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research paper

Laboratory studies on the effect of temperature on epizootic shell disease in the American lobster, *Homarus americanus*

[1] *Virginia Institute of Marine Science, The College of William & Mary, P.O. Box 1346, Gloucester Point, Virginia 23062.*

[2] *Massachusetts Division of Marine Fisheries, 1213 Purchase St., New Bedford, Massachusetts 02740.*

[3] *Massachusetts Division of Marine Fisheries, 30 Emerson Ave., Gloucester, Massachusetts 01930.*

* *Corresponding author email: <jeff@vims.edu>, telephone: 804-684-7128, fax: 804-684-7186.*

Brittnee N Barris [1]
Jeffrey D Shields [1]*
Hamish J Small [1]
Juan Pablo Huchin-Mian [1]
Patricia O'Leary [1]
Josephine V Shawver [1]
Robert P Glenn [2]
Tracy L Pugh [3]

ABSTRACT.—Epizootic shell disease (ESD) is a persistent threat to the population of American lobsters, *Homarus americanus* H. Milne-Edwards, 1837, in Long Island Sound and off southern New England, USA. ESD is caused by a bacterial dysbiosis that occurs in association with increased water temperature and exposure to anthropogenic stressors. Temperature is a leading factor driving the severity and incidence of ESD. Our objective was to quantify disease progression and dynamics in relation to host molting and mortality at three rigorously controlled temperatures (6, 12, and 18 °C) over a 5–6-mo period. Lobsters were photographed at various time points and image analysis was used to examine changes in lesion development over time. The disease progressed at all three experimental temperatures, but it had a significantly faster growth rate at 18 °C. Mean progression rates varied from 8.6–10.4 $mm^2\ d^{-1}$ at the lower temperatures to >25.6 $mm^2\ d^{-1}$ at 18 °C. The mean daily growth rates give conservative estimates for individual progression from light to moderate disease states; i.e., approximately 233 d at 6 °C and 95 d at 18 °C. We show that increased temperature leads to rapid progression of ESD, but individual variation, presumably modulated through immune defenses, can slow the disease and possibly enhance survival of affected lobsters.

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Kari Lavalli, Richard Wahle
Guest Section Editor:
Donald Behringer

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The population of American lobsters, *Homarus americanus* H. Milne-Edwards, 1837, off southern New England is threatened by epizootic shell disease (ESD). The long-term impact of the disease on the fishery has been hard to quantify, but ESD has proven to be persistent and expansive, spreading to most of the nearshore lobster population in Long Island Sound (LIS) and off southern New England (Shields 2013) (approximately 41°8.162′N, 72°40.136′W). The direct effect on the fishery is that lobsters with ESD are not marketable in the lucrative live trade due to extensive necrosis of the carapace and claws; instead their meat is processed in the much less lucrative

canned meat trade. Indirectly, and more importantly, the disease has affected commercial landings (Cobb and Castro 2006, Castro and Somers 2012, Howell 2012) through reductions in egg production (Wahle et al. 2009) resulting from mortality of ovigerous females (Hoenig et al. 2017). The concern is that the disease will continue unabated, effectively thwarting recovery of the southern New England stock.

The etiology of ESD has been characterized as a bacterial dysbiosis that occurs in concert with exposure to environmental (increasing temperatures, decreasing pH, hypoxia) and anthropogenic (nutrient-driven hypoxia, contaminants) stressors (Tlusty et al. 2007, Chistoserdov et al. 2012, Laufer et al. 2013, Shields 2013). Increased temperature is a major environmental component of ESD (Glenn and Pugh 2006); however, the role of temperature and other environmental stressors associated with ESD have not been fully elucidated. High prevalence levels in lobsters from eastern LIS and Buzzards Bay, Massachusetts, suggests that widespread phenomena, such as increased temperature or reduced pH, are involved in the disease, rather than point-source contaminants or local issues with water quality. Nonetheless, some contaminants, notably alkylphenols and heavy metals, have been found in lobsters and sediments from the region, but their roles in ESD are not clear (Jacobs et al. 2012, Leblanc and Prince 2012, Laufer et al. 2013). Our present understanding of the etiology is that increased temperatures negatively affect host defensive responses, with contaminants potentially weakening the cuticle by interfering with sclerotization, making it more susceptible to a dysbiotic bacterial community brought about by anthropogenic effects to water quality (Shields 2013). Lower temperatures may limit the spread of ESD (Glenn and Pugh 2006), but this has not been examined experimentally.

The sustainability of the lobster fishery off southern New England is at significant risk due to the emergence of epizootic shell disease. Our goal is to understand and quantify how temperature affects lobsters with ESD and to better understand the disease dynamics in relation to increasing temperatures in the region. Our objectives were to undertake experimentally-controlled temperature experiments using lobsters with ESD to examine how temperature modulates disease progression and severity, host molting, and disease-associated mortality. We quantified essential disease processes in relation to different environmentally realistic temperatures and associated these processes with important host factors.

Materials and Methods

Temperature Treatments.—Two shipments of lobsters were acquired in late May 2015 from M Trainor, Massachusetts Division of Marine Fisheries, from the region west of Vineyard Sound (around 41.36°N, 70.94°W). Due to some mortalities from these shipments, a third shipment arrived in mid-June. The number of animals in each treatment was, therefore, staggered to account for different start dates. Upon arrival, the animals were randomly assigned to one of three different temperature treatments (6, 12, or 18 °C). Sixty-five female and fourteen male lobsters were randomly assigned by stratum (uninfected, light, moderate, or heavy) to each temperature treatment (Table 1). At the time of their assignment to a treatment group, each animal was assessed for disease and categorized according to Landers (2005). Disease states were: no visible disease = healthy, no scarring; light = shell disease <10% of body surface with lesions; moderate = shell disease 11%–50% of the body; and heavy

Table 1. Disposition of lobsters in the final design of the study, sorted according to temperature treatment and initial disease status (light, moderate/heavy, none). Note that 11 animals were excluded of the 79, due to mortality within the acclimation period of the study (<30 d), and insufficient data. Sixty-eight animals were included in the data analysis, 50 of which were diseased. (*) denotes number of animals dying during shipment and acclimation period <14 d. These animals were excluded from data analysis (n = 11).

Treatment	Light	Moderate	None	Total
6 °C	12 (*3)	13 (*2)	5 (*1)	30
12 °C	14 (*1)	4 (*2)	7	25
18 °C	10	6 (*1)	8 (*1)	24
Total	36 (*4)	23 (*5)	20 (*2)	79 (*11)

= with shell disease >50% of the body. Because several heavily infected lobsters died during transportation and acclimation, for statistical purposes, the moderate and heavily infected animals were pooled for the analysis. Animals were also assessed at the end of the experiment, and the change in their disease state was noted.

Lobsters were housed individually in aquaria and given an acclimation period of 8–10 d prior to any additional handling. Lobsters dying during the acclimation period were replaced (n = 11). After the acclimation period, the animals were measured, sexed, photographed, and assessed for disease and injury. The dorsal aspect of each lobster was photographed (Olympus Tough 3 digital camera) with a ruler at a 90° angle. Of the 79 lobsters received in three shipments, 20 were identified as control animals, i.e., they had no signs of shell disease.

Each lobster was held individually in a 34-L static aquarium equipped with a single recirculating bio-filter (Tetra Whisper 40, filled with activated charcoal), an aerator, and a benthic substrate of approximately 3 cm crushed coral. Aquaria were filled with artificial seawater (Marine Mix) at 33–35 and fitted with custom-fitted plastic lids to reduce aerosol contamination. Lobsters were fed twice per week, alternating between cut fish (spot, *Leiostomus xanthurus* Lacépède, 1802) and squid. Uneaten food was noted and removed after 24 hrs. Ammonia (total ammonia as NH_3-H), nitrate, nitrite, pH (water quality kits, http://www.hach.com), and salinity (refractometer) were monitored three times per week on three randomly chosen tanks within each temperature treatment. All aquaria had 50% water changes at least once per week to maintain water quality. Electric pumps were equipped with varimax current adaptors to maintain control of the flow rates used to move clean artificial seawater from 210-L reservoir tanks into aquaria during water changes. Siphons, hoses, and all gear used for water changes or for handling animals were disinfected in 1% bleach in fresh water for 5–30 min between uses. Two to three separate sets of cleaning gear were cycled through the bleach disinfection to minimize contamination among aquaria.

Due to space and temperature limitations, lobsters were held in two separate rooms in two different types of systems. Lobsters held in the 6 °C treatment were housed in static aquaria equipped as above on racks in a large walk-in refrigerated environmental chamber. Pieces of R-11 insulation were cut to shield lobsters from seeing each other in adjacent aquaria. For the 12 and 18 °C treatments, two large recirculating freshwater bath systems partially filled with chlorinated tap water (recharged approximately weekly) were used to house individual aquaria and maintain temperatures consistently within each treatment. Each water bath system consisted of a large H-frame rack holding an upper and lower 800-L fiberglass tank (capable of holding 12, 38-L aquaria in each tank) joined by a recirculating water pump connected to a

heat exchanger (Aqualogic) set for either 12 or 18 °C. The heat exchanger was connected to the cold-water system supplied to the building. To reduce the ability of the lobsters to see each other, a single piece of landscaping cloth was cut and glued (silicone sealant) onto each aquarium such that one side and end were screened by the cloth. A HOBO temperature data logger set inside an individual aquarium with a lobster was used to monitor temperature once per hour in each system. Temperature was also monitored via the thermistors on the heat exchangers and via a single glass thermometer placed in the whisper filter of a randomly selected aquarium in each system.

During the study period, animals were monitored according to a standard operating procedure that was followed daily. Lobsters were checked one to two times per day and any changes in their health (i.e., molt status, morbidity, mortality, feeding activity) and temperature were recorded daily throughout the study period. After approximately 1 and 2 mo into the experiment, all of the animals were photographed and assessed for disease status as above. For those animals that molted during the study period, additional shell scrapings were collected and photographs taken of the old instar. At the end of the study period, lobsters were photographed and assessed for disease and injury, prior to dissection.

Image Analysis.—Image analysis was used to calculate disease area for measurements of progression and lobster area for calculations of the relative area of the lobster affected by disease. We did not compensate for the curvature of the carapace in the analysis because the dorsal surface of an infected animal bears the brunt of the infection with ESD (Shields et al. 2012), each photograph was handled in the same manner, and the 2-D representation is highly correlated with the 3-D estimation (Stevens 2009). Because each animal was photographed from the same angle and with a scale, the comparisons between periods were internally consistent. If a lobster molted, the molt was photographed and the area of disease assessed as below. For statistical assessments of disease progression, photographs taken during the study period were analyzed by image analysis (ImageJ; https://imagej.nih.gov/ij/). An electronic drawing tablet was used to help digitize disease area and lobster area for progression analysis. In some cases, photographs were imported into Adobe Photoshop to enhance the image of the lobster for calculation of areas prior to the analysis of the diseased area.

Three methods were used to analyze measurement error associated with our image analysis technique. First, we graphed the lobster area on carapace length (CL) to examine for potential outliers and unusual cases. Photographs of lobsters whose area measurements were identified as outliers were re-examined and corrected as appropriate. In a few cases, the photographs did not match with the area measurements. For these, we removed the outlier and used the mean of the two remaining measurements (typically time at start and time at end), because individual lobsters were photographed two or three times during the experiment. If an animal did not molt, then it did not increase or decrease in size. Mean values for the total lobster area were used for statistical analysis. Second, we examined the means and standard deviations for all of the lobster area calculations for consistency and for outliers. Outliers were reassessed as above. Third, we measured and analyzed the photographs of three lobsters three times each and calculated a standard error and coefficient of variation for

Table 2. Analysis of measurement error associated with calculating lobster area and disease area from photographs (jpg files) using Image J. The standard deviations and corresponding coefficients of variation (CV) were typically below our arbitrary limits (<5% CV). * An outlier was identified in the disease area for B4. The image was re-examined and the measurement removed due to measurement error resulting in a lower CV.

Lobster ID	Lobster area (SD)	Lobster CV (%)	Disease area (SD)	Disease CV (%)
A3	22,180 (335)	1.85	7,412 (369)	4.98
AA28	22,726 (330)	1.78	2,290 (103)	4.50
*B4	24,709 (515)	2.56	682 (2)	0.35

lobster area and for disease area (Table 2). This analysis indicated that the coefficient of variation was within our arbitrarily accepted limit of 5%.

Analyses of disease area, lobster area, lobster survival, and molting were analyzed using Systat 11.0. Temperature and disease severity were analyzed using two factor ANOVA. Disease severity at the start of the experiment was used to analyze progression. Data transformations (noted within each analysis) were used to reduce variance in the data. Time-to-event analysis (survival analysis) was used to evaluate host molting and mortality over time by temperature treatment and by disease status. The Tarone-Ware log-rank test and chi-square values were used to evaluate differences in treatments in the time-to-event analyses.

Results

Temperature and Water Quality Parameters.—Lobsters were held separately in three temperature treatments: 6, 12, and 18 °C. Minor corrections were made to the long-term temperature data because short-term, ephemeral spikes were evident in the HOBO data during water changes. When these short spikes were removed as outliers, the corrected temperatures were very close to the nominal treatment values (Table 3). The standard deviations showed little variation in the chiller systems, heat exchangers, and data loggers; moreover, the upper and lower quartiles were within a degree of the nominal temperature. The temperature data indicated that lobsters were held at or very close to the experimental temperatures during the course of the study. There were, however, two exceptions in the 18 °C treatment. On days 61 and 65, the circulation pump in the system began failing; it was replaced on day 68. Temperatures in this treatment spiked twice to >25 °C during this period. No mortalities occurred in this treatment during this period nor did any occur subsequently.

Table 3. Nominal temperature treatments with uncorrected and corrected means and standard deviations (in parentheses) in temperature (°C) over the duration of the experiment. Corrected means had minor outliers removed that occurred as a result of weekly water changes coinciding with automated temperature measurements. HOBO temperature gauges took measurements every hour, glass thermometer measurements were recorded daily, and thermistor settings on the water exchangers were recorded daily (not shown). Quartiles are given for the corrected temperature data from the HOBO gauges.

Treatment group	Uncorrected mean	Lower quartile	Corrected mean	Upper quartile	Glass thermometer mean
6 °C	7.5 (0.3)	7.2	7.4 (0.2)	7.6	6.4 (0.6)
12 °C	13.0 (1.5)	12.0	12.5 (1.4)	13.7	12.8 (1.5)
18 °C	18.3 (1.7)	17.6	18.0 (0.5)	18.3	17.8 (1.0)

Table 4. Monthly means and standard deviations (in parentheses) for water quality parameters monitored during the course of the study. Measurements were taken from three randomly sampled aquaria within each temperature treatment three times per week. Ammonia, nitrate, and nitrite are in ppm, temperature values are from a glass thermometer in each system.

Treatment/month	pH	Ammonia	Nitrate	Nitrite	Salinity	Temperature
6° (mean)	7.8 (0.2)	4.5 (3.8)	46.9 (32.5)	1.4 (1.7)	34.9 (2.0)	6.5 (0.9)
June	7.9 (0.2)	5.5 (2.7)	26.8 (29.8)	3.6 (2.6)	33.2 (2.3)	7.5 (1.2)
July	7.8 (0.1)	8.0 (2.8)	24.4 (21.1)	2.0 (1.1)	34.6 (2.3)	6.9 (1.1)
August	7.9 (0.1)	6.3 (3.0)	55.5 (30.3)	1.6 (0.7)	35.4 (1.3)	6.2 (0.4)
September	7.8 (0.2)	2.8 (3.4)	55.0 (38.4)	0.5 (1.0)	36.0 (1.7)	6.4 (0.7)
October	7.6 (0.2)	0.5 (0.5)	67.8 (15.3)	0.1 (0.2)	34.9 (1.3)	6.0 (0.0)
November	7.7 (0.2)	0.4 (0.3)	73.3 (10.3)	0.4 (0.5)	34.6 (0.9)	6.0 (0.0)
12° (mean)	7.8 (0.2)	2.3 (3.3)	54.6 (37.3)	1.8 (2.5)	35.1 (1.4)	12.8 (1.6)
June	7.9 (0.1)	7.2 (1.7)	61.2 (39.6)	4.4 (1.3)	34.7 (1.5)	12.5 (1.7)
July	7.9 (0.3)	4.4 (3.9)	40.1 (28.0)	1.9 (1.1)	34.6 (1.6)	12.9 (1.3)
August	7.8 (0.2)	0.4 (0.7)	52.8 (32.2)	1.9 (4.4)	35.5 (0.8)	14.6 (1.6)
September	7.8 (0.2)	0.2 (0.4)	52.7 (29.6)	0.4 (0.9)	34.9 (1.6)	13.1 (1.1)
October	7.7 (0.2)	0.3 (0.3)	52.4 (24.2)	0.3 (0.5)	35.7 (1.1)	11.6 (0.8)
November	7.7 (0.2)	0.4 (0.3)	77.4 (63.9)	2.0 (2.1)	35.5 (0.9)	11.9 (0.5)
18° (mean)	7.8 (0.2)	1.9 (3.1)	55.4 (39.7)	1.5 (1.8)	35.4 (1.3)	18.0 (1.2)
June	7.8 (0.2)	6.3 (2.6)	50.5 (42.0)	3.7 (1.8)	34.7 (1.3)	18.1 (0.9)
July	7.9 (0.2)	2.0 (3.3)	39.5 (28.5)	1.6 (0.9)	34.9 (1.6)	17.7 (1.1)
August	7.8 (0.2)	0.3 (0.2)	62.2 (40.2)	0.9 (1.0)	35.9 (0.9)	17.6 (0.5)
September	7.8 (0.3)	0.3 (0.5)	67.4 (41.4)	0.7 (1.7)	35.3 (0.7)	18.6 (2.4)
October	7.8 (0.2)	0.2 (0.2)	67.2 (37.1)	0.2 (0.3)	36.4 (1.3)	18.0 (0.4)
November	7.8 (0.2)	0.3 (0.1)	40.3 (41.8)	1.0 (1.6)	35.9 (1.1)	18.0 (0.0)

Water quality varied considerably during the preliminary months of the study. The profile for total ammonia and nitrite in the 6 °C treatment indicated somewhat high levels of ammonia during the first months of the experiment (means = 5.5–8.0 from June to August), with it cycling down starting in August. This was expected as we did not precondition the tanks to avoid introducing foreign bacteria to these systems prior to the introduction of each lobster. Nonetheless, pH levels were consistently between 7.6 and 7.9 (Table 4), ensuring that ammonia, nitrate, and nitrite levels were not toxic to the lobsters. Ammonia started cycling to lower levels after approximately 2 mo in each temperature, with nitrate and nitrite taking somewhat longer to cycle (Table 4). [N.B., the levels of unionized ammonia in our study systems were never above 0.24 mg L^{-1} (Hach.com DOC326.98.00007), which is more than an order of magnitude below the LD_{50} reported for adult lobsters (Young-Lai et al. 1991). Our values for nitrate and nitrite were several orders of magnitude lower than those reported as toxic for other crustaceans (Romano and Zeng 2013).]

Effects of Temperature on Molting and Survival.—The presence of ESD was significantly associated with the time to molting for the affected hosts (Fig. 1A). Approximately 40%–50% of the diseased lobsters molted during the study, whereas those without disease did not molt (χ^2 = 10.723, df = 2, P = 0.005). There were no differences in molting between lobsters with light or moderate disease severity (χ^2 = 0.300, df = 1, P = 0.584). In addition, there was no difference in the time-to-molt among temperature treatments (Fig. 1B) (χ^2 = 2.025, df = 2, P = 0.363). Lobsters molted throughout the experiment, but their molt stage was not determined prior

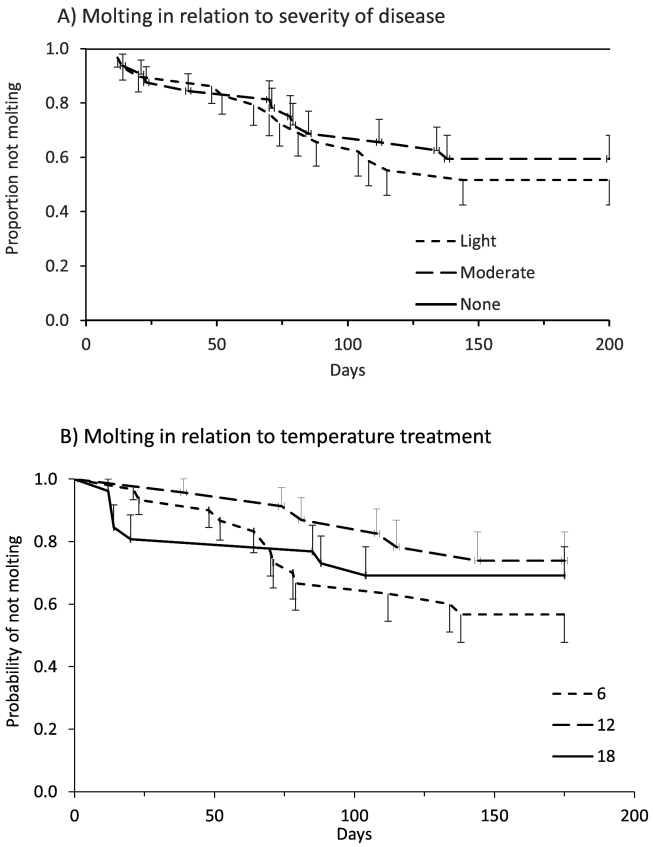

Figure 1. Time-to-event analyses for molting in relation to (A) severity of disease and (B) temperature treatment. Severity is given as none, light, or moderate. Temperature was either 6, 12, or 18 °C. Only the upper or lower standard error bars are shown for clarity. Diseased lobsters molted significantly more than healthy lobsters, but there was no effect of temperature over the study period.

to entering the experiment. With two exceptions, lobsters molting out of the disease did not reacquire shell disease.

Lobsters in the experiment showed high survival in general, and this did not differ between temperature treatments (Fig. 2A) ($\chi^2 = 1.176$, df = 2, $P = 0.555$). An a priori power analysis for sample sizes for mortality and molting indicated a power >80% for detecting a difference in survival with an effect size >30% between treatments. Lower effect sizes rapidly increase the necessary sample size to detect a true difference within the experiment. Survival was relatively high with respect to disease severity, but did not differ among lobsters with different severity levels (Fig. 2B) ($\chi^2 = 2.380$, df = 2, $P = 0.304$). Although uninfected lobsters showed the highest survival, there were no differences in survival between healthy and diseased lobsters. There were no differences in mortality between different disease states, but relatively fewer animals were present in the moderate category (*see* below) than the light category at the start of the study.

Disease Progression.—Of the 36 lobsters classified with light infections at the start of the experiment, 16 had progressed to moderate infections by the end of the

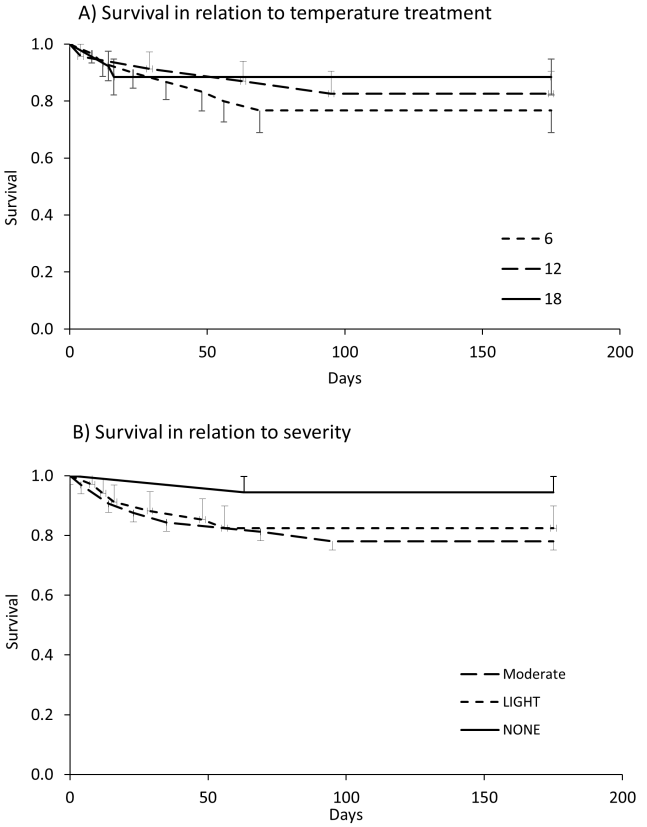

Figure 2. Time-to-event analyses for survival/mortality in relation to (A) temperature treatment and (B) severity of disease. Only the upper or lower standard error bars are shown for clarity. There were no statistical differences in either analysis.

experiment. Of these, five were in the 6 °C treatment, five in the 12 °C treatment, and six in the 18 °C treatment. There were no differences in the proportion of animals converting from light to moderate between treatments (Mantel Hanzel χ^2: $P > 0.05$). Twenty-three lobsters were classified with moderate infections at the start of the experiment. Two of the animals that molted reacquired light infections of ESD.

Changes in Disease Area and Daily Progression.—Disease progression was measured as the lesion area at the end of the study minus the initial lesion area at the beginning of the study. The molts of animals that had molted during the study were analyzed, but the new instar was assessed as healthy in all cases. The day of molting was used for calculating progression rates. Unadjusted disease progression over the course of the experiment varied significantly with temperature treatment, but not with severity of infection (light vs moderate infections) (ANOVA: $P = 0.001$, $P = 0.129$, respectively), and the interaction between these factors was not significant ($P = 0.490$) (Fig. 3). Unadjusted disease progression was slower in the 6 and 12 °C treatments compared to the 18 °C treatment (Fig. 3). Mean values for unadjusted disease progression indicated that increases in lesion area occurred in all treatments, but that the 6 °C treatment had the slowest progression [mean = 930 (SE 263) mm],

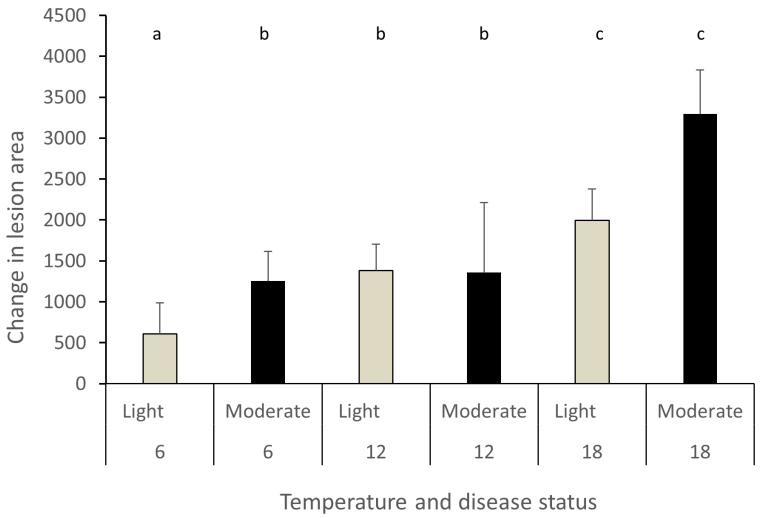

Figure 3. Change in lesion area in relation to disease severity during the study. Disease status (light and moderate) and temperature treatments (6, 12, 18 °C) were main factors. Change in lesion area was calculated as the lesion area at the end minus the lesion area at the start. Treatments with different letters are significantly different from each other. Bars indicate standard error.

that was not significantly different than that for the 12 °C [mean = 1371 (SE 456) mm], but both were lower than that for the 18 °C treatment [mean = 2645 (SE 330) mm]. Although the variance was relatively high in the 12 °C treatment, a square-root transformation of the data yielded the same result.

Daily change in lesion area, or daily progression rate, was calculated as the final lesion area minus the initial lesion area divided by the number of days with disease. The latter variable was based on either (1) possessing disease at the end of the experiment, (2) the number of days before molting out of the disease, or (3) the number of days with disease prior to death. The daily progression rate varied significantly with temperature treatment, but not with disease severity (light vs moderate infections) (ANOVA: $P = 0.003$, $P = 0.857$, respectively) (Fig. 4A), and the interaction between these factors was not significant ($P = 0.483$) (Fig. 4B). Daily progression was slower in the 6 and 12 °C treatments compared to the 18 °C treatment (Fig. 4B). Least squares–adjusted mean values for daily disease progression indicated that increases in lesion area occurred most significantly in the 18 °C treatment [mean = 25.6 (SE 3.6) mm^2 d^{-1}]; albeit daily growth was noted in both the 6 [mean = 10.4 (SE 2.9) mm^2 d^{-1}] and 12 °C [mean = 8.6 (SE 4.9) mm^2 d^{-1}] treatments. Least-squares mean values for daily disease progression between light and moderate infections were not different. Given the relatively large variances in the mean daily progression rates, a square-root transformation was performed, but the pattern was largely unchanged, with temperature the only significant factor in both analyses.

From the daily progression rate, we estimated the time that the disease would take to initiate as a light infection and progress to become a moderate infection (Table 5). Moderate infections are defined as >10% of the lobster body area covered with lesions; that is, infections arbitrarily transition from light to moderate at 10% of the body area of the lobster. Given the daily progression rates of 8.65–25.62 mm^2 d^{-1}, depending on temperature, we estimated the number of days using the simple formula:

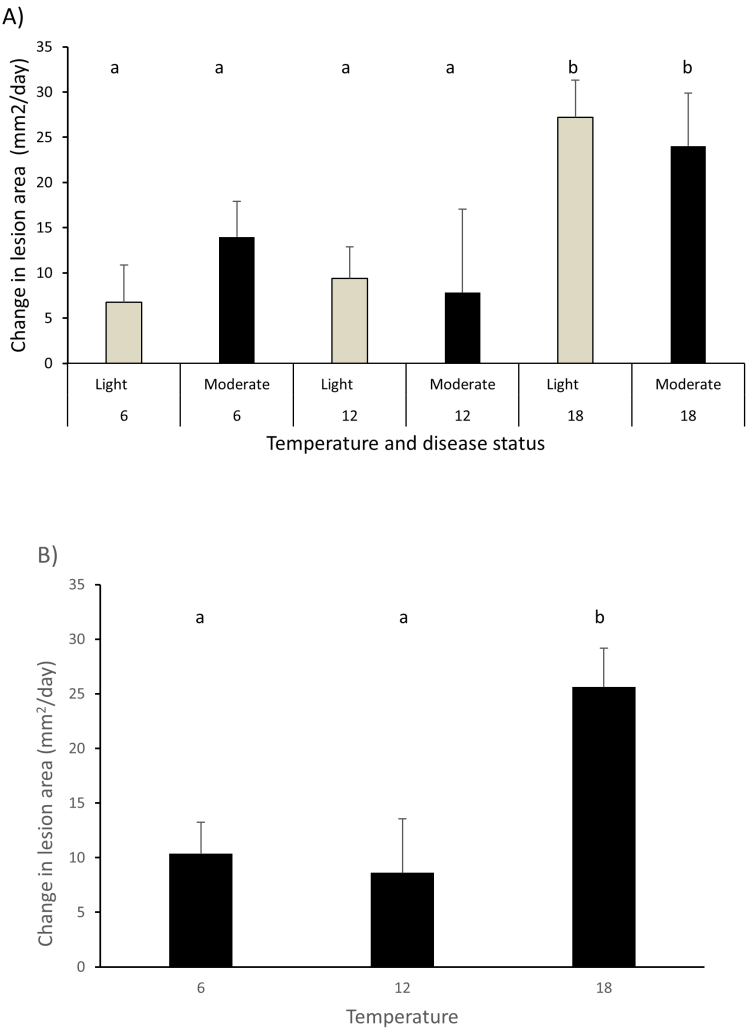

Figure 4. Progression rate as change in lesion area per day (A) in relation to disease severity, and (B) as least squares adjusted means in relation to temperature. Disease status (light and moderate) and temperature treatments (6, 12, and 18 °C) were main factors. Progression rate equals the change in lesion area divided by the number of days with disease. Treatments with different letters are significantly different from each other. Bars indicate standard error.

Table 5. Least squares adjusted means for the daily progression of disease ($mm^2\ d^{-1}$) and estimated number of days for an 80–88 mm carapace length lobster to transition from lightly infected to moderately infected (>10% of the body area covered with disease lesions). Within columns, groups with the different superscript letters were significantly different (two-way ANOVA: $P < 0.003$).

Treatment	*n*	Progression (SE)	Number of days (SE)
6 °C	10	10.4 (2.9) [a]	232.9 (28.0) [a]
12 °C	14	8.7 (4.9) [a]	274.4 (28.5) [a]
18 °C	10	25.6 (3.6) [b]	94.7 (9.6) [b]

0.10 (the proportional area affected) × lobster body area (mm^2) / daily progression rate ($mm^2\ d^{-1}$). Although the study was not designed to estimate transition in disease state from uninfected to lightly infected, once an animal had a light infection, approximately 3 mo would be required at 18 °C for it to progress to a moderate infection (Table 5). Individual trajectories in disease progression highlighted the variability in lesion growth rates over time (Fig. 5). ESD on some lobsters progressed very quickly, whereas on other lobsters it was much slower, showing little if any significant increase. This was particularly true for animals having moderate infections (>10% of their body area or lesions >2100 mm^2).

Discussion

We undertook an independently replicated, strictly controlled laboratory experiment to gauge the effect of temperature on epizootic shell disease (ESD) and different host processes. Mature lobsters (80–90 mm CL) were held at three different temperatures to examine the relationships between temperature, lobster growth, and the progression of epizootic shell disease. We found that the disease progressed at all three temperatures (6, 12, and 18 °C). Surprisingly, progression occurred in the 6 and 12 °C treatments, albeit it was much slower than that in the 18 °C treatment. Because our experiment was done in a well-controlled system, we were able to calculate the daily rate of progression of the disease at different temperatures. Prior to this experiment, the daily progression had not been examined in a controlled fashion. Mean progression rates varied from 8.6 to 10.4 $mm^2\ d^{-1}$ at the lower temperatures to >25.6 $mm^2\ d^{-1}$ at 18 °C. These rates allow us to estimate the number of days for ESD to progress from the lightly to moderately infected state (*see* Table 5). They represent the first estimates of ESD progression in adult lobsters under controlled laboratory conditions.

Three field and three previous laboratory studies have examined temperature effects on different types of shell disease in lobsters. For the field studies, Glenn and Pugh (2006) showed a significant latitudinal trend in ESD associated with temperature. The trend was thought to coincide with reductions in molting frequency (increased instar duration) associated with maturation in female lobsters that occur in Long Island Sound (Briggs and Mushacke 1979, Landers et al. 2002). In other field studies, Landers et al. (2011) and Howell (2012, with Landers' temperature data) showed that the prevalence of ESD increased markedly in eastern LIS in relation to the persistence of annual degree days above 20 °C in contiguous years. Our finding of rapid progression at 18 °C is consistent with the findings of these other studies; however, our findings of consistent but slow progression at 6 and 12 °C are noteworthy because lesions are not thought to develop and progress at these temperatures.

In a laboratory study, Stevens (2009) examined the outcomes of lobsters with ESD held communally in a flow-through, ambient seawater system. Their study analyzed changes in disease progression in relation to ambient water temperature averaged over all of the animals with ESD. Their quantitative disease index was based on percent changes in area affected and, therefore, not directly comparable to the direct estimates ($mm^2\ d^{-1}$) in our study. Importantly, in Stevens' (2009) study, some lesions exhibited regression of disease with winter temperatures, several animals molted during the winter months, and some deaths were due to ESD. Three lobsters in our study appeared to have regression of disease, but this may have been an artifact of

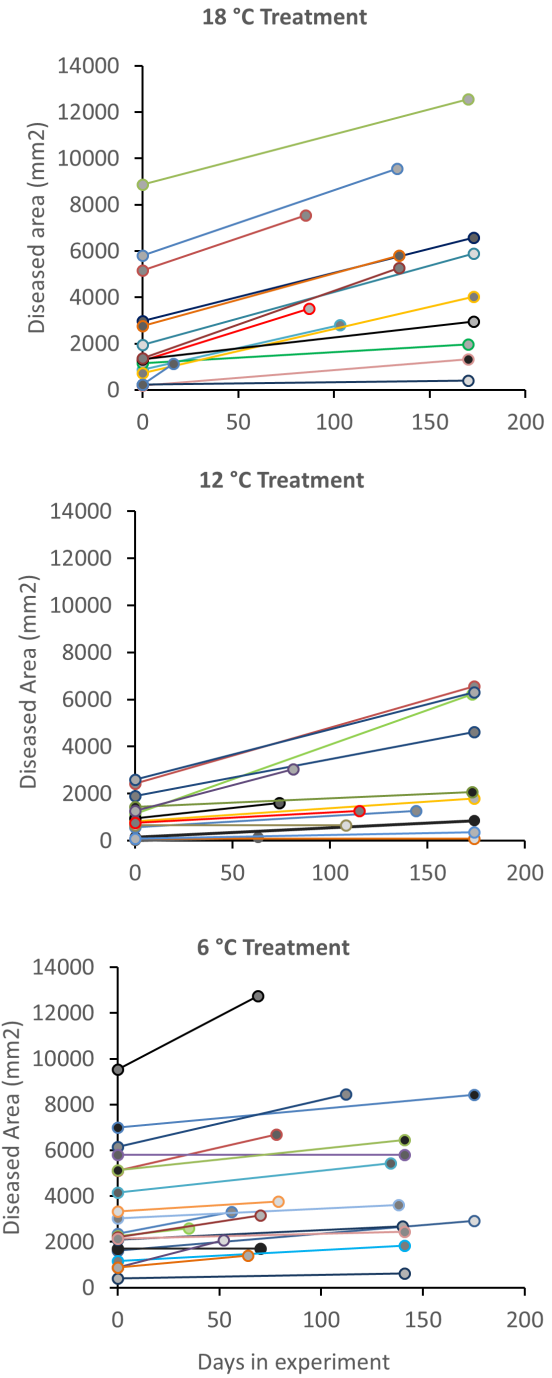

Figure 5. Individual trajectories (as different colored lines) for disease progression for lobsters in different temperature treatments from the start of the experiment to the end of the experiment, death or molting. Light infections are generally <2100 mm^2. Individual variation in response to the disease is apparent. A higher rate of disease progression is also apparent for animals with moderate infections (i.e. >2100 mm^2 of disease) at 12 and 18 °C.

measurement error introduced by the image analysis methods. In our experiment, 16 animals transitioned from light to moderate infections over the course of the experiment.

Tlusty and Metzler (2012) presented data on the effects of temperature (10, 15, 20 °C) on laboratory-induced shell disease in small juvenile lobsters as a model system. Their lobsters (10–20 mm CL) were held separately, but within common recirculating systems. In addition, lobsters were initially fed a diet devoid of plant matter for 6 mo, and then a subset of animals were switched to a diet with astaxanthin for 1 mo to induce pigmentation. They found that temperature effects were U-shaped, with shell disease occurring most frequently in animals held at 15 °C. Progression rates were not calculated per diem but were given as relative changes over each molt period. The U-shaped effect on lesion development was thought to arise from a metabolic trade off with temperature and microbial growth. In our experiment, ESD had a different dynamic with respect to temperature. Lesions grew at all temperatures, but they progressed most rapidly at 18 °C. Lobsters in our study were mature adults and had obtained ESD naturally prior to entering the study.

Quinn et al. (2012) exposed small juvenile lobsters to *Aquimarina "homari"* Miyazaki et al. 2010[1], *Thalassobius* sp., and *Pseudoalteromonas gracilis* Gauthier et al. 1995 held in separate recirculating systems at 10, 15, and 20 °C. These bacteria were considered potential agents of ESD. The carapace of animals in exposure treatments were abraded and inoculated with the different bacteria (different species on right or left side). Lesions developed within abraded areas that were exposed to *A. "homari"* and *Thalassobius* sp., but not to *P. gracilis*. Nonetheless, because animals were held in recirculating systems, all of the bacteria were found on animals serving as controls. Although progression was not quantified, the carapace sides abraded and exposed to *A. "homari"* and *Thalassobius* sp. developed laboratory-induced shell disease; however, multiple exposures were necessary to establish lesions characteristic of shell disease. In a recent study, Quinn et al. (2017) considered *Aquimarina macrocephali* subsp. *homaria* Miyazaki et al. 2010 the valid scientific name for the species of interest on the lobster.

Increased bottom temperatures are clearly important in the etiology of ESD, but other factors may also be involved. Alkylphenols, which are widely used as surfactants and in plastics, have a widespread distribution in lobsters from New England region (Jacobs et al. 2012); however, there are no clear associations with ESD, primarily because lobsters are highly mobile, thus reducing the likelihood of spatial correlations with contaminants. Alkylphenols can interfere with cross-linking of tyrosine, a process important for sclerotization, or tanning, in the newly molted cuticle of lobsters, and can delay tanning in affected lobsters (Laufer et al. 2012, 2013). Our present understanding of the etiology is that increased temperatures affect host defensive responses, with contaminants such as alkylphenols potentially weakening the cuticle, making it more susceptible to a dysbiotic bacterial community brought about by anthropogenic effects to water quality (Shields 2013).

Increased temperature can alter lobster immune defenses, thereby increasing susceptibility to pathogens and increasing their severity. Indeed, the hallmark for the emergence of new diseases in lobsters is the inducement of factors that impinge upon temperature homeostasis. Temperatures >20 °C have a marked effect on cellular defenses through immune suppression and altered acid-base homeostasis (Dove et al.

[1] *Aquimarina homari* has recently been shown to be *Aquimarina macrocephali* var. *homari.*

2005, Laufer et al. 2005). For example, hemocyte densities are lower in lobsters acclimated at 15 °C than in those held at 10 °C (Battison et al. 2003) and in vitro phagocytosis by hemocytes is significantly reduced at 22–23 and 4 °C than at 16 °C (Paterson and Stewart 1974, Dove et al. 2005). However, phagocytosis is sustained for longer periods in vivo at 5 and 8 °C than at 15 °C, indicating that temperature relationships are complex and probably vary in relation to acclimatization and nutritional state of the lobster (Stewart and Zwicker 1972).

ESD changes the normal pattern in molting in mature lobsters. In our study, molting only occurred in lobsters with ESD. None of the control animals molted during the experiment. To avoid laboratory-induced shell disease, our experiment lasted for only 5–6 mo. Nonetheless, our findings give direct support to Laufer et al. (2005) and Castro et al. (2006) that the disease has altered the endocrinology of molting and changes the timing and potentially the molting interval of this important physiological process. Ecdysone levels are significantly elevated in lobsters with ESD, including in ovigerous females (Laufer et al. 2005). Lobsters with ESD also tend to molt earlier and have a smaller size than uninfected lobsters (Castro et al. 2006). ESD likely causes a change in molting due in part to the injury to the cuticle, stimulating wound repair, and inducing the molting pathway (Laufer et al. 2005, 2012).

The lobster fishery off southern New England has declined as a result of poor recruitment due in part to ESD (Pearce and Balcom 2005, Shields 2013). Therefore, developing an understanding of the impacts of this disease is a high priority for the region's iconic lobster fishery. Our findings provide baselines for estimating the spread of the disease in relation to increasing temperatures in the Gulf of Maine. Several questions about the effect of temperature on ESD remain unresolved. To this end, our basic laboratory experiments provided estimates for how temperature affects molting rates and progression rates of disease and other aspects of how ESD impinges on the ecology of the lobster.

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