**Defense of the Mineral Fine Structure of the**

**American Lobster Cuticle**

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**Abstract:** The integument of a metazoan separates critical internal organs from the external environment. Some biologists would concur that the most important role of the integument is protection from microbes. Calcite and amorphous calcium carbonate are the most abundant minerals in lobster cuticle; they are the most acid vulnerable of minerals and thus require protection from an acidified environment. Here we show that calcite is an investment in neutralizing an acidifying environment and this neutralizing function is modulated in this role by the epicuticle. Another more minor cuticle mineral component is carbonate apatite, aka bone. Based on its location and form, lobster bone is proposed to play critical roles in the integuments protective function. Carbonate apatite of lobster exhibits a flexible composition, its least soluble forms protect the environmentally most exposed structures, dermal gland- and neuronal-canals. A trabecular-like carbonate-apatite structure similar to spongy bone illustrates efficient use of phosphate and likely provides the hardness exhibited in the phenolicly-crosslinked inner exocuticle region. We introduce a schematic model of the cuticle emphasizing regional diversity. A thin outer calcite layer provides a dense microbial barrier that dissolves slowly through the epicuticle, providing an external alkaline unstirred layer inhibitory to bacterial movement and metabolism. We show that injury to the epicuticle covering this mineral cuticle surface unleashes a strong flux of alkalinity providing a further general immune response accentuating the normal alkalinity of the antimicrobial unstirred layer. The mineral fine structure of lobster cuticle is described from the perspective of its structural protective role and antimicrobial function.

**Keywords:** *Homarus americanus*, calcite, carbonate-apatite, bone, electron microprobe, ion flux, Scanning Ion Electrode Technique SIET, unstirred-layer

**Running Title:** Calcite and Apatite in Lobster Cuticle

**Introduction**

Arthropod cuticle is a classic object of study by paleontologists, morphologists, cytologists, physiologists and biochemists (Dennell 1947; Richards 1951; Roer & Dillaman 1984; Willis 1999; Locke 2001; Havemann et al. 2008). More recently, materials scientists have viewed crustacean cuticle as an example of a time tested natural composite material (Raabe et al. 2005). The organic polymer nature of the layered cuticle has been described as a twisted plywood pattern (Bouligand 1972, 1986). The mineral contribution to this composite has not been as well elaborated but this detail is now yielding to micro-chemical and physical measurements (Hild et al. 2009; Tao et al., 2009; Seidl 2011). It is clear that crustaceans combine minerals with organic polymers in their exoskeleton to create an effective durable protective covering for a taxonomic group that has survived hundreds of millions of years invading salt and fresh water as well as land. The variety of cuticle composites is able to be studied among 15,000 extant species of Decapods worldwide with a species discovery curve far from flattening out (Martin et al. 2009). Arguments exist about the relative importance of carbonate and phosphatic minerals in the evolution of Decapod cuticle structure with controversy over how one could switch between the two (Vega et al. 2005; Buckeridge & Newman 2006). We show that the two minerals coexist alongside each other in separate cuticle domains. The fresh water and ocean environments in which these composite materials need to survive has recently changed relatively rapidly on an evolutionary time scale due to anthropogenic pressures (Turley et al. 2007; Ries et al. 2009) and we need to evaluate the properties of these vital skeletal organs in the light of those changes and extrapolate to the future. In order to do this extrapolation we need a model of how the cuticle is designed. Modeling from a structural engineering point of view Nikolov and coworkers (2010) have computed general cuticular properties by a hierarchical averaging method. This averaging of properties hides the importance of unique properties of regional specializations. Our approach is to focus rather on the importance of diversity of regional properties with the surface structural properties being most important in a defense against external microbial attack.

Diseases affecting cuticular structure of the American lobster, *Homarus americanus* H. Milne-Edwards, 1837, could provide clues to how a composite design is vulnerable and how the vulnerabilities might be attacked and defended. We are proposing a model of mineralized American lobster cuticle using arguments for how the cuticle defends its owner against chemical and microbial attacks such as seen in lobster impoundment (Smolowitz et al. 1992) and epizootic shell disease, fig 1A (Hsu & Smolowitz 2003).

The general marine environment is becoming more acidic which may exacerbate shell diseases that both erode the mineral and polymeric structure of the cuticle in local environments that are already at extremes of the organism's tolerance: epizootic shell disease is found most frequently in the southern extreme of the American lobster's range and impoundment shell disease is typically found in the abnormal lobster pound environment. It is not yet clear what are the critical factors encouraging symptoms of epizootic shell disease at the southern boundary of the lobster’s range, temperature, pH, pollution, … nor is it clear that the area south of Cape Cod will remain the boundary of the disease. Our intent is to discover forces that might play on cuticular weakness. It is not yet obvious how the attack on the cuticle starts but points of attack develop into small circular lesions, fig 1A, which enlarge and coalesce into lesions that can cover the entire animals cuticle. The theory upon which we are proceeding suggests that vulnerabilities develop at points in the cuticle (Tlusty et al. 2007). We propose a new model of cuticle mineral structure as part of the search for points of vulnerability.

Decapods (shrimp, lobsters and crabs) go through numerous molting cycles during their life that require regular wholesale replacement of the polymers and minerals of their exoskeleton. While early larval and juvenile molting cycles occur frequently enough (a few to several molts per year) to allow replacement of worn and damaged cuticle, later mid- and later-life molting cycles of the lobster must provide enduring protection for one to several years. What design features are associated with a long duration intermolt including a resistance to attack by shell disease organisms? The minerals in the decapod cuticle have traditionally been associated with the hardness and physical strength of the cuticle as if that structural property were their major role. Clearly the hardness of the decapod cuticle defends them against physical attack by major predators during the long intermolt period. It is possible that the minerals also independently participate in a chemically based defense against microorganisms. It is well known that pH controls prokaryotic (i.e. bacterial) growth and physiology (Palmer et al. 1997, Bombelli and Wright 2006). The pH of the ocean macro-environment is controlled mainly by the equilibria of primarily the carbonate ion (Jacobson 2005) in which the current pH is near 8.14 with environmental pressure predicted to be downward (Orr et al. 2005). Local environments are determined by local conditions which may change the local pH making it substantially different from the open ocean average, for instance the bottom of the ocean tends to be more acidic due to the greater number of living organisms there who are all secreting metabolic acids. Even more local on a micro-level, carbonate mineral containing structures that can dissolve into the ocean will contain a superficial layer of ocean water (~100 um) on their surface whose pH is dominated by the dissolution chemistry of the structure. The major mineral of the lobster cuticle is calcium carbonate that appears as calcite and amorphous calcium carbonate (Becker, et al. 2005). Calcite is most often discussed with respect to the strength of the lobster cuticle (Bouligand, 2004) despite the fact that calcite is a relatively soft mineral (3 on the MOHs scale of hardness). We suggest that the forms of calcium carbonate here shown to exist in the lobster exoskeleton gradually dissolve in the ocean water to produce an unstirred layer (Pohl et al. 1998) that approaches the pK of CaCO3 dissolved in water, which is pH 9.0. Magnesium as a minor constituent is known as a hardening factor for crustacean calcite and fluoride for apatite structures (Mirtchi et al. 1991). Classic (Richards 1951) to modern investigators have reported small fractions of phosphatic mineral as components of crustacean cuticle, including carbonate-hydroxylapatite. A general role for phosphatic minerals in the crustacean cuticle has not been established and it has been somewhat ignored due to its reported relative minor compositional percentage (Lowenstam 1981; Boßelmann et al. 2007). The phosphatic mineral, carbonate-apatite (aka bone), has been identified in the mineralized plates of a particular barnacle, *Ibla quadrivalvis* (Whyte 1988; Lowenstam & Weiner 1992), a crustacean with more distinctly hardened structures in its integument. Other barnacles however are reported not to use this method of hardening. We here establish a strategy for the distribution of multiple mineral forms of carbonate-apatite in the lobster cuticle and propose functions for them. More details on the variety of uses of carbonate apatite are being published elsewhere (Kunkel and Jercinovic, 2011).

We propose that a microbial attack on the mineral component of the cuticle begins from the outside and continues using secretion of acid to dissolve the cuticular minerals until the organic layers are exposed enough for proteolytic and chitinolytic enzymes to be brought to bear to form microscopic and then macroscopic lesions such as seen in fig 1A. Mineral structural and chemical mechanisms may be used to resist lesions. Simple abrasion or establishment of small circular artificial lesions, fig 1B, does not lead to shell disease progress. The initial processes to create latent micro-lesions that develop into epizootic or empoundment shell disease remain unknown. It could be based on a specialized microbe that successfully penetrates the lipid and waxy layer of the epicuticle to selectively lay bare surface of the outer calcite rich layer. Such microbes with lipolytic, proteolytic and chitinolytic enzymes have been suggested (Tlusty et al. 2007) and identified elsewhere in this volume (Meres et al. 2011, Chistoserdov et al. 2011). Our strategy is to explore the cuticle mineral microstructure where lesion initialization is defended-against at the micro level and hope to identify structures whose imperfections might allow vulnerabilities to develop. Our analysis focuses on calcium carbonate and phosphate chemistry with additional attention given to trace element content that might affect mineral properties.

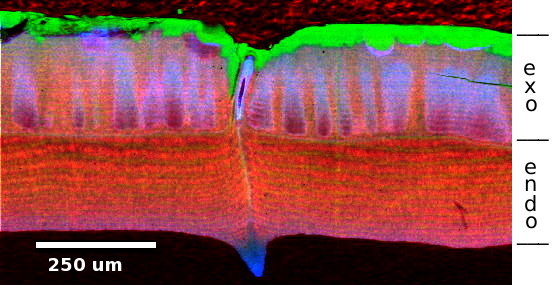


Figure 3. Intermolt cuticle cross-section parallel to canal path viewed via EMP of Ca (green), P (blue) and Cl (red) content. A thin calcite layer is green. Purple canal wall has Ca/P of 4; blue nipple has Ca/P of 3.5 and exocuticle trabeculae have Ca/P of 7.

**Material and Methods**

Lobsters were obtained from several locations. Lobsters symptomatic for empoundment shell disease were obtained May 2008 from the Maine State Aquarium at Booth Bay Harbor. Earliest studies of asymptomatic lobsters and those symptomatic with shell disease were obtained in 2004 from trawls by the NOAA Ship Albatross IV at the mouth of Narragansett Bay as well as in canyons at the edge of the continental shelf directly south of Narragansett Bay. Non-symptomatic lobsters were obtained June 2007 or 2008 from the State of Maine Ventless Trap Program from Casco Bay ME to Isle of Shoals NH. An equal number of asymptomatic lobsters and symptomatic epizootic shell diseased lobsters were obtained in 2008 and 2009 from Narragansett Bay above The Claiborne Pell Bridge. Lobsters obtained in Maine were maintained until used in running 15 C fresh seawater at the University of New England Marine Science Center or in recirculating 15 C artificial seawater at UMass Amherst. RI lobsters were maintained until use in recirculating 15 C artificial seawater at UMass Amherst. Lobsters were fed during workdays M-F with frozen scallop muscle ad lib for a period of ½ hour. Uneaten scallop was removed.

Shells of the Atlantic Jacknife Razor Clam, *Ensis directus* Conrad, 1843, were obtained from Pinepoint Beach, Scarborough ME shortly before use.

To evaluate the role of mineralization in the defense of the cuticle we treat the cuticle as a moist geological specimen (Kunkel et al. 2005b). Excised small cuticle squares are plunge frozen in liquid nitrogen cooled propane; then the frozen water is substituted with liquid-nitrogen-chilled-acetone, and the pieces are slowly brought to room temperature. The cuticle was embedded in Epo-Thin Resin (Buehler). The plastic-embedded cuticle specimen is ground and polished with graded carborundum and diamond abrasive (METADI® SUPREME 6um – 0.25um) suspended in polishing oil on TRIDENT™ polishing cloths to prevent movement of any water soluble components (Kunkel et al. 2005b). The specimens were examined in a Cameca Ultrachron Electron Microprobe or in a Cameca SX-50 Electron Microprobe.

Ionic flux from the cuticle is measured using the Scanning Ion Selective Electrode Technique (SIET) (Kunkel et al. 2005a). To measure flux emanating *in situ* from living cuticle a form-fit tygon observational arena was glued to the cephalothorax of lobsters using *Krazy-Glue® Gel*, fig 2. A Teflon nut was also glued to the cephalothorax to provide a basis for attaching a stereotactic holding device to maintain the lobster immobile enabling electrodes to be brought within 100 um of the cuticle surface for flux measurements, fig 2B. A minimal-artificial-seawater was formulated which included only the ions Na, K, Cl, Ca and Mg in close to normal amounts compared to natural sea water, or selectively reduced experimentally, that served as the medium for measuring ionic flux to and from the cuticle surface. Both the lobster holding chamber and the measuring chamber buffer were continuously cooled to 15 C using a purpose-built Peltier cooling system. Measurements of flux simultaneously with two SIET electrodes, Ca2+ and H+, held in a Dual Probe Holder (Biomedizinische Geräte, Germering, Germany) were made under ASET software control (ScienceWares, Falmouth, MA) using dual SIET amplifiers and motion control electronics (Applicable Electronics, Forestdale, MA).

Artificial lesions of lobster and mollusk shells were created with a Microlux® Variable Speed Drill Press (MicroMark) with a digital depth finder.

**Results**

In typical asymptomatic intermolt cuticle we see: i- A dense thin birefringent crystalline calcite layer on the outside, figs 3,4; ii- a carbonate-apatite, aka bone, lining of dermal gland and neurite canals, figs 4, 5; and iii- trabecular carbonate-apatite used in the exocuticle providing hardness, fig 4A,B. The properties of these three features are derived from electron probe microanalysis (EPMA) compositions of intermolt lobster cuticle seen in figs 4 and 5, each of which illustrates different aspects of calcium and phosphorous mineral distribution in the exo-cuticle of the intermolt lobster. Fig 4A shows a dermal gland canal which has a canal lining of relatively high P:Ca ratio which is typical of canals close to the surface of the cuticle. A cross-section of a canal seen in fig 5 illustrates a dermal gland canal which has two regions of distinct P:Ca ratios, an outer luminal layer P:Ca is 2 while the cuticle sided P:Ca is 3.51. The calcite vs bone signals are differentiated as Principal Components (PC) of the composition variation among pixels. PC-1 points to calcite variation while PC-2 identifies carbonate apatite as two levels of blue and green in the fig 5C’s PC-2 panel. The two ratios of P:Ca are also seen as distinct slopes of Ca:P pixels with ratios of 2 and 3.51 in fig 5D. Both of these ratios represent typical bone ratios of P:Ca as seen in Table 1. A P:Ca of 2 represents bone with a composition Ca10(PO4)5(CO3)(OH)3 • H2O , one of the highest phosphate contents exhibited by bone.

We find that the outermost mineral surface of the lobster intermolt cuticle consists mainly of a smooth dense layer of calcite, fig 4A, that has a calcium Kα intensity close to mollusk shell, which is 95-98% CaCO3, fig 5D. This birefringent outer layer of calcite appears continuous but is punctuated at regular, but widely spread, i.e. over-disperse, intervals by cuticular organules, fig 6, a term popularized by Lawrence (1966) for small organs such as dermal glands and bristles, which are formed from single epidermal mother cell dividing to form a small groups of cells, the organule. The cuticle of the lobster is studded with organules of simple dermal glands and interspersed with compound organules that combine sensory bristles with accompanying dermal lands as seen in fig 6 as surface structures and accompanying clustered carbonate-apatite lined canals. During the molting process cuticular organules are moved apart from each other during the generally isometric expansion of the cuticle surface. The expansion of the cuticle in lobsters varies but is approximately 1.1 fold increase in linear dimensions which represents a 21% increase in cuticle area. New organules need to develop and be inserted into the widening expanses of cuticle devoid of organules.

A single organule canal or bristle cell secretes a canal cuticle surrounding a narrow canal which allows environmental outlet for secretion from an organule gland cell or forms a protective sheeth about neurites innervating sensory organules such as mechano- or chemo-sensory bristles at the surface of the cuticle as seen in fig 4A. The canal lumen in the bristle or gland canal is continuous with the outside environment and could represent a path of attack by microbes. In all arthropods these canal passages are for carrying secretions to the cuticle surface or protecting sensory neurites traveling through the cuticle to a sensory ending at a bristle tip. The canals have been shown in other arthropods to be lined with an epicuticle similar in structure to the general cuticle's epicuticle (Kunkel 1975). The lobster cuticle has canals with 10-20 um openings leading into 400-1000 um long canals in the lobster cuticle into which bacteria could invade and form colonies. Such canal-bacterial colonies could build up substantial populations and establish significant local focused acid gradients, potentially damaging to a calcium carbonate based cuticle. How do the two distinct surfaces, outer cuticle and canal lining, perform when confronted with a bacterial colonization in the lobster?

When observed using EPMA, figs 3, 4A, 5, the calcium Kα intensity of the calcite layer is close to that of crystalline calcite of mollusk shell, used as a standard, fig 5D. The calcite layer thus has limited space remaining for organic polymer. When this thin calcite layer has a small artificial lesion drilled into it, no pro-phenoloxidase (PPO) is activated. Only when the calcite layer is breached and the inner-exocuticle is reached is PPO activated. The crystalline calcite layer can be observed in polarized light as a uniformly birefringent layer which can turn corners, indicating its controlled orientation, fig 3 arrows, most likely deposited along polymer fibrils or bundles. Consistent with this, the numerous pore canal filaments represent microvillar extensions of the epidermal cells course through the physiologically living endo- and exo-cuticle but are excluded from the calcite layer (seen in unshared AFM figures). The remnant spaces, formerly occupied by live filaments, are surrounded by organic and mineral layering observed with EPMA and by AFM but essentially end at the inner surface of the calcite layer. The crystalline density, seen in figs 4 and 5, of the calcite layer does not afford a spatial avenue for bacterial attack unless the density were first compromised by erosion or cracking. The calcite layer also extends down like a collar along the canal wall exterior at the organule canal intersection, seen in cross-section in fig 4A and in tangential section in fig 4B and 5B. The canal wall abuts the calcite collar closely leaving a relatively small space between the mineral faces, particularly visible in the calcium image, fig 5B. The calcite layer and its boundary with the apatite tubes might allow access into the cuticle by microbes down to the typical size of a bacterium (0.2-2 µm) and we may need to know the tolerances of this space under stress as predicted by our model. We do not know the organic composition of that thin space but when other ions are analyzed by EMP it is seen to contain Cl and K which would indicate it is or was a cellular compartment. The flat surface of the general cuticle surface does not encourage the buildup of a pH gradient from a bacterial colony growing on an open surface. This is because protons in water as hydronium ions have an anomalous 12-times faster diffusion than their diameter predicts (Kunkel et al. 2001). This is the physical reason that a calcite surface is a sufficient material to create a physical barrier to microbial attack … protons created by a point source group of bacteria will disperse rapidly and not accumulate enough titer to do damage to the calcite. The calcite layer of the cuticle is also covered by a waxy epicuticle that is an efficient protection from bacterial attack for another reason: during the slow solubilization of calcite through the epicuticle into the ocean water, carbonate and Ca ions are released. The carbonate takes a proton from water becoming bicarbonate and releasing a hydroxyl and thus results in a widely based alkaline zone in an unstirred layer (Pohl et al. 1998) adjacent to the cuticle. The unstirred layer gradient properties are a result of the speed with which diffusion of an ion establishes a nearby high pH gradient despite competing bulk flow of nearby ocean water that eventually undoubtedly will affect the gradient depletion. The high pH gradient, approaching pH 9, is dispersed as it merges with the bulk of the adjacent seawater (pH 7.8-8.2) but a thin alkaline aqueous surface unstirred layer remains on any calcitic shell as an antibiotic to bacterial growth and attack (Palmer 1997; Bombelli & Wright 2006). Calcite itself would not be an effective material choice as a lining to the cuticular organule canals because in the enclosed small diameter, but extended length, of a gland or neurite canal, proton gradients could be established by bacteria that could easily dissolve a calcite lining. Rather, the organule’s canal is fortuitously lined by a phosphatic mineral of moderate high density that is more acid resistant than calcite. Chemical analysis of this layer by EPMA demonstrates, figs 4, 5, that in Ca:P ratio it conforms to the flexible composition of CAP, aka bone, Table 1 (Wopenka & Pasteris 2005), being resistant to acid solubilization. The only other calcium phosphate that could theoretically apply is tetracalcium phosphate, a mineral not described in living tissues (Dorozhkin & Epple 2002). Either would provide the ratio of Ca:P of approximately 2 which is observed in some neurite- and gland-canal walls. But, interestingly, carbonate-apatite as seen in the two locations in lobster cuticle exhibits several of the generally accepted formulations of cabonate apatite, Table 1, that have flexible Ca:P ratios that can include 2:1, 2.25:1, 2.67:1, 3.5:1, 4:1 and 7:1 which are all relatively discretely observed (figs 4, 5) in canal tubes with Ca:P ratios predicted by the bone formula. The discreteness of the carbonate-apatite formulations is often emphasized by finding two Ca:P ratios in a single cross-section, fig 5A,D.

The composition of the calcite, inner-exocuticle and endocuticle layers were studied in greater detail using 12 quantitative transects of lobster intermolt cuticle using the SX-50 electron microprobe. Care was taken to include transects through trabecular as well as non-trabecular regions of the cuticle which changed the chemical profiles mainly in the inner-exocuticle which the trabeculae populate. With the SX-50 EMP we were able to measure the content of the other Group 2 Alkaline Earth Metals, providing additional detail on the regional heterogeneity of the lobster cuticle. Fig 7 shows the relative titers of Mg, Ca, Sr, Mn, Ba. Of particular note is the precipitous decline in Mg, Sr, Mn and Ba together in the calcite layer toward the surface of the cuticle. This may represent a clue to the role of the minor divalent ions in the calcite layer function. While Mg is known for its hardening properties in calcite composition it is also known for higher solubility in the ocean. A more rapid dissolution of MgCO3 in the surface oriented calcite could provide early postmolt protection via the mechanism of raising the unstirred zone’s pH as will be discussed later.

The two distinctly different forms of shell disease, impoundment shell disease and epizootic shell disease, attack at different points of the cuticle, impoundment disease attacks at the dermal gland canals and epizootic shell disease on the plane between dermal gland canals. This difference of point of attack allows us to create alternative hypotheses about the progression of shell disease based on predictable vulnerabilities of our model, fig 8. In pursuit of this theory we describe how our cuticle model might provide a protective role using a major component, the minerals, and then use the model's explicit mineral properties to suggest hypothetical modes of attack. Our morphological mineral model based on some key exemplar physical evidence, e.g. figs 3-5, suggest a protective rationale and a structural role for key minerals of the lobster cuticle that may have provided a selective force during its structural evolution.

The potential vulnerable aspects of the dermal glands are derived from inspecting our model of the lobster cuticle. First, the phosphatic wall of the canal needs to tightly interface with the cuticle's calcite layer, fig 4A,B. The visible space between the two mineral structures seen particularly in fig 5B is filled with the anion chloride. These Cl filled spaces around canals are also seen as red in fig 4B. What are the acceptable tolerances of these spaces? There is a possibility of a failure in the phosphate or carbonate chemistry for this juncture particularly in the crowded and altered water quality of lobster pounds and rearing facilities after which the disease is named. There is also ample evidence of the limiting nature of available phosphate in the North Atlantic arena (Wu et al. 2000; Zubkov et al. 2007). In the altered temperature, pH, salinity, nutrients and microbial environment of a lobster pound or aquarium the chemistry could clearly be a source of problems. Lowering the pH for instance makes the energy necessary for retrieving carbonate from bicarbonate more costly to the lobster. If the dermal gland and neurite canals phosphatic lining were thinner or incomplete for some reason, the ability of bacteria to access and attack the underlying chitin and protein linkages could be encouraged. In addition, an acidified seawater environment might allow a pH gradient produced by a bacterial colony to be more effective given a lower imposed pH environment at the opening of the organule canals. These hypotheses can be pursued in a tightly defined chemostat arena or a relatively simple marine life-table flow-through environment by adjusting the conditions that interact to allow the impoundment type or epizootic type shell disease to develop.

The study of epizootic shell disease with respect to our model is perhaps less straightforward. Efforts must be taken to avoid the conditions of impoundment shell disease from occurring. The evidence from light microscope sections of de-mineralized cuticle from early shell diseased lobsters suggests that the newest lesions develop in the calcite-plane region between the organules producing early pillars of relatively undigested cuticle. We suggest that these pillars occupy the spaces between the more acid resistant phosphatic trabeculae of the inner exocuticle, fig 4A. Our work with oxygen electrodes on artificial lesions of the cuticle suggest that until the thin dense calcite layer is compromised there is no response of oxygen utilization by the lesioned cuticle. Once the calcite layer is breached, the cuticular prophenol oxidase is activated, oxygen starts being utilized and melanin accumulates at the lesion. Therefore the first hypothetical point of attack in epizootic shell disease would be an imperfect calcite layer. This might occur because the calcite layer was not properly developed during the time shortly after ecdysis, during the phase that the cuticle provisionally hardens or it could result from the attack of this layer after it had been formed imperfectly or cracked because of improper development of the more rigid underlying trabecular layer.

Our application of SIET to study the flux of ions from the cuticle and early stage lesions was frustrated by very few examples of early lesions to which we could apply our measurement of Ca- and proton-flux. However from the few such lobsters, fig 2A being one, it was evident that there was a slow flux outward of Ca2+ and inward of H+ from asymptomatic lobster cuticle and a substantial increase of that flux over lesions. This observation immediately rejected our earlier naive hypothesis that we should detect early latent- or developing-lesions from the secretion of protons by a microbial film or colony at the site of a future lesion. Ionic flux from the lobster cuticle weather asymptomatic or symptomatic of shell disease is dominated by calcium carbonate dissolution from the superficial calcite layer of the exocuticle. The entire surface of the lobster carapace is slowly leaching CaCO3 from the calcite layer creating a basic unstirred antimicrobial surface environment.

More productive information was obtained by our creation of graded artificial lesions such as seen in fig 1B. Such lesions allowed us to examine numerous lobsters with different degrees of lesion reaching various levels in the cuticle. These lesions were examined using dual Ca- and H-LIX microelectrodes simultaneously at essentially the same location, fig 9. The pattern was highly reproducible. The Ca-flux was the mirror opposite of the H-flux. Ca2+ appeared to exit the lesion and H+ appeared to enter the lesion. This is explained in our model by the dominant equilibrium reaction occurring at the pH of the lobster’s environmentHH:

CaCO3 + H2O <=> Ca2+ + HCO3- + OH-

The dissolution of CaCO3 reaction produces Ca2+ and OH-, and we measure the outward diffusing Ca2+ and OH- as outward diffusing Ca2+ and inward diffusing H+. This opposite behavior of Ca-flux and H-flux is a signature of dissolving calcite or aragonite or amorphous calcium carbonate, ACC. This would distinguish CaCO3 dissolution from CaCl dissolution, for instance, or from simple Ca2+ export linked to some other balanced transport.

When the calcite layer is penetrated by an artificial lesion a stronger linked flux of Ca2+ and H+ is measured because the dissolution of ACC has a lower energy requirement and thus it dissolves more quickly. The design of a calcite layer underlain by an ACC layer can be seen as selectively advantageous in fighting a progressive shell disease lesion. This is in apposition to the role that ACC has in generating calcite (Pouget et al., 2009) during the post-molt when the calcite layer is being established. In our model the ACC deposits between the trabeculae in the inner-exocuticle and in the general endocuticle serve as a reservoir of quickly deployable calcium carbonate that can aid in responding to injury by lowering pH in the ~100 µm unstirred layer above an injury.

Broadening our understanding of this phenomenon we see in the lobster, we have studied mollusk calcite shells that are covered by a protective periostracum, a protein polymer layer laid down and sometimes actively maintained by the mollusk mantle tissue. This periostracum is analogous to the epicuticle of the lobster in that it protects the calcite layer from rapid dissolution. We demonstrate, fig 10, that the artificial lesions created in the mollusk periostracal covering release a stream of Ca2+ and OH- ions similar to those we have observed emanating from our lobster artificial lesions. In this instance the ratio between the flux at the center versus the edge of the lesion over intact periostracum is 8.9 ± 1.9 se fold. This ~9 fold increase over a shell lesion is probably closer to an accurate measure since it is easier to get close to the inert shell surface that the live lobster cuticle surface.

Study of the mollusk and lobster cuticle artificial lesions demonstrate that the Ca- and H-fluxes mirror each other in direction and strength. The H-flux into the lesions represents the expected flux created by the opposite flow of hydroxyls created by reaction of carbonate, from the dissolved calcite, combining with a proton from water to release a hydroxyl, as diagrammed in fig 6. The artificial removal of the epicuticle doubles the fluxes measured from un-lesioned cuticle even at the 100 um distance from the surface, thus demonstrating that the epicuticle serves as a modulator of calcite dissolution. The pH at the shell surface in the low um space occupied by bacterial films is likely to be close to pH 9, the pK of CaCO3.

**Discussion**

The most interesting discovery made during our fine structure study of the lobster cuticle mineralogy was the identification of a variety of forms of carbonate apatite, aka bone, in the cuticle architecture. In the bone pipes that form the protective canals for dermal glands and neurites, there are often two distinct carbonate apatite formulae applied to discrete adjacent layers in the tube. The rule so far observed is that the outermost bone layer has the higher Ca:P ratio. Since the tube is most likely produced by a single specialized cell of the organule cluster, it is likely that the outer (cuticle-side) layer of the tube is laid down later as the cell regresses from the cuticle surface and more inner layers of the cuticle are being laid down. This may represent a natural reduction in the available phosphorous as the organism starts growth and cell division that require phosphorous for nucleic acid and protein synthesis.

Carbonate apatite had been identified as a minor component in X-ray diffraction powder patterns of lobster cuticle (Boßelmann et al. 2007), Crustacea, Malacostraca. It is a major constituent of the *Ibla* barnacle valve plates in the Malacostracan sister group Thecostraca. However the importance of carbonate-apatite in all Malacostraca cuticles has been minimized. The role of carbonate-apatite as a lining of the gland and neurite canals of lobster cuticle could be a major selective advantage in this crustacean's resistance to microbial attack via the canal lining. In the lobster the most exposed canal linings are closer to 2:1 Ca:P in carbonate-apatite composition which make it least sensitive to acid attack (Baig et al. 1999).

While our lobster cuticle model, fig 8, is derived from earlier observations from various research groups including our own, it relies heavily on a novel motivating principle reinforced by observations made during our current research. Our new principle is that cuticle minerals function chemically to protect against environmental attacks by microorganisms. Our structural model is of intermolt cuticle and we expect it to provide a basis for understanding the relatively long-term resistance to disease experienced by the lobster during its extended intermolt. The cuticle’s natural immune properties are testimony to the difficulty that experimentalists have had in transmitting shell disease between symptomatic lobsters and asymptomatic aquarium mates during the intermolt period.

The model, fig 8, based on EMP measurements, polarized light microscopy, and ionic flux studies has an outer crystalline calcite layer covering a trabecular carbonate apatite exocuticle layer with intervening amorphous calcium carbonate between the trabeculae. The spongy-bone-like trabecular structure brings up the question of what cuticular feature is responsible for the slow progressive hardening of lobster carapace cuticle as described by Waddy and coworkers (1995). Based on our mineralization maps and our model and the hardness tests of Raabe and associates (2005) it seems that the hardness would not coincide with the outer calcite density. In their progressive indentation tests the outermost layer, corresponding to the calcite layer of our studies in thickness, is a moderately soft outer layer. That layer would need to be established relatively quickly for self-protective reasons based on our model. The slower, development-of-hardness layer would correspond to the zone of the phosphatic trabeculae, i.e. the inner exocuticle, which would develop more slowly depending on available phosphorous. Living in the Northern Atlantic phosphorous poor environment (Wu et al. 2000, Zubkov et al. 2007) the American lobster may have developed a strategy of using its limited phosphorous availability by slowing down the cuticle hardening process as we know it (Waddy et al. 1995).

The lobster trabeculae are possibly convergent with the trabeculae of vertebrate spongy bone in ways beyond chemical structure. The dynamics of development of the lobster trabeculae may well be based on stress. The dorsal carapace is the site of many thoracic muscle attachments and the stress provided by those attachments could result in the massive hardness that develops at the dorsal lateral carapace *vs.* the thinner lateral ventral carapace sides that cover the branchial cavity. A stress model of lobster bone development may also apply to the thickness of cuticle chelae that could be behaviorally adjusted by how the lobster uses its crusher vs cutter chelae.

Amorphous calcium carbonate is found between trabeculae of the exocuticle perhaps associated with chitin and protein fibrils as demonstrated in a marine isopod (Seidl et al. 2011); this inherently soft mineral form is similar in electron density to the amorphous calcium carbonate found in the endocuticle that is measureably the softest layer of the lobster cuticle based on Raabe and associates (2005) measurements. This amorphous calcium carbonate may be an essential reserve of available calcium carbonate that can provide the calcium for trabecular development and also respond to cuticular injury by dissolving to form a flush of alkalinity in the unstirred layer that is an antimicrobial shield for the cuticle. This interpretation extends the role of ACC to more than being a precursor to crystalline calcium carbonate forms as previously proposed (Pouget et al., 2009). Exocuticle composed of pure carbonate-apatite would first be a waste of scarce phosphate but also would not serve as a ready source of antimicrobial alkalinity.

Cuticle mineralization in Decapods for Ca2+ and CO32- is acknowledged to be accomplished from the epidermal side of the cuticle after ecdysis (Compere et al. 1993; Wheatly 1999). It requires an investment of energy since the source of CO32- in the cuticle is bicarbonate in the hemolymph that requires a proton to be exported into the hemolymph as a carbonate reaches the cuticle, fig 8. Our model of the intermolt lobster cuticle would be compatible with the calcification process in expecting creation of the calcite layer early after ecdysis, which would establish microbial invulnerability of the surface soon after the calcite layer was made continuous. The early dissolution of MgCO3 from the calcite layer, leaving its outermost surface lower in Mg may provide an early intense alkalinization of the unstirred layer that provides additional bacterial resistance. The energy by which the lobster expels a proton into the hemolymph is the investment in deposition of calcite that becomes the investment in the integrity of the cuticle. Our model also provides a separate imperative for enough phosphate to be invested (1) as organule tubes protecting secretion and neuro-sensory communication and (2) as phosphatic trabeculae to be associated with the well-described (Waddy et al. 1995) gradual hardening of the lobster cuticle that occurs after ecdysis. The hardness of the inner exocuticle based on proper development of the phosphatic trabeculae would provide a more rigid base that would prevent potential brittle failure of the calcite layer due to flexing. As in insects, there is an initial hardening via crosslinking of the exocuticle after ecdysis and expansion by inflation of the new cuticle in Decapods (Dennel, 1947). After the size of the new exoskeleton has been achieved the deposition of the calcite layer must be accomplished and terminated. The calcite layer is relatively thin compared to its potential thickness given the thickness of the procuticle (i.e. the exo-cuticle defined by the time of ecdysis), fig 3, 4A. A relatively sharp border separates the antimicrobial function of the calcite layer from the physical hardness established by the phosphatic trabecular development. The sharpness of the border may be programmed by the existence of phosphoproteins at the border, which are known in other shellfish to organize and regulate crystal growth (Myres et al., 2007). Furthermore, in our interpretation the two layers cooperate in the objective of providing an antimicrobial barrier. The structural and chemical information of this model provides predictions about how the distinct cuticle minerals function during the intermolt period and the predictions can be used to develop hypotheses that will drive future research. For instance, our model predicts that a successful infection of the cuticle could be pioneered by more alkali resistant strains of organisms. Furthermore, the antimicrobial function for calcium carbonate based integuments may play a general role in both arthropod and mollusk shellfish that has not been previously appreciated. The role in carbonate conservation by the epicuticle and periostracum of shells may have as important a role in marine arthropods and molusks as water-conservation by the epicuticle of the integument has for terrestrial arthropods (Beament 1961, Moore & Francis, 1985).

It is of some interest to discuss how other immune factors might interplay with the calcite based antimicrobial function. For instance, pro-phenoloxidase (PPO) is known to be activated by injury. To what extent does PPO provide similar or additive immunity from microbial attack? Clearly PPO has a role in immune responses to lesions in cuticle for a broad selection of arthropods. In the artificial lesions created in our experiments the melanization of the cuticle was visible by 24 hours after the lesion was made through the calcite layer. The calcite dissolution response is immediate. The encapsulation of the lesion by melanization is relatively slow based on our measurement of oxygen utilization by those lesions. No increased oxygen utilization was measureable within hours of lesion initiation. This is perhaps due to the need for the relatively slow enzymatic activation of the PPO. After 24 hours a dramatic increase in oxygen utilization has developed and one can actually see melanization product in the lesion. Clearly the melanization has had some role in stabilizing the lesion by crosslinking the cuticle proteins and if it were a microbial lesion the microbes may well be inhibited in further aggression in the lesion. However, the alkalinization of the unstirred layer is a constant factor already in the unlesioned cuticle and is immediately dramatically increased after a lesion occurs. In addition, after the lesion penetrates through the calcite layer the underlying amorphous calcium carbonate is yet more easily solublized creating a stronger alkaline flux into the unstirred layer. How effective this is and how it interacts with the PPO activation is yet to be established. It is clear that the alkalinization has its effects on bacterial cells in general but probably has little effect on eukaryotic microbes (Palmer et al. 1997). Therefore, other immune mechanisms must be present to defend the cuticle from non-bacterial microbes such as fungi that are targets of antimicrobial peptides, AMPs, which have been described in Decapods (Rosa and Barracco, 2010) but have been more associated with more advanced breaches into the haemocoel.

Last but not least, the rational structure of the lobster cuticle carbonate-apatite structures allow it to serve as a stepwise model for bone synthesis in a non-vertebrate system which might make it invaluable for insights into bone synthesis in general.

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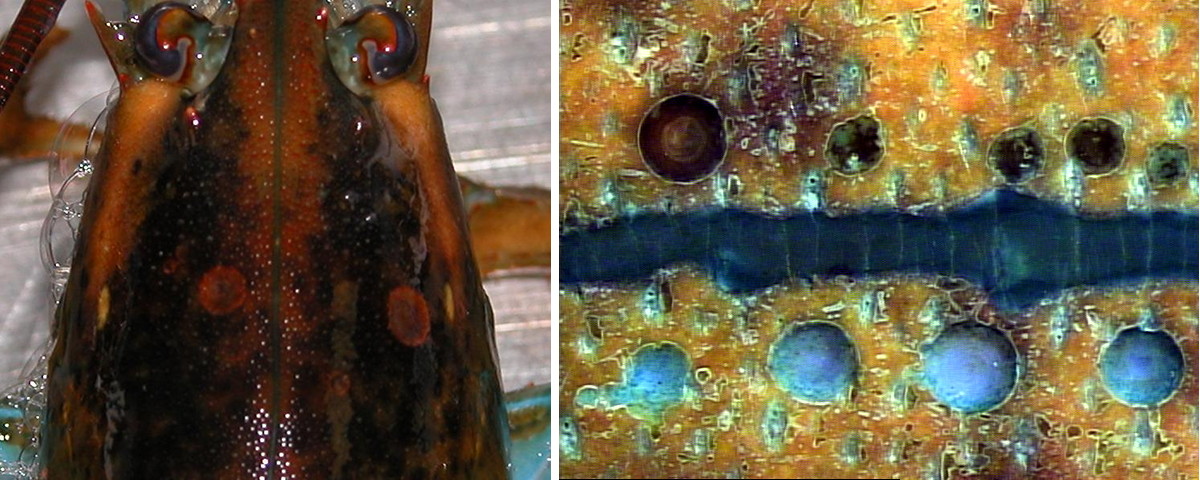


Figure 1. Natural and artificial lobster shell lesions. **A.** American lobster, *Homarus americanus*, with very mild epizootic shell disease lesions. Two circular lesions are indicated by arrows. **B.** Nine artificial lesions imposed on a live lobster carapace using a drill press serve as models of the lesion process. The flux of ions derived from the dissolving shell is followed measuring differential concentrations over short distances with the specific ion electrode technique (SIET). The lesions in B are separated by the mid dorsal suture. The 5 upper, more melanized, lesions were produced 1 month earlier than the bottom 4 lesions.

A B



Figure 2. A lobster with a Tygon observation chamber enclosing carapace cuticle and a Teflon-nut glued to adjacent carapace. (**A)** The observation chamber encloses a region of cuticle with a lesion and holds a measured amount of minimal-ASW. This lobster is studied in (**B)** with dual SIET probes for proton and calcium flux. The large chamber in B holding the lobster is temperature regulated with Peltier cooled ASW that allows the lobster to be probed for hours and afterwards reserved to be probed on succeeding days. This way the progression of shell lesions can be followed over days or months. The objective is to identify candidate early lesions and extrapolate back to the origin of a lesion.

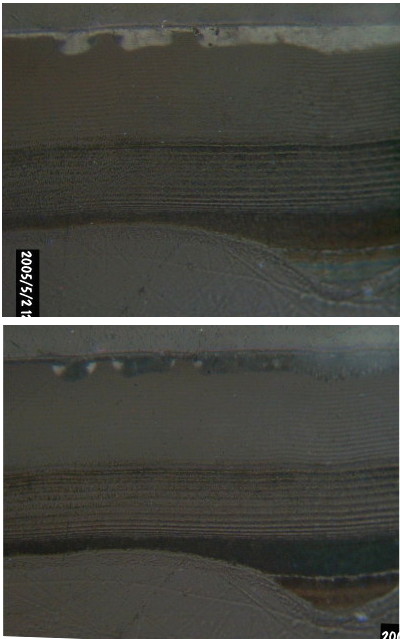


Figure 3. Birefringent Calcite of healthy lobster cuticle follows surface sculpturing. Top and bottom image are same view with analyzer rotated 90°. Arrows show complementary birefringence of calcite layer on 90° rotation of analyzer.

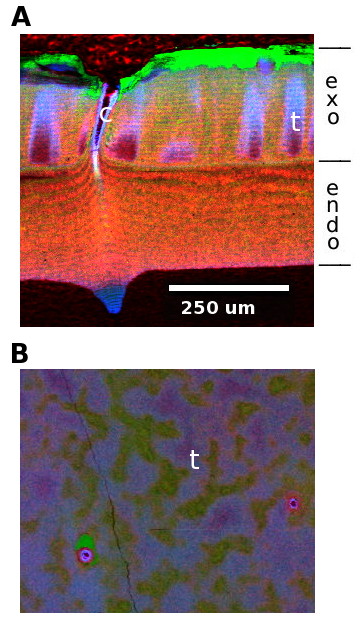


Figure 4. Lobster calcite and carbonate-apatite structures interpreted as false color from three EMP of Ca (green), P (blue) and Cl (red) content. **A.** Intermolt cuticle cross-section parallel to neurite canal, **c**, path showing a Ca:P composition canal wall. A thin green calcite layer colored by Ca alone. A blue nipple area at the cuticle-epidermis interface has Ca:P of 3.5. The exocuticle trabeculae, **t**, have Ca:P of 7. **B.** A tangentially polished section of intermolt exocuticle just under the calcite layer shows Ca (green) intrusions of calcite, P (blue) and Cl (red). Purplish trabeculae, **t**, with Ca:P of 7 are separated by fields of amorphous calcium carbonate seen as greenish since it combines Ca with Cl. Deeper red areas indicate Cl-rich spaces surrounding the dermal and neurite canals from the background cuticle layers.

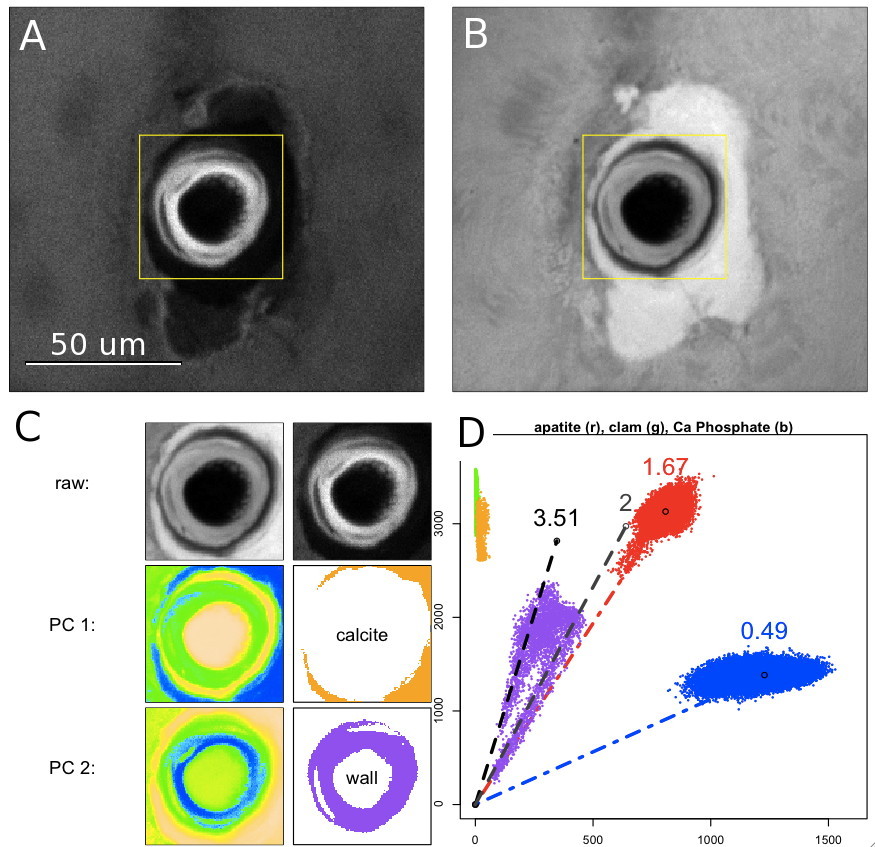


Figure 5. Tangental polished cuticle surface showing organule canal sectioned perpendicular to its long axis illustrating a calcite collar devoid of phosphate and carbonate-apatite lining of the canal. **A.** Phosphorous (Kα) X-ray map. **B.** Calcium (Kα) X-ray map. Note the gap between the canal wall and the **Cal** socket. **C.** rows top to bottom – **raw:** selected areas of Ca, P. **PC 1:** Calcite PC used to choose calcite pixels. **PC 2:** Wall PC used to choose wall pixels. **D.** Calcite and Wall pixels are plotted showing their Ca/P ratios relative to clam calcite (green), HAP (red) and mono-calcium phosphate (blue). The brightness of images **A,** **B** and **C-raw** scales linearly with X-ray intensity, and therefore approximately with concentration which are plotted to produce ratios in **D**. A 50 um calibration bar is seen in panel A.

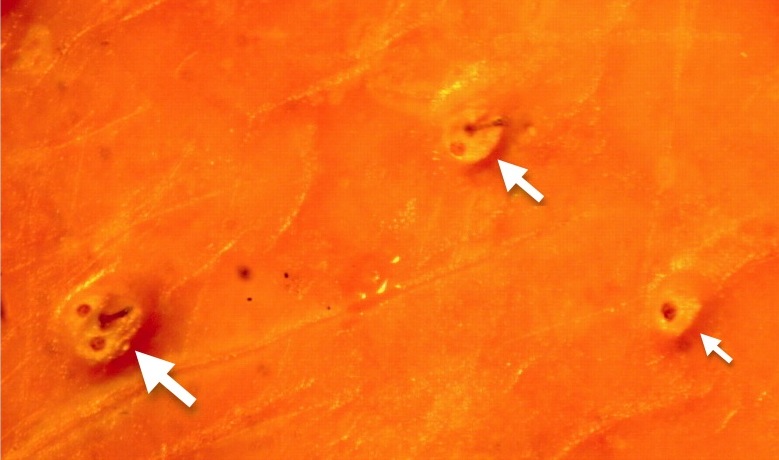


Figure 6. Primary, secondary and tertiary organule cuticle structures on the dorsal carapace of an intermolt lobster. Three size and complexity levels for organules are presented pointed to by increasing sized arrows. The first level is a simple gland tube opening, the second combines a bristle and a gland opening and the third level has more organule components.

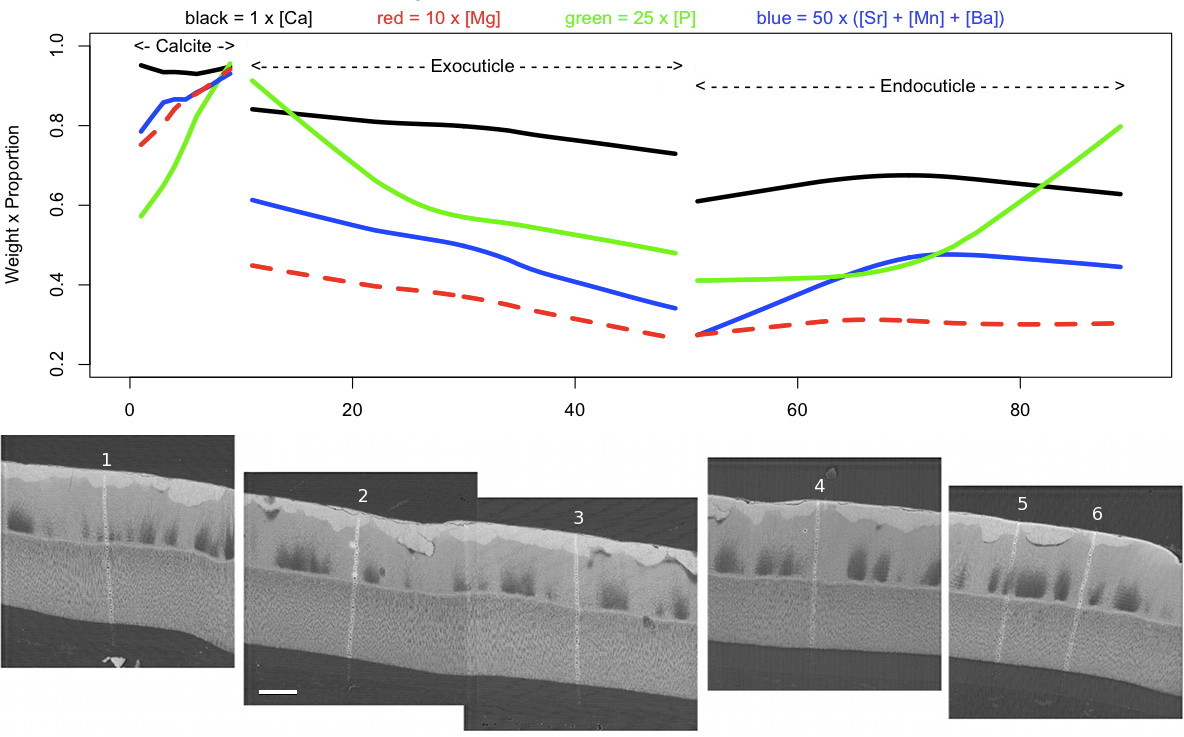


Fig 7. Divalent Cation (Mg, Ca, [Sr+Mn+Ba]) content of the three distinct lobster intermolt cuticle layers: Calcite, Inner-exocuticle, Endocuticle. The average relative molar composition, as measured by EPMA, was plotted as sample-wise points from 100 point transects of the approximately 400 um thick cuticles. The paths of the six labeled transects are shown in five electron backscatter images. A hundred micron calibration bar is presented with the image of scan 2.

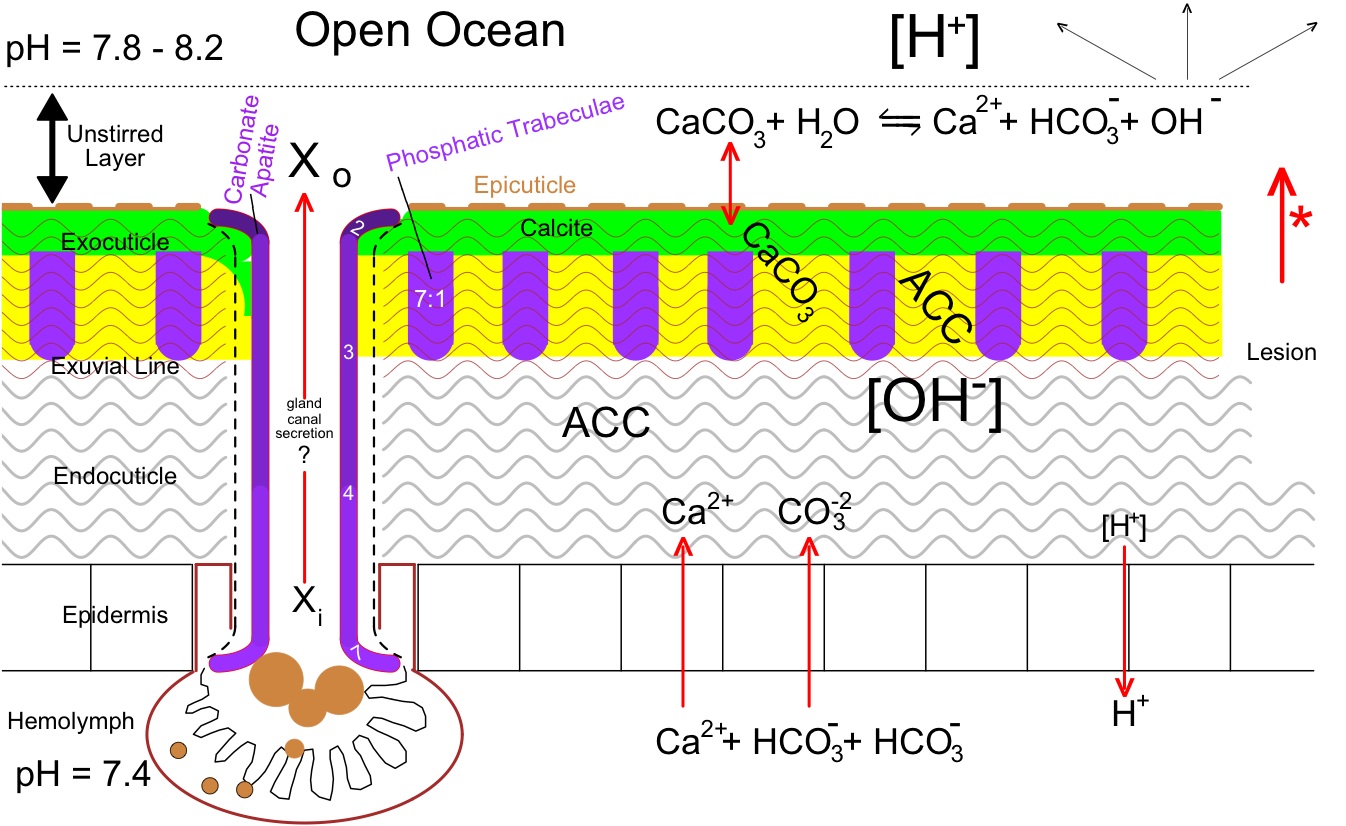


Figure 8. Schematic model of lobster cuticle and basal and apical environment including a dermal gland with graded (2:1 – 7:1 Ca:P) carbonate apatite lined canal secreting a product Xi → Xo. Phosphatic trabeculae are 7:1 Ca:P. During cuticle production Ca2+ and CO3-2 are imported into the cuticle space from the hemolymph side balanced by the expulsion of a proton. At the cuticle outer surface the epicuticle regulates a slow dissolution of the calcite layer that produces a hydroxyl in the unstirred layer when the CO3-2 combines with water. A lesion (asterisk arrow) makes more soluble ACC available for antimicrobial alkalinization.

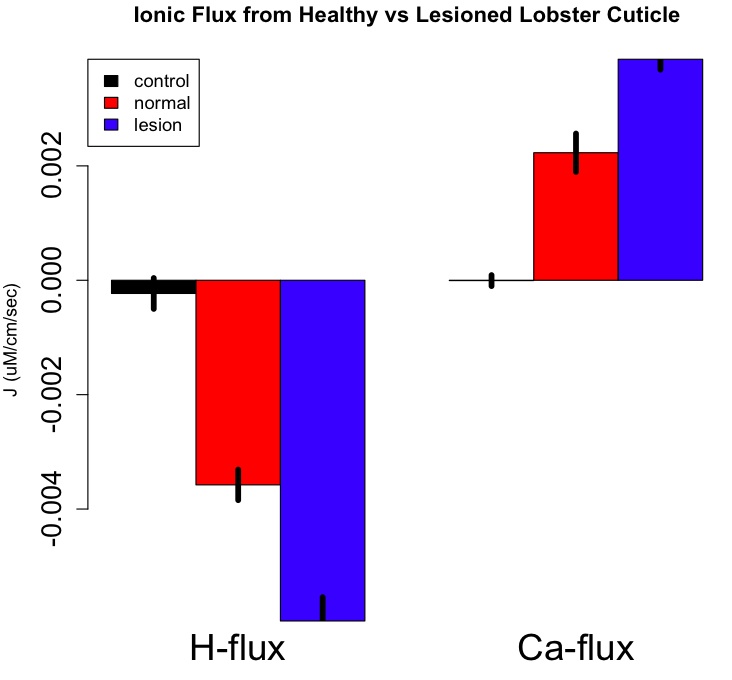


Figure 9. Ca- and H-flux from artificial lesions in lobster cuticle. The Ca and proton flux mirror each other above normal and artificial lesions as predicted for a calcite source by our model, fig 8.

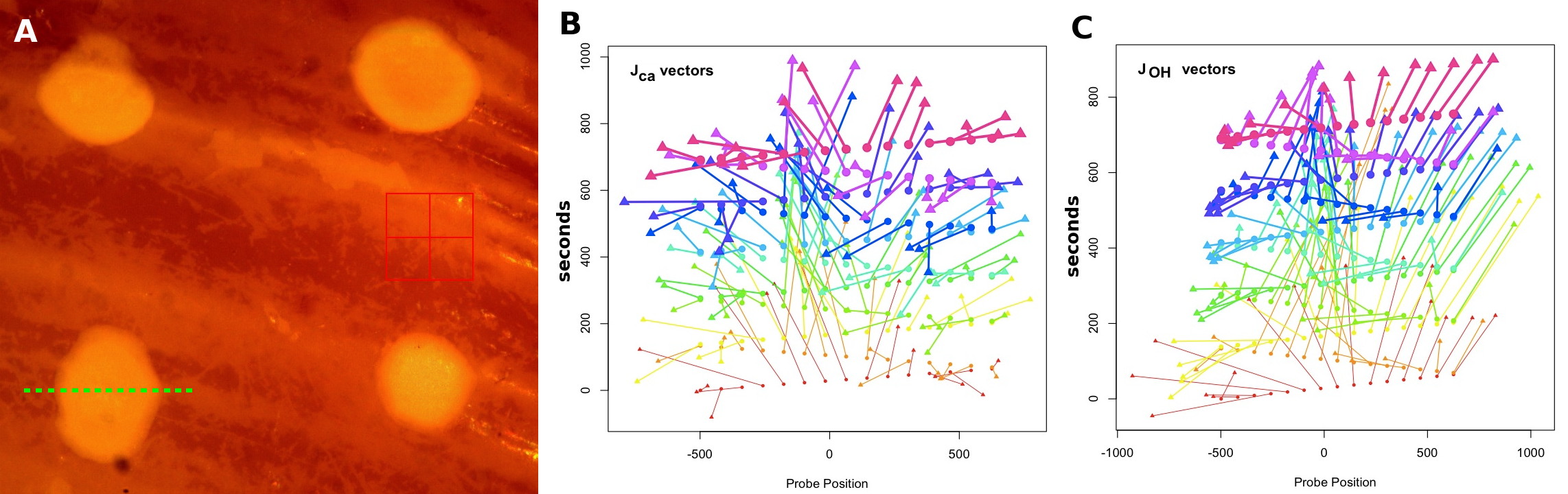


Figure 10. Ca2+ and H+ Flux vectors emanating from an artificial lesion through the periostracal protective layer of a razor clam. **A.** Image of the artificial lesions drilled in the surface of a shell. The dashed green line shows the 1200 um long path of the dual Ca- and H-microelectrodes which were scanned repeatedly across that same path measuring the flux of those tow ions in both the X-direction parallel to the path and the Z-direction perpendicular to the shell surface. These directional fluxes are plotted in panel **B** for the Ca-flux and panel **C** where H-flux is interpreted as the complementary OH-flux. The earliest vectors are depicted thin and become thicker as time progresses. The origin of each vector is a filled circle while the apex of the vector is indicated as a triangle. The earlier fluxes are stronger because they are responding to a larger differential concentration and as the difference is narrowed with dissolution the fluxes moderate. This is an automated governor on dissolution rate associated with lesions.

Table 1. Calcium Phosphate and Carbonate Apatite Formulae and Ca/P Ratios after Wopenka and Pasteris (2005). In the general formula the proportion of phosphates replaced by carbonates plus hydroxyls varies as well as the number of calciums and balancing hydroxyls to produce a balanced formula. In reality chlorides and fluorides may replace hydroxyls to provide bone with modified properties such as with fluoride-based hardening of bone (Mirtchi et al. 1991).

