

Gonadal Antioxidant Responses to Seawater Acidification and Hypoxia in the Marine Mussel *Mytilus Coruscus*

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Abstract

This study investigated the combined effects of seawater acidification and hypoxia on the gonadal antioxidant response of the thick shell mussel *Mytilus coruscus* mainly distributed along the Shengsi Island, East China Sea, where hypoxia and pH fluctuations frequently occur in summer. Mussels were exposed to three pH levels (8.1, 7.7 and 7.3) and two dissolved oxygen (DO) levels (6 and 2 mg L⁻¹) for 21 days following a 10-day recovery. Activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione (GSH), glutathione S-transferase (GST) and malondialdehyde (MDA) in gonad and gonad surface area (GSA) were measured at day 21 and 31. Among all the parameters, there was no significant change in SOD activity. GSA and the activity of CAT and GST were decreased under acidification and hypoxia, but GPX, GSH and MDA were increased. PCA showed that the changes were influenced by pH more than DO. Interaction between acidification and hypoxia was found significant on GPX activity and GSA. Integrated biomarker response (IBR) analysis demonstrated that acidification and hypoxia impaired mussel's antioxidant system and increased oxidative damage. Our results clearly showed that acidification and hypoxia synergistically exert negative impact on the antioxidant system and gonad development of mussels, and the effect of acidification was more significant.

1. Introduction

Ocean acidification (OA) refers to the process in which absorption of excess carbon dioxide from the atmosphere causes a chemical reaction to lower the pH of the ocean (Birchenough et al., 2015; Orr et al., 2005; Feely et al., 2004). The atmospheric CO₂ concentration has increased from 280 ppm to 380 ppm since the industrial age. Moreover, it may reach 1200 ppm and the pH may reduce 0.4 units by the end of this century according to an earth biochemical model (Caldeira, K., & Wickett, M. E., 2003). Nowadays, OA has caused global concern for its effects on marine ecosystem and organisms (Noor & Das, 2019; Jin et al., 2020). The effects on marine bivalves are especially significant (Tan & Zheng, 2020). OA exerts plenty of impacts on survival, calcification, growth and reproduction of marine bivalves by breaking the acid-base balance and reducing the saturation state of CaCO₃ (Anthony et al., 2008; Doney et al., 2009; Nakamura et al., 2011; Orr et al., 2005). Since different species and even various life stages of the particular specie showed different responses to OA (Ries et al., 2009; Melzner et al., 2009), the understanding of these impacts is still weak.

Hypoxia is also a world-wide phenomenon in coastal waters over the past decades (Deutsch et al., 2011; Zhang et al., 2010; Schmidtko et al., 2017; Breitburg et al., 2018), which is mainly caused by greenhouse gas-driven global warming and eutrophication in marine ecosystems (Breitburg et al., 2018). Over 400 hypoxia zones were observed in the coastal ocean since 2008 and a large number of 245000 square kilometers oceanic area was affected, including the East China Sea which has been reported to lack oxygen since 1950s (Diaz & Rosenberg, 2008; Li et al., 2011; Chen et al., 2020).

Studies have showed the expressions of oxidative stress-related genes and antioxidant enzyme activities of marine mussels were affected by hypoxia (Woo et al., 2013; Sui et al., 2017; Nogueira et al., 2017). Antioxidant enzyme activities in gill and hemocyte of mussels such as superoxide dismutase (SOD) activity, catalase (CAT) activity and glutathione peroxidase (GPX) of marine mussels were also activated under OA (Huang et al., 2018; Sui et al., 2017). Moreover, the production of reactive oxygen species (ROS) in the cells was increased under low oxygen concentration (Ekau et al., 2010; Levin et al., 2009), indicating the potential risk of oxidative damage (Hermes-Lima et al., 2015).

Some studies have showed the inhibited gonadal development in marine invertebrates under low pH or hypoxia (Parker et al., 2018; Mos et al., 2016; Kurihara et al., 2013; Uthicke et al., 2014; Aguirre-Velarde et al., 2019). Acidification and hypoxia can change the energy distribution (Sui et al., 2016), subsequently may affect reproduction and population dynamics through reducing gonadal performance. In addition, the combined effects of hypoxia and acidification that have occurred simultaneously on marine mussels are not well clarified. In this study, marine mussels *M. coruscus* collected from the Shengsi Island, East China Sea, where this mussel experiences low dissolved oxygen and OA in their cultured area in the wet season (Sui et al., 2017), were exposed to acidification and hypoxia to evaluate the gonadal antioxidant responses as well as its correlation with reproduction.

2. Materials And Methods

2.1. Animals

Thick shell mussels *M. coruscus* (80 ± 10 mm shell length, 160 ± 20 mg dry tissue weight) were sampled from the Shengsi island, East China Sea ($30^{\circ}33'00.945''\text{N}$, $121^{\circ}49'59.757''\text{E}$) and immediately transferred to the laboratory. Undamaged mussels were selected and acclimated in 500L aquaria containing aerated seawater with similar salinity (25psu), temperature (25°C), oxygen concentration (6 mg L^{-1}) and pH value (8.1) as the waters from where the animals were collected. Carbonate parameters in each treatment during the experiment were measured and summarized in Table 1. The light regime is 12 h light/12h dark. Mussels were fed with microalgae *Chlorella spp.* every 12 h ($25,000 \text{ cells mL}^{-1}$).

Table 1

Seawater carbonate system chemistry parameters during the experimental period (mean \pm SD, n = 3). Seawater pH, temperature, salinity and total alkalinity (A_T) were measured. Partial CO_2 pressure (pCO_2), dissolved inorganic carbon (DIC), aragonite and calcite saturation states (respectively Ω_{ar} and Ω_{ca}) were calculated using CO_2SYS software.

Treatments	DO	pH	Salinity	Temperature	A_T	DIC	pCO_2	Ω_{ca}	Ω_{ar}
pH*DO	(mg L ⁻¹)		(psu)	(°C)	(μ mol Kg ⁻¹)	(μ mol Kg ⁻¹)	(matm)		
8.1*6.0	6 \pm 0.05	8.11 \pm 0.01	25.1 \pm 0.1	25.1 \pm 0.1	2308 \pm 9	2044 \pm 9	366 \pm 9	5.26 \pm 0.08	3.36 \pm 0.05
7.7*6.0	6 \pm 0.03	7.71 \pm 0.02	24.9 \pm 0.6	25.2 \pm 0.1	2291 \pm 8	2200 \pm 16	1060 \pm 58	2.35 \pm 0.10	1.50 \pm 0.07
7.3*6.0	6 \pm 0.02	7.31 \pm 0.01	25.0 \pm 0.1	25.1 \pm 0.1	2209 \pm 6	2238 \pm 6	2697 \pm 31	0.96 \pm 0.01	0.61 \pm 0.01
8.1*2.0	2 \pm 0.01	8.11 \pm 0.02	24.9 \pm 0.0	24.9 \pm 0.1	2310 \pm 7	2050 \pm 9	370 \pm 19	5.21 \pm 0.19	3.32 \pm 0.12
7.7*2.0	2 \pm 0.05	7.70 \pm 0.01	25.1 \pm 0.1	25.0 \pm 0.2	2288 \pm 7	2198 \pm 11	1062 \pm 37	2.33 \pm 0.06	1.49 \pm 0.04
7.3*2.0	2 \pm 0.04	7.30 \pm 0.01	24.8 \pm 0.1	25.0 \pm 0.2	2208 \pm 5	2240 \pm 6	2730 \pm 65	0.94 \pm 0.02	0.60 \pm 0.01

2.2 Experimental design

Prior to the exposure, the mussels were kept under the above conditions for two weeks. Then the mussels were exposed to six combinations of three pH (8.1, 7.7 and 7.3) and two DO (6 and 2 mg L⁻¹). Every treatment had three replicates (tanks), each replicate contained 30 mussels. pH 8.1 is the present pH value. pH 7.7 is the reduced pH predicted for the year 2100 and the minimum of present natural variability at the sampling site (Li et al. 2014), and pH 7.3 is the expected pH by 2300 (Caldeira and Wickett, 2005). DO 6 mg L⁻¹ is the normal value in the seawater around the Shengsi island and DO concentrations in the East China Sea were reported to reach 2–3 mg L⁻¹ in summer (Chen et al., 2007a, 2007b). pH was maintained by aerating CO_2 , the flow rate of which is controlled by pCO_2 /pH feedback STAT systems (DAQ-M) operated by CapCTRL software (Loligo Systems Inc, Tjele, Denmark). DO was maintained by inputting N_2 and air via an O_2 regulator (Loligo Systems Aps, Tjele, Denmark). The gas flow was controlled by a valve, which was dominated by a computer connected to an O_2 regulator allowing maintaining a constant DO level in each aquarium. Salinity was detected daily with a multiparameter instrument (model 5200A, YSI, USA) and total alkalinity (A_T) was calculated by titration method. pCO_2 ,

saturation states of calcite (Ω_{ca}), aragonite (Ω_{ar}) and dissolved inorganic carbon (DIC) were determined using CO₂SYS software. After the treatment, there is a 10-day recovery period.

2.3 Tissue collection and preparation

Gonads for the antioxidant analysis were collected at the 21st and the 31st day. For each treatment, 6 samples were dissected and gonads were carefully excised, surface dried with tissue paper, thoroughly washed with phosphate buffer (50 mM; pH 7.4), and kept in tubes on ice. The gonad surface area (GSA) was measured and the results were expressed as square millimeter (mm²). Aliquots from each tissue pool were immediately frozen in liquid nitrogen and stored at -80°C for further analysis. The gonads were thawed on ice and homogenized (1:4, w-v) in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA, 1 mM Dithiothreitol (DTT, Sigma) and 40 µg mL⁻¹ Aprotinin (Sigma). Homogenization was performed at 4°C using 12–15 strokes of a motor driven Teflon Potter-Elvehjem homogenizer. Homogenized samples were sonicated for 2 min at 0°C with a Braun Labsonic U sonifier at 50% duty cycles and then centrifuged at 12000g for 45 min at 4°C. Supernatants were collected for biochemical analysis.

2.4 Antioxidant assays

Commercial kits from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) were used for antioxidant assays. Optical density values were measured using a microplate reader (Flexstation® 3, Molecular Devices, California, USA). The protein content of enzyme crude extract was determined using Coomassie Brilliant Blue (G-250) method.

2.4.1 Superoxide dismutase (SOD) assay

SOD activity was measured using the nitro blue tetrazolium (NBT) method according to Sun et al. (1988). NBT is reduced to blue forms by O₂⁻ generated by the xanthine/xanthine oxidase system, which has a strong absorbance at 560 nm. One unit (U) of SOD is defined as the amount that inhibits the rate of NBT reduction by 50%.

2.4.2 Catalase (CAT) assay

CAT activity was measured using a spectrophotometric assay of hydrogen peroxide based on the formation of its stable complex with ammonium molybdate at 405 nm. Briefly, a H₂O₂ degradation reaction catalyzed by CAT was terminated by adding ammonium molybdate, and the intensity of a yellow complex formed by molybdate and H₂O₂ at 405 nm was measured. One U of enzyme activity is defined as the degradation of 1 µmol H₂O₂ per second per mg of protein.

2.4.3 Glutathione peroxidase (GPX) assay

The activity of GPX was quantified as the rate of H₂O₂-induced oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG). A yellow product with absorbance at 412 nm can be formed as GSH reacted

with dithiobisnitrobenzoic acid (Xia and Zhou, 1987). One U of GPX is defined as the amount that reduces the level of GSH by $1\mu\text{mol L}^{-1}$ in 1 min per mg of protein.

2.4.4 Malondialdehyde (MDA) assay

MDA was assessed in gonad by measuring the thiobarbituric acid reactive substances (TBARS). The TBARS formed was measured in a microplate reader at 532 nm and quantified as malondialdehyde equivalents using 1,1,3,3-tetramethoxypropane as the standard (Ohkawa et al., 1979). MDA content in the extract is expressed as $\text{nmol TBARS mg}^{-1}$ protein.

2.4.5 Glutathione S-transferase (GST) assay

The GST activity was determined according to the method of Habig et al. (1974), using 2 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 2 mM reduced glutathione in 0.1 M potassium phosphate buffer, pH 7.0. The absorbance was monitored for 2 min at 30°C at 340 nm for a better detection in the microplate reader. One U of GST activity is the amount which catalyze the conjugation of 1mM of substrate per minute.

2.4.6 Glutathione (GSH) assay

Levels of GSH were measured according to the method of Ringwood et al. (1999) by reading the optical density of the yellow substance formed when 5,5'-dithio-2-nitrobenzoic acid is reduced by GSH at 412 nm. GSH content in the extract was determined as nmol mg^{-1} protein by a standard curve generated with GSH at diverse concentrations.

2.5 Integrative biomarker indices

Six biomarkers (SOD, CAT, GPX, MDA, GST, GSH) were integrated by IBR index according to Beliaeff and Burgeot (2002). Steps are as follows: (1) calculation of mean and standard deviation for each biomarker; (2) standardization of data for each sample: $Y = (X - m)/s$, where Y = standardized biomarker response, X = response value of each biomarker, m = mean value of the biomarker, and s = standard deviation of the biomarker; (3) Z was then calculated using the formulae as $Z = Y$ or $Z = -Y$ for biomarker induced or inhibited; (4) Score for the biomarker was computed as $S = Z + |\text{min}|$, where $S \geq 0$ and $|\text{min}|$ = absolute minimum value of Y for each biomarker; (5) Scores were visualized in a star plot and correspond to radial coordinates. $\text{IBR} = \text{Sin}\alpha [(S_1 \times S_{i+1})/2 + (S_{i+1} \times S_{i+2})/2 + \dots + (S_{n-1} \times S_n)/2]$, where α = angle formed by each two consecutive scores, and n = the number of biomarkers.

2.6 Statistical analysis

Shapiro-Wilk test was used to test the normality of the data, and Leven's test was used to test the homogeneity of the variance of the data using SPSS 18.0 before statistical analysis. The effects of pH, hypoxia and their interactions on SOD, CAT, GPX, GSH, GST and MDA were analyzed by two-way analysis of variance (ANOVA). One-way ANOVA was used to analyze the significant effect of pH value at each fixed DO level, and Tukey's HSD test was performed for post hoc test. The Student's t-test was used to analyze the significant effect of DO on pH level. Principal component analysis (PCA) was conducted

using Origin 2018. For all analysis, results were expressed as mean \pm SEM and $P < 0.05$ was a significant difference.

3. Results

3.1 Gonad surface area (GSA)

At day 21 and 31, acidified seawater significantly decreased GSA at both DO level but low DO just significantly decreased GSA under pH 8.1 at the 21st day and pH 7.1 at the 31st day (Fig. 1). There was a significant interaction between DO and pH level (Table 2&3).

Table 2
 Summary of two-way ANOVA testing effects of pH and dissolved oxygen (DO) on SOD, CAT, GPX, MDA, GST, GSH and GSA in the gonad of *M. coruscus* at day 21.

Sources		pH	DO	pH + DO
	df	2	1	2
SOD	MS	1152.725	4055.217	255.146
	F	2.627	9.241	0.581
	P	0.089	0.005	0.565
CAT	MS	0.761	0.501	0.061
	F	9.924	6.530	0.802
	P	< 0.001	0.016	0.458
GPX	MS	111.234	108.116	87.260
	F	32.802	31.883	25.733
	P	< 0.001	< 0.001	< 0.001
GSH	MS	97.457	52.493	11.250
	F	9.491	5.112	1.096
	P	< 0.001	0.031	0.347
GST	MS	1218.627	37.319	55.764
	F	142.138	4.353	6.504
	P	< 0.001	0.046	0.005
MDA	MS	101.857	0.595	14.596
	F	20.267	0.118	2.904
	P	< 0.001	0.733	0.070
GSA	MS	19120.090	7327.132	3660.906
	F	21.510	8.243	4.118
	P	< 0.001	0.007	0.026

Table 3
 Summary of two-way ANOVA testing effects of pH and dissolved oxygen (DO) on SOD, CAT, GPX, MDA, GST, GSH and GSA in the gonad of *M. coruscus* at day 31.

Sources		pH	DO	pH + DO
	df	2	1	2
SOD	MS	301.336	415.817	132.890
	F	0.587	0.810	0.259
	P	0.562	0.375	0.774
CAT	MS	< 0.001	0.002	< 0.001
	F	0.088	1.231	0.090
	P	0.916	0.276	0.914
GPX	MS	88.815	399.039	106.971
	F	17.630	79.209	21.234
	P	< 0.001	< 0.001	< 0.001
GSH	MS	0.855	0.038	8.643
	F	0.064	0.003	0.003
	P	0.938	0.958	0.531
GST	MS	1476.412	16.854	24.704
	F	52.513	0.599	0.879
	P	< 0.001	0.445	0.426
MDA	MS	1.109	0.051	0.505
	F	0.258	0.012	0.118
	P	0.774	0.914	0.889
GSA	MS	14099.556	1345.728	2799.864
	F	23.343	2.228	4.635
	P	< 0.001	0.146	0.018

3.2 Antioxidant assays

There was no significant difference in SOD activity during either the exposure or recovery period except at the 21st day under pH 8.1 and DO 2 (Fig. 2). CAT activity was affected just by pH level. At the 21st day, it was significantly decreased at the extreme pH 7.3 (Fig. 3). At day 31, the exposure group recovered and

showed no significant difference compared with the control group (Table 2&3). GPX activity was significantly affected by pH, DO and their interaction. At day 21, GPX activity was significantly increased by pH 7.7 under DO 6 mg L⁻¹ and pH 7.3 under DO 2 mg L⁻¹. Low DO significantly increased GPX activity at pH 8.1 and 7.3. At day 31, pH 7.7 significantly decreased GPX activity but pH 7.3 increased it. Low DO significantly increased GPX activity under low 7.3 (Fig. 4). The interaction between DO and pH was found significant at two sampling times (Table 2&3).

During the exposure period, only pH had a significant effect on GSH activity. Low pH significantly increased GSH activity at DO 6 mg L⁻¹. The GSH activity of all exposed mussels returned to normal levels after a ten-day recovery (Fig. 5).

pH 7.3 significantly decreased GST activity at day 21 and pH 7.7 decreased it under DO 6 mg L⁻¹. After recovery, mussels recovered except those exposed to pH 7.3 (Fig. 6). The interaction between DO and pH was found significant only at day 21 (Table 2).

Significant increase of MDA level was found in mussels exposed to low pH at day 21. Low DO significantly decreased MDA level only at pH 8.1. After recovery, MDA levels returned to normal in all exposed treatments (Fig. 7).

3.3 Integrated biomarker response

Six biomarkers (SOD, CAT, GPX, MDA, GST, GSH) were selected and standardized in axes of star plot. All the biomarkers were responsive to exposure but did not show a similar pattern. The IBR value decreased in all exposed treatments except the group exposed to pH 7.3 and DO 2 mg L⁻¹. The lowest value was observed in the treatment exposed to pH 7.7 and DO 2 mg L⁻¹ at day 21 and pH 7.3 DO 6 mg L⁻¹ at day 31, respectively (Fig. 8).

3.4 Principal component analysis (PCA)

PCA showed that PC1 expressed 64.94% and PC2 expressed 13.08% of total variance after exposure (Fig. 9). After exposure, PC1 was influenced by biochemical indexes (CAT, GST, GPX, GSH, MDA and GSA). This axis allowed to distinguish between mussels exposed normal pH and decreased pH. SOD activity contributed most to the PC2. CAT and GST were grouped together and negatively correlated with GPX, GSH and MDA.

4. Discussion

When stimulated by the environmental stressors, the dynamic balance between the generation and removal of reactive oxygen species (ROS) in organisms will be disturbed. Excessive ROS can be removed by the enzymatic reaction of antioxidant enzymes. Moreover, peroxidation mostly occurs on the polyunsaturated fatty acids in the plasma membrane phospholipids, and the change of ROS can be judged by detecting MDA, the product of lipid peroxidation (Diguiseppi et al., 1984). The purpose of this study is to clarify the antioxidant response in the gonad of marine mussel *M. coruscus* under ocean

acidification and hypoxia and its connection with gonad development. By exposing mussels to acidified and hypoxic seawater, a series of antioxidant changes were observed.

The relative contribution of each antioxidant enzyme to protect against oxidative stress during exposure period is not well known, and the relationships among reactive oxygen species (ROS) levels and activities of antioxidant enzymes are complex. In this study, the decrease of CAT and GST and the increase of GPX, GSH and MDA under acidification and hypoxia were observed, but no significant changes in SOD.

As a vital scavenger of H_2O_2 , CAT is considered as a second line of antioxidant defense (Chelikani et al., 2004). Although CAT activity often increases as a result of the increased H_2O_2 under oxidative stress (Marcelo Hermes-Lima., 2004), multiple stressors may change this trend (Matozzo et al., 2013). As we found, CAT activity was significantly reduced during short-term acidification and hypoxia exposure. Woo et al. (2013) also found that CAT activity decreased when mussels *Mytilus galloprovincialis* were exposed to hypoxia. The decline may be GPX-related.

GPX not only participates in the conversion of H_2O_2 to water and molecular oxygen, but also plays a vital role in the use of GSH as a reducing agent to convert other lipids to non-toxic products (Sies et al., 1997). In our study, GPX activity was significantly increased by acidification and hypoxia. In some other researches, the effect of pH or hypoxia was quite different, mainly due to different matching stresses and test subjects. Lima et al. (2019) found that the GPX activity in oyster *Crassostrea gasar* exposed to combined effects of acute pH changes and phenanthrene for 96 h showed no significant change. Johannsson et al. (2018) found GPX rose in the brain and gills of Characid fish *Cyphocharax abramoides* during hypoxia. Although both GPX and CAT could catalyze the decomposition of H_2O_2 , their different changes suggested that GPX is more capable of scavenging free radicals than CAT, which is consistent with previous research (Dorval et al., 2003).

GSH is a major thiol compound that acts as a protective agent for a variety of toxic substances through thiol groups. Its functions include the direct removal of oxy compounds and catalysis of organic hydrogen peroxide or H_2O_2 , maintaining membrane protein thiols and acting as a substrate for GPX and glutathione reductase (Habig et al., 1974; Moreno et al., 2005). Some studies have shown that OA and hypoxia or the combination with other stressors either decrease or increase GPX activity, depending on the species and specific tissue (Huang et al., 2018; Khan & Ringwood., 2016). Hu et al. (2015) found GSH in gill and digestive gland of mussels exposed to acidification increased significantly. Similar in our experiment, the GSH activity was increased by pH under $DO\ 6\ mg\ L^{-1}$.

In the process of oxidative stress, GST greatly increases on the basis of sulfhydryl reaction metabolites and the reduction of oxygen species, playing a fundamental role in the process of chemical detoxification (Aniya et al., 1993; Sheehan et al., 2001). Lopes et al. (2018) found GST activities in the soft coral *Veretillum cynomorium* did not change significantly under warming and acidification. The GST activity in our experiment significantly decreased under pH 7.3, indicating that extreme low pH disrupted the balance of GST. The similar phenomenon was also found in the study of Lima et al. (2019).

MDA content is an important parameter reflecting the body's potential antioxidant capacity, which can reflect the body's lipid peroxidation rate and strength, and indirectly reflect the degree of tissue peroxidation damage. In our results, MDA level was significantly increased, meaning that oxidative damage occurred to mussels.

At the end of the recovery period, all enzyme activities returned to normal except GPX and GST, which coincidentally were also the two enzymes that were significantly affected by the combination of acidification and hypoxia during the exposure period. When acting on GPX and GST, acidification and hypoxia coordinated, preventing the mussels from recovering in the short term. Whether long-term recovery will be curable remains to be determined.

As enzyme activities are inhibited and induced under pollution conditions, and the responses of various enzymes to biotoxicity exposure are not synchronous, different enzymes have different sensitivity to pollution. Therefore, the analysis of several enzymes and even other biomarkers can be combined to evaluate the pollution situation more effectively. The IBR index is such a powerful tool. It has been widely used in many studies to assess stress responses and ecological risks (Cao et al., 2019; Damiens et al., 2007; Xie et al., 2016). In this study, among the exposure groups, the group DO 2 mg L⁻¹ and pH 7.7 had the highest IBR/n value, which reflected this group was the most highly impacted one. When put sights on the GSA, it seemed more reasonable. GSA decreased the most at extreme pH level. There is a hypothesis that mussels exhibit physiological trade-offs, and that, under increased pressure from climate change scenarios, they may allocate energy from reproduction to costly physiological defenses (Petes et al., 2008; Béguel et al., 2013). The combined stress of extreme pH and hypoxia put severely negative effects on mussel and threatened their survival, so they had to spend a large part of the energy originally used for gonadal development to cope with the stress. Taking all these results into account, we can confirm that acidification and hypoxia exposure have adverse effects in mussel gonadal antioxidant system, and consequently impact mussel gonad development.

5. Conclusion

Our results suggested that exposure to acidification and hypoxia impaired gonadal antioxidant system of *M. coruscus*. Acidification and hypoxia decreased the activity of CAT and GST, but increased GPX, GSH activity and MDA level. pH showed a more significant effect on the antioxidant response than DO. The imbalance of the antioxidant system seemed to be closely related to the decrease of GSA. A more specific link between the antioxidant system and gonad development is worth exploring in the future.

Declarations

Ethics approval and consent to participate

Animals were used following international, national, and/or institutional guidelines.

Consent for publication

The authors consent to publish.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JY, MH, HK and YD conceived the study. GX, HK, XC and HC carried out the experiment. HK and GX analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

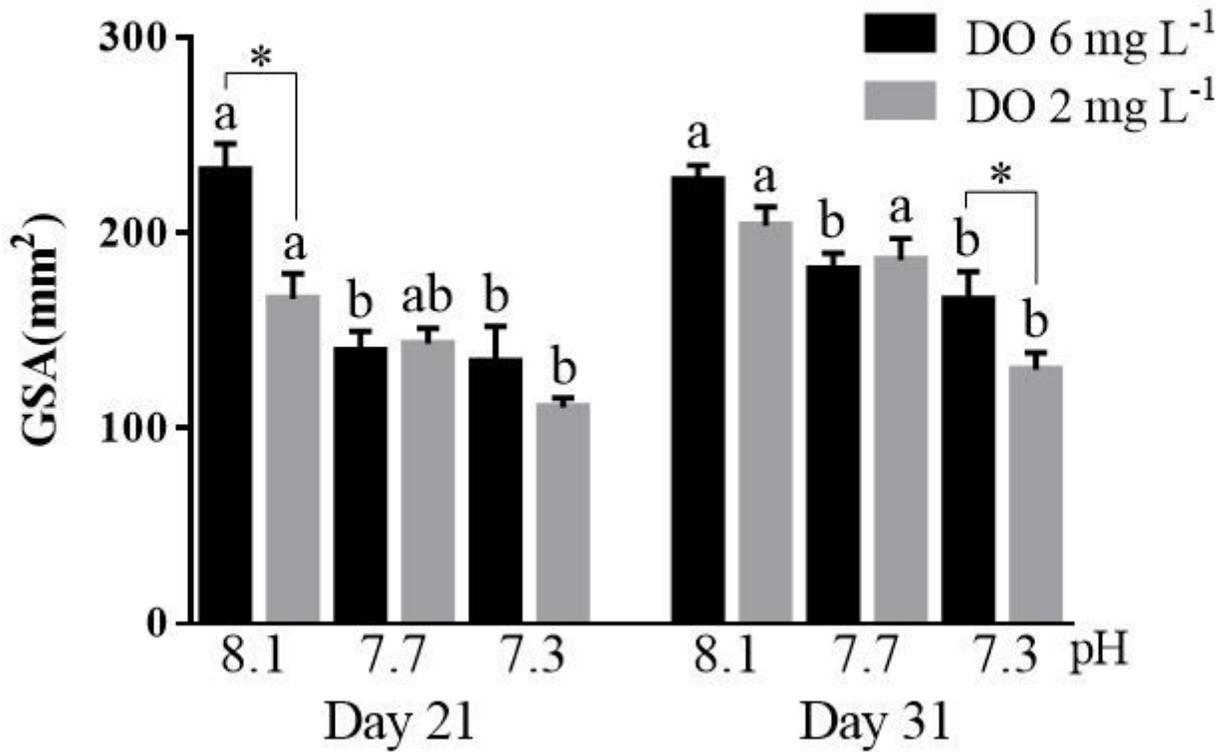


Figure 1

Gonad surface area (GSA) of *M. coruscus* exposed to different DO concentrations (6 and 2 mg L⁻¹) and pH levels (8.1, 7.7 and 7.3) at day 21 and 31. The means denoted by different letters at each fixed pH are significantly different among three pH levels at each sampling time ($P < 0.05$). The means sharing the asterisk between two DO concentrations at each pH level are significantly different at each sampling time ($P < 0.05$).

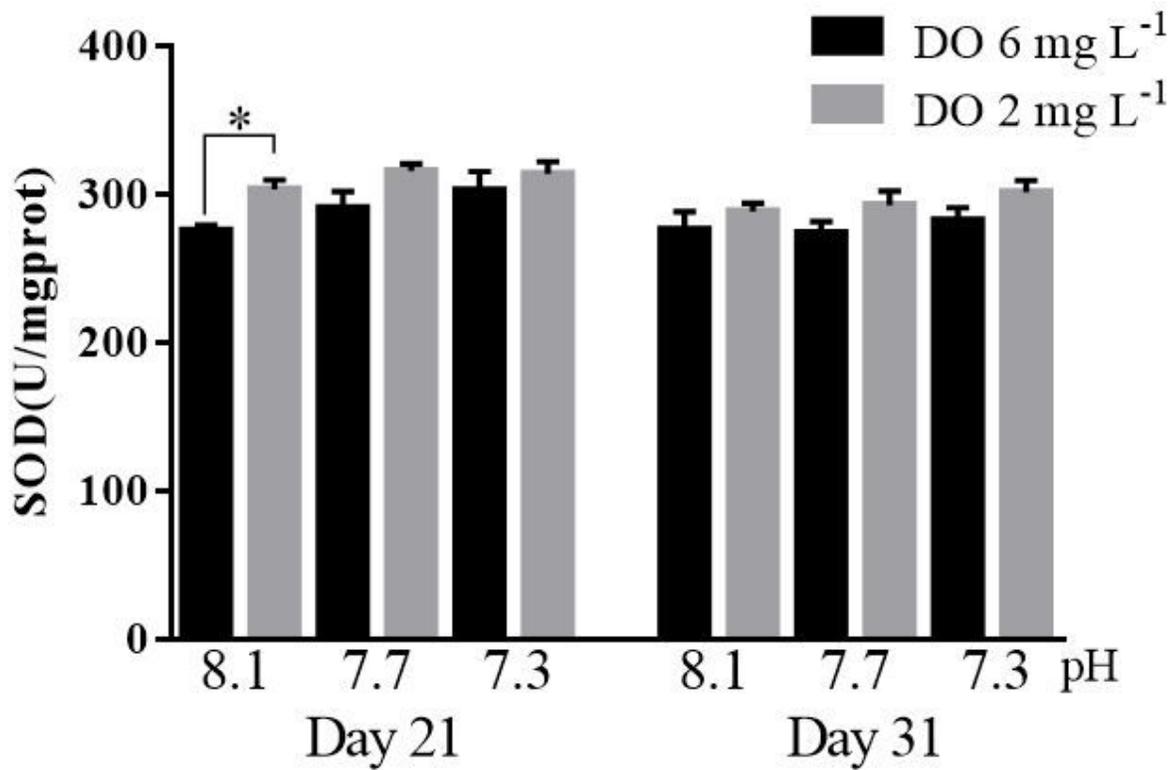


Figure 2

Superoxide dismutase (SOD) activity in the gonad of *M. coruscus* exposed to different DO concentrations (6 and 2 mg L⁻¹) and pH levels (8.1, 7.7 and 7.3) at day 21 and 31. The means sharing the asterisk between two DO concentrations at each pH level are significantly different at each sampling time (P<0.05).

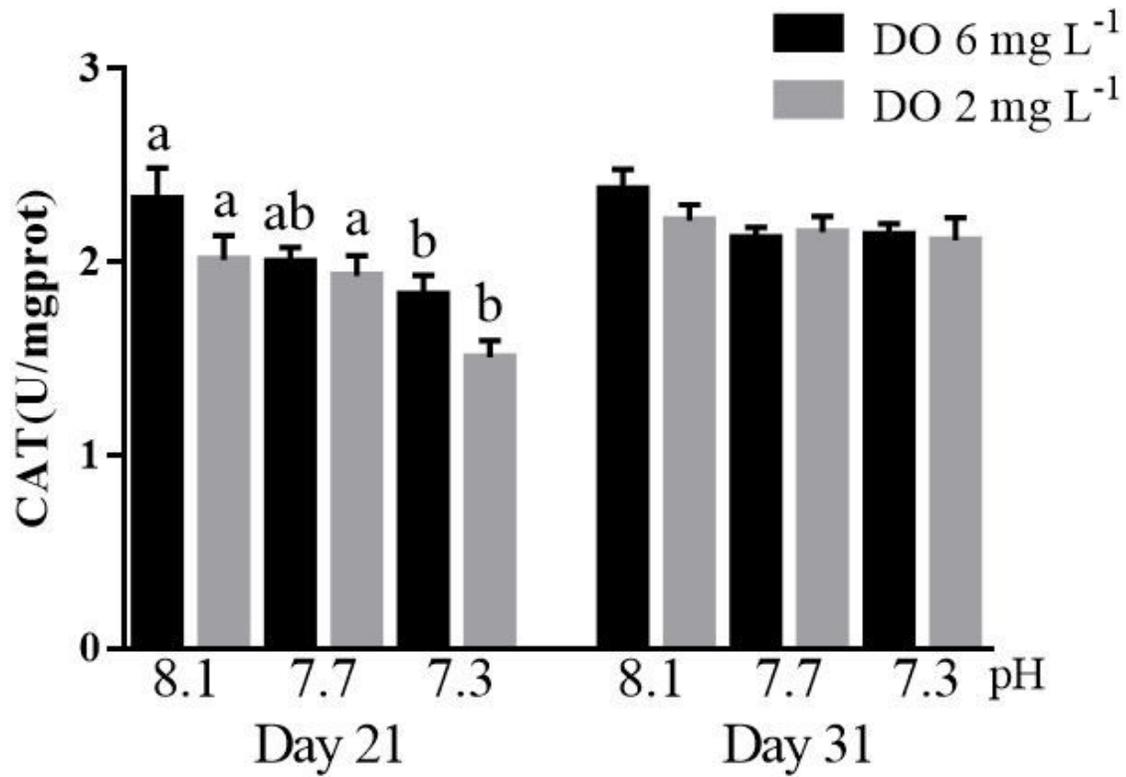


Figure 3

Catalase (CAT) activity in the gonad of *M. coruscus* exposed to different DO concentrations (6 and 2 mg L⁻¹) and pH levels (8.1, 7.7 and 7.3) at day 21 and 31. The means denoted by different letters at each fixed pH are significantly different among three pH levels at each sampling time ($P < 0.05$). The means sharing the asterisk between two DO concentrations at each pH level are significantly different at each sampling time ($P < 0.05$).

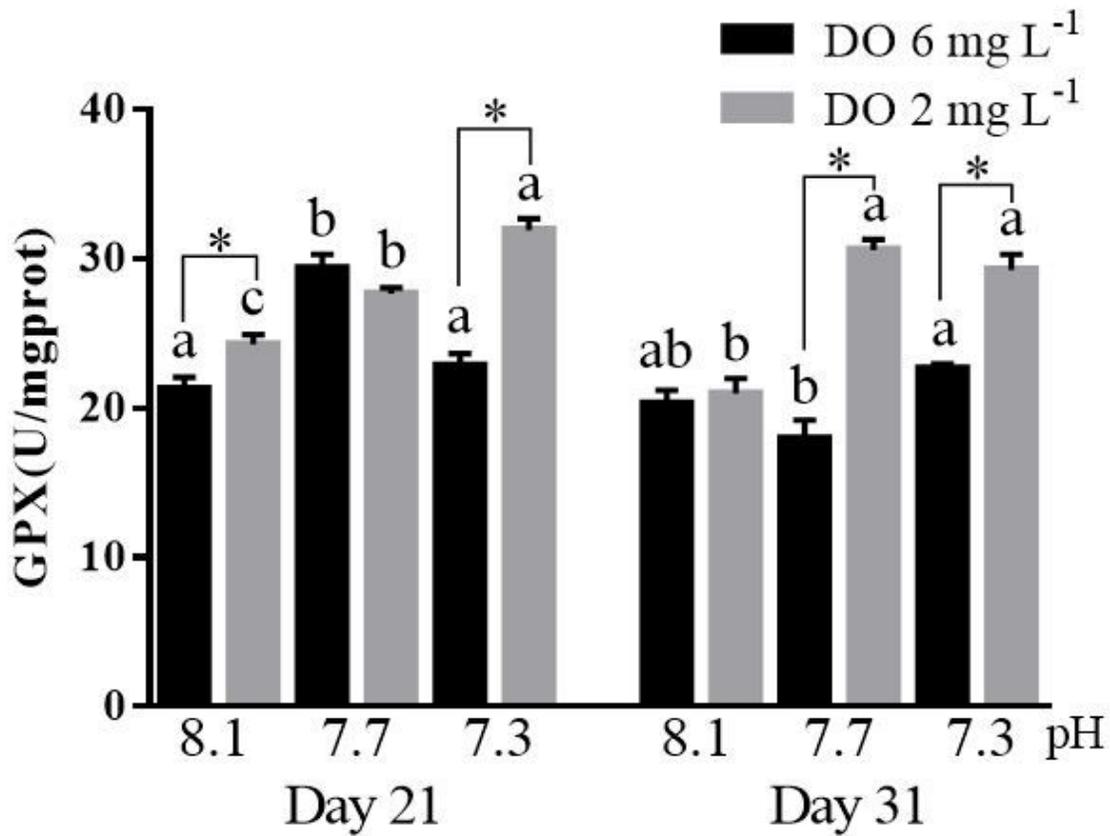


Figure 4

Glutathione peroxidase (GPX) activity in the gonad of *M. coruscus* exposed to different DO concentrations (6 and 2 mg L⁻¹) and pH levels (8.1, 7.7 and 7.3) at day 21 and 31. The means denoted by different letters at each fixed pH are significantly different among three pH levels at each sampling time ($P < 0.05$). The means sharing the asterisk between two DO concentrations at each pH level are significantly different at each sampling time ($P < 0.05$).

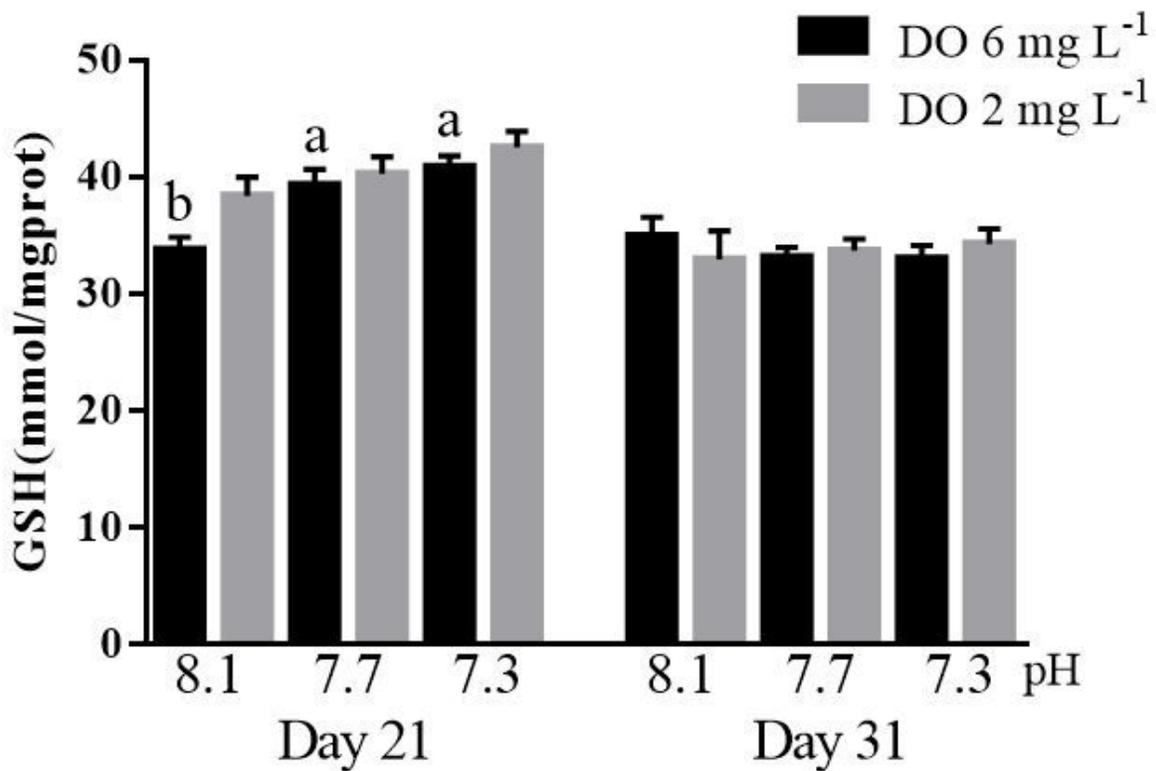


Figure 5

Glutathione (GSH) activity in the gonad of *M. coruscus* exposed to different DO concentrations (6 and 2 mg L⁻¹) and pH levels (8.1, 7.7 and 7.3) at day 21 and 31. The means denoted by different letters at each fixed pH are significantly different among three pH levels at each sampling time ($P < 0.05$). The means sharing the asterisk between two DO concentrations at each pH level are significantly different at each sampling time ($P < 0.05$).

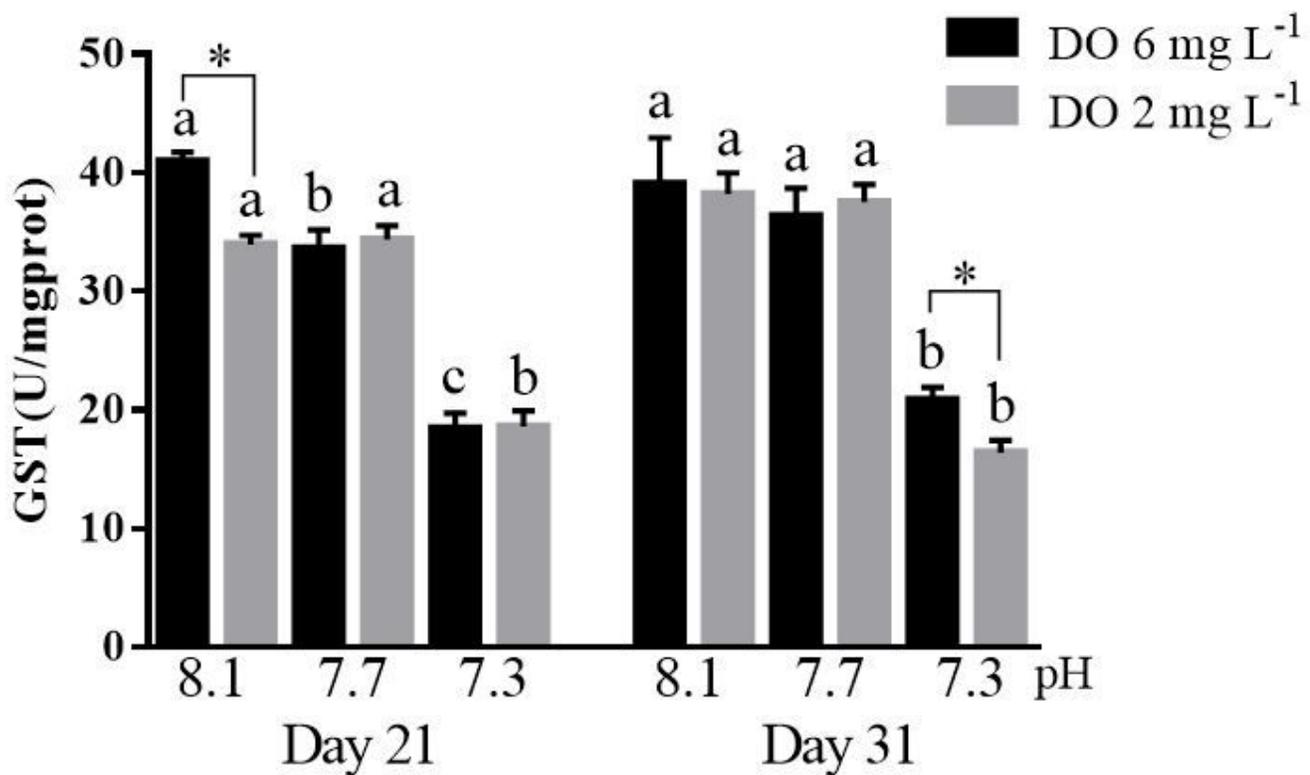


Figure 6

Glutathione S-transferase (GST) activity in the gonad of *M. coruscus* exposed to different DO concentrations (6 and 2 mg L⁻¹) and pH levels (8.1, 7.7 and 7.3) at day 21 and 31. The means denoted by different letters at each fixed pH are significantly different among three pH levels at each sampling time (P<0.05). The means sharing the asterisk between two DO concentrations at each pH level are significantly different at each sampling time (P<0.05).

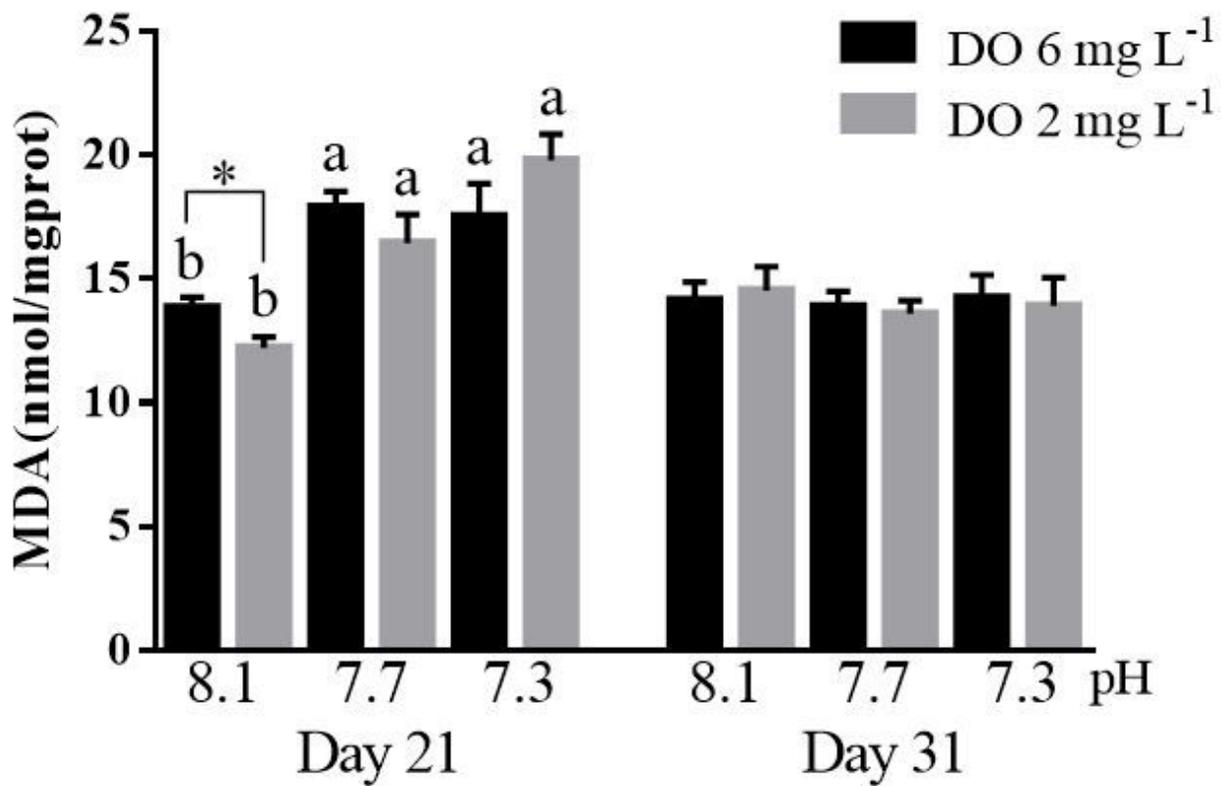


Figure 7

Malondialdehyde (MDA) activity in the gonad of *M. coruscus* exposed to different DO concentrations (6 and 2 mg L⁻¹) and pH levels (8.1, 7.7 and 7.3) at day 21 and 31. The means denoted by different letters at each fixed pH are significantly different among three pH levels at each sampling time ($P < 0.05$). The means sharing the asterisk between two DO concentrations at each pH level are significantly different at each sampling time ($P < 0.05$).

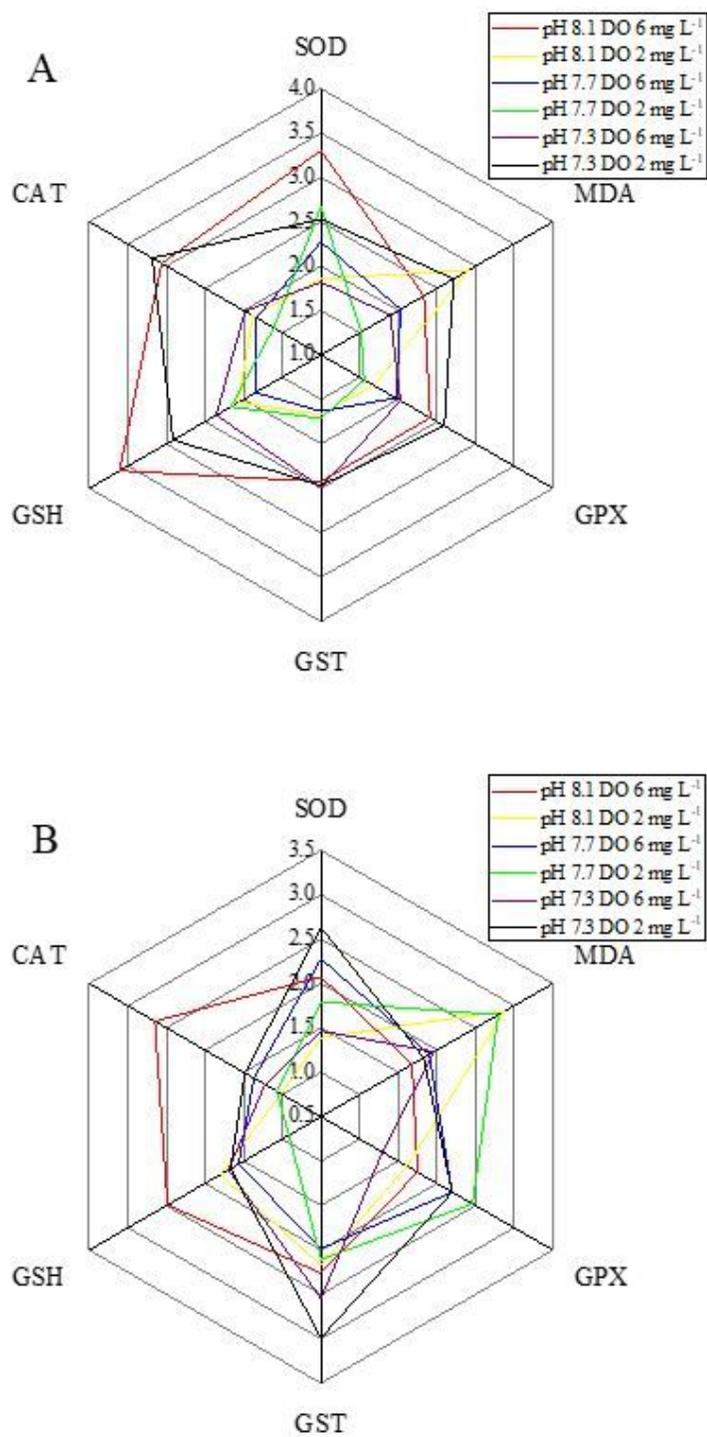


Figure 8

IBR star plots of the biomarkers in *M. coruscus* exposed to different DO concentrations (6 and 2 mg L⁻¹) combined with pH levels (8.1, 7.7 and 7.3) for 21 days (A) and after recovery (B).

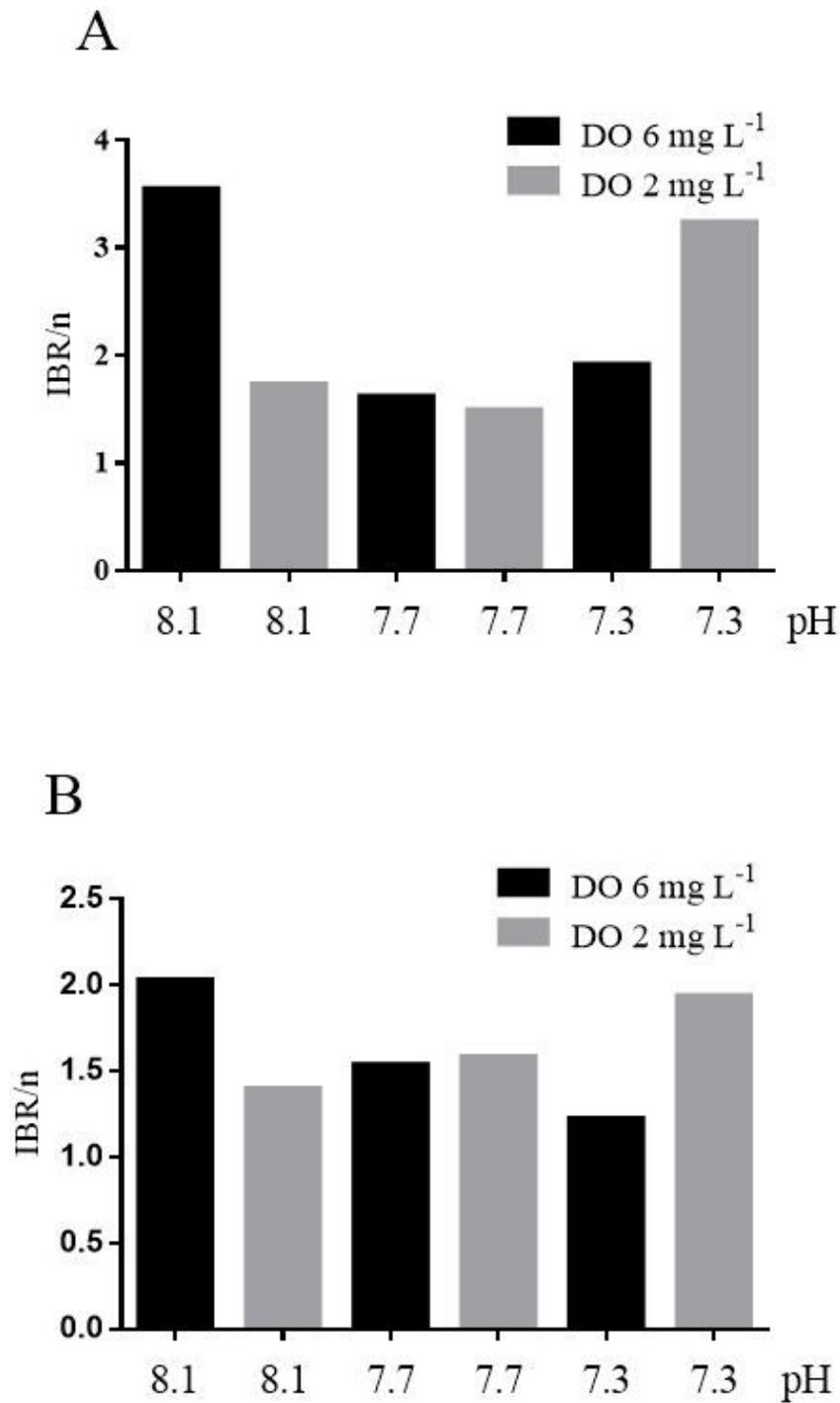


Figure 9

Integrative biomarker response index (IBR/n) in *M. coruscus* exposed to different DO concentrations (6 and 2 mg L⁻¹) combined with pH levels (8.1, 7.7 and 7.3) for 21 days (A) and after recovery (B).

Biplot (axes PC1 and PC2: 64.94 %)

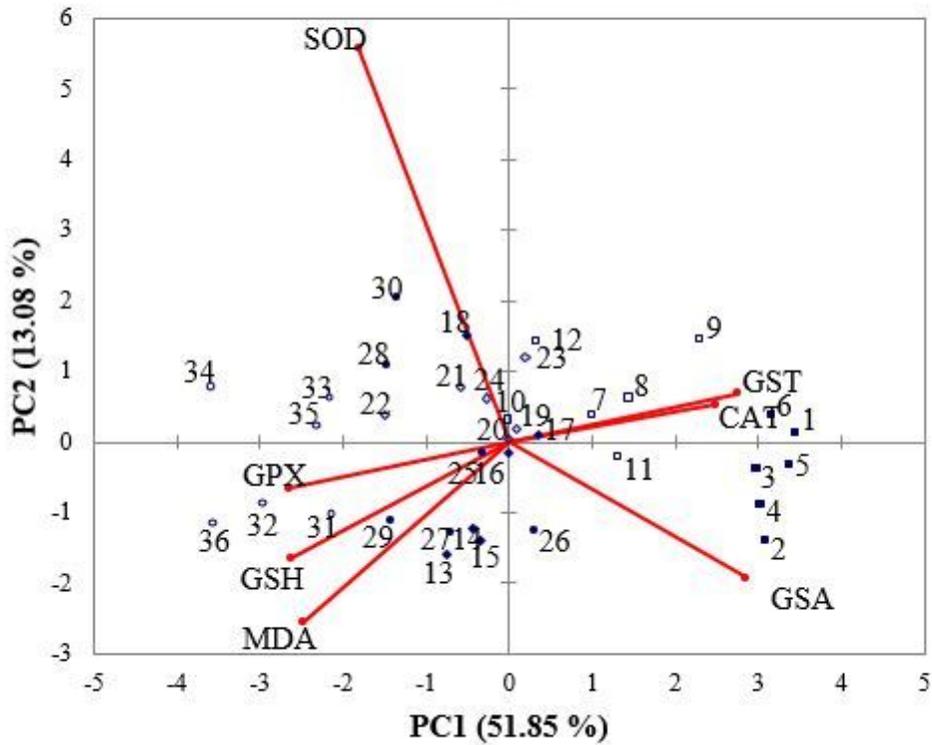


Figure 10

Biplot originating from principal component analysis integrating all measured variables (SOD, CAT, GPX, GSH, MDA, GSA) in six different treatments (■ DO 6 mg L⁻¹ × pH 8.1, □ DO 2 mg L⁻¹ × pH 8.1, ▨ DO 6 mg L⁻¹ × pH 7.7, ▩ 2 mg L⁻¹ × pH 7.7, ▲ DO 6 mg L⁻¹ × pH 7.3, △ 2 mg L⁻¹ × pH 7.3).