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A PROLONGED THERMAL STRESS EXPERIMENT ON THE AMERICAN LOBSTER, *HOMARUS AMERICANUS*

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ABSTRACT Two groups of lobsters were maintained for 31 days at temperatures environmentally realistic for Long Island Sound to investigate the effects of prolonged thermal stress on the physiology of lobsters. One group was held at 16°C, representative of late spring (controls), and the other group at 23°C, representative of late summer/early fall (treatments). *In vivo* hemolymph pH and samples for serum chemistry analysis were taken before and after temperature exposure. Hemolymph samples were taken before, during and after temperature exposure to investigate effects on hemocyte phagocytic activity assay and total hemocyte counts. Treatment lobsters developed a significant pH acidosis. Other serum index changes included marked hyperchloremia and hyperproteinemia. Phagocytic activity of hemocytes was significantly depressed (~60%) in treatment lobsters after 14 days and remained so until the end of the experiment. Similarly, total hemocyte counts increased strongly in the thermal stress group after 14 days, and remained so until the end of the experiment. Results suggest that late summer temperatures in the bottom waters of Long Island Sound may have profound deleterious effects on the physiology of lobsters. Recent changes in water temperature regimes in the bottom waters of Long Island Sound suggest that it may in the long term become inhospitable for lobster survival.

KEY WORDS: lobster, *Homarus americanus*, thermal, temperature, climate, stress, disease, serum chemistry, immunocompetence

INTRODUCTION

Temperature is a dominant controlling force in the physiology of poikilothermic species. Whereas temperature increases can modulate metabolic rate, growth and reproduction (Leffler 1972, LeMoullac & Haffner 2000), all species remain constrained to a fundamental niche defined by their tolerance limits for environmental variables, including temperature. Many species have homeostatic mechanisms that allow them to cope with stress from transient excess of any tolerance limit, but prolonged exposure to such stressors may result in rapid deterioration in physiologic state (LeMoullac & Haffner 2000).

Thermal anomalies and longer-term trends in bottom water temperatures have been proposed as one potential cause of the decline in populations of the American lobster (*Homarus americanus* H. Milne Edwards, 1837) in Long Island Sound (LIS) (Dove et al. 2004), the southernmost inshore population of this largely Boreal species. Both the severe mortality event of late 1999 in western LIS and a less pronounced event in late 2002 in eastern LIS were correlated with thermal anomalies in the bottom water layer. These anomalies were characterized by strongly elevated winter water temperatures and concomitant, though weaker, increases in summer water temperature (Robert Wilson, pers. comm.)

We earlier described excretory calcinosis of the lobster (Dove et al. 2004), a disease characterized by metastatic calcium deposition in the gills and antennal glands. We proposed that this chronic disease results from prolonged thermal stress and its interference with the physiology of the lobster, specifically, disruption of acid-base status and calcium metabolism. In this experiment we studied the effects of prolonged thermal stress on select lobster physiology indices: acid-base status and serum chemistry. In addition, we measured the effects of chronic thermal stress on immunocompetence as measured by total hemocyte counts and phagocytic activity of hemocytes *in vitro*. Previous studies have shown these to be useful measures of the effectiveness of cellular defense mechanisms in other poikilotherms (Carballal et al. 1997, LeMoullac & Haffner 2000, Allam et al. 2001, Tort et al. 2004)

MATERIALS AND METHODS

Experimental Design

Two groups of 12 lobsters each were used in a repeated measures experimental design, whereby interindividual variability between experimental subjects was removed through analysis of before-after change in individual animals. Three animals were maintained in each of 8 recirculating tank systems of 150 L each. Four tanks were held in each of two immersion troughs with a 0.50 hp chiller, used for downregulation of water temperature. A single 200 W heater was used in each tank for upregulation of temperature. Water temperature was checked daily using a digital thermometer, and true average temperature, over the course of the experiment, was reconstructed for statistical analysis *a posteriori* using Tidbit temperature loggers (Onset Corp.) deployed in each tank. Two treatment tanks and two control tanks were assigned in each trough to avoid trough as a confounding factor. Lobsters were donated by a LIS lobsterman at a time when water temperatures were approximately 16°C, the temperature of the control group. All lobsters were males with a carapace length exceeding 80 mm. Lobsters were molt-staged using the methods of Aiken (1980); all were C4 or D0 (intermolt) stage. All lobsters were acclimated in tanks at this temperature for 2 wk prior to the start of the experiment. Two early mortalities, one each in a control and a treatment tank, reduced group numbers to 11 each.

After acclimation, but prior to the start of the experiment, *in vivo* hemolymph pH was measured for all lobsters in post branchial blood in the dorsal cardiac sinus using a 16 ga combination needle pH microprobe (Microprobes Inc.). A sample of hemolymph (1.5 mL) was also drawn for serum chemistry analysis. A second sample of hemolymph was drawn from a prebranchial sinus (base of the 3rd walking leg) as described later for the

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phagocytosis assay. The values for these samples were used as the "before" and "T0" measurements in statistical analyses or blood chemistry and immunocompetence, respectively.

After acclimation, control tanks were maintained at 16°C for a further 31 days. Treatment tanks were raised gradually to 23°C over the course of 7 days. Once at this temperature, treatment lobsters were maintained for a further 24 days. On day 14 (the 7th day at 23°C) a sample of hemolymph was drawn for phagocytosis assay as described later, to provide a midpoint in this measurement ("T1"). It was not possible to collect a midpoint sample for serum chemistry analyses because the volume of blood required was considered too stressful. The experiment was terminated on day 31 ("T2") following complete mortality in one 23°C tank, reducing the treatment group from 11 animals to 8.

Blood Processing

Hemolymph samples for serum chemistry analysis were allowed to clot in a Vacutainer tube (Becton Dickinson, no additive), for at least 1 h held on ice. Clotted hemolymph samples were then disrupted with a glass rod and aliquots of the homogenate transferred to 2 mL microcentrifuge tubes and centrifuged at ×12,100*g* for 7–15 min, depending on the degree of clotting. Serum was decanted to fresh microcentrifuge tubes and frozen at −80°C until the time of analysis.

Serum panel analyses were performed at the New York State Veterinary Diagnostic Laboratory, Cornell University, using a Hitachi 917 blood chemistry analyzer. We used a small companion animal panel consisting of the following indices: sodium (Na), potassium (K), chloride (Cl^-), bicarbonate (HCO_3), anion gap, Na:K, urea, creatinine, calcium (Ca), phosphate (PO_4), magnesium (Mg), total protein, albumin, globulin, albumin/globulin ratio, glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, gamma glutamyltransferase (GGT), total bilirubin, direct bilirubin, indirect bilirubin, amylase, cholesterol, creatine kinase (CK), iron (Fe), transferrin iron binding capacity (TIBC), % iron saturation (%Sat), lipemia, hemolysis and icterus. Of these, creatinine, the 3 bilirubin indices, GGT, amylase, hemolysis and icterus displayed no detectable signal and will not be considered further here.

Immunocompetence Studies

For each lobster on each sampling occasion, 0.6 mL hemolymph was harvested and directly subdivided into 2 fractions: 0.1 mL was diluted in 10% formalin solution in seawater and used for total hemocyte quantifications, and 0.5 mL immediately diluted in 4.5 mL ice-cold Lobster Hemolymph Media (LHM, Paterson & Stewart 1974) supplemented with L-cystine (45 mg/mL).

Hemolymph samples diluted in LHM-cystine were then centrifuged at ×20*g* for 10 min, and sedimented hemocytes were resuspended in 0.5 mL LHM (without cystine). Resuspended hemocytes (100 µL) were placed in each well of a 96-well black microplate (Becton Dickinson), then added to each of three well replicates and incubated for 1 h to allow cells to adhere to the bottom of the wells. The supernatant was then replaced with 100 µL of test particles in LHM. Test particles were *Vibrio parahaemolyticus* cells, previously labeled with fluorescein isothiocyanate (FITC) as described by Allam et al. (2001). Hemocyte to bacteria ratio was in the order of 1/50. Plates were then covered and incubated for 2 h at 16°C or 23°C, according to the treatment group. A fourth well containing dead (formalin fixed) hemocytes was incubated with bacteria as earlier mentioned and used as a negative control.

After incubation, the supernatant was removed and 100 µl of trypan blue (250 µg/mL, pH 4.4 in citrate buffer) was added to quench extracellular fluorescence. After 60 s, the dye was removed and the fluorescence intensity (relative fluorescence unit, RFU) was immediately determined at 485/530 nm excitation/emission wavelength on a microplate reader. Results presented here correspond to the average of RFU from the three experimental wells minus RFU from the control well. All RFU were *a posteriori* adjusted to correct for differences in total and differential hemocyte counts.

Statistical Analyses

Statistical analyses were carried out using Minitab (Minitab Inc.). Before-after analyses were carried out using paired *t*-tests for each analyte. Comparisons of mean analyte change between groups were made using 2-sample *t*-tests. Analysis of phagocytosis data was made using 1-way ANOVA on log10 transformed data and deviant data identified with Fisher multiple pairwise comparisons. In the ANOVA, all T0 (initial) observations were combined, regardless of temperature group, because no significant difference between groups was detected at the start of the experiment. Differences were considered significant at $P < 0.05$.

RESULTS

The effects of experimental conditions on blood chemistry parameters in the control group are shown in Table 1. The effects of experimental conditions and prolonged thermal stress on the treatment group are shown in Table 2. The differences in blood chemistry between the control and treatment groups, and thus the effects of prolonged thermal stress, are shown in Table 3. These results show the strongest changes in acid-base indices, particularly depression of hemolymph pH, consistent with hyperthermic acidosis.

TABLE 1.

Significant before-after differences in serum chemistry-control group.

Analyte	Mean Before	Mean After	*t*-Value	*P*-value
Anion gap	6.1	−25.5	8.88	<0.0001
Mg	19	38	−6.61	<0.0001
TIBC	14.0	48.3	−6.00	<0.0001
Na	438	420	4.50	0.001
Alkaline phosphatase	17.8	4.7	4.65	0.001
Cl	434.5	451	−4.42	0.002
"Albumin"*	2.58	2.29	3.14	0.012
Creatine kinase	2.0	9.7	−3.03	0.014
Ca	67.2	71.6	−2.93	0.017
pH	7.28	7.41	−2.18	0.019
Total protein	3.53	3.24	2.35	0.043
HCO_3	4.1	2.5	2.3	0.047

* The albumin value supplied by the diagnostic laboratory was derived from total protein minus the globulin fraction. Because lobsters do not have albumins, this fraction is probably composed principally of hemocyanin and other high molecular weight proteins.

TABLE 2.

Significant before-after differences in serum chemistry high temperature group.

Analyte	Mean Before	Mean After	*t*-Value	*P*-value
Anion gap	7.25	−19.5	11.42	<0.0001
Cl	397	435	−7.50	<0.0001
Mg	13.38	30.0	−11.07	<0.0001
"Albumin"*	2.36	1.78	5.73	0.001
pH	7.4	7.2	5.07	0.001
Alkaline phosphatase	18.63	0.56	4.68	0.002
HCO_3	6.62	2.50	4.31	0.004
TIBC	13.25	28.25	−4.08	0.005
Total protein	3.2	2.64	3.81	0.007
Creatine kinase	2.0	12.38	−3.42	0.011
PO_4	0.76	1.15	−2.87	0.024
Ca	61.81	68.44	−2.6	0.034
K	4.92	5.40	−2.52	0.04

* See note in Table 1.

Additional changes were observed in serum electrolytes and transferrin ion binding capacity.

The effects of prolonged thermal stress on phagocytic activity (corrected for total hemocyte counts) are shown in Table 4 and Figure 1. Control and treatment lobsters began with statistically-similar phagocytic activity. There was strong depression of phagocytic activity over the course of the experiment in the treatment group. Some loss occurred in the control group by the end of the experiment but at both monitored time points the effect was significantly stronger among treatment group animals. The effects of prolonged thermal stress on total hemocyte count are shown in Table 5 and Figure 2. Hemocyte counts increased over the course of the experiment and much more so in treatment than in control animals.

DISCUSSION

Several significant changes were evident over the course of the experiment in both groups, suggesting that captive conditions in recirculating systems affect the physiologic state of lobsters. Significant differences occurred, however, between treatment and control animals in serum chemistry and immunocompetence, allowing us to separate captivity effects from the effects of prolonged thermal stress. Interpretation of serum chemistry is here discussed by analogy with vertebrate systems, but it should be stressed that functional homology between serum analytes in lobsters with those in vertebrates has not yet been demonstrated. Indeed, in some instances (particularly enzyme analytes), it has not been demonstrated that the test is in fact measuring the lobster equivalent of the vertebrate enzyme (i.e., cross-reactivity, specificity and sensitivity have not been shown). The discussions herein, particularly those relating to enzyme indices, should instead be regarded as new and interesting hypotheses to be tested in future studies.

TABLE 3.

Significantly different delta analyte values between control and treatment groups.

Analyte	Mean Change – Control (16°)	Mean Change – Treatment (23°)	*t*-Value	*P*-value
ΔpH	+0.13	−0.20	−5.35	<0.0001
ΔNa	−17.5	+4.4	−3.70	0.002
ΔCl	+16.5	+37.5	−3.44	0.003
ΔTIBC	+34.3	+15.0	2.68	0.017
ΔHCO_3	−1.67	−4.13	2.19	0.044
Δ"Albumin"*	−0.29	−0.59	2.15	0.047

* See note in Table 1.

TABLE 4.

ANOVA results for effects of prolonged thermal stress on standardized phagocytic activity assay.

Source (df)	MS	F Statistic	*P* Value	Significant Pairs
Time (4)	0.3827	6.75	<0.001	T0 > T1(23)
				T0 > T2(23)
				T1(16) > T1(23)
				T1(16) > T2(23)
				T2(16) > T1(23)
				T1(16) > T2(16)
Error (49)	0.0567			

In vivo pH measurements showed the development of a marked hemolymph acidosis in treatment animals. This result is consistent with field data showing development of acidosis in animals from eastern LIS in late August and September, when water temperatures are highest (Dove et al., data not shown). According to Stewart et al. 1966, (cited again in Martin & Hose 1995) lobsters regulate their hemolymph pH at 7.6, but our results agree more with Rose et al. (1998) that resting hemolymph pH in lobsters is closer to 7.75–7.78. The finding of a significant pH acidosis in this study and in our field study suggests substantial loss of acid-base homeostasis at these temperatures.

When compared with pH, other acid-base indices gave results that were more difficult to interpret. Anion gap, a calculated index of acid-base status incorporating several electrolyte measurements,

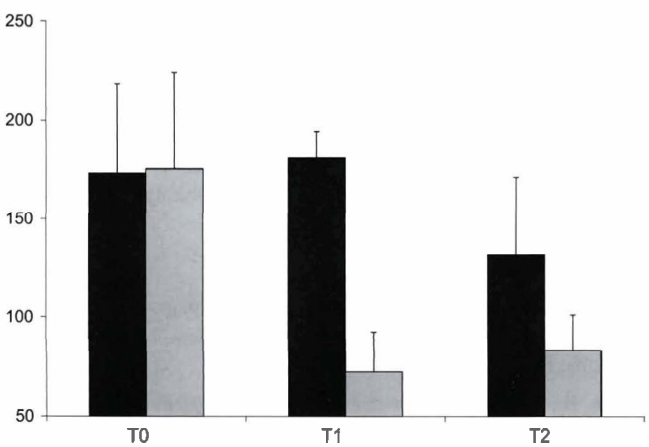

Figure 1. Change in standardized phagocytic activity (arbitrary relative fluorescence units) during prolonged thermal stress in the lobster, *Homarus americanus*. Error bars are standard error of the mean. Dark = 16°C, light = 23°C

TABLE 5.

ANOVA results for effects of prolonged thermal stress on total hemocyte count.

Source (df)	MS	F Statistic	*P* Value	Significant Pairs
Time (4)	0.1457	2.88	0.032	T0 < T1(23) T1(16) < T1(23) T2(16) < T1(23)
Error (49)	0.0506			

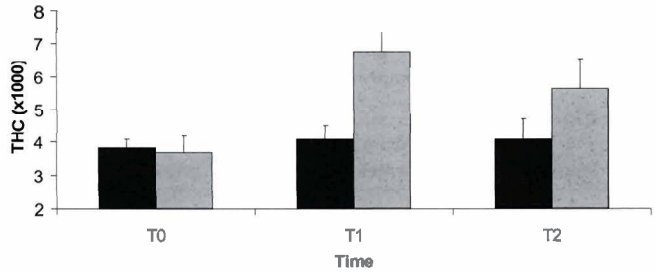

Figure 2. Effects of prolonged thermal stress on total hemocyte count of the lobster, *Homarus americanus*. Dark = 16°C, light = 23°C

became strongly negative over the course of the experiment in control and treatment animals. Negative anion gap is an unusual finding in vertebrate animals and is usually associated with hyperchloremia or other hyperhalogen conditions. Our data showed strong idiopathic increase in serum chloride in all animals in the absence of concomitant increase in serum sodium and was thus consistent with the causes of negative anion gap in vertebrate systems. It seemed that hyperchloremia under these experimental conditions may have masked any utility of the anion gap measurement for understanding acid-base status in lobsters. Similarly, bicarbonate concentration in treatment and control animals dropped to below the testing detection limits. In vertebrate systems, this analyte measures buffering capacity of the blood. The occurrence of a slight increase in hemolymph pH in control animals with concurrent decrease in bicarbonate is cognitively dissonant. Clearly acid-base status in lobsters cannot be simply explained using vertebrate tests. The presence of an unequivocal pH acidosis, however, is species-independent and demonstrates a strong disruption of acid-base homeostasis in the high temperature treatment group, perhaps by excess lactate production from anaerobic metabolism.

The next most significant changes in blood chemistry related to the ions sodium and chloride. Our field data (not shown) suggest that these two ions are always tightly correlated in wild lobsters ($r^2 = 0.98$), and their divergence in our experimental animals of both groups over the course of the experiment is intriguing. In particular, the marked hyperchloremia suggests exogenous chloride inputs from some other salt source. Given that both groups showed significant increases in magnesium, we suspect that this ion may have been the cation conjugate for much of the excess chloride, though the source remains unclear.

The increase in "albumin" is more correctly an increase in nonglobulin proteins in the hemolymph, because lobsters lack albumins and the albumin figure is derived by subtraction as total protein minus directly-measured globulins. This indirectly-measured fraction of hemolymph protein is probably dominated by hemocyanin and its increase may reflect a coping mechanism for increased metabolic oxygen demand from increased temperature.

Our experiment showed significant effects of prolonged thermal stress on total hemocyte count and standardized phagocytic activity. After a week of increasing temperatures 1°C per day and a further week held at 23°C, phagocytic activity of lobster hemocytes was reduced more than 60%. Presumably, such a change would have a profound effect on the ability of the lobster to defend against bacterial and other pathogens. This notion is certainly consistent with the results of other researchers who have showed depression of immunocompetence from thermal stress in lobsters. Steenbergen et al. (1978) showed that at 6°C, 16°C, 18°C and 20°C *in vitro* phagocytic activity of *H. americanus* hemocytes was normal but that at 22°C and 24°C, activity was depressed 75%. They concluded that temperatures above 22°C exceed the immunocompetence range of mature lobsters; indeed, they cautioned against exposing adult lobsters to temperatures above 20°C for this reason. Similarly, Paterson & Stewart (1974) showed that temperatures of 20°C and above are "completely unsatisfactory" for the *in vitro* maintenance of lobster hemocytes.

We demonstrated, using a simple repeated measures experiment, significant deleterious effects of prolonged thermal stress on American lobsters, *Homarus americanus*. The temperatures and durations used in our treatment group occur in Long Island Sound in late summer and fall, suggesting that it is very likely a stressful environment for lobsters at these times. Recent warm winters associated with climate change phenomena (1998, 1999, 2002) may exacerbate the problem, not by increasing the absolute maximum water temperature to which lobsters are exposed, but by increasing the duration of exposure. Because we have shown that duration of exposure may be as important as the actual stressful temperature, we propose that climate change phenomena may make LIS inhospitable to lobster survival in the long term.

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