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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 16ºC. 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 20.6ºC. These temperatures were respectively lower and higher than the threshold values of 16°C at which the disease generally occur. At 20.6ºC, exposed oysters were characterized by a reduction in energetic reserves (carbohydrates and triglycerides) together with a decrease in protein content. Sterols 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.

Highlights

► We study the energetic physiology of infected and healthy oysters at 2 temperatures. ► Healthy oysters increase their energetic reserves together with higher temperatures. ► Infection and high temperatures lead to mortalities and lowered energetic reserves. ► Infected and healthy oysters have similar key energetic enzyme activities. ► OsHV-1 µvar promotes a metabolic disorder above its lethal temperature threshold.

Keywords : *Crassostrea gigas* ; Energetic reserves ; Energetic metabolism ; Enzyme activity ; OsHV-1 ; Temperature

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; Renault, 2011; Martenot et al., 2011; Peeler et al., 2012).

The onset of mass mortality generally occurs when seawater temperature reaches 16-17ºC (Pernet et al., 2012), which is in contrast to the summer mortality phenomena characterized by a temperature threshold of 19ºC (Samain and McCombie, 2008). The effect of temperature on OsHV-1 transmission and related mortalities of oysters has received much attention (Dégremont, 2011; Samain and McCombie, 2008; Garcia et al., 2011; Pernet et al., 2012; Sauvage et al., 2009). Recently, cohabitation experiments shows 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 13ºC 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 mortalities events.

Summer mortality usually occurs when energetic resources are lowest and energy demand and reproductive effort are highest (Samain and McCombie, 2008; Berthelin et al., 2000; Delaporte et al., 2006; Enríquez-Díaz et al., 2009; 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., 2010, 2012, 2014). Conversely, OsHV-1 induce 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 analysed 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 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 1µm.

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 20.6ºC in 23 L plastic replicate tanks for each condition for 40 days (n=2). Food concentration was maintained at 1500 μm^3 μl^{-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 onto 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 were 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 analysed 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 were 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-phospho-threonine 172 AMPKα antibody (dilution 1:1000; CST #2535; Ozyme, Cell Signaling Technology). To ensure for cross-reactivity of such heterologous antibody, Cell Signaling Technology technical services blasted and confirmed that the antigenic region of this commercially available anti-phospho-threonine 172 AMPKα antibody was present at 100 % in the *C. gigas* protein (Cell Signaling Technology; personal communication). Blots were then revealed using an HRP-linked secondary goat anti-rabbit antibody (dilution 1:5000) and a HRP detection kit (Biorad). The relative amount of protein detected was quantified using MULTI-ANALYST software (Biorad), with the background signal removed. The value obtained is expressed in OD/mm^2 and represents the band intensity expressed as mean count per pixel, multiplied by the band surface. To ensure that identical amounts of total protein samples were loaded into the gels, membranes were dehybridized after visualization and signals quantified by incubation for 1 h at room temperature in dehybridizing buffer (100 mM glycine, 100 mM NaCl pH 3.2), and rehybridized with an anti-histone H3 antibody (dilution 1:5000; # 9175; Cell Signaling Technology), as described in Fabioux et al. (2009).

2.4. Statistics

Effects of sanitary status (exposed vs naïve), temperature and their interaction onto the protein, carbohydrate and neutral lipid levels, as well as for measured enzyme activities were analyzed by two-way analysis of variance (ANOVA). Normal distribution of data was checked by Kolmogorov-Smirnov test and homogeneity of variances evaluated using Levene's test. Data for CS, PK and HK activities utilized in statistical analysis were ln+1 transformed to achieve homogeneity of variances. All statistical analyses were performed using SPSS 19 software. Significance level threshold was set at $p < 0.05$.

3. RESULTS

3.1. Protein and carbohydrate content

Protein and carbohydrate contents varied as a function of temperature × sanitary status of oysters (Figure 1a, 1b respectively, Table 1). Protein and carbohydrate content of naïve oysters increased by 30% with temperature. In contrast, in exposed oysters, these two proxies decreased slightly (although not significantly) with increasing temperature (Figure 1a, 1b). It is noteworthy that at 20.6°C, a temperature compatible with disease mortality of oysters, carbohydrate level was lower in exposed relative to naïve oysters.

3.2. Lipid class

Triacylglycerol (triglycerides) significantly varied as a function of temperature and sanitary status of oysters (Figure 1c, Table 1). Exposed oysters had 54 and 33% less triglycerides than naïve ones at 13 and 20.6ºC respectively. Level of triglycerides increased with rising temperatures in the same manner for both exposed and naïve. None of tested factors had a significant effect on the free fatty acid concentration of tissues (Figure 1d, Table 1). Finally, only sanitary status was found to be significant for measured sterols levels (Figure 1e, Table 1). In this case, and as it was observed for the triglycerides content, exposed oysters displayed lower values than naïve individuals.

3.3. PK, HK, AMPK and CS activities

Overall, PK activity did not change with temperature and sanitary status of oysters (Figure 2a, Table 1). Although HK activity between exposed and naïve oysters maintained at 13°C and 20.6°C was found to be non-significant, (Figure 2b, Table 1), p-values were close to significance (p=0.085) since exposed oysters tended to have higher HK activities (2.7 mU mg^{-1} protein) than naïve individuals (1.5 mU mg^{-1} protein). As observed for HK, the AMPK THr172/histone H3 (Figure 2c, Table 1) and CS activity (Figure 2d, Table 1) was generally higher in exposed oysters maintained at both 13°C and 20.6°C, however this difference was not significant.

4. DISCUSSION

The present study investigates the physiological changes of *C. gigas* oysters exposed to a pathogen responsible for mass mortalities. Pacific oysters that had been bred and reared under controlled conditions and then transferred to a farming area where mortalities were occurring became positive for detection of OsHV-1 DNA µVar (Petton et al., 2013). As a result, groups of these 'exposed' animals suffered high mortalities.

The advantage of our protocol is that we avoided high-dose intramuscular injection of pathogens, which may short-circuit some potentially important lines of defense and disturb normal host immune response. Exposed oysters were naturally infected in the field, and the disease they contracted is clearly the one related to the mass mortality phenomenon that occurred in the field.

Overall, at 20.6°C, levels of carbohydrate and triglycerides in exposed oysters which exhibited 54% mortality were lower than those in naïve oysters which showed no mortality. Utilization of energy reserves is a common feature related to infectious responses in bivalves (Genard et al., 2013; Pernet et al., 2012, 2014; Plana et al., 1996). Recent studies show that bivalves exposed to pathogenic bacteria allocate a part of their energetic reserves to sustain immune, antioxidant and cytoprotection processes (Genard et al., 2013). For example, oyster larvae exposed to pathogenic *Vibrio coralliilyticus* had lower triacylglycerol and protein content, the two main energetic reserves in bivalve larvae, and higher abundance of transcripts of antioxidant enzymes and immune-related proteins than healthy larvae (Genard et al., 2013). Therefore, differences in energetic reserves between exposed and naïve oysters in the present study may reflect differences in the cost of immune response and disease expression. Alternatively, these energetic reserves may also be used for the synthesis of macromolecules that are used in virus assembly, as previously reported for triglycerides (Diamond at al., 2010; Lerat et al., 2009).

Protein content of naïve animals increased with temperature while in exposed oysters, the inverse pattern was observed. Proteins have been reported to be utilized as an energetic source during starvation, overwintering or in lean oysters (Whyte et al., 1990). Indeed, it is a common feature among mollusks subjected to nutritional stress (Laing, 1993; Lane, 1986; Du and Mai, 2004; Albentosa et al., 2007; Liu et al., 2010; Telahigue et al., 2013). Protein mobilization in bivalves generally occurs after the depletion of glycogen (see references above). Such preferential utilization of glycogen respect to proteins is seen as a mechanism of protection against the loss of a structural component of the animal (Lane, 1986). Therefore, the use of proteins as an energetic fuel by exposed oysters in present experiments reflects the profound scope of the disease, capable of mimicking food deprivation periods. Proteins may be used in exposed oysters for producing energy or for the synthesis of molecules used in virus assembly.

Carbohydrate content of naïve oysters increased with temperature, while in exposed oysters, carbohydrate reserves decreased with increasing temperature from 13.0°C to 20.6°C, a temperature where disease mortality of oysters occurred. This interaction effect on carbohydrate reserves was not accompanied by changes in key enzyme activities of the glycolysis (HK, PK). This result suggests that although carbohydrate content decreased in exposed oysters at 20.6°C when disease occurred, its utilization was maintained at the same rate as in naïve animals. Additionally, AMPK, showed similar levels in exposed and naïve oysters. Since AMPK is the sensor of the energetic status of the cell in vertebrates (Hardie, 2011) and in *C. gigas* (Guevelou et al., 2013), these results indicate that exposed oysters were insensitive to decreasing reserves.

Exposed and naïve oysters exhibited similar levels of free fatty acids. Free fatty acids usually result from degradation of triglycerides or phospholipids by lipase (Derewenda, 1994; Sewell, 2005). Then, these free fatty acids enter the β-oxidation pathway to yield acetyl-CoA which fuels the Krebs cycle to produce energy (Bartlett and Eaton, 2004; Agnihotri and Liu, 2003). Interestingly, this metabolic pathway has been recently been hypothesized to be activated in the case of *C. gigas* larvae infected with *Vibrio coralliilyticus* (Genard et al., 2013). These authors showed that infected larvae exhibit lower level of triglycerides and higher level of free fatty acids than healthy larvae, likely reflecting degradation of triglycerides by lipase. In our study, the fact that levels of free fatty acids were similar in exposed and naïve oysters may reflect the possibility that fatty acids are intermediate metabolites rapidly used, and are therefore detected in trace amounts regardless of disease expression.

Exposed and naïve oysters exhibited similar CS activity. By contrast, CS activity, which generally correlates with respiration rate in marine invertebrates (Moran and Manahan 2004; Meyer et al., 2007), decreased during a disease mortality event in *C. gigas* larvae (Genard et al., 2013). Similarly, CS activity decrease with the development of brown ring disease symptoms in Manila clam *Ruditapes philippinarum* (Flye-Sainte-Marie et al., 2007). However, in the Thau lagoon (Mediteranean sea, France), CS activity of oysters in which OsHV-1 DNA was detected was similar to that of animals in which OsHV-1 DNA was not detected (Pernet et al., 2012).

In our study, exposed oysters showed lower sterol content than naïve animals. Since oysters cannot synthesize any sterol *de novo* (Teshima and Patterson, 1981; Trider and

Castell, 1980), lower level of sterol in exposed oysters may reflect a decrease in their capacity to acquire it from the diet. This suggests that food ingestion or absorption might have been affected by pathogens. In support to this hypothesis, both *R. philippinarum* and *C. gigas* have been shown to diminish their ingestion rates when infected with bacteria from the genus *Vibrio* (Flye-Sainte-Marie et al., 2007; Genard et al., 2013). Additionally, mussels infected by *Martelia* sp. exhibit lower absorption and higher food consumption than healthy animals (Anestis et al., 2010).

In conclusion, our data show that exposed oysters present a metabolic disorder at the level of storage of energy reserves which could facilitate the herpesvirus expression at 20.6°C. Virus have the ability to interrupt the normal functioning of cells, take control of cell production machinery, utilize it to their own purposes (production of more virus), and escape hosts immune system detection (Hay and Kannourakis, 2002). Herpesvirus have been recently shown in mammals as being capable to induce a Warburg like effects by enhancing glucose uptake and glycolysis in infected cells (Warburg et al., 1924; Yu et al., 2011). It was recently reported that metabolic changes resembling the Warburg effect are induced by white spot syndrome virus (WSSV) in infected shrimp (Chen et al., 2011). At the moment, the differential proteomic signature of infected and naïve oysters have been studied by means of 2D gels approach (Corporeau et al., submitted). Studies dealing with the regulation of the energetic metabolic pathways that lead to the reserves depletion described in present manuscript at various different levels (gene and protein expression, i.e.) would be of high interest. Whether the metabolic disorder observed in present experiments resemble the Warburg effect, in any case, would need further research.

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CAPTIONS

Figure 1. Protein (a) carbohydrate (b), triacylglycerol (TAG; c), free fatty acids (FFA; d), and sterol (ST; e) content (µg per mg of tissues) in exposed (black) and naïve (white) *Crassostrea gigas* maintained at 13°C and 20.6ºC. Values are means ± SD (n=2 replicate tanks). Different letters indicate significant differences (n.s.; not significant difference).

Figure 2. Pyruvate kinase activity (PK; mU per mg of protein) (a), hexokinase activity (HK; mU per mg of protein) (b), AMPK content (AMPK; AMPK THr172 : histone H3) (c), and citrate synthase activity (CS; mU per mg of protein) (d) in exposed (black) and naïve (white) *Crassostrea gigas* maintained at 13°C and 20.6ºC. Values are means ± SD (n=2 replicate tanks). Different letters indicate significant differences (n.s.; not significant difference).

Figure 1

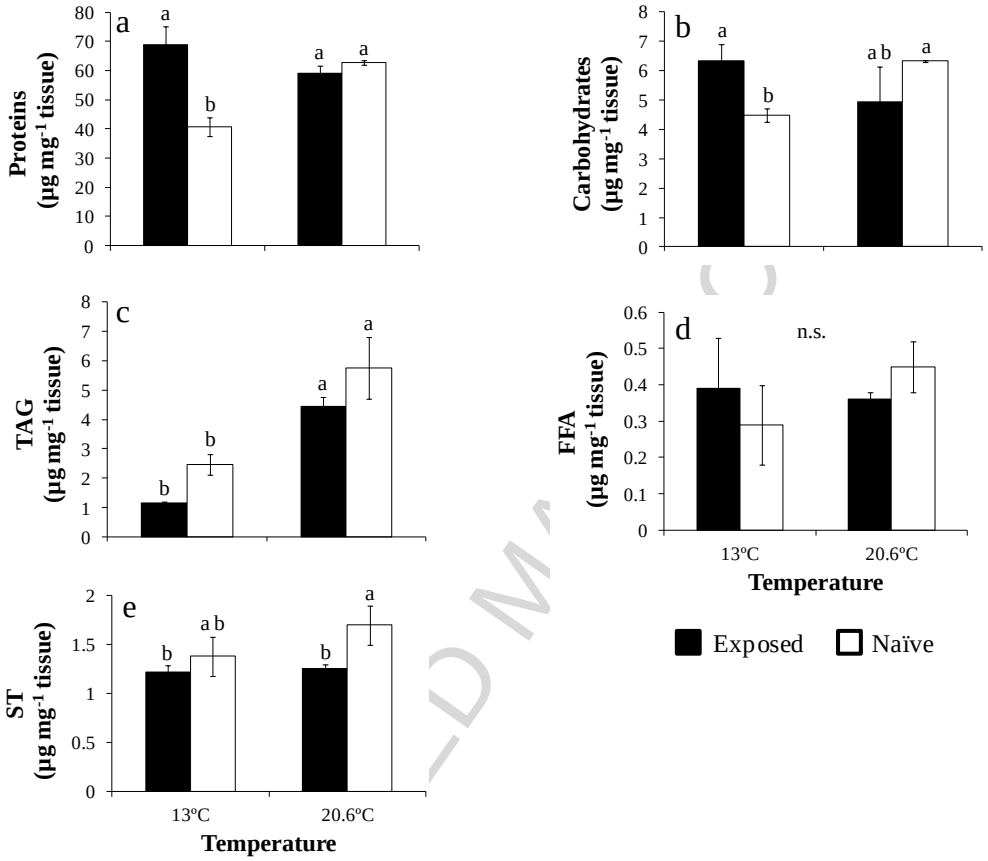

Figure 2

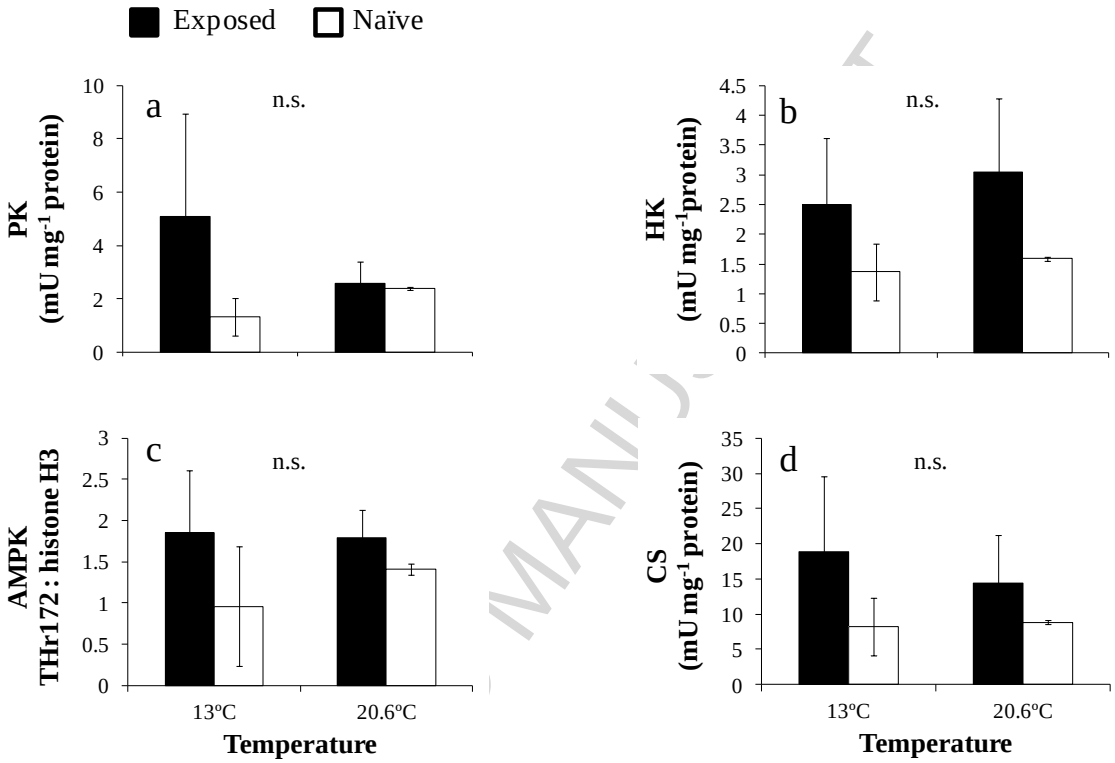

Table 1. Summary of two-factor ANOVA testing significant effects of oyster sanitary status (exposed and naïve) and exposition temperature of *Crassostrea gigas* spat (n=2) on: protein content (µg/mg of tissues), carbohydrate content (µg/mg of tissues), triacylglycerols (µg / mg of tissues), free fatty acids (µg / mg of tissues), sterols (µg / mg of tissues), transformed pyruvate kinase activity (ln+1) (PK; mU / mg of protein), transformed hexokinase activity (ln+1) (HK; mU / mg of protein), AMPK THr172 : histone H3 and transformed citrate synthase activity (ln+1) (CS; mU / mg of protein)

Parameter	Sources of variation	df	MS	SS	F	p
Proteins	Sanitary status	1	76.8	76.8	5.7	0.075
	Temperature	1	305.0	305.0	22.8	0.009
	Interaction	1	512.0	512.0	38.3	0.003
	Error	4	13.4	53.5		
Carbohydrates	Sanitary status	1	0.099	0.099	0.216	0.667
	Temperature	1	0.104	0.104	0.225	0.660
	Interaction	1	5.298	5.298	11.535	0.027
	Error	4	0.459	1.837		
Triacylglycerols	Sanitary status	1	3.458	3.458	10.197	0.033
	Temperature	1	21.451	21.451	63.250	0.001
	Interaction	1	4.999×10^{-5}	4.999×10^{-5}	1.474×10^{-4}	0.991
	Error	4	0.039	1.357		
Free fatty acids	Sanitary status	1	1.12×10^{-4}	1.12×10^{-4}	0.011	0.922
	Temperature	1	0.009	0.009	0.881	0.401
	Interaction	1	0.015	0.015	1.481	0.290
	Error	4	0.010	0.041		
Sterols	Sanitary status	1	0.174	0.174	7.814	0.049
	Temperature	1	0.061	0.061	2.750	0.173
	Interaction	1	0.039	0.039	1.760	0.255
	Error	4	0.022	0.089		
PK	Sanitary status	1	0.416	0.416	2.707	0.175
	Temperature	1	0.001	0.001	0.005	0.945
	Interaction	1	0.339	0.339	2.202	0.212
	Error	4	0.154	0.615		
HK	Sanitary status	1	0.317	0.317	5.211	0.085
	Temperature	1	0.030	0.030	0.496	0.520
	Interaction	1	0.001	0.001	0.020	0.895
	Error	4	0.061	0.244		
AMPK THr172 : histone H3	Sanitary status	1	0.811	0.811	2.693	0.176
	Temperature	1	0.073	0.073	0.241	0.649
	Interaction	1	0.134	0.134	0.445	0.541
	Error	4	0.301	1.204		
CS	Sanitary status	1	0.654	0.654	3.548	0.133
	Temperature	1	0.006	0.006	0.034	0.863
	Interaction	1	0.061	0.061	0.331	0.596
	Error	4	0.184	0.737		