

# Oxidative stress and antioxidant enzymes activities in the African catfish, *Clarias gariepinus*, experimentally challenged with *Escherichia coli* and *Vibrio fischeri*

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**Abstract** The impacts of bacterial infection on cultivated fish species, African catfish, were investigated using oxidative stress biomarkers [lipid peroxidation (LPO) and protein carbonylation] and the activities of important antioxidant/detoxifying enzymes [catalase and glutathione S-transferase (GST)]. Fish were inoculated via oral gavage with one of the following treatments:  $1 \times 10^5$  CFU/ml of *Escherichia coli* (EC1),  $2 \times 10^5$  CFU/ml of *E. coli* (EC2),  $1 \times 10^5$  CFU/ml of *Vibrio fischeri* (V1),  $2 \times 10^5$  CFU/ml of *V. fischeri* (V2), gavaged with distilled water and not gavaged. Fish were maintained in the laboratory for 7 days after the bacterial inoculation, and the levels of LPO, protein carbonylation, GST, and catalase activities were determined in the muscle, gills, and liver of fish. Fish inoculated with bacteria (either *E. coli* or *V. fischeri*) had a significant higher levels of tissue LPO, protein carbonylation, and GST activities in a tissue-specific pattern (liver > muscle > gills). This appears to be related with the levels of bacterial inoculation, with effects more pronounced in fish inoculated with either EC2 or V2. The catalase activity did not differ significantly between the inoculated and fish that were not inoculated. The results of this study indicate that bacterial inoculation

could result in oxidative stress in fish, and liver has a higher rate of oxidative stress per mg tissue compared to the gills and the muscle.

**Keywords** African catfish · Lipid peroxidation · Protein carbonylation · GST activity · Catalase activity · Bacterial infection

## Introduction

In the last few years, the cultivation of African catfish has grown tremendously and there are projections indicating even a higher trend in the next few years (Adewumi and Olaleye 2011). This could be due to the greater demand for its cheap source of protein, especially among low- and middle-income households in several African countries. The African catfish is widely cultivated in Nigeria and of course in many other countries because of its distinctive characteristics that stand it out among cultivable fish species; the characteristics are as follows: tolerance to extreme environmental conditions, ease of cultivation, high-quality flesh, distinctive taste and texture, relatively low fat and absence of intramuscular spines (Khwanjai et al. 1997; Luckhoff 2005; El-Shebly 2006). The earthen pond system of catfish cultivation is quite common especially among the local fish farmers. This aquacultural practice is quite cheap compared to other aquacultural practices; however, there are reports of

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higher chance of infection of fish with bacteria and other pathogens under this system (Ampofo and Clerk 2010).

The use of oxidative stress biomarkers like lipid peroxidation, protein carbonylation, and changes in the activities of antioxidant enzymes has been used to show the responses of aquatic organisms to various environmental stressors such as heavy metals, salinity fluctuations, and herbicides (Dorts et al. 2009; Adeyemi and Klerks 2012). Oxidative stress occurs when the rate of production of free radicals such as hydroxyl ions, superoxide anions, hydrogen peroxides exceeds the scavenging ability of the antioxidants. While there are reports on the possibility of bacterial infection to compromise the immune systems of fish, as shown by various studies that have used hematological profiling (Zorriehzakra et al. 2010; Carraschi et al. 2012; Adeyemi et al. 2013), there is relatively little information on whether or not bacterial infection could result in oxidative stress in fish.

Understanding the mechanisms of oxidative stress could provide the first-line evidence that fish have been infected and could help to provide information on certain diseases of aquatic life that has been reported. For example, pansteatitis disease of catfish and crocodiles in many South African rivers has been shown to be directly related to oxidative stress. This present study is therefore aimed at using various oxidative stress biomarkers to show the impacts of inoculation with various inoculums of *E. coli* and *V. fischeri* that were isolated from water samples. While both *E. coli* and *V. fischeri* are generally assumed to be nonpathogenic in human, however, there are reports of tendencies of virulence in both species in fish and some aquatic invertebrates (Ruby and McFall-Ngai 1999; Gupta et al. 2013). Since the two bacteria have a high occurrence in aquatic environment, the evaluation of their potential toxicity especially in terms of causing oxidative stress is required.

## Materials and methods

### Experimental organisms

Adult catfish ( $n = 36$ ) of average weight  $300 \pm 10$  g were collected from a commercial fish hatchery located in Abere, Osun State, Nigeria ( $07^{\circ}43'55\text{N}$ ;  $004^{\circ}31'07\text{E}$ ). They were then transported live in clean, sterilized plastic kegs to the Microbiology Laboratory of the Department of Biological Sciences of the Osun State University, Osogbo, Nigeria. In the laboratory, fish were divided into six groups (one for each treatment) of six fish each maintained in 250-l bowls. Fish were acclimatized to laboratory conditions for 7 days prior to the commencement of experiments. During acclimatization, a 50 % water change was done every other day, by carefully pouring out the water with clean plastic bowl and replenishing with freshwater. Fish were fed with commercial fish pellets twice daily. At the end of the 7-day acclimatization period, fish in each bowls were later divided into three groups such that each bowl contains two fish, thus making three replicates per treatment. During the latter process of fish splitting, care was taken so that the fish were carefully handled ensuring that stress due to fish handling was minimized.

### Isolation of bacteria

Bacterial isolates used in this study were *V. fischeri* and *E. coli*. The bacteria species were isolated from water samples collected from a stream around Oke Baale, Osogbo, Osun State, Nigeria ( $07^{\circ}38'56\text{N}$ ;  $004^{\circ}29'43\text{E}$ ).

### Isolation of *V. fischeri*

The water sample was cultured on thiosulfate citrate bile sucrose (TCBS) agar at  $37^{\circ}\text{C}$  for 48 h. Bacterial

**Table 1** Morphological and biochemical characterization of *V. fischeri*

Colonial morphology						Gram reaction	Catalase	Oxidase	Indole	Methyl red	Glucose	
Shape	Edge	Elevation	Color	Opacity	Pigment						Acid	Gas
Round	Entire	Raised	Yellow	Luminescent	+	Gram-negative short rod	+	+	+	+	+	-

The plus (+) sign indicates positive while the minus (-) sign indicates negative

colonies that fermented the TCBS agar to give a yellow color were subcultured on nutrient agar and incubated at 37 °C for 24 h. Further characterization was based on colonial morphology, Gram stain reaction, oxidase test, catalase test, indole test, methyl red test, and glucose fermentation test (Table 1).

#### Isolation of *E. coli*

The water sample was cultured on eosin methylene blue (EMB) agar at 37 °C for 24 h. Bacterial colonies that grew on the EMB plate, which showed the characteristic metallic green sheen color, were subcultured on nutrient agar plate. Further characterization was based on colonial morphology, Gram stain reaction, oxidase test, catalase test, indole test, methyl red, citrate test, and lactose fermentation test (Table 2).

#### Bacterial challenge

Before bacterial inoculation, fish were mildly anaesthetized with benzocaine. Fish were inoculated with 2 ml of each bacteria inoculum via oral gavage. There were six different treatments; they were as follows  $1 \times 10^5$  CFU/ml of *E. coli* (EC1),  $2 \times 10^5$  CFU/ml of *E. coli* (EC2),  $1 \times 10^5$  CFU/ml of *V. fischeri* (V1),  $2 \times 10^5$  CFU/ml of *V. fischeri* (V2), gavaged with distilled water (GW) and not gavaged (NG). There were three replicates of each treatment and two sets of control: gavaged with distilled water and not gavaged; this is to show whether oral gavaging procedure produces any stress or not.

#### Sample preparation

After the 7-day period of bacterial inoculation, fish were euthanized with an overdose of benzocaine, carefully dissected out, and the gills, liver, and muscle were excised. A portion of the tissue samples was then

homogenized in ice-cold 0.05 M phosphate buffer, pH 7.4, and the homogenates were further processed for different enzymatic and biochemical assays.

#### Lipid peroxidation (LPO) assay

Lipid peroxidation was measured spectrophotometrically by the method reported by Dorts et al. (2009) with minor modifications. LPO results in the formation of malondialdehyde, which reacts with thiobarbituric acid to form a colored product, thiobarbituric acid reactive substance (TBARS), which is then quantified spectrophotometrically. Briefly, the tissue homogenates were added 1:1 (v/v) to 5 % trichloroacetic acid and then incubated for 15 min on ice. The resulting solution was then mixed in a ratio of 2:1 with 0.67 % thiobarbituric acid and centrifuged at  $2,200 \times g$  for 10 min at 4 °C. The supernatant was then boiled for 10 min and allowed to cool to room temperature. Absorbance was then measured at 535 nm. The amount of LPO was expressed as  $\mu\text{M}$  TBARS/mg protein in the tissue using a molar extinction coefficient of  $156,000 \text{ M}^{-1} \text{ cm}^{-1}$ .

#### Protein carbonylation assay

Carbonyl content was quantified as described by Resnick and Packer (1994). Two samples of the homogenates from each tissue were placed in two different glass tubes. To one tube, 10 mM DNPH in 2.5 M HCl was added, while to the other tube 2.5 M HCl was added. Tubes were left for 1 h of incubation at room temperature in the dark. Then, 20 % TCA (w/v) was added in both tubes, left in ice for 15 min, and centrifuged at  $6,000 \times g$  to collect the protein precipitates. The precipitates were dissolved in 6 M guanidine hydrochloride and were left for 10 min at 37 °C. Absorbance was read at 390 nm using an absorption coefficient of  $22,000 \text{ M}^{-1} \text{ cm}^{-1}$ . Results were expressed as  $\mu\text{mol}$  carbonyl per mg proteins.

**Table 2** Morphological and biochemical characterization of *E. coli*

Colonial morphology						Gram reaction	Catalase	Oxidase	Indole	Methyl red	Citrate	Lactose	
Shape	Edge	Elevation	Color	Opacity	Pigment							Acid	Gas
Round	Entire	Raised	Cream	Transparent	–	Gram-negative short rod	+	–	+	+	–	+	+

The plus (+) sign indicates positive while the minus (–) sign indicates negative

### Glutathione S-transferase (GST) activity assay

The glutathione S-transferase activities in the gills, liver, and muscle were determined following the method of Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay was performed in a test tube by adding 1.7 ml of phosphate buffer to 0.1 ml of 30 mM CDNB. The mixture was allowed to incubate for 5 min at 37 °C, after which 0.1 ml of the homogenate was added, and absorbance was read after min at 340 nm. The specific activity of GST was expressed as  $\mu\text{mol}$  of GSH–CDNB conjugate formed/min/mg protein, using the molar extinction coefficient of  $9.6 \text{ M}^{-1} \text{ cm}^{-1}$ .

### Catalase activity assay

Catalase activity in tissue (gill, liver, and muscle) homogenates ( $n = 6$ ) was quantified spectrophotometrically by the method described by Ellerby and Bredesen (2000). It is based on the rate of decomposition of hydrogen peroxide by catalase. One unit of the enzyme is defined as the amount of enzyme that decomposes 1  $\mu\text{mol}$  of hydrogen peroxide in 1 min at 25 °C. Briefly, 0.6 ml of 0.05 M phosphate buffer was transferred into a cuvette and then 0.3 ml of 0.03 M hydrogen peroxide solution was added. Subsequently, 20  $\mu\text{l}$  of the whole-fish homogenate was added and the change in absorbance after 1 min was recorded. Absorbance was read at 240 nm using a UV/Vis spectrophotometer (Ultrospec 3000, Pharmacia Biotech). Catalase activity was expressed as Units/mg protein using a molar extinction coefficient of  $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ .

### Protein estimation

The protein content of aliquots of the tissue extracts was determined by a Bradford assay (Bradford 1976) using bovine serum albumin as a standard.

### Statistics

The data were first checked for normality using the Shapiro–Wilk test. The data did not deviate significantly from normal, so parametric tests were further carried out on the data. The data were first subjected to a two-way mixed model analysis of variance, factors being the treatment group and experimental bowls

(since multiple bowls were used per group). Because the experimental bowl effect was not significant, this factor was eliminated from the model. The tissue LPO level, protein carbonyl contents, GST activity, and catalase activity data were analyzed with one-way analysis of variance, in order to detect the differences among the means of the different treatment groups. This is followed by Tukey's multiple comparison tests whenever there was a significant difference. All statistics were performed using JMP version 9.0 software (SAS Inc. 2010). For reporting purposes, data were expressed as mean  $\pm$  SD and statistical significance was assumed at  $p \leq 0.05$ .

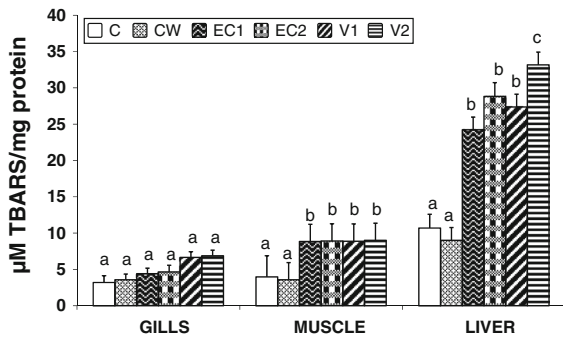
## Results

### Lipid peroxidation assay

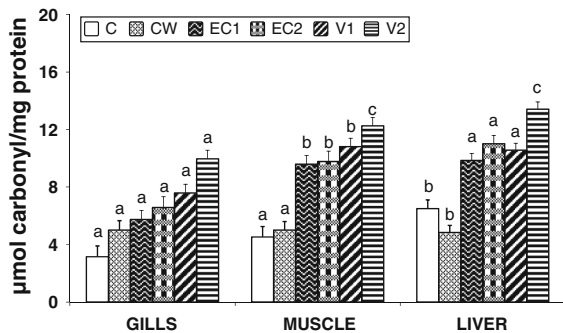
There was no significant difference in the gill LPO levels among the groups ( $F_{5, 30} = 1.6811$ ,  $p = 0.2264$ ) while the LPO levels in the liver and muscle were significantly different among the groups ( $F_{5, 30} = 3.9532$ ,  $p = 0.0307$ ;  $F_{5, 30} = 3.3265$ ,  $p = 0.0482$  for liver and muscle, respectively). Bacterial inoculation resulted in increased LPO levels in the gills, liver, and muscle with effects being highest in the liver, followed by the muscle and lowest in the gills. In all the tissues, LPO levels correlated with the concentration of bacterial inoculums and the fish were infected with higher bacteria inoculums. Fish that were infected with higher bacteria inoculums has higher LPO levels. There seems to be no difference in LPO levels in fish infected with either *E. coli* or *V. fischeri* (Fig. 1).

### Protein carbonylation assay

The levels of protein carbonyl contents followed the trends observed for the LPO levels in all the tissues. In the gills, protein carbonylation did not differ significantly among the groups ( $F_{5, 30} = 3.1265$ ,  $p = 0.0589$ ) but the liver and muscle LPO levels differ significantly among the groups ( $F_{5, 30} = 9.6877$ ,  $p = 0.0014$ ;  $F_{5, 30} = 6.2415$ ,  $p = 0.0070$  for liver and muscle, respectively). Fish inoculated with bacteria has higher levels of protein carbonyl contents especially in the liver and in the muscle. Again, the data did not suggest any significant difference between the



**Fig. 1** The LPO levels in the different tissues of *C. gariepinus* inoculated with different treatments of bacteria:  $1 \times 10^5$  CFU/ml of *E. coli* (EC1),  $2 \times 10^5$  CFU/ml of *E. coli* (EC2),  $1 \times 10^5$  CFU/ml of *V. fischeri* (V1),  $2 \times 10^5$  CFU/ml of *V. fischeri* (V2), gavaged with distilled water (GW) and not gavaged (NG). Each bar is mean  $\pm$  SD ( $n = 6$ ). Bars with different letters are significantly different

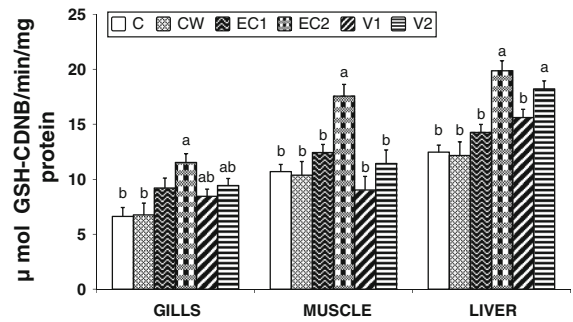


**Fig. 2** The carbonyl protein contents in the different tissues of *C. gariepinus* inoculated with different treatments of bacteria:  $1 \times 10^5$  CFU/ml of *E. coli* (EC1),  $2 \times 10^5$  CFU/ml of *E. coli* (EC2),  $1 \times 10^5$  CFU/ml of *V. fischeri* (V1),  $2 \times 10^5$  CFU/ml of *V. fischeri* (V2), gavaged with distilled water (GW) and not gavaged (NG). Each bar is mean  $\pm$  SD ( $n = 6$ ). Bars with different letters are significantly different

fish that were infected with *E. coli* and those infected with *V. fischeri* (Fig. 2).

#### Glutathione S-transferase (GST) activity assay

GST activity differs significantly in the gills ( $F_{5, 30} = 5.8925$ ,  $p = 0.0086$ ) and in the muscle ( $F_{5, 30} = 11.9482$ ,  $p = 0.0006$ ) but not in the liver ( $F_{5, 30} = 1.1661$ ,  $p = 0.3895$ ) among the treatment groups. GST activity was highest in the liver, followed by the muscle and least in the gills. Although activity levels



**Fig. 3** The GST activity levels in the different tissues of *C. gariepinus* inoculated with different treatments of bacteria:  $1 \times 10^5$  CFU/ml of *E. coli* (EC1),  $2 \times 10^5$  CFU/ml of *E. coli* (EC2),  $1 \times 10^5$  CFU/ml of *V. fischeri* (V1),  $2 \times 10^5$  CFU/ml of *V. fischeri* (V2), gavaged with distilled water (GW) and not gavaged (NG). Each bar is mean  $\pm$  SD ( $n = 6$ ). Bars with different letters are significantly different

appeared to be highest in fish inoculated with higher inoculums, the difference was not significant (Fig. 3).

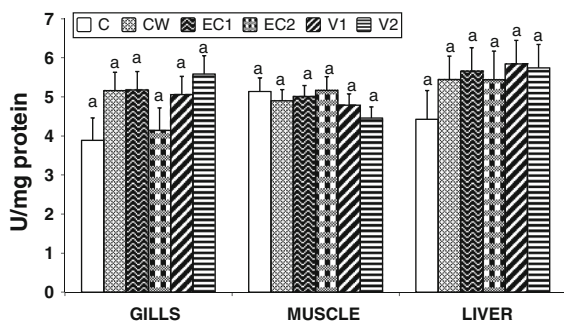
#### Catalase activity assay

There was no significant difference in catalase activity in the gills ( $F_{5, 30} = 1.5485$ ,  $p = 0.2598$ ), muscle ( $F_{5, 30} = 0.7432$ ,  $p = 0.6087$ ), and liver ( $F_{5, 30} = 0.5405$ ,  $p = 0.7422$ ) among the treatment groups. Catalase activity data did not follow any regular pattern and did not show any effect of bacterial inoculation in all the treatment groups (Fig. 4).

#### Discussion

Fish farming is becoming more popular in Africa and of course in almost all the nations. This could be partly due to increasing global demand for fish protein in response to the ever increasing global population. Since there is the possibility of infection of cultured fish with environmental pathogens, especially fish raised in the earthen ponds (Eissa et al. 2010), it therefore becomes imperative to ascertain the health status of fish prior to consumption. One way of relating the health status of cultured fish to the effects of bacterial infection is by using various biomarkers of oxidative stress such as lipid peroxidation, protein carbonylation, and activity and expression levels of antioxidant enzymes. Usually, stressed fish would exhibit more symptoms of oxidative stress compared





**Fig. 4** The catalase activity levels in the different tissues of *C. gariepinus* inoculated with different treatments of bacteria:  $1 \times 10^5$  CFU/ml of *E. coli* (EC1),  $2 \times 10^5$  CFU/ml of *E. coli* (EC2),  $1 \times 10^5$  CFU/ml of *V. fischeri* (V1),  $2 \times 10^5$  CFU/ml of *V. fischeri* (V2), gavaged with distilled water (GW) and not gavaged (NG). Each bar is mean  $\pm$  SD ( $n = 6$ ). Bars with different letters are significantly different

to unstressed fish (Welker and Congleton 2004; Adeyemi and Klerks 2012).

Oxidative stress due to various environmental perturbations such as heavy metal pollution and salinity fluctuations has been reported in fish (Bopp et al. 2008; Park et al. 2011). However, only few studies have shown oxidative stress in fish that were exposed to biotic stressors like bacteria. In this study, we provide evidence of induction of oxidative stress in the African catfish that have been exposed to two different environmental bacteria such as *E. coli* and *V. fischeri*. *E. coli* is a Gram-negative, rod-shaped bacterium that may or may not be pathogenic. This bacterium is commonly found in the gut and has been isolated in different aquatic environments, in association with fecal contamination. Since most local farmers depend on rivers or streams that may have fecal contamination as the source of water for their ponds, this practice exposes fish raised under this system to infection with *E. coli* (Howgate 1998; Nwabueze 2012). Aside *E. coli*, one other bacterium that has been widely isolated in aquatic environments is *Vibrio fischeri* (Thompson et al. 2004). *V. fischeri* is also a Gram-negative bacterium, although considered not to be pathogenic to human but are potentially harmful to fish and some aquatic invertebrates (Ruby and McFall-Ngai 1999).

Oxidative stress occurs when the rate of production of reactive oxygen species (free radicals) exceeds the scavenging rate by antioxidant molecules. The resultant effect is oxidation of biomolecules, for example

peroxidation of lipids and carbonylation of proteins. In this study, bacterial infection resulted in increased level of lipid peroxidation in the gills, muscle, and liver, with effects more pronounced in the liver. This is consistent with the results of similar studies in which fish exposed to copper showed a higher level of liver lipid peroxidation compared to the control (Adeyemi and Klerks 2012). The higher lipid peroxidation levels in the liver suggest that the rate of production of reactive oxygen species in the liver could be higher than in the gills or the muscle and are therefore at more risk of exposure to oxidative stress. Another indicator of oxidative stress in fish is protein carbonylation. The levels of protein carbonylation assumed a similar trend just as the levels of tissue lipid peroxidation, with levels considerably higher in the liver compared to the gill and the muscle. This again could be an indication that pro-oxidation activity is quite low in the gills compared to the liver. One would have expected that effects would be more pronounced in the gills or in the skin, since they are the sites of primary contact with environmental toxicants in fish (Hollis and Playle 1997).

The activity of antioxidant and detoxifying enzymes has been widely used as indices of oxidative stress in aquatic organisms that were exposed to xenobiotics (Dorts et al. 2009; Sabatini et al. 2009). Glutathione S-transferase is a phase II detoxifying enzyme that has been implicated in the metabolism of various endogenous substances. Organisms are sometimes exposed to a wide array of deleterious reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals, and hydrogen peroxide as a result of various metabolic processes. The protective role of GST against oxidative stress has been speculated by its ability to detoxify some of the secondary ROS produced when ROS react with cellular constituents. A high GST activity is assumed to be strongly correlated with increased resistance to oxidative stress (Fournier et al. 1992). *C. gariepinus* that were exposed to either *E. coli* or *V. fischeri* showed a higher GST activity than the control fish, and the activity was higher in the liver and in the muscle. This higher activity level is an indication of protective role of GST in combating the oxidative stress that has been initiated by bacterial infection since GST is usually induced as a first-line protective mechanism against exposure to xenobiotics. Another important antioxidant enzyme that is associated with oxidative stress is

catalase. Catalase is involved in the decomposition of toxic hydrogen peroxide to water and oxygen, thus helping to detoxify hydrogen peroxide. In the present study, there is no evidence of involvement of catalase in protection against oxidative stress in *C. gariepinus* that were infected with bacteria in all the tissues. The catalase activity levels did not differ significantly among the treatment groups for all the tissues. This could be an indication that different forms of free radicals from hydrogen peroxides are associated with oxidative stress due to bacterial infection in fish.

In conclusion, the findings of this study show that cultured fish species that are infected with environmental microbes may be physiologically impaired, and this could be shown by using various oxidative stress biomarkers. The degree of oxidative stress is dependent on the extent of microbial infection. Fish with higher inoculums show greater oxidative stress. The effect of oxidative stress due to bacterial infection is more pronounced in the liver, followed by the muscle and least in the gills. The degree of oxidative stress was not dependent on the kinds of bacterial infection, although caution should be made when making this inference since effects were considered using just *E. coli* and *V. fischeri* in this study.

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