Metabolomics (2010) 6:250–262
DOI 10.1007/s11306-009-0194-y

ORIGINAL ARTICLE

Metabolomic analysis of Atlantic blue crab, *Callinectes sapidus*, hemolymph following oxidative stress

Tracey B. Schock · David A. Stancyk · Lindy Thibodeaux · Karen G. Burnett · Louis E. Burnett · Arezue F. B. Boroujerdi · Daniel W. Bearden

Received: 4 June 2009 / Accepted: 14 December 2009 / Published online: 20 January 2010

Abstract The Atlantic blue crab, *Callinectes sapidus*, is an economically, ecologically, and recreationally valuable decapod crustacean that inhabits estuaries along the Atlantic and Gulf coasts of the United States. In their natural environment, blue crabs are exposed to many stressors including anthropogenic contaminants, viruses and bacteria. Bacterial infection results in the depression of oxygen uptake, and impairs normal metabolic function in a manner that has not yet been fully elucidated. Our laboratory is developing NMR-based metabolomic tools for environmental research to discover metabolomic biomarkers of stress in marine organisms. We have used NMR spectroscopy to compare the response of the crab metabolome to depression of aerobic metabolism by injection of the bacterium *Vibrio campbellii*, versus elevation of aerobic metabolism by treatment with 2,4-dinitrophenol (DNP), a known uncoupler of oxidative phosphorylation. The corresponding NMR spectral variations between treatments were evaluated using chemometric tools for pattern recognition and biomarker identification, including principal components analysis and partial least-squares analysis. Metabolic changes were identified in crab hemolymph 30 min after injection with *V. campbellii* and DNP. Glucose, considered a reliable indicator for biological stress in crustaceans, and lactate, a metabolite indicating anaerobic respiration, provided the largest variations in the metabolomes, respectively. While biological variability and/or tight regulation of the hemolymph masked subtle metabolic changes at individual time-points, metabolic trajectory analysis revealed clear differences between the two modes of oxidative stress, providing insight into the biochemical pathways involved.

Keywords Metabolomics · Metabonomics · NMR · Environmental · Blue crab · Oxidative stress · *Callinectes sapidus* · Hemolymph

1 Introduction

Metabolomics is a systematic study of the endogenous, small-molecule metabolites involved in specific biological processes, providing an assessment of the physiological status of an organism. Metabolomic analysis is currently applied in medicine, toxicology and environmental sciences. The development of metabolomics as an environmental research tool holds great promise for contributions to environmental risk analysis (Ekman et al. 2008), discovery of new biological insights (Bundy et al. 2002), and for developing environmental system models (Viant 2008; Bundy et al. 2008). Here we have applied NMR-based metabolomics to assess the response of the Atlantic blue crab, *Callinectes sapidus*, to different oxidative stresses.

The Atlantic blue crab is economically, ecologically, and recreationally valuable along the Atlantic and Gulf coasts of the United States. The blue crab population is currently declining due to heavy fishing, pollution and

Electronic supplementary material The online version of this article (doi:10.1007/s11306-009-0194-y) contains supplementary material, which is available to authorized users.

T. B. Schock · A. F. B. Boroujerdi · D. W. Bearden (✉)
Hollings Marine Laboratory, Analytical Chemistry Division, National Institute of Standards and Technology, 331 Ft. Johnson Rd, Charleston, SC 29412, USA
e-mail: dan.bearden@nist.gov

D. A. Stancyk · L. Thibodeaux · K. G. Burnett · L. E. Burnett
Grice Marine Laboratory, College of Charleston, Charleston, SC 29412, USA

slowly warming waters (Zohar et al. 2008). Methods for evaluating the health of these crustaceans can provide important data for natural resource managers who are charged with maintaining robust natural populations of blue crabs. Such assessments of crustacean health also reflect coastal ecosystem health and may serve as early warning indicators of ecosystem failure.

In the wild, crabs are constantly exposed to anthropogenic stressors (toxicants from pollution) and natural stressors (variations in salinity, temperature, and oxygen and viral and bacterial infections). Bacteria are abundant in the ocean reaching $\sim 10^9$ culturable bacteria l^{-1} seawater (Fuhrman 1999) and are found in 75% of "unstressed" healthy crabs that are considered marketable (Welsh and Sizemore 1985). Many of the bacteria that infect crabs are of the *Vibrio* species. Burnett et al. (2006) determined that crabs injected with *Vibrio campbellii* displayed a 43% *decline* in the overall flux of oxygen into the crab within 30 min to 1.5 h postinjection. This decrease in oxygen uptake was associated with a significant reduction in oxygenation of hemolymph at the gills. The reduced oxygen uptake may be a consequence of the crab's immune response, in which hemocytes rapidly aggregate in the presence of foreign particles. These aggregates become trapped in the fine vasculature of the gill, where they melanize (Martin et al. 2000) and consequently compromise the ability of the gill to exchange gases (Burnett et al. 2006). Long-term effects may result in reduced survival performances such as migration, feeding and predator avoidance, with an increased effect when organisms are located in hypoxic environments.

In contrast to the effects of bacterial stress, exposure of *C. sapidus* to a chemical uncoupler of oxidative phosphorylation, inhibiting the production of ATP, resulted in an *increase* of oxygen uptake (Fox and Rao 1978). Environmental contaminants such as 2,4-dinitrophenol (DNP) and pentachlorophenate (PCP) cause this toxic biochemical effect. Tjeerdema et al. (1991) used in vivo ^{31}P NMR and gas chromatography (GC) to show that injection of PCP in the foot muscle of red abalone (*Haliotis rufescens*) led to an accumulation of tricarboxylic acid (TCA) cycle intermediates (citrate, succinate, and malate) and glycolytic products (lactate, alanine, and glycerol 3-phosphate). Chemical pollutants, such as DNP and PCP, are capable of affecting the normal metabolic function of marine organisms (Martello et al. 2000).

In this study, we tested the hypothesis that changes in aerobic metabolism will exhibit the different modes of action associated with bacterial infection versus chemical uncoupling of oxidative phosphorylation in crab hemolymph. The corresponding NMR spectral variations between individual crabs were investigated using chemometric tools for pattern recognition and biomarker identification, including principal components analysis (PCA) and partial least-squares analysis (PLS).

2 Methods

2.1 Test animals and preparation

Adult male blue crabs (90–235 g) were trapped daily in tidal creeks feeding Charleston Harbor, Charleston, SC, from June through the end of July 2008. Crabs were held and kept alive at the Hollings Marine Laboratory in recirculating seawater at 30 parts per thousand (ppt) (w/v) salinity and 20–22°C with a 12-h light cycle for at least 3 days and no longer than 10 days prior to experimentation. Frozen squid (bait or consumption grade, depending on availability) was fed to the crabs on a daily basis, but food was withheld at least 24 h prior to the experiment.

A standard timeline was used in all experiments (Fig. 1). Twenty-four hours prior to injection, each crab was prepared for experimentation by drilling a 1.6 mm port through the carapace over the heart for test injections and two 1.6 mm ports over the pericardium from which hemolymph could be drawn. A small sheet of latex rubber was glued into place over each port using cyanoacrylate glue to form a diaphragm. The crabs were weighed and then placed into individual holding tanks with 1.5 l well-aerated 30 ppt (w/v) seawater and a thin layer of sterilized gravel where they would remain throughout the experiment. Immediately following preparation, a sample of hemolymph was extracted from a pericardial port to assess baseline hemocyte counts and to quantify the number of culturable bacteria for each crab (Macey et al. 2008). Approximately, 30% of the sampled crabs had detectable levels of culturable bacteria and were not used in the experiment, although hemolymph was collected for comparison (see below). Four hours after preparation ($t = -20$ h in our protocol), hemolymph was sampled for baseline NMR analysis and processed as described below.

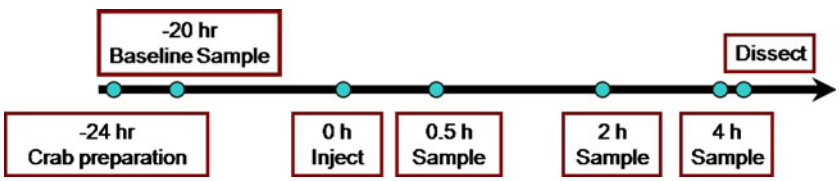

Fig. 1 Experimental design of blue crab oxidative stress NMR metabolomic study

2.2 2,4-Dinitrophenol (DNP) preparation and dosage

A 2 g l^{-1} stock solution of DNP (Sigma-Aldrich, CAS:51-28-5) was prepared in 10 mmol l^{-1} HEPES 2.5% (w/v) saline, pH 6.9. Crabs were injected with 3 µl DNP stock solution g^{-1} crab, which delivered a final dose of 6 µg DNP g^{-1} crab. This dose was previously used by Fox and Rao (1978) and is assessed for effectiveness in the oxygen uptake experiment below. Control animals received an injection of 1 µl g^{-1} crab of 10 mmol l^{-1} HEPES 2.5% (w/v) saline adjusted to pH 7.5. Injection volumes never exceeded 450 µl.

2.3 Oxygen uptake analysis

To determine the in vivo oxidative response due to DNP exposure, oxygen uptake was measured in blue crabs following injection of DNP compared with saline-injected controls. Six crabs, not otherwise involved in the metabolomic study, were prepared for injection as described previously and placed in individual containers 24 h before experimentation. At the start of the experiment, an individual crab was placed in a flow-through 1.8 l circular, plexiglass respirometry chamber (17 cm wide, 8 cm high) lined with sterile gravel, covered and left undisturbed for 2 h during which time well-oxygenated seawater (30 ppt salinity) was passed through the chamber. The chamber was then opened and the crab was injected with the appropriate dose of saline or DNP directly into the ventricle. The chamber was resealed, and oxygen uptake was measured continuously for 4 h at 25°C. The Po_2 of seawater entering and leaving the chamber was measured every 10 s using an oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) and a Sable System (Las Vegas, NV) data acquisition system. Filtered seawater entering the chamber was saturated with 30% oxygen (31 kPa) to ensure that the oxygen pressure within the chamber did not drop below 80% air saturation (16.5 kPa) when an animal was present and consuming oxygen. Flow rates typically ranged between 80 and 150 ml min^{-1}. This procedure was repeated for each of the six crabs.

2.4 *Vibrio campbellii* preparation and dosage

Vibrio campbellii was prepared for injection as per Macey et al. (2008). From a frozen working stock, *V. campbellii* was streaked onto tryptic soy agar (TSA) plates supplemented with 2.0% NaCl (w/v), 100 µg ml^{-1} kanamycin A (Sigma-Aldrich) and 5 µg ml^{-1} chloramphenicol (Sigma-Aldrich) and grown overnight at 25°C. Bacteria from the streaked plate were resuspended in 5 ml of sterile 10 mmol l^{-1} HEPES 2.5% (w/v) saline and adjusted to an optical density (OD) of 0.1 ± 0.005 at 540 nm, which equals a density of 1×10^8 colony-forming units (CFU) (Mikulski et al. 2000). Crabs were injected with 2.5×10^4 *V. campbellii* g^{-1} crab. This dose assumes a hemolymph volume of 0.25 ml g^{-1} body weight (Gleeson and Zubkoff 1977), thus achieving a circulating dose of 1×10^5 CFU of bacteria g^{-1} crab weight (Burnett et al. 2006). This bacterial dose is approximately 1/25th of the LD_{50}/48 h of *V. campbellii* for *C. sapidus* (6.25×10^5 g^{-1}), which is high enough to cause a significant depression of the oxygen consumption rate and low enough to minimize the risk of mortality from infection during this experiment (Burnett et al. 2006; Thibodeaux et al. 2009).

2.5 Intracardiac injections of treatments and experimental procedure

Crabs selected for metabolomics measurements, collected over a 3-week period, were randomly assigned one of the following treatments: (1) Control: 1 µl g^{-1} crab HEPES buffered saline, (2) DNP: approximately 6 µg g^{-1} crab DNP, or (3) *Vibrio*: 2.5×10^4 *Vibrio campbellii* g^{-1} crab with a target of $n = 8$ for each treatment (Table 1). At approximately 24 h after preparation, the designated treatments were injected directly into the ventricle of the heart to ensure rapid distribution throughout the circulatory

Table 1 Blue crab NMR metabolomic samples

	Field-infected	*Vibrio*-injected	DNP-injected	Saline-injected (control)	Total
Number of crabs	8	8	7	7	30
Number of NMR samples (four time-points)	8	32	28	28	96

Note: The table shows the final number of crabs and NMR samples analyzed for this report. The target for the experiment was to have $n \approx 8$ for each treatment and the control group and it was anticipated that field conditions could affect the number of available crabs. The infection pre-screening procedure identified 10 field-infected crabs representing 30% of the tested crabs; one of these field-infected crabs had abnormally high acetate levels and one crab was lost in work-up resulting in eight final crabs being included in the analysis. One DNP-injected crab exhibited very high lactate and was found to be an outlier in all applied tests, reducing the final number in this class to seven. Due to difficulty collecting crabs, only seven crabs were in the saline-injected control group as a result of the randomized class assignment procedure

system (Fig. 1). Hemolymph was sampled over a time course with sampling at $t = -20$ h (baseline samples, 4 h after crab preparation), $t = 30$ min, $t = 2$ h, and $t = 4$ h for each crab. At each sampling time-point, approximately 1 ml of hemolymph (roughly 2–4% of the total hemolymph volume) was withdrawn and placed in a microcentrifuge tube containing 100 µl of 0.6 mmol l^{-1} hippurate in H_2O as an internal standard. The sample was immediately centrifuged at 16,000×*g* for 15 s at 4°C to pellet hemocytes. The supernatant was transferred to a pre-conditioned (washed 2× with 0.1 M NaOH, and 8× with water) centrifuge filter tube (Millipore™ Amicon Ultra-4 30 K NMWL) and allowed to clot at room temperature for 10 min. The tube was centrifuged at 5,100×*g* for 10 min at 4°C. If a sufficient volume did not pass through the filter, the clotted hemolymph was mixed with a spatula and spun again for 5 min. The filtrate was flash frozen in liquid nitrogen, lyophilized and stored at −40°C until analysis.

2.6 NMR sample preparation

The lyophilized crab hemolymph samples were rehydrated with 1.0 ml 99.9 atom% D_2O containing 1.0 mmol l^{-1} sodium 3-trimethylsilyl-2,2,3,3-d_4-propionate (TMSP, CAS: 24493-21-8), an internal chemical shift standard. In addition to the baseline, treatment, and control samples, the final sample set also included baseline samples from crabs with field bacterial infection ($n = 8$). Six hundred microliters of each sample was analyzed by NMR. A final total of 96 (Table 1) crab hemolymph samples are analyzed for this report.

2.7 NMR spectroscopy

NMR spectra of crab hemolymph samples were measured at a temperature of 305 K using a Bruker Avance II 700 MHz spectrometer fitted with a cryogenically cooled probe. The data were acquired with 65,536 real data points across a spectral width of 14 ppm. Spectra were acquired with a 1-D pulse sequence for water suppression based on the standard NOESY (noesypr1d) pulse sequence with 16 steady-state scans, 80 transients, a mixing time of 100 ms and 3 s of relaxation delay for a total repetition time of 6.34 s. The spectra were processed by multiplying the free induction decay by an exponential line broadening function of 0.3 Hz and the data were zero-filled to 65536 complex points prior to Fourier transformation. Manual phasing, baseline correction and calibration of the standard TMSP peak at 0.000 ppm were performed. Peaks were assigned by comparison to chemical shift databases (Cui et al. 2008; Ulrich et al. 2008) including an in-house compiled database, as well as the SBASE-1-1-2 database included in AMIX (3.8.3, Bruker Biospin, Inc., Billerica, MA), a software program for statistical and spectroscopic analyses. Metabolite identification was confirmed by 2D ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC).

2.8 Data analysis

2.8.1 *Statistical approach*

The primary tools used for statistical analysis were AMIX and SIMCA-P + 12.0 (Umetrics Inc., Kinnelon, NJ). Spectra were normalized to constant total spectral area of the included regions and the bins (0.005 ppm) were mean-centered. No scaling was applied to the bins. For principal component analysis (PCA) of treatment pairs, the PC scores were evaluated for significant differences between groups using a two-tailed, unequal variance Student's *t*-test with a weighted Bonferroni multiple test correction (overall $\alpha = 0.05$) (Rosenthal and Rubin 1984; Benjamini and Hochburg 1997) with weights based on the fractional explained variance for each PC. The data was also assessed by supervised PCA (sPCA) where hybrid loadings for identification of biomarkers were calculated using a procedure where the loadings for the two principal components with largest differences between group means in score space are geometrically combined (Rousseau et al. 2008). In the case of PCA of simultaneous multiple treatments, scores results were assessed for significant differences by ANOVA followed by Tukey–Kramer test of multiple comparisons (overall $\alpha = 0.05$).

Both AMIX and SIMCA were used for supervised analysis via PLS and partial least-squares discriminant analysis (PLS-DA). In SIMCA, the number of components for the model was optimized using cross validation. The model quality was evaluated based on the residuals (R^2X, R^2Y) and the model predictive ability parameter (Q^2) determined through the default leave 1/7th out cross validation. The model was tested for overfitting using y-table permutation testing ($n = 499$) and an ANOVA based on the cross-validated predictive residuals (CV-ANOVA) ($\alpha = 0.05$) (Eriksson et al. 2008).

2.8.2 *Control samples*

Multivariate analysis was performed on the NMR spectra of the control (saline-injected) crab hemolymph samples from $t = 30$ min, 2 and 4 h ($n = 21$) by separating the spectra into bins (0.005 ppm bin size) from 0.2 to 5.3 ppm with the exclusion of water (4.7–5.2 ppm). Further exclusions include the peaks at 3.97 ppm (hippuric acid, added as an internal standard) and 3.25 ppm (trimethylamine-*N*-oxide, TMAO), as well as the glycerol region (3.44–3.68 ppm). The reported analysis used these exclusions and a final total of 953 bins.

2.8.3 Baseline samples (uninfected versus field-infected)

The baseline hemolymph samples ($t = -20$ h) from uninfected crabs ($n = 22$) were compared against the baseline samples from field-infected crabs ($n = 8$) using PCA.

2.8.4 Challenge samples

The hemolymph from crabs challenged with *Vibrio* and DNP-injection were analyzed by time-point (30 min, 2 and 4 h) against the corresponding saline-injected controls by PCA. The analysis was performed on pairwise data (treatment versus control) and on all the data at the given time-point (simultaneous treatments versus control).

Following unsupervised analysis, the data were analyzed with PLS-DA to discriminate between specific treatments (seven DNP and eight *Vibrio*-injected crabs) and controls (22 baseline and 7 saline-injected crabs). The classes were modeled with a 4 dummy-variable Y-table. The mean and standard error were calculated from the scores of each challenge at all time-points.

2.8.5 Trajectory analysis

Following the previous analyses, response trajectories were generated for male blue crabs using PLS. All crabs (seven saline, seven DNP and eight *Vibrio*-injected), including the baseline hemolymph samples ($t = -20$ h, $n = 22$), were incorporated in the analysis and the Y-table was populated with the time of hemolymph sampling. The mean and standard error were calculated from the scores of each challenge at all time-points.

3 Results and discussion

Because this is the first NMR-based metabolomic study of a marine decapod crustacean, it was necessary to develop methods for preparing viscous, protein-rich crab hemolymph samples suitable for NMR analysis. Several variations were tested: with or without pelleting the hemocytes, with or without centrifuge filtration (2 types), with or without hemolymph clotting, and with or without lyophilization. Centrifuge filtration tubes yielded the best NMR samples by removing large proteins and lipids that overlapped and masked many small metabolite NMR signals (Fig. 2). Deproteinization of serum samples by filtration has been used frequently to prepare samples for metabolomics (Viant et al. 2003), although some investigators still prefer to use pure serum and suppress protein signals by using the Carr–Purcell—Meiboom–Gill (CPMG) NMR pulse sequence (Tiziani et al. 2008). However, the CPMG sequence creates difficulties in suppressing water and leads

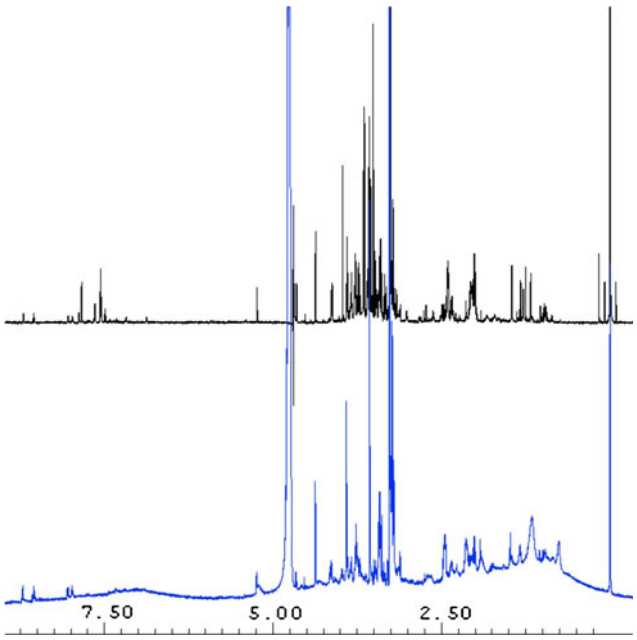

Fig. 2 NMR spectra of crab hemolymph for methods development optimization. *Top*: Cell-free hemolymph, centrifuge filtered through a 30 K NMWL filter, lyophilized and rehydrated in D_2O + 1.0 mmol l^{-1} TMSP and analyzed with a NOESYPR1D NMR sequence. *Bottom*: cell-free hemolymph, unfiltered, diluted with D_2O + 1.0 mmol l^{-1} TMSP and analyzed with a Carr–Purcell–Meiboom–Gill (CPMG) NMR pulse sequence

to distorted baselines due to spin–spin coupling resulting in unsuitable spectra. The filtration technique yielded quality NMR data for analysis.

3.1 Effect of 2,4-dinitrophenol on oxygen uptake

Measurement of oxygen consumption not only indicates overall metabolic rate, but also provides an indication of stress conditions. The in vivo oxidative stress response due to DNP exposure in *C. sapidus* was demonstrated before starting the trials. Oxygen uptake in all crabs was not statistically different between individuals prior to injection (Student's *t*-test; $\alpha = 0.05$, $p = 0.512$). Injection of DNP caused a significant and sustained elevation in oxygen uptake over 4 h compared to saline-injected crabs (Fig. 3; two-way repeated measures ANOVA; $\alpha = 0.05, p < 0.001$). Oxygen uptake in DNP-injected crabs was elevated by 42% above saline-injected crabs after only 20 min and remained elevated for 4 h after injection when monitoring was stopped.

3.2 NMR spectral analysis of crab hemolymph metabolites

NMR spectra of *C. sapidus* hemolymph contained a large number of metabolite peaks (Fig. 4) including amino acids (e.g., alanine and glutamine), organic acids (e.g., acetate

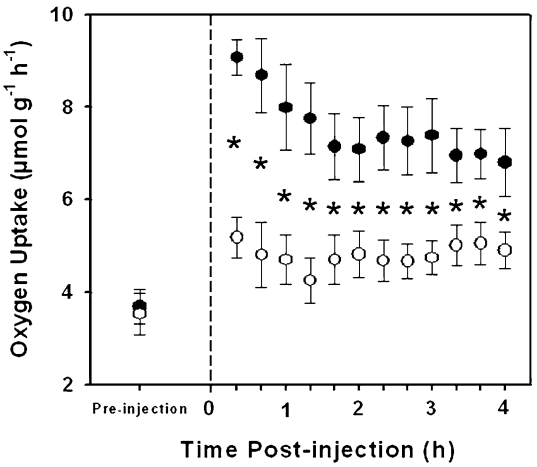

Fig. 3 Oxygen uptake in *C. sapidus* following injection of 2,4-dinitrophenol (6 µg g^{-1} crab; *closed circles*) or control (control = *open circles*). Oxygen uptake was measured at 25°C using flow-through respirometry. Values represent averages over 20 min time intervals for 6 animals ± SEM. There was a significant treatment effect on oxygen uptake following injection (two-way repeated measures ANOVA; $p < 0.001$). * A significant difference in oxygen uptake between treatments at individual time-points (Holm–Sidak post-hoc comparisons; $p < 0.005$)

and 3-hydroxybutyrate), carbohydrates (e.g., glucose), glycolytic products (e.g., lactate), and organic osmolytes (e.g., betaine and TMAO). Several peaks within the spectra shift from hemolymph sample to hemolymph sample which is presumably caused by small changes in pH or ionic strength and this required us to expand the exclusion range for our analysis. Shifting peaks may have been avoided by rehydration of the lyophilized hemolymph with a phosphate buffer to maintain a common pH, but even then, pH related shifts are known to occur. Our starting samples were highly saline, as expected for hemolymph from a marine organism, resulting in large proton pulse widths. We decided not to rehydrate in buffer since it would further increase the salt concentration, degrading instrument performance.

3.3 Multivariate analysis of oxidative stress on blue crab hemolymph

3.3.1 Control samples

Control samples (saline-injected) were initially analyzed to develop a compact control group. There was great individual metabolite variation from crab to crab. Statistical analysis of the multivariate loadings of control samples at $t = 30$ min, 2 and 4 h ($n = 21$) provided evidence that several metabolites varied in concentration as the experiment progressed. As a result, we chose to exclude the olefinic and aromatic region (5.3–10 ppm) and peaks at 3.97 ppm (hippuric acid, added as an internal standard) and

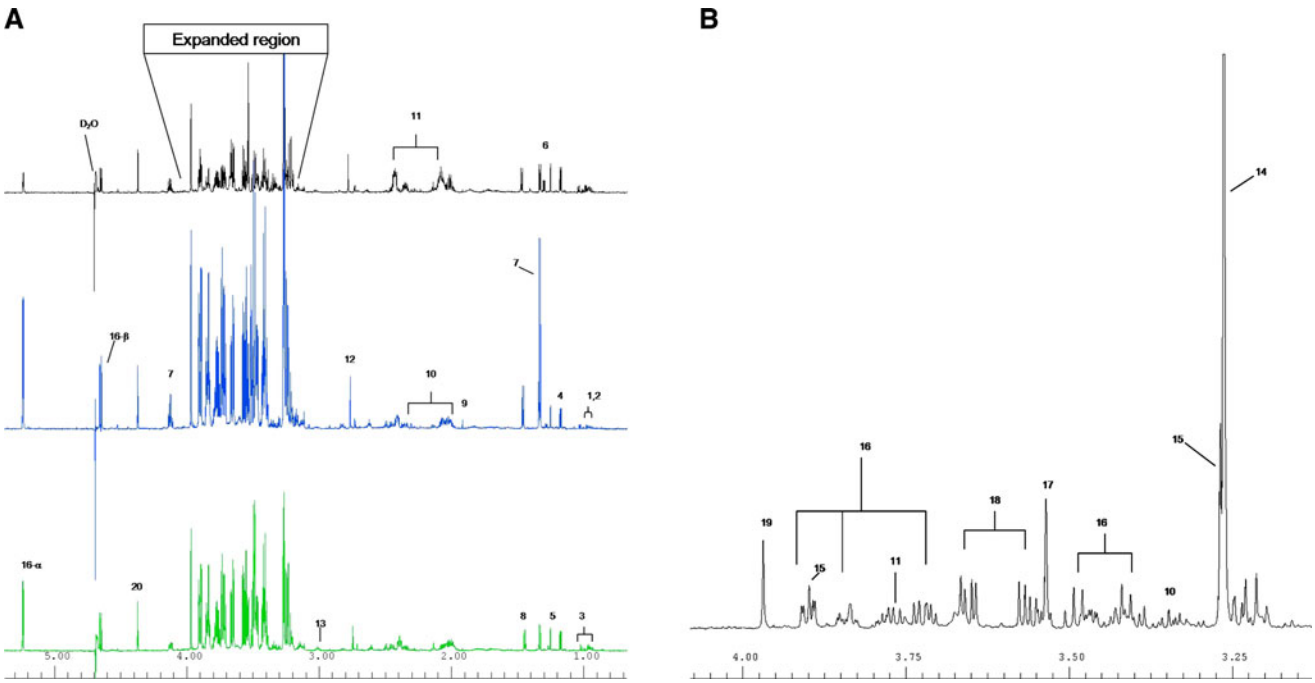

Fig. 4 Representative one-dimensional ^{1}H NMR spectra of hemolymph from individual Atlantic blue crabs sampled at 4 h. **a** *Top*: control hemolymph. *Middle*: DNP-treated hemolymph. *Bottom*: *Vibrio campbellii*-treated hemolymph. **b** Expanded region of the control treated hemolymph spectrum. Key to spectrum: 1. Isoleucine, 2. leucine, 3. valine, 4. 3-hydroxybutyrate, 5. unidentified metabolite, 6. threonine, 7. lactate, 8. alanine, 9. acetate, 10. proline, 11. glutamine, 12. hypotaurine, 13. lysine, 14. TMAO, 15. betaine, 16. glucose, 17. glycine, 18. glycerol, 19. hippurate (internal standard), 20. homarine

3.25 ppm (trimethylamine-*N*-oxide, TMAO), as well as the glycerol region (3.44–3.68 ppm) which contained a shifting glycine peak.

3.3.2 *Baseline samples (uninfected versus field-infected)*

Approximately 30% of the crabs collected for this study were identified as field-infected, already possessing a culturable bacteria population in the hemolymph. A comparison of the baseline samples ($t = -20$ h) from field-infected ($n = 8$) and uninfected crabs ($n = 22$) revealed no statistically significant separation in any of the scores (Fig. 5a) based on the weighted Bonferroni multiple test correction. Using a supervised principal component analysis (sPCA) technique (Rousseau et al. 2008), we calculated the weighted hybrid loading plot which gives the best separation between infected and uninfected groups based on the loadings for PC1 and PC17 which show the two largest differences in group means (Fig. 5b). The group separation was driven by an increase in lactate in the bacteremic crabs, according to the hybrid loadings plot (Fig. 5c). This result suggests that the infected crabs are utilizing anaerobic mechanisms of metabolism for

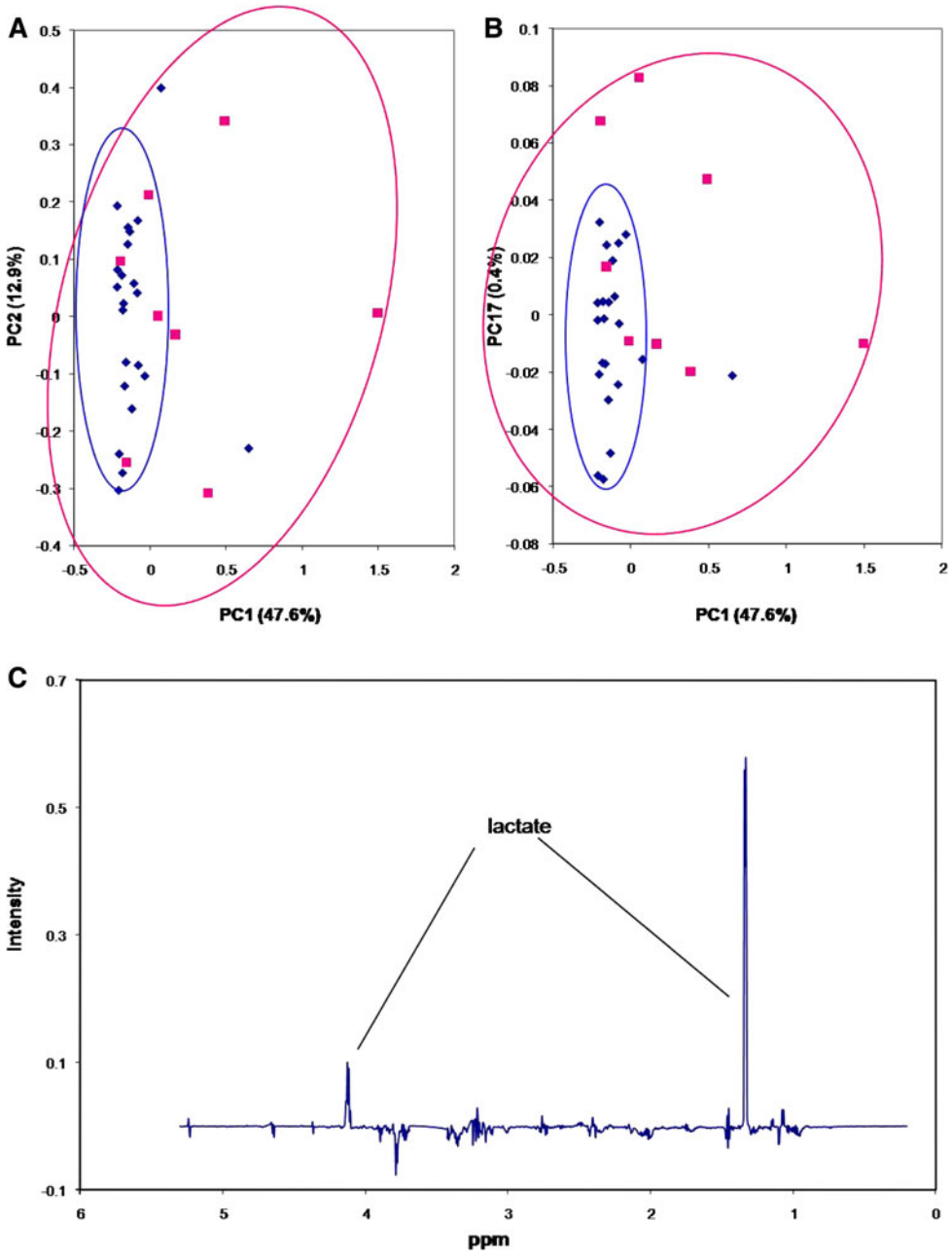

Fig. 5 **a** PC1 versus PC2 scores plot and **b** PC1 versus PC17 scores plot from the PCA of baseline hemolymph samples from healthy (◆) and field (■) infected crabs. The ellipses represent the 80% confidence interval of each group. **c** Loadings plot of the hybrid loading determined from PC1 and PC17 highlighting lactate peaks that contribute to the largest difference in means

energy production. Scholnick et al. (2006) reported that *Vibrio*-injected shrimp (*Litopenaeus vannamei*) experience a large depression in aerobic metabolism and a small, but significant increase in anaerobic metabolism as judged by the accumulation of lactate in the tissues of injected shrimp. Unfortunately, employing lactate as a biomarker of bacteremia alone may not be practical given that the situation may be similar to oxygen depletion in organisms in hypoxic environments or exercising crabs with hypoxic tissues. However, lactate may be an excellent common marker of metabolic depression in environmentally stressed crustaceans.

3.3.3 Challenge samples

The central objective of this study was to assess the time-dependent metabolic response to different mechanisms of oxidative stress. The DNP and *Vibrio*-injected treatment groups were analyzed against the corresponding control group (saline-injected) at each time-point. In this pairwise comparison, PCA yielded some significant differences for some of the scores when tested using $\alpha = 0.05$ for each PC score. To more thoroughly account for the effects of multiple testing, the alpha for a given PC was adjusted proportionally to the explained variance for each PC. With this modification, no statistically significant differences were found; nevertheless, the data was further analyzed using a principal component analysis (sPCA) technique where weighted hybrid loadings giving the best separation between groups are calculated (Rousseau et al. 2008). Scores plots from PCAs of pairwise data (treatment versus control or treatment versus treatment) at 30 min post-injection are shown in Fig. 6 along with the calculated hybrid loading. Analysis of the loadings showed that the difference between control and *Vibrio*-injected crabs can be attributed primarily to an increase in glucose production (Fig. 6d). Environmental stress and infection have been shown to cause elevation of glucose levels (Eddy et al. 2007; Hall and van Ham 1998). Lorenzon et al. (1997) reported an increase in hemolymph glucose in several crustacean taxa following injection of lipopolysaccharide (LPS). LPS triggers the release of crustacean hyperglycemic hormone leading to disturbances in the regulation of carbohydrate metabolism (Lorenzon et al. 1997).

In the case of DNP-injected crabs, lactate is the major contributor to the difference between the treatment and control groups (Fig. 6e). The change in lactate concentration is most likely caused by a switch to anaerobic metabolism. The differences between DNP and *Vibrio* treatments (Fig. 6f), were primarily due to changes in lactate and glucose. In all comparisons, changes were observed in proline and alanine (Fig. 6d, e and f). These amino acids are considered glucogenic, where their break-down generates energy and glucose through gluconeogenesis in starved or energy depleted organisms (Brosnan 2003).

Hemolymph sampled at 2 and 4 h from *Vibrio* and DNP-injected crabs clustered in the same multivariate space as the controls in this time-point by time-point analysis. There were no significant differences in any of the PCs. We hypothesize that the similarity of the metabolite profiles at 2 and 4 h for *Vibrio*-injected hemolymph and controls can be attributed to the fact that crustaceans injected with bacteria or foreign materials rapidly clear more than 90% of the exogenous particles from their hemolymph within the first 10 min following injection (Holman et al. 2004; Macey et al. 2008). A majority of intact bacteria have been found to accumulate in the lymphoid organ in the Pacific white shrimp, *Litopenaeus vannamei* (Burgents et al. 2005); however, crabs do not contain lymphoid organs. It is believed that the bacteria are engulfed and degraded in the hepatopancreas and/or phagocytized in the gill by hemocytes (Martin et al. 2000; Burnett et al. 2006; Macey et al. 2008); thus a lack of significant differences in the hemolymph in the scores is not profound in the 4 h experiment reported here.

Injection of DNP triggers elevated oxygen uptake rates over 4 h compared to saline-injected control crabs ($\mu mol\ g^{-1}\ h^{-1}$) (Fig. 3). This observed effect is consistent with the mechanism of action for DNP, which binds to mitochondrial membrane proteins, uncoupling oxidative pathways from ATP production (Edsall 1934). Fox and Rao (1978) demonstrated that the resulting decrease in ATP pools has multiple downstream effects on hepatopancreas enzymes of *C. sapidus* including inhibition of carbohydrate metabolism, energy production, lipid metabolism, ion transport, and protein metabolism. These impaired mechanisms should be reflected in metabolomic differences in the hemolymph of DNP-injected crabs compared to control crabs. The lack of significant changes in the metabolome of these two groups suggests that there may be rapid circulation of hemolymph, or that the primary effects are in other tissues, or that our sample size is not large enough to observe subtle changes in the metabolic profiles.

The data were also analyzed with a three-class PCA model to visualize time-dependent differences between DNP-injected, *Vibrio*-injected and control crabs ($n = 22$). The overall differences between the metabolic profiles at 30 min post-injection were confirmed to be significant in PC1 ($p = 0.03$) via ANOVA of PC scores (Fig. 7). A Tukey–Kramer post hoc comparison test revealed a significant difference between control crabs and DNP-injected crabs. Lactate contributes as the metabolite causing the greatest variability between these samples, suggesting a metabolic switch to anaerobic respiration in the DNP-treated crabs. Differences were not observed between treatments and control samples at the later time-points.

The data were also assessed using a supervised PLS-DA with four classes (baseline, saline, DNP, and *Vibrio*-injected crabs) for all time-points (Fig. 8). Baseline hemolymph samples were included in this analysis, and the large number of baseline samples ($n = 22$) results in a compact quiescent crab metabolic fingerprint. The individual variability of the baseline samples (Fig. 8a) could be an indication that the 4 h (from $t = -24$ h until $t = -20$ h) equilibration time may not have been sufficient for the crabs to become quiescent after the initial carapace preparation. Sampling baseline hemolymph immediately before the challenge injection ($t = 0$), 24 h after preparation, could have resulted in a more representative baseline data set. However, an alternative explanation is that this variation may be primarily due to intrinsic biological variability in the wild caught crab population. It has been suggested that laboratory stabilization increases metabolic variability, masking the metabolic changes associated with applied stresses (Hines et al. 2007) and sampling directly from the environment provides more informative metabolic profiles due to natural stresses (i.e., hypoxia, pollution).

The PLS-DA scores plot showed clear distinction between all groups, especially when the groups were averaged (Fig. 8b). The predictive quality of the optimized PLS-DA model was low ($Q^2 = 0.15$, with three components; $R^2X = 0.53$, $R^2Y = 0.28$). Model validation with both y-table permutation ($n = 499$) and CV-ANOVA shows that some of the treatments are modeled better than others (Table 2 and Supplemental material). Correlation between the permutation assessment criteria and the

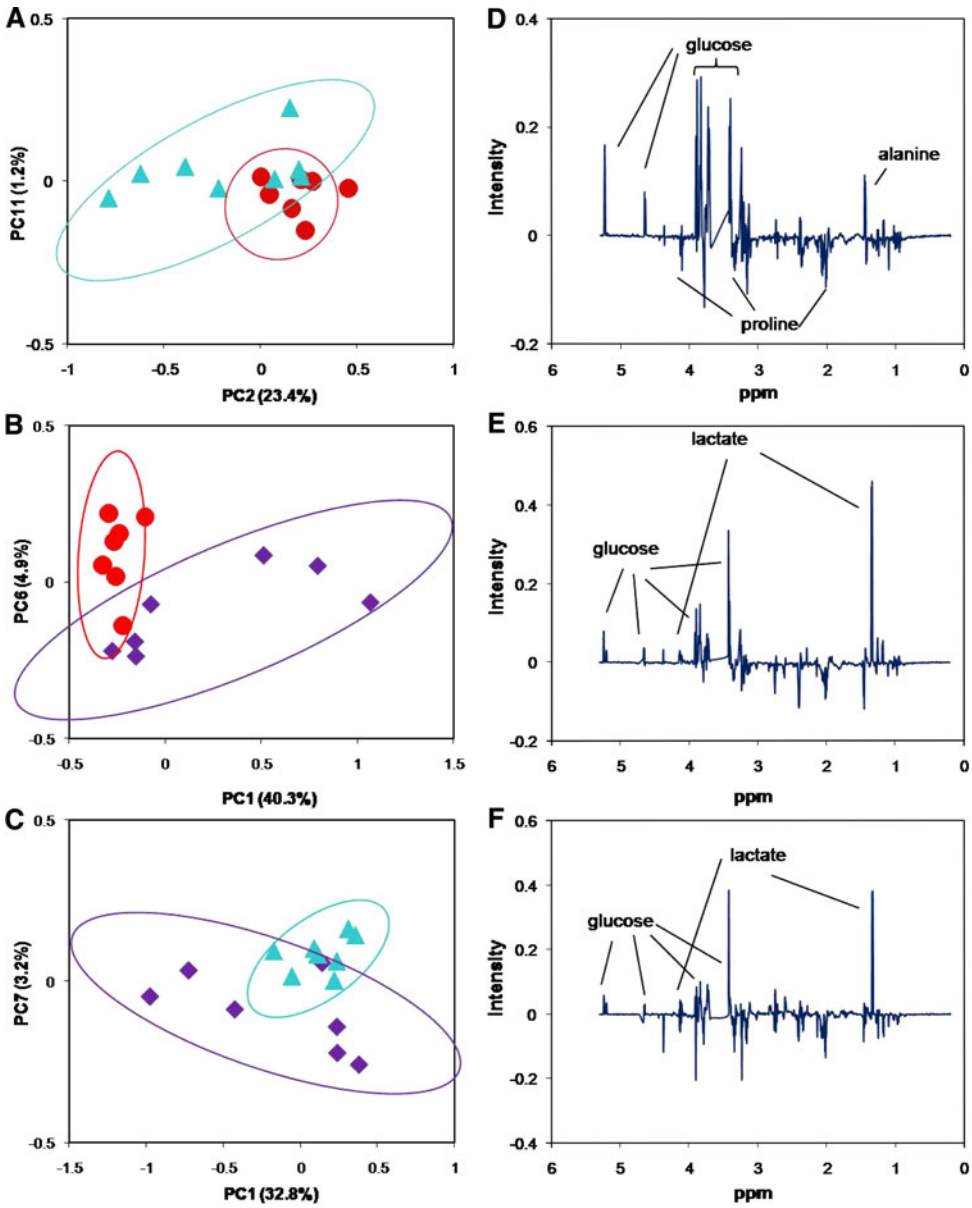

Fig. 6 Scores plots from the PCAs of hemolymph sampled at 30 min postinjection of **a** control (●) versus *Vibrio campbellii* (▲) samples; **b** control versus DNP (◆) samples; and **c** *V. campbellii* versus DNP samples. PCs with the largest difference in means were determined by a supervised principal components analysis (sPCA). The ellipses represent the 80% confidence interval of each group. **d–f** The hybrid loading for each sPCA shows the candidate biomarkers contributing to the means' difference

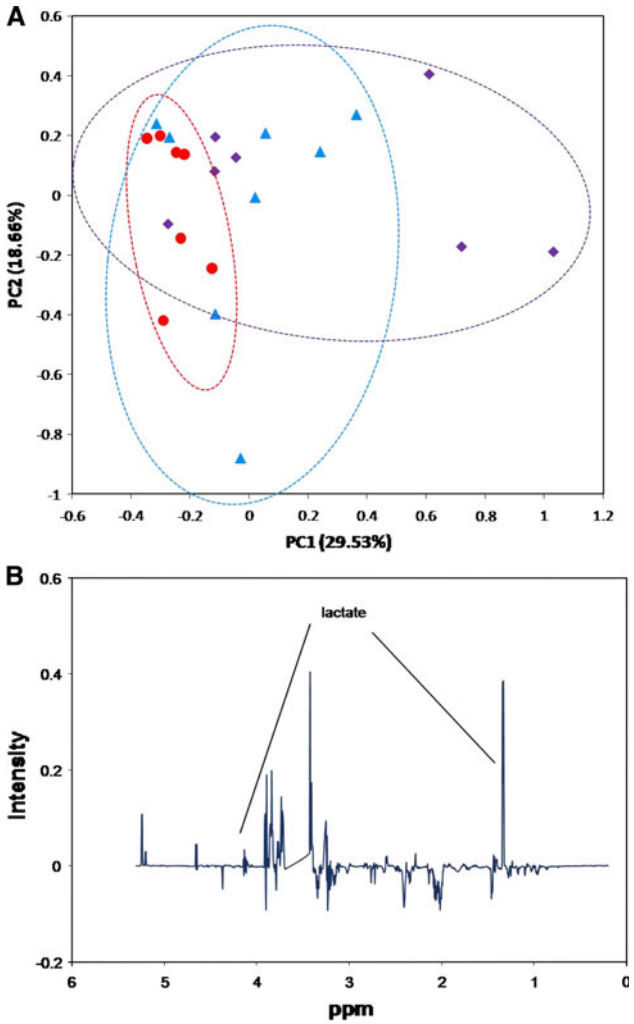

Fig. 7 **a** Scores plot from the PCA of control (●), *Vibrio campbellii* (▲) and DNP (◆) samples showing significant separation in PC1 (ANOVA, $\alpha = 0.05$, $p = 0.03$) at 30 min postinjection. Specifically, control and DNP-injected crabs separate significantly (Tukey–Kramer) in PC1. The ellipses represent the 80% confidence interval of each group. **b** PC1 loadings

p-value criteria from CV-ANOVA is excellent (Table 2), showing the utility of the CV-ANOVA cross-validation assessment technique. The results for the control samples indicate that changes that occur in the saline-injected crabs are not well modeled. Often in metabolomic studies, the control group shows the most inter-individual variation and the metabolic responses from treatments appear more uniform (Parsons et al. 2007; Parsons et al. 2009). Analysis of the loadings showed metabolites responsible for the treatment separations did not change from PCA to PLS-DA, i.e., lactate correlates with DNP-injected separations and glucose correlates with *Vibrio*-injected separations. Time-dependent changes are apparent in the control and *Vibrio*-injected samples.

3.3.4 Trajectory analysis

The time-dependence of the metabolic profiles was assessed using PLS with the Y-table populated with elapsed time after injection (Fig. 9). The optimized model was a two component model with acceptable predictive capability ($Q^2 = 0.30$) and no significant overfitting, with permutation testing giving a Y-intercept $= -0.18$ and a CV-ANOVA having a *p*-value <0.001 (Table 2). Based on the cross-validation significance assessment, the PLS model based on time was more predictive than the PLS-DA model based on treatment, reported above.

Paths have been sketched to illustrate the dynamics of the control and the treatment groups over the 4 h experiment duration. These paths represent metabolic profile trajectories as the experiment progresses. The direction and magnitude of the movement of the profile can provide information on the mode of action of a chemical or disease (Keun et al. 2004). The metabolic response seen in the control hemolymph is an indication of the sensitivity of NMR-based metabolomics. The presumably benign control treatment showed a physiological response to the minimal physical handling during injection of the treatments, an indication of the absolute necessity of controls in biological studies. The trajectories for control and *Vibrio*-injected crabs have a similar shape and direction and seem to reach equilibrium between 2 h and 4 h on a latent variable map (Fig. 9). The parallel paths suggest that the bacterial injections and control injections elicited similar effects on the crabs' system. However, the magnitude differences in the trajectories indicate a difference in response to the injections. The variation from the bacterial injection in PC1 can be attributed primarily to a change in glucose. A visible difference was also observed at the 30 min time-point of the DNP-injected hemolymph trajectory when compared to the controls. The path of this trajectory, however, has a different geometry than the control and *Vibrio*-treated hemolymph trajectories, providing evidence of different mechanisms of action. Additionally, the movement in the downward direction of the DNP-treated hemolymph trajectory at 2 h and 4 h indicates a continuing physiological response, whereas the trajectories for the control and *Vibrio* challenge seem to stabilize around the 2 h and 4 h time-points. This analysis suggests that the crab hemolymph metabolic responses to *Vibrio* and DNP treatment conform to the known mechanisms of action and physiological responses of the organism (decreased and increased aerobic metabolism, respectively).

While subtle differences in the time-point by time-point analysis were observed, differences between control and treated hemolymph metabolic profiles became more apparent with PLS-DA and trajectory analysis. With these supervised analyses, a larger number of spectra ($n = 88$)

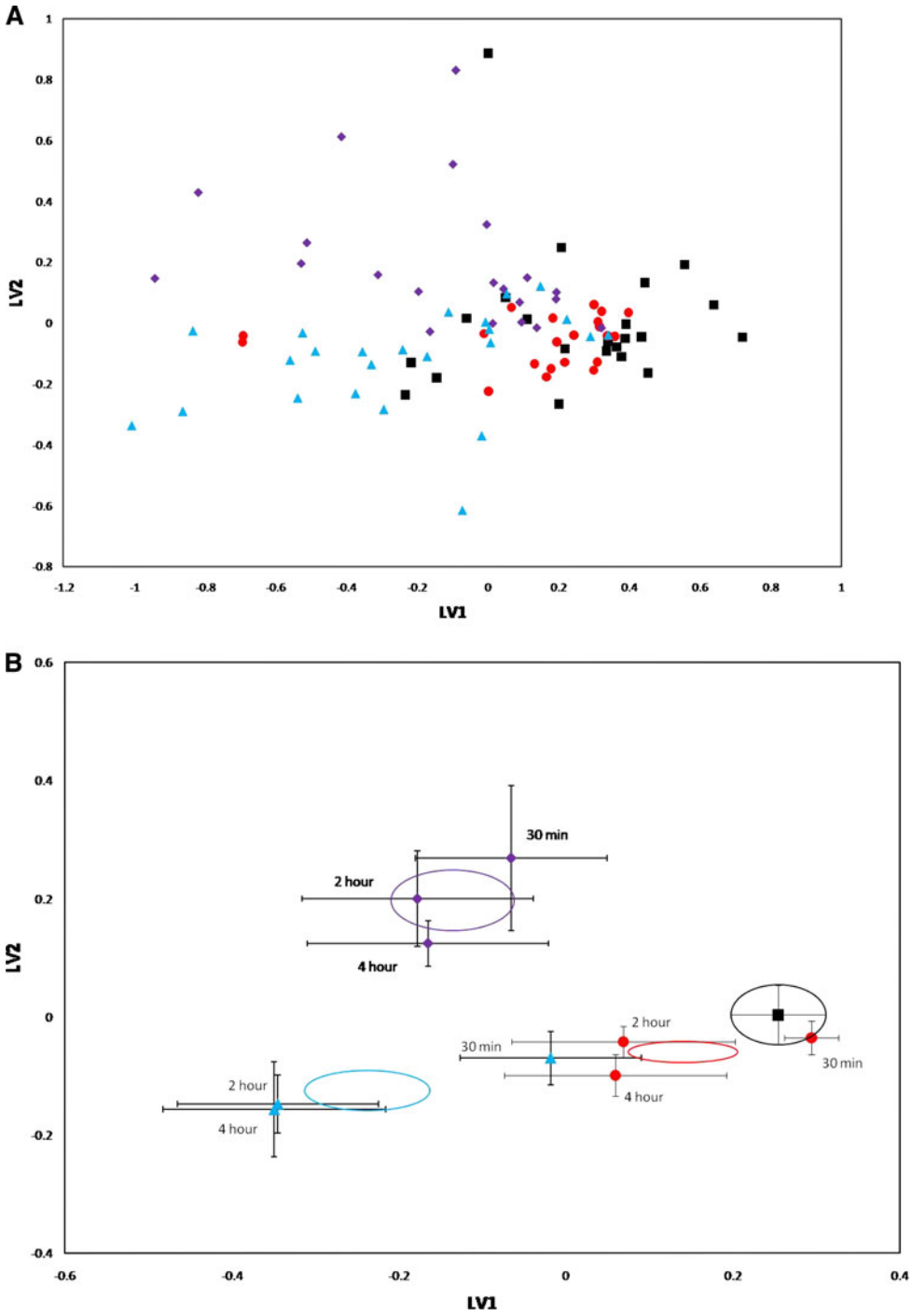

Fig. 8 PLS-DA analysis of blue crab hemolymph NMR data obtained after challenge with control (●) ($n = 7$), DNP (◆) ($n = 7$), and *Vibrio campbellii* (▲) ($n = 8$). ■ = baseline hemolymph samples ($n = 22$). **a** Scores plot. **b** Mean of scores per time-point per treatment. Error bars represent ±1 SE for each time-point average. Ovals are centered at the mean for each treatment group and are scaled to denote the standard error of each treatment

were compared which included the baseline hemolymph samples ($n = 22$). PCA time-point by time-point analysis was performed on a much smaller sample size ($n = 14$ and 15, 2-class PCA; $n = 22$, 3-class PCA) and thus was statistically weaker. The PLS-based trajectory analysis fully demonstrates the dynamic differences between treatments and controls in time (which are dominated in PC1 by glucose and PC2 by lactate) especially at the 30 min time-point where crab metabolism has been acutely affected.

4 Conclusions

Blue crab hemolymph metabolomic analysis provided evidence that physiological changes occurred in response to oxidative stresses, corresponding to what we observe in the whole organism. The differences between treatments and control were slight, however. The lack of dramatically significant changes in the hemolymph metabolome observable in single time-point analysis contrasts with the results

Table 2 PLS-DA and PLS cross validation significance results

Model	Y-component	Results from Y-table permutation			Results from CV-ANOVA
		Test A	Test B	Test C	*p* Value
Y-table with 4 pseudo-components for control samples and treatments	Baseline	OK	−0.196	OK	1.6×10^{-3}
	Vibrio treatment	OK	−0.201	NO	7.7×10^{-3}
	DNP treatment	~OK[a]	−0.183	NO	0.031
	Control treatment	NO	−0.174	NO	0.72
Y-table with time post injection	Time	OK	−0.177	OK	7×10^{-5}

Note: The table shows the results of model validation for the PLS-DA and PLS models using the complementary techniques of y-table permutation and CV-ANOVA. For Y-table permutation ($n = 499$) the tests to assess model significance are: Test A—Is Q^2(cum) for the model larger than all the results for permuted models; Test B—Is the y-intercept of the Q^2(cum) fit negative; Test C—Is R^2Y(cum) for the model larger than all the results for permuted models (Eriksson et al. 2008). For CV-ANOVA assessment of significance, a *p*-value less than 0.05 may be taken as significant

[a] One of the 499 permutations was at or above the model value of 0.125

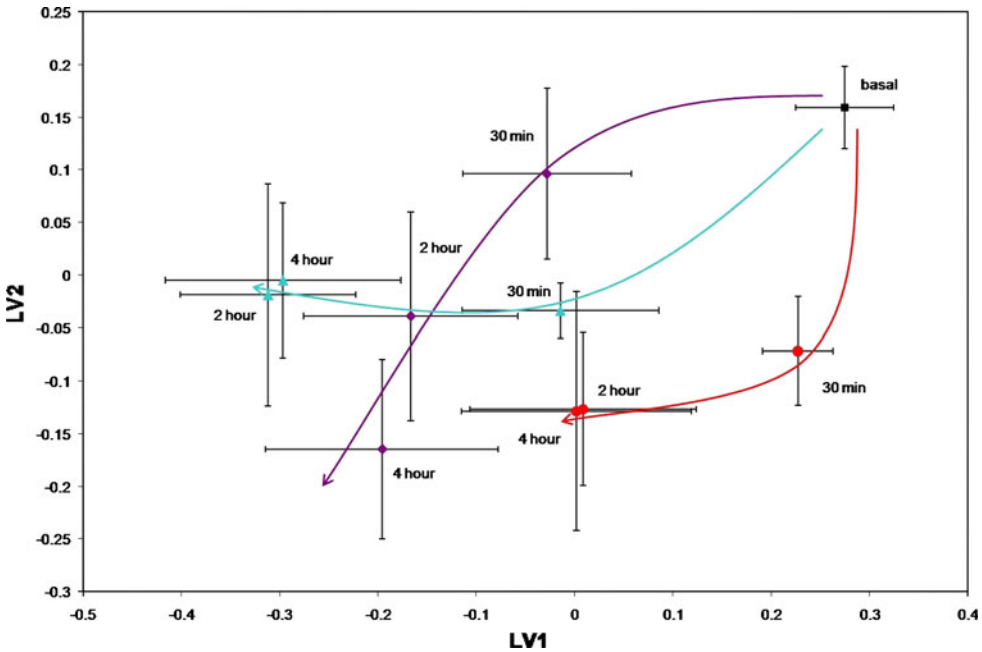

Fig. 9 Trajectory analysis of blue crab hemolymph NMR data obtained after challenge with control (●) ($n = 7$), DNP (◆) ($n = 7$), and *Vibrio campbellii* (▲) ($n = 8$). ■ = baseline hemolymph samples ($n = 22$). Error bars represent ±1 SE for each time-point average. The hand-drawn trajectories display the path of the metabolic profiles in response to challenge

from metabolic trajectory analysis over 4 h. The trajectory analysis aided in elucidating the subtle changes in the hemolymph metabolome and dynamics of the stress responses. The relatively large volume of hemolymph (25% of body weight) circulating in blue crabs, combined with a high cardiac output (151 ml kg^{-1} min^{-1}) (Booth et al. 1982) and the open nature of its circulatory system where hemolymph bathes tissues directly, are factors that likely contribute to the subtle nature of the metabolic differences. A more significant metabolic response may be observed in the metabolic fluctuations in organs and tissues.

Acknowledgments We acknowledge the support of the Hollings Marine Laboratory NMR Facility. The report is based in part upon research supported by the National Science Foundation under grants IOS-0725245 and DBI-0244007.

Disclaimer Commercial equipment or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by NIST, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

References

Benjamini, Y., & Hochburg, Y. (1997). Multiple hypotheses testing with weights. *Scandinavian Journal of Statistics, 24*, 407–418.

Booth, C. E., Mcmahon, B. R., & Pinder, A. W. (1982) Oxygen uptake and the potentiating effects of increased hemolymph lactate on oxygen transport during exercise in the blue crab *Callinectes sapidus*. *Journal of Comparative Physiology,* 148, 111–121.

Brosnan, J. T. (2003). Interorgan amino acid transport and its regulation. *Journal of Nutrition, 133*, 2068S–2072S.

Bundy, J. G., Lenz, E. M., Bailey, N. J., Gavaghan, C. L., Svendsen, C., Spurgeon, D., et al. (2002). Metabonomic assessment of toxicity of 4-fluoroaniline, 3,5-difluoroaniline and 2-fluoro-4-methylaniline to the earthworm *Eisenia veneta* (Rosa): Identification of new endogenous biomarkers. *Environmental Toxicology and Chemistry, 21*, 1966–1972.

Bundy, J. G., Sidhu, J. K., Rana, F., Spurgeon, D. J., Svendsen, C., Wren, J. F., et al. (2008). 'Systems toxicology' approach identifies coordinated metabolic responses to copper in a terrestrial non-model invertebrate, the earthworm *Lumbricus rubellus*. *BMC Biology, 6*, 25.

Burgents, J. E., Burnett, L. E., Stabb, E. V., & Burnett, K. G. (2005). Localization and bacteriostasis of Vibrio introduced into the Pacific white shrimp, *Litopenaeus vannamei*. *Developmental and Comparative Immunology, 29*, 681–691.

Burnett, L. E., Holman, J. D., Jorgensen, D. D., Ikerd, J. L., & Burnett, K. G. (2006). Immune defense reduces respiratory fitness in *Callinectes sapidus*, the Atlantic blue crab. *Biological Bulletin, 211*, 50–57.

Cui, Q., Lewis, I. A., Hegeman, A. D., Anderson, M. E., Li, J., Schulte, C. F., et al. (2008). Metabolite identification via the Madison Metabolomics Consortium Database. *Nature Biotechnology, 26*, 162–164.

Eddy, F., Powell, A., Gregory, S., Nunan, L. M., Lightner, D. V., Dyson, P. J., et al. (2007). A novel bacterial disease of the European shore crab, *Carcinus maenas* molecular pathology and epidemiology. *Microbiology, 153*, 2839–2849.

Edsall, G. (1934). Biological actions of dinitrophenol and related compounds: A review. *New England Journal of Medicine, 211*, 385.

Ekman, D. R., Teng, Q., Villeneuve, D. L., Kahl, M. D., Jensen, K. M., Durhan, E. J., et al. (2008). Investigating compensation and recovery of fathead minnow (*Pimephales promelas*) exposed to 17alpha-ethynylestradiol with metabolite profiling. *Environmental Science and Technology, 42*, 4188–4194.

Eriksson, L., Trygg, J., & Wold, S. (2008). CV-ANOVA for significance testing of PLS and OPLS models. *Journal of Chemometrics, 22*, 594–600.

Fox, F. R., & Rao, K. R. (1978). *Effects of sodium pentachlorophenate and 2,4-dinitrophenol on hapatopancreatic enzymes in the blue crab, Callinectes sapidus*. New York: Plenum Press.

Fuhrman, J. A. (1999). Marine viruses and their biogeochemical and ecological effects. *Nature, 399*, 541–548.

Gleeson, R. A., & Zubkoff, P. L. (1977). The determination of hemolymph volume in the blue crab, *Callinectes sapidus*, utilizing 14C-thiocyanate. *Comparative Biochemistry and Physiology. Part A, Physiology 56*, 411–413.

Hall, M. R., & Van Ham, E. H. (1998). The effects of different types of stress on blood glucose in the giant tiger prawn Penaeus monodon. *Journal of the World Aquaculture Society, 29*, 290–299.

Hines, A., Oladiran, G. S., Bignell, J. P., Stentiford, G. D., & Viant, M. R. (2007). Direct sampling of organisms from the field and knowledge of their phenotype: Key recommendations for environmental metabolomics. *Environmental Science and Technology, 41*, 3375–3381.

Holman, J. D., Burnett, K. G., & Burnett, L. E. (2004). Effects of hypercapnic hypoxia on the clearance of Vibrio campbellii in the Atlantic blue crab, *Callinectes sapidus* Rathbun. *Biological Bulletin, 206*, 188–196.

Keun, H. C., Ebbels, T. M., Bollard, M. E., Beckonert, O., Antti, H., Holmes, E., et al. (2004). Geometric trajectory analysis of metabolic responses to toxicity can define treatment specific profiles. *Chemical Research in Toxicology, 17*, 579–587.

Lorenzon, S., Giulianini, P. G., & Ferrero, E. A. (1997). Lipopolysaccharide-induced hyperglycemia is mediated by CHH release in crustaceans. *General and Comparative Endocrinology, 108*, 395–405.

Macey, B. M., Rathburn, C. K., Thibodeaux, L. K., Burnett, L. E., & Burnett, K. G. (2008). Clearance of *Vibrio campbellii* injected into the hemolymph of *Callinectes sapidus*, the Atlantic blue crab: The effects of prior exposure to bacteria and environmental hypoxia. *Fish & Shellfish Immunology, 25*, 718–730.

Martello, L. B., Friedman, C. S., & Tjeerdema, R. S. (2000). Combined effects of pentachlorophenol and salinity stress on phagocytic and chemotactic function in two species of abalone. *Aquatic Toxicology, 49*, 213–225.

Martin, G. G., Quintero, M., Quigley, M., & Khosrovian, H. (2000). Elimination of sequestered material from the gills of decapod crustaceans. *Journal of Crustacean Biology, 20*, 209–217.

Mikulski, C. M., Burnett, L. E., & Burnett, K. G. (2000). The effects of hypercapnic hypoxia on the survival of shrimp challenged with *Vibrio parahaemolyticus*. *Journal of Shellfish Research, 19*, 301–311.

Parsons, H. M., Ekman, D. R., Collette, T. W., & Viant, M. R. (2009). Spectral relative standard deviation: A practical benchmark in metabolomics. *Analyst, 134*, 478–485.

Parsons, H. M., Ludwig, C., Gunther, U. L., & Viant, M. R. (2007). Improved classification accuracy in 1- and 2-dimensional NMR metabolomics data using the variance stabilising generalised logarithm transformation. *BMC Bioinformatics, 8*, 234.

Rosenthal, R., & Rubin, D. B. (1984). Multiple contrasts and ordered Bonferroni procedures. *Journal of Educational Psychology, 76*, 1028–1034.

Rousseau, R., Govaerts, B., Verleysen, M., & Boulanger, B. (2008). Comparison of some chemometric tools for metabonomics biomarker identification. *Chemometrics and Intelligent Laboratory Systems, 91*, 54–66.

Scholnick, D. A., Burnett, K. G., & Burnett, L. E. (2006). Impact of exposure to bacteria on metabolism in the penaeid shrimp *Litopenaeus vannamei*. *Biological Bulletin, 211*, 44–49.

Thibodeaux, L. K., Burnett, K. G., & Burnett, L. E. (2009). Energy metabolism and metabolic depression during Exercise in *Callinectes sapidus*, the Atlantic Blue Crab: Effects of the bacterial pathogen *Vibrio Campbellii*. *Journal of Experimental Biology*, in press.

Tiziani, S., Emwas, A. H., Lodi, A., Ludwig, C., Bunce, C. M., Viant, M. R., et al. (2008). Optimized metabolite extraction from blood serum for 1H nuclear magnetic resonance spectroscopy. *Analytical Biochemistry, 377*, 16–23.

Tjeerdema, R. S., Fan, T. W., Higashi, R. M., & Crosby, D. G. (1991). Sublethal effects of pentachlorophenol in the abalone (*Haliotis rufescens*) as measured by in vivo 31P NMR spectroscopy. *Journal of Biochemical Toxicology, 6*, 45–56.

Ulrich, E. L., Akutsu, H., Doreleijers, J. F., Harano, Y., Ioannidis, Y. E., Lin, J., et al. (2008). BioMagResBank. *Nucleic Acids Research, 36*, D402–D408.

Viant, M. R. (2008). Recent developments in environmental metabolomics. *Molecular Biosystems, 4*, 980–986.

Viant, M. R., Rosenblum, E. S., & Tieerdema, R. S. (2003). NMR-based metabolomics: A powerful approach for characterizing the effects of environmental stressors on organism health. *Environmental Science and Technology, 37*, 4982–4989.

Welsh, P. C., & Sizemore, R. K. (1985). Incidence of bacteremia in stressed and unstressed populations of the blue crab, *Callinectes sapidus*. *Applied and Environmental Microbiology, 50*, 420–425.

Zohar, Y., Hines, A. H., Zmora, O., Johnson, E. G., Lipcius, R. N., Seitz, R. D., et al. (2008). The Chesapeake Bay blue crab (*Callinectes sapidus*): A multidisciplinary approach to responsible stock replenishment. *Reviews in Fisheries Science, 16*, 24–34.