Recognizing Incipient Epizootic Shell Disease Lesions in the Carapace of the American Lobster, *Homarus americanus*

Running title: Recognizing Incipient Epizootic Shell Disease in American Lobster

Joseph G. Kunkel, Melissa Rosa, Brian Tarbox,
Sabine Hild, Michael J. Jercinovic, Ali N. Bahadur

- joe@bio.umass.edu, UNE Biddeford, ME 04005
- mrosa2@une.edu, UNE Biddeford, ME 04005
- btarbox@smccme.edu, SMCC South Portland, ME 04106
- sabine.hild@jku.at, Johannes Kepler University, Linz, AT A-4040
- mjj@geo.umass.edu, University of Massachusetts Amherst, MA 01003
- Ali.Bahadur@bruker.com, Bruker BioSpin Corp., Billerica, MA 01821
ABSTRACT: Causal factors leading to Epizootic Shell Disease (ESD) lesions in American Lobster, *Homarus americanus*, are still not well understood. We explore the structural and physiological bases for development of ESD to early visible stages from earlier stages invisible to unaided eye. We suggest a lobster shell model which develops structural functional vulnerability and explains the origin of ESD. Medallions of carapace cuticle are obtained from carapace fixed with various protocols to minimize movement of mineral and macromolecular components. Minimal processing of medallions is used to encourage large sample sizes compatible with environmental surveys. One- and two-dimensional analytic maps of polished sections of the cuticle, obtained with an Electron Microprobe, describe the composite mineral and polymeric structures. MicroRaman Spectroscopy identifies bonding types of phosphates and carbonates as well as signatures of organic structures. The frequency and properties of identified structures can be monitored through the lobster molting cycle using high throughput application of micro-Computed Tomography (µCT) to quantify the frequency of structures of interest. We observed density differences in the calcite layer, inner exocuticle and endocuticle and the frequency and structure of CaCO\(_3\) structures in the endocuticle and membranous layer of carapace cuticle during stages of the molting cycle. The correlative microscopy and µCT of shell structures will lead to an improved understanding of the lobster cuticle structure. Detailed structural differences quantified though development and under different environmental conditions can provide insight into causes and vulnerabilities associated with ESD.
INTRODUCTION

American lobster populations experienced increased and variable incidence of Epizootic Shell Disease (ESD) (Smolowitz et al. 2005, Glenn and Pugh 2006) starting in 1978 (Castro et al. 2006, 2012). It is not clear what the basis is of observed localized and variable ESD rates. Numerous studies and correlations have been quoted in pursuit of causal factors including an early observed association with rising mean temperatures (Tlusty et al. 2007). Record harvests of American lobster have been recorded in both American and Canadian waters correlated with rising temperatures in the Gulf of Maine and lowered populations of top predators (Steneck and Wahle 2013). The phenomenon of global warming is being imposed on arrays of populations of species of Northern Hemisphere organisms that have responded with shifts northward (Perry et al. 2005). However in the American lobster, part of moving northward includes a population collapse south of Cape Cod which has been accompanied by, and many think caused by, ESD. While ESD frequency has increasing markedly in the Gulf of Maine from historically low levels, the official opinion is that the general population is relatively healthy with ESD incidence in general below 1 percent. There are however reports of hot spots of ESD found in particular Gulf of Maine locations where frequencies of 1 in 5 lobsters were found to have ESD at the peak in late fall. There is some urgency for practical as well as theoretical reasons in being able to detect shell disease at its earliest stages. We are focusing on the structural and functional aspects of the shell in order to understand their potential for predicting the onset of ESD. The dorsal carapace is the typical location where ESD is first seen and thus it was chosen as the principle structure to be studied. Harvesting a population sometime prior to peak ESD development could yield a substantially healthier crop that might be less marketable later in the season. Strategies for bringing product to market earlier or avoiding impoundment, during which shell disease might develop, could lessen the economic
impact of ESD in particular seasons if the potential incidence could be predicted. We establish here the chemical and physical basis for understanding the structure of the lobster carapace cuticle and, based on that structure, demonstrate a rapid approach to high throughput analysis of a population's vulnerability to ESD.

**MATERIALS and METHODS**

*Animals*

American lobster non-shell diseased individuals were obtained from an area with a low incidence of ESD, Inner Casco Bay. American lobster shell diseased individuals were obtained from traps at a location just outside Casco Bay, latitude, longitude, which from past years experience has exhibited a high incidence of ESD.

*Tissue Preparation*

Medallions of cuticle were obtained using a drill press (Micro-Mark MicroLux® Benchtop Variable Speed Mini Drill Press) with a 6 mm diamond coring bit. Medallions were freeze fixed in a -40C acetone bath with BioBeads to scavenge any released water and gradually returned to room temperature over a 24 hour period with several changes of anhydrous acetone. The acetone was allowed to evaporate leaving a dry medallion. Some medallions were affixed to 26 mm plastic blanks and polished with diamond polish (6µ to 0.25µ) to reveal planar views of cuticle structures at the polished face.

*Data Collection*

Selected areas of diamond polished surfaces of lobster cuticle (Kunkel et al. 2012) and standards were submitted to both electron microprobe (EMP, Cameca SX-50 and SX-Ultrachron,
UMass Geosciences Microprobe Facility) and micro-Raman spectrometry (Horibe Jobin Yvon Aramis, JKU Linz AT, Polymer Science Institute). A limited number of area integral spectra were collected with the Thermo-Fisher Scientific DXR Raman Microscope.

Seven medallions of lobster cuticle were submitted to μCT voxel data collection as described (Kunkel et al. 2017). The collection of μCT voxel data at 8 or 16 bit density resolution from the 6 mm medallions requires substantial memory and computation power. In addition the Analyzer Pro, ImageJ and R software analysis require substantial memory and computation power. In many cases data sets were divided into Regions of Interest (ROI) for further analysis. When rotation of the data was necessary, freely available DataViewer software was used to subset rotate and save data to a new stack of voxel slices in the new orientation. When rotation was not necessary, ImageJ was used to load desired voxel slices and crop them to the desired ROI and saved as a stack with the same resolution or reduced resolution using ImageJ library functions.

Matrices of multivariate data were collected from Electron Microprobe by raster elemental analysis at 0.3 μm spacing (Kunkel and Jercinovic, 2012) and by raster collection of 2800 element μm⁻¹ spectra by Raman spectrometer at typical 2 μm spacing from 0.25 μm diamond polished sample surfaces. The multivariate data were processed by matrix algebra in R which allowed use libraries that access multicores of a computer for actions that can use parallel processing. Singular Value Decomposition (SVD), sometimes referred to as Principle Component Analysis, using R basic library functions. Cluster analysis was also performed on multivariate data using K-means clustering. PhotoSticher software was used in instances to create smooth montages of images grabbed at limiting resolutions of the equipment.
Results

Chemical analyses

Chemical properties of lobster carapace cuticle architecture were obtained by applying two chemical analytic tools to polished cuticle surfaces. As an example, the pattern of carbonate apatite trabeculae in the lobster carapace exocuticle is clearly demonstrated by measuring its elemental composition using the hi-resolution electron micro-probe on tangential sections of the exocuticle layer. In Fig 1 the phosphate detected, illustrated in Fig 1A, is seen to be in a trabeculum-like pattern on a more uniform pattern of Ca$^{2+}$ seen in Fig 1B. In some phosphate rich areas of Fig 1A apparent cortexes of structures have medullas with lowered phosphate density. In corresponding regions of Fig 1B one can see corresponding lowered Calcium densities. This suggests organized trabecular cortex and medulla structures with cortexes of higher Ca and P but lower at their core. This analytic approach was extended to examine the fine resolution of tangentially cut structures such as the dermal gland canals as seen in Supplementary Fig S1, where an organule canal wall is seen to use two distinct ratios of Ca:P in discrete layers.

The patterns of elements used in constructing the carapace can be teased apart using k-means clustering of pixel-wise composition matrices producing a map of clustered pixel types and displayed in raster or bar-chart format, as in supplementary Fig S2. K-means clustering uses a Monte Carlo approach which requires several iterations of clustering to establish a consistent result. However its output provides additional insights into the carapace structure, e.g. chloride distribution in the endocuticle is seen to oscillate significantly in synchrony with the Bouligand layers of cuticle, Fig S2C, while the calcium, phosphate and Ca:P ratios are not significantly different, Fig S2B,D,E. It is likely
that the chloride content is a basis for endocuticle Bouligand layers being clustered differently, which is repeated in all iterations of the k-means clustering algorithm. Three of the k-means clusters span the phosphate rich trabeculae seen as the three bars labeled *canal*, *nip* and *lam2* because they respectively here, in Fig S2, characterize (1) the majority of the length of the dermal gland canal wall and the core of the trabeculae (yellow). (2) the next most phosphate rich structure, the nipple structures, which characterize and surround the exit of the dermal gland canals on the basal side of the cuticle. (3) the lowest phosphate rich structures (brown) which here form a continuum with the endocuticle clusters and share with them an enrichment with chloride. The k-means clustering also creates cartoons of the trabecular cortices and medullas referred to in the previous paragraph. The yellow cortex around a lavender medulla surrounding the dark-brown inner medulla is seen as a cartoon replicated through the exocuticle of Fig S2A and characterizing the trabecular layered structures which are compositionally different enough to be clustered. The light brown stalactite material extending from the calcite layer toward the endocuticle typically merges with the dark brown material of the endocuticle layers. Both light and dark brown clusters are lower in chloride.

Given that this analysis only covers the three elements Ca, P and Cl, it is possible that the distinct properties of the structures are established by other elements and likely chitin and layer-specific protein polymers that participate in the structures. It is known, for instance, that fluoride modifies the luminal surface of dermal gland canals (Kunkel et al. 2013) perhaps participating in a similar hardening of the carbonate apatite as recognized for vertebrate teeth and bones.
A major insight into the chemical differences between carapace cuticle structures can be obtained from micro-Raman spectroscopy. The laser wavelength of the probe stimulus is re-emitted as characteristic shifted Raman shift photons which characterize the molecular vibration emitting the photon. The bonding signatures Raman shifts of carbonate, phosphate and organic structures were evaluated in polished sections of carapace cuticle at an approximate 1 um resolution using the Labram Aramis micro-Raman spectrometer with auto-focus and auto-cosmic-ray-filter. Fig 2 illustrates average spectra identified from raster collected spectra that were identified using SVD analysis. The canal wall of a dermal gland, Fig 2A, was identified using SVD and a mask created to pick spectra to average. The canal wall spectral averages, Fig 2D-c, showed spectral signals at v1, v2, v4 of PO$_4^{3-}$ and v1, v2, of calcite consistent with carbonate apatite, Supplementary Table S1.
Similarly the canal lumen exhibited spectra with a dominant peak at wavenumber 1318 cm\(^{-1}\) which when averaged, Fig 2D-a, suggest the canal contains the CH bonding of protein chains. The rings surrounding the canal wall represent alternating phosphate rich and \(\text{CaCO}_3\) rich bands which when averaged provide respectively the trabecular-like spectra, Fig 2D-e, and \(\text{CaCO}_3\) spectra, Fig 2D-d. The bone and aragonite samples are provided as reference samples to provide positive controls of standard Raman spectral shifts.

The signatures of chitin and protein polymer residues are more clearly seen in supplementary Fig S3 which has its wavelength axis extended to 3500 cm\(^{-1}\) and includes the broader ch-stretch regions surrounding 2883 (chitin), 2935 (CHO polymers), and 2965 (protein sidechains). In addition this Fig S3 demonstrates the Raman spectra of the carapace calcite layer showing its lack of any substantial
chitin spectral signal, which characterizes it as a cuticular pre-chitin-synthesis product, marking it as part of the epicuticle.

**3-D Density analyses**

The main objective of this study is to develop a rapid high-throughput approach to evaluating lobster carapace architecture. Using μCT, an X-ray based approach to exploring 3-dimensional voxel densities, promises to provide the type of diagnostic information that as it has for medical problems. A high thoughput μCT protocol for lobsters was planned to study the carapace cuticle of a select number of lobsters, chosen to provide contrasts of potential targets to advance understanding of the role of cuticle structure in vulnerability to ESD and to allow its early detection. Seven medallions of cuticle were sampled from lobster, six were sampled from lobster carapaces. One medallion was collected from a chela of a carapace-sampled lobster to contrast the carapace to the alternate object of lobster cuticle study, the chela. Two medallions were collected from a lobster with advancing ESD, one with a barely visible lesion and one medallion from (superficially) lesion-free cuticle. One lobster was sampled pre-molt initiation (D4), a patch applied to allow molting, and sampled again one week post molting.

All medallions were freeze fixed, and dehydrated in anhydrous acetone and either a planar viewing surface was polished for chemical analytic analysis as described above or medallions were viewed directly as a whole medallion in a μCT as described (Kunkel et al. 2017).

A  μCT provides a stack of voxel slices that can be analyzed slice by slice or as integrated volumes via analytic software, which can provide insights into the underlying structure beyond the limits of slices. Of particular interest to this study are the surface structures, the epicuticular calcite layer and its intersection with the carbonate apatite organule canals, interruption of either of which would be immediate signs of vulnerability. Additional interest is focused on underlying structures such as the exocuticle trabeculae which support the integrity of the surface structures.
The surface of the carapace seen on a one week post-molt medallion shows regularly spaced organules. Supplementary Figure S4 shows the entire medallion scanned at 2.5 µm. The freeze fixation was able to catch an active secretion process from many of the dermal glands of primary and tertiary organules which may correspond to the ‘cement’ layer (Wigglesworth 1948, Locke 1961) being applied to the epicuticle. It is clear that a secretion, dense enough to be profiled by the X-ray beam, is being applied, virtually sprayed, onto the epicuticle surrounding each dermal gland. Fig 3A illustrates a region-of-interest of the Fig S4 medallion processed with the Volume Viewer plugin showing a tertiary organule with 4 streams of material emanating from dermal gland canals. In Fig 3B the voxels are analyzed by the Thickness routine of the BoneJ plugin of ImageJ, which fits spheres of largest possible radius into voxel volumes defined in a 3-D binary segmentation of the X-ray densities. The diameters of circles which fit into the 3D segmented spaces are presented in graded colors from small (purple) to large (yellow). This segmentation technique and filling the segmented spaces with graded circles is time consuming in machine time but carried out on select samples they provide insight into the uniformity of structures and their thicknesses. The Fig 3 demonstration of a thickness measure is carried out in ImageJ using a density segmentation set of binary interpretations of the greyscale density after various levels of smoothing of the voxel data. The thickness of the elements of the circle-filled 3-D binary structures is then characterized by statistics on the circle diameters or visually as in Fig 3B.

Figure 3. ROI of an actively secreting organule with secretion streams captured as X-ray densities. A. Oblique view of the ROI interpreted with ImageJ VolumeViewer plugin. B. Oblique view of the ROI interpreted with the ImageJ Bone Thickness plugin with lighter toned structures are thicker.
This segmentation and Thickness analysis, applied to the 7 day post molt data, suggests that the calcite layer of the post molt carapace is relatively uniform in thickness throughout the viewed surface despite it being from a prior ESD individual. That protocol was applied to three medallions illustrated in Fig 4. One sample was from a C3 lobster that did not display any lesions, Fig 4A. A second medallion, Fig 4B, was from a lobster 7 days after a molt that did display lesions in the prior instar. A third medallion, Fig 4C, did not displaying lesions itself but was from a C3 lobster that was displaying ESD lesions. It is clear that the Fig 4C animal, with active ESD, had thinned calcite regions in its apparently non-lesioned areas of the carapace. The apparently healthy lobster cuticle, Fig 3A, shows relatively uniform thickness calcite layer and the recently molted medallion sample, Fig 3B, from the formerly ESD animal, also showed a uniform thickness calcite layer. The serious degree of thinning of the Fig 3C medallion is emphasized by the Supplementary Fig S5 movie displaying the entire stack of the thickness interpretation of that medallion.

Both the Volume Viewer plugin and Bone Thickness plugin of ImageJ provide their insight at a substantial time cost, partly due to many of the decisions needing to be made by a human operator and partly due to the immense number of calculations that need to be done when the operator chooses a direction to take. Similarly, a commercial medical software, Analyzer Pro was able to segment the data into density defined surfaces that provide useful insights into the carapace architecture by creating stl files, which can be used to display the surfaces selectively as digital images. For example, Fig 5
illustrates Analyzer Pro produced stl files of the calcite layer and of the trabeculae of the late intermolt, stage C3, carapace medallion of an ESD lobster (the medallion only superficially lesion free) shown in supplementary Fig S5. The stl file plots can be embellished with outlines of structures obtained with ImageJ line tracing functions. One feature of Fig 5 and the video of the rotating structure, provided as supplementary Fig S6, is that the calcite layer, as segmented, can be seen to be very thin in several areas where one can see through the calcite-segmentation to what would be the next layer down, nominally the exocuticle stalactites, but which are not included in Fig 5. However one can see the trabeculae more clearly further below in the supplementary Fig S6 video when it is in motion. Again, this segmentation approach producing stl files is expensive in time, taking on the order of two computation days for each medallion examined on a fast computer (2.5GHz, 8 cores) with large memory (16Gb). While some economy of effort might be achieved when the segmentation of interest is reduced to routine, it is unlikely that this approach would provide successful high-throughput discrimination between healthy cuticle vs. ESD prone. It does however direct one, in this case, to focus on developing a protocol to measure the thickness of the calcite layer in a random enough way to capture the thinness that may be associated with vulnerability to ESD.

Figure 5. Structural surfaces of lobster carapace cuticle defined by stl files extracted from µCT of ESD infected American lobster carapace medallions. A and B are from a medallion that show no apparent lesions. C and D are from a medallion with a 500 µm diameter ESD lesion. A. 2.5 µm resolution µCT scan that needed to be divided into two hemi-medallion data sets to be analyzed in AnalyzerPro and re-assembled in Meshlab. B. stl files of the calcite and trabeculae are viewed obliquely using an R-library to read and plot stl defined surfaces. C. ROI of the ESD lesion as seen with stl file defined density structures of calcite layer, trabeculae, membranous layer with outlines of recognizable canal paths. D. Same as C viewed through an imposed transparency of the calcite layer.
A more rapid measure of thickness that can be used is a standard protocol of ImageJ records the density along a linear transect. It uses the ImageJ decision path, Analyze / Plot Profile, to record a linear path constructed perpendicular to the cuticle surface through all the layers and occasionally including the reference crystal material, Fig 6. This is extremely fast and can be automated using a Java Macro defined as an ImageJ plugin. Such an approach produces data transects (as stored csv files) of voxel density in a linear path through the cuticle, which can be viewed individually, e.g. Fig 6 A and B, or used to provide statistics on the average calcite layer thickness or density distribution for that medallion.

Figure 6. X-ray density of voxels in a µCT of American lobster carapace medallion. Two linear transects are displayed which transect the cuticle from epicuticle through the endocuticle and including two standards, K Bipthalate - density 1.636 g/cm$^3$ shown as a blue line and KH$_2$PO$_4$ - density 2.338 g/cm$^3$ shown as a green line. The calcite layer is seen to approximate the green line density, sections of endocuticle are seen to approximate blue line density.

**Recognizing incipient ESD lesions**

In order to recognize incipient ESD lesions one needs to extrapolate back to the earliest ESD lesions recognizable by the naked eye and characterize how they develop. One way to do that is to take a recognizable ESD infected individual (e.g. the carapace of Fig S5) and look on the periphery of the well developed ESD lesions to see smaller lesions that, from past experience, will develop into larger ESD lesions. We did that with an individual stage C3 lobster in which an approximate 500 µm lesion was included in a carapace medallion and another medallion superficially free of lesions was also examined. In addition a medallion was sampled from the large chela which also had a small ESD
lesion. An ImageJ Volume Viewer interpretation of the μCT data view of the surfaces of these medallions is provided in Fig 7.

The smallest clearly recognizable ESD lesion we have visualized with μCT, Fig 7A, was interpreted with a Volume Viewer protocol. The same protocol applied to a larger lesion on the chela of the same animal is seen in Fig 7B. The conical lesion has a diameter at the chela cuticle base surface of 250 μm and widens to 1500 μm at the cuticle surface. The chela lesion is dominated by endocuticle erosion which is a late stage in the ESD process. Further effort was made to search for lesions in the cuticle sampled from the carapace of a shell diseased lobster that had surrounding cuticle with advanced ESD lesions. One, somewhat questionable, much smaller potential lesion is seen as a departure from regular texture interpreted by Volume Viewer in supplementary Fig S7. That lesion is possibly interesting in that it potentially shows a decrease in mineral density ballooning internal to the outer surface, which could signal some type of penetration though a narrow imperfection in a tight surface.

Discussion

The major message of this study is that μCT can be tremendously informative in exploring the structure and development of the lobster cuticle. Particularly, μCT of medallions of American lobster carapace cuticle is the approach of choice to study lobster ESD-susceptible cuticle. Chela cuticle makes a poorer object to obtain hi-resolution structural data due to its particular thickness plus
dominance of its endocuticle in its total structure, which likely causes shadow-blurring of the information in the epicuticle- and exocuticle-computed voxel densities given the radial X-ray exposures to collect the data.

Thinness of the calcite layer may be diagnostic of vulnerability to ESD. In our study, calcite layer thinness is seen to be correlated with ESD and may be diagnostic in a predictive sense in individuals that do not yet display ESD in other regions of their cuticle. To establish this prediction one would need to apply this approach in a truly high though-put manner in populations that predictably would develop high ESD incidence in lobsters that currently are superficially ESD free.

Seeing a confirmable smallest ESD lesion may not be a realistically achievable objective. The protocols to analyze the data to see those smallest lesions are too time consuming in our experience. This may change with faster more powerful computers. Rather, currently, characterizing the calcite layer thinness may be the diagnostic feature that defines lobster cuticle health and the ability to predict the vulnerable fraction of the lobster population that will develop shell disease.

A healthy calcite layer has an approximate density of $\text{KH}_2\text{PO}_4$, used as a calibration standard. This approximate density was seen in all the so-called healthy cuticles observed in this study. Clearly it could be tested as a standard by studying a variety of carapace medallions from more populations of established health. This density feature, easily evaluated in high throughput samples using $\mu$CT, in itself may be a standard of cuticle health that can be used diagnostically.

Using Raman spectrometry, which did not detect chitin in the calcite layer of the carapace cuticle, the calcite layer is now clearly shown to be a part of the lobster carapace epicuticle. That layer clearly develops its mineralization after ecdysis and an application of cement from the dermal glands, which agrees with the concept of the arthropod cuticle being a living compartment of the organism that develops in many ways post ecdysis (Wigglesworth 1948, Locke 1961). This opens further the questions about functions of macromolecules associated with lobster epicuticle/calcite layer formation
(Kunkel, 2013). Of particular interest would be characterizing the macromolecules, organic materials and potential mineral components added to the epicuticle from the dermal glands which are seen to be X-ray dense enough to be clustered as a cuticular component in canal contents (Kunkel et al. 2017 Fig 6A) as well as above, Figs 2Da and 3A,B.

Irrespective of cuticle microbial flora (Feinmann et al, 2017), it is likely that vulnerability of the cuticle to widely distributed flora will control whether ESD actually develops. If environmental stresses such as ocean acidification creates a population of lobsters with increased vulnerability (e.g. due to thinness of the calcite layer) it is more likely that an opportunistic bacterium can nucleate the original subliminal lesion needed to allow ESD to develop.

The nature and causes of the vulnerability needs to be studied further. The newest model of the lobster carapace cuticle provided here, Fig 8, includes our latest insights into lobster shell that is the target of ESD. The features of this model were developed from observations on the cuticle made by many past investigators (reviewed by Kunkel, 2013) with newer insights reported recently (Kunkel et al. 2017) as well as here. Many features of the model could contribute to vulnerability and development of shell disease. The immediate cause of shell disease may be the local thinness of the calcite layer which allows a specific microbe or a combined to attack, causing a subliminal lesion that
is widened into a recognizable lesion by a greater variety of microbes (Bell et al. 2012).

However the underlying cause of ESD may derive from various sources. One cause could be the lack of sufficient CaCO$_3$ and carbonate apatite precursors that needed to be accumulated in the prior stage that would allow a healthy new cuticle to be established in a timely fashion that would promote an ESD resistant cuticle. Another avenue to vulnerability could be an improper application of the cement layer that was seen to stream onto the new cuticle from the dermal glands. The composition of this applied component of the epicuticle has been shown to be of sufficient density to be detected with the X-ray dose that images the main mineral cuticle. In fact the density of the canal contents is similar to that of the lobster’s endocuticle (Kunkel et al. 2017). That cement is also independent of the contribution that is traditionally considered to be applied to the epicuticle from the pore canals which service the epicuticle from the inside (Locke, 1961).

The observation that a seemingly healthy-thick calcite layer was seen in a new cuticle sampled from a prior stage ESD lobster, Fig4B, argues that environmental erosion of the calcite during the early phase of the new stage may be important to ESD vulnerability. Measuring the post-ecdysial thinning of the calcite layer, due to ocean acidification, is one potential avenue of research that may provide insight into that possibility. Using a high throughput procedure like µCT to follow carapace cuticle development and correlating it with local contemporaneous historical environmental data, such as temperature, calcium availability and pH in that lobster’s experience, may be the only way we can understand how and why ESD develops.

This approach depends on both a high-throughput data sampling method, such as the particular model of µCT used here, which provides high-sample-throughput; but also on a rapid high-throughput-analysis. We examined several methods which were informative but which were too time-consuming to be applied as high-throughput and compared them to methods that are directed at rapidly establishing statistics such as thinness and density of the calcite layer. Collection of µCT data from
lobster carapace medallions sampled from lobsters of different developmental stages from different populations of variable ESD vulnerability would create a database of truly big data. It would allow tailor made rapid questions to be asked using fast ImageJ macros that could mine the Big Data to test hypotheses on determinants of ESD. We argue here for establishing such a database with its appropriate metadata on age and environmental parameters that can be mined to better understand current lobster populations as they change.

Acknowledgements

Vertebrate bone reference material was provided kindly dissected and provided by Paola Divieti Pajevic.

Literature Cited


Supplementary Material

Supplementary Table S1. Raman spectral regions Wavenumber(s) of interest (i – vi) covering PO$_4^{3-}$, CO$_3^{2-}$, and chitin and carbohydrate polymers. Critical types mentioned in the text and highlighted in figs 2D and S4D are given lower case Roman numbers.

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</table>

Figure S1. Selected area analyses of Ca and P for a secondary organule seen in a tangental polished section of lobster cuticle through the inner exocuticle. 

A. Phosphate image. B. Calcium image. C-F. Selected areas of panels A and B analyzed by singular value decomposition which allow apatite and calcite pixels to be identified. G. A scatter-plot of apatite pixel Ca and P vs each other with a standard of fluoroapatite (red with Ca/P 5:3 =1.67), clam shell (green with Ca/P of 1:0) and Ca(H₂PO₄)₂ (blue with Ca:P slope 0.5). The CAp of the large canal, E & F, displays Ca:P dual ratios of 2 and 2.67 (orange) while the smaller canal in G & H, displays a single ratio of 3.5 (purple). The Ca rich enclosing sheath of the canal seen in B and E is likely an organule socket cell product.
Figure S2. K-means clustering analysis of carapace cuticle EMP alternate of Kunkel and coworkers Fig 3 (2013) data. A. One of 25 independent iterations of a 10-mean clustering of EMP compositional data of American lobster C3 carapace polished cuticle section. Six clusters (4 ignored clusters were external) span the cuticle structures which are given colors and names typical of their location. The calcite cluster is red. The canal cluster is yellow. The nipple cluster is violet. The stalactite cluster is light brown. The endo1 cluster is green. The endo2 cluster is dark brown. Barcharts comparing the cluster properties are presented as panels B-E. B. Ca/P ratios of the cluster pixels. C. Chloride content of cluster pixels. D. Calcium content of cluster pixels. E. Phosphate content of cluster pixels.
Fig S3. Raman spectra of selected areas of lobster cuticle in tangential polished section. A. Neonatal mouse cancellous bone. B. LM image of dermal canal. C. SVD V7 contrasting canal wall to other surrounding structure. D. Raman Spectra a-f as intensity vs cm$^{-1}$: a. Outside canal wall. b. Canal wall. c. alpha-chitin crystal. d. neonatal mouse bone. e. calcite crystal. f. Lobster calcite layer (Thermo Fisher DX). Grey highlighted zones: i. v1 PO$_4^-$, ii. v2 PO$_4^-$, iii. v3 PO$_4^-$, iv. v1 CO$_3^-$, v. v2 CO$_3^-$; vi. v3 CO$_3^-$. 
Fig S4. One-week post molt carapace cuticle medallion. A. Four sectors of data were analyzed separately using identical protocols in ImageJ VolumeViewer because the full 2.5 μm voxel resolution 6 mm diameter medallion 16 bit data set was too large to be analyzed together. The resultant images were saved and combined with GIMP software. B. A ROI of the same medallion scanned at 1 μm voxel resolution showing the organule of Fig 3 and Fig 4B as a stereo pair which allows one to see more clearly the organule canal freeze-fixed-secretion streaming in the space above the organule depression onto the epicuticle surface. The stereo pair can be viewed effectively by uploading into a phone browser and viewing with a VR viewer or by cross-eye viewing, URL:
http://www.bio.umass.edu/biology/kunkel/LabWiki/images/thumb/a/ab/Ha7_1um_b_stereo.jpg
A further URL directed toward a video of the organule is also instructive:
http://www.bio.umass.edu/biology/kunkel/LabWiki/images/3/38/Ha7_2.5_crop2A_TMMacrCx0.6.avi
Figure S5. Exemplar ESD shell diseased lobster. A. Carapace (right half) molted from an advanced ESD infected American lobster. B. Montage of hi-res LM images grabbed from center region of S5-A lobster combined via PhotoSticher with organules and large and small lesions. It is difficult to differentiate the smallest ESD lesions from large high-complexity organules at this level or resolution. Raw PhotoSticher output file available at URL:
http://www.bio.umass.edu/biology/kunkel/LabWiki/images/c/c8/Montage_stereograph100.jpg
… to allow evaluation of effectiveness of viewing at this resolution.

Figure S6. Video of ROI, Fig 5C,D and 7A, 50 um ESD lesion, based on 1 µm resolution µCT data from American lobster medallion viewed in Fig 5A and Fig 7C,D.

http://www.bio.umass.edu/biology/kunkel/pub/lobster/3D/XRT/Ha4_1um/AVI/intep/Ha4_1um_16b_transp_0-325_237.avi

Figure S7. Video of American lobster 6 mm hemi-medallion viewed in Fig 5A,B illustrating a potential subliminal ESD lesion. The calcite layer is yellow, the trabeculae are green and some canals and organule depression contents were characterized as red. A few structures that did not conform to the criteria of calcite, trabeculae, stalactites or canal contents in the epicuticle and exocuticle region were defined as white and one such large such object is seen at about 2 o’clock on the face of this S7 video, URL:

http://www.bio.umass.edu/biology/kunkel/pub/lobster/3D/XRT/Ha5_2.5/16bit/AVI/intep/Ha5_2.5um_16b_semiMedalionA3.avi

...this object was not able to be fully understood and is a potential subliminal ESD structure.