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research paper

# Increasing prevalence of epizootic shell disease in American lobster from the nearshore Gulf of Maine

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ABSTRACT.—Epizootic shell disease (ESD) is a significant concern to the southern New England lobster fishery. Although ESD has been reported in the southern Gulf of Maine off Massachusetts, there are few reports from Maine waters. We report on the occurrence and distribution of ESD in American lobsters from nearshore Gulf of Maine from the Maine Commercial Lobster Sea Sampling Program. Overall, average prevalence levels of ESD by trip were very low (<0.16%) through 2010, then increased from 2011 to the present, reaching 1.2% in 2013. As with previous studies, recent prevalence levels in legal and sublegal (<127 mm CL) animals were higher (6%–7%) in egg-bearing females than in males and non-ovigerous females. This pattern was amplified in oversized (>127 mm CL) lobsters, regardless of sex and reproductive state, with much higher prevalence levels (up to 22%). Spatially, prevalence levels of ESD were significantly higher in western regions of the Gulf of Maine than off eastern Maine. Using histology and microbiome analyses, the etiology of the disease was investigated and common signs of lobsters with ESD were described. *Aquimarina homari,* a bacterium associated with ESD, was significantly more prevalent on lobsters with lesions and abundance was correlated with severity of ESD. Our report indicates that ESD is present on lobsters throughout the nearshore waters of the Gulf of Maine. Given the effect of the disease on lobsters from southern New England and its increasing prevalence over time, further monitoring of ESD in the Gulf of Maine is warranted.

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The American lobster, *Homarus americanus* H. Milne-Edwards, 1837, supports one of the largest shellfish fisheries in the world. Maine lobster landings reached a peak in 2016 worth more than US$533 million ex-vessel value and Maine has landed >55,000 t of lobster annually since 2012. The state's landings provided on average 84% of the total US American lobster harvest in that time period (ACCSP 2016, Maine DMR 2016a). The lobster population in the Gulf of Maine, as a component of the Gulf

of Maine and Georges Bank lobster stock (GOM/GBK), has increased rapidly over the last decade, reaching a record high in abundance in the last stock assessment (ASMFC 2015). This increase in GOM/GBK stock is happening as the southern New England (SNE) stock has collapsed to record low abundance levels (ASMFC 2015). The decline in the SNE stock is due to poor recruitment, possibly related to the effect of epizootic shell disease (ESD) on ovigerous lobsters (ASMFC 2015, Hoenig et al. 2017). The GOM/GBK stock has always been a larger population than the SNE stock, but the southern stock experienced similar relative record highs in the late 1990s before collapsing in the early 2000s (ASMFC 2015).

ESD is a complex disease characterized as a bacterial dysbiosis that occurs in conjunction with environmental stressors, particularly temperature, and possibly contaminants (Tlusty et al. 2007, Chistoserdov et al. 2012, Shields 2013). High prevalence levels of ESD in lobsters from eastern Long Island Sound (LIS) and Buzzards Bay, Massachusetts, indicate that widespread phenomena, such as increased temperature, influence prevalence of the disease, rather than point-source contaminants or local problems with water quality. Alkylphenols, heavy metals, and other contaminants have been found in lobsters and sediments from the region, but with the exception of alkylphenols, there are no clear associations with ESD (Jacobs et al. 2012, LeBlanc and Prince 2012, Laufer et al. 2012, 2013). As an environmental disease, the etiology of ESD is thought to arise as increased temperatures negatively affect hosts' defensive responses, with contaminants potentially weakening the cuticle after molting, making it more susceptible to a dysbiotic bacterial community arising from anthropogenic driven deterioration of water quality (Laufer et al. 2012, 2013, Shields 2013). Increased temperature (>16 °C) is a major driver of ESD and lower temperatures apparently limit the spread of it on individual lobsters, and may reduce prevalence in affected populations (Glenn and Pugh 2006).

Classical, enzootic, or endemic shell disease was first described on the American lobster by Hess (1937). It is typically present at low levels in lobster populations and only rarely develops into a severe infection (Taylor 1949, Estrella 1991, Cobb and Castro 2006). Scrapes and cuts to the cuticle from entering traps or from handling provide a portal of entry for chitinoclastic bacteria to invade the underlying, chitin-rich layers of the exo- and endocuticle. In lobsters, classical shell disease has been categorized based on underlying causalities: impoundment shell disease (Smolowitz et al. 1992), diet-induced shell disease (Tlusty et al. 2008), and trauma-induced, or endemic shell disease (Smolowitz et al. 2014).

ESD is different from classical shell disease, as it develops rapidly, has a high prevalence in affected populations, and is clearly associated with environmental stressors (Castro et al. 2006). ESD presents as an extensive necrosis of the lobster cuticle (Smolowitz et al. 2005, Shields et al. 2012). In the early manifestation of ESD, bacterial invaders burrow rapidly through the exocuticle into the endocuticle, forming distinctive pits visible to the naked eye. The intact endocuticle around the pits appear histologically as pillars that eventually ablate revealing large affected areas of eroded endocuticle. The proximate causality of ESD is a rapid degradation of the exocuticle and endocuticle by a suite of bacteria, most notably including the bacterium *Aquimarina macrocephali* subsp. *homari,* as well as several other chitinoclastic bacteria (Chistoserdov et al. 2012, Meres et al. 2012, Quinn et al. 2017).

ESD first emerged in lobsters from eastern Long Island Sound (LIS) and Buzzards Bay, Massachusetts, in the late 1990s. From 1997 to the present, it became more

prevalent in lobsters off SNE, especially from eastern LIS, Block Island Sound, Narragansett Bay (Castro and Angell 2000, Castro et al. 2005, Landers 2005, Howell et al. 2005, Castro and Somers 2012, Howell 2012), and Buzzards Bay, and to a lesser extent, more northward in waters from Cape Cod Bay to Cape Ann (Glenn and Pugh 2006). Lobsters with severe shell disease have been found in the Gulf of Maine, but at low levels (Taylor 1949, Wilson 2005). ESD is most prevalent (>65%) in egg-bearing females from waters off Rhode Island and in eastern LIS, presumably because they do not molt for 2 yrs (Castro et al. 2005, Howell et al. 2005, Landers 2005, Castro and Somers 2012). ESD makes lobsters unmarketable in the live trade due to extensive necrosis of the carapace and claws. More importantly, the disease has been associated with a reduction in the affected lobster population (Castro and Somers 2012, Howell 2012) through reductions in egg production (Wahle et al. 2009) due to high mortality rates in diseased ovigerous females (Hoenig et al. 2017).

The objectives of the present study were to: (1) report the occurrence of ESD in American lobsters caught in Maine; (2) analyze the prevalence data in relation to time, space, and other abiotic factors to uncover possible associations with the disease; (3) identify the nature and causality of ESD in lobsters from Maine using histological and molecular techniques; and (4) establish baseline information on the bacterial community in lobsters with and without shell disease using molecular techniques.

## Methods

Maine Commercial Lobster Sea Sampling Program.—Starting in 1985, Maine's Department of Marine Resources (DMR) began collecting fishery dependent biological data on legal and discarded lobsters from the nearshore Maine commercial lobster fishery. In 1998, the DMR expanded the data collection to its now standard three trips per zone (A–G) per month from May to November (Fig. 1). As part of these data collection efforts, Maine's Commercial Lobster Sea Sampling (LSS) Program places trained observers on lobster boats to collect and record biological data on both the harvested and discarded portions of the catch. The data collected on every lobster observed includes carapace length (CL), sex, presence and stage of eggs, relative molt stage, V-notch presence and characterization, claw status, and since 2003, the presence and intensity of shell disease (Maine DMR 2016b). Since 2003, the LSS program has collected biological data on between 100,000 and 250,000 kept or discarded lobsters annually.

Lobsters available for harvest are determined by minimum and maximum CL limits and reproductive status. For size, legal lobsters must have a CL of >83 and <127 mm. For reproductive status, females bearing eggs or possessing a V-notch are prohibited from harvest. A V-notch is a conservation tool used in the lobster fishery. Lobstermen cut a V-shaped mark in a specific uropod defined by regulation on each egg-bearing females to indicate it was a successful breeder. Once eggs have hatched, the presence of the V-notch legally prohibits harvest and protects the female lobster to reproduce again in the future.

The LSS Program samplers collect relative shell stage data by determining if a lobster molted within the calendar year and defining it as new or old shell. The dorsal carapace of each animal is pressed inwards and claw or carapace scars are assessed (Maine DMR 2016b). The program considers all lobsters old shell as of January 1 of

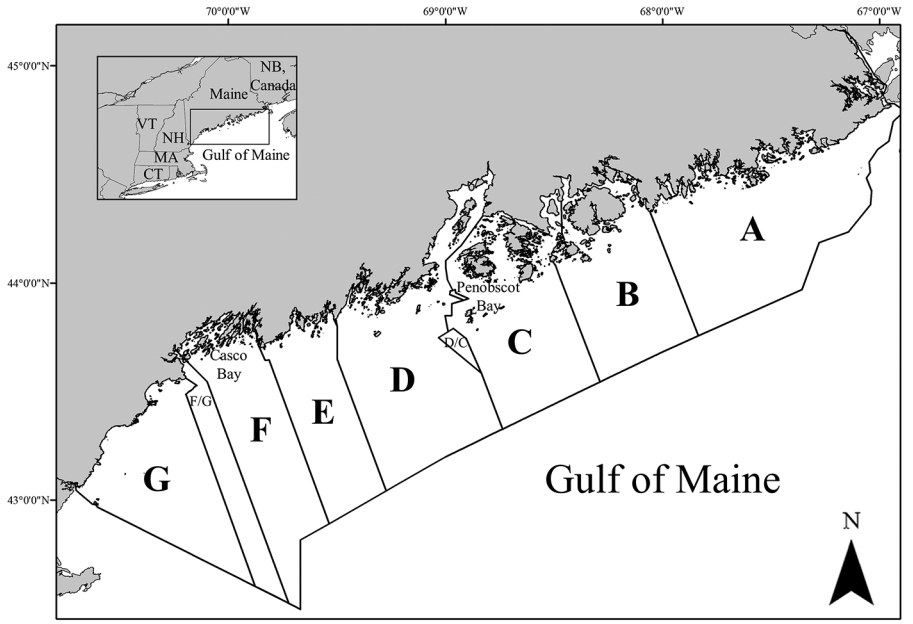


Figure 1. Map of Maine's Lobster Management Zones in Lobster Management Area 1 with locations of collections for dissections and histology.

the sample year. The data provide an old shell proportion by trip found in the combined harvested and discarded catch.

ESD data collection was initiated in the LSS Program in 2003. ESD is visually assessed by LSS observers as a categorized percentage of carapace covered by the characteristic lesions and assigned a standardized indexed level of coverage (0%, 1%–10%, 11%–50%, and >50%) (Landers 2005, Maine DMR 2016b). Samplers are trained using pictures and examples of relative coverage.

Although not randomly selected, monthly sampling trips are distributed spatially within each zone by port and boats are typically sampled only once per year providing a spatially representative data set across the state and within each zone for the months sampled. The Maine lobster fishery is territorial, so general trap locations can be predicted by home port or previous sampler experience. Spatial data is approximated for groups of 10–20 traps and collected via handheld GPS while depth is estimated for the group of traps using each boat's depth finder. Participation in the program is not mandatory, but the list of available license holders includes more than 300 volunteer captains and the program continues to add new participants annually.

Model Development.—Generalized additive models (GAM) were used to investigate the statistical significance of annual, spatial, and seasonal trends of ESD prevalence or mean CL. For these analyses, only the data noting the presence or absence of disease were utilized. To determine the prevalence of ESD, we used the number of symptomatic vs healthy animals by trip to assure a standard sample per year and by spatial area or zone. The biological data considered in these analyses included the proportion of lobsters with ESD (i.e., prevalence) and the proportion of lobsters with ESD binned by size and reproductive status. The minimum and maximum sizes

were used to classify the samples into three size categories: sublegal (<83 mm), legal (83–127 mm), and oversize (>127 mm) animals. All samples were classified into three reproductive categories: male, female without eggs, and female with eggs. V-notch presence was not considered. The three reproductive categories were also used to investigate differences in mean CL trends over time for both ESD and healthy lobsters. Other biological specific data included the proportion of old shell lobsters by trip.

The GAMs were estimated using R package mgcv. The response variables calculated by trip were total ESD prevalence, ESD prevalence by size and sex categories, and mean CL by sex. The factors considered were year, zone, month, proportion old shell, and average trip depth. The reference factors were defined as year 2003, Zone A, October, male sublegal, and mean size of healthy lobsters for each sex because these were the categories with the lowest ESD. There were many trips with no ESD so a Tweedie error distribution was necessary to accommodate the zero inflated prevalence data (Shono 2008). Variables were retained if statistically significant ($P < 0.05$) in at least some levels as in Chang et al. (2010). Figure means and error structure were calculated by bootstrapping the trip values 10,000 times for each year but raw data was used for model runs.

Lobster Dissection and Histology.—To assess the pathology and causality of ESD in Maine waters, approximately equal numbers of diseased and healthy animals were sampled from two different areas in Maine in June and July 2013 and shipped on blue ice to the Virginia Institute of Marine Science (VIMS) for etiological investigations. Lobsters were collected by personnel from the DMR using commercial lobster pots. Lobsters were not sampled randomly; they were visually selected based on the severity of their shell disease, with uninfected animals from the same regions serving as relative controls. Animals representing the eastern regions of the Gulf of Maine were captured in Penobscot Bay, Maine. Animals from the western regions were captured in Casco Bay, Maine. At VIMS, lobsters were assessed for morbidity and processed as described below.

Standard protocols were used for health assessments as in Shields et al. (2012). Briefly, animals were visually examined for sex, CL, shell condition, and injuries. They were then photographed (Olympus 3000) and evaluated for shell disease (no shell disease, light, moderate or heavy infections) as in Landers (2005). For the histological component, samples (approximately 1 cm square pieces) of cuticle, gill, hepatopancreas, heart, muscle, antennal gland, and gonad were excised, placed in cassettes and fixed in Z-fixative (Fisher Scientific) or 10% neutral buffered formalin. Shell pieces were decalcified overnight using the formic acid-sodium citrate method (Luna 1968). Tissues were then processed using paraffin histological techniques as in Shields et al. (2012). Prepared sections were examined with an Olympus BX51 compound microscope and photographs were taken using a Nikon DXM1200 digital camera with the aid of the ACT-1 computer program (Nikon).

During lobster dissections, pieces of the shell (carapace, abdomen, telson, uropods, pleopods, claws, and legs) of each lobster were taken from diseased areas, from adjacent to diseased areas, and from healthy areas of individual lobsters and frozen at −80 °C for later analyses. Cuticle samples were shipped overnight on dry ice to George Mason University, Manassas, Virginia, for molecular assessments.

Microbiome Analysis.—For the molecular assessment of the bacterial flora, cuticle samples were processed as in Meres et al. (2012). Briefly, cuticle pieces were

placed in EDTA and Proteinase K to isolate all microorganisms from the surface and subsurface. Total DNA was extracted using the FastPrep Bio101 kit (Qbiogene/MP Biomedicals LLC, Solon, Ohio). The polymerase chain reaction (PCR) was employed to amplify the bacterial genes from the first two hyper-variable regions of the 16s ribosomal RNA using universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′), and 355R (5′-GCT GCC TCC CGT AGG AGT-3′) (Invitrogen Corp.). Length Heterogeneity PCR (LH-PCR) fingerprinting (Suzuki et al. 1998) was used to rapidly survey samples and standardize the community amplification. Multitag Pyrosequencing (MTPS) was used to characterize the taxa in the microbiome of the carapace samples as Gillevet (2006) and Meres et al. (2012). A set of 96 fusion primers containing emulsion PCR linkers (454 Life Sciences) was generated with different 7-base "barcodes" on either 27F or 355R universal 16S rRNA primers. Each sample of lobster DNA was amplified with a unique set of tagged forward and reverse 16S rRNA primers, pooled, subjected to emulsion PCR, and pyrosequenced using a GS-FLX pyrosequencer as per manufacturer's instructions (Roche). Sequence data were sorted into bins based on barcodes using custom PERL scripts. Sequence reads were identified using the Bayesian analysis in the Ribosomal Database Project (Cole et al. 2008). A custom PERL script was used to calculate the normalized abundance of taxa in a sample based on the total reads in each sample.

Comparisons of the microbial community sequences derived by MTPS were analyzed using the software program Quantitative Insights into Microbial Ecology (QIIME) (Caporaso et al. 2010). The similarities in the microbiome structure were analyzed using Unifrac neighbor-joining trees (Lozupone and Knight 2005) that graphically displayed similarities between each lobster sampled. Discriminant analysis using the default parameters was performed with PASW v18 (IBM, Chicago, IL). Principle coordinate analysis was performed using MultiVariate Statistical Package (Kovach Computing Services, Anglesey, Wales) using a Bray Curtis distance metric.

For standard PCR protocols, we followed the protocol for *A. macrocephali* subsp. homaria as in Quinn et al. (2017). Briefly, shell pieces were scraped with a sterile blade, the shavings were added to DNA extraction buffer, and the DNA was isolated and purified using standard kits (Qiagen). The 16s primer set was 341fM (5′-CCTACGGGDGGCWGCAG-3′, *Escherichia coli* position 341 bp) and 907rM (5′-CCGYCWATTCMTTTGAGTTT-3′, *E. coli* position 907) as in Quinn et al. (2013). The protocol described by Quinn et al. (2013) was used for DNA amplification. Briefly, each 20-µl reaction contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 0.5 unit of Taq polymerase (Applied Biosystems), and 1 µl DNA. The amplification cycle consisted of 35 cycles of 95 °C for 30 s, 51 °C for 30 s, and 72 °C for 45 s, followed by a final extension of 72 °C for 3 min. Amplified products were visualized by agarose gel electrophoresis (1.5% w/v), stained with ethidium bromide and viewed under a UV light source.

## Results

Fishery Trends in Epizootic Shell Disease (ESD).—The LSS Program completed 2210 sampling trips from 2003 through 2016 with about 150 trips annually. The total number of trips with observations of ESD was 9% in 2003. The percentage of ESD positive annual trips ranged between 10% and 32% until 2011 when the number increased to 66%; it has remained above that level peaking at 86% in 2013

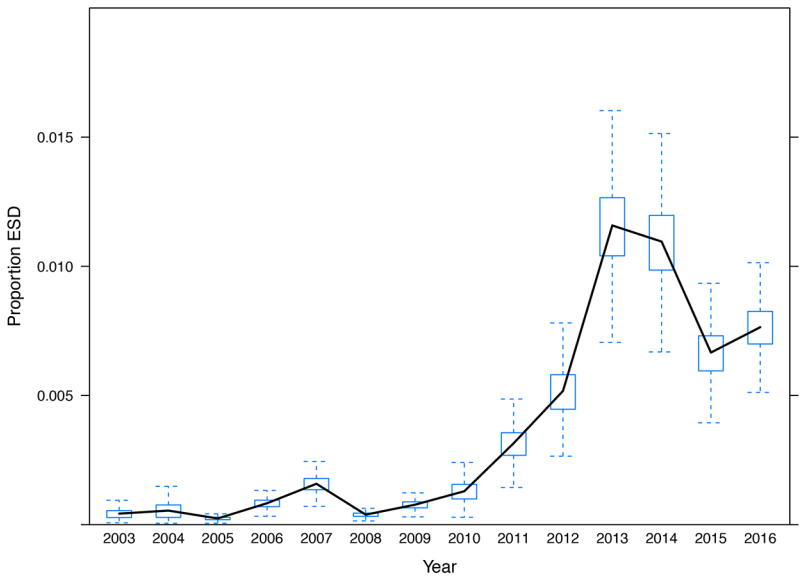


Figure 2. Bootstrapped annual trip mean prevalence of ESD by in all observed lobsters, 2003–2016, with 95% confidence intervals.

and 2014. The prevalence of ESD per trip was very low (<0.16%) through 2010, then increased to moderately low levels (>0.31% and <1.2%) between 2011 and 2016 with a maximum level in 2013 (Fig. 2). Between 2003 and 2010, the sampling program observed 100,000 and 150,000 lobsters per year, with 44–120 lobsters exhibiting signs of ESD annually. Starting in 2011, the total number of lobsters sampled (>200,000) increased, as did those with ESD ranging from 428 (2011) to 1464 (2013).

Spatially, ESD was more prevalent in the western fishery management zones (E–G, *see* Fig. 1 for management zones), but increased prevalence was observed along the entire coast (Fig. 3). Overall zone levels of ESD peaked in lobster management zones F and G with mean values of 2.9% per trip in 2013, but then dropped to lower levels in subsequent years. Eastern zones remained <0.5%, with an outlier in zone A in 2014 (0.7%). The proportion of ESD was greater relative to the sampling effort in

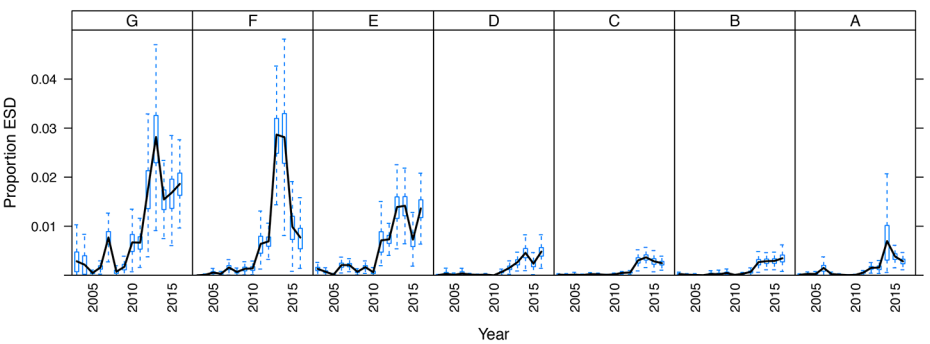


Figure 3. Bootstrapped annual trip mean prevalence of ESD in lobsters by Lobster Management Zone for years 2003–2016 with 95% confidence intervals. The zones run west to east along the coast (G–A).

shallowest depths (<20 m) in the western zones, but was similar to sampled effort in the eastern management zones.

The GAM of the total ESD prevalence determined significance in time (year), space (zone), and season (month) with significant smoothing factors of depth and proportion old shell explaining 62.5% of the deviance from the reference factors (M1 in Table 1). All years were significantly different from 2003 except 2004, 2005, 2008, and 2009. The years after 2010 were all strongly significant ($P < 0.001$). Using zone A as the reference level, zones E, F, and G were strongly significantly different. Zones C and D were not statistically different from zone A, while zone B was significantly different ($P < 0.05$). All months except May were significantly different from the reference month of October with the highest significance in the June through August period. The proportion of lobsters in the old shell condition was highly correlated with month (–0.79), but was also a highly significant smoothing factor. The prevalence of ESD decreased with lower proportions of old shell lobsters, which is also a seasonal process. In addition, depth was a significant smoothing factor with higher prevalence of ESD at the shallowest and deepest values, although less data were available at greater depths yielding higher variability.

While the overall prevalence levels of ESD have remained low, certain segments of the population have experienced higher levels and shown clear increasing trends (Fig. 4). The greatest change and highest prevalence levels, from 5% in 2007 to over 22% in 2016, were observed in oversize males. In 2013, the other oversize categories

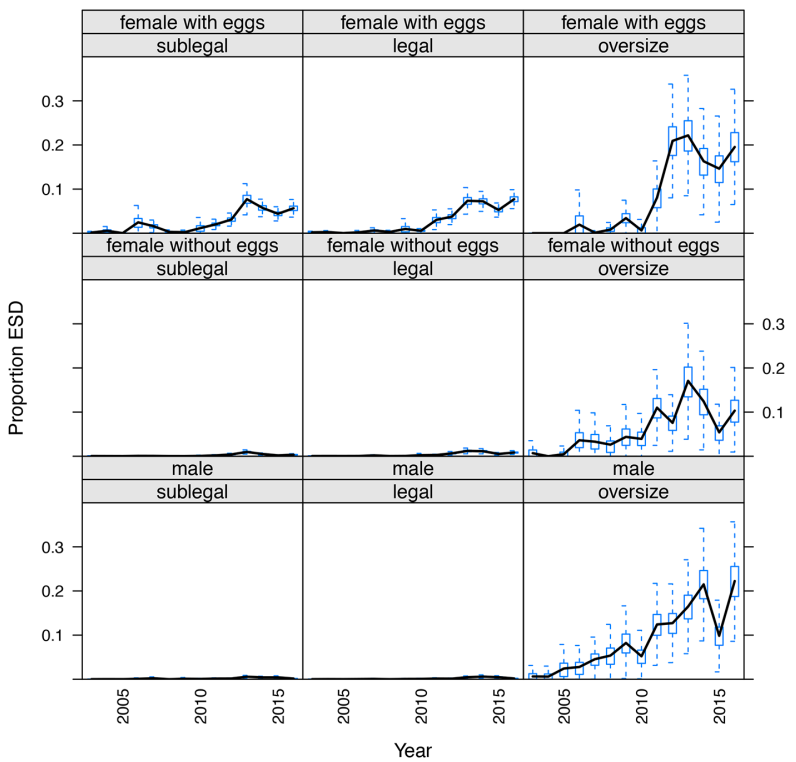


Figure 4. Bootstrapped annual trip mean prevalence of ESD in lobsters for each size (sublegal: <83 mm CL, legal: >83 mm CL and <127 mm CL, and oversize: >127 mm CL) and sex (female with eggs, female without eggs, and male) category with 95% confidence intervals.

Table 1. *P*-value results for categorical and smoothing factors in generalized additive models (GAMs) for total epizootic shell disease (ESD) prevalence (M1) and ESD prevalence by size and sex category (M2). Bold indicates significance at $P < 0.05$. For year, 2003 was the reference year; for zone, Zone A was the reference zone; for month, October was the reference month; and for size and sex category, sublegal male was the reference category.

| Variables | M1 (total ESD) | M2 (ESD by size and sex) |
|---|---|---|
| Year | | |
| 2004 | 0.900 | 0.945 |
| 2005 | 0.052 | 0.673 |
| 2006 | **0.002** | **<0.001** |
| 2007 | **<0.001** | **<0.001** |
| 2008 | 0.339 | **<0.001** |
| 2009 | 0.336 | **<0.001** |
| 2010 | **0.010** | **<0.001** |
| 2011 | **<0.001** | **<0.001** |
| 2012 | **<0.001** | **<0.001** |
| 2013 | **<0.001** | **<0.001** |
| 2014 | **<0.001** | **<0.001** |
| 2015 | **<0.001** | **<0.001** |
| 2016 | **<0.001** | **<0.001** |
| Zone | | |
| Zone B | **0.037** | 0.179 |
| Zone C | 0.055 | **0.020** |
| Zone D | 0.535 | 0.970 |
| Zone E | **<0.001** | **<0.001** |
| Zone F | **<0.001** | **<0.001** |
| Zone G | **<0.001** | **<0.001** |
| Month | | |
| May | 0.052 | **<0.001** |
| June | **<0.001** | **<0.001** |
| July | **<0.001** | **<0.001** |
| August | **<0.001** | **<0.001** |
| September | **0.001** | 0.285 |
| November | **0.033** | 0.516 |
| Size and sex category | | |
| Male | | |
| Legal | | 0.472 |
| Oversize | | **<0.001** |
| Female without eggs | | |
| Sublegal | | 0.327 |
| Legal | | **<0.001** |
| Oversize | | **<0.001** |
| Female with eggs | | |
| Sublegal | | **<0.001** |
| Legal | | **<0.001** |
| Oversize | | **<0.001** |
| Smoothing factors | | |
| Percent old shell | **<0.001** | **<0.001** |
| Depth | **0.029** | 0.050 |
| Sample size (*n*) | 2,199 | 15,068 |
| Deviance explained | 62.5% | 50.1% |

Table 2. *P*-value results for categorical and smoothing factors in generalized additive models (GAMs) for mean size of healthy and diseased animals by female with eggs (M3), female without eggs (M4), and male (M5). Bold indicates significance at $P < 0.05$. For year, 2003 is the reference year. ESD = epizootic shell disease.

| Variables | M3 (female with eggs) | M4 (female without eggs) | M5 (male) |
|---|---|---|---|
| Year × ESD | | | |
| 2004 | 0.271 | 0.576 | 0.251 |
| 2005 | 0.926 | 0.094 | **0.029** |
| 2006 | 0.276 | **0.031** | 0.904 |
| 2007 | 0.101 | **<0.001** | **0.002** |
| 2008 | 0.094 | **<0.001** | **<0.001** |
| 2009 | 0.058 | **<0.001** | **<0.001** |
| 2010 | 0.314 | **0.009** | **<0.001** |
| 2011 | 0.817 | **<0.001** | **<0.001** |
| 2012 | 0.991 | 0.064 | **<0.001** |
| 2013 | 0.680 | 0.170 | **0.002** |
| 2014 | 0.679 | **0.034** | **0.001** |
| 2015 | 0.680 | **0.033** | **0.015** |
| 2016 | 0.684 | **0.005** | **0.000** |
| Smoothing factors | | | |
| Percent old shell | - | **0.011** | **<0.001** |
| Depth | **<0.001** | **<0.001** | **<0.001** |
| Sample size (*n*) | 2,736 | 2,819 | 2,627 |
| Deviance explained | 27.6% | 37.4% | 48.6% |

also had the highest peaks at 22% for oversize females with eggs and 17% for oversize females without eggs. For both legal- and sublegal-sized lobsters, the ovigerous females had higher prevalence levels of ESD at 7.5% in 2016 and 7.6% in 2013, respectively, compared to the males or females without eggs of these sizes (<1%). Maximum ESD levels in sublegal males and females without eggs occurred in 2013 and were 0.5% and 0.9%, respectively. The highest ESD levels for legal sized males and females without eggs were 0.5% in 2014 and 1.1% in 2013.

The GAM of ESD prevalence for each size and sex as the response variable with factors of year, zone, month, and the nine size and sex categories with old shell proportion and depth as smoothing variables, explained 50.1% of the deviance (M2 in Table 1). Patterns for the size and sex combinations were similar to the total ESD prevalence, where 2006–2016 were highly significantly different from 2003; the western zones E, F, and G were highly significantly different from zone A; and seasonally, May through August were highly significantly different from October. For the actual size and sex combinations, all of the oversize and female with eggs categories were significantly different from the male sublegal levels. Legal sized females without eggs also were significantly different from male sublegals, while legal males and sublegal females without eggs were not significantly different.

The models for mean size were simplified to an interaction of year and a binomial ESD factor (healthy or ESD lobsters) with smoothing factors of depth and proportion old shell (M3–M5 in Table 2). Each GAM separately tested for significant difference of mean size by presence/absence by year for the categories of male, female with eggs, and female without eggs, with 2003 as the reference year. The model for females with eggs (M3) explained only 27.6% of the deviance and the mean size was

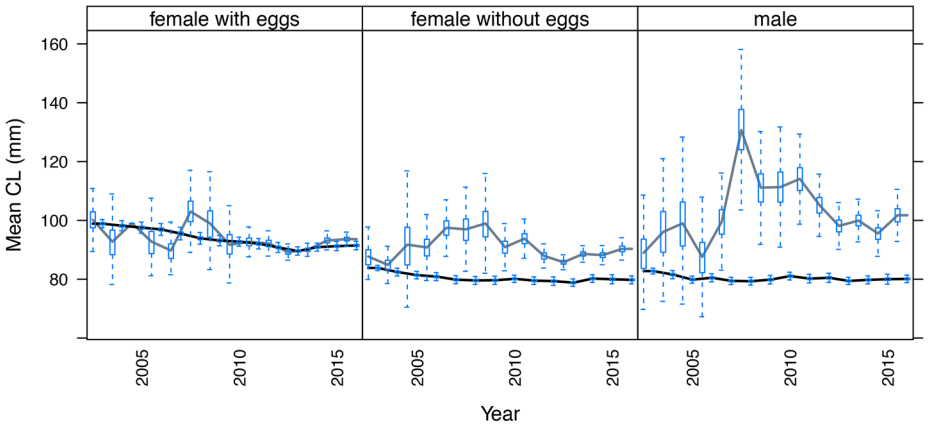


Figure 5. Bootstrapped annual trip mean size for symptomatic (gray line) and healthy (black line) lobsters for each sex (female with eggs, female without eggs, and male) category with 95% confidence intervals. The two data sets are staggered by year and represent trips from 2003 to 2016.

not significantly different in any year. For this model, the proportion old shell by trip was not a significant smoothing factor and was removed from the model. The GAM for female without eggs mean size (M4) explained 37.4% of the deviance and was significantly different from 2003 in all but four years (2004, 2005, 2012, and 2013). The model for mean size of males (M5) explained 48.6% of the deviance and was significant different from the reference year in all years except 2004 and 2006. The mean size of lobsters with ESD was always larger than healthy animals for females without eggs and males (Fig. 5). For all ESD and sex categories, the variability and range of the 95% confidence intervals were high in the earlier years but narrowed with increased samples sizes of diseased animals in time. The mean size of males with ESD peaked in 2008 (130.7 mm CL) then decreased to approximately 100 mm CL (Fig. 5).

Disease Causality.—Lobsters were selected based on sex and infection status. There were 29 females and 6 male lobsters. Of these, 19 were from the Down East region (Penobscot Bay) and 14 were from the Southern Coast (Casco Bay). Lobsters with ESD presented with gross signs of the disease. There were 19 females and no males with ESD. Of the animals with ESD, 6 had light infections, 9 had moderate infections, and 4 had heavy infections. The lesions were identical to ESD on lobsters from LIS. Externally, the lesions were friable, discolored, or with signs of intensive melanization. The lesions varied considerably in size, with some covering the entire dorsal carapace. Animals characterized as having light cases of ESD had small burn-like areas that in some cases had coalesced into 1–2 cm lesions on the carapace or abdomen. Animals with moderate ESD had more extensive lesions, typically 2–3 cm areas of involvement, generally with coverage on both the dorsal carapace and the dorsal abdominal somites (Fig. 6). Animals with heavy infections had extensive coverage of the carapace and abdomen, with >3 cm lesions, often with involvement of the claws and extensive involvement of the dorsal carapace.

Histologically, the pathology of lobsters with shell disease lesions was virtually identical to that described by Smolowitz et al. (2005) and Shields et al. (2012). Deep penetrating pits occurred on the cuticle of animals with ESD and the pitted areas contained friable remnants of the cuticle with sinuous pillars of endocuticle. Zones

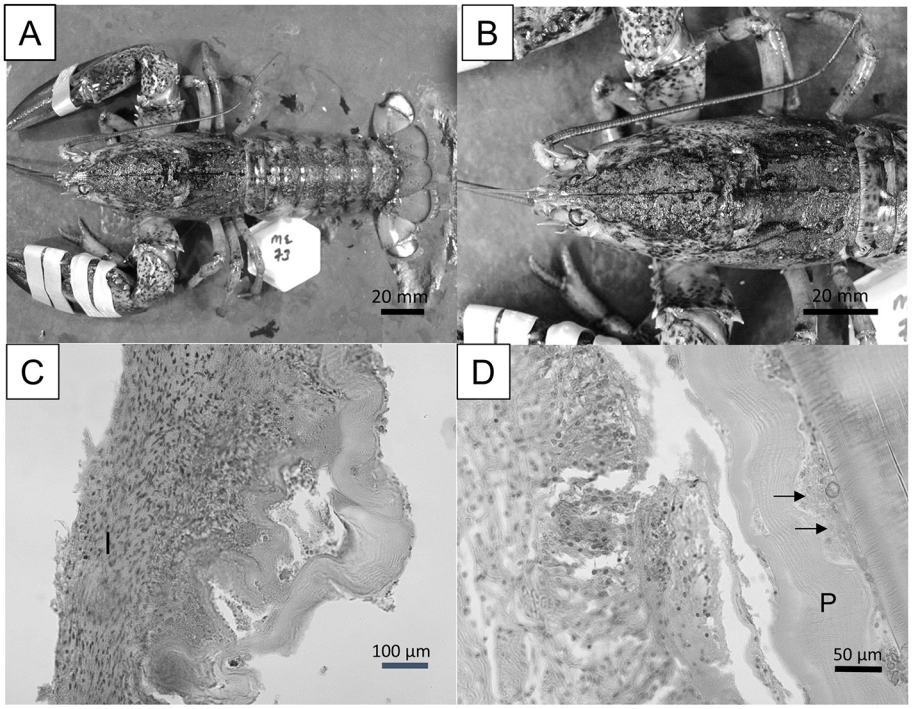


Figure 6. (A, B) Lobster ME 73 from Penobscot Bay exhibiting a heavy case of epizootic shell disease. Note the extensive, coalesced lesions on the dorsal carapace. (C) Histological preparation showing extensive cellular infiltration (I) in connective tissue scaffold under a severely eroded cuticle. (D) Pseudomembrane (P) between the cuticle to the right and the underlying soft tissues to the left. Note the extensive nodulation (arrows) and melanization.

of affected cuticle often contained microbial organisms medially within the pillars. In moderate and heavy infections, melanization was extensive, occurring within the membranous layer, the endocuticle, and the exocuticle (Fig. 6). In areas adjacent to the lesions, the layers of the cuticle appeared to have become delaminated from their normal lamellar appearance. In moderate and heavy infections, the membranous layer of the cuticle had varying degrees of nodulation with infiltration of hemocytes into the adjacent epidermal area in some animals. Nodulation was often surrounded by melanized areas and was clearly associated with pseudomembranes. Pseudomembranes were observed in several animals with moderate and heavy ESD (Fig. 7). In two animals in premolt, the lesions had penetrated through cuticle with clearly formed lesions in the newly developing cuticle. In most cases, the underlying musculature was not affected, but at least two lobsters showed extensive infiltration of hemocytes into subepidermal areas that included muscular attachments.

The microbiome on the lobster cuticle was comprised of a diverse bacterial community. Lobsters from Maine had a floral community that included at least 16 different phyla of bacteria (for more information, *see* Online Supplementary Material). Members of the Proteobacteria were the most abundant members of the community followed by members of the Fusobacteria, the Bacteriodetes, and the Actinobacteria. A more detailed analysis of the genera within the Bacteroidetes, Actinobacteria, and a few other phyla show a very high level of diversity with a large number of

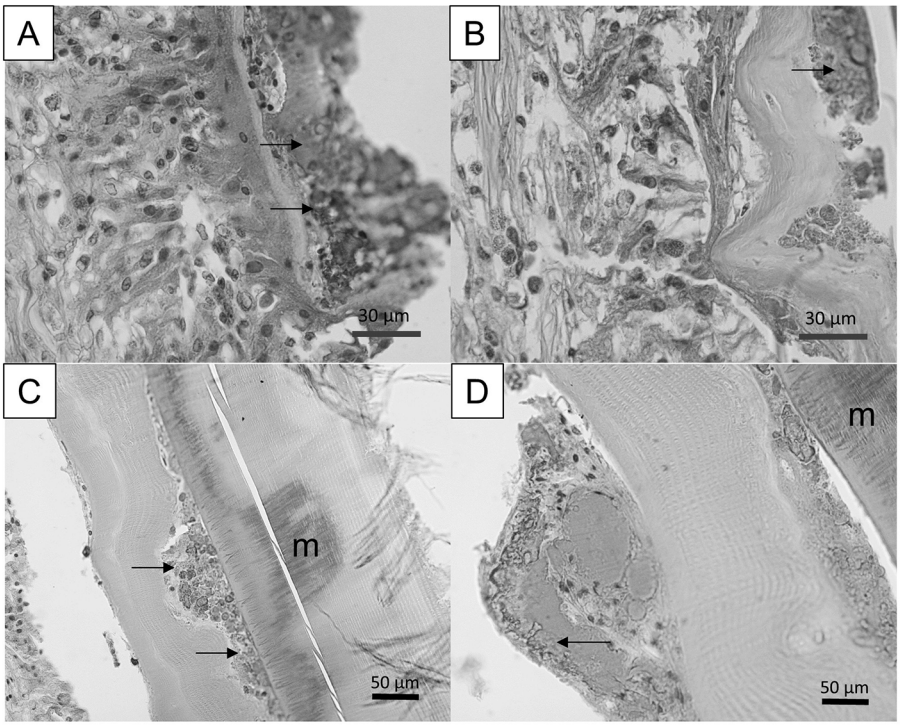


Figure 7. Detail of lesions from lobster ME 73. (A, B) Epidermis showing basophilic epidermal cells indicative of cells generating new cuticle and melanized nodulation (arrows). (C, D) Pseudomembrane with nodulation and melanization (m).

constituent taxa. Analytically, the bacterial flora of healthy lobsters was not distinguishable from that on diseased lobsters using principal components analysis. There was no obvious dysbiotic shift in the bacterial flora in association with the disease. There were no clear distinctions in the communities based on the disease status of the host lobsters. There were no associations with the bacterial community in relation to disease, severity, region, external damage to the lobsters, maturity, CL, sex, or sample type. No patterns were apparent in any of these analyses.

Although the microbiome did not show apparent dysbiosis, we analyzed the relative abundance of *Aquimarina* spp. on individual lobsters from the data obtained with high-throughput sequencing. In terms of presence or absence, *Aquimarina* spp. had a significantly higher prevalence (72%) on diseased animals than on healthy animals (35%) (Table 3). The bacterium also had a higher prevalence (72.7%) on diseased shell pieces than on healthy shell pieces (40%) from diseased animals. However, there were no differences in prevalence between healthy shell pieces from healthy animals and healthy shell pieces on diseased animals (Mantel-Haenszel statistic = 0.250, Mantel-Haenszel chi-square = 2.648, $P = 0.104$). As expected from the prevalence analysis, *Aquimarina* spp. had a higher relative abundance on diseased animals than on healthy animals (Fig. 8). However, there were no clear associations with relative abundance and disease severity, and this likely resulted from using small, relatively standardized pieces of diseased shell. The high throughput sequencing did not differentiate among species of *Aquimarina*. At least two closely-related species were

Table 3. Frequency of occurrence of *Aquimarina* spp. on cuticle of *Homarus americanus* (as presence or absence) in relation to disease status of the shell. Row percentages are given in parenthesis. Note healthy cuticle pieces from diseased animals are included in this analysis. *Aquimarina* has a significantly higher prevalence on diseased shell pieces. Pearson chi-square = 6.762, df = 1.00, $P = 0.009$.

| | Absent | Present | Total | *n* |
|---|---|---|---|---|
| Disease | 6 (27.3%) | 16 (72.7%) | 22 | 22 |
| Healthy | 18 (64.3%) | 10 (35.7%) | 28 | 28 |
| Total | 24 (48.0%) | 26 (52.0%) | 50 | |

present. Therefore, in addition to the high throughput sequencing, we used standard PCR amplifications to further confirm the presence of *A. "homari"* in a subsample of lobsters. We tested 7 of 8 lobsters using primer sets specific for *A. "homari."* These amplifications were positive for the presence of the bacterium on diseased shell pieces.

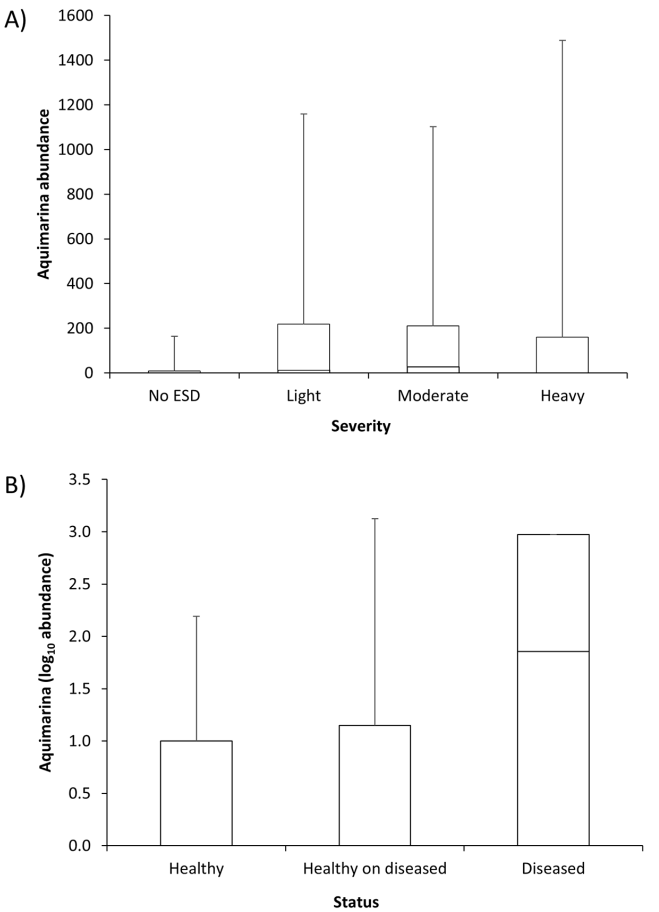


Figure 8. (A) Abundance of *Aquimarina* spp. in relation to severity of epizootic shell disease. (B) Abundance (log10) of *Aquimarina* spp. on healthy lobsters vs healthy shell on diseased lobsters vs diseased shell on diseased lobsters. Key: box = 75% quartile, line in box = median, bar = range.

## Discussion

We confirm that ESD is present at low levels in lobsters from Maine's nearshore waters of the Gulf of Maine using a fishery dependent data set standardized by trip and spatial area. Unsurprisingly, the prevalence increased in ovigerous females of all sizes and in oversize lobsters at much higher levels than in the overall population. Higher prevalence levels in these population components mirror those observed in the SNE stock in animals with the longest intermolt interval (Castro et al. 2006, Glenn and Pugh 2006, Hoenig et al. 2017). The correlation between ESD prevalence and lobster size also suggests that the overall prevalence along the coast of Maine is driven by increasing disease levels in oversize and ovigerous females. The mean size of males with the ESD and, to a lesser extent, the females without eggs, was higher than the mean size of the healthy population, indicating that the signs of ESD are relatively rare except in the larger animals. On the other hand, the mean size of healthy and ESD lobsters were not significantly different.

Spatially, the western regions and management zones of the Gulf of Maine have higher prevalence of ESD and this was not a factor of sampling intensity. Water temperature has been considered a strong predictor for the prevalence of ESD (Glenn and Pugh 2006) and a higher proportion of lobsters were found in the shallowest water in western Maine. The increases after 2010 could have been driven by the general warming of the Gulf of Maine and the peaks observed in 2013 and 2014 immediately followed the 2012 warm water anomaly (Pershing et al. 2015). Interestingly, deeper bottom water temperatures outside of the inlets and bays in the western Gulf of Maine can be colder than eastern Maine in the summer months, making temperature a complicated factor to characterize in the Gulf of Maine. The regional oceanographic conditions driven by the Eastern Maine Coastal Current may explain these spatial differences. The strong coastal current creates conditions in eastern Maine where the water column is well mixed and cooler throughout, whereas in the western Gulf of Maine, it is highly stratified (Churchill et al. 2005, Pettigrew et al. 2005).

The Northeastern Regional Association of Coastal Ocean Observing Systems (NERACOOS) buoys provide water temperature at depth in eastern, central, and western nearshore regions of the Gulf of Maine. Although attempted within these GAMs, the temperature, categorized by geographic area, both as degree days lagged by a year and observed water temperatures at depth, did not contribute significantly to explain the deviance. With more of the fishing activity occurring in shallower waters in the western zones, further analysis is needed to determine the appropriate temperature proxy for the shallow portions of the western Gulf of Maine.

The existing depth data from the LSS Program provides a possible confounding variable for these regional differences where more of the ESD lobsters in western regions were observed in <20 m as compared to the total catch. In eastern Maine, the ESD lobsters followed similar patterns as the total catch over depth. While depth was a significant smoothing factor in the GAMs, the possible interaction between geographic area and depth was not characterized and needs future analysis to further clarify its importance.

In addition to temperature and depth, there are other factors that should be explored to better elucidate possible drivers for increases in ESD. There is evidence that the onset of the annual molt has shifted in the Gulf of Maine, especially after 2010 (K Reardon unpubl data). In the earlier period 2003–2010, the mean monthly catch

of symptomatic lobsters closely followed the seasonal catch proportions of old shell lobsters. This is apparent in the GAMs where the prevalence of ESD in the summer months was significantly different from those in the fall. In the more recent period 2011–2016, more diseased lobsters were caught in late summer and fall (K Reardon unpubl data). These patterns need further exploration on an annual basis to determine annual and seasonal variability in molt timing, the impact of reproductive status, and other potential factors. It is very likely that molt phenology is also driven by temperature. Other factors could include substrate type, shifts in pH, freshwater input, contaminants, or population density.

The gross and histological evidence indicates pathology identical to that described for the disease in animals from LIS. The lesions associated with epizootic shell disease in LIS are comprised of large friable areas of shell that coalesce into extensive, broad ulcers particularly on the dorsal aspect of the affected animal (Smolowitz et al. 2005, Shields et al. 2012). This also describes observed lesions on Maine lobsters. The pathology is identical including extensive nodulation of the epidermis and membranous layer, the development of a pillar-like appearance of the cuticle arising from burrowing activities of microbial action, and extensive development of pseudomembranes in response to the disease. In addition, the presence of *A. homari* is further evidence that the bacterium, which has been implicated in ESD in LIS, is associated with the disease in Maine. That we found the bacterium present in and adjacent to the lesions on diseased lobsters, but not on healthy lobsters, is consistent with the evidence of its involvement with ESD (Quinn et al. 2017).

In conclusion, we show an increasing prevalence of ESD over time in the nearshore waters of the Gulf of Maine lobster fishery. The shifting patterns in relation to time, region, and fishery factors indicate that the disease has a complex epidemiology in the Gulf of Maine. Nonetheless, our findings indicate that ESD is present on lobsters throughout the nearshore waters of the Gulf of Maine. It is more prevalent on larger animals that are not molting as frequently as smaller animals, and it is more prevalent in animals from the western Gulf of Maine regions than those from the eastern region. Given the effect of the disease on lobsters from SNE, and its increasing prevalence over time, further monitoring of ESD in the Gulf of Maine is warranted.

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