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Study of the antioxidant capacity in gills of the Pacific oyster *Crassostrea gigas* in link with its reproductive investment

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Abstract:

Energy allocation principle is a core element of life-history theory in which “the cost of reproduction” corresponds to an acceleration of senescence caused by an increase in reproductive investment. In the “theory of aging”, senescence is mainly due to the degradation of lipids, proteins and DNA by reactive oxygen species (ROS), by-products of oxidative metabolism. Some studies have shown that oxidative stress susceptibility could be a cost of reproduction. The present study investigates the effect of reproductive investment on antioxidant capacity in the gills of a species with a very high reproductive investment, the Pacific oyster *Crassostrea gigas*. We used RNA interference targeting the *oyster vasa-like gene* (*Oyvlg*) to produce oysters with contrasted reproductive investment. Antioxidant capacity was studied by measuring the mRNA levels of genes encoding major antioxidant enzymes, and the activity of these enzymes. The highest reproductive investment was associated with the highest transcript levels for glutathione peroxidase and extra-cellular and mitochondrial superoxide dismutase. In contrast, lipid peroxidation did not show any sign of oxidative damage whatever the reproductive investment. Up-regulation of certain genes encoding enzymes involved in the first step of ROS detoxification could therefore be a part of the organism's strategy for managing the pro-oxidant species produced by heavy reproductive investment.

Keywords: Antioxidant capacity ; *Crassostrea gigas* ; Reproduction ; RNA interference ; ROS

1. Introduction

Oxygen (O_2) is an essential molecule for life because it allows cellular respiration and thus the synthesis of ATP, the main energy source for cells. Most oxygen used in cellular respiration undergoes a tetravalent reduction, producing water in the inner mitochondrial membrane. However, when it forms reactive oxygen species (ROS), oxygen can also be toxic for cells. Around 2% of O_2 will experience a partial reduction leading to the production of ROS (Han et al. 2001). ROS species can be pro-oxidant radicals, such as hydroxyl radicals ($^{\cdot}OH$), superoxide radicals ($O_2^{\cdot-}$), peroxy radicals ($RO_2^{\cdot}$) or non-radical species such as hydrogen peroxide (H_2O_2). All of these molecules are highly reactive species that have deleterious effects on molecules that are rich in high electron density sites, such as molecules with high nitrogen content (DNA, RNA and proteins), or that have a large number of carbon double-bonds (fatty acids and phospholipids); ROS therefore damage cells (Cadenas 1989). To cope with ROS production, organisms possess a complex arsenal of antioxidant defense species based on vitamins (C and E), glutathione, zinc, selenium, metallothioneins or specific enzymes (Hermes-Lima 2005). The principal ROS-scavenging enzymes are catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione S-transferase (GST) (Hermes-Lima 2005).

Under optimal physiological conditions, a balance exists between pro- and antioxidant species. Oxidative stress situations create an imbalance in favour of pro-oxidant species, leading to oxidative damage to cells. Several studies have demonstrated the involvement of oxidative stress in the process of cellular aging (Finkel et al. 2000). In such cases, organisms have to cope with the management of a respiration / ROS-production conflict, whose regulation involves many antioxidant species and whose efficiency ensures the survival of the organism. ROS overproduction can be induced by many stressful conditions, such as those caused by heat shock, UV exposure, pollution, oxygen availability changes or pathogens (Lesser 2006). Essential metabolic functions requiring high metabolic activity, such as reproduction, could also be a source of oxidative stress (Alonso-Alvarez et al. 2004). Several studies have shown that increasing reproductive investment causes an acceleration of senescence, which contributes to “the cost of reproduction” (Stearns 1992). The concept of cost of reproduction is fundamental in life-history theory models based on the principle of energy allocation: individuals should allocate resources optimally among maintenance, growth and reproduction in order to maximize their fitness (Stearns 1992). In addition, according to the “theory of ageing”, some studies (Alonso-Alvarez et al. 2004; Alonso-Alvarez et al. 2006) have hypothesized that decrease of antioxidant capacities together with or without an increase of ROS production could be considered as cost of reproduction because it accelerates senescence. Relationships that could represent physiological trade-offs between reproductive investment and antioxidant capacities have been observed in a few species. In *Drosophila melanogaster*, an increase in egg production resulted in decreased resistance to oxidative stress, leading to higher mortality rate after exposure to paraquat, a compound that generates oxygen radicals (Salmon et al. 2001). In the zebra finch (*Taeniopygia guttata*), a negative correlation was observed between reproductive investment and antioxidant defences, with an increase of reproductive investment causing a reduction of SOD and GPX activities (Wiersma et al. 2004).

ROS production is an important component of the stress response for aquatic organisms exposed to various stressful conditions (Lesser 2006). Sessile organisms in particular, such as the Pacific oyster *Crassostrea gigas*, live in variable and potentially stressful natural ecosystems and have no choice but to endure changing environmental conditions. Over the last ten years, populations of the Pacific oyster, which is the most important aquaculture resource in France, have suffered mass mortalities during spring and summer, the period of its maximum reproductive activity. The research program MOREST (MORtalités ESTivales), led by IFREMER from 2001 to 2006, revealed that this phenomenon was determined by a multifactorial interaction between oysters, their environment and opportunistic pathogens (Samain et al. 2008). The physiological status of oysters is suspected to play a major role in these interactions, and reproduction has often been viewed as a main contributing factor (Koganezawa 1974; Mori 1979; Perdue et al. 1981; Samain et al. 2007). Indeed, a negative correlation was found between reproductive effort and survival (Ernande et al. 2004; Huvet et al. 2010) . This might be partly due to the fact that marine bivalves have a high reproductive allocation. At maturity, the volume of an oyster gonad can

reach 70% of the volume of the animal (Enríquez-Díaz et al. 2009). Such an investment requires a large production of ATP and, therefore, an increase in energy metabolism during gametogenesis (Van der Veer et al. 2006). However, enhancing the activity of the energy metabolism implies an increase in cellular respiration and, therefore, an increase in ROS production. Management of oxidative metabolism could be a component of oyster survival in stressful conditions during the reproductive period. Indeed, a difference between the constitutive pathways of antioxidant defense was suggested as an underlying difference between lines of oysters that were resistant or susceptible to summer mortality (Delaporte et al. 2007; Samain et al. 2007; Fleury et al. 2010; Fleury et al. 2012).

During the breeding season, there may be a conflict between the production of germ cells - which mobilizes the endogenous energy reserves of the animal - and other physiological functions, such as immunity or detoxification of pro-oxidant species. A reduction of the “haemolymph profile” (i.e. cells are fewer, smaller and less complex; phagocytosis and phenoloxidase activity are low, associated with high basal reactive oxygen species production) has previously been reported during gonad maturation in abalone (Travers et al. 2008). Similarly, reduced phagocytosis activity of haemocytes was reported during oyster gametogenesis (Delaporte et al. 2006) and a higher intra-haemocyte ROS production in oysters was found to be correlated with higher reproductive investment (Delaporte et al. 2007).

In this context, the present study aimed to determine whether antioxidant capacities of *C. gigas* are modified with reproductive effort. To achieve this goal, we used RNA interference (RNAi) to knockdown the *oyster vasa-like gene* (*Oyvlg*) (Fabioux et al. 2009), a gene known to be implicated in germ cell proliferation in *C. gigas* germline cell development (Fabioux et al. 2004). This innovative method enabled us to produce oysters with contrasted reproductive investment, which we investigated for their antioxidant defence mechanisms. We then exposed these oysters to a heat shock to provoke metabolism over-activation to observe whether this had an impact on their antioxidant capacities according to their reproductive investment. We aimed to study the expression of genes encoding major antioxidant enzymes (SOD, CAT, GPX, GR, and GST) and the activity of these enzymes. We also measured the lipid peroxidation as a marker of oxidative stress. All these tests were made in the gills, the organ responsible for oyster respiration but also the first in contact with the animal environment. Thus they are a key organ for studying environmental stresses such as thermal stress (Farcy et al. 2009).

2. Material and methods

2.1. Biological material

Oysters aged two and a half years were obtained in February 2010 from stocks issued from natural spat, cultured at Plougastel-Daoulas (Rade de Brest, France). The oysters were transferred to the IFREMER experimental hatchery in Argenton (France) where they were placed in 600-L raceways filled with 20-µm filtered seawater. All the oysters were in early initiation of gametogenesis stage (stage 1) at the beginning of the experiment. They were acclimated for one week and then placed in optimal conditions for germ cell maturation (see Fabioux et al., (2005), for experimental procedure), fed on a diet of two algae species with a ratio equal to 12% dry weight algae/dry weight oyster per day (50% *Chaetoceros gracilis,* 50% *T-Isochrysis galbana*) and water temperature maintained at 19 ± 1°C. Raceways were drained and cleaned twice a week.

2.2. RNA interference targeting the *oyster vasa-like gene*

2.2.1. dsRNA synthesis

Two fragments of *oyvlg* cDNA (GenBank accession n° AY423380) from positions 495 to 1020 (*oyvl4*) and 29 to 906 (*oyvl5*) were amplified by RT-PCR using total RNA extracted from female gonad as template. PCR fragments were extracted from agarose gel and purified using a QIAquick® Gel Extraction Kit (Qiagen). O*yvl4*- and *oyvl5*-dsRNA were produced according to the protocol previously described in Fabioux et al. (2009). To assess the quality and synthesis of non-degraded unique fragments of 525 bp (oyvl4) and 877 bp (oyvl5), dsRNA were run by electrophoresis on a 1% agarose gel. *Oyvl4*- and *oyvl5*-dsRNAs were mixed in equi-proportion and diluted in Tris/NaCl to obtain a final concentration of 1 $\mu g.\mu L^{-1}$.

2.2.2. dsRNA administration

Oysters were anesthetized in $MgCl_2$ solution (3/5 fresh water, 2/5 seawater and 50 $g.L^{-1}$ $MgCl_2$) for 3 hours. Anesthetized oysters were injected at T0 (February, beginning of conditioning) and at T19 (19 days after the beginning of the conditioning) directly into the gonad with 100 µL saline solution containing 15 µg (Oyvl 15, n = 20) or 75 µg (Oyvl 75, n = 20) dsRNA. On the same days, 20 oysters were injected with 100 µL saline solution without dsRNA to provide injection controls (IC, n = 20). The remaining oysters were kept as non-injected controls (NIC, n = 40). After injection, the oysters were maintained in raceways in optimal conditions for gonadic maturation (see Fabioux et al.(2005)).

2.2.3. Heat shock challenge and sampling

At T48 (48 days), half of the oysters from each treatment were heat-shocked by putting them in water at 28 ± 1°C for 48 h. Two days later, at T50 (50 days), all oysters were dissected. Gills were sampled, frozen in liquid nitrogen and ground cryogenically using a mixer mill MM400 (Retsch) to obtain a homogenized powder, which was separated into three parts: 50 mg for RNA extraction, 50 mg for lipid peroxidation measurement and 125 mg for protein extraction and enzymatic assays. For 10 oysters non-injected at T0 and all the oysters at T50, a 3 mm-thick section of the visceral mass was cut above the pericardic region and fixed in modified Davidson's solution (Latendresse et al. 2002) at 4°C for 48 h for later histological analysis.

2.3. Histological analysis

The visceral mass sections were dehydrated in an ascending ethanol series, cleared with the xylene substitute Microclearing (DiaPath), and embedded in paraffin. Five µm-thick sections were then cut using a microtome, mounted on glass slides, stained with Harry's hematoxylin-Eosin Y (Martoja et al. 1967), and protected with a cover slip. Slides were examined under a light microscope to determine the sex and reproductive stage of the oysters according to Steele and Mulcahy's (1999) classification. The quantification of reproductive investment was measured as the occupation of the gonad relative to the total surface of the visceral mass, i.e. gonadic occupation (GO), determined by image analysis software (IMAQ Vision Builder, National Instrument) as described in Fabioux et al. (2005).

2.4. Assay of transcript level of genes coding major antioxidant enzymes

2.4.1. RNA extraction and DNase treatment

To extract total RNA, 50 mg of gill powder was homogenized in 1 mL of TRI reagent (Sigma-Aldrich) using an Ultra-Turrax® (IKA), according to manufacturer's instructions. RNA extracts were diluted in 150 µL ultra pure water. Six µg RNA were then treated with DNase RQI (0.5 U/µg total RNA, Promega) to prevent DNA contamination and then diluted in 10 µL ultra pure water. RNA concentrations were measured using a Nanodrop spectrometer (Thermo Scientific) at 260 nm, using the conversion factor 1OD = 40 $\mu g.mL^{-1}$ RNA. RNA quality was checked using RNA nanochips and Agilent RNA 6000 nanoreagents (Agilent Technologies), according to manufacturer's instructions.

2.4.2. Reverse transcription and real-time polymerase chain reaction

From 1 µg of total RNA, reverse transcription was carried out in a 20 µL final volume, using the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas) with $Oligo(dT)_{18}$ Primers. A reverse transcription was also performed in the same conditions using RNA extracted from the entire tissues of 10 wild oysters to serve as a reference sample. The transcript levels of *Catalase* (*CAT*), cytoplasmic *SOD* (*cy-SOD*), mitochondrial *SOD* (*Mn-SOD*), extra-cellular *SOD* (*ec-SOD*), *Glutathione peroxidase* (*GPX*), *Glutathione reductase* (*GR*), *Glutathione S-transferase* (*GST*), *Heat shock protein 70* (*Hsp70*) and *Hypoxia-inducible factor α* (*Hif-α*) were investigated in the samples by real-time PCR, using the Applied Biosystems® 7500 Real-Time System. Amplification of oyster *Elongation Factor I* (*EFI*) (BQ426516) cDNA was made to confirm the steady-state expression of a housekeeping gene and to provide an internal control for gene expression (CV < 5%). PCR efficiency (E) was determined for each primer pair by making standard curves from serial dilutions of control cDNA (from 1/10 to 1/10 000) and using the following formula: $E = 10^{(-1/slope)}$. For each gene, primer sequences, GenBank accession numbers and PCR efficiencies are listed in Table 1. Real-time PCR assays were performed in triplicate, with 3.5 µL cDNA (1/25 dilution) in a total volume of 10 µL, with 0.75 µL of each primer at 1 µM and 5 µL ABsolute™ Blue QPCR *SYBR*® *Green ROX* Mix (Thermo Fisher Scientific). The cycling conditions consisted of Taq polymerase activation for 15 min at 95°C, then 45 cycles consisting of denaturation at 95°C for 15 sec and annealing/elongation at 60°C for 1 min. For each individual sample, a melting curve program was applied from 60°C to 95°C by increasing temperature at a ramp rate of 1%. Each run included the reference cDNA and water controls. Negative reverse-transcription controls (each total RNA sample after DNase treatment) were run to assess the absence of DNA carryover. Calculation of the relative expression ratio (R) of a target gene made using the following formula: $[(E_{\text{target gene}})^{(Ct(\text{target gene, reference cDNA})-Ct(\text{target gene, sample}))}]/[(E_{EFI})^{(Ct(EFI, \text{reference cDNA})-Ct(EFI, \text{sample}))}]$ (Pfaffl 2001).

2.5. Enzymatic analysis of the principal antioxidant enzymes

2.5.1. Total protein extraction and assay

Total protein extraction for subsequent SOD and CAT enzymatic assays was realised using 50 mg of gill powder homogenized in 250 µL lysis buffer [NaCl 150 mM, Tris HCl 10 mM, EDTA 1 mM, EGTA 1 mM, Phosphatase inhibitor cocktail II (Sigma) 1%, Triton® X-100 1%, CA-630 Igepal® (Sigma) 0.5% and 1 tablet/25 mL of Complete EDTA free Protease inhibitor cocktail (Roche)] with an Ultra-Turrax® (Corporeau et al. 2011). The samples were then centrifuged for 45 min at 10 000 g at 4°C and the supernatant collected, aliquoted and stored at -80°C. Total protein extractions for subsequent GR and GST enzymatic assays were realised using 75 mg of gill powder

homogenized in 250 µL extraction buffer [Phosphate Buffer Saline (PBS) solution, EDTA 1 mM, Triton® X-100 0.1%] with an Ultra-Turrax®. The samples were then centrifuged for 10 min at 15 000 g at 4°C and the supernatant was collected, aliquoted and stored at -80°C. Protein assays were performed in triplicate using the DC™ protein assay (Bio-Rad), following the supplier's instructions. Samples used in SOD and CAT enzymatic assays were diluted 10 times in lysis buffer those used in GR and GST enzymatic assays were diluted 10 times in extraction buffer. Results were expressed in mg proteins.mL^{-1}.

2.5.2. Enzymatic assays

For SOD enzymatic assays, the Sigma-Aldrich SOD determination kit was used, following the supplier's instructions. Samples were diluted 20 times in dilution buffer and a standard solution was used to obtain results in $U.mL^{-1}$. For CAT enzymatic assays, the Amplex® Red Catalase Assay Kit (Invitrogen) was used, following the supplier's instructions, and absorbance was measured at 560 nm to obtain results in $U.mL^{-1}$. Samples were diluted 1000 times in dilution buffer. For GR enzymatic assays, samples were diluted 1.5 times in phosphate buffer (100 mM, pH 7.6 at 25°C). Twenty µL of sample were added to 180 µL of reaction buffer (phosphate buffer, EDTA 1 mM, NADPH 0.1 mM, oxidized glutathione 1 mM, DTNB 0.75 mM). Starting immediately after mixing, absorbance was read at 412 nm every 42 sec for 7 min. GR enzyme concentration was determined in $U.mL^{-1}$ using the delta absorbance per minute and the DTNB molar extinction coefficient (14.15 $mM^{-1}.cm^{-1}$). For GST enzymatic assays, samples were not diluted. Ten µL of sample were added to 190 µL of reaction buffer (D-PBS, L-glutathione reduced 2 mM, CDNB 1 mM). Starting immediately after mixing, absorbance was read at 340 nm every 41 sec for 7 min. GST enzyme concentration was determined in $U.mL^{-1}$ using the delta absorbance per minute and the CDNB molar extinction coefficient (9.6 $mM^{-1}.cm^{-1}$). For each sample, assays were performed in duplicates in 96-well microplates and a blank was run. Using the protein concentration results, enzymatic results were then expressed in $U.mg^{-1}$ proteins.

2.6. Measurement of oxidative damage using the malondialdehyde (MDA) assay

The malondialdehyde (MDA) assay method is based on the formation of a MDA/TBA (thiobarbituric acid) to measure lipid peroxidation in gill samples. Approximately 50 mg of gill powder were homogenized in 1 mL Milli-Q water containing 2% of a 1% butylhydroxytoluene methanol solution, using an Ultra-Turrax® (IKA). Then, 3 mL of a 1% phosphoric acid solution and 1 mL of a 1% thiobarbituric acid solution were added. The mix was heated for 30 min at 100°C, cooled on ice and mixed with 5 mL of butanol before being centrifuged for 10 min at 6000 g at 4°C. The supernatant was used to assay the MDA/TBA complex at 532 nm, and MDA quantified using a standard curve. Results were expressed in nmol $MDA.g^{-1}$ wet tissue.

2.7. Statistical analyses

Statistical analyses were performed using STATGRAPHICS® software. According to the Shapiro-Wilk test, results were not normally distributed. Therefore, statistical comparisons between groups of oysters were made by Kruskal-Wallis tests, followed by a Nemenyi-Damico-Wolfe-Dunn post hoc tests for histological analyses and Mann-Whitney tests for gene transcript levels, enzymatic activities and the malondialdehyde assay. The threshold of statistical significance was set at $P < 0.05$. In analyses designed to compare parameters according to reproductive investment, oysters were pooled and divided into quantiles on the basis of their GO to obtain a low GO group (the 25% of males, n = 8, and 25% of females, n = 12, with the lowest GO; mean GO ± SD = 17.66 ±

8.97%) and a high GO group (the 25% of males, n = 8, and 25% of females, n = 12, with the highest GO; mean GO ± SD = 69 ± 6.69%).

3. Results

3.1. Production of oysters with contrasted phenotypes for reproductive investment

Analysis of the gonadic occupation rate (GO) on visceral mass sections from oysters showed that mean GO at the start of the experiment (T0; mean GO ± SD = 10.54 ± 4.12%) was significantly lower than the mean GO at T50 for oysters injected with 15 µg *oyvl*-dsRNA (Oyvl15; 43.67 ± 16.40%), injection controls (IC; 48.10 ± 20.69%) and non-injected controls (NIC; 53.53 ± 18.40%) (Fig. 1). At T50, oysters injected with 75 µg *oyvl*-dsRNA showed a mean GO (Oyvl75; 24.62 ± 17.67%) that was statistically similar to the mean of the T0 group, but significantly lower than the mean GO of Oyvl15, NIC and IC groups (*P = 7.574e-08*). At the individual level, 12 out 19 of the oysters injected with 75 µg *oyvl*-dsRNA showed a reduction in their GO of 50% or more compared with the average in the controls (NIC+IC; 51.63%). Only 2 out of 18 of the oysters injected with 15 µg *oyvl*-dsRNA showed such a reduction. At T50, when all of the oysters from all of the injection treatments were considered, we found that we had successfully obtained a broad range of GO, from 4.45% to 80.40%. The T50 oysters from the different treatments were therefore pooled and tested together in the subsequent analyses.

3.2. Effects of the heat shock treatment on antioxidant parameters, Hsp70 and Hif-α transcripts and MDA levels

The survival rate of the oysters was 100% throughout the heat-shock treatment (48 h at 28°C). The oysters subjected to the heat shock (n = 39, with n = 16 males and n = 23 females) were compared with those not subjected (n = 38, with n = 15 males and n = 23 females) for transcript levels of *CAT*, *cy-SOD*, *Mn-SOD*, *ec-SOD*, *GPX*, *GST*, *Hif-α* and *Hsp70*; enzymatic activities of SOD, CAT, GR and GST; and malondialdehyde (MDA) level in gills. The transcript level of *ec-SOD* was 1.8-fold higher in oysters that had not been exposed to a heat shock than in those that had (*P = 0.027*). There were no significant differences in the other parameters between the oysters that had or had not been exposed to a heat shock. Because we used randomized selection to choose the oysters that were exposed to heat shock, we subsequently found that the exposed group of oysters had a significantly lower mean GO (mean GO ± SD = 38.05 ± 19.83%) than the group of oysters that had not been exposed (48.02 ± 21.41%) (*P = 0.045*). In consequence, all the oysters were grouped and we divided them into quantiles on the basis of their GO to obtain a low GO group (the 25% of males, n = 8, and 25% of females, n = 12, with the lowest GO; mean GO ± SD = 17.66 ± 8.97%) and a high GO group (the 25% of males, n = 8, and 25% of females, n = 8, with the highest GO; mean GO ± SD = 69 ± 6.69%) (Fig. 2). Transcript levels of *CAT*, *cy-SOD*, *Mn-SOD*, *ec-SOD*, *GPX*, *GR, GST, Hif-α* and *Hsp70,* enzymatic activities and MDA level were compared between heat-shocked and non heat-shocked oysters in the low and high GO groups. No differences in the transcript levels, enzyme activities or level of MDA were observed in either the low GO group or the high GO group (Table 2). As a consequence, for the subsequent analyses, oysters were grouped, as described above, to form low GO and high GO groups.

3.3. Effects of gonadic occupation rate on antioxidant parameters and MDA level

The oysters of the low GO group were compared with the oysters of the high GO group for transcript levels of *CAT*, *cy-SOD*, *Mn-SOD*, *ec-SOD*, *GPX*, *GST*, enzymatic activities of SOD, CAT, GR and GST, and MDA level in gills. Oysters of the high GO group showed transcript levels almost 1.90-fold ($P = 0.020$), 1.42-fold ($P = 0.0065$) and 1.27-fold ($P = 0.043$) higher than oysters of low GO group for *ec-SOD*, *Mn-SOD* and *GPX*, respectively (Fig. 3). For the other parameters, no significant differences were observed between oysters of the high and low GO groups (Table 3).

3.4. Effects of sex and gonadic occupation on antioxidant parameters and MDA level

To examine the effect of sex, we considered all the previous analyses according to sex and GO group. When males were compared with females at the same level of GO (low or high) for all the parameters, no significant differences were observed between sexes (Table 3). Males of the low GO group (n = 8; mean GO ± SD = 25.41 ± 9.23%) were then compared with males of the high GO group (n = 8; mean GO ± SD = 71.61 ± 5.12%) and females of the low GO group (n = 12; mean GO ± SD = 12.50 ± 3.46%) were compared with females of the high GO group (n = 12; mean GO ± SD = 67.33 ± 7.26%). Only the *Mn-SOD* values for females showed a significant difference according to GO group (Fig. 3). The transcript level of *Mn-SOD* in females was 1.46-fold higher in the high GO group than in the low GO group ($P = 0.040$). The transcript levels of *Mn-SOD* for males and of *GPX* and *ec-SOD* for both males and females also showed higher values in oysters of high GO groups, but without any significant difference (Fig. 3). No significant variations were observed between high and low GO groups for the transcript levels of the other genes, the enzymatic activities or the MDA level (Table 3).

4. Discussion

Many studies have shown that reproduction is an expensive function in terms of the energy expenditure needed for germ cell production and breeding (Zera et al. 2001; Ernande et al. 2004; Pouvreau et al. 2006). High investment in reproduction can provoke a trade-off in the allocation of energy to the detriment of important physiological functions, such as immunity or defence to stress (Harshman et al. 2007). The energy demand of reproduction is satisfied by an increase in overall metabolic rate during reproductive period (Angilletta et al. 2000). This leads to increased cellular respiration, which results in an elevation of ROS production. If this rise is not counteracted by an equivalent rise in antioxidant capacities, a rupture in the antioxidant/pro-oxidant equilibrium can occur, leading to oxidative stress. Thus, during the reproductive period, organisms have to cope with an elevation of ROS production due to an increase in metabolic activity, and the possibility of an increased susceptibility to stress. Studies on a possible link between reproduction and sensitivity to oxidative stress are very scarce (Salmon et al. 2001; Wang et al. 2001; Alonso-Alvarez et al. 2004; Wiersma et al. 2004). The aim of the present study was to determine whether the extend of reproductive investment can influence antioxidant capacities or susceptibility to oxidative stress in the Pacific oyster *Crassostrea gigas*. For the first time, we manipulated the reproductive investment of oysters by using an *in vivo* RNA interference technique to target *Oyster vasa-like* gene, a germ-cell specific gene, according to the protocol set up by Fabioux et al. (2009). We estimated oyster reproductive investment by measuring gonadic occupation rate, estimated by examining the median part of the visceral mass (Royer et al. 2008). The dose-dependent effect and high intra-group variability we observed in response to *oyvlg*-dsRNA injection allowed us to obtain oysters with a wide range of reproductive investment, corresponding to mosaic knock-down phenotypes, as shown in Fabioux et al. (2009).

To exacerbate any putative difference in pro-oxidant production and/or antioxidant capacities between oysters with contrasted reproductive investment, we performed a 48 h heat shock to increase overall metabolic activity

(Bougrier et al. 1995; Gillooly et al. 2001) and thus potentially increase ROS production to stimulate the antioxidant system. The antioxidant system did not appear modulated after this heat shock, except for *ec-SOD*, which was over-expressed in control oysters compared with heat-shocked oysters. However, the level of expression of *ec-SOD* according to "heat shock treatment" cannot be dissociated from a GO effect because the oysters of the non heat-shocked group had higher GO than the oysters of the heat-shocked group due to the randomness of the experimental design. Moreover, no oxidative damage (estimated by MDA assay) was observed after heat shock, at least for lipids. Finally, no heat stress was revealed by the transcript levels of *Hif-α* and *Hsp70*, both markers of thermal stress (Hamdoun et al. 2003; Kawabe et al. 2012), which were not up-regulated in the oyster gills in response to thermal shock. These results can be explained if the delta of temperature (from 19°C to 28°C) and the maximum temperature used in this study were too low to induce a real thermal stress in *C. gigas*. However, it is interesting to note that a recent paper identified 88 *Hsp70* genes in the oyster genome (Zhang et al. 2012) illustrating the complexity of studying heat shock response in *C. gigas*. It would be interesting to apply a more severe stress to oysters to investigate whether this could induce different antioxidant responses depending on reproductive investment.

Nevertheless, reproduction alone can be enough to reduce antioxidant activity. Wiersma et al. (2004) demonstrated that antioxidant protection was reduced in the pectoral muscles of birds rearing large broods compared with those rearing small broods. In our study, the transcript levels of three major antioxidant proteins, GPX, ec-SOD and Mn-SOD*,* appeared significantly higher for the group of oysters with the highest reproductive investment compared with the group of oysters with the lowest reproductive investment for both males and females, which could relate to an activation of the antioxidant system in gills of oysters with high reproductive investment. GPX catalyses glutathione oxidation, allowing the reduction of hydrogen peroxide to H_2O, and can protect cells by a ROS-scavenging action. It has been seen to be activated at transcription level after different stresses, e.g. following cadmium exposure in oysters (Jo et al. 2008) or a change of salinity in olive flounders (Choi et al. 2008) . In our study, GPX activation may correspond to a mobilisation of glutathione to eliminate high H_2O_2 levels. H_2O_2 may be produced directly by the rise in metabolic rate associated with gonadic maturation or as a product of dismutation of O_2^-, the precursor of most ROS, by SOD enzymes (Pamplona et al. 2011). In our study, catalase, which also eliminates H_2O_2, did not appear up-regulated in oysters with high reproductive investment. The differential regulation between these two enzymes is likely due to their different properties: catalase has less affinity for H_2O_2 than GPX and is useful during peaks of H_2O_2 production, whereas GPX is most useful for the small amounts of H_2O_2 produced continuously by cell metabolic activities (Pamplona et al. 2011), as may occur during active gametogenesis in oysters. In addition to GPX and CAT, SOD is the third type of direct ROS-scavenging enzyme. The expression of *ec-SOD* we measured in the gills likely represented the expression of *ec-SOD* in the infiltrated haemocytes. In fact, it has been shown that *ec-SOD* is expressed only in the haemocytes of oysters (Gonzalez et al. 2005), and gills are bathed by infiltrated haemocytes. Haemocytes are considered to be the main cellular mediators of the bivalve defence system against pathogens and environmental stressors (Cheng 1996). Gonzales et al. (2005) demonstrated that ec-SOD protein could assume different functions, like elimination of microorganisms, and could improve the resistance of haemocytes to oxidative damage. The higher *ec-SOD* transcript levels we observed in the gills of oysters with higher reproductive investment could indicate an important role of haemocytes in ROS detoxification. The activity of the Mn-SOD is restricted to the mitochondria and could play an important role in cell protection in *C. gigas* under oxidative stress conditions (Park et al. 2009). In this work, the differences in *Mn-SOD* transcript levels in gills between oysters with contrasted reproductive investment is very relevant considering that, in the "theory of ageing" by mitochondrial oxidative stress, mitochondria are the first targets of their ROS (Miquel 1998). Thus, ROS are more likely to damage mitochondria first, which is why Mn-SOD appeared to be important in scavenging ROS. Moreover, it has been shown that mitochondria are highly solicited during reproduction in bivalves (Kraffe et al. 2008).

Finally, the apparent stimulation of expression of the direct ROS scavenging enzymes GPX, ec-SOD and Mn-SOD, at transcription level, was likely a response to elevated ROS production due to a high energetic demand associated with high reproductive investment. ROS production was not, however, measured in this study because they are very labile species with a short half-life, from microseconds to nanoseconds (Pamplona et al. 2011). But

no sign of oxidative stress was detected in gills by measuring lipid peroxidation, whatever the level of reproductive investment. In future studies, protein or DNA degradation could be measured to test whether any cellular damage occurred. The transcript level increase of *GPX, ec-SOD* and *Mn-SOD* did not lead to an increase of their enzyme activities. These results are not in accordance with those of Wiersma et al. (2004) on birds, in which antioxidant capacities decreased with reproductive investment. Nevertheless, for SOD, we analysed the overall SOD activity rather than the different SOD isoform specific activities of ec-SOD and Mn-SOD. This is why it would be relevant to assay enzymatic activities of the different isoforms of SOD in future studies on the antioxidant system of oysters, though this will require development of specific protocols. Moreover, a transcriptional activation is not necessarily followed by a translational activation (Chen et al. 2002). We could thus hypothesise that the higher transcription rate for gene encoding antioxidant enzymes observed in gills of oysters with higher reproductive investment would result from a slightly higher production of ROS over the gametogenesis period for these oysters compared to oysters with lower reproductive investment. Nevertheless, a post-transcriptional repression or degradation of these mRNA may occur if the antioxidant activity is sufficient to offset this production of ROS. The mRNA produced would then constitute a stock of transcripts ready for translation in case of the occurrence of an additional production of ROS resulting from a stress to protect oysters with high reproductive investment from oxidative stress.

Our results suggest that oysters have developed enzymatic antioxidant system with basal levels higher enough to cope with elevated metabolism rate resulting from reproduction. As a result, the germ cell production does not seem to lead to a reduction of antioxidant capacity in oyster gills (males or females). Studies have shown that *C. gigas* protein extract was able to protect human epithelial cells against oxidative stress and elevate the level of glutathione and GST activity in several organs of rats (Gaté et al. 1998; Gaté et al. 1999), suggesting that there is a high basal antioxidant level in *C. gigas* oyster. This could be due to the ecology of *C. gigas*, since this intertidal species is accustomed to daily re-oxygenation after low tide, leading to an increase of ROS production, in a manner resembling reperfusion-ischemia injury (Storey 1996). This mechanism could explain the great resistance and adaptation of *C. gigas* to unfavourable environments that stimulate high ROS production. The recent data on oyster genome and transcriptome in response to environmental stressors have confirmed important genomic adaptations of *C. gig*as to life in highly stressful environments (Zhang et al. 2012).

To our knowledge, our study is the first that has aimed to determine a possible link between reproductive investment and management of oxidative metabolism in marine bivalves. To conclude, our data suggest that reproductive investment alone is not sufficient to significantly interfere with antioxidant capacity or susceptibility to oxidative stress in *C. gigas*.

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Tables

Table 1: Sense and anti-sense sequences of primers used for real-time PCR analysis. PCR efficiencies were determined for each primer pair by determining the slopes of standard curves obtained from serial dilution analysis of the reference cDNA.

GenBank access	EST name or annotation	Abbreviation	Forward primer 5'-3'	Reverse primer 5'-3'	PCR Efficiency %
GenBank ID: **BQ426516**	Elongation Factor 1	EFI	ACCACCCTGGTGAGATCAAG	ACGACGATCGCATTTCTCTT	101.7
GenBank ID: **AM853618**	Catalase	CAT	TTCGTCATATCGGGTTTACTTCTG	CCTTGTCACGTCCTGCCATT	101.9
GenBank ID: **AJ496219**	Superoxide dismutase	Cy-SOD	GACGATTGGCTTGTGGAGTGA	GGACCGAGTGGAGGCTAGTG	100
GenBank ID: **DQ010420**	Hemocyte extracellular superoxide dismutase	Ec-SOD	CTTCATGCCAGGCAACCT	TGACGTTGAATCCGGTCA	100.2
GenBank ID: **EU420128**	Manganese-superoxide dismutase	Mn-SOD	AGTCTGGTCGCACATTCTTGT	CATGTGCCAATCAAGATCCTC	104.7
GenBank ID: **AM856731**	Glutathione peroxidase	GPX	GGACTACCCGATGATGAACGA	GGAAGGAAACCCGAGAACCA	99.1
GenBank ID: **CU685269**	Glutathione reductase	GR	TTCGCCCTGCTGCTATGG	TTGCCCTGGGAGATGTTTG	99
GenBank ID: **FP091130**	Glutathione S-transferase	GST	GGATTCAAACGCCTCAAACAG	TGGGCAGTTGGCAAAAAGA	101.1
GenBank ID: **AM854673**	Heat shock protein 70	HSP70	CCTGCAATATGGAGTGATTCG	CTTCCGGTTCATAAGCCATC	101.8
GenBank ID: **AB289857**	Hypoxia-Inducible factor α	HIF-α	ACAGCCGCACCCAGAAAC	TCAGGCTTGGATTCTCAATTCC	99.2

Table 2: Comparison of relative mRNA levels, enzymatic activities and lipid peroxidation in gills (mean ± standard error) between heat-shocked and non heat-shocked oysters for high or low gonadic occupation. P-value obtained with the Mann-Whitney test.

			Relative mRNA levels									Enzymatic activities				Lipid peroxidation
			cy-SOD	*ec-SOD*	*Mn-SOD*	*CAT*	*GPX*	*GR*	*GST*	*Hif-α*	*HSP70*	SOD U/mg	CAT U/mg	GR mU/mg	GST mU/mg	MDA nmol/g of wet tissue
Low GO group	Heat shock/	n=14	9.86 ± 3.11	1.70 ± 0.41	3.13 ± 0.36	5.06 ± 1.14	20.61 ± 3.12	7.80 ± 1.26	29.92 ± 5.43	19.83 ± 2.99	1.31 ± 1.29	19.92 ± 1.06	131.41 ± 15.05	1.37 ± 0.16	7.88 ± 1.09	40.98 ± 2.84
	No heat shock	n=6	8.74 ± 4.75	1.32 ± 0.62	3.20 ± 0.55	4.93 ± 1.74	21.48 ± 4.77	10.49 ± 1.92	20.85 ± 8.30	28.28 ± 11.64	0	22.48 ± 1.62	133.19 ± 22.99	1.09 ± 0.24	6.02 ± 1.67	44.54 ± 4.34
		P-value	0.84	0.54	0.97	0.84	0.30	0.15	0.34	0.97	0.38	0.17	0.90	0.34	0.59	0.96
High GO group	Heat shock/	n=6	9.43 ± 4.80	1.79 ± 0.50	4.85 ± 0.96	5.42 ± 0.78	21.04 ± 4.08	6.57 ± 1.52	27.98 ± 2.89	16.45 ± 3.87	5.55 ± 5.52	21.79 ± 1.31	91.58 ± 13.90	0.99 ± 0.29	5.43 ± 1.64	37.99 ± 4.70
	No heat shock	n=14	15.22 ± 3.44	3.53 ± 0.78	4.32 ± 0.24	5.64 ± 0.99	28.98 ± 2.16	9.37 ± 1.22	26.60 ± 3.01	15.56 ± 2.24	11.93 ± 8.06	21.66 ± 1.11	109.86 ± 12.01	1.25 ± 0.21	6.34 ± 1.05	51.10 ± 5.30
		P-value	0.48	0.13	0.97	0.71	0.09	0.17	0.77	0.84	0.79	0.97	0.59	0.39	0.65	0.17

Table 3: Comparison of relative mRNA levels, enzymatic activities and lipid peroxidation in gills (mean ± standard error) between oysters, depending on gonadic occupation group and/or sex. P-value obtained with the Mann-Whitney test.

			Relative mRNA levels				Enzymatic activities				Lipid peroxidation
			cy-SOD	*CAT*	*GR*	*GST*	SOD U/mg	CAT U/mg	GR mU/mg	GST mU/mg	MDA nmol/g of wet tissue
	Low GO group/	n=20	9.52 ± 2.24	5.02 ± 0.85	8.61 ± 1.00	27.20 ± 4.17	20.69 ± 0.83	131.94 ± 11.34	1.28 ± 0.12	7.32 ± 0.80	41.92 ± 2.87
	High GO group	n=20	13.48 ± 2.80	5.57 ± 0.72	8.53 ± 0.99	27.01 ± 2.24	21.70 ± 0.85	104.37 ± 9.38	1.17 ± 0.17	6.07 ± 0.87	47.17 ± 4.13
		P-value	0.44	0.24	0.95	0.60	0.51	0.08	0.18	0.23	0.34
Low GO group	Females/	n=12	5.72 ± 2.27	5.77 ± 1.29	8.42 ± 1.55	27.61 ± 6.07	20.51 ± 1.32	130.59 ± 16.14	1.33 ± 0.18	7.75 ± 1.20	44.37 ± 4.41
	Males	n=8	15.23 ± 3.75	3.89 ± 0.79	8.89 ± 1.04	26.58 ± 8.14	20.94 ± 0.70	133.97 ± 16.05	1.22 ± 0.12	6.69 ± 0.92	38.54 ± 3.04
		P-value	0.10	0.15	0.97	0.67	0.73	0.79	0.97	0.67	0.53
High GO group	Females/	n=12	13.05 ± 3.64	6.19 ± 1.08	8.10 ± 1.07	24.08 ± 2.70	21.93 ± 1.25	100.26 ± 11.96	0.95 ± 0.14	4.92 ± 0.92	46.19 ± 5.77
	Males	n=8	14.12 ± 4.69	4.65 ± 0.75	9.19 ± 1.97	31.41 ± 2.84	21.34 ± 1.09	110.55 ± 15.85	1.51 ± 0.35	7.79 ± 1.54	48.63 ± 6.06
		P-value	0.97	0.51	0.79	0.15	0.67	0.73	0.15	0.15	0.67
Males	Low GO group/ High GO group	P-value	0.96	0.19	0.79	0.56	0.87	0.37	0.87	0.71	0.19
Females	Low GO group/ High GO group	P-value	0.34	0.62	0.88	0.98	0.51	0.17	0.09	0.13	0.95

Figures

Figure 1. Gonadic occupation rate (percentage surface occupied by the gonad / total area of the visceral mass) depending on the injection treatment (mean ± standard deviation). T0 = oysters sampled at T0; T50 = oysters sampled at 50 days post injection: NIC = non-injected controls (n = 26), IC = injection controls (n = 15), Oyvl15 = injection with 15 µg *oyvl*-dsRNA (n = 18), Oyvl75 = injection with 75 µg *oyvl*-dsRNA (n = 19). Multiple comparisons were made between groups using a Kruskal-Wallis test followed by a Nemenyi-Damico-Wolfe-Dunn post hoc test at the 5% level; homogenous groups share letters.

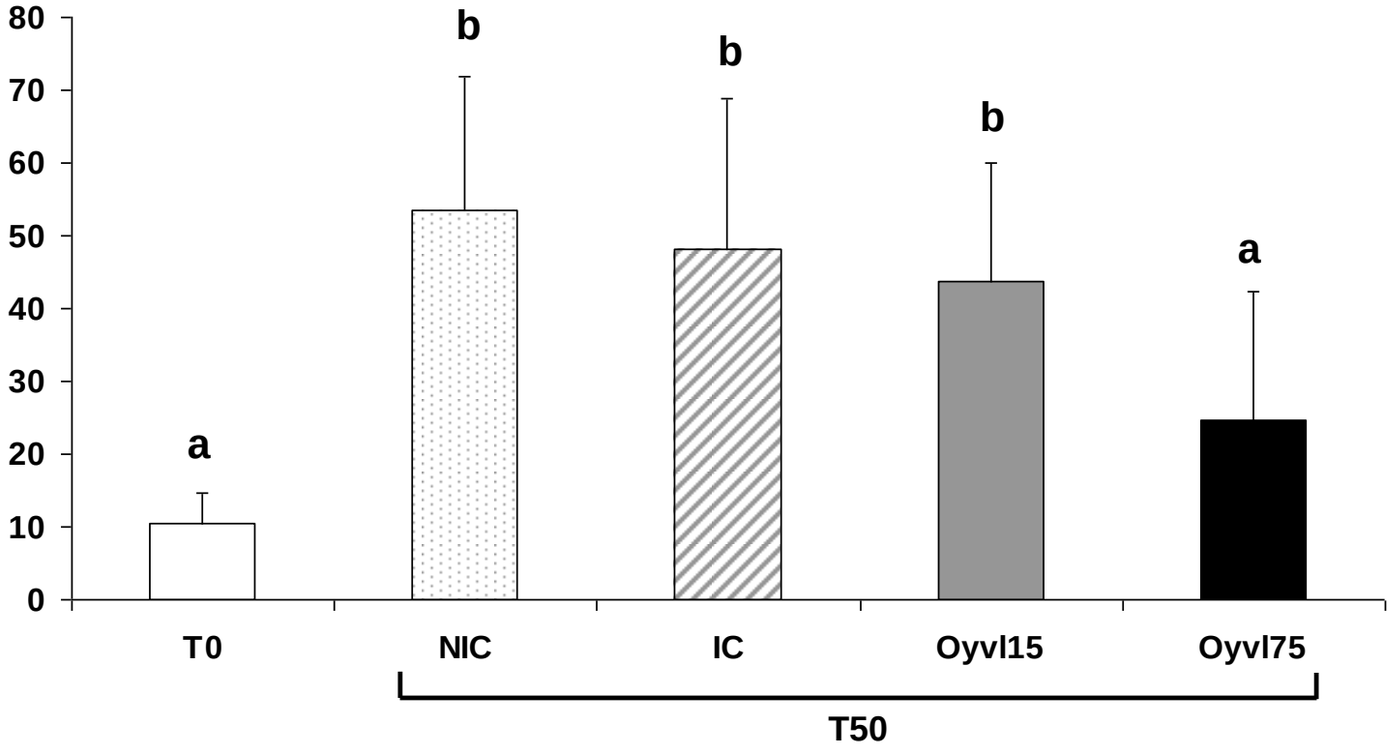


Figure 2. Individual gonadic occupation rate (percentage surface occupied by the gonad / total area of the visceral mass) at T50 for the non injected controls (white and grey dots), injection controls (white and grey striped), oysters injected with 15 µg *oyvl*-dsRNA (grey) or 75 µg *oyvl*-dsRNA (black). M = males and F = females. Asterisks indicate oysters subjected to the heat-shock. Oysters selected on the basis of their gonadic occupation rate to form low and high GO groups are indicated by brackets.

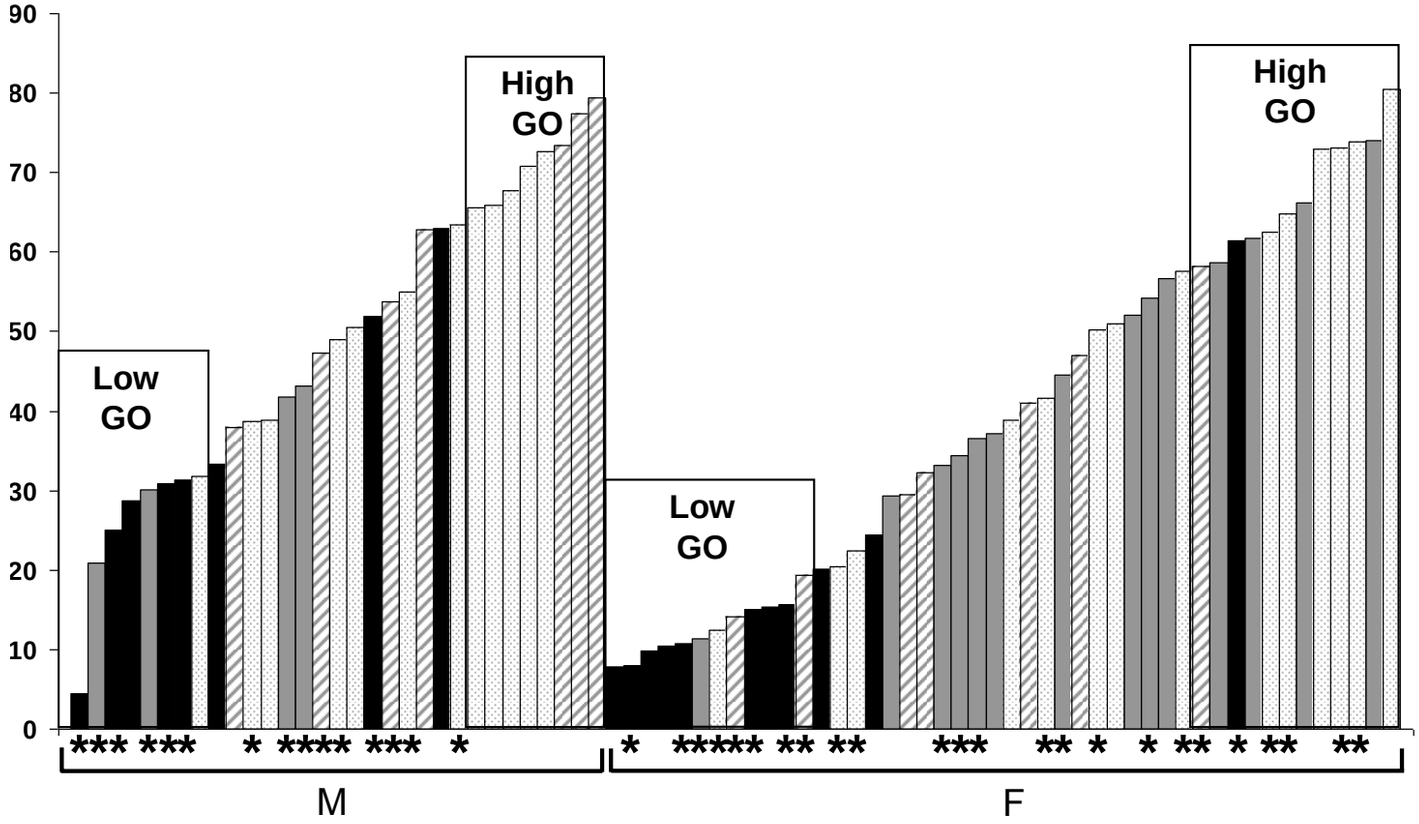
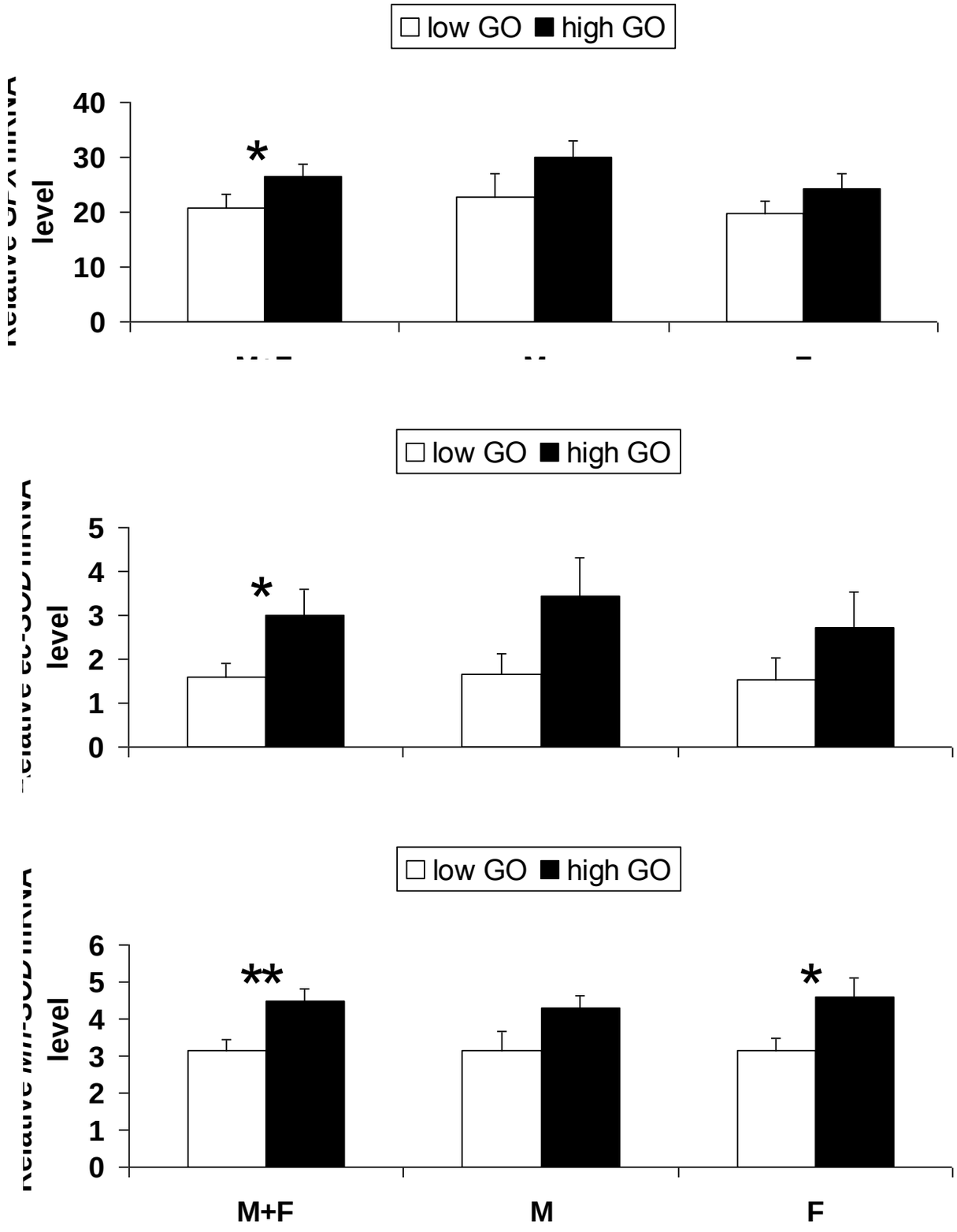


Figure 3. Level of *GPX* (a), *ec-SOD* (b) and *Mn-SOD* (c) transcripts relative to *Elongation Factor I* transcripts in the gills of oysters from the high and low gonadic occupation groups. M = males and F = females. Vertical bars represent standard errors. One asterisk indicates a P-value < 0.05, two asterisks indicate a P-value < 0.01 (Mann-Whitney test).


low GO
high GO
level
40
30
20
10
0
*
low GO
high GO
level
5
4
3
2
1
0
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low GO
high GO
level
6
5
4
3
2
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*
M+F
M
F