Modeling the Calcium and Phosphate Mineralization of American Lobster Cuticle

Joseph G. Kunkel

Abstract: Bottom-up modeling of lobster cuticle explains architecture and function *ab initio*, from first principles, starting with synthesis of component polymers and progressively building composite structure which should explain observed properties. A top-down perspective decomposes the lobster cuticle starting at the top level of structural complexity and function aiming to descend to the finest detail. Both approaches aim to ultimately model the same cuticle structure. Current bottom-up models of the cuticle do not succeed in explaining key structural and functional detail identified by top-down approaches. Top-down identified structures and associated functions are valuable as bases for potential vulnerabilities to microbial attack. An immediate objective is to inform the bottom-up approach of top-down identified model components critical to cuticle function. Top-down features include detail of protein expression and mineral heterogeneity and their function in observed structures. This function-directed approach provides a better understanding of the distribution and roles of minerals in relation to their immediate cuticle environment. The top-down identified features can hopefully be included in *ab initio* models to improve our understanding of cuticle design.

Keywords: *Homarus americanus*, model building, biomineralization, ab initio, carbonate apatite, calcite, amorphous calcium carbonate, cuticle protein, microvilli, pore canals, organules

Introduction

American lobster, *Homarus americanus*, cuticle can be viewed from multiple perspectives due to research carried out in the past decades. Several approaches have added contrasting dimensions to our understanding of cuticle structure. Recent morphological views of the cuticle separates lobster cuticle into its traditional zones associated with the molting cycle, epicuticle, exocuticle, endocuticle (Smolowitz et al. 2005) with their respective mineral content, fig 1 (Kunkel et al. 2012; Kunkel and Jercinovic 2013). Calcium carbonate is the dominant calcium compound associated in all crustacean cuticles (Luquet 2011). And phosphatic minerals have been largely ignored except in special situations (Kunkel et al. 2012). Gene expression associated with the moulting cycle is enriching our understanding of how proteins may be involved in cuticle structure and development in crustaceans (Kuballa and Elizur 2008; Kuballa et al. 2011). Homologies and analogies with the broader arthropod literature provide a rich source of gene and protein data associated with cuticle structure (Willis 2010).

Synthetic approaches have developed the biochemical and biophysical principles underlying the traditionally understood cuticle construction from protein and chitin. One leading "bottom-up" approach is based on a background of existing biochemical and ultrastructural features of arthropods in general with the addition of bioengineering stress tests (Nikolov et al. 2011). This approach constructs an *ab initio* model of the developing laminar structure of the cuticle, including biochemical, biophysical and ultrastructural knowledge of polymer components, arriving at a relatively homogeneous view of the general cuticle that assumes a uniform matrix of amorphous calcium carbonate (ACC). While a complete bottom-up perspective is a desirable goal, the current achievements in that direction provide only a general appreciation of arthropod cuticle structure and are far from useful in understanding the real world

Submitted June 1, 2013

Joseph G. Kunkel
Center for Land-Sea Interaction
Marine Science Center
11 Hills North Road
University of New England,
Biddeford Maine 04005
joe@bio.umass.edu

vulnerabilities of American lobster cuticle (Tlusty et al. 2007). A contrasting "top-down" approach has identified diverse mineral components in the cuticle structure whose properties suggest important chemical as well as physical roles that minerals may play in the lobster cuticle (Kunkel and Jercinovic 2013). This approach includes analysis of the mineralogy of uniformly spaced sensory and secretory organules that contrasts with the mineralogy of an intervening pavement-type cuticle between the organules. This pavement cuticle itself is demonstrated to have diverse mineralogy in its different layers. The prior dominant role of calcium carbonate in American lobster cuticle is now known to be complemented by different forms of calcium carbonate plus focal uses of carbonate apatite, CAp (Kunkel et al. 2012). The top-down approach to clawed lobster cuticle depends on CAp to contribute to rigidity and greater microbial resistance to the overall structure. The resultant conceptual model, fig 1, exhibits predictable resistances to microbial attack.

Structural integrity is achieved in the face of limiting resources of carbonate and phosphate in an environment increasingly hostile to $CaCO_3$ structures. The major feature of this top down model is an adaptive heterogeneity of the minerals used to achieve the observed 3-dimensional cuticle structure. This contrasts with the explanation of amorphous calcium carbonate as the sole mineral of importance to the bottom-up model of lobster cuticle (Nikolov et al. 2011). However a recent *ab initio* result has shown that Mg substitutions of Ca in calcite stiffens the resultant calcite (Elstnerová et al. 2010). This of particular interest because of the wide presence of calcite in crustacean and mollusk shells. The *ab initio* approach can be powerful when the conclusions are integrated to a meaningful level and when direct experimental observations of the properties are difficult to achieve directly on the natural source. Here we show that the strained fit of the current *ab initio* models of cuticle (Nikolov et al. 2010, 2011) need to consider some major aspects of our accumulated knowledge of proteins and minerals of arthropod cuticle.

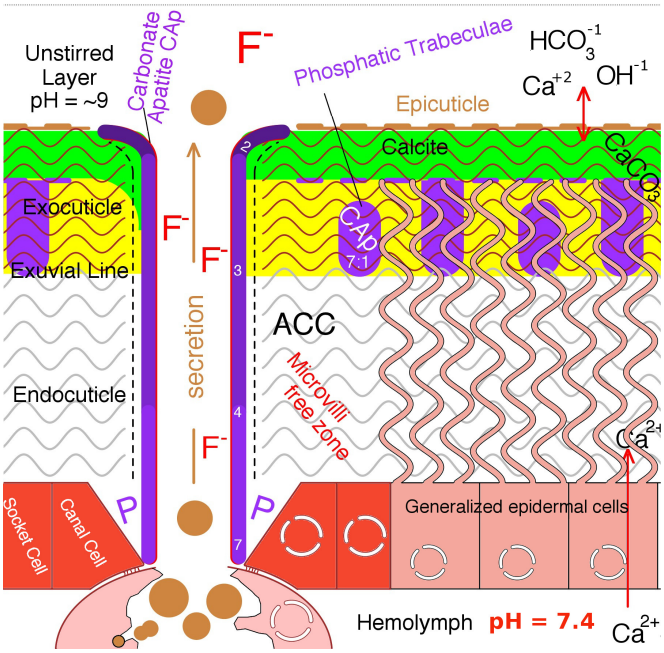

Figure 1. Model of American lobster cuticle and surrounding environment. The epidermal layer illustrates generalized epidermal cells and specialized cells of a simple organule composed of a Canal Cell, Socket Cell and Gland Cell with their overlying cuticles. The generalized epidermal cells produce a pavement type cuticle which spans between the organules. Calcite (green layer) and and amorphous calcium carbonate (ACC), are forms of calcium carbonate. Phosphatic mineralization is indicated in colored fields of purple. Trabeculae of CAp (purple lozenge shapes) are interconnected to provide rigidity. In the canal wall Ca:P ratios are indicated going from 2 to 7 out to in the CAp (CAp) trabeculae marked with Ca:P ratio of 7:1. A dashed purple line beneath the calcite layer indicates phosphate deposits. Horizontal wavy lines indicate the closer layering of the chiitin protein in the exocuticle than the endocuticle. Solubilization of the calcite through the epicuticle produces Ca^{2+}, HCO_3^- and OH^- plus a high pH unstirred layer providing an antimicrobial effect at the cuticle surface. Fluoride F^- diffuses in from the environment to create fluoridated CAp.

Observations on Cuticle Proteins.

To start I must reiterate the advice of thoughtful arthropod researcher Judy Willis (1999) who encouraged the expansion of the pioneer work of Dorothy Skinner on crustacean cuticle proteins as well as her advice to crustacean workers to pay attention to accumulating examples from the insect literature. We must understand the properties and function of the proteins in the different cuticle layers and structures. Another pioneer in insect protein research has recently proposed a curious property of cuticle proteins in general which may create problems in homologizing the function of proteins ... intrinsically disordered regions are seen in a large fraction of published cuticle protein structures (Andersen 2011).

Identification of crustacean cuticle proteins has proceeded from the 1997-2004 technical phase (Kragh et al. 1997; Andersen 1999; Willis 1999) to the modern molecular era in which many cuticle proteins are listed in online protein archives and databases (Magkrioti et al. 2004; Willis 2010) and their amino acid sequences searchable for common structural motifs (Punta et al. 2012; Kuballa and Elizur 2008; Kuballa et al. 2011). Through these resources numerous lobster proteins have been homologized with proteins identified in other arthropods through common motifs, which include cuticle-specific motifs, chitin-binding motifs, hemocyanin domains and small-

organic binding motifs (Table I).

The hemocyanin, HbCy, family of proteins are particularly important proteins in crustaceans and their cuticle (Terwilliger et al. 1999). This family of proteins in crustaceans is part of the larger hexamerin family which is found in all arthropods (Telfer and Kunkel 1991). The hexamerins have ancient roots that include origins from the copper binding active site of phenol-oxidases which evolved into the oxygen binding site of hemocyanins, which further evolved into serum storage proteins and cuticle precursors (Telfer and Kunkel 1991; Willis 1999). Several hexamerins remain as important components in lobster cuticle structure and function. All hexamerin type proteins are basically large with ca. 650 to 690 amino acid residues per subunit (Table I). They all have three domains identified as N, M and C, which sequentially span the ~670 aa. The M domain is the historical site of the copper binding site. If any hexamerin were fragmented, e.g. in extraction from a cuticle, its parts could be identified by partial homology as seen with the specific HbCy-A fragment which has only two of the 3 HbCy domains. Besides being large and having identifiable domains, the hexamerins have a general property of assembling into hexamers of 670 aa monomers. This creates high molecular weight particles with very large Stokes radii which allow the proteins to remain in the arthropod serum without being filtered out of the hemolymph by clearance processes (Duhamel and Kunkel 1987) a property also observed in decapods (Terwilliger et al. 2005). This specific tendency of hexamerin type subunits to associate into higher order structures has not been specifically referenced and quantified in the bottom-up modeling of cuticle structure. A hexamerin of lobster, cryptocyanin, is a major hemolymph protein and eventually becomes located in the cuticle as demonstrated for other decapods (Terwilliger et al. 2005; Kuballa et al. 2007, 2011). The deposition and modification of cryptocyanins, apparently by phenolic crosslinking, in the crab *M. magister* has been demonstrated (Terwilliger et al. 2005).

The titer of cryptocyanin (Terwilliger et al. 2005), as with insect arylphorins (Telfer and Kunkel 1991, Duhamel and Kunkel 1983; Karpells et al. 1990), increases in the hemolymph during the premolt phase of the instar and drops during the molt phase as the protein is transported across the epidermis and deposited in the cuticle becoming a major part of exocuticle protein. HbCy on the other hand maintains a relatively even titer throughout molting. Cryptocyanin is immunologically detectable in the endocuticle but apparently does not exhibit the extreme crosslinking seen in the exocuticle after ecdysis that seems to obscure immunological identification (Terwilliger et al. 2005). It, like the arylphorins of insects, is likely a major protein to participate in cross-linking to create the initial stabilized exoskeletal shape of the newly molted crustacean sclerites, this despite cryptocyanin having only a modest increase in phenolic residues in its structure, Table I. Expert opinion suggests that the mechanism of cross-linking in crustaceans is likely to be different from that in insects (Andersen 2010).

Three astaxanthin binding proteins have been identified as cuticle proteins, alpha-crustacyanin and beta-crustacyanin from exocuticle and crustochrin from the epicuticle layer. The crustacyanins contain the Lipocalin motif of binding proteins which is consistent with their being a carrier of astaxanthins. The protein quarternary structure of alpha-crustacyanin has been determined and the basis of its blue color explained (Dellisanti et al. 2003). Crustacyanins plus astaxanthin complexes provide the cuticle colors of the decapod cuticles (Tlusty and Hyland 2005; Wade et al. 2009). They also contain a higher level of phenolic amino acids (Table I) which may play a role in their acting as chromophores in the decapod cuticle.

The expression of many cuticle proteins during the different phases of crustacean molting cycle have been examined (Terwilliger 2012; Seear et al. 2012). It is not clear which proteins in the lobster cuticle participate in the crosslinking typical of the quinone-based tanning seen in insects. However, it is clear that when crustacean cuticle is injured that pro-phenoloxidases are activated and lead to a melanization of the surrounding cuticle (Cerenius and Söderhäll 2004) but in lobsters only when the calcite layer has been penetrated and protein rich inner exocuticle abraded (Kunkel et al. 2012). Curiously the crustacyanins are the cuticle proteins that may be the best candidates for being crosslinked by injury-induced quinones based on their own phenolic content.

The arylphorin category of hexamerins of insects have higher tyrosine and phenylalanine content that distinguish them from more typical hexamerins and cuticle proteins tabulated from the protein literature (Table I) (Telfer and Kunkel 1991). They are the dominant serum proteins transported to the cuticle during molting in insects and become

phenolically crosslinked . The cryptocyanins may be the major cuticle protein in decapods but due to their modest phenolic content, they may be crosslinked by a different mechanism during ecdysial hardening (Andersen 2010). Other candidates for cuticle proteins include all the hexamerin family proteins including phenoloxidases, hemocyanins, and pseudohemacyanins which all have modestly high phenolic residues. It is of some interest that hemocyanins in a variety of crustaceans and arachnids have been shown to exhibit phenoloxidase activity when treated with detergent or protease (Decker et al. 2001). It is possible that they might develop a phenoloxidase function during transport across the epidermis into the cuticle space or by subsequent denaturation as part of an injury to the cuticle.

Chitin-protein interaction motifs have been identified in various lobster proteins. Proteins with a Rebers-Riddiford (R&R) domain in their sequence, a hallmark of their chitin-binding ability, have been identified in the American lobster particularly in the antimicrobial proteins (AMPs). AMP1A, AMP1B, AMP2, AMP3, AMP4 and AMP5 each contain one chitin-binding type R&R domain (Rebers and Willis 2001). While these AMPs are considered cuticle proteins they are products of haemocyte cells and as such their chitin-binding might be a part of the arthropod immunological defense.

All cuticle proteins will not necessarily be associated with hard or mineralized cuticle and proteins associated with flexible cuticle have been identified including resilin, one of the first cuticle proteins to be described (Andersen 1963). Now resilin-like proteins are being found across the entire animal and plant world. A class of flexible cuticle proteins besides resilin has been identified and typified as cecropia protein HC CFP12 (Table I), which has a chitin binding domain and a moderate increase in phenolics.

Another series of cuticle proteins contain motifs in the Pfam data base with simple designation 'cuticle protein 4' (Table 1). This group of cuticle proteins is partly distinguished by having no chitin binding domain; they are localized to the epicuticle or outer exocuticle of various insects (Papandreou 2010). The epicuticle is said to consist of protein devoid of chitin thus it would be logical to identify lobster proteins devoid of a chitin binding motif to populate the epicuticle.

To date, interest in lobster cuticle as a composite material has concentrated on the general cuticle (Romano et al. 2007) and not on the more specialized features produced by organules (Lawrence 1966; Merrit 2007), which are the special structures such as dermal gland canals and sensory bristles. Interest in the underlying principles of generalized crustacean cuticle continues a long line of theoretical interest in arthropod cuticle. The theoretical models based on understanding of molecular properties of cuticle components, start with the twisted plywood structure that we now recognize as a basic part of arthropod cuticle design (Bouligand 1986). Eventually an *ab initio* approach combined accumulated knowledge into four (Neugebauer et al. 2010) or seven levels (Raabe et al. 2005) of hierarchical structure to provide a theoretical model of the general pavement type of cuticle of the lobster. This pavement type of cuticle is produced by generalized epidermal cells, fig 1. The origin of this model is linked to an interest in crustacean cuticle as a time-tested composite material that might provide insight into design of novel composite materials (Raabe et al. 2005; Romano et al. 2007). This focus on the pavement cuticle ignores the complications of intervening dermal glands and sensory hairs; but these structures are so abundant in the clawed lobsters that they must be considered in future models of a functional cuticle. A more realistic model will integrate all the decidedly important structures and proteins which contribute to the stability and vulnerabilities of the cuticle in its roles as an exo-skeleton. The problem is to identify those important structural components.

The bottom-up approach is a necessary process in integrating current aspects of the composite nature of the lobster's pavement cuticle into a model. Some top-down identified assemblages of proteins and minerals that are judged to have favorable properties or motifs should be incorporated into future cuticle models. They will contribute substantially to understanding properties of the native cuticle. The top-down identified features include proteins, lipids and minerals which may add to our understanding of the American lobster cuticle as a defense against environmental insults.

Mineralogy of the Lobster Cuticle

A Schematic Model

The micro-distribution of the basic minerals of the lobster cuticle has been recently described, fig 1 (Kunkel et al. 2012; Kunkel and Jercinovic 2013).

Briefly, the cuticle of lobster sclerites is made up of broad planes of a relatively smooth pavement cuticle, produced by underlying generalized epidermal cells, separating evenly spaced organules, whose cuticle is produced by two specialized epidermal cell types, the canal cell and the socket cell, fig 1. Epidermal cell microvilli play roles in the creation of the cuticle layers (Locke 2001; Schwarz and Moussian 2007). In some models they play that role mainly at the epidermal cell cuticle interface, but there are also microvillar extensions deep into the formed cuticle post molt (Modla 2006). The pore canals of lobster cuticle persist in both the exo- and endocuticle, fig 2. The extent to which the pore canals in lobsters retain microvillar extensions throughout the intermolt phase is not known. In the lobster the socket and canal cells are overlain by a microvillar-free zone and seem to be devoted primarily to the production of an integrated organule cuticle that serves either a cuticular gland canal or a sensory neurite canal, each of which have a luminal cuticle surface that is distinctly different from the pavement cuticle surface. This organule cuticle may be structured more like the general epicuticle, being a surface sculpturing and depend on the chitinised exo- and endocuticle for general support.

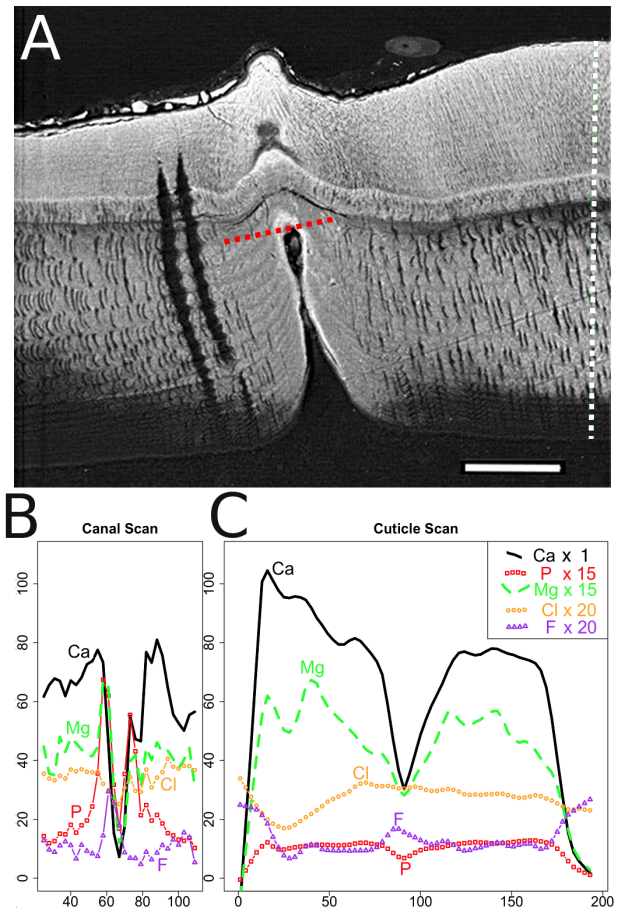

Figure 2. Mineral composition of American lobster canal wall *vs* pavement cuticle. **A.** Electron backscatter image of ventro-lateral carapace cuticle cross-section from a 42 hr post molt *Homarus americanus* adult. The black dotted line shows the transect through a neurite canal structure while the white dotted line show the transect through the general pavement cuticle. Pore canals are seen sinuously penetrating into the cuticle from the lower epidermal surface of the cuticle up into the exocuticle layer as vacancies of electron density. Pore canals are missing in the cuticle directly surrounding the neurite canal where the twisted plywood pattern of cuticle deposition is more obvious, suggesting that the canal cell and perhaps the socket cell do not maintain microvilli that continue to penetrate the cuticle in general. Scale bar = 50 µm.
B. Relative abundance of atoms of Ca, P, Mg, Cl and F in a transect through an organule neurite canal in A. A multiplier is applied to the molar content listed on the Y-axis that as shown in the legend is 1x for Ca, 15X for P, ... and 20X for F. Mg and F are elevated in the CaP rich wall of the neurite canal. Mg is calculated to replace Ca 7% of the time while F^- may cooperate with CO_3^{-2} in replacing PO_4^{-3} in 2% of the CAp and mainly at the lumenal surface of the CAp canal wall. Significantly Cl^- does not follow the F^- contour in the wall structure.
C. Pavement cuticle transect from A shows similar relative abundance of atoms in the transect from surface to epi-dermal side. The Mg conforms more regularly with the Ca content and F with the Cl content. A mineral poor cleft at the exocuticle endocuticle boundary is significantly enriched in fluoride but not chloride showing a level of differ-entiation of minerals in the general cuticle other than Ca and P. P is seen to be relatively low throughout this lateral transect of pavement cuticle which is missing phosphatic trabeculae and more pliable than more dorsad cuticle.

Canal Apatite Formulae

The canal wall is composed of various discrete formulations of CAp, which in vertebrates is called bone. In vertebrates bone is generated in association with a template, collagen, in an extracellular process but research is still active on the actual process of the role of the type 1 collagen in the inorganic chemicals coming together to create CAp (Landis and Jacquet 2013). In vertebrates fluoride affects both bone synthesis and bone density (Li 2003). It is not known if fluoride has any such effect on CAp in the lobster

cuticle. In lobsters the CAp takes on ratios of Ca:P from the pure 1.76 of mineral apatite (i.e. no apparent carbonate replacing phosphate) in the outermost segment of the canal wall but uses increasing but discrete ratios up to a Ca:P ratio of 7 at the innermost segment of the canal, fig 1. A major question is how is this regulated? Is there a specific protein that nucleates CAp formation produced by the organule canal cell? Do the organule cells create the cuticle environment in which CAp spontaneously crystalizes given the inorganic precursors?

From chemistry we know that when a phosphate's -3 charge is replaced with a carbonate ion's -2 charge an additional monovalent anion needs to be added to allow for balance of the charges. In bone that monovalent replacement anion can be a hydroxyl, a chloride or fluoride (Kunkel et al. 2012). Fluoride provides an increased hardness to the resultant CAp . In the lobster canals we also see the addition of fluoride particularly at the luminal surface of the CAp canal wall, fig 2, and illustrated in fig 1 as a thin red layer. As indicated in fig 1, it is likely that the fluoride is added to the canal wall surface from an environmental source in the ocean seawater after molting when the new cuticle comes into direct contact with the seawater for the first time. Magnesium is also associated with the inner surface of the canal wall which indicates it also may be a post-molt modification consistent with the high molar content (50 mM) of Mg in seawater. The phenomenon of Mg substitution of Ca in apatite has been observed analytically and experimentally by biologists and material scientists (de Silva nun *et al.* 2010; Laurencin *et al.* 2011; Aina et al. 2012 ;Shepherd *et al*. 2012). It also occurs in calcite (Becker et al. 2005; Elstnerová et al. 2010). Our understanding of the role of doping with other divalent cations, or substantial replacement of formula

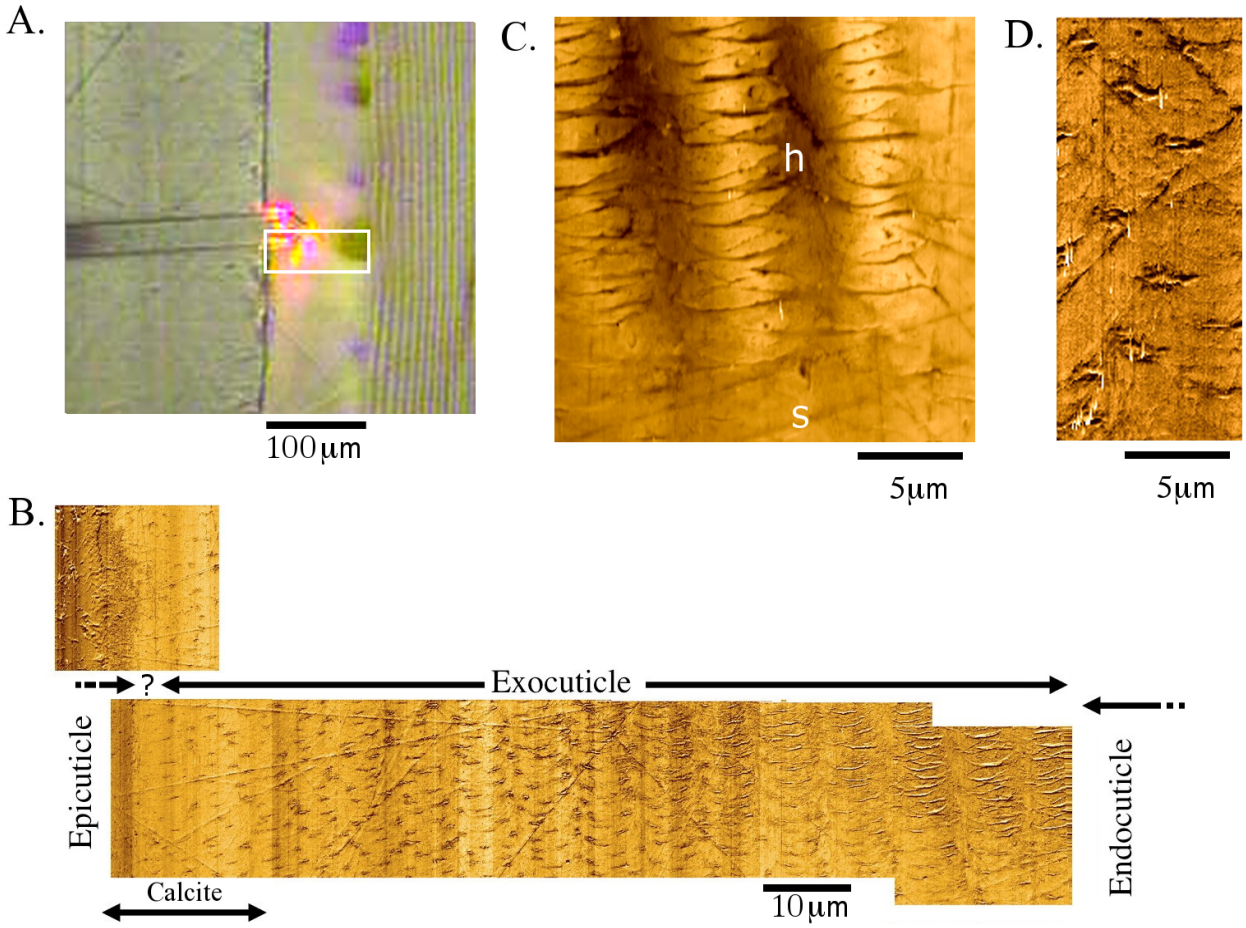

Figure 3. Atomic Force Microscope (AFM) view of American lobster exocuticle. A. View of the AFM probe tip with the scan area of B delimited in white outline. **B.** Montage of texture scans of the exocuticle showing stark pore canal tracks in the inner exocuticle but only slight scars in the calcite layer. The montage of overlapping scans was alligned using fortuitous scoring of the sample during its polishing. **C.** Higher resolution textural scan of three cuticle laminae in a trabecular transect which illustrates the hard, **h**, vs. soft, **s,** texture contrast between the layer levels due to trabecular structure. **D.** Higher resolution AFM structural scan of pore canals which appear as semilunar scars..

components by alternate ions such as fluoride, will clearly benefit by continued research involving observation in nature, experimentation by materials scientists plus understanding by *ab initio* construction of the structures.

Procuticle

The procuticle is cuticle that is produced under the existing cuticle prior to ecdysis. It starts with the secretion of a trilaminar membrane at a microvillar surface of the epidermal cells which have detached from the old cuticle (Locke 2001). This procuticle represents the surface of the exoskeleton for the next stage with all its sculpturing and is designed so that at ecdysis it expands to attain the form of the next stage. The procuticle develops thickness of its outermost layer, the epicuticle, from transported proteins which do not have a chitin binding motif (Willis 2010, 2012) which is logical since the epicuticle is devoid of chitin (Schawarz and Moussian 2007). The epicuticle is suffused with wax and overlain with a so-called cement layer (Smolowitz et al. 2005). The epicuticle protein is crosslinked (after expanding in ecdysis) and would presumably include the pigmented protein Crustacyanin C with its astaxanthin pigment (Tlusty and Hyland 2005). This layer would be the earliest to be constructed and its protein(s) the earliest to be transported across the epidermis.

Below the epicuticle in the procuticle, the exocuticle would be secreted with its twisted plywood construction that includes layers of oriented chitin and protein. At least some of the protein would have chitin binding domains as opposed to the protein laid down in the epicuticle. Other cuticle proteins would consist of cryptocyanin types which have self assembly capability. This is the layering that is partially modeled by the bottom up approach (Nikolov *et al.* 2011). At ecdysis the old cuticle is shed and the procuticle is expanded by hydraulic inflation fueled by the swallowing of water into the lobster stomach. At the point of maximal inflation the cuticle is stabilized by some form of crosslinking of the proteins (Andersen 2010). The inflation causes stretching in the plane of the cuticle and resultant compression of the proecdysial layering; and this allows one to distinguish layers produced before *vs.* after ecdysis.

The outer layer of the pavement exocuticle develops dense crystalline calcite in early days after molting when it shows characteristic birefringence (Kunkel et al. 2012) and shows traces of collapsed pore canals which have ablated the epidermal microvilli, presumably mechanically, during the calcite crystal growth. This exclusion of the microvilli (and/or their pore canal paths) from the calcite layer has been observed using an atomic force microscope (AFM) which can measure the texture of surfaces. In fig 3 we see an AFM scan of the lobster exocuticle in which a favorable polishing of an intermolt specimen shows tangental intersections with helical pore canals, which are substantially suppressed in the calcite region. The birefringence of the calcite layer follows contours of the cuticle sculpturing that suggests the crystals coalesce about an underlying polymeric structure whose axis itself follows the sculpturing (Kunkel et al. 2012, fig 3). There are undoubtably unknown unidentified proteins that are involved in the guidance of the calcite coalescence. However, the finding of immuno-identifiable tubulin and actin in the exoskeleton is more likely a demonstration of the presence of microvilli or their remnant components in the cuticle rather than the suggested participation in the cuticle architecture itself (Mykles et al. 2000).

The function of this calcite layer has been suggested to be antimicrobial in two ways (Kunkel and Jercinovic 2013): The density of the calcite layer is close to the density of mollusk shell which allows for very little organic polymer within the layer and meager access to resources for the incursion of microbes attempting to access organic substrate. In addition, the calcite, as demonstrated, dissolves slowly across the epicuticle, which may thus be thought of as a governor of shell dissolution. This ionic flux emanating from calcite or amorphous calcium carbonate dissolving through the epicuticle can be recognized by a measureable outward calcium flux that is mirrored by an apparently opposite proton flux, which is generated by the reaction of the dissolved carbonate with water releasing a hydroxyl (recorded by a proton electrode) (Kunkel et al. 2012). The production of hydroxyls by the cuticle's $CaCO_3$ dissolution creates a high pH in the unstirred layer close to the cuticle surface. This high pH is intrinsically inhibitory to the metabolism and motility of bacteria and represents an hypothesized major function of the $CaCO_3$ found in the shells of crustaceans and mollusks. Even if a bacterium attaches to the epicuticle it will have a diminished capability of movement and metabolism because those functions depend upon a proton-motive force which is kept low at the elevated pH in the unstirred layer. The calcite layer is less soluble than the underlying

amorphous calcium carbonate for two reasons. First it is overlain by the epicuticle which moderates its solubility and second because its dissolution also requires additional investment to counter the energy of crystalization of calcite (Radha et al. 2010). If the epicuticle and the calcite layer are both broached by a lesion, the underlying ACC dissolves more readily. At the moment, this added role of the ACC is conjecture that needs rigorous testing.

Another property of the calcite layer is the potential role that magnesium plays in replacing the abundant calcium of that layer. Magnesium could play several functional roles in the calcite layer. First, the solubility of magnesium-calcite is higher than that of normal calcite. In the intermolt cuticle the proportion of Mg measurable as the Mg:Ca ratio declines in the calcite layer from a peak at the inside edge, where Mg can be 10% of the Ca abundance, to 5-6% of the Ca abundance at the calcite layer outer surface (Kunkel et al 2012). This state could be achieved by $MgCO_3$ dissolving or being replaced faster from the calcite outer face. A $MgCO_3$ dissolution-component of calcite could function to allow a greater increase in the cuticle's outer unstirred layer pH during the early phase after ecdysis, but before the rigidity of the cuticle is established via development of the trabeculae. The physical reality of this suggested role needs testing by measuring the Mg gradient in cuticle at different stages after ecdysis.

A second effect of Mg was demonstrated by *ab initio* simulation of the structure of $Mg/CaCO_3$ crystals, predicting Mg-substituted calcite to have a measurably higher stiffness than normal calcite (Elstnerová et al. 2010).

Inner-Exocuticle trabecular layer

Underneath the calcite layer lies the inner exocuticle, which is the least homogeneous layer of the American lobster cuticle (fig 1). It clearly does not conform to the relatively uniform twisted plywood model of hierarchical structure suggested by the Dusseldorf models (Romano et al. 2007; Nikolov et al. 2011). With light microscopy of unstained cross-sections the exocuticle shows profiles of amber sclerotised regions which correspond to the phosphatic trabeculae that are seen in the electron microprobe (Kunkel et al. 2005; Kunkel et al. 2012) and with a higher textural density in AFM (fig 3A,C). This trabecular architecture has a most important role in the top-down model of the lobster cuticle. The trabeculae exhibit a low-phosphorous CAp formula with Ca:P ratio of ~7 (Kunkel and Jercinovic 2013). This trabecular structure is proposed to gain its strength from the 3-dimensional connectivity of the CAp struts which may provide a mechanical advantage over a uniform deployment of the same material over the entire cuticle volume. That strength may also be integrated with crosslinked proteins which seem to correspond visually with the trabecular phosphatic content and AFM textural difference in the cuticle. This trabecular organization is similar to a strategy used to produce the light-weight bones of birds. In agreement with this proposal, the physical indentation tests published by the Dusseldorf group (Raabe et al. 2005) indicate that the trabecular layer of the cuticle provides the majority of the rigidity of the American lobster cuticle. The outer calcite layer would be expected to be relatively soft unless it were combined with polymers (as with nacre in mollusks). Indentation tests suggest that the calcite layer, which is not identified as calcite by Raabe and coworkers (2005), is a relatively soft cuticle layer. The mineral composition of the exocuticle between the trabeculae is markedly similar to the mineral composition of the endocuticle (Kunkel et al. 2012). This exocuticle trabecular area is different from the endocuticle in two ways. It is invested with CAp and it is the region with the most obvious indication of cross-linking. The trabeculae are therefore proposed to be the origin of the hardening and rigidity which develops after molting (Waddy et al. 1995). The origin of the trabecular *vs* inter-trabecular areas is open to speculation but could very likely be based on the patterned deposition of the cuticle proteins cryptocyanin or crustacyanins, which are deposited in the exocuticle (Terwilliger et al. 1999).

Exocuticle microvilli and pore canals

The lobster general pavement cuticle is penetrated by microvilli emanating from the epidermal pavement cells which run through what are called pore canals, fig 1. The microvilli, while they persist, are capable of communicating material back and forth along their actin filaments powered by myosin motors. The microvilli and pore canals are part of the phenomena that keeps the majority of the cuticle within an active metabolic pool for arthropods (Locke 2001). Analysis of the hypodermal microvillar membranes of the blue crab (*Callinectes sapidus*) has shown the membranes rich in n-6 phospholipids, which form a tighter membrane for Ca transport, and varies during the molt cycle to maintain high Ca^{2+} in the cuticle matrix during its deposition (Williams et al. 2004).

Preparations of such membranes may be valuable resources for investigating the way that the microtubules regulate cuticle synthesis. The microvilli being extensions of the epidermal cells may transport and release small molecules directly and perhaps in a more focused way, e.g. phenols for use by endogenous cuticular prophenoloxidases that may be activated by a lesion into the exocuticle. Glycolytic intermediates have been shown to regulate ACC crystallization (Sato 2011) and could serve as extracellular signals of organismal health affecting the animals cuticle maintenance (Weiss 2011). Small molecule release and extracellular sensory-domains of enzymes on the microvilli are two ways that microvillar membranes can sense the condition of and modulate cuticle mineralization (Weiss et al. 2013). In addition the ACC in the exocuticle and endocuticle are poised to be used if needed in a metastable state that can be mobilized or transformed to another state such as calcite (Foran et al. 2013).

In the American lobster the epicuticle or outer exocuticle becomes invested with highly oriented calcite that retains very little measurable density ascribable to anything but calcite. It is of interest that a modest level of cuticle polymer persists. Are there discretely different proteins in this layer? Given that the calcite layer is almost devoid of organic material it appears that as the calcite crystals grow they displace any organic material. Is there epicuticle material above and below the calcite layer or is the calcite region delimited by the epicuticle? Something covers the calcite layer (perhaps phenolically linked epicuticle protein?), because when the cuticle surface is breached by an artificial lesion, the calcite flux increases dramatically (Kunkel et al. 2012, fig 8). The pore canals, as possible paths of microvilli, terminate just below the calcite layer which may have mechanically excluded them during the calcite deposition phase early after ecdysis as described above. At the interface of calcite with the inner-exocuticle there are often plaques of calcium phosphate which may be involved in the capping of further crystallization of calcite at its inner surface. Regulating the calcite layer growth may be a microvillar function, the microvilli allowing the cuticle to remain 'alive' in active communication with both the epidermal cells and cuticle. The role of the microvilli in modifying the cuticle after deposition must be investigated in light of the mineralogy and chemistry which is now being uncovered. Is there continued maintenance of the calcite layer from the inner-exocuticle using ACC as an intermediate? Are their adjustments to the Ca/Mg CO_3 to a lower Mg substitution formulation of the calcite layer directed from the outside environment? Is their a turnover and maintenance of the CAp of the canals and the trabeculae during the extended intermolt phase of the molting cycle as is suggested by the fluoride enriched wall surface of the canals seen in fig 2? All these questions are as yet unanswered. They could have significant effects on a model of cuticle from a functional point of view. Could an appropriate *ab initio* model predict properties that would answer these questions? Some of these questions might be answered piecemeal by *ab initio* modeling, as was done by predictions on Mg-substituted calcite (Elstnerová et al. 2010).

Deficiencies of the current bottom-up model

The bottom-up models of the American lobster cuticle represent laudable efforts in advancing our ability to predict the physical properties of cuticle given the contributions of polymer and mineral composition that are included in these models (Romano et al. 2007; Nikolov et al. 2010, 2011). Indeed, a*b initio* constructions may be the only approach feasible when the natural structures can not be readily prepared or are complicated by other factors. Calculations for such models require an engineering understanding of the physical and chemical properties of the cuticle constituents. The Dusseldorf led research group has made efforts to explain the tensile properties of cuticle, relating it to a hypothetical content, observed ultrastructural organization and physical measurements. Their models do not, to this point, include mineral contributions beyond spherical ACC as a 70% matrix for the cuticle polymers which conflicts with demonstrations that the calcite layer may exhibit 85% of its density as Ca/Mg calcite (Kunkel et al. 2012). Crustacean cuticles have also been shown to include a variety of divalent cations (Becker et al. 2005; Hild et al. 2008, 2009; Neues et al. 2011). The layer that is suggested to provide the maximum stiffness to the cuticle based on hardness tests is the inner exocuticle (Raabe et al. 2005). That layer also contains phosphatic trabeculae that, in our view, provide stiffness to the cuticle through the heterogeneity of their mineral content (Kunkel et al. 2012). There is clearly a challenge in introducing new levels of organization into an *ab initio* model. The current bottom-up model predicts stiffness, as indicated by Young's modulus, using ACC mineral

density levels (Nikolov et al. 2011) which may not be realistic; and indeed the molecules are required to behave optimally for the cuticle's observed Young's modulus to lie within the bounds of the model's predicted values. The modeling concludes that the properties of the constituent proteins are important and that their interaction with the matrix of ACC is important for the cuticles to achieve the tensile properties indicated by the predicted Young's modulus. As described above there are numerous proteins to be considered with perhaps variable amounts of inherent intermolecular binding among the hexamerins as well as variable amounts of phenolic content to allow for cross-linking. These factors need substantial research to evaluate the physical chemistry of potential forces available in the construction of a bottom-up model, perhaps more heterogeneous, but closer to typical lobster cuticle.

The hierarchical model including only ACC as a matrix presents no particular physical model for mineral interaction with chitin and protein polymers. From fig 3, AFM finds textural differences between the CAp phosphatic trabeculae and intervening cuticle in which ACC predominates. The use of trabecular architecture, which makes efficient structural use of a low phosphate content and availability, is an important structural difference that needs to be included in any calculation of the sufficiency of a Young's modulus to measure overall stiffness.

The existence of several identified cuticle phenomena as discussed above are outside of the bounds of the current bottom-up models: (1) heterogeneity of mineral types, (2) potential for different protein interactions in the inner exocuticle, (3) potential crosslinking agents and substrates, (4) the new structural concept of apatite trabeculae, and (5) microvillar control of the cuticle ionic milieux. It is possible that with the inclusion of these proposed cuticle phenomena, that the native cuticle's structure can reach its observed levels of stiffness using average performance of the polymers involved in the current *ab initio* bottom-up models. Or, adding all those components could also reduce Young's modulus if they only weakly interact or bind with ACC.

Implications from top-down cuticle models

Another model of decapod cuticle has included top-down morphological concepts (Tarsitano et al. 2006). In that model the sculpturing of the cuticle in several lobster families are used to define functions of the macro-tubercles that are suggested to provide a horizontal crack blunting function. The twisted plywood laminae function provide vertical crack blunting. It is possible that the organule pits of the clawed lobsters, the Nephropidae, function in a similar way since the organules are relatively abundant and evenly spaced (Kunkel and Jercinovic 2013). The larger compound organules include sensory bristles plus glands and thus require multiple hardened CAp canals traversing the cuticle thickness.

It is important for the bottom-up modelers of the cuticle to be cognizant of the properties which have been identified by the top-down view. Together these approaches will identify important aspects of the cuticle structure and function. Future bottom-up models will need to incorporate phosphatic trabecular structure in a ACC plus fibrillar matrix in an attempt to approximate the physical properties of American lobster cuticle. It is likely necessary that the CAp canals could be modeled separately from the CAp trabeculae and calcite layer before being joined in a more inclusive model.

Acknowledgements

This research was funded in part by grants from Rhode Island Seagrant and MIT Seagrant. Funding for a 2009-2010 sabbatical at JKU in Linz Austria was provided by a grant to Sabine Hild from JKU and afforded valuable interaction with several investigators of Crustacean cuticle who participate in the European Crustacean Group. The thoughtful comments of a reviewer are credited with substantial improvements in this MS.

References

Aina, V., Lusvardi, G., Annaz, B., Gibson, I. R., Imrie, F. E., Malavasi, G., Menabue, L., Cerrato, G. and Martra, G. 2012. Magnesium- and strontium-co-substituted hydroxyapatite: the effects of doped-ions on the structure and chemico-physical properties. J. Mater. Sci. Mater. Med. **23***:* 2867—2879.

Andersen, S. O. 1963. Characterization of a new type of cross-linkage in resilin, a rubber-like protein. Biochim Biophys Acta **69:** 249—262.

Andersen, S. O. 1999. Exoskeletal proteins from the crab, *Cancer pagurus*. Comp Biochem Physiol A **123:** 203—211.

Andersen, S. O. 2010. Insect cuticular sclerotization: A review. Insect Biochem. Mol. Biol. **40:** 166—

178.

Andersen, S. O. 2011. Are structural proteins in insect cuticles dominated by intrinsically disordered regions? Insect Biochem. Mol. Biol. **41**(8): 620—627.

Becker, A., Ziegler, A., and Epple, M. 2005. The mineral phase in the cuticles of two species of Crustacea consists of magnesium calcite, amorphous calcium carbonate, and amorphous calcium phosphate. Dalton Trans. **21**(10): 1814—20.

Binger, L. C. and Willis, J. H. 1994. Identification of the cDNA, gene and promoter for a major protein from flexible cuticles of the giant silkmoth Hyalophora cecropia. Insect Biochem. Mol. Biol. **24**(10): 989—1000.

Bouligand, Y. 1986. Theory of microtomy artifacts in arthropod cuticle. Tissue Cell **18**: 621–643.

Burmester, T. 1999. Identification, molecular cloning, and phylogenetic analysis of a non-respiratory pseudo-hemocyanin of *Homarus americanus*. J. Biol. Chem. **274:** 13217-13222.

Cerenius, L. and Söderhäll, K. 2004. The prophenoloxidase-activating system in invertebrates. Immunol. Rev. **198:** 116-126.

Christie, A. E., Rus, S., Goiney, C. C., Smith, C. M., Towle, D. W. and Dickinson, P. S. 2007. Identification and characterization of a cDNA encoding a crustin-like, putative antibacterial protein from the American lobster *Homarus americanus*. Mol. Immunol. **44**(13): 3333—3337.

Decker, H., Ryan, M., Jaenicke, E. and Terwilliger, N. 2001. 'SDS-induced phenoloxidase activity of hemocyanins from *Limulus polyphemus*, *Eurypelma californicum*, and *Cancer magister.* J Biol Chem **276**(21), 17796—17799.

Dellisanti, C. D., Spinelli, S., Cambillau, C., Findlay, J. B. C., Zagalsky, P. F., Finet, S. and Receveur-Bréchot, V. 2003. Quaternary structure of alpha-crustacyanin from lobster as seen by small-angle X-ray scattering. FEBS Lett. **544**(1-3), 189—193.

Duhamel, R. C. and Kunkel, J.G. 1983. Cockroach Larval-specific Protein, a Tyrosine-rich Serum Protein. J. Biol. Chem. **258**: 14461-14465.

Duhamel, R. C. and Kunkel, J.G. 1987. Molting cycle regulation of hemolymph protein clearance in cockroaches: Possible size-dependent mechanism. J. Insect Physiol. **33**: 155—158.

Elstnerová P., Friák, M., Fabritius, H. O., Lymperakis, L., Hickel, T., Petrov, M., Nikolov, S., Raabe, D., Ziegler, A., Hild, S. and Neugebauer, J.. 2010. *Ab initio* study of thermodynamic, structural, and elastic properties of Mg-substituted crystalline calcite. Acta Biomater. **6**(12): 4506—4512.

Ferrari, M., Folli, C., Pincolini, E., McClintock, T. S., Rössle, M., Berni, R. and Cianci, M. 2012. Structural characterization of recombinant crustacyanin subunits from the lobster *Homarus americanus*. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. **68**(Pt 8): 846—853.

Foran, E., Weiner, S. and Fine, M. (2013), Biogenic fish-gut calcium carbonate is a stable amorphous phase in the gilt-head seabream, *Sparus aurata*. Sci. Rep. **3**, 1700.

Hauton, C., Hammond, J.A., Smith, V.J. 2005. Real-time PCR quantification of the in vitro effects of crustacean immunostimulants on gene expression in lobster (*Homarus gammarus)* granular haemocytes. Dev. Comp. Immunol. **29**: 33-42.

Hild S., Marti, O. and Ziegler, A . 2008. Spatial distribution of calcite and amorphous calcium carbonate in the cuticle of the terrestrial crustaceans *Porcellio scaber* and *Armadillidium vulgare*. J .Struct. Biol. **168**: 426—436.

Hild, S., Neues, F., Znidarsic, N., Strus, J., Epple, M., Marti, O., and Ziegler, A. 2009. Ultrastructure and mineral distribution in the tergal cuticle of the terrestrial isopod *Titanethes albus*. Adaptations to a karst cave biotope. J. Struct. Biol. **168**: 426-436.

Horst, M. N., Golden, R. J., Walker, A. N. and Mayorov,V. I. 2009. Cloning and sequencing of the gene for chitin synthase 2 from the American lobster, *Homarus americanus*. Genbank **ACS45330**.

Horst, M. N., Lee, D. A., Mayorov, V. and Walker, A. N. 2007. Pesticide induced alterations in hepatopancreatic chitinase from the American lobster, *Homarus americanus.* Genbank **ABQ59096**.

Karpells, S. T., Leonard, D. E. and Kunkel, J. G. 1990. Cyclic fluctuations in arylphorin, the principal serum storage protein of *Lymantria dispar*, indicate multiple roles in development. Insect Biochem. **20**: 73-82.

Kragh, M., Mølbak, L. and Andersen, S. O. 1997. Cuticular proteins from the lobster, Homarus americanus. Comp Biochem Physiol B Biochem

Mol Biol **118**(1): 147—154.

Kuballa, A. V., Merritt, D. and Elizur, A. 2007. Gene expression profiling of cuticular proteins across the moult cycle of the crab *Portunus pelagicus*. BMC biology **5**(1): 45.

Kuballa, A. V. and Elizur, A. 2008. Differential expression profiling of components associated with exoskeletal hardening in crustaceans. BMC genomics **9**(1): 575.

Kuballa, A. V., Holton, T. A., Paterson, B. and Elizur, A. 2011. Moult cycle specific differential gene expression profiling of the crab *Portunus pelagicus*. BMC genomics **12**(1): 147.

Kunkel, J. G., Nagel, W. and Jercinovic, M. J. 2012. Mineral Fine Structure of the American Lobster Cuticle. Journal of Shellfish Research **31**(2): 515-526.

Kunkel, J. G., Jercinovic, M. J., Callaham, D. A., Smolowitz, R., and Tlusty, M. 2005. Electron Microprobe Measurement of Mineralization of American lobster, *Homarus americanus*, Cuticle: Proof of concept. In (Tlusty, M.F., Halvoarson, H.O., Smolowitz, R. and Sharma, U., eds) Lobster Shell Disease Workshop. Aquatic Forum Series 05-1. New England Aquarium, p. 76-82.

Kunkel, J. G. and Jercinovic, M. J. 2013. Carbonate apatite formulation in cuticle structure adds resistance to microbial attack for American lobster. Marine Biology Research **9**(1): 27-34.

Landis, W. J. and Jacquet, R. 2013. Association of Calcium and Phosphate Ions with Collagen in the Mineralization of Vertebrate Tissues. Calcif. Tissue Int. (in press).

Laurencin, D., Almora-Barrios, N., de Leeuw, N. H., Gervais, C., Bonhomme, C., Mauri, F., Chrzanowski, W., Knowles, J. C., Newport, R. J., Wong, A., Gan, Z. and Smith, M. E. 2011. Magnesium incorporation into hydroxyapatite. Biomaterials **32**(7), 1826—1837.

Lawrence, P. A. 1966. Development and determination of hairs and bristles in the milkweed bug, *Oncopeltus fasciatus* (Lygaeidae, Hemiptera). J. Cell Sci. **1**: 475–498.

Li, L. 2003. The biochemistry and physiology of metallic fluoride: action, mechanism, and implications. Critical Reviews in Oral Biology and Medicine **14**(2): 100–114.

Li, W. and Riddiford, L. M. 1992. Two distinct genes encode two major isoelectric forms of insecticyanin in the tobacco hornworm, *Manduca sexta*. Eur. J. Biochem. **205**(2): 491—499.

Luquet, G. 2012. Biomineralizations: insights and prospects from crustaceans, ZooKeys **176**: 103-121.

Locke, M. 2001. The Wigglesworth Lecture: Insects for studying fundamental problems in biology. J. Insect Physiol. **47**: 495-507.

Magkrioti, C. K., Spyropoulos, I. C., Iconomidou, V. A., Willis, J. H. and Hamodrakas, S. J. 2004. cuticleDB: a relational database of Arthropod cuticular proteins. BMC Bioinformatics **5**: 138.

Merritt, D. J. 2007. The organule concept of insect sense organs: Sensory transduction and organule evolution. Advances in Insect Physiology **33**: 192–241.

Modla, S. 2006. The effect of fixation on the morphology of the late premolt and early postmolt cuticle of the blue crab, *Callinectes sapidus*. Thesis, University of North Carolina Wilmington, 114pp.

Mykles, D. L., Haire, M. F. and Skinner, D. M. 2000. Immunocytochemical localization of actin and tubulin in the integument of land crab (*Gecarcinus lateralis*) and lobster (*Homarus americanus*). J. Exp. Zool. **286**(4): 329—342.

Neues, F., Hild, S., Epple, M., Marti, O. and Ziegler, A. 2011. Amorphous and crystalline calcium carbonate distribution in the tergite cuticle of moulting *Porcellio scaber* (Isopoda, Crustacea). J Struct Biol **175**(1): 10—20.

Nikolov, S., Fabritius, H., Petrov, M., Friák, M., Lymperakis, L., Sachs, C., Raabe, D. and Neugebauer, J. 2011. Robustness and optimal use of design principles of arthropod exoskeletons studied by *ab initio*-based multiscale simulations., J. Mech. Behav. Biomed. Mater. **4**(2): 129—145.

Nikolov, S., Petrov, M., Lymperakis, L., Friák, M., Sachs, C., Fabritius, H.-O., Raabe, D. and Neugebauer J. 2010. Revealing the design principles of high-performance biological composites using *ab initio* and multiscale simulations: the example of lobster cuticle. Adv. Mater **22**(4): 519—526.

Nousiainen, M., Rafn, K., Skou, L., Roepstorff, P. and Andersen, S. O. 1998. Characterization of exoskeletal proteins from the American lobster, Homarus americanus., *Comp Biochem Physiol B*

Biochem Mol Biol **119**(1): 189—199.

Papandreou, N. C., Iconomidou, V. A., Willis, J. H. and Hamodrakas, S. J. 2010. A possible structural model of members of the CPF family of cuticular proteins implicating binding to components other than chitin. J. Insect Physiol. **56**(10): 1420—1426.

Pomés, A., Melén, E., Vailes, L. D., Retief, J. D., Arruda, L. K. and Chapman, M. D. 1998. Novel allergen structures with tandem amino acid repeats derived from German and American cockroach. J. Biol. Chem. **273**(46): 30801—30807.

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J., Heger, A., Holm, L., Sonnhammer, E. L. L., Eddy, S. R., Bateman, A. and Finn, R. D. 2012. The Pfam protein families database. Nucleic Acids Res **40**(Database issue), D290—D301.

Raabe, D., Sachs, C., and Romano, P. 2005. The crustacean exoskeleton as an example of a structurally and mechanically graded biological nanocomposite material. Acta Materialia **53**: 4281-92.

Radha, A. V., Forbes, T. Z., Killian, C. E., Gilbert, P. U. P. A. and Navrotsky, A. 2010. Transformation and crystallization energetics of synthetic and biogenic amorphous calcium carbonate. P.N.A.S. **107**(38): 16438-43.

Rebers, J. E. and Willis, J. H. 2001. A conserved domain in arthropod cuticular proteins binds chitin. Insect Biochem. Mol. Biol. **31:**1083-1093.

Romano, P., Fabritius, H., and Raabe, D. 2007. The exoskeleton of the lobster *Homarus americanus* as an example of a smart anisotropic biological material. Acta Biomater **3**(3): 301—309.

Sato, A., Nagasaka, S., Fuhirata, K., Nagata, S., Arai, S., Saruwatari, K., Kogure, T., Sakuda, S., and Nagasawa, H. 2011. Glycolytic intermediates induce amorphous calcium carbonate formation in crustaceans. Nature Chemical Biology **7**: 197–199.

Schwarz, H. and Moussian, B. 2007. Electron-microscopic and genetic dissection of arthropod cuticle differentiation. In (A. Méndez-Vilas and J. Díaz, Eds.) Modern Research and Educational Topics in Microscopy. 1: 316–325.

Seear, P. J., Goodall-Copestake, W. P., Fleming, A. H., Rosato, E., and Tarling, G. A. 2012. Seasonal and spatial influences on gene expression in Antarctic krill *Euphausia superba*. Mar. Ecol. Prog. Ser. 467: 61—75.

Shepherd, J. H., Shepherd, D. V. and Best, S. M. 2012. Substituted hydroxyapatites for bone repair. J. Mater. Sci. Mater. Med. **23**(10): 2335—2347.

Smolowitz, R., Chistoserdov, A., and A. Hsu. 2005. Epizootic shell disease in American lobster, *Homarus americanus*. In (Tlusty, M.F., Halvoarson, H.O., Smolowitz, R. and Sharma, U., eds) Lobster Shell Disease Workshop. Aquatic Forum Series 05-1. New England Aquarium, p. 1—11.

Tarsitano, S. F., Lavalli, K. L., Horne, F. and Spanier, E. 2006. The constructional properties of the exoskeleton of homarid, palinurid, and scyllarid lobsters, *In* K. Martens and M. Thessalou-Legaki, ed., Issues of Decapod Crustacean Biology, Springer Netherlands, pp. 9—20.

Telfer, W. H. and Kunkel, J. G. 1991. The Function and Evolution of Insect Storage Hexamers. Ann. Rev. Entomol. **36**: 205—28.

Terwilliger, N. B. 2012. Gene expression profile, protein production, and functions of cryptocyanin during the crustacean molt cycle. Invertebrate Reproduction and Development **56**(3): 229—235.

Terwilliger, N. B., Dangott, L. and Ryan, M. 1999. Cryptocyanin, a crustacean molting protein: evolutionary link with arthropod hemocyanins and insect hexamerins. PNAS U.S.A. **96**(5): 2013—2018.

Terwilliger, N. B., Ryan, M. and Phillips, M. R. 2006. Crustacean hemocyanin gene family and microarray studies of expression change during eco-physiological stress. Integr. Comp. Biol. **46**(6): 991—999.

Terwilliger, N. B., Ryan, M. C. and Towle, D. 2005. Evolution of novel functions: cryptocyanin helps build new exoskeleton in Cancer magister., J. Exp. Biol. **208**(Pt 13): 2467—2474.

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

Tlusty, M. F., Smolowitz, R., Halvorson, H. and DeVito, S. 2007. Host susceptibility hypothesis for lobster shell disease. Journal of Aquatic Animal Health **19**: 215—225.

Wade, N. M., Tollenaere, A., Hall, M. R. and Degnan,

B. M. 2009. Evolution of a novel carotenoid-binding protein responsible for crustacean shell color. Mol. Biol. Evol. **26**(8): 1851—1864.

Waddy, S. L., Aiken, D.E., deKleijn, D. P. V. 1995. Control of growth and reproduction. In: Factor JR, editor. Biology of the Lobster Homarus americanus. New York,NY: Academic Press, p 217—66.

Weiss, I. M. 2011. Biomaterials: metabolites empowering minerals. Nat Chem Biol **7**(4): 192—193.

Weiss, I. M., Lüke, F., Eichner, N., Guth, C. and Clausen-Schaumann, H. 2013. On the function of chitin synthase extracellular domains in biomineralization. J. Struct. Biol. (in press).

Williams, E. E., Anderson, M. J., Miller, T. J. and Smith, S. D. 2004. The lipid composition of hypodermal membranes from the blue crab (Callinectes sapidus) changes during the molt cycle and alters hypodermal calcium permeability., Comp. Biochem. Physiol. B Biochem. Mol. Biol. **137**(2): 235—245.

Willis, J. H. 1999. Cuticular Proteins in Insects and Crustaceans. Amer. Zool., **39**: 600-609.

Willis, J. H. 2010. Structural cuticular proteins from arthropods: Annotation, nomenclature, and sequence characterization in the genomics era. Insect Biochem. Molec. Biol. **40**: 189-204.

Willis, J. H. 2012. Cuticular Proteins. Chap. V in **Insect Molecular Biology and Biochemistry**. Ed. By L. I. Gilbert. Academic Press pp. 134-165.

Wu, C. H., Lee, M. F., Liao, S. C. and Luo, S. F. 1996. Sequencing analysis of cDNA clones encoding the American cockroach Cr-PI allergens. Homology with insect hemolymph proteins., J. Biol. Chem. **271**(30): 17937—17943.

Table Legend

Table I. Proteins relevant to American lobster cuticle proteins. The list includes proteins from other crustaceans and insects when relevant. The protein name has a superscript which provides a reference to find the protein in a database or discussed in a publication. Columns tabulate: 1) the phenolic amino acids tyrosine plus phenylalanine mole percent determined by counting the residues in the sequence in the NIH database accessed at URL: http://www.ncbi.nlm.nih.gov/protein/ and Pfam motifs were searched for using the Welcome Trust online database at URL: http://pfam.sanger.ac.uk/search . 2) presence of cuticle protein motifs 1 or 4. 3) presence of a chitin binding motif. 4) presence of HbCy domaines N, M, and C. 5) presence of a Lipocalin motif. 6) presence of any Pfam A motif with stringency 1.0.

Table I.

Protein	Species	a.a. residues	Tyr+Phe mole %	cuticle protein	Binds Chitin	HbCy	Lipo-calin	Pfam
Cryptocyanin [a]	*M. magister*	653	10.11	-	-	N,M,C	-	+
Cryptocyanin 2 [b]	*M. magister*	674	9.64	-	-	N,M,C	-	+
Hemocyanin 1 [b]	*M. magister*	662	9.67	-	-	N,M,C	-	+
Hemocyanin 2 [b]	*M. magister*	663	9.8	-	-	N,M,C	-	+
Pseudo-hemacyanin [c]	*H. americanus*	684	9.65	-	-	N,M,C	-	+
Hemocyanin alpha [d]	*H. americanus*	672	9.67	-	-	N,M,C	-	+
Hemocyanin beta [e]	*H. americanus*	223	11.21	-	-	N,M	-	+
Pro-phenoloxidase [f]	*H. americanus*	683	8.64	-	-	N,M,C	-	+
Pro-phenoloxidase [g]	*H. gammarus*	683	8.49	-	-	N,M,C	-	+
AAB0962 allerg [h]	*P. americana*	685	16.5	-	-	N,M,C	-	+
ACY40651.1 allerg [i]	*B. germanica*	657	16.59	-	-	N,M,C	-	+
Crustacyanin A [j]	*H. americanus*	181	14.36	-	-	-	+	+
Crustacyanin B [j]	*H. americanus*	181	14.36	-	-	-	+	+
Insectacyanin [k]	*M. sexta*	206	12.14	-	-	-	+	+
Chitin-synthase 2 [l]	*H. americanus*	267	10.11	-	-	-	-	+
Crustin-like [m]	*H. americanus*	112	7.14	-	-	-	-	+
HA-AMP1A, 1B, 4 [n]	*H. americanus*	105	9.52	-	+[s]	-	-	+
HA-AMP2, 3 [n]	*H. americanus*	105	10.47	-	+	-	-	+
HA-AMP5 [n]	*H. americanus*	114	8.77	-	+	-	-	+
HACP188 [n]	*H. americanus*	184	13.04	-	+	-	-	+
HACP142 [n]	*H. americanus*	129	11.62	-	+	-	-	+
HC CPF12 [o]	*H. cecropia*	105	9.52	-	+	-	-	+
HACP127 [n]	*H. americanus*	116	15.52	-	-	-	-	-
HACP202B [n]	*H. americanus*	189	10.58	-	-	-	-	-
HACP116a [n]	*H. americanus*	111	8.11	1	-	-	-	+
HACP116b [n]	*H. americanus*	111	8.11	1	-	-	-	+
CG8541[p]	*D. melanogaster*	279	9.09	4	-	-	-	+
HACP93 [n]	*H. americanus*	85	14.12	-	-	-	-	-
chitinase [q]	*H. americanus*	243	4.94	-	+	-	-	+
Vitellogenin [r]	*H. americanus*	2583	7.01	-	-	-	-	+

[a] Terwilliger et al. 1999. [b] Terwilliger and Ryan 2006. [c] Burmester 1999. [d] Kusche and Burmester 2001. [e] Horst et al. 2007. [f] Hauton et al. 2005. [h] Wu et al. 1996. [i] Pomés 1998. [j] Ferrari et al. 2012. [k] Li and Riddiford 1992. [l] Horst et al. 2009 [m] Christie et al. 2007. [n] Nousiainen et al. 1998. [o] Binger and Willis 1994. [p] Papandreou et al. 2010. [q] Horst et al. 2007. [r] Hui et al. 2007. [s] HA-AMP1B contains DUF4480.