Relationship between Temperature and Shell Disease in Laboratory Populations of Juvenile American Lobsters (*Homarus americanus*)

Author(s): Michael F. Tlusty and Anita Metzler
Source: Journal of Shellfish Research, 31(2):533-541. 2012.
Published By: National Shellfisheries Association
DOI: http://dx.doi.org/10.2983/035.031.0213
URL: http://www.bioone.org/doi/full/10.2983/035.031.0213

Journal of Shellfish Research, Vol. 31, No. 2, 533–541, 2012.

RELATIONSHIP BETWEEN TEMPERATURE AND SHELL DISEASE IN LABORATORY POPULATIONS OF JUVENILE AMERICAN LOBSTERS (*HOMARUS AMERICANUS*)

MICHAEL F. TLUSTY* AND ANITA METZLER
New England Aquarium, 1 Central Wharf, Boston, MA 02110

ABSTRACT Epizootic shell disease is affecting American lobster (*Homarus americanus*) populations and the associated fisheries significantly. Historical movement of the disease suggests that temperature has been a critical factor in driving the susceptibility of lobsters to this disease. Here, juvenile lobsters were held in the laboratory at 10°C, 15°C, or 20°C for up to 1,021 days. Survival, growth, and shell disease were tracked throughout, and a separate experiment was conducted to assess the rate at which dietary white lobsters deposited pigment to the cuticle at the different temperatures. In the laboratory, shell disease was tracked as an initial ''spot,'' the melanization of the cuticle in response to the bacterial ingress, or, as it progressed, a lesion, which was a visible erosion of the shell. The length of the molt cycle (number of days from one molt to the next consecutive molt) was inversely associated with temperature. Average growth (increase in weight and carapace length per molt) at 10°C and 15°C was equivalent and higher than at 20°C, whereas shell disease was greatest at the intermediate temperature. The amount of initial melanization responses to bacteria (spots) was similar across temperatures, but the number of lesions was greatest at 15°C. This is likely a result of the conditions that allow for a combination of longer molt cycles and active bacterial growth, which allowed the disease to progress to the more advanced lesioned state. At the highest temperature, lobsters molted the compromised shell before severe lesion formation could occur. No gross differences in shell structure were observed at the different temperatures. The lack of a linear relationship between shell disease and temperature does not discount temperature as a critical factor in the onset of this disease in lobsters, but signifies that additional factors are important and the disease is complicated and multifactorial in nature. The continued use of a laboratory model system to study this disease further is important to be able to control the multitude of complicating factors appropriately.

KEY WORDS: American lobster, *Homarus americanus*, astaxanthin, *Aquimarina*, bacteria, host susceptibility, laboratory model, shell disease, stress

INTRODUCTION

American lobster (*Homarus americanus,* Milne Edwards) landings in the United States are at a near record, yet geographically identifiable stocks have experienced severe decreases, with the most notable declines occurring toward the southern extent of the lobster's range. In 1999, a massive die-off in the Long Island Sound fishery impacted 1,300 fishermen and decreased landings by 92% (McKown et al. 2005). Similarly, the Rhode Island fishery experienced a 50% decline in landings value ($32 million to $16 million) between 1999 and 2003 (National Marine Fisheries Service, Fisheries Statistics Division, Silver Spring, MD, pers. comm.), in addition to a loss of 9 million individuals in the 1997 oil spill (Gibson et al. 1997). Although various environmental factors are implicated in these population declines, disease issues are pervasive in these populations, and are indicative that the long-term health of the lobster stock may be compromised.

One of the more noticeable diseases affecting lobsters in the southern extent of their range is epizootic shell disease (ESD). ESD, along with impoundment shell disease (ISD) and burn spot, is 1 of 3 degenerative cuticular diseases exhibited by lobsters (Smolowitz et al. 2005). ESD lesions are caused by bacteria (Chistoserdov et al. 2005), with *Aquimarina* '*homaria*' (family Flavobacteriaceae) a potential initiator (Quinn et al. 2012). Once the bacteria compromise the shell, other bacteria, fungi, and protists can enter the lesions and degrade the shell further, leading to more advanced erosions into the exo- and endocuticular layers (Smolowitz et al. 2005). For years, ESD occurred at low levels of prevalence and only with minor severity (Glenn & Pugh 2005). However, beginning in the mid to late 1990s, lobsters with severe ESD signs were being reported in higher numbers (Castro et al. 2006). Currently, lobsters with ESD range throughout southern New England, with lower prevalence in Maine and Canada. The cause of the increasing prevalence and distribution of ESD among lobsters is currently unknown.

Although bacteria initiate the disease, bacterial presence alone does not explain the etiology of ESD. Sneiszko's (1973) model of disease requires 3 factors—the environment, the host, and the pathogen—to be of sufficient status to permit the disease. Castro et al. (2006) conceptualized this model to include population-level impacts and feedback loops. Tlusty et al. (2007) developed a model for shell disease from the standpoint of host susceptibility, and discussed how changes in individuals could lead them to become more susceptible to disease agents. These models point out that for ESD in lobsters, multiple factors aid the onset of the disease state. Nonetheless, parameters have to be investigated individually to understand their potential roles prior to attempting to understand these interacting effects.

Temperature is an environmental factor that may play a role in the etiology of ESD. ESD is more common in southern regions of the lobsters range, and is extremely infrequent in Maine and Canada. Even at smaller spatial scales, there is a positive correlation between temperature and ESD prevalence, as seen in eastern Long Island Sound (Castro et al. 2006), and Massachusetts (Glenn & Pugh 2006). In Massachusetts, ESD showed a yearly northward progression taking approximately 5 y to travel from Buzzard's Bay in the south to Cape Ann in the north (Glenn & Pugh 2005, Glenn & Pugh 2006). In addition, high temperatures in excess of 20°C in one year leads to a high incidence of ESD the next year (Glenn & Pugh 2005, Glenn & Pugh 2006). Thus, the high temperature affects the lobsters, but it takes time for the disease to be realized in the lobsters.

*Corresponding author. E-mail: mtlusty@neaq.org
DOI: 10.2983/035.031.0213

Temperature is intriguing as a driver of ESD because there are different underlying routes in which increased temperature can lead to an increased disease state. Temperature can affect deposition and growth of the cuticle, as shell and other biocomposites have been shown to be thinner at warmer temperatures (Fletcher 1995, Trussell & Etter 2001, Nagarajan et al. 2006, Doyle et al. 2010), which could lead to easier ESD infection. Alternatively, increased disease at warmer temperatures may be a more direct result of an increased rate of bacterial growth leading to greater shell degradation (Fig. 1). In either case, the result is that, at higher temperatures, the cuticle exhibits an increased diseased state compared with a cuticle at lower temperatures. Structural deficiency and increase bacterial growth are not mutually exclusive explanations and may operate in consort.

To assess the potential structural or bacterial mechanisms by which temperature may influence the onset and progression of ESD, juvenile lobsters were grown in a laboratory at three temperatures (10°C, 15°C, and 20°C). The lobsters were held for 1,021 days at these temperatures and growth, mortality, and ESD prevalence and intensity were monitored. To determine whether temperature influences the structure of the shell, differences in the thickness and in the layering of the cuticle were analyzed by scanning electron microscopy. The ability of lobsters to mobilize nutrients into their shell was examined by assessing the rate at which lobsters deposited pigment into their cuticle (Tlusty 2005). We hypothesized that the lobsters exposed to the warmest temperature (20°C) would exhibit the most shell disease as a result of a decrease in shell integrity in the face of increased bacterial activity.

MATERIALS AND METHODS

Lobster Rearing and Experiment Setup

Lobsters were hatched and raised at the Lobster Research and Rearing Facility (LRRF) at the New England Aquarium (Boston, MA). Ovigerous females were collected from Boston Harbor and held individually in tanks (99 × 57 × 25 cm, 150 L) within a 4,000-L semiclosed recirculation system. Upon hatching, larvae were maintained communally in 40-L kreisel tanks and fed a combination of *Spirulina*-enriched adult frozen *Artemia* sp. (San Francisco Bay Brand, San Francisco, CA) and Cyclop-eeze (Argent Chemical Laboratories, Redmond, WA). When larvae metamorphosed to the postlarval stage, they were moved into individual round containers with holes to provide water circulation (diameter, 3 cm; height, 3.5 cm) and held in shallow tanks (193 × 53 × 7.5 cm, 390 L each) within the same semiclosed system. At this point, lobsters were fed Economac, an extruded diet with highly unsaturated fatty acids (Aquafauna Bio-Marine Inc, Hawthorne, CA). Economac contains low levels of the carotenoid pigment, astaxanthin (AXT; 25 ppm), that provides lobsters with shell coloration. Because of the low levels of AXT, lobsters fed Economac for relatively extended periods of time lose shell coloration over several molts and demonstrate a white phenotype (Tlusty & Hyland 2005).

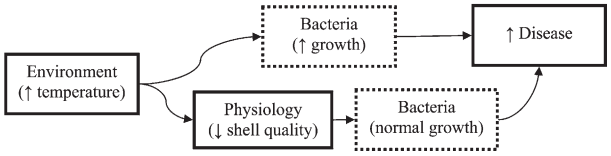

Figure 1. The relationship between the environment and shell disease in lobsters either through an influence on bacteria growing on the cuticle or through the shell quality of the individual lobster. Solid boxes refer to those parameters that were identified in the model by Castro et al. (2006), whereas the stippled boxes indicate the role bacteria have in the onset of shell disease.

Lobsters were moved into larger round mesh containers (diameter, 5.5 cm; height, 9.5 cm; and diameter, 9 cm; height, 9 cm) and rectangular slotted containers (13.5 × 7 × 7 cm and 19 × 6 × 9 cm) as dictated by their growth. At approximately 9 mo of age, the diet regimen was modified by adding dissolved gelatin to the Economac (3 g gelatin in 38 mL water for 30 g Economac) to provide a sinking food that remains intact. Lobsters were fed daily a portion of food half the size of their last abdominal segment, which is standard feeding protocol for the LRRF. After several hours, uneaten food was removed. Lobsters were also checked daily for molt occurrences.

In August 2007, 81 phenotypic white lobsters lacking shell disease, 1.5–2 y old, were separated into 3 temperature treatments: 10°C, 15°C, and 20°C ($n = 27$ per treatment). Lobsters were measured for carapace length (CL; Mitutoyo IP67 calipers) and weight (in grams; OHaus Galaxy 160) prior to placement into temperature treatments, and current shell disease status as described later was also noted. Lobsters continued to be checked daily for molt occurrences and were fed daily to satiation. Six months after the start of the experiment, diet regimens were altered for 10 lobsters in each temperature treatment. The lobsters were fed a diet containing the carotenoid pigment AXT. The diet (AXT diet) consisted of 98.5% Economac, 1.5% Naturose (natural astaxanthin) bound with dissolved gelatin (3 g gelatin in 38 mL distilled water for 30 g total ingredients), which results in a 100-μg/g final concentration of AXT. This concentration provided enough AXT for the lobsters to develop wild-type shell coloration (Tlusty & Hyland 2005).

Temperatures were monitored daily as well as logged every 5 min using Tidbit v2 data loggers (Onset Computer Corporation, Bourne, MA).

Growth

CL and weight were monitored throughout the course of the experiment. After ecdysis, lobsters were allowed at least 1 wk for sclerotinization before measurements were taken. Because the lobsters had different initial CLs and weights at the start of the experiment, growth was compared by calculating percent change in both. Percent change in growth for each molt was calculated by taking the difference in premolt measurement from postmolt measurement and dividing by the premolt measurement multiplied by 100. For each lobster, an average of these values for the course of the experiment was calculated and compared by temperature treatment.

Shell Disease Monitoring

Lobsters were allowed to acclimate to the temperature treatments for 7 mo, after which both white and AXT-fed lobsters were monitored for shell disease every 2 wk by visual inspection by eye and with a stereomicroscope when needed. Lobsters exhibited early stages of shell disease as small melanized

areas, but without visible shell erosion by the naked eye or by microscope at 20× ("spots"). As time progresses, these spots can eventually exhibit noticeable shell erosion, at which point they are considered to be lesions that are the typical symptoms of ESD. Lesions can also form without prior spot development; however, it is unknown whether spot-to-lesion development occurred rapidly and therefore went undetected. Other melanized areas resulting from physical trauma (cracks or breaks in the shell) were noted but not included as shell disease. Some lesions would become severe enough to cause parts of the shell to break away, such as the uropods of the tail region. In these cases, the lesions were still counted and considered as such, even though parts of the shell were missing.

Twenty five dorsal and lateral areas of the lobster were analyzed for shell disease. These areas included the tips of each claw ($n = 2$); the inside of each claw ($n = 2$); claws ($n = 2$); claw "shoulders" ($n = 2$); rostrum ($n = 1$); around each eye ($n = 2$); left, center, and right carapace ($n = 3$); each abdominal segment ($n = 6$); and each tail uropod ($n = 5$). Although the chelae were included in analysis, the remaining 4 pairs of pereiopods were not. Because shell disease is a progressive disease, lesions can enlarge over time. Therefore, lesions close in proximity can enlarge and overlap (coalesce), forming 1 large lesion. Because of this, the number of spots or lesions can be a misleading value with regard to the severity of shell disease on the lobster. As such, an index was created to determine shell disease severity based on the percentage of body areas that exhibited shell disease. The percent coverage was determined out of the 25 areas listed here. However, if a lobster lost a limb, this would lower the total possible number of body areas inspected. Thus, occasionally, the percent coverage is out of fewer that 25 total areas. Shell disease indices are presented as percent spots and lesions, which are the percent of the areas exhibiting spots and lesions, respectively. Results are also presented as total shell disease, which is the percent total body areas exhibiting any shell disease symptom, either spots and/or diseased lesions.

Shell Disease Data Analysis

Because shell disease is a progressive disease, clinical signs become the most severe just before a lobster is about to molt. Right after ecdysis occurs, the lobster shell generally has no lesions. To ensure data analyzed was in a time frame when shell disease was most severe, data collected only within 80–100% of the molt cycle were used. Percent of the molt cycle is equal to the number of days postmolt the shell disease analysis occurred divided by the total number of days in that particular molt cycle multiplied by 100.

To determine differences in shell disease in the dietary white lobsters compared with AXT-fed lobsters, data from all temperatures were pooled for each molt period. The difference throughout the course of the experiment for which there was sufficient data (molts 3–8) was determined by repeated-measures ANOVA for data on spots, lesions, or total shell disease, where the individual lobsters were the repeated measure.

Correction for Molt Cycle

Because molt occurrences are temperature dependent, the temperature treatments were hypothesized *a priori* to affect molt cycle length, which may also affect shell disease severity. Shell disease indices were corrected for the discrepancies in the molt cycle by dividing the shell disease indices by the number of days postmolt the data were collected. Results based on corrected data are described e described as such.

Color Change Experiment

To determine temperature effects on protein binding of carotenoid pigment in the shell, 34 dietary-induced white lobsters, 9 mo old, were introduced into the 3 temperature treatment tanks (10°C: $n = 10$; 15°C: $n = 12$; 20°C: $n = 12$). The lobsters were introduced into the treatment tanks within 1 wk of a molt event. Lobsters were acclimatized to the temperature treatments until the next consecutive molt. During this time, they continued to be fed the gelatin-bound Economac diet, which maintained their white coloration. After molting, the lobsters were fed the AXT diet, which introduced the pigment astaxanthin to the lobsters and initiated color changes in the shell.

Digital images were taken weekly of the lobsters to monitor shell color changes. Images were taken using a Nikon Coolpix 5700 digital camera with ring light attachment for even illumination. The camera settings were manual, with macro, F-stop 4.5, and a shutter speed of 1/30. Images were taken of the uropods and last abdominal segment on a Behr Pure White color sample.

Images were analyzed using ImageJ software (rsbweb.nih.gov/ij/). For each image, an area on one of the endopodites and the last abdominal segment of the lobster was highlighted and analyzed for red and blue, and light intensity. Areas with glare, and edges of the shell, were avoided for analysis. Images were first corrected for discrepancies in white coloration by analyzing a section of the Behr Pure White background. Pure white is equal to values of 255 each for blue, red, and intensity. If the white background area analyzed in the image varied from pure white values, the blue, red, and intensity values on the endopodite and abdominal segment were corrected accordingly by dividing the color or intensity value by the corresponding value in the white area and multiplying by 255. Images were analyzed by correcting for the percent completion through the molt.

For each lobster image, both the red/blue (R/B) ratio and intensity for the endopodite and abdominal segment were analyzed. The R/B ratio indicates the distribution of the pigment in the lobster. Lobsters incorporate AXT into either the dermal layer or into the shell. In the dermal layer, the AXT remains in free form and is red in color. In the shell, AXT binds to protein and becomes blue (Tlusty & Hyland 2005). Therefore, analysis of R/B ratios provides information on whether more AXT was incorporated into the dermal layer or into the shell. Higher R/B ratios indicate the red component of the color is greater proportionately, and the AXT has been absorbed more in the dermal layer than in the shell. Intensity values indicate the overall darkness of the lobster, with lower values signifying a darker, more pigmented lobster.

To determine the amount of AXT absorption into the cuticle, maximum R/B ratios and minimum intensity values (Tlusty 2005) were identified for each individual lobster along with its corresponding point in the lobster's molt cycle. These values were analyzed by 1-way ANOVA for the R/B or intensity value along with the slope and the percent in the molt cycle at which the extreme value occurred. Rate of AXT absorption was determined by calculating the difference in maximum R/B ratio from initial R/B ratio, and dividing by the number of days to

reach the maximum R/B ratio. Data were analyzed similarly for intensity, which had a negative rate.

Shell Structure by Scanning Electron Microscopy

Seven months from the start of the experiment, 3 white lobsters from each treatment were sacrificed for shell structure analysis. In addition, 3 lobsters from the LRRF system of similar age and size were also sacrificed as controls. Lobsters were anesthetized then bisected between the carapace and abdomen and placed in 70% ethanol. These samples were sent to the Max Plank Institute (Germany) where Dr. H. Fabritius measured from the claw and carapace the shell thickness, relative contribution of the calcite (J. Kunkel, pers. comm.), exocuticle and endocuticle layers to the overall thickness (all analyzed as 1-way ANOVAs respective to separate body locations), and the stacking height of the bouligand layers (Raabe et al. 2005). Temperature differences in stacking height within the exo- and endocuticle were determined by analyzing the total thickness of the shell for the claw and carapace using a 2-way ANOVA (temperature and body location/cuticle layer).

RESULTS

Water temperatures throughout the course of the 1,021-day experiment averaged 10.06 ± 0.97°C (10°C treatment), 14.59 ± 1.17°C (15°C treatment), and 20.2 ± 1.25°C (20°C treatment). Over this time frame, 29 lobsters died, some as a result of system problems (i.e., water flow).

Water temperature affected both molt cycles and growth in the lobsters. Molt cycles during the course of the experiment averaged 136, 96, and 62 days at 10, 15, and 20°C, respectively. Because of this temperature-mediated difference in the length of the molt cycle, during the maximum of 1,021 days on experiment, lobsters at 10°C only realized an average of 7 molts, whereas those at 15°C and 20°C averaged 10 and 14 molts, respectively (Fig. 2). For growth at the 6th molt, temperature influenced significantly the increase in both CL ($F_{2,35} = 10.97$, $P < 0.001$) and weight ($F_{2,35} = 20.57$, $P < 0.001$). Although the lobsters at 20°C molted more frequently, they averaged less growth per molt than at the other temperatures (Tukey's HSD, %CL, q > 3.48, $P < 0.05$; %WT, q > 6.55, $P > 0.001$). Lobsters at 10°C on average grew 10.36 ± 1.60% (SE) in CL and 39.4 ± 1.57% in weight per molt. Lobsters at 15°C and 20°C grew less than the 10°C lobsters and also grew more equivalently to each other, with average growths in CL of 8.26 ± 0.82% and 5.52 ± 1.08% and in weight of 25.29 ± 2.09% and 25.52 ± 1.83°C, respectively.

Without correcting for the molt cycle, shell disease was generally greater at 15°C than at the other temperature treatments. Only molts 3–8 could be compared across all treatments because a sufficient number of lobsters were measured repeatedly (Fig. 2). Overall, temperature influenced the total amount of shell disease (sum of spots and lesions) lobsters exhibited (2-way RM ANOVA, $F_{2,42} = 14.44$, $P < 0.001$; Fig. 2C). Lobsters exhibited the greatest percentage total shell disease at 15°C (35.52 ± 2.52%, $\bar{X}$ ± 1 SE), although it was not significantly greater than the percentage exhibited at 10°C (28.23 + 2.56%, Tukey's test, q = 3.00, $P > 0.09$). Lobsters at 20°C exhibited significantly less total shell disease than lobsters at the 2 lower temperatures (10.63 ± 4.39%, Tukey's test, q > 4.80, $P < 0.005$). The total shell disease (measured as a percentage) was not influenced by molt number

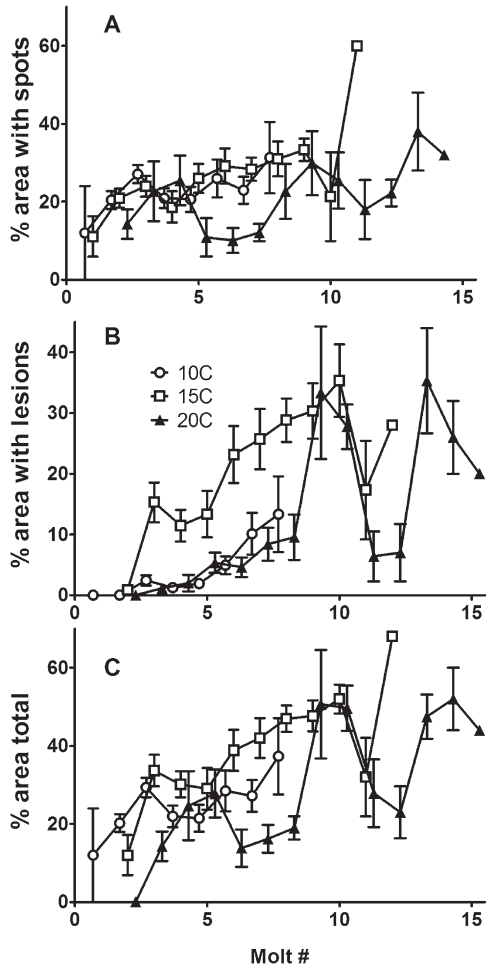

Figure 2. (A–C) Average percent body areas with shell disease (±SE) in juvenile lobsters reared at 3 temperatures—10°C (○), 15°C (□), or 20°C (▲) for a maximum of 1,021 days. Shell disease was monitored for spots (A), lesions (B), and total shell disease (C) just prior to molting.

(2-way RM ANOVA, $F_{5,177} = 1.74$, $P > 0.1$), and there was no temperature × molt interaction (2-way RM ANOVA, $F_{10,177} = 1.62$, $P > 0.1$).

The percentage of areas with lesions was influenced by both temperature (2-way RM ANOVA, $F_{2,42} = 26.78$, $P < 0.001$) and molt (2-way RM ANOVA, $F_{5,177} = 5.42$, $P < 0.001$), and there was no significant interaction (2-way RM ANOVA, $F_{10,177} = 0.76$, $P > 0.6$; Fig. 2b). In this case, lobsters at 15°C had the greatest percent cover of lesions (18.50 ± 1.36%), which was significantly greater (Tukey's test, q < 7.50, $P < 0.001$) than either lobsters at 10°C (6.30 ± 1.53%) or lobsters at 20°C (2.54 ± 2.63%). There was a trend for the number of lesions to increase with molt, and the molts clustered into a group (molts 3, 4, and 5) with a low percentage of lesions (5.1 ± 2.1%, 3.9 ± 1.8%, and 6.1 ± 1.7% respectively), and molts 7 and 8, which had 14.8 ± 2.0% and 15.9 ± 2.5% coverage, respectively.

The percent areas with spots exhibited a significant temperature × molt number interaction (2-way RM ANOVA, $F_{10,177} = 2.06$, $P > 005$; Fig. 2A). There was no significant temperature treatment effect for molt numbers 4 and 5. At the third molt, the lobsters at 10°C (27.1 ± 2.5%) had a greater percent spot coverage than lobsters at 20°C (6.5 ± 5.9%, Tukey's test, q = 3.82,

$P < 0.02$), but not those at 15°C (20.9 ± 2.6%). For the final 3 molts (6, 7, and 8), lobsters at 15°C and 10°C had an equivalent percent coverage of spots, and both were greater than those at 20°C.

Correcting for molt cycles did not alter the results (Fig. 3). Overall, the lobsters at 15°C had the most shell disease in terms of percent total body areas with shell disease and percent areas with lesions, whereas the lobsters at 10°C had the lowest levels of shell disease in spots, lesions, and total areas. These results were not a simple matter of differential length of molt cycles.

Mortality was observed in 7 lobsters in the 20°C treatment, 2 in the 15°C treatment, and 1 in the 10°C treatment. The percent total body areas with shell disease of the moribund lobsters was 54.3 ± 12.9% (mean ± 95% CI, 20°C), 56.0 ± 0% (15°C), and 88% (10°C) for the lobsters at the respective temperatures. Although these averages were higher than what was observed for surviving lobsters with shell disease (Fig. 2C), there was no difference between shell disease coverage in surviving and moribund lobsters (based on overlap of the 95% CIs; Fig. 2C). Furthermore, the maximum shell disease experienced by a lobster did not cause mortality immediately. Only 5 of these 10 lobsters died immediately after their maximum recorded severity of shell disease. The other 5 molted after the most severe episode, only to perish later (an average of 166.8 ± 68.6 days after the maximum observed shell-disease episode).

The trend in shell structure was for the total shell thickness to be least at the highest temperature (Fig. 4A), whereas the outer calcite layer tended to be thinnest at 15°C in both the claw and carapace (Fig. 4B, C). However, these trends were not statistically significant (for all analyses: 1-way ANOVA, $F_{2,6} < 3.0$, $P > 0.05$). For all temperatures, the stacking of the bouligand

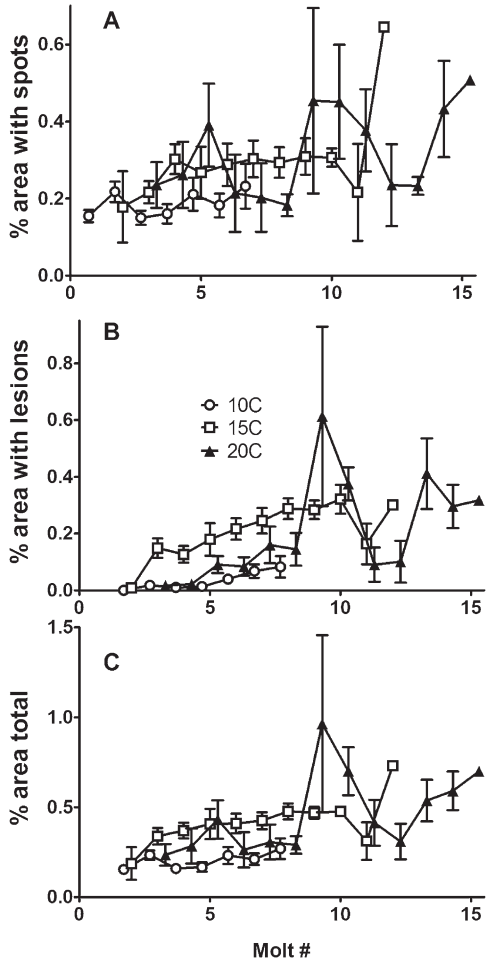

Figure 3. (A–C) Average percent body areas with shell disease corrected for the length (days) of the molt cycle in juvenile lobsters reared at 3 temperatures—10°C (○), 15°C (□), or 20°C (▲)—for a maximum of 1,021 days. Shell disease was monitored for spots (A), lesions (B), and total shell disease (C) just prior to molting.

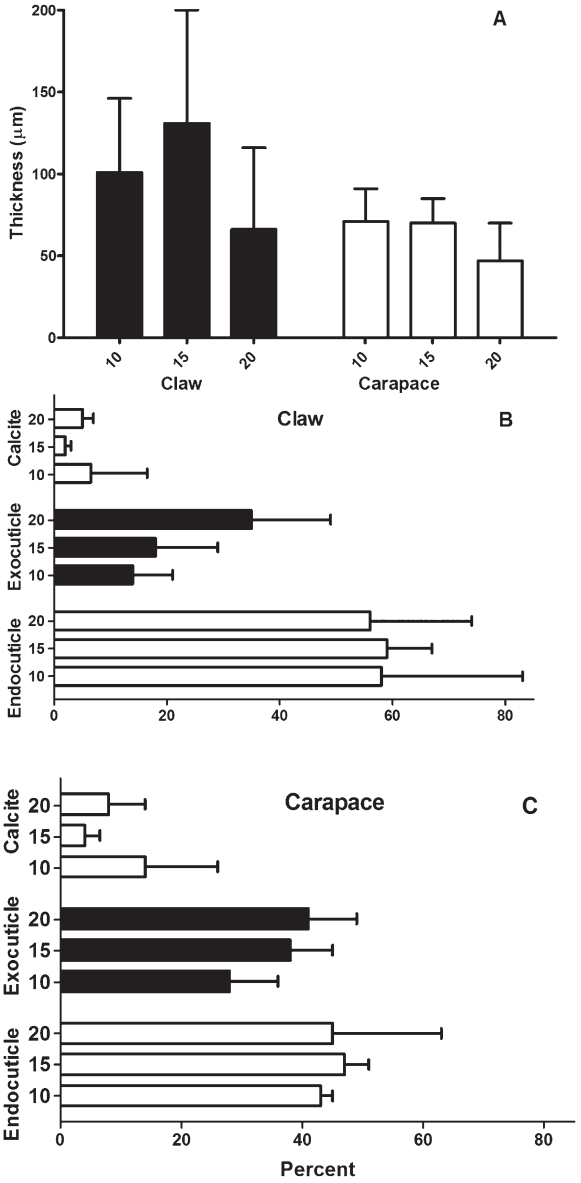

Figure 4. Shell thickness of juvenile lobsters reared at 3 temperatures—10°C, 15°C, or 20°C—for 7 mo. (A) The thickness of the entire cuticle in the claw and carapace. (B, C) The composition (percentage of total thickness) of the calcite, exocuticle, and endocuticle layers of the claw (B) and carapace (C). Error bars are 95% confidence intervals. Temperature had no effect on total shell thickness or on the thickness in the shell layers (1-way ANOVA, $F_{2,6} < 3.0$, $P > 0.05$).

layers was largest in the endocuticle of the claw compared with the exocuticle of the claw and the endo- and exocuticle of the carapace (2-way ANOVA, $F_{3,24} = 53.30$, $P < 0.001$, Fig. 5). None of the stacking heights of any of the bouligand layers differed with temperature (2-way ANOVA, $F_{2,24} = 0.31$, $P > 0.7$).

The addition of AXT to the cuticle varied with temperature. The lobsters in this experiment were carried through a single molt, which lasted 105 ± 23 days, 71 ± 13 days, and 52 ± 10 days (average ± 95% CI) for temperatures of 10°C, 15°C, and 20°C respectively. The lobsters at 20°C had lower R/B ratios than the other 2 temperatures, indicating that the blue component (AXT in the cuticle) was greatest at this temperature (Fig. 6). Yet although they had lower R/B ratios, the slope of the increase in R/B through the molt cycle per day was not significantly different, but tended to be intermediate for lobsters at 20°C compared with those at 15°C or 10°C (1-way ANOVA, $F_{2,31} < 2.27$, $P > 0.1$; Fig. 6). The final color intensity tended to be lower for the lobsters at 10°C (indicating a darker overall color and thus more AXT absorption), although this was not significantly lower (for all comparisons within value and percent molt cycle, 1-way ANOVA, $F_{2,31} < 2.7$, $P > 0.08$). The change in intensity per day was significantly different for the different temperatures (1-way ANOVA, $F_{2,31} > 3.7$, $P < 0.05$), with the shallowest slope being at 10°C, indicating that the change in intensity was slower for this temperature than the other temperatures. Overall, these results indicate that deposition of pigment to the cuticle was fastest at 15°C and 20°C, with the 15°C treatment trending slightly greater than the 20°C treatment.

White lobsters had slightly but not significantly more total shell disease than did AXT-fed lobsters (paired t-test, $P > 0.08$; Fig. 7C). However, this was a result of detection of spots in a greater number of locations on individual lobsters (paired t-test, $P < 0.005$; Fig. 7A). The detection of lesions was similar across the two phenotypes (paired t-test, $P > 0.8$; Fig. 7B).

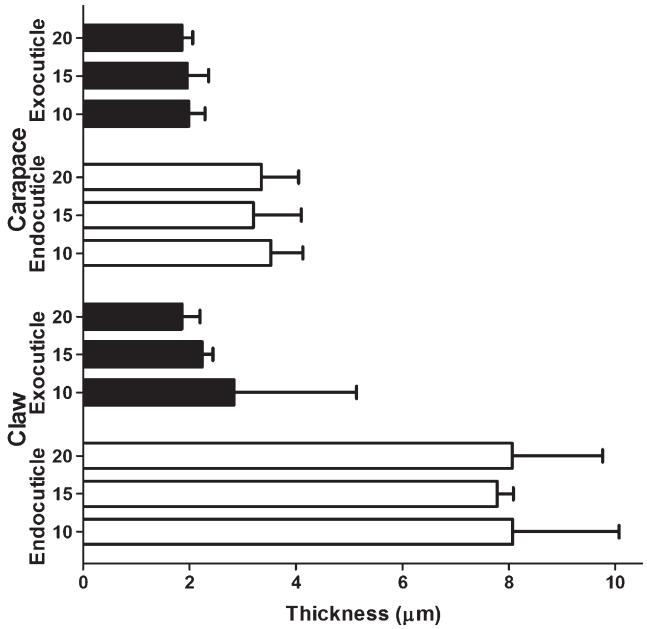

Figure 5. Stacking height thickness of the exocuticle and endocuticle for the claw and carapace in juvenile lobsters reared at 3 temperatures—10°C, 15°C, or 20°C—for 7 mo. Temperature had no affect on stacking height thickness (2-way ANOVA, $F_{2,24} = 0.31$, $P > 0.7$). For all temperatures, the stacking height of the endocuticle layer was the thickest of all the layers (2-way ANOVA $F_{3,24} = 53.30$, $P < 0.001$).

DISCUSSION

One particularly intriguing hypothesis for the recent increase in the prevalence of ESD is that the environment has changed in a manner that is now more permissive to the disease (Castro et al. 2006, Tlusty et al. 2007). In many animals, problems in the growth of shells are linked directly to environmental factors (Wiesner & Iben 2003). For example, juvenile oyster disease occurs only if the temperature is greater than 22°C (Bower & Meyer 1999, Ford & Borrero 2001). In American lobsters, health is greatly impacted by multiple environmental factors (Evans 2001), and anthropogenic impacts can exacerbate any preexisting condition. Humans are rapidly changing the environment, and one of the more significant and recent environmental threats is global warming. Increased temperatures are affecting all of earth's ecosystems, and impacts to the oceans can be quite severe (Millennium Ecosystem Assessment 2005). Because lobsters are exothermic, they are susceptible to changes in temperature through changes in metabolic functions such as growth, and enzymatically driven processes such as protein binding to chitin and pigments in the cuticle.

Shell disease in wild populations of American lobsters was observed to be related to temperature, with increased temperature leading to greater severity of shell disease (Glenn & Pugh 2006). Although shell disease appears to be linked to temperature, high temperatures do not always result in an increased prevalence of ESD. Castro et al. (2006) did not find a correlation between bottom temperature and ESD for Narragansett Bay. Furthermore, not all lobsters within a population get ESD. In the Upper East Passage of Rhode Island in 2002, the highest prevalence of shell disease was 45% (Castro et al. 2006). Yet, these observations do not imply automatically that there is no correlation between temperature and ESD, and as pointed out by Castro et al. (2006), this "may reflect different environmental conditions for Narragansett Bay, or may be an artifact of insufficient data." There is also the issue of selection for resistance to disease, as may have occurred to animals in western Long Island Sound (Howell et al. 2005).

This laboratory experiment confirmed some aspects of the hypothesis that increased temperatures exacerbate shell disease. This model system allows for shell disease to be examined in its true progressive nature. Here, spots are observed and, upon fully breaching the outer cuticle, a lesion forms, leading to the highest prevalence and the most severe signs being identified prior to molting (Tlusty et al. (2008), but see also Glenn and Pugh (2005) for a similar result from a wild population). Progression to lesions was greatest at the intermediate temperature of 15°C, a result that may be dependent on at least 2 nonexclusive factors. First, the molt cycle length was shortest for lobsters in the 20°C treatment. Therefore, the amount of total time available for the bacteria to affect the shell to the state of a full lesion is limited, and thus the disease does not progress to the more advanced state. Second, 20°C may be beyond the optimal temperature for the causative agent, and thus could not progress at the same rate as 15°C (A. Chistoserdov, pers. comm.). There were no statistically significant trends in shell

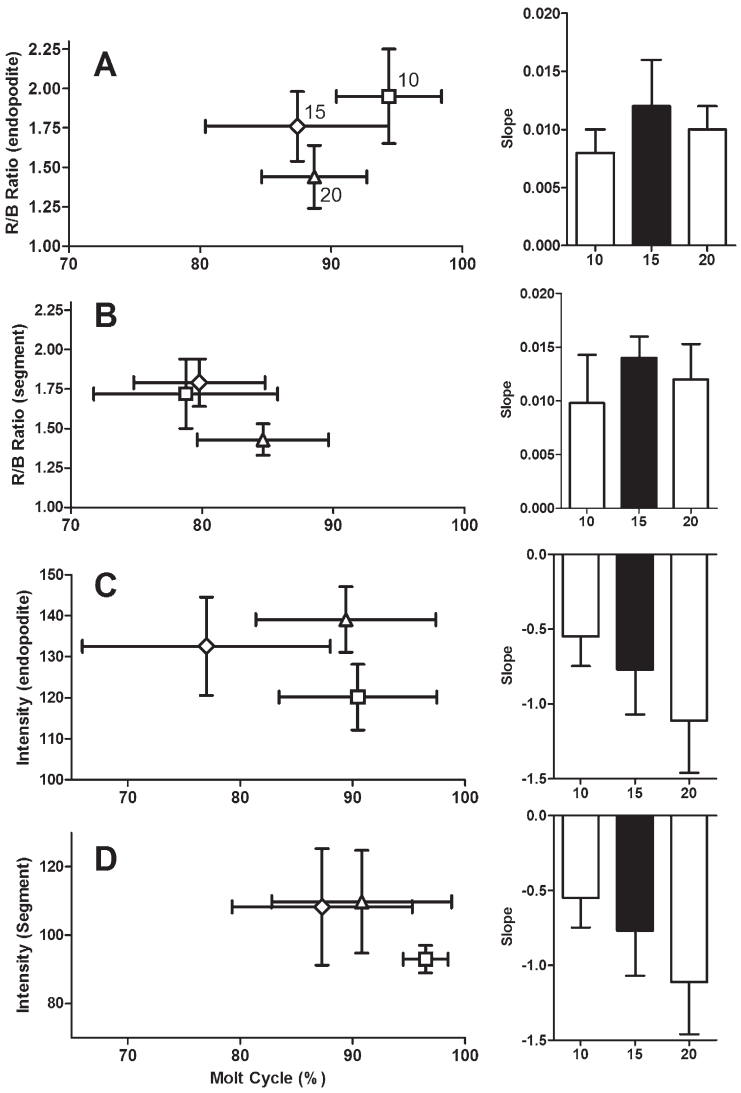

Figure 6. (A–D) Percent in the molt cycle for maximum astaxanthin (AXT) absorption in juvenile lobsters reared at 3 temperatures—10°C (□), 15°C (◇), or 20°C (Δ). The maximum red/blue (R/B) ratio, indicating the maximum AXT absorption into the dermal layer (A, B), and the minimum intensity, indicating the most overall AXT absorption (C, D), for the endopodite (A, C) and abdominal segment (B, D). The average slope from first measure to the extreme value corrected for the number of days in the molt cycle is provided on the right portion of the figure. Values are means ± 95% CI. Only the slope of the intensity values were found to be significant (1-way ANOVA, $F_{2,31} > 3.7$, $P < 0.05$). Slope of R/B values and comparisons of maximum R/B values and minimum intensity values showed no significance (1-way ANOVA, $P > 0.05$ on all tests).

structural composition or incorporation of pigment, indicating no relationship between gross structure and disease. However, this does not rule out finer scale changes in structure and chemistry (see Kunkel et al. (2012)).

Based on the fact that the low temperature treatment (10°C) resulted in the lowest disease, the temperature regime is likely to be a critical factor in the onset of shell disease both in the laboratory (this study) and in the wild (Glenn & Pugh 2006). However, it is not the only predisposing factor (Tlusty et al. 2007). In this study, over the different temperatures, there was a concave-downward response, with peak shell disease intensity and severity occurring at 15°C. However, although this laboratory study examined lobster responses to static temperatures, in nature, temperatures are variable. The metric used by Glenn and Pugh (2006) was indicative of the number of days the lobsters were in excess of 20°C, but at the same time, the lobsters were also experiencing fluctuating temperatures, many of which would be in the 15°C range. Thus, the high temperatures exceeding 20°C may have caused increased stress to the lobsters, but the significant damage by bacteria to the cuticle was likely when temperatures were in the range of 15°C. Lobsters can have an upper lethal temperature as great as 30°C (van Olst et al. 1980), and Steenbergen et al. (1978) did not observe a decrease in immune function until lobsters were held at 22°C or greater. However, when faced with multiple stressors, lobster will have a significantly lower maximum lethal temperature. Robohm et al. (2005) found that 19.5°C was stressful enough to cause mortality when presented in hypoxic conditions. The 20°C used in this experiment was at the threshold of being a significant stressor, and it is possible that the lobsters had a decreased immune

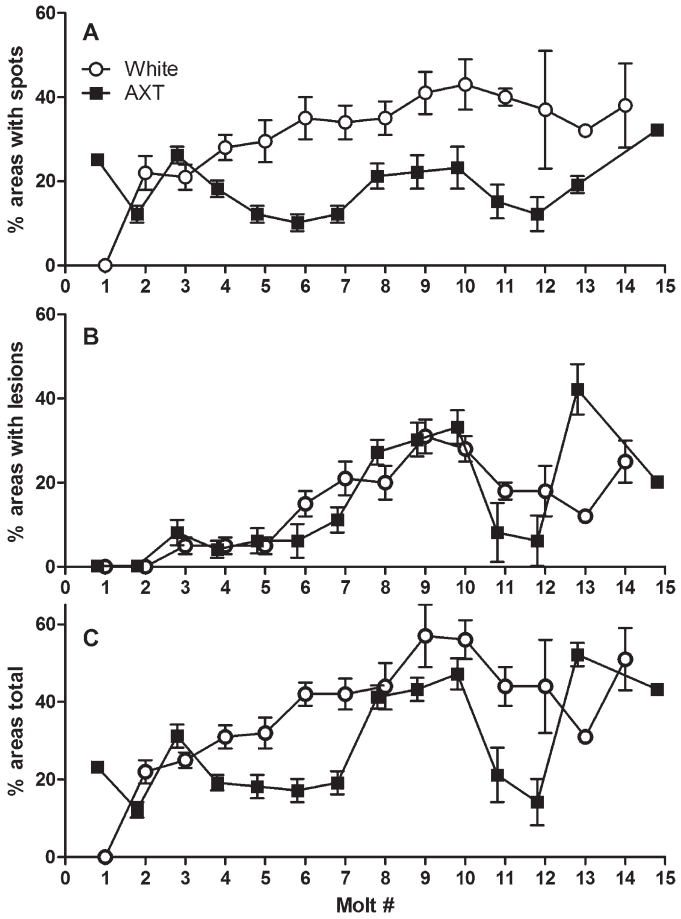

Figure 7. (A–C) Average percent body areas with shell disease in juvenile lobsters reared on 2 diet regimens—without (○) or with (■) astaxanthin (AXT) diets, resulting in different shell phenotypes, white and wild-type color, respectively. Data from lobsters from 3 temperatures (10°C, 15°C, and 20°C) were pooled. Shell disease was monitored for spots (A), lesions (B), and total shell disease (C) just prior to molting. White lobsters had a significantly higher percent of shell-disease spots than wild-type-color lobsters (paired *t*-test, $P < 0.005$), but no significant differences were found in shell-disease lesions or total shell disease (paired *t*-tests, $P > 0.8$, $P > 0.08$, respectively).

function (Homerding et al. 2012) in this treatment, leading to a decreased prevalence and intensity of shell disease.

Stevens (2009) observed a fitness consequence to shell disease in adult lobsters, where the lobsters with the highest shell disease index grew the least during a molt. Over the expanse of temperatures examined in this study, a negative association of shell disease and growth was not observed. Growth was least in the 20°C treatment, whereas the lobsters at 15°C exhibited the most severe signs of shell disease (lesions). At this macro level, biological performance was not compromised by a shell disease state (or vice versa). Furthermore, Stevens (2009) indicated a significant negative fitness consequence of shell disease, which led to mortality 24% of the time. In the current study, total mortality was 35%, and mortality did not always immediately follow the lobster's presentation of its maximum level of shell disease. Thus, temperature is affecting lobsters in more ways than solely through the increase of disease and subsequent decrease in growth.

The laboratory model presented in the current study (see also Tlusty et al. (2008)) provides a novel means to examine shell disease. Historically, shell disease has been difficult to model in laboratory populations (Malloy 1978). Here, success was achieved in part because of the ability to create susceptibility in the host lobster to the bacteria (Tlusty et al. 2007) through nutritional means (Tlusty et al. 2008). Following individual juvenile lobsters through subsequent molts and disease events allowed for observation of the onset of shell disease in this laboratory population, and ultimately led to the realization of an initial stage of the infection process. As bacteria first settle on and invade the shell, the lobster responds via a phenolic (tanning) response to contain the invading bacteria. Detection of the initial stage of infection, observed as spots, was simplified by the use of the dietary white lobsters. Spots were observed to be more numerous in the white lobsters compared with normal lobsters, a result attributed to the ease of detection. When the bacteria overcome this defense, then the disease progresses to a lesioned state. There was no difference in the number of lesions on the lobsters between white and wild colored lobsters. The identification of this initial spot phase on the pigment-free animals allowed for a better exploration of the bacteria involved in initiating this disease (Quinn et al. 2012). The continual development of this laboratory model allows for a better understanding of the factors that may allow shell disease to progress to the lesioned state.

This series of experiments determined that a critical factor in shell disease was the length of the molt cycle, in that at the

highest temperature, the abbreviated molt cycle likely limited the damage that the bacteria could do to the cuticle. Temperature is an important component of the onset and progression of shell disease in American lobster, but is not the only factor. As shown by the other research presented in this volume, there are a multitude of factors that can act to increase the probability of onset or to increase the severity of this disease. How these singular factors interact with one another remains to be examined.

ACKNOWLEDGMENTS

We thank A. Shantz and N. Keeney for assistance with photographing and color-scoring lobsters. A large number of interns and volunteers assisted with husbandry and data collection, and we thank them all. The presentation of the information was improved by comments from K. Castro, B. Somers, M. Gomez-Chiarri, and two anonymous reviewers on a draft of this manuscript.

LITERATURE CITED

Bower, S. & G. Meyer. 1999. Effect of cold water on limiting or exacerbating some oyster diseases. *J. Shellfish Res*. 18:296–304.

Castro, K. M., J. R. Factor, T. Angell & D. F. Landers. 2006. The conceptual approach to lobster shell disease revisited. *J. Crustac. Biol*. 26:646–660.

Chistoserdov, A. Y., R. M. Smolowitz, F. Mirasol & A. Hsu. 2005. Culture-dependent characterization of the microbial community associated with epizootic shell disease lesions in the American lobster, *Homarus americanus*. *J. Shellfish Res*. 24:741–748.

Doyle, S., B. MacDonald & R. Rochette. 2010. Is water temperature responsible for geographic variation in shell mass of *Littorina obtusata* (L.) snails in the Gulf of Maine? *J. Exp. Mar. Biol. Ecol*. 394:98–104.

Evans, L. H. 1999. Lobster health and disease: basic concepts. In: L. H. Evans & J. B. Jones (eds). International symposium on lobster health management held September, 19–21, 1999, Adelaide, South Australia. Curtin University, Bentley, Western Australia, pp. 3–8.

Fletcher, C. R. 1995. Microgeographical variation in shell strength in the flat periwinkles *Littorina obtusata* and *Littorina mariae*. *Hydrobiologia* 309:73–87.

Ford, S. E. & F. J. Borrero. 2001. Epizootiology and pathology of juvenile oyster disease in the Eastern oyster, *Crassostrea virginica*. *J. Invertebr. Pathol*. 78:141–154.

Glenn, R. & T. L. Pugh. 2005. Observations on the chronology and distribution of lobster shell disease in Massachusetts coastal waters. In: M. F. Tlusty, H. O. Halvorson, R. M. Smolowitz & U. Sharma, editors. Lobster shell disease workshop. Aquatic Forum Series 05-1. Boston, MA: New England Aquarium. pp. 141–155.

Glenn, R. P. & T. L. Pugh. 2006. Epizootic shell disease in American lobster (*Homarus americanus*) in Massachusetts coastal waters: interactions of temperature maturity, and intermolt duration. *J. Crustac. Biol*. 26:639–645.

Homerding, M., A. McElroy, G. T. Taylor, A. Dove & B. Allam. 2012. Investigation of epizootic shell disease in American lobsters (*Homarus americanus*) from Long Island Sound: II. Immune parameters in lobsters and relationships to the disease. *J. Shellfish Res*. 31:495–504.

Howell, P., C. Giannini & J. Benway. 2005. Assessment and monitoring of the American lobster resource and fishery in Long Island Sound. Semi-Annual Performance Report. NOAA Grant #NA16FW1238.

Kunkel, J. G., W. Nagel & M. J. Jercinovic. 2012. Mineral fine structure of the American lobster cuticle. *J. Shellfish Res*. 31:515–526.

Malloy, S. 1978. Bacteria induced shell disease of lobsters (*Homarus americanus*). *J. Wildl. Dis*. 14:2.

McCay, D. P. F, M. Gibson & J. S. Cobb. 2003. Scaling restoration of American lobsters: combined demographic and discounting model for an exploited species. Marine Ecology Progress Series 264:177–196.

McKown, K., R. Burgess & P. Nunnenkamp. 2005. Incidence of shell disease in American lobster (*Homarus americanus*) in New York waters. In: M. F. Tlusty, H. O. Halvorson, R. M. Smolowitz & U. Sharma, editors. Lobster shell disease workshop. Aquatic Forum Series 05-1. Boston, MA: New England Aquarium. pp. 173–176.

Millennium Ecosystem Assessment. 2005. Ecosystems and human well-being: synthesis. Washington, DC: Island Press. 137 pp.

Nagarajan, R., S. Lea & J. Gosscustard. 2006. Seasonal variations in mussel, *Mytilus edulis* L. shell thickness and strength and their ecological implications. *J. Exp. Mar. Biol. Ecol*. 339:241–250.

Quinn, R. A., A. Metzler, R. M. Smolowitz, M. Tlusty & A. Y. Chistoserdov. 2012. Exposures of *Homarus americanus* shell to three bacteria isolated from naturally occurring epizootic shell disease lesions. *J. Shellfish Res*. 31:485–493.

Raabe, D., C. Sachs & P. Romano. 2005. The crustacean exoskeleton as an example of a structurally and mechanically graded biological nanocomposite material. *Acta Mater*. 53:4281–4292.

Robohm, R. A., A. F. J. Draxler, D. Wieczorek, D. Kapareniko & S. Pitchford. 2005. Effects of environmental stressors on disease susceptibility in American lobsters: a controlled laboratory study. *J. Shellfish Res*. 24:773–779.

Smolowitz, R., A. Y. Chistoserdov & A. Hsu. 2005. A description of the pathology of epizootic shell disease in the American lobster, *Homarus americanus*, H. Milne Edwards 1837. *J. Shellfish Res*. 24: 749–756.

Snieszko, S. F. 1973. The effects of environmental stress on outbreak of infectious diseases of fishes. *J. Fish Biol*. 6:197–208.

Steenbergen, J. F., S. M. Steenbergen & J. C. Schapiro. 1978. Effects of temperature on phagocytosis in *Homarus americanus*. *Aquaculture* 14:23–30.

Stevens, B. 2009. Effects of epizootic shell disease in American lobster *Homarus americanus* determined using a quantitative disease index. *Dis. Aquat. Organ*. 88:25–34.

Tlusty, M. F. 2005. Use of digital colour analysis to assess variation within individual adult American lobsters (*Homarus americanus*) and the process of addition of colour in white lobsters. *N. Z. J. Mar. Freshw. Res*. 39:571–580.

Tlusty, M. & C. Hyland. 2005. Astaxanthin deposition in the cuticle of juvenile American lobster (*Homarus americanus*): implications for phenotypic and genotypic coloration. *Mar. Biol*. 147:113–119.

Tlusty, M., A. Myers & A. Metzler. 2008. Short- and long-term dietary effects on disease and mortality in American lobster *Homarus americanus*. *Dis. Aquat. Organ*. 78:249–253.

Tlusty, M. F., R. M. Smolowitz, H. O. Halvorson & S. E. DeVito. 2007. Host susceptibility hypothesis for shell disease in American lobsters. *J. Aquat. Anim. Health* 19:215–225.

Trussell, G. C. & R. J. Etter. 2001. Integrating genetic and environmental forces that shape the evolution of geographic variation in a marine snail. *Genetica* 112:321–337.

van Olst, J. C., J. M. Carlberg & J. T. Hughes. 1980. Aquaculture. In: J. S. Cobb & B.F. Phillips, editors. The biology and management of lobsters. Vol. II. Ecology and management. New York: Academic Press. pp. 333–384.

Wiesner, C. S. & C. Iben. 2003. Influence of environmental humidity and dietary protein on pyramidal growth of carapaces in African spurred tortoises (*Geochelone sulcata*). *J. Anim. Phys. Anim. Nutrit*. 87(1–2):66–74.