Electron Microprobe Measurement of Mineralization of American lobster, *Homarus americanus,* Cuticle: Proof of concept.

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INTRODUCTION

Previously the histological study of lobster cuticle has been done primarily in the de-mineralized state. Tissue is fixed with an aqueous fixative, the minerals are leached out with an acidic bath and the resultant de-mineralized tissue embedded for traditional sectioning (Smolowitz et al., 1992). This project aims to provide a proof of concept that minerals can be measured *in situ* if the cuticle is treated for the electron microprobe as an aqueous soluble specimen. This could allow qualitative and quantitative analysis of the cuticle's mineral components as has been done for the land crustacean *Porcellio* (Ziegler, 2002) potentially to the micron level. This would provide a way to determine to what extent mineral content is a factor in the vulnerability of lobsters to shell disease.

The regular pattern of arthropod cuticle has been an important structural feature and subject of analysis. While the biochemical basis of some of the mineral features of crustacean cuticle has been recently studied (Ziegler, 2002; Becker et al., 2003), the fine structural basis is almost entirely lacking. This report outlines the progress we have made in preparing the lobster cuticle for electron microprobe analysis of mineral content.

METHODS

Sampling: American lobsters (*Homarus americanus*) with epizootic shell disease lesions were collected for sampling by bottom trawl on the NOAA Ship Albatross IV at two randomly picked stations at the mouth of Naragansett Bay on the Spring 2004 Groundfish Bottom Survey AL0403. The six selected animals included one with no apparent shell disease lesions. Digital photos were acquired of the lesions at sea briefly after capture and preliminary diagnosis, fig 1. They were maintained in a circulating seawater tank until reaching port when they were packed in wet seaweed in a cooler for transport to UMass Amherst and fixation after 48 hours.

A second sample of lobsters was obtained at the NE Aquarium lobster rearing facility. The lobster samples included: (1) One nutritionally based blue lobster with shell disease (not epizootic) plus recently shed cuticle. (2) Two fourth stage swimming larvae (3) Two third stage swimming larvae and (4) One second stage swimming larva. These were transported to UMass Amherst in a cooler and fixed within 24 hours.

Fixation: Small (< 1 cm sq) sectors of the cuticle including lesion and non-lesion surface were plunge frozen by plunging into liquid propane cooled to –185°C with liquid nitrogen. The frozen tissue was then transferred into dry acetone at –86°C. The tissue was held at –80°C for 36 hours while the acetone substitutes for solid water, and then allowed to come to room temperature over a 12-hour period after which the acetone was replaced with fresh anhydrous acetone.

Embedding: Embedding for treatment as a geological specimen required experimentation since little prior experience was available for this material. Sample 1 was embedded using Spur's resin. Sample 2 was embedded using Epo-Thin (Buehler) resin. Samples were equilibrated in 50% resin/acetone mixture on a rocking table for 15 minutes before embedding in pure resin under room temperature vacuum.

Embedded cuticle was sawed out of the initial blocks with a jewelers saw and reoriented for grinding and polishing in 25 mm diameter molds which fit the microscope carriage in groups of 4. Cuticle from several specimens was reoriented and embedded in parallel in order to allow comparison under the same microscopic conditions.

Grinding and Polishing: The plastic embedded material was next treated like an aqueous soluble specimen for microprobe analysis. Initial coarse grinding with 180 grit discs on an 8" diameter grinding-wheel allowed approaching the correct cross-sectional level in the specimens. The grit level was changed to 320 and then 600 grit (Buehler) using non-aqueous polishing oil as a lubricant. At this point the specimens were polished further with Metadi II diamond pastes on low to high nap lapping cloths (Buehler Trident and Whitefelt) using an oil lubricant. Washing and rinsing of blocks between grinding phases was done with anhydrous isopropanol and acetone to inhibit ion displacement.

Light Microscope Analysis: The blocks of embedded tissue were photographed with a 24-bit 2.1 megapixel digital camera (Kodak MDS 290) under various magnifications of a stereomicroscope (Parco) or Leitz Ortholux polarizing microscope under epi-illumination. A digital image record provides orientation of cuticular structure relative to shell disease lesion features on and below the polished surface. The shell disease severity scale devised for demineralized specimens (Smolowitz et al., 1992) was adapted (e.g. immune cell criteria are in general not visible although the epidermal layer can be discerned).

Electron Microprobe Analysis: The analysis of geological and biological samples is being pursued using technology that has been described in detail for other subjects (Goldstein et al, 2003; Ziegler, 2002). Our specimen preparation needs to approach as good a polish as possible in order to achieve the highest resolution of analysis possible. The Spur's resin specimens were able to be polished using 3 μm diamond paste, but no further. The EpoThin-embedded-specimens can be further polished with 1 μ and 0.25 μ diamond polish and may soon be examinable with the microprobe. For quantitative analysis by X-ray backscatter, the highest degree of flatness and polish are necessary. That has not been achieved as yet.

PRELIMINARY RESULTS

Gross Specimen Observations: Sample 1 from the mouth of Narragansett Bay was made up of individuals with mild to severe shell disease lesions centered in frequency on the anterior dorsal midline of the carapace (fig 1a). Sample 2 consisted of one shell diseased adult blue lobster (nutritionally generated) with its exuvium reared in the NE Aquarium lobster rearing facility (fig 1b). In addition, 4th instar stage larvae (fig 1c) from the NE Aquarium rearing facility were dissected in seawater and freeze substituted.

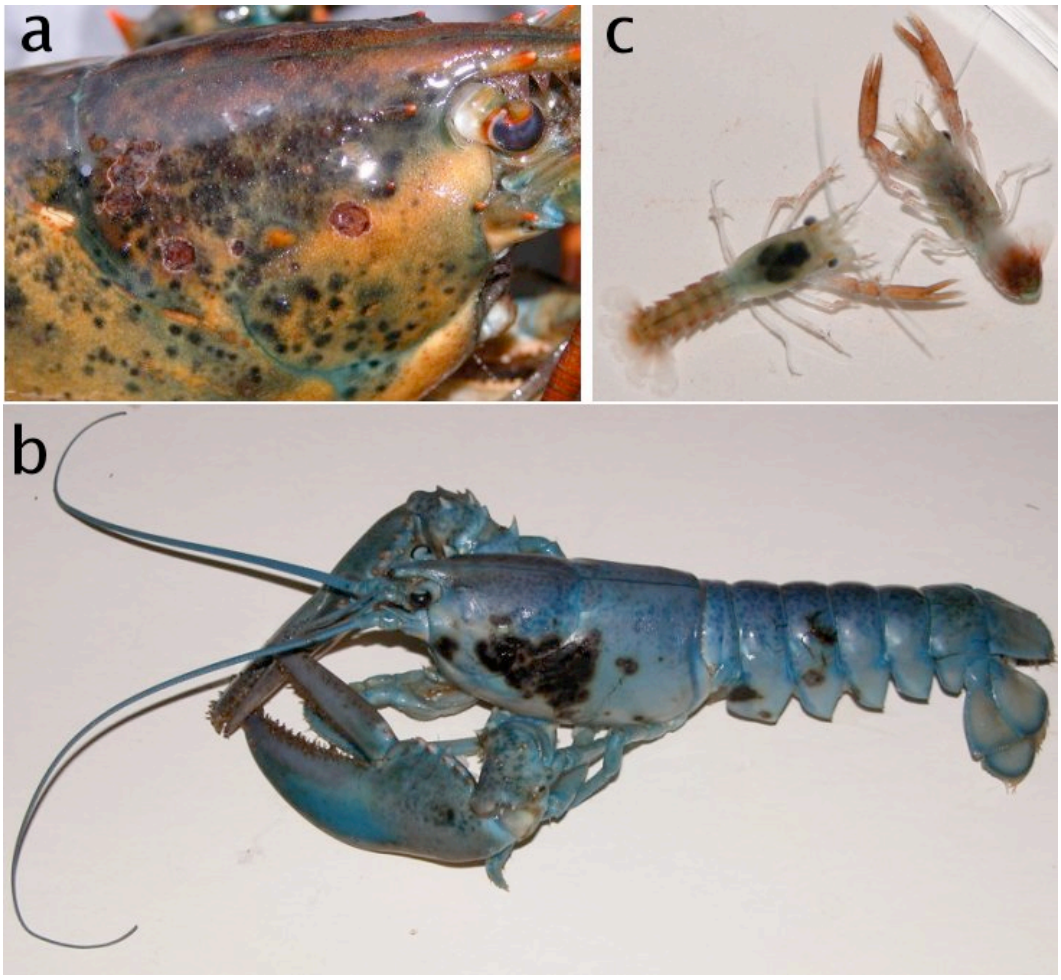

Figure 1. American lobster, *Homarus americanus*, specimen sources for cuticle analysis. a) 1 of 6 epizootic shell diseased lobsters from RI coastal waters. b) Single blue lobster from NE Aquarium shell disease (not epizootic). c) Pair of 4th instar larvae.

Light Microscope Observations and Organization *en bloc*: Fixed and plastic embedded cuticle specimens (fig 2a-d) were realigned and remounted in groups to provide experimental contrasts, which could be ground and polished together. They were observed in a stereomicroscope to view the general anatomy of a lesion on the cuticle as well as the gross structure of the surrounding epi- exo- and endo-cuticle at a certain level of grinding and polishing (fig 2b). The pairs would provide contrasts within the same block providing internal controls for the quantitative phase. Digital images of the cuticle at the level of the light microscope were taken of each specimen at stages of the polishing process in order to be able to relate the lesion geometry to the surrounding cuticle.

Light Microscopy: The block faces were observed in the stereo light microscope to identify cuticle features in control and shell-diseased lobsters. Lobster VI, with a Grade III erosion of the cuticle, below the focal plane, is viewed at several levels of magnification (fig 2b-d). Epicuticular lined dermal gland and sensory nerve canals may be critical features around which to measure mineralization. They occur at a less than one per epidermal cell since they correspond 1 to 1 with dermal glands and sensory structures. Epidermal cell features are not visible in this mode of viewing but can be

seen using a compound microscope with a reduced iris diaphragm to increase refraction.

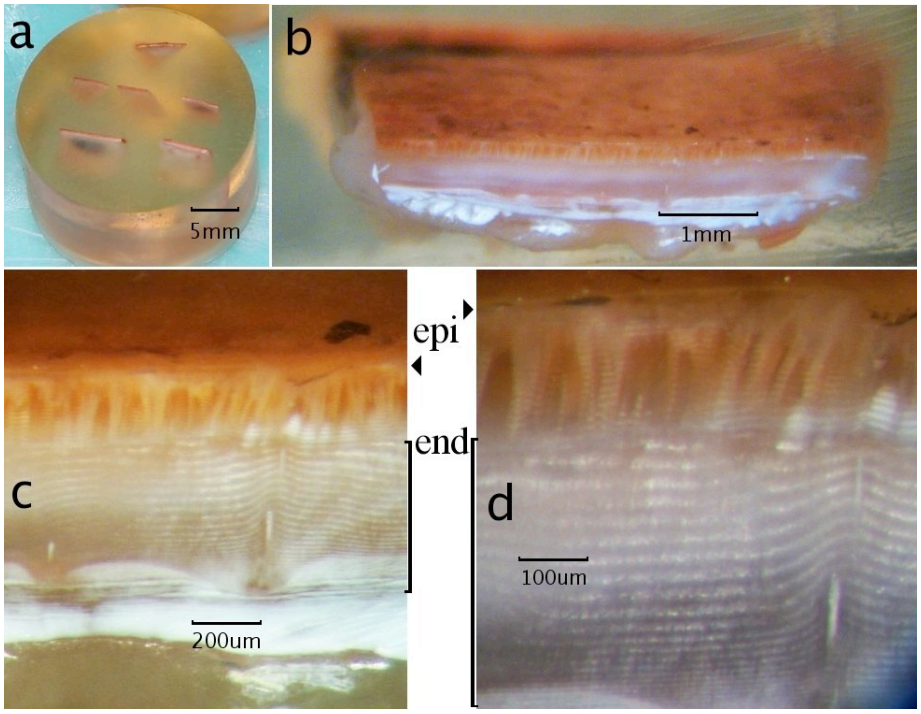

Figure 2. Lobster VI. a) block 7 specimens. b) single cuticle specimen VI epidermis near a Grade III erosion. c) polished cuticle face with 2 epicuticular lined canals. d) single epicuticular lined canal. Cuticular layers: epicuticle (arrowheads **epi**), endocuticle (brackets **end**).

The examination of *en bloc* polished lesion-associated mineralization patterns may help us understand the lesion process. Fig 3 illustrates a Grade IV(+) erosion with associated melanization of adjacent cuticle. Higher resolution examination of the polished cuticle under polarized light reveals pore canals that contain cellular extensions that wind through the cuticle at a higher density than dermal glands and sensory canals (Fig 4) and end at the interface of the exo- and endocuticle.

STRATEGY TO IDENTIFY INITIAL INFECTION CONDITIONS: Using the above *en bloc* approach to studying the mineral content of the lobster cuticle:

1) Examine normal healthy lobster mineralization pattern.
2) Examine lobsters with Grade I – V lesions, studying the neighborhood of the spreading lesions to see if the advancing front exhibits abnormal mineralization levels associated with cuticle features such as dermal glands, sensory channels and pore canals.
3) Examine the Ca:Mg:P ratios for the cuticle regions to determine the major players in the mineralization process. A high Ca but low Mg and P content would suggest $CaCO_3$ as the most likely form of calcium.
4) Measure Na, K, Mg and Ca levels in the endocuticle to see if the earliest lesions show an encroachment of the seawater pattern of minerals adjacent to regions such as the dermal glands or pore canals.
5) Is the cuticle of lobsters from shell-diseased regions as dense with minerals as healthy lobsters from shell-disease-free regions?
6) Why is melanization not able to prevent the encroachment of a lesion?

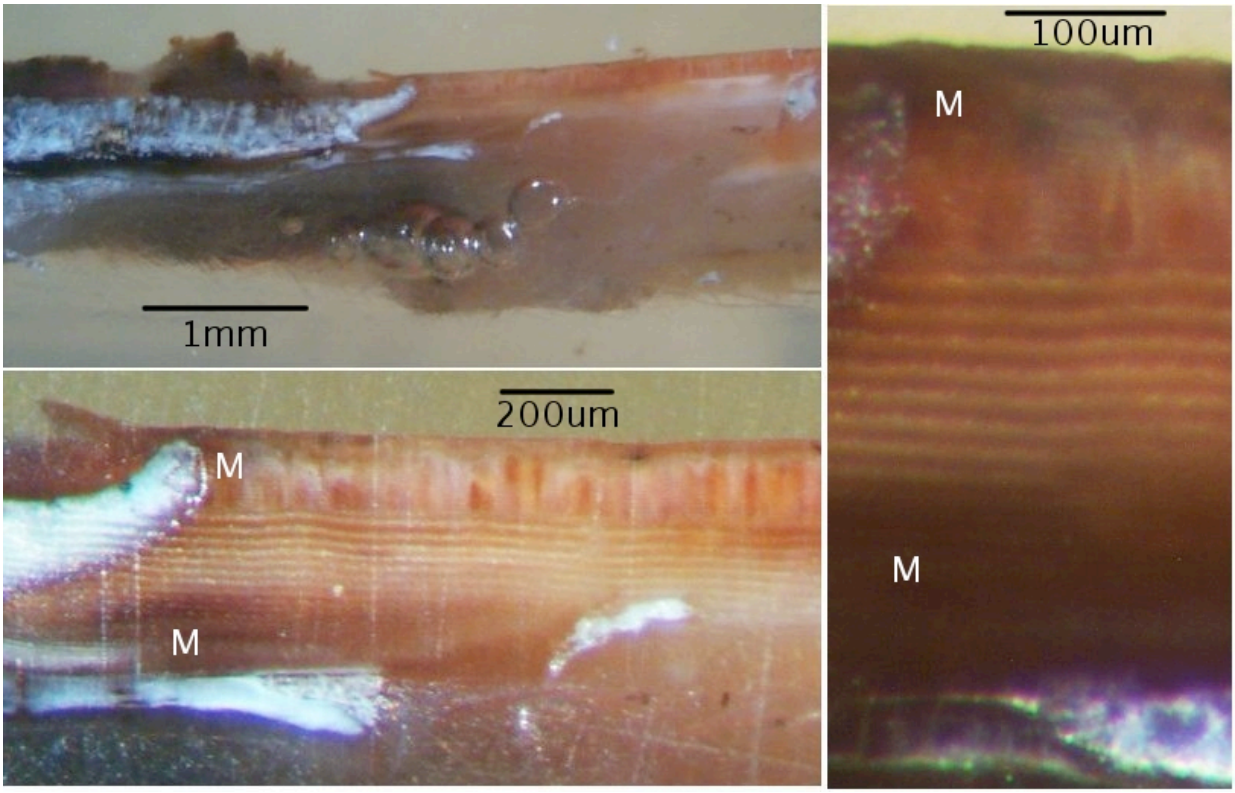

Figure 3. Three images of Grade IV(+) erosion of lobster cuticle at increasing magnification. Melanization (M) of cuticular regions in the neighborhood of an advanced shell disease lesion. Hyper-melanization is seen adjacent to the epidermis in the newly deposited endocuticle and adjacent to the cuticular surface in the exocuticular layer.

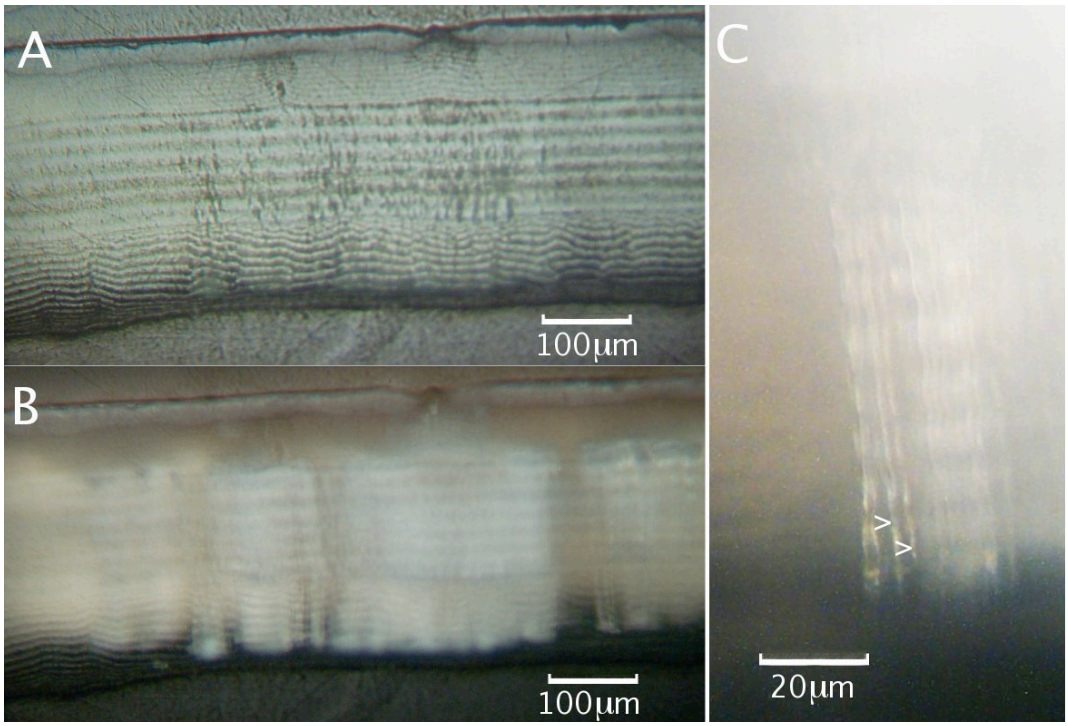

Figure 4. Visualizing pore canals. **A.** Cuticular layers visible via reflected light microscopy of *en bloc* polished surface. **B.** Surface of '**A**' viewed in epi-illuminated polarized light. **C.** Detail of the view between crossed-polarizers indicates that the structures revealed are the quasi-helical reflective lipid and ester-wax filled channels (>) of the pore canal system (Locke, 1964).

DISCUSSION

A strategy to identify the initial departure from healthy mineralization of the lobster cuticle is proposed. This involves freeze-substitution fixation of the cuticle, which immobilizes all molecules and ions combined with embedding in plastic and preparing the cuticle to be observed via electron microprobe microscopy. The ultimate objective is to be able to prepare the tissue for X-ray backscatter analysis of the mineral content. The resolution of this technique depends on the degree of refinement in specimen preparation that can be achieved.

Currently, the plastic embedded specimens are well disposed for examining the cuticle surrounding shell disease lesions. Examining the *en bloc* cuticle with a stereomicroscope and polarizing light microscope allows details including dermal gland canals, sensory canals and pore canals to be identified. Previously the pore canals in the lobster had not been observed routinely possibly due to the rigors of acid extraction that needed to be used for observing sections of demineralized cuticle. The density of the pore canals (~8-10 per 20 μm) is consistent with approximately 100 pore canals arising from each epidermal cell. In addition, other features of light microscopic sectioning diagnosis of the stages of shell disease lesioning (Smolowitz et al., 1992) such a super-melanization and surface incursions of microbial growth can be observed and recorded *en bloc*. Moreover, a compound microscope is capable of imaging details of cuticle structure below the polished surface by optical sectioning either with transmitted light, reflected light or polarized reflective light. The freeze substitution approach has provided an improved way of viewing aspects of the undisturbed morphology of the cuticle and hopefully the mineral composition in relation to its morphology.

Quantitative analytical work on the plastic embedded cuticle of this study is in its early stages of development. X-ray backscatter analysis is the ultimate objective using an EM Microprobe facility. Prior work with the minimally calcified cuticle and calcium storage deposits of terrestrial isopods, *Porcellio sp.,* was able to achieve limited area mineral analysis. Substantial variation in the type of deposits between species and between deposit locations was observed (Ziegler, 2003). The substantially more heavily mineralized cuticle and hypothetically finer structural detail being targeted in the lobster required modifying the protocols applied. Change to the geologist's embedding medium of choice for irregular surfaces, EpoThin resin, has allowed lap polishing the *en bloc* cuticle down to the 0.25 μm diamond polish required for high resolution. This approach should allow quantitative estimation of the insoluble and immobilized aqueous soluble mineral content in small sub-regions of the specimen. This high resolution is needed to allow statistics within a region (n-pixels square) to be calculated. This can lead to a 2-D contour map of the mineral content of a cross-section of the specimen. The resolution of such a contour map depends on the degree of flatness one can achieve in polishing the plastic embedded specimen. Another option for studying the lesions is a microfocus X-ray CT-scanner, which is available to use at neighboring Amherst College. This approach produces a 3-D estimation of the mineral content of the structure, which can be interpreted as a series of slices or as a 3-D contour map. The current problem remains how to calibrate the maps produced so that one could compare experimental or lesioned specimens *vs* control or normal contours.

Early signs of vulnerability of lobster cuticle have included accumulations of immune cells near the base of dermal gland or sensory neuron canals (Smolowitz et al., 1992) in Grade I shell diseased lobsters. It will be interesting to see if abnormalities of localized or general mineralization of the cuticle precede, are concomitant with or lag the

earliest signs of shell disease lesions. It is also useful that features of the pore canal system are able to be routinely observed at the light microscope level using polarized light on the *en bloc* observed cuticle. That means that all of the important potential avenues of attack through the lobster cuticle (dermal gland canals, sensory neuron canals and pore canals) can be observed at the tissue preparation level. Both the dermal gland canals and the sensory neuron canals are similarly epicuticle lined structures. This reduces the question of mechanism of microbial attack to a few surface types. All surfaces are protected by an epicuticular layer. The pore canals end blindly at the exo-endocuticular border as viewed by polarized light in intermolt cuticle but it is clear from other arthropods that they function as channels for providing wax, other hydrocarbon based molecules and perhaps other small molecules to the developing cuticle layers. This ability to recognize the fine structures of the cuticle will be of immense help in choosing sample cuticle surfaces to be examined by electron microprobe.

Other participants in the Lobster Shell Disease Workshop have noted that the American lobster's mineralization of the cuticle is slower than in some other decapod species which produce a hard cuticle shortly after molting and are also relatively free of shell disease. These observations make an understanding of the timing and pattern of normal mineralization of the cuticle a desirable goal. Our freeze substitution protocol is designed to provide that information in the near future.

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