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Physiological changes in Pacific oyster *Crassostrea gigas* exposed to the herpesvirus OsHV-1 μVar

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ABSTRACT

Since 2008, mass mortality events of *Crassostrea gigas* have been occurring along the French coast when seawater temperature exceeds 16 °C. These mortality events are related to a particular genotype of the ostreid herpesvirus named OsHV-1 μVar. The present study aimed to detail various physiological aspects underlying the onset of the disease. In the laboratory, both exposed (infected) and naïve (healthy) oysters were maintained at 13.0 °C and 20.6 °C. These temperatures were respectively lower and higher than the threshold values of 16 °C at which the disease generally occurs. At 20.6 °C, exposed oysters were characterized by a reduction in energetic reserves (carbohydrates and triglycerides) together with a decrease in protein content. Sterol levels were lower in exposed oysters than in naïve individuals, irrespective of temperature. Finally, activities of some key enzymes related to energetics were similar in exposed and naïve oysters and did not change with temperature. This result suggests that although energetic reserves were being diminished in infected oysters, their metabolic activities remained similar to that of healthy animals.

1. Introduction

Since 2008, mass mortality events of *Crassostrea gigas* of less than one year old have been occurring along all oyster rearing sites on the French coast (EFSA, 2010; Jolivel and Fleury, 2012), threatening the exploitation of this valuable resource at an unprecedented scale. Summer oyster mortalities had been reported for decades in many countries (Samain and McCombie, 2008) and invoked to be the consequence of a multifactorial interaction among pathogens, environmental conditions and oyster physiological status.

Mass mortality of oysters were found to be associated to a particular genotype of the ostreid herpesvirus 1 (OsHV-1) named μVar (Segarra et al., 2010) that affects preferentially less than one-year old individuals (Clegg et al., in press; Dégremont, 2011; Paul-Pont, 2013; Peeler et al., 2012; Pernet et al., 2010, 2012). Additionally, bacteria of the genus *Vibrio* are generally reported concomitantly to OsHV-1 in mortality events occurring in the field, further complicating the understanding of the action mechanisms that lead to oyster mortality (Dégremont, 2011; Pernet et al., 2012; Petton et al., 2013; Saulnier et al., 2010). Although mass mortalities of oysters have been mostly reported in France, this phenomenon has also been reported in the UK, Australia and New Zealand (EFSA, 2010; Martenot et al., 2011; Peeler et al., 2012; Renault, 2011).

The onset of mass mortality generally occurs when seawater temperature reaches 16–17 °C (Pernet et al., 2012), which is in contrast to the summer mortality phenomena characterized by a temperature threshold of 19 °C (Samain and McCombie, 2008). The effect of temperature on OsHV-1 transmission and related mortalities of oysters has received much attention (Dégremont, 2011; Garcia et al., 2011; Pernet et al., 2012; Samain and McCombie, 2008; Sauvage et al., 2009). Recently, cohabitation experiments show that healthy oysters exposed to field areas where mortalities were occurring could become infected by OsHV-1 μVar (Petton et al., 2013). In addition, disease transmission and related oyster mortality vary with seawater temperature. Indeed, when field exposed oysters were maintained at 13 °C for 40 days in the hatchery, they exhibited no additional mortality, were negative for OsHV-1 and did not transmit the disease to healthy oysters (Petton et al., 2013). These results further highlight the importance of temperature in recent mass mortality events.

Summer mortality usually occurs when energetic resources are lowest and energy demand and reproductive effort are highest (Berthelin et al., 2000; Delaporte et al., 2006; Enríquez-Díaz et al., 2009; Samain and McCombie, 2008; Soletchnik et al., 1997, 2006). Energetic status has been shown to play a major role in bacterial infection and disease expression in bivalves (Flye-Sainte-Marie et al., 2007; Genard et al., 2011, 2013; Paillard et al., 2004; Plana et al., 1996). Also, the energetic status of oysters prior to the onset of mass mortality events plays a role in OsHV-1 transmission and related mortalities (Pernet et al.,

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2010, 2012, 2014). Conversely, OsHV-1 induces physiological changes such as differential regulation of several cell processes in relation with the disease such as immune response, cell signaling and reception pathways, and, apoptosis and cell cycle regulation as reported in several transcriptomic studies (Fleury and Huvet, 2012; Jouaux et al., 2013; Renault et al., 2011).

In this study, we investigated the interactive effects of temperature and disease exposure on physiological parameters of oysters. In a previous paper (Petton et al., 2013), we showed that mortality of healthy (naïve) oysters is influenced by the temperature during their cohabitation with oysters previously exposed to field conditions where mortalities occurred (hereafter referred to as "exposed"). Here we analyzed the energetic reserves and activities of key metabolic enzymes on exposed and naïve oysters.

2. Matherial and methods

2.1. Animals

Wild individuals were collected in Fouras (Marennes-Oléron, France) in August 2008, placed in mesh bags in February 2009, then transferred to Paimpol (northern Brittany, France, 48°48′24.49″N, −87 3°0′22.84″W) until February 2010. Then, these animals were moved to the Ifremer grow-out farm located in Aber-Benoît (northern Brittany, France, 48°34′29.976″N, 4°36′18.378″W). These animals were exposed to the disease during the spring of 2009 and suffered ca. 75% mortality.

In April 2010, 60 individuals (3 year-old, 80 mm shell length) were transferred to the Ifremer marine station located at Argenton (Brittany, France) for conditioning. These animals were held in 500 l flow-through tanks for 6 weeks, with seawater at 19 °C and enriched with a phytoplankton mixture which consisted of *Isochrysis affinis galbana* (T-ISO) and *Chaetoceros gracilis* (1:1 in dry weight) at a ration equivalent to 6% of the oyster dry weight. Seawater was treated with UV radiation and filtered at 1 μm. Gametes from 13 males and 27 females, obtained by stripping, were mixed at 50 spermatozoids per oocyte on 9 June 2010. The D-larvae were reared in flow-through systems (Rico-Villa et al., 2008) at 25 °C for a proper larval performance (Ben Kheder et al., 2010). After 16 days, competent larvae were collected on a 225 μm sieve and allowed to settle on cultch. Post-larvae were maintained in downwelling systems where they were continuously supplied with enriched seawater (see above for conditions) as described elsewhere (González Araya et al., 2012). After 10 days, the cultchless spat were collected on 400 μm mesh and reared at 25 °C in downwellers for 90 days until late August 2010. Throughout this time, the oysters were free of any abnormal mortality and OsHV-1 DNA was not detected.

2.2. Experimental design

On 26 August 2010, juvenile oysters (2500 individuals) were placed in mesh bags and transferred to a farming area located in the Bay of Brest where mortalities were occurring among local oysters (Petton et al., 2013). Shell length of juvenile oysters varied between 15 and 30 mm, average whole body wet weight was 0.3 g and age was 3 months. Exposed oysters were maintained for 16 days in the field before returning to the marine station. At that time, these animals were infected by OsHV-1 μVar (Petton et al., 2013). The unexposed oysters (hereafter referred to as "naïve" animals) were kept in the Ifremer marine station with UV treated seawater filtered at 1 μm.

Following exposure in the field, animals were returned to the laboratory, where an equal number of 200 individuals for both exposed and naïve oysters were placed at 13.0 and 20.6 °C in 23 l plastic replicate tanks for each condition for 40 days (n = 2). Food concentration was maintained at 1500 $\mu m^3\ \mu l^{-1}$ algae. It was controlled every day by means of a Counter Coulter Multisizer 3 at the inflow and outflow of all experimental tanks.

Ten days after exposure to the temperature treatment, the whole tissues of 5 pooled oysters for each condition (n = 2 sampling tanks per condition) were dissected, and immediately stored into liquid nitrogen. Concomitantly, dead and alive oysters were counted and recorded daily thereafter. Survival analyses are detailed elsewhere (Petton et al., 2013). Briefly, at the time of sampling, exposed oysters exhibited 54% mortality against 0% in naïve oysters at 20.6 °C, whereas at 13.0 °C, no mortality occurred in both exposed and naïve animals (Petton et al., 2013).

2.3. Biochemical analysis

Soft tissues of five pooled oysters were grounded in liquid nitrogen with a MM400 homogenizer (Retsch), and the resulting powders were subsampled and stored at −80 °C until biochemical analysis (Pernet et al., 2012).

2.3.1. Proteins, carbohydrates and neutral lipids

For protein extraction, 200 mg of fine powder was placed in Eppendorf tubes filled with 1 ml ice-cold lysis buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% Igepal, 2 mM PMSF, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 100 mM sodium fluoride, 10 nM sodium pyrophosphate, and 2 mM sodium orthovanadate; pH 7.4). Proteins were extracted as described by Corporeau et al. (2011). Protein lysates were quantified using a DC protein assay (Bio-Rad, Hercules, CA, USA) and diluted at the same concentration in ice-cold lysis buffer.

For carbohydrate, samples of 100 to 200 mg of fine powder were placed in Eppendorf tubes containing 1.5 ml nano-pure water, homogenized and diluted according to carbohydrate concentration. Carbohydrates were quantified following DuBois et al. (1956) as described in Pernet et al. (2010).

Samples of 100 mg of powder were placed in glass vials filled with 6 ml dichloromethane/methanol (2:1, v/v), and total lipids were extracted according to Folch et al. (1957). Neutral lipid classes were further analyzed by HPTLC (high performance thin layer chromatography) using a CAMAG system, consisting of a sampler (TLC Sampler 4) and a reader (TLC Scanner 3) (Ben Kheder et al., 2010). Identified compounds were sterols (ST; μg mg^{-1} tissues), free fatty acids (FFA; μg mg^{-1} tissues) and triacylglycerol (TAG; μg mg^{-1} tissues).

2.3.2. Enzyme activities

The activity of citrate synthase (CS; EC 4.1.3.7), was assayed using the method of Childress and Somero (1979). The assay reaction is based on the reaction of 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) with the reactive −SH group of the free co-enzyme A. The 2-nitro-5-thiobenzoate (NTB) produced is yellow and its maximum absorbance is at ~412 nm. The activities of hexokinase (HK; EC 2.7.1.1), and pyruvate kinase (PK; EC 2.7.1.40) in oyster tissues were determined as described by Greenway and Storey (1999). Increase in NADPH or decrease in NADH was followed by monitoring the absorbance at 340 nm. All assays were made in triplicate at room temperature and initiated by mixing the enzyme buffer with 20 μl homogenate in a 220 μl total microplate-well volume. Absorbance was measured using a BioTek microplate reader (BioTek Instrument). All enzymatic measurements were related to the total protein concentration of each sample.

2.3.3. AMPK phosphorylation

For each oyster pool, 90 μg of protein lysate was denatured by Laemmli solution (0.6 M Tris–HCl, 1 ml glycerol, 10% w/v SDS, 0.5 ml 0.1% w/v bromophenol blue, 0.5 ml β-mercaptoethanol) and loaded onto 10%/SDS/polyacrylamide gel. Proteins were then transferred to a PVDF membrane (Biorad) for immunodetection on western-blot. Immunodetection was performed as described in Guévélou et al. (2013) with a rabbit monoclonal anti-phosphothreonine 172 AMPKα antibody (dilution 1:1000; CST #2535; Ozyme,