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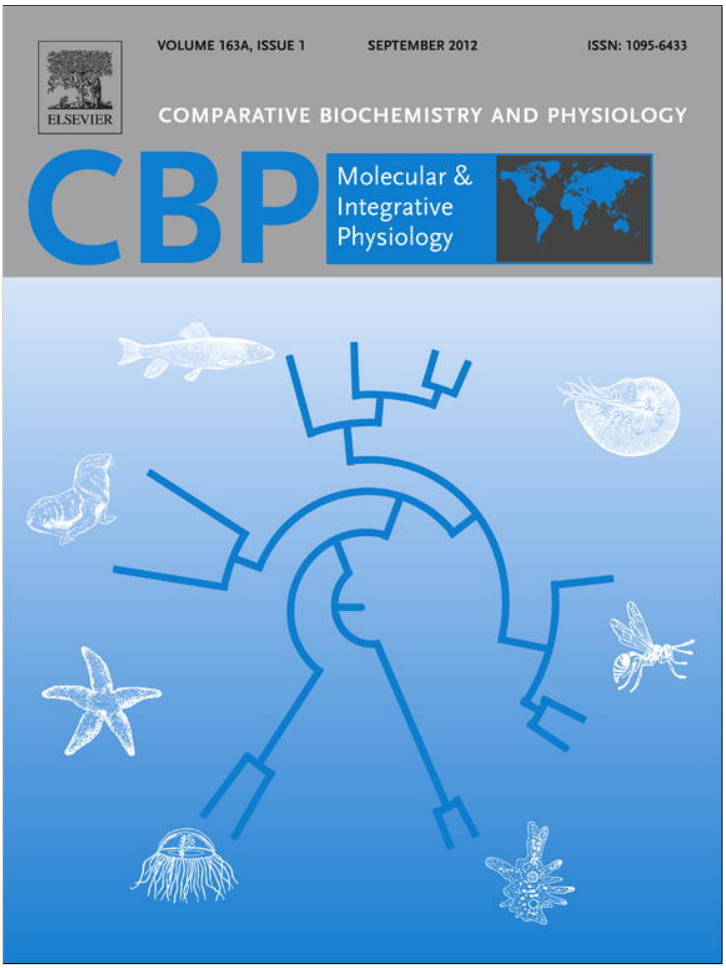

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Physiological biomarkers of hypoxic stress in red swamp crayfish *Procambarus clarkii* from field and laboratory experiments

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ABSTRACT

The crayfish industry in Louisiana is the largest in the United States, with crayfish frequently harvested from waters that experience episodic or chronic hypoxia (dissolved oxygen [DO] ≤ 2 mg/l). We examined physiological biomarkers (hemolymph lactate, glucose, and protein concentrations) of hypoxic stress in the red swamp crayfish *Procambarus clarkii* from chronically hypoxic natural habitats and laboratory hypoxia experiments. *P. clarkii* from normoxic and hypoxic areas in the Atchafalaya River Basin were sampled monthly from April to July 2010. Laboratory experiments subjected *P. clarkii* to severe hypoxia (1 mg/l DO), moderate hypoxia (2 mg/l DO), or normoxic conditions (control: DO > 7.5 mg/l) for 12, 24, and 48 h. *P. clarkii* from normoxic and hypoxic natural habitats did not display significantly different hemolymph lactate or glucose concentrations; however, mean hemolymph protein concentration was significantly lower in crayfish from hypoxic areas. *P. clarkii* exposed to severe hypoxia in laboratory experiments had significantly higher hemolymph lactate and glucose concentrations for all three exposure times, whereas large differences in protein concentrations were not observed. These results suggest that elevated hemolymph lactate and glucose concentrations are responses to acute hypoxia in *P. clarkii*, while differences in protein concentrations are the result of chronic hypoxic exposure.

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1. Introduction

Crayfish are ecologically important taxa in the United States and economically important to the southeastern US region. Conservation status of the 363 crayfish species listed by the American Fisheries Society Endangered Species Committee recently reported 174 taxa (47.9%) as vulnerable (54), threatened (52), endangered (66), or possibly extinct (2; Taylor et al., 2007). Populations can be assessed regarding their overall health and risk to extirpation by use of physiologically based conditional indices and biomarkers of acute and chronic stressors. However, such indices of condition and information on how these parameters relate to individual health are not yet established for crayfish. Crayfish physiology is an important component of health due to issues of water quality, habitat alteration, and presence of diseases. Anthropogenic stressors put populations at risk and could also threaten an important agricultural industry with a total value of more than US$181 million in Louisiana (LSUAC, 2011).

Over 90% of the wild crayfish harvest in Louisiana comes from the Atchafalaya River Basin (ARB) located in south-central Louisiana (Isaacs and Lavergne, 2010). The ARB is the largest bottomland hardwood river-floodplain system in North America (Lambou, 1990) and its mosaic of bayous, petrochemical canals, lakes, and swamps, all affected by the annual Atchafalaya River flood pulse, provide a diversity of ideal habitats and conditions for a variety of crayfish species. However, anthropogenic modifications (levee and canal construction) have altered water circulation and flow patterns that facilitate the formation of hypoxic waters (dissolved oxygen [DO] ≤ 2 mg/l) that can persist for several weeks to months throughout extensive areas of the ARB during the annual flood pulse (Sabo et al., 1999).

Environmental hypoxia is not uncommon in many aquatic habitats, and the biota occupying these environments must adapt and acclimate to temporary or extended periods of reduced oxygen concentrations in order to survive. Crayfish routinely encounter hypoxic conditions in aquatic or burrow habitats and have developed several physiological adaptations to cope with periods of suboptimal oxygen concentrations (Mauro and Thompson, 1984; McMahon, 1986; Reiber, 1995; Morris and Callaghan, 1998; Reiber and McMahon, 1998; Fujimori and Abe, 2002; McMahon, 2002; Silva-Castiglioni et al., 2010). Behaviorally, crayfish often move to the air–water interface in hypoxic areas to take advantage of higher DO concentrations and may move out of the water completely (Taylor and Wheatly, 1981; McMahon and Wilkes, 1983; McMahon and Hankinson, 1993; McMahon and Stuart, 1999). Physiologically, crayfish have developed compensatory mechanisms such as hypometabolism, bradycardia, hyperventilation, and modulation of hemocyanin oxygen affinity that allow them to maintain homeostatic balance in hypoxic water. Although physiological adaptations and

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tolerance thresholds to hypoxia vary among crayfish species, the red swamp crayfish *Procambarus clarkii* is able to tolerate lower oxygen concentrations compared to other species (Nyström, 2002) and can maintain high survival rates in oxygen poor environments (Powell and Watts, 2006). Although compensatory mechanisms afford *P. clarkii* and other crayfishes the opportunity to tolerate hypoxic conditions, prolonged exposure could lead to detrimental population effects such as reduced survival (Avault et al., 1975; Melancon and Avault, 1977; McClain, 1999; Sladkova and Kholodkevich, 2011) and growth (Jussila and Evans, 1997; McClain, 1999; Reynolds, 2002; McClain et al., 2007).

Hypoxic stress elicits multiple physiological responses in crayfish. Hemolymph lactate, glucose, and protein concentrations in crayfish have been shown to fluctuate in response to hypoxia (Gäde, 1984; Mauro and Thompson, 1984; Morris and Callaghan, 1998; Fujimori and Abe, 2002; Silva-Castiglioni et al., 2010) and quantifying these biomarkers allows for examination of physiological responses during hypoxic stress. However, most of this research has been conducted in the laboratory in the absence of confounding biotic and abiotic factors, which can significantly influence physiological responses to hypoxia in natural systems. Therefore, the purpose of the present study was to examine hemolymph lactate, glucose, and protein concentrations in *P. clarkii* from normoxic (DO > 2 mg/l) and chronically hypoxic natural habitats in the ARB, as well as in individuals exposed to simulated hypoxia in the laboratory.

2. Materials and methods

2.1. Field collections

Adult intermolt *P. clarkii* were collected from two hypoxic and two normoxic sites in the southeastern ARB during floodplain inundation with five pillow traps per site baited with 150 g of Purina® manufactured bait that were fished overnight. We tried to minimize handling stress and possible hemolymph lactate and glucose increases associated with aerial exposure and processing time by sampling no more than two individuals per trap. Crayfish hemolymph was collected by pericardial cavity puncture (20-gauge needle) and transferred via capillary tube into a microcentrifuge tube and placed immediately on ice, allowed to clot, and stored at −4 °C in the laboratory. We recorded carapace length (mm), weight (g), and sex of sampled crayfish. Five hemolymph samples per site ($n = 10$ normoxic, $n = 10$ hypoxic) were collected on 12 April, 7 May, 1 June, and 1 July 2010. We were unable to attain *P. clarkii* hemolymph samples after 1 July due to receding floodplain water levels and a retreat of crayfish into burrows. Temperature (°C), DO (mg/l), and pH were recorded with a handheld multiparameter water quality sonde (YSI model 6820, Yellow Springs, OH) at each sample site. Additionally, a continuous recording water quality sonde (YSI model 6600, Yellow Springs, OH) was deployed from 21 to 27 April 2010 at one of the sampled hypoxic sites to record DO concentrations at 15-min intervals ($n = 96$ observations per day).

2.2. Laboratory experiments

P. clarkii were collected from the ARB and acclimated to laboratory conditions in a recirculating raceway for a minimum of 4 weeks prior to experimentation. While in raceways, crayfish were fed algal wafers ad libitum semi-weekly. We recorded the carapace length, weight, and sex of adult intermolt crayfish then placed them into individual 1-l polyethylene amber bottles with 0.635-cm holes over the entire bottle to allow uninhibited water circulation. Bottles restricted access to atmospheric oxygen at the air–water interface and eliminated conspecific interactions. Individual crayfish in bottles were placed into nine 30-l aquaria filled with ARB water and equipped with a standpipe and under gravel filter powered by an air stone. Each aquarium was aerated with atmospheric air (DO > 7.5 mg/l) and contained six crayfish (three males and three females), which were allowed to acclimate in the aquaria for 24 h before the beginning of experimental trials. Three DO concentrations were randomly assigned to the nine aquaria: control (DO > 7.5 mg/l), moderate hypoxia (DO ~ 2 mg/l), and severe hypoxia (DO ~ 1 mg/l). Control aquaria were kept at full DO saturation by bubbling air through the under gravel filter. Moderate and severe hypoxia conditions were created by bubbling in nitrogen or a nitrogen/air mixture. Because bubbling nitrogen can decrease water temperatures, heaters (Stealth 50, Marineland Aquarium Products, Cincinnati, OH) were added to all aquaria to maintain consistent temperatures among treatments (Table 1).

Three separate experiments were performed in which *P. clarkii* were exposed to aforementioned experimental DO concentrations for 12, 24, and 48 h ($n = 54$ crayfish per time trial). DO levels in moderate and severe hypoxia aquaria were decreased at similar rates and a time trial began when the desired DO concentrations were attained (usually within 2.5 h). Temperature, DO, and pH were recorded with a handheld multiparameter water quality sonde. Crayfish hemolymph was extracted from individuals at the conclusion of each time trial with the same methods used in the field, with the clotted sample stored at −4 °C.

2.3. Hemolymph processing

Hemolymph clots were broken up, the sample was centrifuged (2415 ×*g* for 15 min at 4 °C), and the supernatant serum was extracted. Spectrophotometric assays were performed in triplicate and read with a BioTek® Synergy 2 Multi-purpose Microplate Reader (BioTek®, Winooski, VT) and interpreted with Gen5™ analysis software (BioTek®, Winooski, VT).

Hemolymph lactate concentration was enzymatically determined with an L-lactate assay kit (Eton Bioscience Inc., San Diego, CA). Each sample was diluted 50-fold with deionized water. Twenty microliters of diluted samples and lactate standards were added to 96-well microplates. Fifty microliters of lactate assay solution was then added to each well, mixed by gentle agitation, and incubated at 37 °C for 30 min. The reaction was then stopped by adding 50 µl of 0.5-M acetic acid to each well. Standard curves were prepared at concentrations of 0, 39, 78, 156, 312.5, 625, 1250, and 2500 µM L-lactate per manufacturer's instructions. Standard curves for lactate assays had R^2 values of 0.99 or greater. Absorbance was measured at 490 nM and reported as mmol/l.

Total protein concentration of crayfish serum was determined with the Pierce Coomassie Plus assay (Thermo Fisher Scientific Inc., Rockford, IL) modified from the Bradford method (Bradford, 1976). Each sample was diluted 100-fold with deionized water. Standard curves were prepared at concentrations of 0, 25, 125, 250, 500, 750, 1000, 1500, and 2000 µg/ml bovine serum albumin per manufacturer's instructions. Standard curves for protein assays had R^2 values of 0.99 or greater. Absorbance was measured at 595 nM and reported as mg/ml.

Hemolymph glucose concentration was determined with a colormetric QuantiChrom™ Glucose Assay Kit as per manufacturer

Table 1
Mean (±SE) of water quality parameters in aquaria ($n = 3$ per treatment) during experimental trials. Asterisks denote a significant difference ($P < 0.05$) within experiments.

Experiment	Treatment	*n*	Dissolved oxygen (mg/l)	Temperature (°C)	pH
12 h	Control	18	8.45 ± 0.06	25.07 ± 0.15	7.44 ± 0.01
	Moderate	18	1.93 ± 0.04	24.67 ± 0.06	7.33 ± 0.02*
	Severe	18	1.03 ± 0.02	25.04 ± 0.15	7.50 ± 0.03
24 h	Control	24	8.01 ± 0.02	24.83 ± 0.09	7.40 ± 0.01
	Moderate	24	2.05 ± 0.03	24.59 ± 0.08	7.28 ± 0.03*
	Severe	24	1.10 ± 0.02	24.40 ± 0.07	7.38 ± 0.02
48 h	Control	18	8.62 ± 0.05	24.28 ± 0.21	7.40 ± 0.01
	Moderate	18	2.09 ± 0.03	23.88 ± 0.06	7.22 ± 0.02*
	Severe	18	0.99 ± 0.05	24.15 ± 0.06	7.42 ± 0.02

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instructions (BioAssay Systems, Hayward, CA, USA). Standard curves were prepared at concentrations of 0, 500, 1000, 2000, and 3000 mg/l $C_6H_{12}O_6$ per manufacturer's instructions. Standard curves for glucose assays had R^2 values of 0.97 or greater. Absorbance was measured at 630 nM and reported as mmol/l.

2.4. Statistical analysis

All statistical tests were performed with SAS 9.1.3, and significance for all statistical tests was determined with a Type I error rate of $\alpha = 0.05$.

2.4.1. Field collections

Analysis of variance (ANOVA) with a Tukey–Kramer post hoc adjustment was used to test for differences in DO concentrations between hypoxic sites as well as between normoxic sites. Additionally, ANOVA with a Tukey–Kramer post hoc adjustment was performed to test for differences in carapace length, weight, and DO between hypoxic and normoxic treatments. Lactate and glucose concentrations from *P. clarkii* hemolymph collected at field sites were $\log_{(e)}$ transformed to improve normality. Analysis of covariance (ANCOVA; PROC MIXED) with a Tukey–Kramer post hoc adjustment was used to examine significance of Julian date (covariate) and to test for differences in hemolymph protein and glucose concentrations between hypoxic and normoxic treatments. Hemolymph lactate concentrations from hypoxic and normoxic sites were not influenced by sampling date (i.e., ANCOVA slopes equal to zero), so an ANOVA with a Tukey–Kramer post hoc adjustment was performed to test for a difference between treatments.

2.4.2. Laboratory experiments

ANOVA with a Tukey–Kramer post hoc adjustment was used to test for differences in temperature and pH among treatments for each time trial experiment. Variances of hemolymph lactate, glucose, and protein concentrations from laboratory experiments were homogeneous (Levene's test). Lactate concentrations were inverse transformed and glucose concentrations were $\log_{(e)}$ transformed to improve normality. A two-level hierarchical ANOVA (PROC MIXED) with a Tukey–Kramer post hoc adjustment was used for each physiological biomarker to examine statistically significant differences among DO treatments for each time trial experiment. Additionally, a two-way ANOVA (PROC GLM) was used to test for differences in physiological responses between sexes.

3. Results

3.1. Field collections

Crayfish size (carapace length) and weight did not differ significantly between hypoxic (48.76 ± 0.86 mm, 21.96 ± 1.35 g) and normoxic (50.19 ± 0.86 mm, 24.25 ± 1.40 g) treatments. Mean DO concentrations did not differ between hypoxic sites and were below 2.0 mg/l on every sample date. Similarly, mean DO concentrations did not differ between normoxic sites and were above hypoxic level on every sample date. However, diurnal mean DO concentrations from hypoxic sites (1.21 ± 0.07 mg/l) were significantly lower than normoxic sites (3.64 ± 0.60 mg/l; $F_{1,14} = 32.22$, $P < 0.0001$). Quarter-hourly sonde measurements taken over a 1-week period at the hypoxic site revealed elevated DO concentrations in the afternoon with concentrations above 1.5 mg/l recorded on 6 days and 2.0 mg/l on 3 days (Fig. 1). These DO concentrations represent total water column DO concentrations based on previous sampling in the ARB, which demonstrated that DO levels are homogenous throughout the floodplain water column (Halloran, 2010).

3.1.1. Hemolymph lactate

Sampling date did not influence *P. clarkii* hemolymph lactate concentrations. Mean hemolymph lactate concentrations from crayfish collected in hypoxic and normoxic areas were 20.26 ± 3.93 mmol/l and 9.99 ± 1.46 mmol/l, respectively; however, there was not a significant difference between treatments. Mean lactate concentrations from hypoxic sites were higher than normoxic sites on every sample date except for 7 May 2010 (Fig. 2A).

3.1.2. Hemolymph glucose

ANCOVA revealed that the slopes of the regressions for hypoxic and normoxic treatments with sampling date were not significantly different. Mean crayfish hemolymph glucose concentrations from hypoxic and normoxic areas were 1.22 ± 0.11 mmol/l and 1.34 ± 0.15 mmol/l, respectively, and were not significantly different (Fig. 2B).

3.1.3. Hemolymph protein

As with glucose, ANCOVA revealed that the slopes of the regressions for hypoxic and normoxic treatments with sampling date were not significantly different. However, crayfish hemolymph protein concentrations from hypoxic areas (49.47 ± 2.71 mg/ml) were significantly lower than those from crayfish collected in normoxic areas (61.61 ± 3.48 mg/ml; $F_{1,77} = 8.89$, $P = 0.0038$). Moreover, mean protein concentrations in crayfish collected from normoxic sites were higher than hypoxic sites on every sample date (Fig. 2C).

3.2. Laboratory experiments

The pH of moderate hypoxia treatment tanks was significantly lower in all three laboratory time trial experiments, although differences were not more than 0.2 pH units (Table 1). There was no significant difference in temperature among treatments in any of the time trials. Crayfish mortalities occurred in each time trial but were limited to only severe hypoxia treatment tanks (12 h, $n = 2$; 24 h, $n = 1$; 48 h,

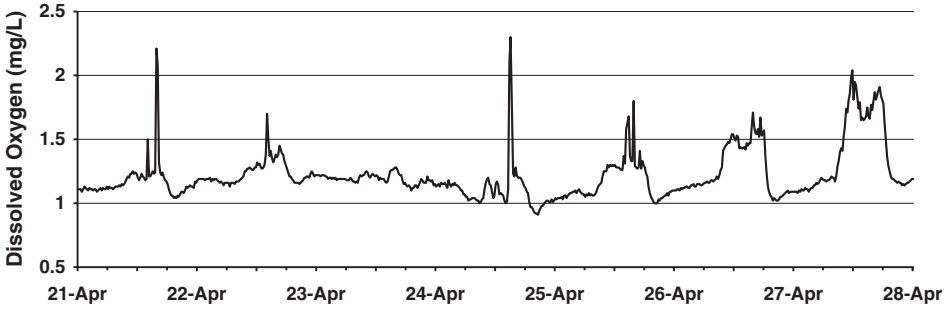

Fig. 1. Quarter-hourly dissolved oxygen sonde measurements from 21 to 27 April 2010 at a chronically hypoxic site in the lower Atchafalaya River Basin.

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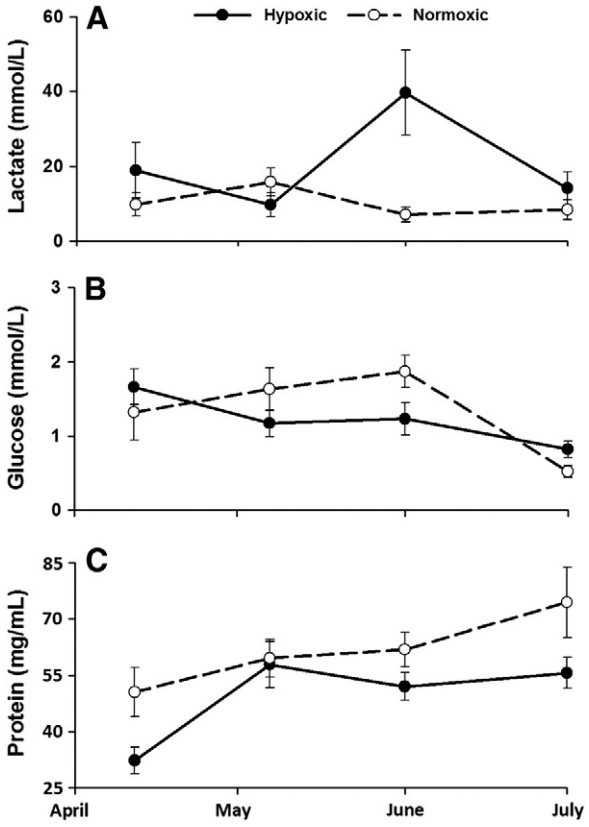

Fig. 2. Mean (±SE) hemolymph (A) lactate, (B) glucose, and (C) protein concentrations in *P. clarkii* from hypoxic and normoxic sites in the Atchafalaya River Basin during 2010 sampling.

$n = 9$). Hemolymph lactate, protein, and glucose concentrations did not differ statistically between male and female *P. clarkii*.

3.2.1. Hemolymph lactate

P. clarkii mean hemolymph lactate concentrations were significantly higher in individuals subjected to the severe hypoxia treatment for the 12, 24, and 48 h experiments (Fig. 3A). Moreover, mean lactate concentration in crayfish from severe hypoxia treatments exhibited an increasing trend with exposure time. Lactate concentrations for the moderate hypoxia and control treatments did not differ statistically in any of the time trials.

3.2.2. Hemolymph glucose

Mean hemolymph glucose concentration from the moderate hypoxia DO treatment was not dissimilar from severe hypoxia and control treatments but severe hypoxia-exposed individuals had significantly higher concentrations compared to control subjects after 12 h. Additional exposure time revealed significantly higher mean glucose concentrations in severe hypoxia treatments relative to moderate hypoxia and control treatments at both 24 and 48 h (Fig. 3B). Mean hemolymph glucose concentrations from control and moderate hypoxia treatments were not dissimilar in the 24 and 48 h experiments.

3.2.3. Hemolymph protein

Mean hemolymph protein concentration from the moderate hypoxia treatment was not significantly different from severe hypoxia and control treatments; however, severe hypoxia exposed individuals had significantly higher concentrations than control subjects in the 24 h experiment. Treatments did not differ statistically in the 12 and 48 h experiments (Fig. 3C).

4. Discussion

P. clarkii frequently inhabits environments that experience extended periods of aquatic hypoxia in the ARB, and have developed behavioral and physiological adaptations in order to successfully exploit these suboptimal DO environments. We observed changes in hemolymph lactate, glucose, and protein concentrations from crayfish exposed to hypoxic water in both field and laboratory experiments. As environmental DO concentrations become unfavorable, some crayfish will switch to anaerobic metabolism in order to maintain homeostasis (Gäde, 1984; Morris and Callaghan, 1998; Fujimori and Abe, 2002; Silva-Castiglioni et al., 2010). Lactate is the major end product of anaerobic metabolism in crustaceans while also enhancing hemocyanin O_2-binding affinity during hypoxia (Truchot, 1980; Järvenpää et al., 1983; Bouchet and Trouchot, 1985; McMahon, 1986; Morris and Callaghan, 1998; McMahon, 2001; Qui et al., 2011). Hemolymph lactate accumulation is related to the duration and severity of hypoxia (Albert and Ellington, 1985) and researchers have documented lactate increases from hypoxia-stressed crayfish (Gäde, 1984; Mauro and Thompson, 1984; Morris and Callaghan, 1998; Jackson et al., 2001; Fujimori and Abe, 2002; Silva-Castiglioni et al., 2010, 2011). Thus, elevated hemolymph lactate concentration appears to serve as a viable biomarker of hypoxic stress in crayfish. We observed significantly higher mean hemolymph lactate concentrations in *P. clarkii*

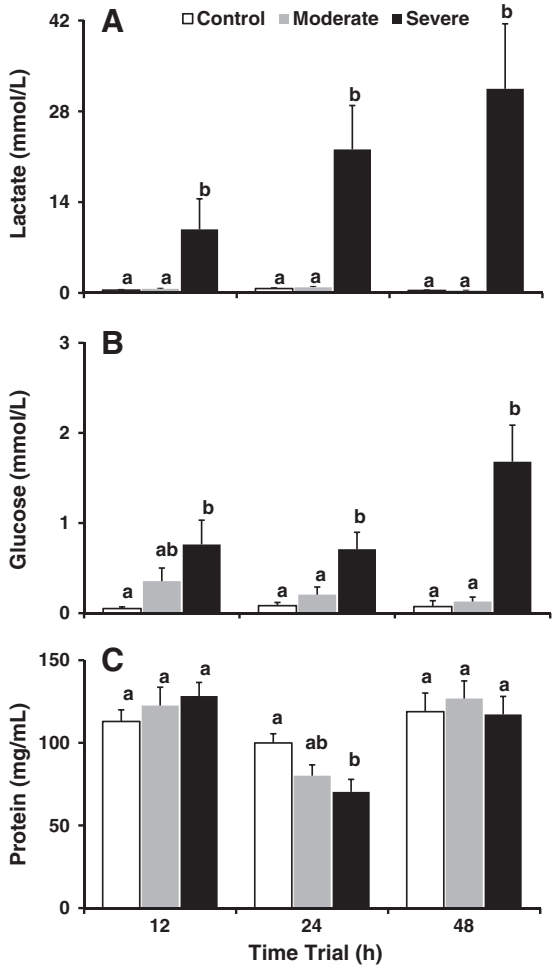

Fig. 3. Mean (±SE) hemolymph (A) lactate, (B) glucose, and (C) protein concentrations in *P. clarkii* subjected to three laboratory simulated dissolved oxygen (DO) concentrations during three exposure time experiments. Control: DO > 7.5 mg/l, moderate hypoxia: DO ~ 2 mg/l, and severe hypoxia: DO ~ 1 mg/l. Different letters indicate a significant difference ($P < 0.05$) for each physiological parameter within a time trial experiment.

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from severe hypoxia treatments in all three laboratory time trial experiments and a 220% increase in lactate concentration from 12 h to 48 h, suggesting activation of anaerobic pathways. Because crayfish were kept in bottles during experimentation, individuals were restricted from using atmospheric air to supplement their oxygen uptake, affirming that the observed lactate concentrations are representative of the physiological response to treatment DO concentrations. Interestingly, hyperlactaemia was not observed in moderate hypoxia treatment crayfish, with mean concentrations similar to control individuals. These results suggest that a DO concentration of 2 mg/l does not elicit anaerobic metabolism activation and therefore does not appear to be physiologically stressful to quiescent *P. clarkii*.

P. clarkii hemolymph glucose concentrations from severe hypoxia treatments were also significantly higher in all three laboratory time trials. Hyperglycemia is commonly recognized as a stress response to hypoxia in decapod crustaceans (e.g., crab, Zou et al., 1996; shrimp, Racotta et al., 2002; lobster, Ocampo et al., 2003; and crayfish, Silva-Castiglioni et al., 2010). During hypoxia, glycogenolysis enhances hemolymph glucose concentrations in order to provide increased substrate for anaerobic glycolysis (Oliveira et al., 2001; Silva-Castiglioni et al., 2010, 2011; Marqueze et al., 2011). The somewhat elevated hemolymph glucose concentration observed in the 12 h moderate hypoxia treatment may be the result of a physiological preparation for impending anaerobic glycolytic demands (Taylor and Spicer, 1987; Zou et al., 1996). Detection of declining DO concentrations by *P. clarkii* at the start of the experimental trial initiated glucose mobilization for use in a potentially stressful hypoxic environment. When DO concentrations stabilized at approximately 2 mg/l, superfluous glucose was presumably synthesized back into glycogen. Although glycogen concentrations were not measured in this study, increasing glycogen associated with decreasing glucose in crustaceans has been previously documented (Oliveira et al., 2001; Morris and Adamczewska, 2002; Patterson et al., 2007; Silva-Castiglioni et al., 2010, 2011; Marqueze et al., 2011). Furthermore, there was a decline in *P. clarkii* mean hemolymph glucose concentration with exposure time in the moderate hypoxia treatment (Fig. 3B).

P. clarkii from normoxic and hypoxic field sites showed no significant difference in mean glucose or lactate concentrations even though individuals from hypoxic sites demonstrated lactate concentrations of more than twice that of crayfish from normoxic sites. This was a result of high lactate concentrations from a few hypoxic exposed individuals on 1 June (Fig. 2A). It is not surprising, however, that hemolymph glucose and lactate concentrations from individuals captured in hypoxic and normoxic areas were similar. Bimodal respiration in crayfish is possible because of trichobranchiate gills, which do not collapse in air and allow for utilization of atmospheric air during reduced DO periods. Total and partial emergence and aerial ventilation increases crayfish hemolymph oxygenation (Taylor and Wheatly, 1981; McMahon and Wilkes, 1983; McMahon and Hankinson, 1993; McMahon and Stuart, 1999) and reduces hemolymph lactate levels (Gäde, 1984). This adaptive strategy could enable *P. clarkii* inhabiting low-oxygen environments to avoid anaerobiosis and accumulation of concomitant metabolites.

Biotic and abiotic characteristics of natural habitats may also mitigate *P. clarkii* physiological stress in hypoxic environments. Hydrophytes that enhance ARB hypoxia by reducing water circulation and increasing benthic decomposition rates may serve as local DO refugia (Miranda et al., 2000; Bunch et al., 2010) for crayfish inhabiting these environments, at least during the day. Fontenot et al. (2001) observed normoxic conditions in littoral macrophyte beds adjacent to hypoxic limnetic areas in the ARB. Aquatic macrophyte stands could help offset environmental anaerobiosis that crayfish would experience in the surrounding hypoxic water.

Many crayfish are oxygen regulators and can maintain normal metabolic rates during reduced ambient oxygen concentrations until a critical oxygen level (P_c) is reached. At oxygen concentrations below P_c, oxyconformation reduces metabolic rates prompting anaerobic metabolism activation and subsequent lactate and glucose accumulation (Morris and Callaghan, 1998). The observed increase in *P. clarkii* lactate and glucose hemolymph concentrations from severe hypoxia treatments in all three time trial experiments suggests that DO concentrations were below P_c and that anaerobic metabolism was initiated. Conversely, *P. clarkii* from moderate hypoxia treatment tanks did not display significantly increased hemolymph lactate and glucose concentrations relative to control individuals. These results suggest that P_c for quiescent *P. clarkii* under laboratory conditions is between 1 and 2 mg/l. This may prove beneficial to crayfish inhabiting hypoxic areas of the ARB. Diurnal oxygen concentrations in hypoxic ARB waters regularly increased to near or above 2 mg/l over the week that we monitored (Fig. 1). If the P_c of *P. clarkii* in the ARB is similar to that indicated by our laboratory results, then diel oxygen fluctuations in hypoxic areas in conjunction with the ability of crayfish to utilize atmospheric oxygen may allow crayfish to escape prolonged exposure to periods below P_c, which would allow for increased metabolic activity and elimination or reduction of accumulated anaerobic metabolites. Although environmental parameters (Staples et al., 2000), activity (Crear and Forteath, 2000), and feeding (Robertson et al., 2002) can all influence P_c, it can still be interpreted as a homeostatic balance point between oxyregulation and oxyconformation and a physiological response to reduced oxygen environments (Reiber, 1995).

Although biotic and abiotic factors may offset hemolymph lactate and glucose concentrations in *P. clarkii* exposed to naturally hypoxic habitats, hemolymph protein concentrations demonstrate marked effects of chronic hypoxic exposure. Protein concentrations in ARB *P. clarkii* from normoxic areas were significantly and consistently higher than individuals sampled from hypoxic areas (Fig. 2C). Although ANCOVA failed to demonstrate date as a significant covariate, the disparity in protein concentrations between normoxic and hypoxic areas appears to intensify through time and extended hypoxic exposure appears to slow increases in hemolymph protein concentrations. In the current study, *P. clarkii* hemolymph protein concentrations from normoxic areas increased throughout the spring and summer presumably due to increased photoperiod, temperatures, and food availability (Sladkova and Kholodkevich, 2011). Conversely, lower protein concentrations in *P. clarkii* from hypoxic areas may be the result of both extrinsic and intrinsic factors. Active foraging and food consumption may be diminished in hypoxic waters (Bailey et al., 1985; Das and Stickle, 1993; Paschke et al., 2010), requiring hemolymph protein concentrations to be used as organic reserves (Oliver and MacDiarmid, 2001) which would in turn reduce protein concentrations in starved individuals (Wen et al., 2007). Silva-Castiglioni et al. (2007) also documented decreased hemolymph protein concentrations in crayfish from natural habitats during reduced DO periods. Additionally, *P. clarkii* occupying hypoxic waters above P_c and utilizing these physiological compensation mechanisms, may fall below P_c as the result of increased oxygen consumption associated with activity, feeding, (Crear and Forteath, 2000) and digestive processes (Du Preez et al., 1992).

Hemolymph protein concentration appears to be a biomarker of chronic rather than acute hypoxic stress in *P. clarkii* as demonstrated by our field and laboratory results. Although *P. clarkii* in the ARB are exposed to chronic hypoxia that can last for periods of weeks to months, laboratory experiments that subjected crayfish to acute hypoxic conditions for a maximum of 2 days failed to elicit consistent significant differences in hemolymph protein concentrations. Changes in hemolymph protein concentrations associated with laboratory hypoxia experiments were also not documented in *Parastacus defossus* after 8 h of exposure (Silva-Castiglioni et al., 2010), *Litopenaeus stylirostris* after 24 h of exposure (Mugnier et al., 2008), *Penaeus vannamei* after 2 weeks of exposure (Racotta et al., 2002), or *Orconectes rusticus* after 3.5 weeks of exposure (Wilkes and McMahon, 1982).

P. clarkii in this study have demonstrated multiple physiological responses to environmental hypoxia. The high oxygen affinity of *P. clarkii* hemolymph (McMahon and Hankinson, 1993; McMahon and Stuart,

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1999; McMahon, 2001; Powell and Watts, 2006) allows this species to maintain normal metabolic functions at relatively low DO concentrations. The short hypoxic exposure time and relatively rapid rate of oxygen decline in laboratory experiments compared to the onset of naturally occurring hypoxia in the ARB (Bonvillain et al., 2011) suggests that hemolymph lactate and glucose concentrations are acute biomarkers of hypoxic stress in *P. clarkii* whereas protein levels are an indicator of chronic hypoxic stress. Furthermore, these results demonstrate that physiological responses by animals to environmental stressors under laboratory simulations can vary markedly from the actual responses experienced in natural environments.

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