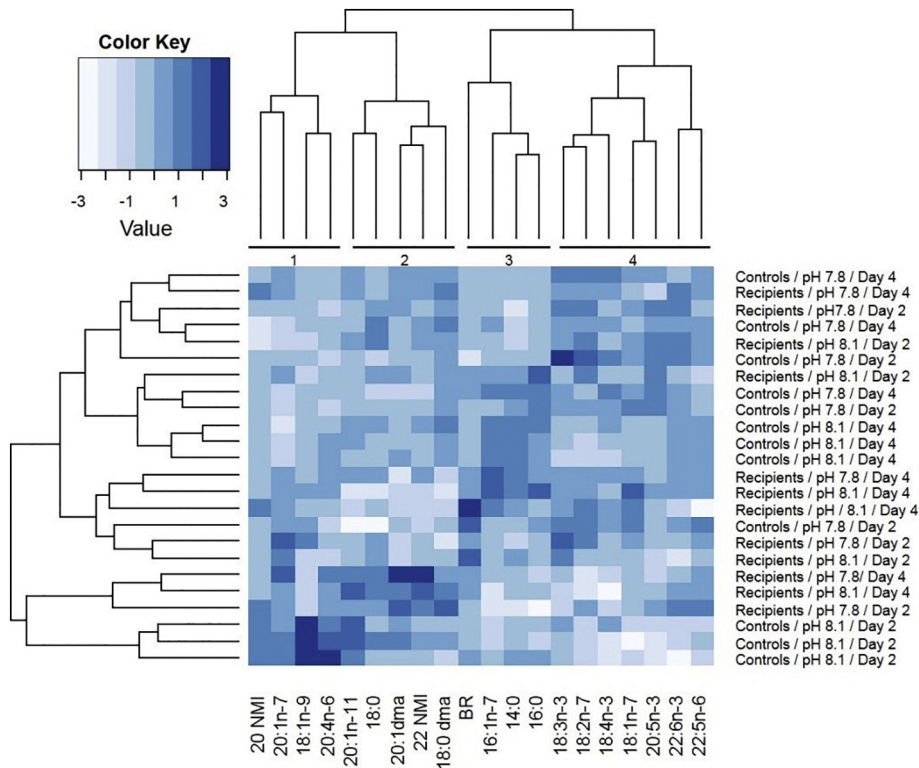


Figure 5

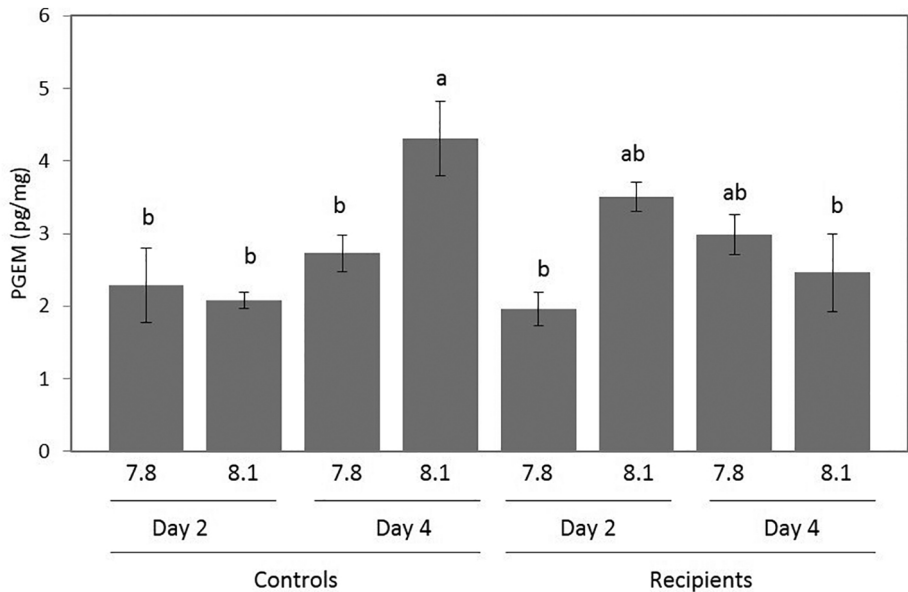


Figure 6