



ELSEVIER

Available online at [www.sciencedirect.com](http://www.sciencedirect.com)

SCIENCE @ DIRECT®

Marine Environmental Research 58 (2004) 787–792

MARINE  
ENVIRONMENTAL  
RESEARCH

[www.elsevier.com/locate/marenvrev](http://www.elsevier.com/locate/marenvrev)

## Effects of hypoxia on gene and protein expression in the blue crab, *Callinectes sapidus*

Marius Brouwer<sup>a,\*</sup>, Patrick Larkin<sup>b,c</sup>,  
Nancy Brown-Peterson<sup>a</sup>, Christina King<sup>a</sup>, Steve Manning<sup>a</sup>,  
Nancy Denslow<sup>b</sup>

<sup>a</sup> Department of Coastal Sciences, University of Southern Mississippi, P.O. Box 7000, Ocean Springs, MS 39566, USA

<sup>b</sup> Department of Biochemistry and Molecular Biology and Center for Biotechnology, University of Florida, P.O. Box 100156 HC, Gainesville, FL 32610, USA

<sup>c</sup> EcoArray LLC, Alachua, FL 32615, USA

---

### Abstract

13 Increases in hypoxic conditions are one of the major factors responsible for declines in  
14 estuarine habitat quality, yet to date there are no indicators for recognizing populations of  
15 estuarine organisms that are suffering from chronic hypoxic stress. Here we test the hypothesis  
16 that alterations in gene and protein expression of antioxidant enzymes and other stress-specific  
17 proteins can be used as molecular indicators of hypoxic stress. Blue crabs, *Callinectes sapidus*,  
18 were exposed to 2–3 ppm DO for 5 days. Gene expression was measured using macroarrays  
19 constructed from cDNA of 10 partial gene transcripts cloned from blue crab hepatopancreas.  
20 Significant ( $p \leq 0.05$ ) down-regulation of gene expression was found for MnSOD, hemocya-  
21 nin, ribosomal S15 and L23. Subtractive hybridization using RNA from control and hypoxic  
22 hepatopancreas tissues also indicated down-regulation of hemocyanin transcription. In con-  
23 trast, Western blotting showed a significant ( $p \leq 0.05$ ) increase of hemocyanin protein in the  
24 hepatopancreas and cross-linking of MnSOD proteins in hypoxia-exposed crabs. Thus, hy-  
25 poxia-responsive cDNA arrays and Westerns may be useful diagnostic tools for monitoring  
26 effects of hypoxia in estuarine crustacea.

27 © 2004 Published by Elsevier Ltd.

28 **Keywords:** Hypoxic stress; cDNA array; Hemocyanin; MnSOD; Molecular biomarkers

---

\* Corresponding author. Tel.: +1-228-872-4294; fax: +1-228-872-4204.

E-mail address: [Marius.brouwer@usm.edu](mailto:Marius.brouwer@usm.edu) (M. Brouwer).

29 Hypoxic conditions in estuarine ecosystems are increasing in frequency, intensity,  
30 duration and extent, and are responsible for serious declines in habitat quality. Ox-  
31 ygen depletion events have occurred in 32 of 38 Gulf of Mexico estuaries (Bricker,  
32 1997), and there is a well-known expansive area of seasonal hypoxia/anoxia on the  
33 Louisiana continental shelf (Turner & Rabalais, 1994). The effects of hypoxia on  
34 biota are often inferred from measurements of low oxygen levels which coincide with  
35 catastrophic mortality of various organisms (Winn & Knott, 1992). Thus, indicators  
36 at the organismal/cellular level are needed that can be used to assess the onset,  
37 duration and severity of hypoxia and its effect on biota. We hypothesize that al-  
38 terations in protein concentration and gene expression of antioxidant enzymes and  
39 other stress-specific proteins can be used as molecular indicators of hypoxic stress.  
40 Here, we examine the effects of low oxygen on the blue crab, *Callinectes sapidus*, an  
41 estuarine organism that frequently encounters hypoxic stress.

42 Five adult blue crabs in 35 L replicate aquaria were exposed to hypoxia (2–3 mg/L  
43 DO) for 5 days in filtered seawater adjusted to a salinity of 15 g/L at 27 °C in an  
44 intermittent flow-through system (Manning et al., 1999). Normoxic controls were  
45 held at 6–8 mg/L DO. Flow rate was sufficient to provide approximately 4.0 volume  
46 additions/day (126 cycles per day of 1 L each cycle) in each treatment aquarium. To  
47 achieve the desired DO of 2–3 ppm, oxygen in the flow-through dilution water was  
48 maintained in a covered headbox at supersaturation (14 ppm). This high DO con-  
49 centration was necessary because of the high rates of oxygen consumption by the  
50 study crabs. The normoxic dilution water was maintained from 18 to 20 ppm before  
51 delivery to normoxia aquaria.

52 Hepatopancreatic tissues from 10 crabs in each treatment were analyzed by  
53 Western blots for stress proteins and RNA was extracted for analysis of gene ex-  
54 pression using cDNA arrays. Ten genes cloned and sequenced from hepatopancre-  
55 atic tissue of wild-caught blue crabs (metallothionein [two isoforms], MnSOD [two  
56 isoforms], Heat Shock Protein 70, hemocyanin, actin and ribosomal proteins S15,  
57 S20 and L23) were PCR amplified, spotted in duplicate onto neutral nylon macro-  
58 arrays and hybridized as previously described (Larkin, Folmar, Hemmer, Poston, &  
59 Denslow, 2003). For each cDNA clone, the general background of each membrane  
60 was subtracted from the average value of the duplicate spots on the membrane. The  
61 values were normalized to the average value of a  $\beta$ -actin cDNA clone. For the  
62 subtractive hybridizations (Clontech, Palo Alto, CA), mRNA samples from hepa-  
63 topancreas of normoxic and hypoxic blue crabs were reverse transcribed into cDNA,  
64 heat denatured and the cDNA pools were then hybridized together. For cloning of  
65 hypoxia-upregulated genes “normoxic cDNA” was used as the driver, whereas for  
66 down-regulated genes “hypoxic cDNA” served as the driver. The cDNAs that re-  
67 mained un-hybridized, which represent putative differentially regulated mRNAs,  
68 were then PCR amplified, cloned and sequenced for identification.

69 Significant decreases (Student's *T*-test,  $p < 0.05$ ) in transcription of one of the  
70 MnSOD isoforms, hemocyanin and ribosomal S15 and L23 genes were observed in  
71 hypoxia-exposed crabs. The decrease in two of the three ribosomal cDNAs analyzed  
72 suggests that protein synthesis may be starting to shut down in these animals. In-  
73 terestingly, while there were no significant differences between hypoxic and normoxic

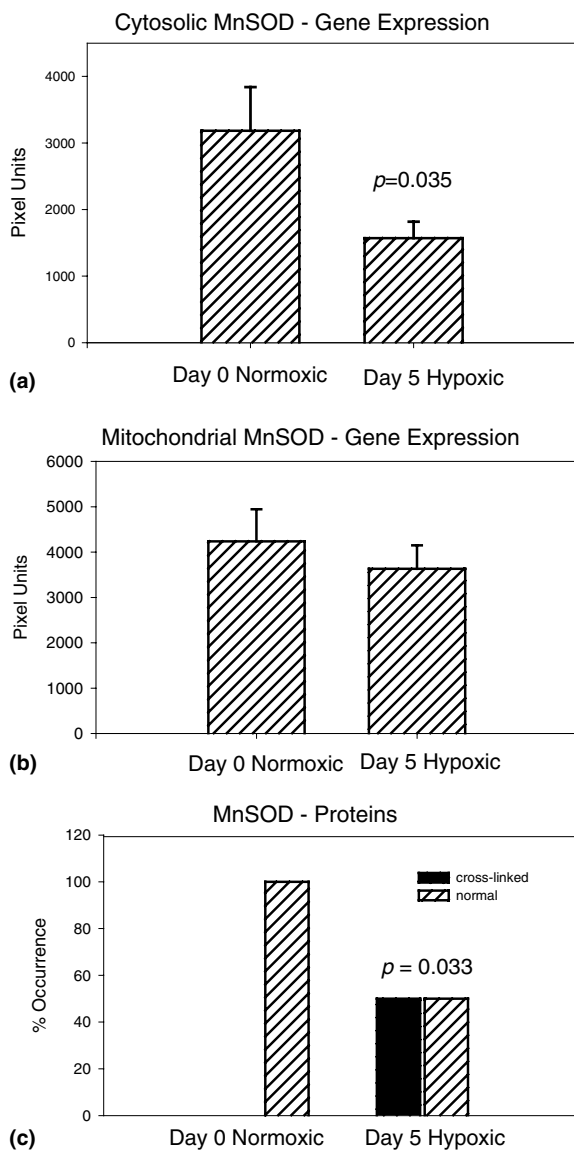


Fig. 1. Results of gene and protein expression of MnSOD in normoxic and hypoxic blue crabs. Gene expression of cytosolic MnSOD (a) and mitochondrial MnSOD (b) was determined from macroarrays. Bars represent mean  $\pm$  SE of nine individuals in each group. Pixel units refer to the intensity of the radiolabeled spots on the macroarrays normalized to the intensity of  $\beta$  actin. Protein expression of both isoforms of MnSOD (c) was determined with Western Blot analysis, using an anti-MnSOD antibody (SOD-111) from Stressgen and chemiluminescence detection. The graph shows the percentage of crabs with normal or cross-linked MnSOD.

74 crabs for the other genes tested, a general trend of down-regulation of gene ex-  
75 pression after 5 days hypoxia was observed (data not shown).

76 Blue crabs lack Cu, ZnSOD and instead have two MnSOD isoforms, one in the  
77 cytosolic compartment and one in the mitochondria (Brouwer, Hoexum-Brouwer,  
78 Grater, Engchild, & Thogersen, 1997). Gene transcription of the mitochondrial  
79 isoform was significantly down-regulated in response to hypoxia ( $p = 0.035$ ; Fig.  
80 1(a)), but transcription of cytosolic MnSOD was not affected ( $p = 0.50$ ; Fig. 1(b)).

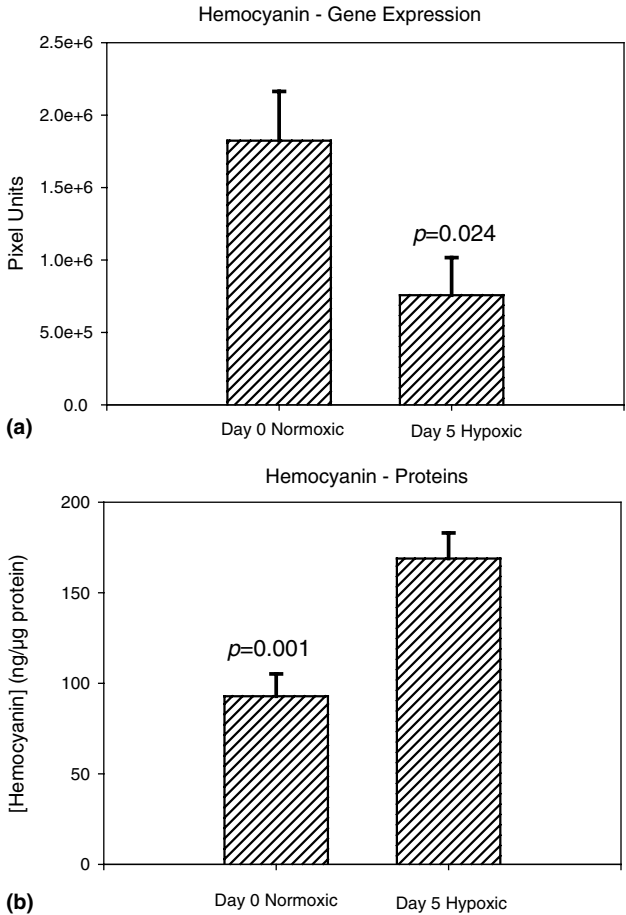


Fig. 2. Results of gene and protein expression of hemocyanin in normoxic and hypoxic blue crabs. Gene expression of hemocyanin (a) was determined from macroarrays. Bars represent mean  $\pm$  SE of nine individuals in each group. Pixel units refer to the intensity of the radiolabeled spots on the macroarrays normalized to the intensity of  $\beta$  actin. Hemocyanin concentration in hepatopancreas (b) was determined with Western Blot analysis of hepatopancreas extracts and purified hemocyanin standards using a rabbit anti-blue crab hemocyanin polyclonal antibody (Duke University Vivarium) and chemiluminescence detection. Total protein was determined with the BCA (Pierce) Protein Assay. Bars represent mean  $\pm$  SE of 10 individuals in each group.

81 The SOD proteins showed a dramatic effect of hypoxia (Fig. 1(c)), with an un-  
82 expected, and as yet unexplained, cross-linking of MnSOD proteins (subunit MW  
83 ~22 kDa) into higher molecular weight (~70–90 kDa) aggregates. Cross-linking of  
84 MnSOD was not seen in the normoxic controls, but there was a significant increase  
85 (Fisher's Exact Test,  $p = 0.033$ ) in high-molecular weight MnSOD after 5 days  
86 exposure to hypoxia. The potential functional significance of this phenomenon is  
87 unknown.

88 Hemocyanin gene expression was significantly ( $p = 0.024$ ) down-regulated after a  
89 5 days hypoxic exposure (Fig. 2(a)). In contrast, hemocyanin protein concentrations  
90 significantly increased ( $p = 0.001$ ; Fig. 2(b)). The down-regulation of hemocyanin  
91 mRNA suggests the crabs are shutting down aerobic metabolism after 5 days ex-  
92 posure to hypoxia. The increased levels of protein still present in the hepatopancreas  
93 suggest that turnover of hemocyanin protein is slow. A shorter-term experiment  
94 might show upregulation of the hemocyanin gene as an immediate response to hy-  
95 poxia, followed by the down-regulation observed here.

96 Results of the subtractive hybridization support data obtained from the macro-  
97 arrays, in that hemocyanin as well as cryptocyanin (a copperless hemocyanin ho-  
98 mologue) appear to be down-regulated in 5 days hypoxic crabs. In addition, it  
99 appears that transcription of cytochrome *c* oxidase subunit I is also down-regulated,  
100 a further indication that the aerobic metabolic pathway may be shutting down. The  
101 genes obtained through the subtractive hybridization will be spotted on macroar-  
102 rays, permitting more conclusive results regarding the effects of hypoxia on their  
103 regulation.

104 The results to date are encouraging in the search for molecular indicators of  
105 hypoxia. However, data presented here represent only one single point in time, and  
106 both shorter and longer-term exposures to hypoxia are necessary to establish the  
107 temporal changes in gene expression and protein synthesis in response to hypoxia.  
108 Finally, the observation that hemocyanin mRNA decreases whereas hemocyanin  
109 protein increases stresses the importance of verifying if gene expression patterns truly  
110 reflect protein responses.

## 111 Acknowledgements

112 T. Brouwer and W. Grater helped with cDNA cloning and sequencing. This re-  
113 search is supported by EPA-STAR grant R 82945801.

## 114 References

- 115 Bricker, S. (1997). *Estuarine Research Federation Newsletter*, 23, 20–21.  
116 Brouwer, M., Hoexum-Brouwer, T., Grater, W., Enghild, J. J., & Thogersen, I. B. (1997). *Biochemistry*,  
117 36, 13381–13388.  
118 Larkin, P., Folmar, L. C., Hemmer, M. J., Poston, A. J., & Denslow, N. D. (2003). *Environmental Health*  
119 *Perspectives, Toxicogenomics*, 111, 29–36.

Manning, C. S., Schesny, A. L., Hawkins, W. E., Barnes, D. H., Barnes, C. S., & Walker, W. W. (1999). *Toxicological Methods*, 9, 201–217.

122 Turner, R. E., & Rabalais, N. N. (1994). *Nature*, 368, 619–621.

123 Winn, R. N., & Knott, D. M. (1992). *Marine Ecology Progress Series*, 88, 161–179.