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Molecular and Organismal Indicators of Chronic and Intermittent Hypoxia in Marine Crustacea

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Introduction and Background

Human population growth in coastal regions and their watersheds, accompanied by agricultural, industrial, and urban development, has led to an unprecedented acceleration of contaminant and nutrient inputs into estuaries. Both the nutrients that fuel primary productivity and the near-coastal hydrodynamics that generate water column stratification contribute to the formation of hypoxic zones. Of the total estuarine area in the Gulf of Mexico that was surveyed in 1994–1995, oxygen depletion (anoxia or hypoxia) events occurred in 32 of 38 estuaries (U.S. EPA, 1999), whereas an expansive area of seasonal hypoxia/anoxia develops yearly on the Louisiana continental shelf (Turner and Rabalais, 1994). Eutrophication and ensuing bottom water hypoxia and anoxia are regarded as major factors responsible for declines in habitat quality and harvestable resources in estuarine ecosystems (Justic et al., 1993; Turner and Rabalais, 1994; Paerl et al., 1998). In addition, increased nutrient loading amplifies cyclic dissolved oxygen (DO) patterns that often develop in shallow waters during the summer months, leading to conditions of intermittent

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hypoxia. Estuarine organisms are therefore not only at risk of being subjected to chronic hypoxic conditions, but also face increases in duration and frequency of hypoxic–normoxic cycles (Ringwood and Keppler, 2002). However, because we lack fundamental information regarding sublethal effects of chronic or intermittent hypoxia on estuarine organisms, indicators of adaptive responses to these conditions are largely unknown.

Currently, the effects of hypoxia on biota are often inferred from measurements of low oxygen levels, which coincide with a reduction of demersal fish and death of benthic fauna (May, 1973; Seliger et al., 1985; Winn and Knott, 1992). The consequences are immediate as well as long term due to the elimination of sensitive species and reduction in overall abundances of food in the benthic communities. Laboratory experiments have shown that, when possible, fish and crustaceans will avoid or move out of hypoxic conditions (Wannamaker and Rice, 2000; Wu et al., 2002). Prolonged exposure to hypoxia results in behavioral or physiological changes such as increased ventilation frequency and cardiac output in both fish and crustaceans (McMahon, 2001; Wu et al., 2002; Robb and Abrahams, 2003). However, while these responses are valuable indices of low oxygen conditions, they cannot serve as effective biomarkers of hypoxia since they involve *in situ* measurements of the organisms, which is not practical for monitoring or management.

Indicators of hypoxia at the organismal/cellular level are needed that can be used to assess the onset, duration, and severity of chronic as well as intermittent hypoxia and its effect on biota. The challenge of scaling these molecular indicator responses to population level responses is addressed later in this chapter. Genes and their products (mRNA and proteins) that respond to hypoxia have great potential to serve as indicators of hypoxic stress, including enzymes of the glycolytic pathway, which increase anaerobic ATP production, glucose transporters, enzymes involved in amino acid metabolism and gluconeogenesis, which maintain blood glucose levels (Semenza et al., 1994; Hochachka et al., 1996; Gracey et al., 2001), and heat shock proteins involved in protein repair and refolding (Hightower, 1993). In general, genes that encode proteins involved in energy production, protein synthesis and degradation, lipid and carbohydrate metabolism, locomotion and contraction, and antioxidant defense are also potential biomarkers of hypoxic stress (Hochachka et al., 1996). While much of the work on gene and protein expression as related to hypoxia has focused on vertebrates, there is growing evidence that these molecular indicators, including ribosomal proteins, antioxidant defense enzymes, and oxygen-carrying proteins, can also effectively indicate hypoxia exposure in invertebrates (DeFur et al., 1996; Mangum, 1997; Choi et al., 2000; McMahon, 2001; Brouwer et al., 2004).

We briefly review the importance of the use of genomics and proteomics in developing indicators for detection of environmental stressors to provide the reader, especially coastal resource managers who are potential end users of these novel indicators, with a background on the importance of this emerging technology. Examples of the use of these molecular indicators for evaluating effects of chronic and intermittent hypoxia in estuarine crustacea, and the link between these indicators and whole animals/populations, are discussed.

Brief Review of Molecular Biomarkers

The utility of molecular biomarkers is that they allow scientists to identify a potential environmental problem prior to any phenotypic or toxicological expression in the organism, thus providing an "early warning system" of a developing problem. Changes in behavior, physiological processes, and metabolic pathways can help determine specific mechanisms of action for a variety of chemical and physical environmental stressors (Denslow et al., in press). Transcriptions of genes that encode specific proteins that can deal with perceived stressors are usually the first measurable biomarkers that can be assessed. However, examining gene regulation by itself does not give a complete picture as it is also necessary to quantify the protein activity to ascertain that increased gene transcription also results in increased protein levels. Changes in both specific gene and protein expression are excellent indicators that the organism has mobilized metabolic pathways in response to a specific stimulus.

The classic method for measuring expression levels of mRNA has been Northern blotting, in which the relative abundance of genes is determined by probing electrophoretically separated RNA bound to a nylon membrane with radiolabeled cDNA that is complementary to the sequence of interest. However,

Northern blotting can only be used for measuring a small number of genes at a time, is less sensitive and quantitative, and has a lower throughput than more recently developed methods for measuring gene expression (Larkin et al., 2003b). Nevertheless, Northern blots have been successfully used to illustrate the downregulation of a variety of genes in aquatic species (Buhler et al., 2000; Funkenstein, 2001; Larkin et al., 2003a).

The recent availability of complete gene sequences for a variety of organisms has resulted in the rapid development of the field of genomics. Emerging technologies now make it possible to screen for changes in up- or downregulation of a large number of different genes at one time. One such technology, quantitative real-time polymerase chain reaction (Q-PCR), emerged in the 1990s (Heid et al., 1996) and is a fairly quick and efficient method to accurately quantify mRNA, allowing the determination of expression levels of the gene or genes of interest. The advantages of this procedure include the sensitivity of the assay, the small amount of total RNA needed, the elimination of radio-isotope labeling, the ability to process a large number of samples, and the potential to measure several genes at once (Larkin et al., 2003b). However, researchers must have already identified the genes they think will be up- or downregulated to effectively utilize this technology. Furthermore, Q-PCR can only be used for genes for which at least a partial sequence has been identified. Q-PCR is a powerful tool that has been used successfully to examine gene expression and induction in a number of aquatic organisms (Trant et al., 2001; Kumar et al., 2001; Dixon et al., 2002).

In situations where scientists want to determine which genes respond to chemical or environmental stimuli, a more global, open-ended technology than Q-PCR is required. Two techniques, subtractive hybridization and gene arrays, can be used to initially identify a large number of genes of interest. Subtractive hybridization enables the researcher to compare mRNA from two different treatments or populations and obtain cDNA clones that are differentially expressed in one group compared to the other (Lisitsyn and Wigler, 1993; Ermolaeva and Sverdlov, 1996). Subtractive hybridization has the potential to identify genetic biomarkers for specific types of environmental contaminants (Larkin et al., 2003b), although this use in aquatic species has only been recently documented (Blum et al., 2004).

Gene arrays, also called micro- or macroarrays, can be used to measure simultaneously the expression of hundreds or thousands of genes affected by a particular compound or exposure condition. These arrays are like reverse Northern blots, in that the RNA is reverse-transcribed with fluorescent or radiolabeled markers and then hybridized with DNA sequences (specific genes) attached to a solid support matrix such as nylon membranes or glass slides (Schena et al., 1998). Up- or downregulation of gene expression can be visualized by the intensity of the spots on the membrane or slide. Arrays are limited in that they may not be sensitive enough to detect rare genes, and the function of many genes spotted onto an array may not be known (Larkin et al., 2003b). However, microarrays are invaluable for identifying a suite of genes that are affected by chemical or environmental stimulation, and several studies suggest that specific contaminants may have their own specific profiles of gene expression (Bartosiewicz et al., 2001; Larkin et al., 2003b). The use of gene arrays to study environmental contaminants in aquatic systems has been applied to fish (Hogstrand et al., 2002; Larkin et al., 2003a; Williams et al., 2003). Furthermore, gene arrays have been successfully used to examine the effects of hypoxia in fish and crabs (Gracey et al., 2001; Ton et al., 2002; Brouwer et al., 2004). This emerging technology will no doubt become routine for assessing a variety of bioindicators in the next decade.

The classic method for quantifying protein levels is Western blotting, the protein analog to Northern blots. Proteins from homogenized tissue samples are electrophoretically separated in denaturing polyacrylamide gels, transferred to a membrane, and then incubated with primary antibodies to the proteins. Secondary antibodies with a covalently linked enzyme, which catalyzes formation of a colored or chemiluminescent product from an appropriate substrate, are used to visualize and quantify the protein–primary antibody complex. Western blots are an excellent choice for confirming protein expression from a limited number of up- or downregulated genes, and they have been used to confirm changes in gene expression in blue crab (Brouwer et al., 2004).

Proteomics refers to the large-scale study of proteins, including understanding the complex interactions among proteins that occur within cells. Expression proteomics is a subfield that focuses on understanding the factors that affect protein expression, and thus is complementary to Q-PCR, subtractive hybridizations, and gene arrays (Denslow et al., in press). Proteomics analysis requires separation of proteins by

two-dimensional (2D) gel electrophoresis or ion-exchange chromatography followed by mass spectrometry of tryptic digests of the separated proteins. A number of proteins can be identified in this manner, but the procedure is costly and labor intensive. Changes in protein patterns and expression in response to environmental and chemical factors have recently been identified in fish (Kultz and Somero, 1997; Bradley et al., 2002) and invertebrates (Shepard and Bradley, 2000; Meiller and Bradley, 2002).

Indicators of Hypoxia in Crustacea

While the ultimate goal of bioindicators is effectiveness and usefulness in the field, the development of new bioindicators requires testing, calibration, and validation under controlled laboratory conditions. As a first step toward attaining this goal, the effects of chronic and intermittent hypoxia on blue crab (*Callinectes sapidus*), brown shrimp (*Farfantopenaeus aztecus*), and grass shrimp (*Palaemonetes pugio*), were examined in the laboratory. The identification of potential molecular bioindicators of hypoxia in blue crab will enable examination of the efficacy of these indicators to identify hypoxic exposure in wild-caught blue crabs.

Materials and Methods

Exposure Methods

Wild-caught, adult male blue crabs (85 to 152 mm carapace width [CW] 42.5 to 191.3 g wet weight [ww]) were maintained in the laboratory at 15‰ and 27 ± 1°C for 6 to 28 days prior to experimentation. During acclimation and experimentation periods, crabs were fed commercial shrimp pellets once daily. For the chronic hypoxia study, six crabs were placed in each of six hypoxic (2 to 3 ppm DO) and two normoxic (6 to 8 ppm DO) 35-L tanks under flowthrough conditions (Manning et al., 1999). Oxygen in the flowthrough dilution water was maintained at supersaturation (14 ppm, hypoxic tanks, and 18 to 20 ppm, normoxic tanks). The high DO concentrations were necessary because of the high rates of oxygen consumption by the study crabs. Ten control (normoxic) crabs were sacrificed at days 0 and 15. Ten hypoxic crabs were sacrificed at days 5, 10, and 15. To examine the effects of cyclic, intermittent hypoxia, five crabs were placed in each of four cyclic DO (2 to 3 ppm DO to 8 to 9 ppm DO cycle every 24 h) and four normoxic (8 ppm DO) 35-L tanks under flowthrough conditions for 10 days. This exposure was conducted by limiting the oxygen addition to the dilution water reservoir during the evening hours, which resulted in a drop in DO in the treatment aquaria to 2 to 3 mg/L by early morning. In the morning the frequency of oxygen aeration was increased in the reservoir, which increased the aquaria to normoxic levels by late morning or early afternoon. Ten control (normoxic) crabs were sacrificed at days 0 and 10 and ten intermittent hypoxic crabs were sacrificed at days 5 and 10. The hepatopancreas of the crabs was removed and frozen at –70°C for protein analysis or stored in RNAlater (Ambion®, Austin, Texas) at –20°C for nucleic acid extraction.

Wild-caught, adult brown shrimp (64 to 138 mm total length, TL, 1.85 to 17.55 g) were maintained in flowthrough tanks in the laboratory at 15‰ and 27 ± 1°C for 16 to 30 days prior to experimentation. Shrimp were fed commercial shrimp pellets once daily. To determine survival of brown shrimp under chronic hypoxic conditions, 12 shrimp were placed in each of 6 hypoxic (2 to 3 ppm DO) and 12 normoxic (6 to 8 ppm DO) 35-L tanks under flowthrough conditions for 12 days. Survival of brown shrimp subjected to cyclic DO variations, as described above, was assessed by placing 12 shrimp in each of 6 cyclic DO (2 to 3 ppm DO to 8 to 10 ppm DO cycle every 24 h) 35-L tanks and 6 normoxic (6 to 8 ppm DO) 35-L tanks under flowthrough conditions for 13 days.

Wild-caught, adult grass shrimp (25 to 38 mm TL, 0.1711 to 0.4925 g) were maintained in the laboratory at 15‰ and 27 ± 1°C for 6 to 182 days prior to experimentation. During acclimation and experimentation periods, grass shrimp were fed twice daily, once with commercial maintenance flake and once with *Artemia* sp. *nauplii* (24 to 48 h post-hatch). Individual grass shrimp were isolated in mesh containers to facilitate observations of molting and egg production. The 25 containers were placed into each of eight hypoxic (2 to 3 ppm DO) and four normoxic (6 to 8 ppm DO) 35-L tanks under flowthrough

conditions for 14 days. The 20 control (normoxic) female shrimp were sampled at day 0, and the 16 hypoxic and 16 normoxic female shrimp were sacrificed at days 3, 7, and 14. After 14 days of exposure, male and female pairs were placed into the mesh containers for an additional 4 weeks; 16 pairs were maintained under hypoxia, 32 pairs consisted of hypoxia-exposed females maintained under normoxia for the reproductive portion of the experiment, and 32 pairs were maintained under normoxia. Female shrimp were sacrificed 2 to 5 days after the appearance of a clutch of eggs. A second experiment, to determine the effects of extreme hypoxia (1 to 2 ppm DO) on grass shrimp, was conducted following the same protocol, with the exception that the reproductive pairs of shrimp were maintained for an additional 6 weeks in hypoxic or normoxic conditions following the initial 14-day exposure. Upon sacrifice, grass shrimp were weighed (0.0001 g) and measured (TL, mm), and eggs were removed and counted. The thorax of the shrimp samples was frozen at –70°C for protein analysis or stored in RNAlater at –20°C for nucleic acid extraction.

Cloning and Macroarrays of Blue Crab Genes

Ten genes, including heat shock protein 70 (Hsp70), mitochondrial and cytosolic manganese superoxide dismutase (MnSOD) (Brouwer et al., 2003), hemocyanin (Brouwer et al., 2002), Cu and Cd-metallothionein (Syring et al., 2000), beta-actin, and ribosomal proteins S15, L23, and S20, were cloned and sequenced from hepatopancreas tissue of blue crab. The blue crab genes were PCR-amplified and then robotically spotted in duplicate onto neutral nylon membranes. Total hepatopancreatic mRNA was extracted from six to nine blue crabs per treatment group using Stat-60® (TEL-TEST, Friendswood, Texas). mRNA was transcribed into radiolabeled cDNA, and hybridized to the membranes. For each cDNA clone, the general background of each membrane was subtracted from the average value of the duplicate spots on the membrane. The values were then normalized to a beta-actin cDNA clone; the resulting values were used to determine which gene transcripts were up- or downregulated by hypoxia (Larkin et al., 2003a).

Subtractive Hybridization for Identification of Hypoxia-Responsive Genes in Blue Crabs and Grass Shrimp

Subtractive hybridizations were performed by EcoArray® LLC (Alachua, Florida). For the blue crab subtractive hybridizations (Clontech® PCR-Select cDNA Subtraction Kit, Palo Alto, California), mRNA samples from seven day 0 (control) and seven day 5 (hypoxic) crabs were reverse-transcribed into cDNA and heat-denatured, and the cDNA pools were then hybridized together. Two subtractive hybridizations were performed to obtain grass shrimp hypoxia-responsive genes. mRNA samples from day 0 normoxic (control) and day 3 and 5 moderate hypoxic (2 to 3 ppm DO) and day 3 severe hypoxic (1.5 ppm DO) shrimp were reverse transcribed into cDNA. The cDNAs that remained unhybridized were PCR-amplified, cloned, and subsequently sequenced for identification. Subtraction libraries were run in both the forward and reverse directions to obtain both up- and downregulated genes. The blue crab clones obtained through subtractive hybridization were spotted in duplicate onto expanded macroarrays as described above.

Western Blots

Homogenized hepatopancreas tissue samples from seven to ten blue crabs from each treatment group were subjected to SDS-PAGE on 10 and 12.5% polyacrylamide gels for hemocyanin and MnSOD, respectively. Separated proteins were transferred electrophoretically to PVDF membranes and visualized using the Pierce SuperSignal West Dura Extend Substrate system for chemiluminescent detection of proteins. The *Callinectes sapidus* antihemocyanin antibody was prepared by DLAR Vivarium (Duke University, Durham, North Carolina). The MnSOD antibody was commercially available from Stressgen Biotechnologies Corporation (Victoria, British Columbia, Canada).

Statistical Analyses

Significant differences in survival between normoxic and hypoxic crabs and shrimp were determined by chi-square analysis of 2×2 contingency tables. Differences in gene expression between control and normoxic crabs were determined with a Student's *t*-test. Percentage data were arcsine square root transformed prior to analysis (Sokal and Rohlf, 1995). Results were considered significant if $p < 0.05$.

Results

Laboratory Survival under Hypoxic Conditions

Blue crab survival decreased during the 15 days of the chronic hypoxia experiment, but there was no significant difference between normoxic and hypoxic crabs (Table 17.1). Survival of crabs in the cyclic DO experiment decreased in both the normoxic and cyclic DO treatments by day 10, with crabs exposed to cyclic DO showing higher, but statistically not significant mortality than those in normoxic conditions (Table 17.1).

TABLE 17.1

Percent Survival of Blue Crabs Exposed to Chronic or Intermittent Hypoxia

	Days of Exposure			
Treatment	**Day 3**	**Day 5**	**Day 10**	**Day 15**
Hypoxic	88	78	55	50
Normoxic	83	77	67	53
Intermittent Hypoxic	96.7	90	47.8	—
Normoxic	80	80	71.4	—

Survival of brown shrimp consistently decreased during the 12-day exposure to chronic hypoxia (Figure 17.1A), and there was a significant difference in survival between normoxic and hypoxic brown shrimp at days 10 and 12. Intermittent hypoxia appeared to affect brown shrimp survival more severely than chronic hypoxia, and there was a significant difference in survival between brown shrimp exposed to normoxic or cyclic DO within 3 days exposure (Figure 17.1B).

Survival of grass shrimp exposed to normoxia or 2 to 3 ppm DO hypoxia over a 6-week period was nearly 100% (data not shown). However, grass shrimp exposed to severe hypoxia (1.5 ppm DO) began to show increased mortality by week 5, which became significant by week 8, when there was only 56% survival of the hypoxic shrimp (Figure 17.2).

Changes in Gene and Protein Expression in Blue Crabs in Response to Hypoxia

Gene macroarrays appear to be an effective method to detect changes in gene expression in blue crabs following exposure to hypoxia. Macroarrays spotted with ten blue crab genes plus several internal controls show that five genes appear to be downregulated (less intense spotting) after 5 days exposure to hypoxia: HSP70, mitochondrial MnSOD, hemocyanin, and ribosomal proteins S15 and L23 (Figure 17.3). Data from all membranes combined show subtle, but significant, differences in gene expression between control and 5 day hypoxia-exposed crabs for hemocyanin, mitochondrial MnSOD, and ribosomal protein S15 (Figure 17.4A). In general, all genes with the exception of Cu-metallothionein showed modest downregulation after 5 days exposure to hypoxia. This generic downregulation of gene expression after 5 days appears to be a transient phenomenon since gene transcription was restored to normal levels after 15 days exposure to hypoxia (Figure 17.4B).

Hemocyanin protein concentrations in the hepatopancreas were significantly elevated after 5 days of hypoxia exposure (Figure 17.5A), but this difference was no longer observed after 15 days of hypoxia exposure (Figure 17.5B). Western blots showed unexpected changes in molecular weight of MnSOD

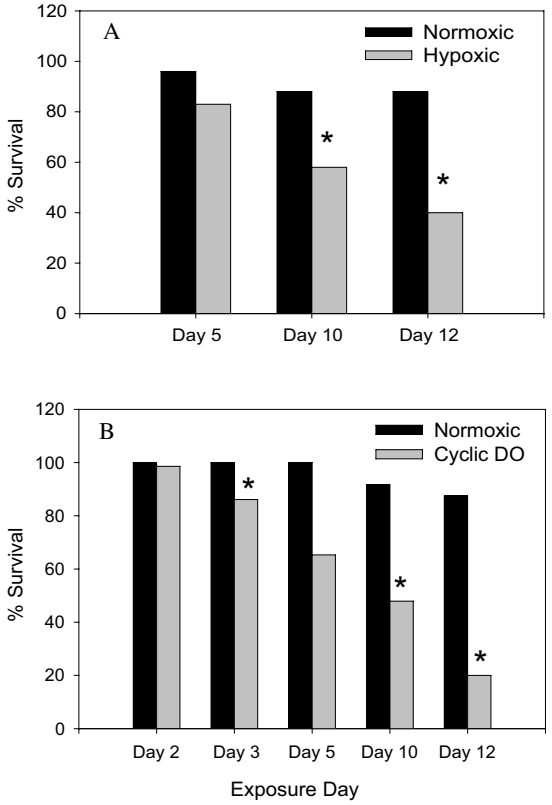

FIGURE 17.1 Survival of brown shrimp, *Farfantopenaeus aztecus*, exposed to hypoxic conditions. (A) Chronic hypoxia (2 to 3 ppm DO). (B) Diurnal fluctuations in hypoxia (2 to 3 ppm DO to 6 to 8 ppm DO over a 24-h period). * = Significantly reduced survival relative to the controls ($p < 0.05$).

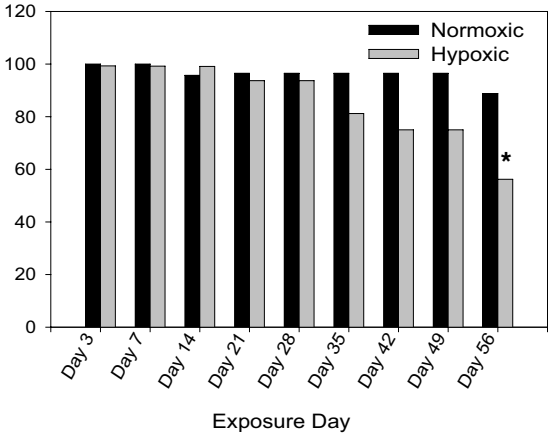

FIGURE 17.2 Survival of grass shrimp, *Palaemonetes pugio*, exposed to severe chronic hypoxia (1.5 ppm DO). * = Significantly reduced survival relative to the controls ($p < 0.05$).

protein in the hepatopancreas of hypoxia-treated crabs. Control, normoxic crabs showed 22- and 25-kDa bands corresponding to the mitochondrial and cytosolic forms of this protein (Brouwer et al., 2003). After 5, 10, and 15 days of exposure to hypoxia, significant cross-linking of proteins occurred, resulting in high-molecular-weight (~70 to 90 kDa) aggregates (Figure 17.6).

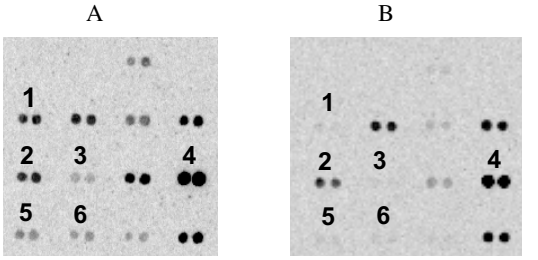

FIGURE 17.3 Gene macroarrays of blue crabs exposed to hypoxia in the laboratory. (A) Normoxia (6 to 8 ppm DO). (B) Chronic hypoxia (2 to 3 ppm DO) exposure for 5 days. 1: HSP 70; 2: actin; 3: mitochondrial MnSOD; 4: hemocyanin; 5: ribosomal protein S15; 6: ribosomal protein L23.

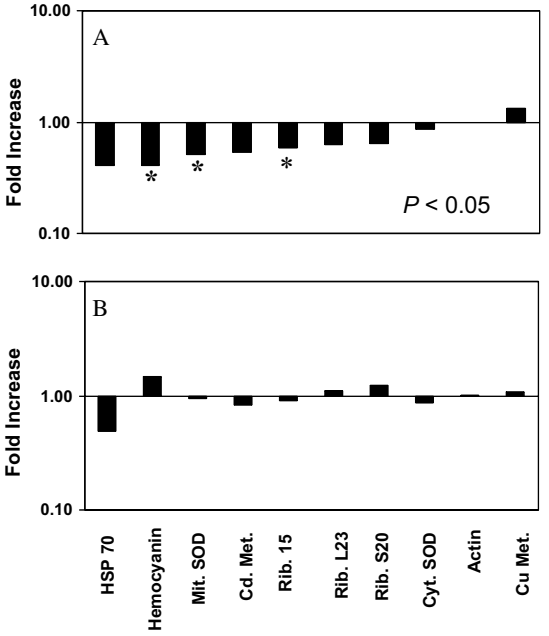

FIGURE 17.4 Fold differences in blue crab gene expression, as determined from macroarray analysis, following chronic hypoxia (2 to 3 ppm DO) exposure. (A) Difference between day 0 normoxia and 5 day chronic hypoxia. (B) Difference between day 15 normoxia and day 15 chronic hypoxia. HSP 70: heat shock protein 70; Mit. SOD: mitochondrial-MnSOD; Cd. Met.: Cd-metallothionein; Rib. 15: ribosomal protein S15; Rib. L23: ribosomal protein L23; Rib. S20: ribosomal protein S20; Cyt. SOD: cytosolic-MnSOD; Cu Met.: Cu-metallothionein. * = Significant difference.

Diurnal fluctuations in DO did not significantly affect gene or protein expression in blue crab, although the overall patterns were similar to those observed for crabs exposed to chronic hypoxia. For example, hepatopancreas hemocyanin protein concentration was higher at day 5 and 10 diurnal hypoxia than in the day 0 and day 10 normoxic controls (data not shown). All genes appear to be slightly downregulated after 5 days of exposure to diurnal DO cycles (Figure 17.7A). Similar to results from the chronic hypoxia study, this generic pattern changes after 10 days of exposure to diurnal DO fluctuations (Figure 17.7B).

Due to only a modest response in gene regulation following exposure to chronic or diurnal hypoxia, subtractive hybridizations were performed to identify more potentially hypoxia-responsive genes in blue

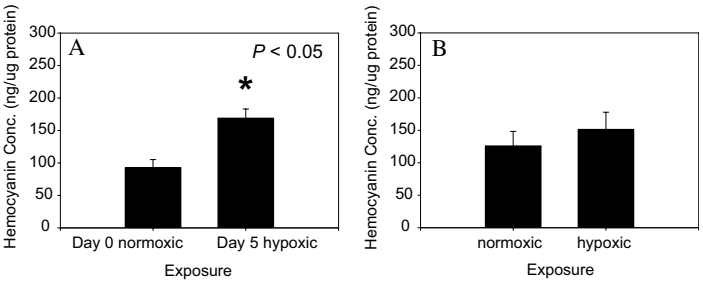

FIGURE 17.5 Hemocyanin protein concentration in hepatopancreas of normoxia- and hypoxia-exposed blue crabs. (A) Short-term (5 day) chronic hypoxia (2 to 3 ppm DO) exposure. (B) Long-term (15 day) chronic hypoxia (2 to 3 ppm DO) exposure. * = Significant difference.

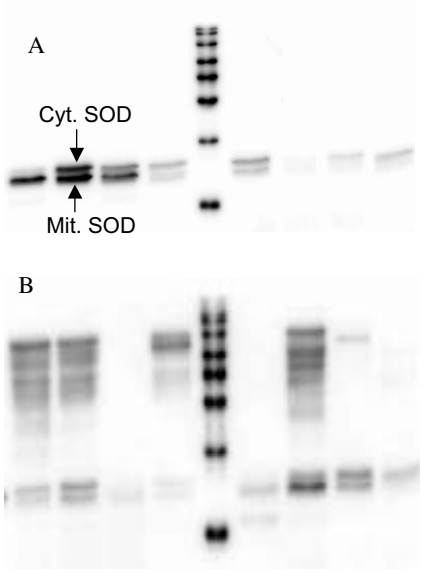

FIGURE 17.6 SDS-PAGE gels of MnSOD protein from hepatopancreas of blue crab, *Callinectes sapidus*. (A) Normoxic crabs showing the cytosolic and mitochondrial forms of blue crab MnSOD. (B) Day 5 and 10 hypoxic crabs showing cross-linked MnSOD. The ladder represents molecular weight markers of 20, 30, 40, 50, 60, 80, 100, and 120 kDa.

crab. An additional 14 genes were identified, and their expression levels determined in blue crabs exposed for 10 days to either normoxic or diurnal DO fluctuations (Figure 17.7B, right half of graph). The new set of genes did not show greater up- or downregulation compared to the first set (Figure 17.7B).

Effects of Hypoxia on Grass Shrimp

Subtractive hybridization using mRNA from grass shrimp exposed to chronic hypoxia for 3 days resulted in a suite of 74 potentially differentially regulated genes. A large number of these genes are related to specific biological functions that are influenced by hypoxic conditions, such as ATP metabolism, oxygen transport, protein synthesis, and gluconeogenesis (Table 17.2). There is a high likelihood that future testing of this suite of genes on macroarrays with shrimp exposed to chronic hypoxia will result in biologically relevant molecular biomarkers of hypoxia.

The effects of chronic hypoxia on grass shrimp fecundity were examined. Grass shrimp exposed to both chronic moderate (2 to 3 ppm DO) and severe (1.5 ppm DO) hypoxia showed significant differences in relative fecundity (number of eggs/g shrimp; Figure 17.8). In both experiments, grass shrimp

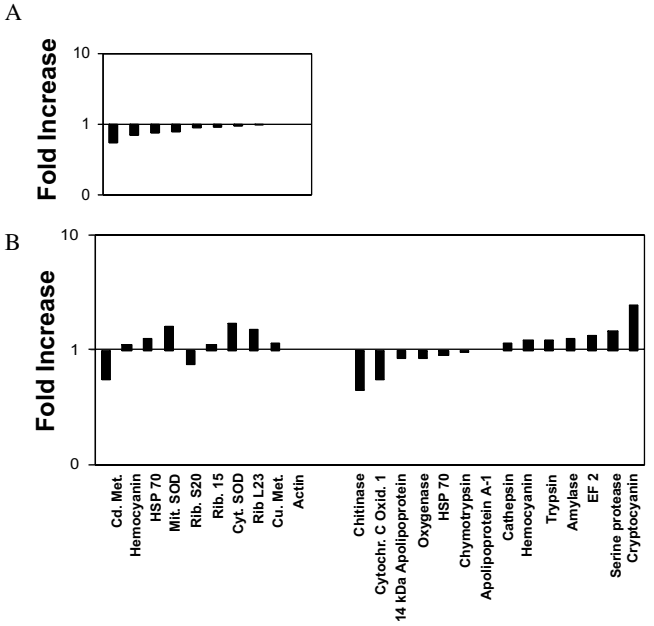

FIGURE 17.7 Fold differences in blue crab gene expression, as determined from analysis of macroarrays, following diurnal hypoxia (2 to 3 ppm DO to 8 to 10 ppm DO) exposure. (A) Difference between day 0 normoxia and 5 day diurnal hypoxia (n = 8). (B) Difference between day 10 normoxia and day 10 diurnal hypoxia (n = 8). Note additional genes identified through subtractive hybridization on right side of graph. Cd. Met.: Cd-metallothionein; HSP 70: heat shock protein 70; Mit. SOD: mitochondrial-MnSOD; Rib. S20: ribosomal protein S20; Rib. S15: ribosomal protein S15; Cyt. SOD: cytosolic-MnSOD; Rib. L23: ribosomal protein L23; Cu. Met.: Cu-metallothionein; Cytochr. C Oxid. 1: cytochrome *c* oxidase subunit 1; EF2: elongation factor 2.

TABLE 17.2

Potential Hypoxia Responsive Genes Identified in Grass Shrimp, *Palaemonetes pugio*, through Subtractive Hybridization

Biological Function	No. of Genes
Energy production—ATP metabolism	7
Energy production—Oxygen transport	6
Protein synthesis	9
Protein degradation	6
Protein folding	3
Lipid metabolism	7
Carbohydrate metabolism	4
Gluconeogenesis	3
Locomotion/contraction	4
Other functions	25

Note: Genes are grouped by general biological function.

maintained in hypoxic conditions for 6 (Figure 17.8A) or 8 (Figure 17.8B) weeks had a significantly higher relative fecundity compared to shrimp maintained in normoxia for the same time, and also compared to shrimp exposed to hypoxia for 2 weeks and then transferred to normoxia for the remaining 4 or 6 weeks (Figure 17.8). However, there was no difference in relative fecundity between grass shrimp exposed to hypoxia for only 2 weeks and those maintained in normoxia for the entire experiment (Figure 17.8).

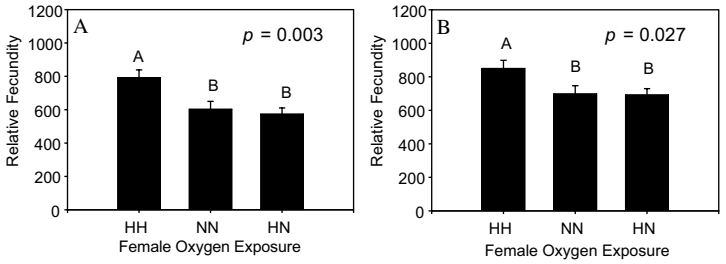

FIGURE 17.8 Relative fecundity ($\bar{x}$ ± 1 SE) of grass shrimp, *Palaemonetes pugio*, exposed to chronic hypoxia: (A) 6-week exposure to moderate (2 to 3 ppm DO) hypoxia; (B) 8-week exposure to severe (1.5 ppm DO) hypoxia. Letters indicate groups that are significantly different. HH, hypoxic females; NN, normoxic females; HN, females maintained in hypoxic conditions during the first 2 weeks of the experiment and then transferred to normoxic conditions.

Discussion

Laboratory data provide convincing evidence of the effects of hypoxia on crustaceans, from the whole-animal to the molecular level. Of the three species examined here, tolerance to chronic and intermittent hypoxia, as measured by survival, decreases from grass shrimp to blue crabs to brown shrimp. For both crabs and brown shrimp intermittent hypoxia appeared to have a greater effect than chronic hypoxia. The dramatic decrease in survival of brown shrimp during both chronic and intermittent hypoxia indicates this species cannot tolerate low DO. Indeed, independent studies have shown that brown shrimp strongly avoided oxygen concentrations of 2 mg/L (Renaud, 1986) and 1 mg/L (Wannamaker and Rice, 2000), whereas blue crabs do not show any significant avoidance behavior (Das and Stickle, 1994). Recent studies have shown that localized shrimp catch is negatively related to the amount of local coverage by hypoxia and that shrimp migration to offshore habitat is blocked by hypoxia (Zimmerman and Nance, 2001). It appears therefore that chronic and intermittent hypoxia may have serious consequences for shrimp fisheries by limiting availability of suitable habitat.

Fecundity of grass shrimp appears to be affected by hypoxia, with shrimp held under chronic hypoxia having a higher fecundity than normoxic animals (Figure 17.8). While this seems counterintuitive, it may represent a strategy of maximizing reproductive output prior to death. The reproductive studies reported here measured fecundity based on the first brood observed. Experiments are planned to determine if second and third broods (if any) decrease in size under hypoxic conditions, and if time between production of broods increases. Combination of whole-animal information such as this with data from molecular biomarkers is crucial for developing models of the effects of hypoxia at the ecosystem level (see below).

Molecular biomarkers could be potentially useful for assessing exposure and effects of hypoxia on estuarine organisms that are hypoxia tolerant, such as blue crab and grass shrimp. Significant differences in both gene and protein expression in crabs were observed after 5 days exposure to chronic hypoxia, indicating measurable changes are occurring on the molecular level that are not manifested at the whole-organism level. One of the first responses to hypoxia is downregulation of protein synthesis to save energy (Hochachka and Lutz, 2001). This was evident in blue crab by the downregulation of gene expression of ribosomal proteins, heat shock protein, hemocyanin, and mitochondrial MnSOD after 5-day exposure to both chronic and intermittent hypoxia (Figure 17.4A and Figure 17.7A). Downregulation of MnSOD is a typical cellular response to hypoxia in both invertebrates and vertebrates (Russell et al., 1995; Choi et al., 2000). After 15 days of hypoxia exposure, the expression level of most of the genes had returned to normoxic levels. This suggests that the initial response to hypoxia is reversible and that the crab has additional regulatory mechanisms to stabilize energy consumption at a new, hypometabolic steady state, as found for other hypoxia-tolerant organisms (Hochachka and Lutz, 2001).

Despite the fact that hemocyanin mRNA levels had decreased after 5 days of hypoxia, hemocyanin protein levels had increased significantly. This observation stresses the importance of validating the results from gene arrays with protein-level determinations. The apparent discrepancy may be explained

as follows. Subtractive hybridization experiments suggest that transcription of the lysosomal proteolytic enzyme cathepsin is downregulated in response to hypoxia. Thus, the existing pool of intracellular proteins may be preserved through deactivation of proteolytic pathways, resulting in discrepancies between mRNA and protein levels.

Intermittent hypoxia and chronic hypoxia affect gene regulation differently. For example, genes for enzymes that detoxify reactive oxygen species produced during reoxygenation, such as MnSOD, are upregulated in response to cyclic DO (Hochachka and Lutz, 2001), but downregulated in response to chronic hypoxia (Russel et al., 1995; Choi et al., 2000). Blue crabs respond in a similar manner, as mitochondrial MnSOD appears to be upregulated after 10 days of intermittent hypoxia (Figure 17.7A), but downregulated in response to chronic hypoxia (Figure 17.4A). The effect of chronic and intermittent hypoxia on MnSOD protein is different as well. Under hypoxic conditions, a cross-linked, high-molecular-weight species is the predominant form of MnSOD, whereas under normoxic and intermittent hypoxic conditions the monomeric form of the protein predominates. It is of interest to point out that cytosolic MnSOD, which occurs in a monomer–dimer equilibrium, is found only in crustacea that use hemocyanin for oxygen transport (Brouwer et al., 2003). The monomer–dimer equilibrium may lend itself to further polymerization, by an as yet not understood mechanism. While the differences we have demonstrated in gene expression appear promising, they are rather subtle and often not statistically significant. Verification of the most promising gene expression results using Q-PCR is therefore necessary to obtain a complete and accurate picture of the molecular responses of blue crab to hypoxia, as well as increase the reliability of the gene arrays as a tool for detection of DO stress in field-collected animals.

The results of the subtractive hybridizations for discovery of hypoxia-responsive genes in grass shrimp are very promising. Most of the genes identified are known to play a role in adaptation to hypoxia or in antioxidant defense. In all, 13 genes are involved in energy (ATP) production, the use of which must be limited to survive prolonged hypoxia/anoxia (Hochachka et al., 1996). Other downregulated genes are those involved in protein synthesis and turnover, which will result in turning protein synthesis rates down to pilot-light levels (Hochachka and Lutz, 2001). Still other genes are involved in lipid and carbohydrate metabolism, including enzymes that are catalyzing the conversion of amino acids into glucose (gluconeogenesis), which maintains blood glucose levels under hypoxic conditions (Gracey et al., 2001). Finally, several proteins involved in locomotion are downregulated, similar to what is observed in hypoxic fish (Gracey et al., 2001). Macroarrays constructed with the identified grass shrimp genes should provide unequivocal biomarkers of hypoxia for this important estuarine species, which is amenable to reproductive studies, which, in turn, will allow us to determine if the hypoxia-responsive gene arrays can be used as predictive indicators of reproductive impairment.

Scaling Molecular Biomarker Responses to Population Responses

The greatest limitation of many indicators of coastal condition is the lack of linkage with the cause or causes for change (Suter et al., 2002). Molecular indicators of stress in individuals have considerable potential to provide information on cause-and-effect relationships, and to serve as indicators of early stages of ecological change. However, for this potential to be realized, it will be necessary to develop modeling tools that will make it possible to scale molecular responses in individuals to higher, ecologically more relevant, levels of biological organization. This scaling up of the indicators can be accomplished by using carefully designed laboratory experiments that provide relationships between indicator values in controlled conditions and laboratory end points such as fecundity and embryo survival.

Predicting the population-level consequences of environmental stressor exposure is difficult because many of the potentially important effects are sublethal. Incorporating sublethal effects into population dynamics models is not currently possible because they lack sufficient detail for such effects to be represented as changes in model parameters (Rose, 2000). However, a suite of appropriately scaled models — physiological/statistical, individual-based (IBM), and matrix projection models — for linking sublethal physiological or behavioral effects to long-term population dynamics is an effective method for addressing this problem (Figure 17.9). This approach has been applied to predict population effects of contaminants (PCB) on fish (Rose et al., 2003), and can be adapted to predict the population-level effects of hypoxia on grass shrimp.

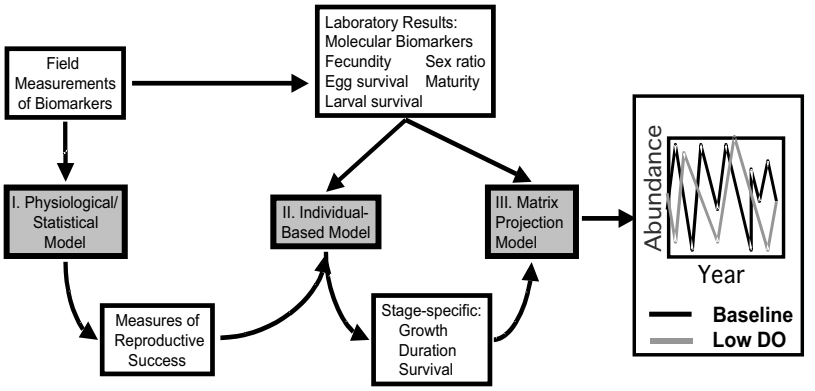

FIGURE 17.9 Conceptual model for scaling individual-level effects (biomarkers) of environmental stressors to the population level.

As shown in Figure 17.9, when correlations between biomarkers and reproductive success are known from laboratory experiments, field-measured biomarkers may be directly converted to relevant inputs to the IBM. When these correlations are not known, it is necessary to model physiological or behavioral responses of individuals to convert biomarkers to measures of reproductive success for inputs to the IBM. The IBMs will link the various measures of reproductive output (from experiments and from the output of the physiological/statistical model) to changes in the inputs of the matrix projection model, which will then simulate the long-term dynamics of the shrimp population. Since both approaches use molecular biomarkers as inputs, they will enable the prediction of ecologically relevant population effects in situations when only biomarkers are measured in shrimp collected in the field.

Conclusions

The data presented here demonstrate that current technology, in the form of gene arrays and subtractive hybridizations, shows considerable promise to provide diagnostic tools for the identification of hypoxic indicators in estuarine crustacea. Additionally, whole-animal indicators of hypoxia, such as mortality and changes in reproductive parameters, are important in combination with the molecular indicators. The true test of these indicators, identified under controlled laboratory conditions, will be their ability to distinguish crustacea exposed to hypoxic conditions in the field. The ability to incorporate information obtained from these molecular and whole-animal indicators into models to predict population-level effects will provide resource managers with sensitive early-warning indicators of incipient ecological change due to chronic and intermittent hypoxia. Such indicators are vitally important for effective management of ecological systems, because they will allow pro-active rather than re-active strategies for restoring ecosystem health. In addition, the indicators will be useful for evaluating the success of remediation efforts in a timely manner.

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