

Provided for non-commercial research and educational use only.
Not for reproduction or distribution or commercial use.



This article was originally published in a journal published by Elsevier, and the attached copy is provided by Elsevier for the author's benefit and for the benefit of the author's institution, for non-commercial research and educational use including without limitation use in instruction at your institution, sending it to specific colleagues that you know, and providing a copy to your institution's administrator.

All other uses, reproduction and distribution, including without limitation commercial reprints, selling or licensing copies or access, or posting on open internet sites, your personal or institution's website or repository, are prohibited. For exceptions, permission may be sought for such use through Elsevier's permissions site at:

<http://www.elsevier.com/locate/permissionusematerial>



ELSEVIER

Journal of Experimental Marine Biology and Ecology 341 (2007) 16–31

**Journal of
EXPERIMENTAL
MARINE BIOLOGY
AND ECOLOGY**

www.elsevier.com/locate/jembe

Molecular and whole animal responses of grass shrimp, *Palaemonetes pugio*, exposed to chronic hypoxia

Marius Brouwer ^{a,*}, Nancy J. Brown-Peterson ^a, Patrick Larkin ^b, Vishal Patel ^c,
Nancy Denslow ^c, Steve Manning ^a, Theodora Hoexum Brouwer ^a

^a Department of Coastal Sciences, The University of Southern Mississippi, 703 East Beach Dr., Ocean Springs, MS 39564, USA

^b EcoArray Inc., 12085 Research Dr., Alachua, Florida 32615, USA

^c Department of Physiological Sciences and Center for Environmental and Human Toxicology, University of Florida,
PO Box 110885, Gainesville, FL 32611, USA

Received 28 July 2006; received in revised form 15 September 2006; accepted 20 October 2006

Abstract

Hypoxic conditions in estuaries are one of the major factors responsible for the declines in habitat quality. Previous studies examining effects of hypoxia on crustacea have focused on individual/population-level, physiological or molecular responses but have not considered more than one type of response in the same study. The objective of this study was to examine responses of grass shrimp, *Palaemonetes pugio*, to moderate (2.5 ppm DO) and severe (1.5 ppm DO) chronic hypoxia at both the molecular and organismal levels. At the molecular level we measured hypoxia-induced alterations in gene expression using custom cDNA macroarrays containing 78 clones from a hypoxia-responsive suppression subtractive hybridization cDNA library. Grass shrimp exposed to moderate hypoxia show minimal changes in gene expression. The response after short-term (3 d) exposure to severe hypoxia was up-regulation of genes involved in oxygen uptake/transport and energy production, such as hemocyanin and ATP synthases. The major response by day 7 was an increase of transcription of genes in the mitochondrial genome (16S rRNA, cytochrome *b*, cytochrome *c* oxidase I and III), and up-regulation of genes encoding proteins involved in iron metabolism. By day 14 a dramatic reversal was seen, with a significant down-regulation of both mitochondrial and Fe-metabolism genes. Validation of the macroarray results with q-PCR showed similar up- or down-regulation at multiple time points for 9 genes. At the organismal level, our studies showed condition factor of grass shrimp exposed to severe chronic hypoxia was lower than normoxic controls during the first 7 days of the experiment, but there were no differences after that time point, or in grass shrimp exposed to moderate hypoxia. Surprisingly, chronic hypoxia appeared to enhance grass shrimp reproduction; females exposed to moderate hypoxia had higher fecundities and a greater percentage produced first, second and third broods than normoxic shrimp. The hypoxic shrimp took longer to produce their first brood than the normoxic controls, although starved larvae from hypoxia-exposed mothers lived longer than normoxic control larvae. Shrimp exposed to severe hypoxia also had higher fecundity than normoxic controls, although embryos from hypoxia-exposed mothers took longer to hatch than normoxic control embryos. The gene expression and reproductive results suggest that expression levels of genes encoding proteins involved in oxygen and electron transport, energy, and iron metabolism may be useful molecular indicators of both short term (<7 d) and moderate (14 d) exposure to severe hypoxia, and that chronic hypoxia may have population-level impacts on grass shrimp.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Gene expression; Hemocyanin; Hypoxia; Macroarray; Mitochondrial genes; Reproduction

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

E-mail address: marius.brouwer@usm.edu (M. Brouwer).

1. Introduction

Chronic and intermittent/cyclic hypoxia is of increasing concern as related to declines in habitat quality in coastal and estuarine environments (Diaz and Rosenberg, 1995; Buzzelli et al., 2002). Hypoxia can lead to rapid as well as long-term cellular, physiological and behavioral changes in a variety of organisms. Because of this, detection of short-term “rescue” responses and long-term adaptive adjustments caused by hypoxic exposure is important in environmental research. 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). Physiologically, aquatic invertebrates respond to hypoxia by regulating oxygen transport by increased cardiac output and hemoglobin/hemocyanin synthesis and expression (Mangum, 1997; Terwilliger, 1998; Paul et al., 2004). At the molecular level, differential gene expression in fishes reflects the metabolic roles of tissues during hypoxia exposure (Gracey et al., 2001; Ton et al., 2002, 2003; van der Meer et al., 2005). Hypoxia-responsive genes and proteins have recently been identified in blue crab, *Callinectes sapidus* (Brown-Peterson et al., 2005), suggesting molecular indicators show promise for identifying signs of hypoxia exposure in estuarine crustacea. However, these molecular signals by themselves do not provide information on the effects of hypoxia on the individual and its ability to help maintain the population. Changes in reproductive parameters in response to hypoxia have population level consequences (Wu, 2002), yet little is known about the effects of hypoxia on reproductive fitness in estuarine organisms routinely exposed to low oxygen conditions. Therefore, the aim of our studies was to link molecular indicators to reproductive endpoints. Understanding the potential relationship between molecular and organismal endpoints could reveal new mechanisms of hypoxia tolerance/adaptation and may help predict ecologically relevant consequences of hypoxia.

We used the hypoxia tolerant, estuarine grass shrimp, *Palaemonetes pugio*, to examine the effects of chronic hypoxia on gene expression and reproduction. This species has been shown to be uniquely physiologically adapted to stressful tidal marsh habitats (Welsh, 1975). The use of a commonly occurring resident species in these studies allows laboratory results to be more easily related and applied to field measurements. In contrast to previous studies which examined global responses of hypoxia on gene expression (Gracey et al., 2001; Ton et al., 2002, 2003; van der Meer et al., 2005), we have taken a directed approach to identify potentially hypoxia

responsive genes through suppression subtractive hybridization. We concentrated on those genes coding for proteins in the mitochondrial electron transport chain, ATP synthesis, oxygen transport, carbohydrate metabolism, protein synthesis/repair/degradation, antioxidant defense and lipid metabolism that are known to be responsive to hypoxic stress (Hochachka et al., 1996; Czyzyk-Krzeska, 1997; Hochachka and Lutz, 2001). We found that the selected genes were significantly up-regulated or down-regulated when grass shrimp were exposed to moderate or severe chronic hypoxia. Furthermore, gene expression varied with duration and severity of dissolved oxygen exposure, and hypoxia exposure resulted in marked effects on shrimp egg production and larval survival.

2. Materials and methods

2.1. Experimental animals, exposure methods and reproduction

Grass shrimp were collected in the vicinity of Ocean Springs, Mississippi in Davis Bayou using dip nets. Adult females and males were segregated by sex based on morphological differences in the first and second pleopods (Meehean, 1936) and maintained in the laboratory at 15 psu and 27 ± 1 °C for 7 to 30 d prior to experimentation. During acclimation, shrimp were held in 296 L tanks with static renewal of seawater. During acclimation and experimentation periods, grass shrimp were fed brine shrimp nauplii once daily and commercial flake food once daily. During all acclimation and experimentation periods, shrimp were held in artificial seawater (Fritz Super Salt, Fritz Industries, Mesquite TX) diluted to 15 psu with non-chlorinated well water.

Four separate laboratory experiments were conducted to determine the effects of moderate (2.5 ppm dissolved oxygen, DO) or severe (1.5 ppm DO) chronic hypoxia on gene expression and reproduction in grass shrimp. The exposures were conducted in a modified intermittent flow-through system previously described (Manning et al., 1999). The flow through test system provided 1 L every 20 min (resulting in 3 complete volume additions/day) to each of the 35 L test aquaria using a separate water delivery partitioner for each of the normoxic and hypoxic treatments. Oxygen levels were controlled by bubbling nitrogen into a holding tank which gravity fed to the partitioner used to deliver flow-through hypoxic seawater. A 24 h timer was used to activate a solenoid valve which controlled nitrogen introduction into the holding tank at intervals that maintained oxygen in the holding tank at a level which

resulted in the desired oxygen concentration when introduced into the test aquaria. An additional partitioner provided flow-through normoxic seawater, and normoxic conditions were maintained by gently bubbling oxygen into the cells of the water partitioner prior to delivery of water to the individual aquaria. In all experiments, oxygen was monitored continuously in one hypoxic flow-through aquaria, and DO, temperature and salinity were measured in all flow-through aquaria once or twice daily using a YSI Model 600XLM data sonde.

Grass shrimp were housed individually or in reproductive pairs in retention chambers constructed from 10 cm Petri dish bottoms with a 15 cm high collar of 500 μ m nylon mesh and a 10 cm diameter disposable Petri dish lid to prevent escape. The mesh walls of the chamber facilitate flow of water into the chamber. Twenty-five chambers were placed into each of the 35 L flow-through glass aquaria in a water bath held at 27 ± 1 °C. The exchange of water within the chambers was assured by fluctuating the water level within the aquaria 8 to 10 cm (18.6 to 23.3 L) periodically with a self-starting siphon. This compartmentalization of the test organisms precluded cannibalism and enabled individual identification and the enumeration of molts of each test animal. Retention chambers were maintained at a minimum depth of approximately 7 ± 1 cm and a maximum depth of 10 ± 1 cm.

2.2. Experiments 1, 2 and 3; sampling for gene expression

One moderate (experiment 1) and two severe (experiments 2 and 3) hypoxia studies were conducted following similar protocols to monitor gene expression as well as reproductive effects of hypoxia. In all experiments, 310 shrimp were isolated individually into 12 aquaria. Male (2 tanks \times 25 shrimp) or female (6 tanks \times 25 shrimp) shrimp were maintained in 8 hypoxic aquaria (2–3 ppm DO, moderate hypoxia or 1.5 ppm DO, severe hypoxia). The normoxic shrimp (6–8 ppm DO) were housed in 1 tank of 35 males and 3 tanks of 25 females. For each experiment, twenty female shrimp (10 normoxic, 10 hypoxic) were sampled at three time points (days 3, 7, and 14) during the course of the studies for analysis of gene expression, for a total of 60 individual shrimp per experiment. An equal number of shrimp were removed from each 35 L aquarium during each sampling event to maintain similar densities of shrimp in each aquarium. At the beginning of each experiment, 10 female shrimp were removed from the test population for the day 0 assessment. Sample selection was made from females at each sampling time that had egg masses, and eggs were removed and counted. Males were not

analyzed for gene expression due to their small size and insufficient tissue for analysis. Shrimp were anesthetized in ice water, and the total length (TL, mm) and egg-free wet weight (ww, 0.1 mg) were recorded for each shrimp. The thorax was removed and stored at -20 °C in RNA later (Ambion, Inc. Austin, TX) for gene expression analysis.

2.3. Experiments 1 and 2; reproductive sampling

At the end of two weeks under hypoxic or normoxic conditions, males and females from the moderate (experiment 1) and one severe (experiment 2) hypoxia exposure were paired into reproductive groups to determine differences in fecundity and survival of the F1 generation based on hypoxia exposure. Hypoxic males were paired with hypoxic females in 16 individual breeding chambers under continued hypoxic conditions (moderate or severe). All other mating pairs (hypoxic males (16) \times hypoxic females (16); hypoxic males (16) \times normoxic females (16); normoxic males (16) \times hypoxic females (16) and normoxic males (16) \times normoxic females (16)) were kept under normoxic DO. Pairs were checked daily for egg production, and sacrificed after the female was determined gravid for a minimum of 2 days. All eggs from each sacrificed female were removed and counted, and 20 viable eggs from each female were incubated individually in sterile seawater in 24-well polystyrene culture plates in a stirring incubator at 27 °C and 60 rpm for 12 d. Culture plates containing embryos were observed daily and percent embryo survival was determined by successful hatch by day 10 post-isolation. The reproductive portion of the experiments lasted for 4 weeks. Sacrificed females from the reproductive portion of the study were processed for gene expression analysis as described above. At the termination of the experiment, all remaining females that had not produced egg masses were sacrificed and processed for gene expression analysis.

2.4. Experiment 4; multiple brood reproduction

A fourth experiment to determine the effects of moderate hypoxia on reproduction was undertaken to examine the production and condition of multiple broods produced by the same female. For this experiment, 100 male: female pairs of shrimp were isolated into each of 4 moderate hypoxic (2–3 ppm DO) aquaria and 4 normoxic (6–8 ppm DO) aquaria for 10 weeks, resulting in 25 reproductive pairs/aquaria. During the study, females with egg masses were isolated into hatching chambers within their aquaria (5 cm petri dish with a 10 cm collar of 1 mm mesh) 7 to 8 d after first observation of the

Table 1

Genes used for construction of macroarrays to detect hypoxia-responsive genes in *Palaemonetes pugio*

<i>Protein folding/repair and translocation</i>		
1	Peptidyl prolyl <i>cis</i> – <i>trans</i> isomerase	P54985 ^{a, b} 5.81 e–24 ^c
2	<i>Hsp70</i> cognate	BAA32395 ^{a, b} 2.80 e–45 ^c
3	Mitochondrial import inner membrane translocase subunit (TIM14)	NP648475 ^{a, b} 1.94 e–27 ^c
4	<i>Hsp70</i>	AAL27404 ^{a, b} 3.00 e–63 ^c
5	<i>Hsp70</i> (AY935982 ^d)	Z15041 ^{a, b} 3.00 e–75 ^c
6	Signal sequence receptor-translocation associated protein	AAK15544 ^a 2.40 e–31 ^c
<i>Protein synthesis</i>		
7	Ribosomal protein L13	Q90Z10 ^a 1.26 e–34 ^c
8	Ribosomal protein L5	Q26481 ^a 7.73 e–19 ^c
9	Ribosomal protein S6	AY769320 ^{a, b} 1.00 e–57 ^c
10	Ribosomal protein L31	Q91A76 ^a 3.09 e–29 ^c
11	Ribosomal protein L27A	BAC54559 ^{a, b} 2.03 e–25 ^c
12	Mitochondrial ribosomal protein S2	NP057118 ^{a, b} 9.66 e–42 ^c
13	Ribosomal protein L21	AAK95147 ^a 2.00 e–98 ^c
14	Ribosomal protein L3	AAH42242 ^{a, b} 2.00 e–78 ^c
15	Elongation factor 2 (<i>EF2</i>)	AAAR01298 ^{a, b} 2.00 e–74 ^c
16	Ribosomal protein L6	NP498584 ^a 1.00 e–15 ^c
17	Ribosomal RNA 16S (<i>16S rRNA</i>)	AF3047 ^{a, b} 1.00 e–18 ^c
18	Ribosomal protein S20 (AY935983 ^d)	NM079697 ^a 1.00 e–27 ^c
19	Ribosomal protein S14 (AY935984 ^d)	D14609 ^{a, b} 9.00 e–16 ^c
<i>Protein degradation</i>		
20	Cathepsin L	BAC65418 ^{a, b} 1.0 e–96 ^c
21	Aminopeptidase N	XP396261 ^a 2.0 e–29 ^c
22	Cathepsin C	BAC57934 ^a 5.2 e–30 ^c
23	Cysteine proteinase	CAA75309 ^{a, b} 9.0 e–11 ^c
24	Trypsin	CAA75309 ^{a, b} 1.8 e–08 ^c
25	Crustapain (cysteine proteinase)	BAC65417 ^{a, b} 3.0 e–56 ^c
26	Aminopeptidase N	AAD09272 ^a 1.7 e–07 ^c
<i>Lipid metabolism</i>		
27	Acetyl CoA binding protein	P12026 ^{a, b} 1.5 e–22 ^c
28	Acetyl CoA dehydrogenase	NP776919 ^a 1.6 e–04 ^c
29	Dehydrocholesterol reductase	BAD51990 ^a 4.0 e–61 ^c
30	Lipase I	046107 ^{a, b} 1.6 e–18 ^c
31	Vitellogenin-1	BAB69831 ^{a, b} 1.0 e–108 ^c
32	Apolipoprotein A-1	CAC34942 ^{a, b} 9.0 e–72 ^c
33	Fertilization envelope	AAD23572 ^{a, b} 1.6 e–05 ^c
34	Vitellogenin-2	AAG17936 ^{a, b} 4.0 e–05 ^c
<i>ATP synthesis and electron transport</i>		
35	Cytochrome <i>c</i> oxidase subunit III	CAB40368 ^{a, b} 5.02 e–32 ^c
36	Cytochrome <i>b</i> (<i>cytB</i>)	NP038299 ^{a, b} 2.17 e–38 ^c
37	ATP synthase d chain (<i>ATPsyn-d</i>)	Q24251 ^{a, b} 9.96 e–23 ^c
38	Cytochrome <i>c</i> oxidase subunit III (<i>Ccox III</i>)	CAB40368 ^{a, b} 2.47 e–26 ^c
39	ATP synthase f chain (<i>ATPsyn-f</i>)	Q9W141 ^{a, b} 4.58 e–41 ^c
40	ATP synthase b chain	AAH61296 ^{a, b} 3.00 e–08 ^c
41	Cytochrome <i>c</i> oxidase subunit I (<i>Ccox I</i>)	CAG26687 ^{a, b} 2.00 e–97 ^c
<i>Oxygen transport and sensing</i>		
42	Hypoxia inducible factor 1a (AY655698 ^d)	XP967427.1 ^{a, b} 4.0 e–122 ^c
43	Haemocyanin I (<i>HcyI</i>)	AAF04148 ^a 1.0 e–129 ^c
44	HEMOCYANIN (<i>HcyII</i>)	P80888 ^{a, b} 8.2 e–32 ^c
45	Hemocyanin (<i>HcyIII</i>)	AAL27460 ^{a, b} 3.0 e–90 ^c
46	Hemocyanin subunit 4 (<i>HcyIV</i>)	CAD56697 ^{a, b} 1.0 e–47 ^c

(continued on next page)

Table 1 (continued)

Carbohydrate metabolism			
47	<i>Drosophila melanogaster</i> CG1637-PA, isoform A	<u>NP727464</u> ^a	8.8 e-04 ^c
48	Amylase I	<u>CAB65552</u> ^{a, b}	1.0 e-129 ^c
49	Alpha-amylase preproprotein	<u>AAO72321</u> ^a	2.0 e-22 ^c
50	Acid beta glucosidase	<u>P17439</u> ^{a, b}	2.4 e-31 ^c
51	PEP carboxykinase	<u>CAB85964</u> ^{a, b}	1.0 e-108 ^c
52	Chitinase	<u>AAN74647</u> ^{a, b}	2.1 e-21 ^c
53	Glycogenin-1	<u>P13280</u> ^a	2.6 e-12 ^c
Cell structure/motility and muscle contraction			
54	Troponin C gamma	<u>NP001011651</u> ^{a, b}	3.0 e-60 ^c
55	Alpha-1-tubulin	<u>AAC47522</u> ^a	6.0 e-87 ^c
56	Cellular myosin	<u>A71144</u> ^a	0 ^c
57	Fast myosin heavy chain	<u>AAA17371</u> ^a	3.0 e-40 ^c
58	Troponin I, fast skeletal muscle	<u>P05547</u> ^a	1.0 e-13 ^c
59	Beta-actin (AY935989 ^d)	<u>AY626840</u> ^{a, b}	4.0 e-139 ^c
Metal binding and anti-oxidant			
60	Heme binding protein	<u>NP956492.1</u> ^{a, b}	4.0 e-11 ^c
61	Ferritin subunit	<u>XP624076</u> ^{a, b}	4.0 e-9 ^c
62	Cytosolic Mn-superoxide dismutase (<i>cyt-MnSOD</i>) (AY211084 ^d)	<u>DQ073104</u> ^{a, b}	3.0 e-88 ^c
63	Mitochondrial Mn-superoxide dismutase (<i>mit-MnSOD</i>) (AY935986 ^d)	<u>AE017283</u> ^{a, b}	4.0 e-142 ^c
64	Cd metallothionein 1 (<i>CdMT1</i>) (AY935987 ^d)	<u>AAB5227.1</u> ^a	5.0 e-18 ^c
Blood Coagulation and Immune function			
65	PmAV	<u>S78774</u> ^{a, b}	3.9 e-05 ^c
66	Clottable protein	<u>AAF19002</u> ^a	3.4 e-37 ^c
67	Beta-1,3-glucan binding protein	<u>AAM21213</u> ^{a, b}	2.0 e-51 ^c
68	Coagulation factor V and VIII	<u>CAC94896</u> ^a	2.0 e-17 ^c
69	Complement C3-S	<u>BAA36621</u> ^a	2.0 e-55 ^c
70	Fibrinogen A	<u>AAH41754</u> ^a	1.2 e-41 ^c
Miscellaneous functions			
71	Cutical protein AMP4	<u>P81388</u> ^a	3.99 e-10 ^c
72	Ornithine decarboxylase antizyme	<u>P70112</u> ^a	2.10 e-12 ^c
73	H3 histone, family 3B	<u>XP235304</u> ^a	8.60 e-44 ^c
74	Fibrillarlin	<u>NP523817</u> ^a	3.00 e-66 ^c
75	Orn decarboxylase antizyme	<u>P55814</u> ^{a, b}	1.80 e-06 ^c
76	Glutamine repeat protein-1	<u>NP032158</u> ^{a, b}	3.80 e-07 ^c

Genes are arranged by biological function.

^a Closest match as identified by BLASTX search.

^b Significantly up-regulated or down-regulated based on macroarray analysis with all normalization techniques.

^c e(xpect) values (Karlin and Altschul, 1990).

^d GenBank nucleotide accession numbers: grass shrimp sequences.

eggs. The hatching chamber was placed in a larger retention chamber of smaller mesh (200 µm) to retain hatched larvae and separate them from the female when the offspring hatched. Following hatch, the female was returned to the original retention chamber with her mate. This method allowed for assessment of multiple broods from each reproductive pair. All hatched larvae were counted, and approximately twenty-five larvae from each female were isolated into a 200 µm mesh chamber for survival assessment. Normoxic and hypoxic larvae from each female and each brood were kept under

normoxic conditions and monitored twice daily until death from starvation. Larvae were not fed during this time to determine whether yolk content may have changed with subsequent broods and to determine if survival differed between normoxic and hypoxic broods.

2.5. Cloning, subtractive hybridization and macroarrays of grass shrimp

Eight genes (Table 1, superscript ^c), including heat shock protein (*Hsp70*), mitochondrial and cytosolic

manganese superoxide dismutase (*mit-MnSOD* and *cyt-MnSOD*), Cd metallothionein (*CdMt1*), hypoxia inducible factor 1 α (*hif-1 α*), β -actin and ribosomal proteins S14 and S20 were cloned and sequenced from grass shrimp thorax tissue containing hepatopancreas using RT-PCR. An additional 68 potentially hypoxia responsive genes (Table 1, superscript ^a) were identified from two suppression subtractive hybridizations (SSH) performed by EcoArray Inc, Alachua, FL. Subtracted libraries were constructed with poly-A mRNA, converted to cDNA, that was isolated from day 0 normoxic (control) and day 3 and 5 moderate hypoxic grass shrimp and with mRNA from day 0 controls and day 3 severe hypoxic shrimp. Subtractive hybridizations were performed in both directions on these samples in order to obtain up-regulated and down-regulated genes. Subtractive hybridizations were performed using the Clontech (Palo Alto, CA) SSH kit following the manufacturer's recommendations. The resultant pool of cDNA clones were shotgun ligated into pGEM T-Easy cloning vector (Promega, Madison, WI), transformed into DH5 α cells, and plated onto Luria-Bertani (LB) agar plates containing ampicillin and oxacillin (100 μ g/mL each). Recombinant colonies were picked from the plates, plasmids were purified and inserts were sequenced.

The resulting 76 genes produced from cloning and SSH were PCR amplified and then robotically spotted in duplicate onto neutral nylon membrane macroarrays together with various controls, including exogenous *Arabidopsis* "spiking" genes, as previously described (Larkin et al., 2003). Genes were arranged on the membrane by functional group (Table 1). Total hepatopancreatic RNA was extracted from 8–10 grass shrimp per treatment group using Stat-60 (Tel-Test, Friendswood, TX). Genomic DNA was removed by DNase treatment and total RNA was transcribed into radiolabeled cDNA and hybridized to the membranes. Background subtraction for each cDNA spot was performed as previously described by Larkin et al. (2003). The values were then log₂ transformed and normalized three different ways (to the mean and median intensity of the array data, as well as α -tubulin). Fold change values, calculated from the mean values of normalized hypoxic and normoxic shrimp for each time point were used to determine which gene transcripts were up-regulated or down-regulated by hypoxia.

To assess consistency of gene signal intensities on the macroarrays, a pooled sample of extracted RNA was reverse transcribed into labeled cDNA and hybridized to 4 separate membranes. Values were log₂ transformed and normalized to the median array intensity, and the coefficient of variation among the 4 membranes was

determined for each gene. Quality of macroarrays was further evaluated by determining correlation coefficients and slopes of scatter plots of duplicate signal intensities for all macroarray combinations.

2.6. Real-time PCR

To validate results from the gene arrays, real-time quantitative RT-PCR (q-PCR) was run for genes that showed significant up-or down-regulation on the macroarrays. These included hemocyanin (3 separate subunits), ATP synthase f chain, cytochrome *c* oxidase (I and III), cytochrome *b*, 16S rRNA and ferritin. The q-PCR was

Table 2
Sequences and percent amplification efficiency of primers used for real-time PCR validation of macroarrays

Gene	Forward primer-5'	Reverse primer-5'	Efficiency
Haemocyanin I (<i>HcyI</i>)	AAA CCA GTA	AGG ACG GGC	94% 92%
	GAA GAG GGC	AGG GAA TTC ^a	
	TTT GCT ^a and	and TGT CAC	
	AGG GCT TTG	GAA CTC TTG	
	CTC CAC ACA	CTA CTC C ^b	
ATP Synthase f (<i>ATPsyn-f</i>)	ATG GAC CGA	AAT TTC CTT	94% 101%
	GAC TTT CCC	CTC CAC CCT	
	AGA T ^a and CGC	CCT T ^a and AAC	
	CGA AGC ATT	TGC CCA TAA	
	GGA GGT G ^b	GGA GCG AAT C ^b	
α -tubulin	AGA CTG TGC	CGA TGA GAC	94% 94%
	CTT CAT GGT	GGT TCA AGT	
	CGA T ^a and GCA	TGG T ^a and AGT	
	GGT CCG CTG	TGG TGT AGG	
	TTG TTG ^b	TGG GTC TC ^b	
HEMOCYANIN (<i>HcyII</i>)	GCA GTC ACT	GGA TAC GGC	98%
	GAT GGA GAA	GAT CAA GAG	
	TAT G ^b	G ^b	
Hemocyanin (<i>HcyIII</i>)	ATA CAG TCC	AAA CCA GGG	92%
	CAA TGC TCA	TCA CGA GTG ^b	
	ATA C ^b		
Cytochrome <i>c</i> oxidase I (<i>CcoxI</i>)	CGG AGC GTG	CGA AGG CGT	94%
	AGC AGG AAT	GGG CTG TAA	
	AG ^b	C ^b	
Cytochrome <i>c</i> oxidase III (<i>CcoxIII</i>)	TTG TTG CCA	CCA GGC TGC	98%
	CAG GAT TCC	TGC TTC AAA	
	ATG ^b	G ^b	
Cytochrome <i>b</i> (<i>CytB</i>)	ACC CTT TTA	CCT TAT TTG	91%
	ACG CCA TAC	TCT GGG ATA	
	ATA C ^b	GAG C ^b	
16S rRNA	TTG TAA GGG	TAC GCT GTT	96%
	TAG CTG TGT	ATC CCT AAA	
	G ^b	G ^b	
Ferritin	CGT GGT GGA	GGT TCA TAC	103%
	AGC ATC AAT	TCT TGG TCA	
	G ^b	CTT C ^b	

Primers designed using ^aPrimer Express Software or ^bBIO-RAD iCycler iQ Beacon Designer software.

also used to validate the utility of α -tubulin for use in normalizing the array data. Primers for these genes were designed using Primer Express software (Applied Biosystems, Foster City, CA) and BIO-RAD iCycler iQ Beacon Designer software. Sequences for these primers are shown in Table 2.

Each primer set was validated for specificity and efficiency by running dissociation and standard curves, respectively. Standard curves included data from a minimum of four serially diluted cDNA samples. The efficiency of amplification for each primer set is shown in Table 2. The amplification efficiency of 18S rRNA, which was used as a normalizing gene, was 94%.

Different protocols were used for q-PCR validation of the moderate and severe hypoxia treatments. For the moderate hypoxia samples, DNase-treated (DNA-free; Ambion, Inc., Austin, TX) total RNA from hypoxic and control grass shrimp was reverse transcribed to cDNA using random hexamers and Multiscript reverse transcriptase according to the manufacturer's instructions (Applied Biosystems). The q-PCR was performed in 25 μ l reactions that contained 100 ng shrimp cDNA, 12.5 μ l SYBR green master mix (Applied Biosystems P/N 4309155; which included SYBR green, buffer, Taq polymerase, and dNTPS), and 50 nM each of the forward and reverse primers. The PCRs using 18S rRNA primers were also performed in a 25 μ l reaction and contained 0.5 ng cDNA, 12.5 μ l SYBR green master mix, and 50 nM each of the 18S rRNA forward and reverse primers (Applied Biosystems). For all of the samples, minus RT controls were run to ensure the removal of all contaminating genomic DNA. An Applied Biosystems 7500 thermocycler was used for the PCR reactions with the following cycle parameters: 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min and 40 cycles of [95 °C for 15 s and 60 °C for 60 s].

For validation of the severe hypoxia samples, DNase-treated (DNA-free, Ambion,) 1 μ g total RNA from hypoxic and control grass shrimp was reverse transcribed to cDNA using random hexamers (Ambion) and Superscript II Reverse Transcriptase according to the manufacturer's instructions (Invitrogen). The cDNA was diluted 40 \times with sterile water for q-PCR. q-PCR was performed in 50 μ l reactions that contained 25 μ l iQ SYBR Green Supermix, (BioRad, cat # 170-8884, which included SYBR Green 1, buffer, iTaq DNA polymerase and d-NTP's), 400 nM each of the forward and reverse primers and 2 μ l of the diluted cDNA. Minus RT controls were run for some samples to ensure the removal of all contaminating DNA, and in all cases no contaminating DNA was found. A BIO-RAD iCycler iQ Multi-Color Real Time PCR Detection System was

used for the PCR reactions with the following cycle parameters: 1 cycle of 95 °C for 2 min, 50 cycles of [95 °C for 15 s and 58 °C for 15 s].

Each sample was run in duplicate for both q-PCR protocols using 5–10 hypoxic or normoxic shrimp. The averaged duplicate C_t value (PCR cycle threshold where target amplification is first detected) was normalized to measured 18S rRNA C_t values for each sample. 18S rRNA values did not fluctuate between treatment groups (data not shown). The comparative C_t ($2^{-\Delta\Delta C_t}$) method of analysis was used to determine changes in gene expression between controls and treated samples (Wong and Medrano, 2005).

2.7. Data analysis

Throughout all experiments, each shrimp in its individual chamber is considered a replicate for both gene expression and reproduction ($n=10$) because maintaining hundreds of shrimp in individual experimental units at the same DO is practically impossible. Additionally, the flow-through system and high volume of water exchange in each experimental tank limited interaction through chemical cues among shrimp in the same tank, whereas the separation into individual retention chambers prevented physical interactions.

A length-weight relationship was calculated for all grass shrimp sampled ($ww=14.84+3.741TL$, $r^2=0.863$, $p<0.001$). The slope of this line was used to calculate condition factor (K) for each individual ($K=ww/TL^{3.74}\times 100,000$; see Murphy and Willis, 1996). There was a significant relationship between shrimp length and number of eggs ($TL=8.39$ eggnum $- 90.63$, $r^2=0.33$, $p=0.002$). Therefore, the relative fecundity of grass shrimp was calculated by dividing the number of eggs or hatched larvae by the ww of the female (expressed as # eggs/g), and is used in all analyses. Reproductive groups from the moderate and severe hypoxia studies were combined into 3 groups, based on the DO history of the females; females continuously exposed to hypoxia (HH), females continuously exposed to normoxia (NN) and females exposed to hypoxia the first 2 weeks of the study but allowed to mate in normoxia (HN). Differences in relative fecundity, K , percentage of hatched embryos, and days to hatch among the 3 treatment groups for the moderate and severe hypoxia studies were tested using ANOVA; differences among groups were evaluated with the Bonferroni post-hoc test. Differences in K , larval survival, interbrood interval, and larval survival between normoxic and moderate hypoxic females in the multiple brood study were tested using student's t-test. Percentage data were arcsine square root transformed prior to analysis

(Sokal and Rohlf, 1995). Data were tested for homogeneity of variance (Levene's test) and normality of distribution (1 sample Kolmogorov–Smirnov test) and were log-transformed if necessary to meet these assumptions. Student's *t*-test and ANOVA were performed with SPSS (version 11.5). Data were considered significant if $p \leq 0.05$.

Gene expression data from macroarrays normalized to the array mean, median, and α -tubulin, and q-PCR data normalized to 18S rRNA were analyzed with Student's *t*-test to determine differences between normoxic and hypoxic grass shrimp for each time point in the moderate and severe hypoxia experiments. Only shrimp continuously exposed to normoxia or moderate/severe hypoxia were used for these analyses; shrimp from the HN reproductive group were not included in these analyses. Changes in gene expression for both macroarrays and q-PCR were considered significant if $p \leq 0.05$. The gene expression data are plotted as fold change, and thus error bars for the control and treated samples are not shown.

3. Results

3.1. Grass shrimp survival and condition

Survival of grass shrimp during the moderate hypoxia experiment was excellent; only 1 out of 225 females died during the course of the 46 d study. Mortality during the 14 d exposure period of severe hypoxia was 2.7% for both hypoxic and normoxic females. During the 4.5 week reproductive portion of the severe hypoxia study, female mortality was 22.6% in hypoxia and 10.3% in normoxia; no males died during the course of this study. Female mortality was higher during the 10 week multiple brood moderate hypoxia experiment, 32% in normoxia and 15% in hypoxia.

There were no significant differences in length or weight between normoxic and hypoxic grass shrimp during the 14 d exposure period or subsequent reproductive periods for any of the experiments. There were also no differences in condition factor between normoxic and hypoxic females for either of the moderate hypoxia studies. However, after 3 and 7 d exposure to severe hypoxia, condition factor of normoxic females was higher than that of hypoxic females ($p=0.049$ and 0.052 , respectively). There were no additional significant differences in condition factor as the study progressed. This suggests severe, chronic hypoxia may have a short-term effect on grass shrimp condition, but they are able to adjust and compensate within 1 week of exposure.

Shrimp in both normoxic and hypoxic exposures routinely molted every 5–8 d. However, differences in

molt frequency between normoxic and hypoxic shrimp were not quantified since shrimp held in normoxia rapidly consumed their molts (often in <24 h) and we feel that molt frequency in normoxic shrimp is thus underrepresented in our observations. It is noteworthy, however, that shrimp held in both moderate and severe hypoxia rarely consumed their molts, which often persisted in the cages for 2–3 d.

3.2. Gene expression

Forty-eight of the original 76 potentially hypoxia-responsive genes showed significant up- or down-regulation for all 3 normalization methods (mean, median, α -tubulin) for at least one time point (Table 1, superscript ^d). Data presented here represent these most robust genes, and are displayed using α -tubulin normalization, since this gene was determined to be a consistent normalization gene (see real-time PCR section below).

Tests of variability of gene signal intensities on the macroarrays due to experimental error showed a coefficient of variation $\leq 20\%$ for the robust genes. Comparisons among the membranes showed slopes of scatter plots of signal intensities of corresponding genes for all six macroarray combinations ranging from 0.835 to

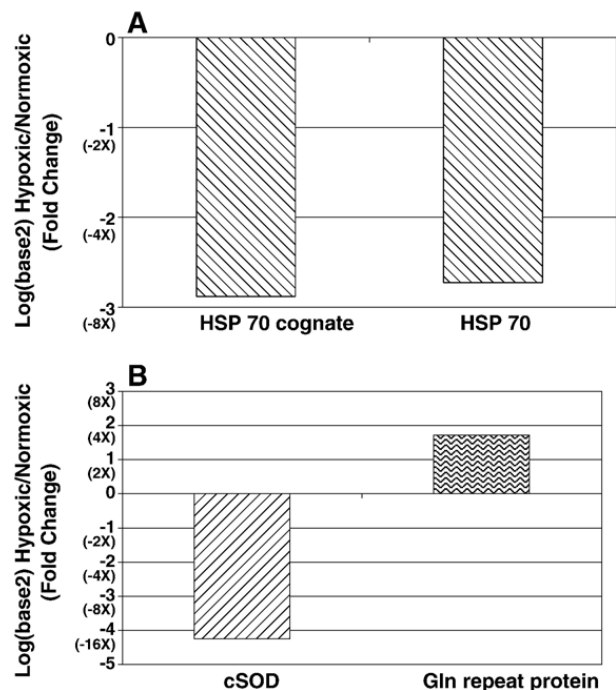


Fig. 1. Fold changes in gene regulation measured by macroarrays in grass shrimp exposed to chronic, moderate (2.5 ppm DO) hypoxia. Data shown are normalized to α -tubulin and all changes are significant (*t*-test, $p \leq 0.05$). Genes are pattern coded by functional group, as defined in Table 1. Genes with values <-1 are down-regulated 2-fold or greater. A. 7 day exposure. C. 14 day exposure.

0.955 and r^2 values from 0.83 to 0.95 indicating good consistency of signal intensity values among membranes. In addition, a plot of mean intensity for each gene from the normoxic controls ($n=23$) of the moderate hypoxia exposure experiment against the mean intensity of corresponding genes from the controls of the severe hypoxia experiment ($n=33$) had a slope of 0.891 and r^2 value of 0.792, indicating good consistency of signal intensity values among shrimp.

There were no significant changes in gene expression after 3 d exposure to moderate, chronic hypoxia. However, after 7 d exposure, there was significant down-regulation of 2 *HSP70* genes (Fig. 1A). After 14 d exposure to moderate, chronic DO, there was a significant 19-fold decrease in expression of the gene encoding the antioxidant enzyme cytosolic Mn Superoxide Dismutase (*cSOD*), which in crustacea has replaced the more commonly found cytosolic Cu, Zn Superoxide Dismutase (Brouwer et al., 2003, 1997). Expression of one other gene, which shows weak sequence similarity (E value = 3.83^{-07}) with a mouse gene encoding for glutamine repeat protein, is up-regulated (Fig. 1B). It appears in general that no genes are robust indicators of moderate chronic DO exposure, with the possible exception of *cSOD*.

In contrast to moderate hypoxia, grass shrimp exposed to severe (1.5 ppm DO), chronic hypoxia showed

significant changes in expression of a number of genes that are potentially robust indicators of hypoxia. Furthermore, gene expression profiles change over the time-course of chronic DO exposure, lending further insight into how the grass shrimp adapt to severe hypoxia. After 3 d exposure to severe hypoxia, significantly up-regulated genes include ATP synthase d and f chains (*ATPsyn-d* and *ATPsyn-f*), 3 hemocyanin genes (*Hcy II*, *Hcy III*, and *Hcy IV*), troponin C and ferritin (Fig. 2A), suggesting an attempt to increase oxygen uptake/transport (hemocyanin), ATP synthesis (ATP synthases) and locomotion (troponin C). After 7 d exposure to severe chronic hypoxia, the adaptation induced by day 3 becomes insufficient, and ATP synthase, hemocyanin and troponin are no longer up-regulated (Fig. 2B). The major response by day 7 appears to be an increase of transcription of genes present in the mitochondrial genome (16S mitochondrial rRNA (*16S rRNA*) and cytochrome *c* oxidase subunit 1 (*Ccox I*); Fig. 2B). Increased synthesis of cytochromes, which are Fe/heme proteins, is accompanied by up-regulation of the genes encoding heme binding protein and ferritin (Fig. 2B). The adaptation seen after 7 d once again becomes insufficient by Day 14, and a dramatic reversal is seen, with a significant down-regulation of transcription of genes in the mitochondrial genome (*16SrRNA*, *Ccox I*, cytochrome *c*

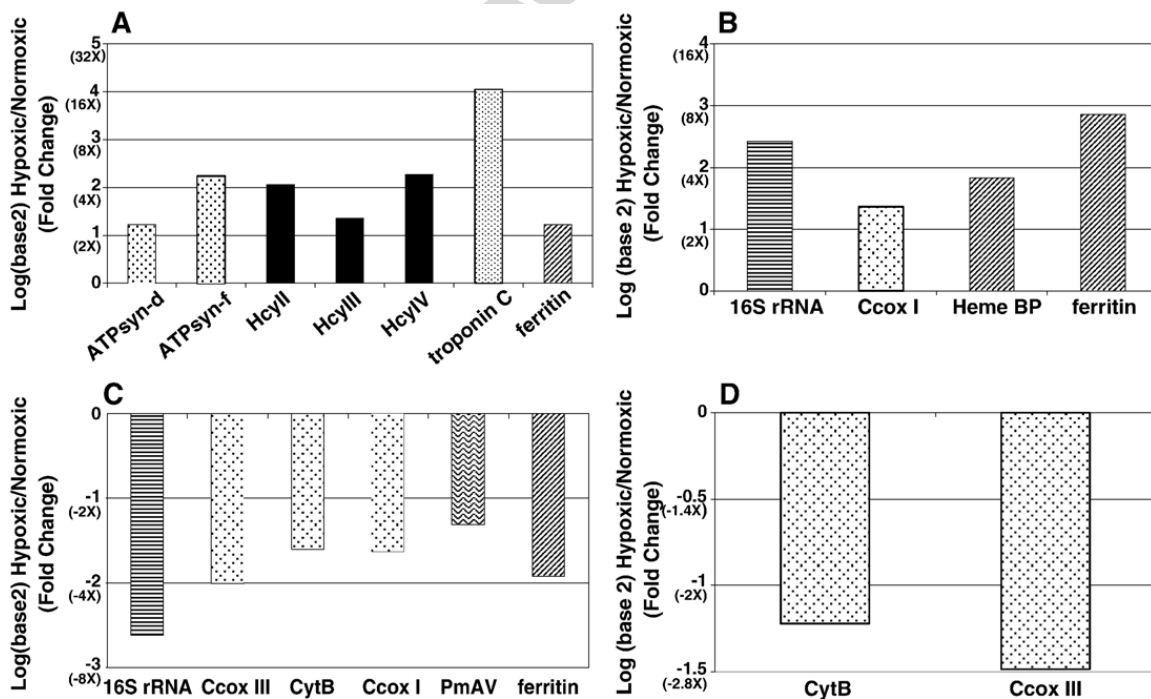


Fig. 2. Fold changes in gene regulation measured by macroarrays in grass shrimp exposed to chronic, severe (1.5 ppm DO) hypoxia. Data shown are normalized to α -tubulin and all changes are significant (t -test, $p < 0.05$). Genes are pattern coded by functional group as defined in Table 1. Genes with values > 1 are up-regulated 2-fold or greater. Genes with values < -1 are down-regulated 2-fold or greater. A. 3 day exposure. B. 7 day exposure. C. 14 day exposure. D. 26–61 day exposure.

oxidase III (*Ccox III*) and cytochrome *b* (*cytB*) as well as ferritin (Fig. 2C). Finally, *PmAIV*, a novel gene shown to be important in virus resistance in the shrimp *Penaeus monodon*, is also down-regulated after 14 d exposure to severe hypoxia, suggesting a potential for increased susceptibility to disease with chronic severe hypoxia exposure. Prolonged 26–61 d exposure to severe hypoxia shows continued down-regulation of mitochondrial proteins *Ccox III* and *cytB* (Fig. 2D). Thus, mitochondrial genes such as *16S rRNA*, *cytB*, *Ccox I* and *Ccox III* as well as hemocyanin and Fe-proteins appear to provide promise as indicators of chronic severe hypoxia exposure in grass shrimp.

3.3. Real-time PCR validation

The q-PCR was used to validate the gene expression results from the macroarrays for both the moderate and severe hypoxia experiments, as well as to verify the utility of using α -tubulin as a normalizing gene. Alpha-tubulin did not change significantly in response to 14 d exposure to moderate hypoxia or to 3, 7 or 14 d exposure to severe hypoxia (Fig. 3). These results, in combination with the consistent appearance of α -tubulin with high intensity values on all membranes, justify the use of α -tubulin for normalization of the arrays.

Moderate hypoxia resulted in few significant changes in gene expression in macroarrays (Fig. 1), and q-PCR also showed no significant changes in expression of α -tubulin, *Hcy I* or *ATPsyn-f*, although the direction of gene regulation (up or down) was the same for these genes in the macroarrays and q-PCR (Fig. 3A).

In contrast to results for moderate hypoxia, the q-PCR showed a greater, but still not significant, up-regulation of 3 separate hemocyanin genes after 3 d exposure to severe hypoxia (Fig. 3B) mirroring macroarray results for the same time point (Fig. 2A). Additionally, *ATPsyn-f* was also up-regulated in both q-PCR (Fig. 3B) and on macroarrays (Fig. 2A). Furthermore, 2 separate groups of shrimp exposed to severe hypoxia for 3 d showed a similar up-regulation of the 3 hemocyanin genes and *ATPsyn-f* measured using q-PCR and macroarray analysis, suggesting this is a robust, consistent response.

There were significant changes in gene expression as measured by q-PCR after both 7 and 14 d exposure to chronic severe hypoxic (Fig. 3C and D). Three genes (*Ccox I*, *Ccox III* and ferritin) were significantly up-regulated at day 7, and two of these (*Ccox I* and ferritin) were also significantly up-regulated on the macroarrays at the same time point (Figs. 2B and 3C). Gene expression changed significantly for *16S rRNA* and ferritin after 14 d exposure to severe hypoxia (Fig. 3D), and

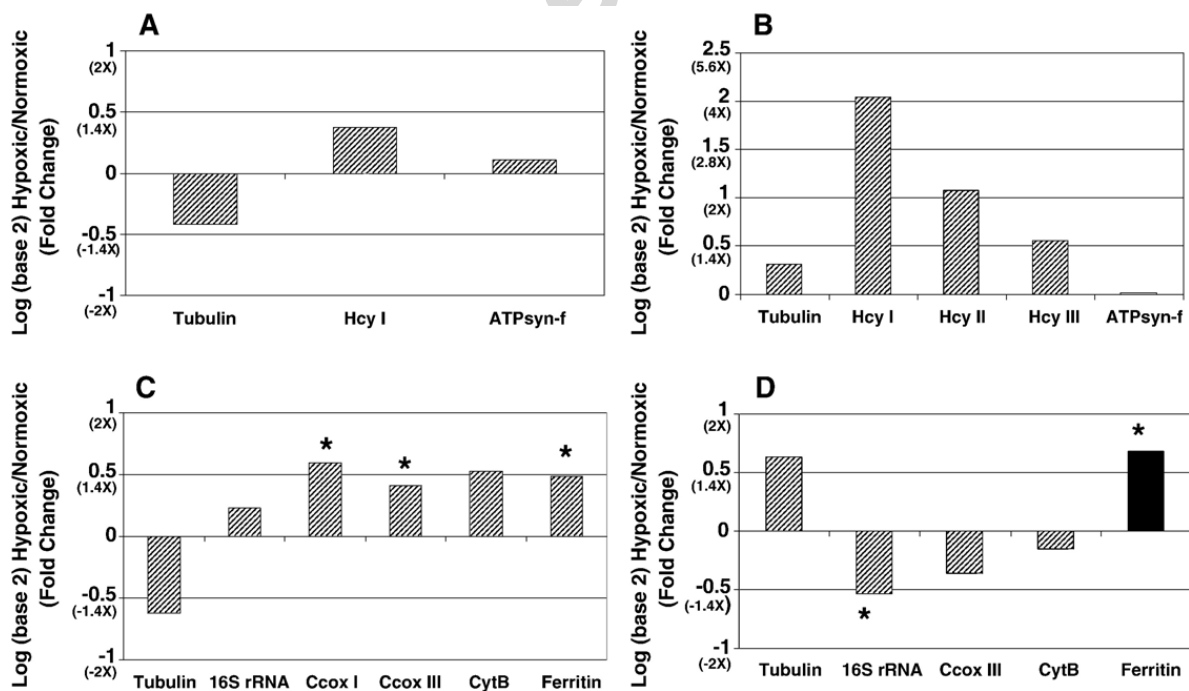


Fig. 3. Fold changes in gene regulation measured by real-time PCR in grass shrimp exposed to chronic hypoxia. Genes with values >1 are up-regulated 2-fold or greater. Genes with values <-1 are down-regulated 2-fold or greater. Striped bars indicate the direction of the fold change was the same for both real-time PCR and macroarrays. Data shown are normalized to 18S rRNA. Significant (*t*-test, $p < 0.05$) real-time PCR changes indicated by *. A. 14 day exposure to moderate (2.5 ppm DO) hypoxia. B. 3 day exposure to severe (1.5 ppm DO) hypoxia. C. 7 day exposure to severe hypoxia. D. 14 day exposure to severe hypoxia.

both these genes were significantly down-regulated on the macroarrays (Fig. 2C). There was only one instance of disagreement among all genes tested using q-PCR and macroarray analysis. Ferritin was up-regulated as measured by q-PCR and down-regulated as measured by macroarrays after 14 d exposure to severe hypoxia. Overall, the similarity in response of 9 genes at multiple time points using both q-PCR and macroarrays validates the macroarray gene expression results.

3.4. Reproduction

There were no significant differences in any reproductive parameters when considering male DO exposure history. Thus, all reproductive groups were analyzed based on female DO exposure history only. A higher percentage of grass shrimp exposed to continuous moderate hypoxia (HH) produced a first brood than females exposed to NN or HN. This difference was not observed in shrimp exposed to severe hypoxia (Table 3). Furthermore, shrimp exposed to moderate hypoxia (HH) took longer to produce a brood (22.7 ± 2.3 d) than those exposed to NN (16.1 ± 1.8 d), and this difference was significant ($t_{24} = 2.24$, $p = 0.035$). However, there was no between-group difference in time to produce a brood for shrimp exposed to severe hypoxia (22.6 ± 3.1 d, hypoxic, 22.6 ± 2.5 d, normoxic; $p = 0.995$). There was a significant difference in relative fecundity among exposure groups for grass shrimp in the moderate hypoxia experiment ($F_{2,35} = 5.317$, $p = 0.010$; Fig. 4A). Grass shrimp exposed to continuous moderate hypoxia (HH) had significantly higher relative fecundities than shrimp exposed to HN ($p = 0.009$, Bonferroni test) and marginally higher relative fecundities than normoxic (NN) shrimp ($p = 0.074$, Bonferroni test). There was a more pronounced difference for grass shrimp exposed to severe hypoxia, with relative fecundity of severe HH shrimp significantly higher than both NN and HN shrimp ($F_{2,47} = 3.935$, $p = 0.027$; Fig. 4B). There were no differences among groups in either percentage of embryos that successfully hatched (81.5–88.5%) or days until hatch

Table 3
Percentage of female grass shrimp producing first broods as a function of hypoxia exposure

Dissolved oxygen	HH (<i>N</i> = 16 pairs)	NN (<i>N</i> = 32 pairs)	HN (<i>N</i> = 32 pairs)
Moderate (2.5 mg/ml)	62.5%	46.9%	34.4%
Severe (1.5 mg/ml)	68.7%	53.1%	62.5%

HH, NN-females exposed to hypoxia or normoxia for the entire experiment; HN-females exposed to hypoxia for 14 d prior to mating, but allowed to mate in normoxia.

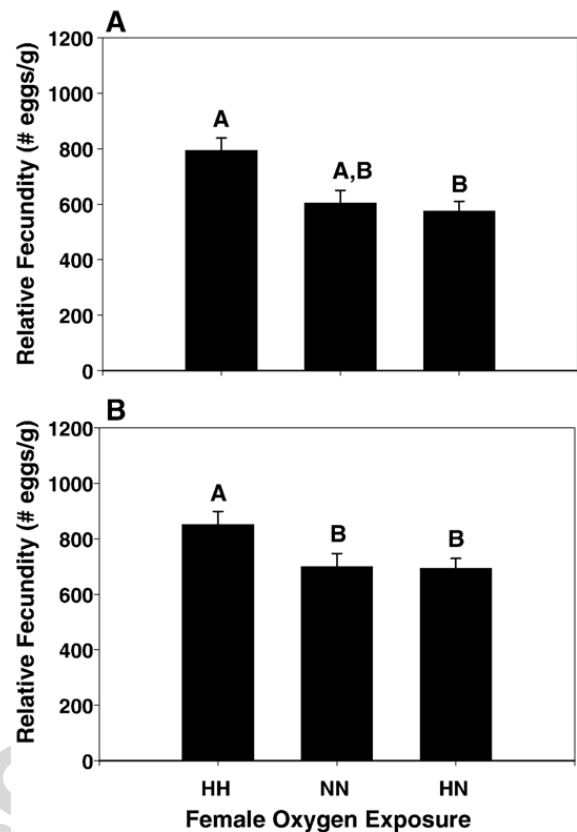


Fig. 4. Relative fecundity (# eggs/g shrimp weight) of grass shrimp exposed to chronic hypoxia, expressed as mean \pm standard error. Different letters indicate means significantly different (ANOVA, $p < 0.05$). A. Moderate (2.5 ppm DO) hypoxia. B. Severe (1.5 ppm DO) hypoxia. HH-females continuously exposed to hypoxia. NN-females continuously exposed to normoxia. HN-females exposed to hypoxia for 2 weeks and then allowed to mate in normoxia.

(11.8–12.1 d) for grass shrimp exposed to moderate hypoxia. However, there was a significant difference in days until hatch in the severe hypoxia experiment ($F_{2,44} = 3.317$, $p = 0.046$), with NN embryos hatching earlier (11.8 ± 0.2 d) than embryos from hypoxia-exposed mothers (12.5 ± 0.2 d). These results suggest exposure to hypoxia results in an increase in fecundity, but does not affect hatching success of embryos. However, these results may only apply to the first brood produced, as shrimp were sacrificed for RNA extraction prior to production of a subsequent brood in these experiments.

Table 4
Percentage of female grass shrimp exposed to chronic moderate hypoxia (2.5 mg/ml DO) over a 70 d period producing multiple broods

Brood number	Hypoxia (<i>N</i> = 100 pairs)	Normoxia (<i>N</i> = 100 pairs)
First	60%	49%
Second	24%	12%
Third	3%	1%

Table 5
Reproductive parameters of female grass shrimp in a multiple-brood, chronic moderate hypoxic study

Parameter	Hypoxia (2.5 pm DO)		Normoxia (8 ppm DO)	
	Brood 1 (N=56)	Brood 2 (N=19)	Brood 1 (N=48)	Brood 2 (N=9)
Relative fecundity (eggs/g)	202±12.9	189±21.3	226±14.3	178±82.8
Interbrood interval (d)	24.2±1.3 ^a	17.6±2.0 ^b	21.1±1.1	13.0±1.9 ^b
Larval survival (d)	6.3±0.11 ^a	6.2±0.15 ^a	5.9±0.09	5.3±0.47

Shrimp were exposed continuously over a 70 d period to hypoxia or normoxia.

^a Significant difference ($p \leq 0.05$) between treatments within a brood.

^b Significant difference ($p \leq 0.05$) between broods within a treatment.

In the multiple brood experiment, a greater percentage of females exposed to moderate hypoxia produced first, second and third broods than normoxic females (Table 4). Furthermore, the percentage of females producing first broods was similar to the previous experiments for HH and NN shrimp (Table 3). There was no significant difference in relative fecundity between normoxic- and hypoxic-exposed females for either the first or second brood (Table 5); too few females produced a third brood for meaningful analysis. Hypoxic females took a longer time to produce their first brood than normoxic females ($t_{100.6} = -1.938$, $p = 0.055$), similar to previous results for moderate hypoxia exposure, but there was no significant difference for the second brood ($p = 0.163$; Table 5). Both normoxic and hypoxic females produced their second brood in a significantly shorter time than their first brood ($t_{55} = 3.75$, $p = 0.003$, normoxic; $t_{73} = 2.82$, $p = 0.009$, hypoxic; Table 5). There was no difference in the percentage of embryos that hatched or days to hatch between normoxic and hypoxic females for either brood. Finally, starved larvae from hypoxic females survived significantly longer than starved larvae from normoxic females for both the first and second broods ($t_{100.6} = -2.488$, $p = 0.014$, first brood; $t_{26} = -2.25$, $p = 0.033$, second brood; Table 5). There were no differences in larval survival between the first and second broods of an individual female for either normoxic or hypoxic larvae ($p > 0.24$). These results suggest that moderate hypoxia impacts the initial ability of females to produce eggs, but that the number of eggs produced is not affected. However, it appears that larvae from moderate hypoxic females have a greater energy store and are able to survive longer without food.

4. Discussion

Our data indicate that changes in gene expression in grass shrimp hold promise as molecular indicators for exposure to chronic hypoxia. Furthermore, grass shrimp reproduction appears to be affected by chronic hypoxia exposure, suggesting population-level implications of long-term hypoxia. Previous studies examining the effects of hypoxia on crustacea have focused on individual/population-level (Coiro et al., 2000; Wu et al., 2002; Bell et al., 2003a,b; Mistri, 2004; Bell and Eggleston, 2005), physiological (DeFur et al., 1990; Tankersley and Wieber, 2000; McMahan, 2001) or molecular (Mangum, 1997; Paul et al., 2004; Brown-Peterson et al., 2005) responses but have not considered more than one type of response in the same study. Thus, our data provide the opportunity to integrate various disciplines in examining the responses of grass shrimp to chronic hypoxia.

Grass shrimp appear to respond differently to moderate vs. severe chronic hypoxia. Significant changes in gene expression were not seen until 7 d exposure to chronic, moderate hypoxia. The down-regulation of expression at day 7 of both the constitutive (HSP70 cognate) and the stress-inducible (HSP70) forms of the chaperone protein HSP70, important in cellular stress defense (Kultz, 2003), was somewhat unexpected. Previous studies on hypoxic responses in vertebrates and invertebrates have shown up-regulation (Benjamin et al., 1990; Ton et al., 2003; van der Meer et al., 2005; Brown-Peterson et al., 2005) or no change (Zarate and Bradley, 2003) of heat shock protein expression. However, both forms of HSP70 are also involved in folding of newly synthesized proteins (Beckman et al., 1990), and a decrease in protein synthesis in response to hypoxia may account for the decrease in expression of genes involved in protein folding. A similar down-regulation in expression of chaperone proteins such as chaperonin 10 and heat shock factor binding protein 1 was observed in gills of hypoxia-exposed zebrafish (van der Meer et al., 2005). Finally, the observed down-regulation of MnSOD in grass shrimp is a typical cellular response to hypoxia in both vertebrates (Russel et al., 1995) and invertebrates (Choi et al., 2000; Brown-Peterson et al., 2005), presumably due to reduced production of superoxide radicals under hypoxic conditions. However, this down-regulation has been previously shown in the mitochondrial form of MnSOD, whereas the unusual cytosolic form of MnSOD was down-regulated in grass shrimp.

The changes observed in gene regulation in response to severe chronic hypoxia lend insight into potential mechanisms grass shrimp may use to survive hypoxic conditions. There were distinct time-course related

differences in expression of genes important in ATP synthesis, oxygen uptake/transport and the mitochondrial electron transport chain. The initial response after short-term (3 d) exposure of grass shrimp to severe hypoxia was upregulation of genes involved in oxygen uptake/transport and energy production, such as hemocyanin and ATP synthases. This suggests an initial attempt to compensate for reduced availability of oxygen by increasing the oxygen transport and ATP synthesis capacity. Similar upregulation of proteins involved in oxygen transport such as myoglobin has also been observed in gill tissue of zebrafish exposed to hypoxia (van der Meer et al., 2005).

After 7 d exposure to severe hypoxia, expression of hemocyanin and ATP synthase genes has returned to normoxic levels. The major response by day 7 appears to be an increase of transcription of genes present in the mitochondrial genome (16S mitochondrial rRNA, cytochrome *c* oxidase 1 (*Ccox I*) and to a lesser extent ($p < 0.1$) cytochrome *b*), together with upregulation of a putative heme binding protein and the iron storage protein, ferritin. This apparent link between mitochondrial electron transport chain and proteins involved in iron metabolism is not unexpected since the mitochondrion is a dynamo of Fe metabolism, being vital not only for heme (cytochrome) biosynthesis but also for the biogenesis of [Fe–S] clusters that are present in more than 10 subunits of enzymes in Complex I, II and III of the respiratory chain (Napier et al., 2005; Taketani, 2005).

The adaptation observed after 7 days apparently becomes insufficient by Day 14, and a dramatic reversal is seen, with a significant downregulation of transcription of genes in the mitochondrial genome (16S rRNA, cytochrome *c* oxidase subunits I and III (*Ccox I and III*) and cytochrome *b*), similar to results from zebrafish exposed to long-term hypoxia (van der Meer et al., 2005). Both ferritin ($p = 0.003$) and to a lesser extent the heme binding protein ($p = 0.062$) are down-regulated as well.

Mitochondrial genes of the grass shrimp are simultaneously down-regulated in response to long-term hypoxia. Similarly, mitochondrial (*Ccox I and II*) and nuclear encoded (*Ccox IV* and *Vb*) subunits of cytochrome *c* oxidase are coordinately down-regulated in mouse and rat cell lines during hypoxia (Vijayasarathy et al., 2003). This suggests control of their transcription is coordinately regulated in response to hypoxia, or it may reflect an overall decrease in mitochondrial biogenesis with a concomitant reduction in the number of mitochondrial genome copies. Mitochondrial DNA, mitochondrial ribosomal RNA and cytochrome *b* mRNA

levels each increase with increased oxidative capacity of muscle tissue (Williams, 1986). These results indicate that the expression of mitochondrial genes in mammalian striated muscle is proportionate to their copy number, suggesting that amplification of the mitochondrial genome relative to chromosomal DNA is an important feature underlying enhanced expression of mitochondrial genes in highly oxidative tissue. Thus, downregulation of these genes in hypoxic situations could be a result of decreased need for oxidative metabolism and therefore reduced copy numbers of mitochondria and mitochondrial genes. Grass shrimp may use this mechanism to cope with hypoxic stress, and save energy by reduction of mitochondrial biogenesis.

Finally, down-regulation of *PmAV*, a gene important in virus resistance in penaeid shrimp (Luo et al., 2003), suggests the grass shrimp immune system may be compromised with chronic exposure to severe hypoxia. Burnett and Burnett (2000) suggested hypoxia results in a depression of the generalized innate immune response in *P. pugio* and *Penaeus vannamei* based on measurements of circulating hemocytes and survival of shrimp exposed to *Vibrio*. A similar conclusion was reached regarding hypoxia exposure in killifish (Boleza et al., 2001). Thus, prolonged hypoxia may have population consequences, as individuals that have already down-regulated their aerobic metabolism also have decreased immune defenses, which could result in high mortality of the population.

Data from macroarray and microarray analysis need to be interpreted cautiously (Kothapalli et al., 2002) and validation of observed changes with additional measurement techniques is desirable. All the genes in this study were identified using SSH, and gene expression results on the arrays mirrored the up- or down-regulation seen with SSH. We also used q-PCR to validate our array data, and found that 9 genes showing differential expression on macroarrays were also differentially expressed with q-PCR. The fold change values, and significance of these values, between macroarrays and RT-PCR were not always directly comparable, but in all but one instance up-regulation or down-regulation was confirmed. Quantitative differences between array data and q-PCR results have been reported previously (Ton et al., 2002, 2003; van der Meer et al., 2005; Brown-Peterson et al., 2005), and which of the two methods is more accurate is debatable (Allison et al., 2005).

Interestingly, moderate chronic hypoxia resulted in more dramatic effects on reproduction in grass shrimp than it did on regulation of our SSH identified hypoxia-responsive genes. Additionally, there was a wider variety of reproductive effects related to moderate, rather

than severe, chronic hypoxia. The surprising results of increased fecundity, percentage of ovigerous females and larval survival of hypoxia-exposed shrimp cannot be readily explained by classic life history theories. However, it has been proposed that *Daphnia* channel more resources into growth and reproduction early in life when faced with sub-optimal environmental/habitat conditions (Weber et al., 2003). Possibly, grass shrimp are following a similar strategy when exposed to chronic hypoxia.

In various fish species, exposure to hypoxia results in a decrease in gonadosomatic index (GSI), an indicator of reproductive readiness and future fecundity (Wu et al., 2003; Thomas et al., 2006) as well as decreases in actual egg number (Landry et al., 2003). Furthermore, embryonic development, hatching success and survivorship decreased with decreasing DO in Florida flagfish, *Jordanella floridae* (Hale et al., 2003), and there was a significant difference in hatching success between embryos exposed to normoxia and moderate (2–3 ppm DO) hypoxia. It appears grass shrimp embryos are more tolerant of hypoxia than Florida flagfish embryos, as there was no difference in hatching success of grass shrimp in moderate hypoxia in either the single brood or the multiple brood experiments. However, embryos of grass shrimp exposed to severe hypoxia took longer to hatch than normoxic embryos, although there was no difference in ultimate hatching success. This contrasts with the Florida flagfish results, where no embryos exposed to severe hypoxia (<1.0 ppm DO) hatched (Hale et al., 2003). Thus, while chronic hypoxia appears to have some adverse effects on grass shrimp reproduction (longer interbrood interval in moderate hypoxia, longer hatch time in severe hypoxia), the higher fecundity, greater percentage of ovigerous females and increased larval survival time of hypoxia-exposed grass shrimp indicates an overall strategy of attempting to maximize reproduction in unfavorable conditions.

Grass shrimp appear to be quite tolerant of moderate hypoxia, based on few significant changes in gene expression and no differences in condition factor. Severe hypoxia appears to have short term effects, based on a decrease in condition factor during the first 7 days of exposure in combination with upregulation of genes important in oxygen transport and energy metabolism. In general, grass shrimp appear to adapt well to both moderate and severe chronic hypoxia with little long-term mortality and few noticeable physiological effects. Indeed, the reaction to hypoxia appears to be an increase in reproduction for this species, an unexpected result. However, this may be an artifact of the laboratory envi-

ronment, where food was not limiting, there was no predation threat, and movements were restricted to a small area.

Our results suggest that gene expression may be a useful indicator for measuring both short term (<7 d) and moderate (7–14 d) exposure to severe chronic hypoxia. Since the genes used in our study were selected for their response to hypoxia, and not for their involvement in control of reproduction, the observed changes in gene expression do not provide insight into the molecular mechanisms through which hypoxia affects reproduction. However, it is in combining the molecular biomarkers with whole animal responses such as fecundity, interbrood interval and embryo/larval survival that the data presented here become most valuable for understanding and predicting population-level effects of hypoxia. A conceptual model for scaling molecular and reproductive biomarkers of environmental stressors to the population level was presented by Brouwer et al. (2005). This model stresses the importance of having both molecular as well as whole-animal inputs to be able to predict ecologically relevant population effects. Thus, the data presented here can be used in the development of physiological/statistical, individual-based (IBM) and matrix projection models (Rose et al., 2003) to gain a better understanding of population-level consequences of chronic hypoxia. Chronic hypoxia of 1–3 d duration is not uncommon in marsh systems where grass shrimp reside during summer months along both the Gulf of Mexico and the Southeastern United States (see National Estuarine Research Reserve water quality data, http://cdmo.baruch.sc.edu/data_summary.cfm), suggesting grass shrimp are exposed to these conditions in their natural environments. Furthermore, a number of mobile, hypoxia tolerant estuarine organisms have been shown to remain in hypoxic conditions (Pihl et al., 1991; Breitbart et al., 1994), suggesting at least some species do not actively avoid such conditions. Thus, our experimental results are applicable to natural marsh systems. Current research is focused on cyclic hypoxia exposures in the laboratory and grass shrimp captured from hypoxic and normoxic field sites to determine if the indicators determined from laboratory experiments are useful in a field situation. Results from these studies will help to further refine development of models, and will continue to demonstrate the importance of combining molecular and whole animal data within the same study.

Acknowledgments

We appreciate the technical expertise of B. Carter, T. Li, W. Grater, C. King and M. Peterson for their help

with subtractive library construction, cDNA cloning, sequencing, shrimp sampling and husbandry and statistical consultation. This research was supported by grants to M.B. from the US Environmental Protection Agency's Science to Achieve Results (STAR) Estuarine and Great Lakes (EaGLe) program through funding to the Consortium for Estuarine Ecoindicator Research for the Gulf of Mexico (CEER-GOM; US EPA Agreement R82945801) and from the National Oceanic and Atmospheric Administration (NAO3NOS4260216). Although the research described in this article was funded wholly or in part by the US EPA, it has not been subjected to the Agency's required peer and policy review and, therefore, does not necessarily reflect the views of the Agency and no official endorsement should be inferred. [SS]

References

- Allison, D.B., Cui, X., Page, G.P., Sabripour, M., 2005. Microarray data analysis: from disarray to consolidation and consensus. *Nat. Rev. Genet.* 7, 55–65.
- Beckman, R.P., Mizzen, L.A., Welch, W.J., 1990. Interaction of Hsp 70 with newly-synthesized proteins: implications for protein folding and assembly. *Science* 248, 850–854.
- Bell, G.W., Eggleston, D.B., 2005. Species-specific avoidance response by blue crabs and fish to chronic and episodic hypoxia. *Mar. Biol.* 146, 761–770.
- Bell, G.W., Eggleston, D.B., Wolcott, T.G., 2003a. Behavioral responses of free-ranging blue crabs to episodic hypoxia. I. Movement. *Mar. Ecol. Prog. Ser.* 259, 215–225.
- Bell, G.W., Eggleston, D.B., Wolcott, T.G., 2003b. Behavioral responses of free-ranging blue crabs to episodic hypoxia. II. Feeding. *Mar. Ecol. Prog. Ser.* 259, 227–235.
- Benjamin, I.J., Kroger, B., Williams, S., 1990. Activation of the heat shock transcription factor by hypoxia in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 87, 6263–6267.
- Boleza, K.A., Burnett, L.E., Burnett, K.G., 2001. Hypercapnic hypoxia compromises bactericidal activity of fish anterior kidney cells against opportunistic environmental pathogens. *Fish Shellfish Immunol.* 11, 593–601.
- Breitburg, D.L., Steinberg, N.D., DuBeau, S., Cooksey, C., Houde, E.D., 1994. Effects of low dissolved oxygen on predation on estuarine fish larvae. *Mar. Ecol. Prog. Ser.* 104, 235–246.
- Brouwer, M., Hoexum Brouwer, T., Grater, W., Enghild, J., Thogersen, I., 1997. The paradigm that all oxygen-respiring eukaryotes have cytosolic CuZn-superoxide dismutase and that Mn-superoxide dismutase is localized to the mitochondria does not apply to a large group of marine arthropods. *Biochem.* 36, 13381–13388.
- Brouwer, M., Hoexum Brouwer, T., Grater, W., Brown-Peterson, N., 2003. A novel cytosolic Mn-superoxide dismutase (MnSOD) has replaced cytosolic Cu, ZnSOD in crustacea that use copper (hemocyanin) for oxygen transport. *Biochem. J.* 374, 219–228.
- Brouwer, M., Brown-Peterson, N.J., Larkin, P., Manning, S., Denslow, N., Rose, K., 2005. Molecular and organismal indicators of chronic and intermittent hypoxia in marine crustacea. In: Bortone, S.A. (Ed.), *Estuarine Indicators*. CRC Press, Boca Raton, FL, pp. 261–276.
- Brown-Peterson, N.J., Larkin, P., Denslow, N., King, C., Manning, C., Brouwer, M., 2005. Molecular indicators of hypoxia in the blue crab *Callinectes sapidus*. *Mar. Ecol. Prog. Ser.* 286, 203–215.
- Burnett, L.E., Burnett, K.G., 2000. The effects of hypoxia and hypercapnia on cellular defenses of oysters, shrimp and fish. *Comp. Biochem. Physiol., B* 126, S20.
- Buzzelli, C.P., Luettich, R.A., Powers, S.P., Peterson, C.H., McNinch, J.E., Pinckney, J.L., Pearl, H.W., 2002. Estimating the spatial extent of bottom-water hypoxia and habitat degradation in a shallow estuary. *Mar. Ecol. Prog. Ser.* 230, 103–112.
- Choi, J., Roche, H., Caquet, T., 2000. Effects of physical (hypoxia, hyperoxia) and chemical (potassium dichromate, fenitrothion) stress on antioxidant enzyme activities in *Chironomus riparius* Mg (Diptera, Chironomidae) larvae: potential biomarkers. *Environ. Toxicol. Chem.* 19, 495–500.
- Coiro, L.L., Pucher, S.L., Miller, D.C., 2000. Hypoxic effects on growth of *Palaemonetes vulgaris* larvae and other species: using constant exposure data to estimate cyclic exposure response. *J. Exp. Mar. Biol. Ecol.* 247, 243–255.
- Czyzyk-Krzaska, M.F., 1997. Molecular aspects of oxygen sensing in physiological adaptation to hypoxia. *Respir. Physiol.* 110, 99–111.
- DeFur, P.L., Mangum, C.P., Reese, J.E., 1990. Respiratory responses of the blue crab *Callinectes sapidus* to long-term hypoxia. *Biol. Bull.* 178, 46–54.
- Diaz, R.J., Rosenberg, R., 1995. Marine benthic hypoxia: a review of its ecological effects and the behavioural responses of benthic macrofauna. *Oceanogr. Mar. Biol. Annu. Rev.* 33, 245–303.
- Gracey, A.Y., Troll, J.V., Somero, G.N., 2001. Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *Proc. Natl. Acad. Sci. U. S. A.* 98, 1993–1998.
- Hale, R.E., St. Mary, C.M., Lindstrom, K., 2003. Parental responses to changes in costs and benefits along an environmental gradient. *Environ. Biol. Fishes* 67, 107–116.
- Hochachka, P.W., Lutz, P.L., 2001. Mechanism, origin and evolution of anoxia tolerance in animals. *Comp. Biochem. Physiol., B* 130, 435–459.
- Hochachka, P.W., Buck, L.T., Doll, C.J., Land, S.C., 1996. Unifying theory of hypoxia tolerance; molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. U. S. A.* 93, 9493–9498.
- Karlin, S., Altschul, S.F., 1990. Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. *Proc. Natl. Acad. Sci. U. S. A.* 87, 2264–2268.
- Kothapalli, R., Yoder, S.J., Mane, S., Loughran, T.P., 2002. Microarray results: how accurate are they? *BMC Bioinformatics* 3, 22.
- Kultz, D., 2003. Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. *J. Exp. Biol.* 206, 3119–3124.
- Landry, C.A., Manning, S., Cheek, A.O., 2003. Hypoxia affects reproduction in Gulf killifish (*Fundulus grandis*). *Integr. Comp. Biol.* 43, 812.
- Larkin, P., Folmar, L.C., Hemmer, M.J., Poston, A.J., Denslow, N.D., 2003. Expression profiling of estrogenic compounds using a sheepshead minnow cDNA macroarray. *Environ. Health Perspect. Toxicogenomics* 111, 839–846.
- Luo, T., Zhang, X., Shao, Z., Xu, X., 2003. *PmAV*, a novel gene involved in virus resistance of shrimp *Penaeus monodon*. *FEBS Lett.* 551, 53–57.
- Mangum, C.P., 1997. Adaptation of the oxygen transport system to hypoxia in the blue crab, *Callinectes sapidus*. *Am. Zool.* 37, 604–611.
- Manning, C.S., Schesny, A.L., Hawkins, W.E., Barnes, D.H., Barnes, D.S., Walker, W.W., 1999. Exposure methodologies and systems

- for long term chemical carcinogenicity studies with small fish species. *Toxicol. Methods* 9 (3), 201–217.
- McMahon, B.R., 2001. Respiratory and circulatory compensation to hypoxia in crustaceans. *Respir. Physiol.* 128, 349–364.
- Meehan, O.L., 1936. Notes on the freshwater shrimp *Palaemonetes paludosa* (Gibbes). *Trans. Am. Microsc. Soc.* 55, 433–441.
- Mistri, M., 2004. Effects of hypoxia on predator-prey interactions between juvenile *Carcinus aestuarii* and *Musculista senhousia*. *Mar. Ecol. Prog. Ser.* 275, 211–217.
- Murphy, B.R., Willis, D.W. (Eds.), 1996. *Fisheries Techniques*, 2nd ed. American Fisheries Society, Bethesda, Maryland.
- Napier, I., Ponka, P., Richardson, D.R., 2005. Iron trafficking in the mitochondrion: novel pathways revealed by disease. *Blood* 105, 1867–1874.
- Paul, R.J., Zeis, B., Lamkemeyer, T., Seidl, M., Pirow, R., 2004. Control of oxygen transport in the microcrustacean *Daphnia*: regulation of haemoglobin expression as central mechanism of adaptation to different oxygen and temperature conditions. *Acta Physiol. Scand.* 182, 259–275.
- Pihl, L., Baden, S.P., Diaz, R.J., 1991. Effects of periodic hypoxia on distribution of demersal fish and crustaceans. *Mar. Biol.* 108, 349–360.
- Rose, K.A., Murphy, C.A., Diamond, S.L., Fuiman, L.A., Thomas, P., 2003. Using nested models and laboratory data for predicting population effects of contaminants on fish: a step toward a bottom-up approach for establishing causality in field studies. *Hum. Ecol. Risk Assess.* 9, 231–257.
- Russel, W.J., Ho, Y.S., Parish, G., Jackson, R.M., 1995. Effects of hypoxia on MnSOD expression in mouse lungs. *Am. J. Physiol., Lung Cell. Mol. Physiol.* 13, L221–L225.
- Sokal, R.R., Rohlf, F.J., 1995. *Biometry*, 3rd ed. W.H. Freeman, New York.
- Tankersley, R.A., Wieber, M.G., 2000. Physiological responses of postlarval and juvenile blue crabs *Callinectes sapidus* to hypoxia and anoxia. *Mar. Ecol. Prog. Ser.* 194, 179–191.
- Taketani, S., 2005. Acquisition, mobilization and utilization of cellular iron and heme: endless findings and growing evidence of tight regulation. *Tohoku J. Exp. Med.* 205, 297–318.
- Terwilliger, N.B., 1998. Functional adaptations of oxygen-transport proteins. *J. Exp. Biol.* 201, 1085–1098.
- Thomas, P., Rahman, S.Md., Kummer, J.A., Lawson, S., 2006. Reproductive endocrine dysfunction in Atlantic croaker exposed to hypoxia. *Mar. Environ. Res.* 62, S249–S252.
- Ton, C., Stamatou, D., Dzau, V.J., Liew, C.-C., 2002. Construction of a zebrafish cDNA microarray: gene expression profiling of the zebrafish during development. *Biochem. Biophys. Res. Commun.* 296, 1134–1142.
- Ton, C., Stamatou, D., Liew, C.-C., 2003. Gene expression profile of zebrafish exposed to hypoxia during development. *Physiol. Genomics* 13, 97–106.
- van der Meer, D.L.M., van den Thillart, G.E.E.J.M., Witte, F., de Bakker, M.A.G., Besser, J., Richardson, M.K., Spaink, H.P., Leito, J.T.D., Batowski, C.P., 2005. Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. *Am. J. Physiol., Regul. Integr. Comp. Physiol.* 289, 1512–1519.
- Vijayarathy, C., Damle, S., Prabul, S.K., Otto, C.M., Avadhani, N.G., 2003. Adaptive changes in the expression of nuclear and mitochondrial encoded subunits of cytochrome *c* oxidase and the catalytic activity during hypoxia. *Eur. J. Biochem.* 270, 871–879.
- Wannamaker, C.M., Rice, J.A., 2000. Effects of hypoxia on movements and behavior of selected estuarine organisms from the southeastern United States. *J. Exp. Mar. Biol. Ecol.* 249, 145–163.
- Weber, A., Vesela, S., Repka, S., 2003. The supposed lack of trade-off among *Daphnia galeata* life history traits is explained by increased adult mortality in *Chaoborus* conditioned treatments: Recent Developments in Fundamental and Applied Plankton Research (Guest Editors: R. Van Donk, M. Boersman and P. Spaak). *Hydrobiologia* 491, 273–287.
- Welsh, B.L., 1975. The role of grass shrimp, *Palaemonetes pugio*, in a tidal marsh ecosystem. *Ecology* 56, 513–530.
- Williams, R.S., 1986. Mitochondrial gene expression in mammalian striated muscle. Evidence that variation in gene dosage is the major regulatory event. *J. Biol. Chem.* 261, 12390–12394.
- Wong, M.L., Medrano, J.F., 2005. Real-time PCR for mRNA quantitation. *BioTechniques* 39, 1–11.
- Wu, R.S.S., 2002. Hypoxia: from molecular responses to ecosystem responses. *Mar. Pollut. Bull.* 45, 35–45.
- Wu, R.S.S., Lam, P.K.S., Wan, K.L., 2002. Tolerance to, and avoidance of, hypoxia by the penaeid shrimp (*Metapenaeus ensis*). *Environ. Pollut.* 118, 351–355.
- Wu, R.S.S., Zhou, D.S., Randall, D.L., Woo, N.Y.S., Lam, P.K.S., 2003. Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. *Environ. Sci. Technol.* 37, 1137–1141.
- Zarate, J., Bradley, T.M., 2003. Heat shock proteins are not sensitive indicators of hatchery stress in salmon. *Aquaculture* 223, 175–187.