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3D-Xray-tomography of American lobster shell-structure. An overview

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ABSTRACT

A total inventory of density defined objects of American lobster cuticle was obtained by using high resolution 3D Xray tomography, micro-computed-tomography. Through this relatively unbiased sampling approach several new objects were discovered in intermolt cuticle of the lobster carapace. Using free and open-source software the outlines of density-defined objects were obtained and their locations in 3D space calculated allowing population parameters about these objects to be determined. Nearest neighbor distances between objects allowed interpretations of structural relationships of and between objects. Several organule types are recognized by their structural outlines and density signatures. A hierarchy of organule types and distribution suggests they develop during sequential molts. New objects (stalactites, Bouligand spirals, and basal granules) are described as mineral structures with well defined morphological character, allowing them to be recognized by their descriptive names and distributions. The three new cuticular objects appear to represent forms of calcium carbonate deposition in the sequential layers of exocuticle, endocuticle and membranous layer and may provide signatures of underlying epidermal cells.

1. Introduction

Research on American Lobster, *Homarus americanus*, cuticle continues to be of interest to material scientists as a composite material, to immunologists as an antigen and to pathologists as the target of shell disease. The current chemical and physical models of arthropod cuticle are based on a long standing interest in modeling the LM, TEM and SEM views of cuticle structure as well as the chemistries of chitin (Richards, 1951), protein polymer and small molecules (Andersen, 2010) in cuticle structure. The primary breakthrough in understanding the apparent layers of the cuticle was made when the illusion of layering was explained as a more gradual rotation of the angle of orientation of chitin fibers in the plane of the cuticle (Bouligand, 1972). When the angle of layered fibers rotates through 180° there is an illusion of a layer in the cuticle. Additional interest has developed in designing biopolymers as biomimetic composite materials (Fabritius et al., 2009; Nikolov et al., 2011), applying engineering principles to understand structural properties (Raabe et al., 2006; Romano et al., 2007; Grunenfelder et al., 2014) associated with the Bouligand (1972) layers of so-called twisted-fiber plywood structure of the cuticle. The structure of lobster cuticle is informed by knowledge of the chitin and protein polymers associated with particular cuticle layers (Kunkel, 2013) and the organic chemistry associated with hardening or flexibility after molting (Andersen, 2010). Departures from the uniform structure of the cuticle exemplified in various Decapod cuticles by regular surface sculpturing, including but not limited to organule

Abbreviations: AFM, atomic force microscopy; AVI, a digital movie file type produced by ImageJ image analysis software; CSV, comma-separated-value type text file is a widely used non-proprietory rectangular data file used to transfer data between software applications; EMP, electron microprobe; ESD, epizootic shell disease is a locally prevalent disease (currently of unknown cause) in natural populations of American lobster differing (in its striking abundance) from endemic shell disease which is found at very low levels in all lobster populations (Smolowitz et al. 2005); FTIR, fourier transform infrared analysis; LM, light microscopy; microCT, micro computed tomography; NA, a reserved word in the R language referring to a missing value; NND, nearest neighbor distance. NND is the euclidean distance of an object centroid to its nearest neighbor's centroid; R, R is a General Public License (GNU) computation environment open-source programming and graphical tool; R rgl, the rgl library is a downloadable add on to R that allows creating 3D rotatable plotting spaces with functions for using those spaces to create plane views stereo views and movies; SEM, scanning electron microscopy; TEM, transmission electron microscopy; μm, micrometer 10^{-6} meters; voxel, the building block of 3D-arrays making up a solid structure; XRT, Xray tomography.

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structures, have been implicated in avoiding structural failure of the cuticle (Tarsitano et al., 2006).

Earlier models of cuticle structure are based on one dimensional analysis of powdered cuticle, e.g. X-ray powder pattern analysis (Lowenstam 1981; Lowenstam and Weiner, 1989) or various two dimensional analyses of sections of de-mineralized cuticle (Smolowitz et al., 2005) or polished surfaces of mineralized cuticle (Hayes and Armstrong, 1961; Fabritius et al., 2009; Kunkel et al., 2012; Kunkel and Jercinovic, 2013; Kunkel 2013) that derive their interpretations from one or two-dimensional data analysis.

One dimensional studies (e.g. chemical analysis of whole cuticle) are biased by focusing on average compositions, ignoring the importance of the abundant but small regular features, such as the organules, which are developmentally related small secretory and sensory organs, first described in insects but present in all arthropods. Most two dimensional studies of biological structure (e.g. AFM, LM, TEM, SEM, EMP, micro Raman and micro FTIR) are biased by investigator selection of ideal views of successful sections or polished surfaces which are more immediately interpretable. Often one sees a small sample of the possible sections, others being rejected as being not representative or not easily fit into a general model. Investigators interested in modeling 'general' cuticle properties have often avoided considering organule structures, e.g. the dermal gland canals and sensory bristles, in their models as being beyond the current level of interest and in hope of reaching an *ab initio* model of the composite material nature of cuticle (Raabe et al., 2006; Fabritius et al., 2009; Nikolov et al., 2011). These one- and two-dimensional studies may not represent an exhaustive or accurate window into the diversity, physical relationships or importance of cuticle structures. Since the available chemical analytical and structural data on lobster cuticle, obtained by one- and two-dimensional studies, does not show the 3D view of the micro-architecture of the cuticle, we implemented a three dimensional study of lobster cuticle based on the density of the cuticle to X-rays using the recently available high resolution X-ray computed tomography or microCT (Naleway et al., 2016). Resultant voxel density data derived from a limited number of lobsters sampled is immense and allows for cataloging all structures observable at the resolution of the technique. Here our immediate focus is to provide an overview of new objects discovered in carapace cuticle by focusing on micro density measurements. The relationships of these new objects with historically described cuticle structures are described, particularly with the developmentally important layers: epicuticle, exocuticle, endocuticle and membranous layer (Bouligand 1972; Waddy et al., 1995), and with the carapace organules (Henke, 1952; Merritt, 2006; Kunkel, 2013).

2. Materials and methods

2.1. Animals

The American lobster cuticles in this study were obtained from specimens collected Spring 2009 during leg three of a Northeast Groundfish Survey of NOAA Ship HB Bigelow on the outer (eastern edge) of Georges Bank in USA territorial waters, which is effectively the eastern edge of the Northeast USA continental shelf. This offshore population of American lobsters sampled was historically free of epizootic shell disease (ESD) (Glenn and Pugh, 2006) and the samples reported on here are considered to be from normal lobsters recently taken from nature. All lobsters were intermolt, stage C4, which is a holding stage before induction of the next molting cycle (Waddy et al., 1995). Sections of medial lateral carapace cuticle (1.5 × 3 cm) were excised from euthanized lobsters and fixed in 50 ml of −40 °C 95% ethyl alcohol, changed twice over 24 h, and subsequently stored in absolute alcohol. The sampled piece of cuticle is biased in avoiding the major suture lines and muscle insertions and apodemes of the carapace, but is unbiased in recording all the density defined objects including the pattern of exoskeleton organules as previously described (Kunkel and Jercinovic, 2013) for the primary site of ESD.

2.2. Cuticle medallion preparation

A drill press with coring drill bit was used to obtain 6 mm medallions of cuticle from dorsal lateral carapace pieces stored in absolute alcohol. Medallions were air dried overnight and attached with Super-Glue® to a 25 mm resin block in triangular arrays for stability while grinding. The triads of medallions were briefly ground with a carborundum disc (600 grit) at high speed on a rotary wheel to approximate desired depth. They were then finely polished with a no-nap lapping cloth (Trident ™) with 6, 3, 1, 0.25 μm diamond (Buehler, Metadi Supreme®) in lapping oil. They were cleaned after each polishing level with anhydrous solvents (100% isopropyl alcohol and 100% acetone recently treated with Molecular Sieve (Sigma-Aldrich) to scavenge water) to remove grinding media and oil. These samples were objects further processed for EMP (Kunkel et al., 2005), microRaman, microFTIR and microCT analysis to characterize structures at various levels in the cuticle.

2.3. MicroCT

Micro-computed-tomography (microCT) scans were performed using the Skyscan 1272 (Bruker MicroCT, Kontich, BE), a high resolution desktop microCT scanner for specimen imaging. The system is capable of scanning samples at resolutions as high as 0.35 μms. All samples (M1C, M2C and M3A, Supplementary Table S1) were portions of 6 mm diameter medallions and scanned at 3.5 μm (low resolution) isotropic voxel resolution, with the x-ray voltage at 40 kV and x-ray current at 222 μA using the 0.25 mm Aluminum filter. The raw acquisitions were collected every 0.2 rotational degree with a 1150 ms exposure time for each image. Sample M3A was scanned at 1.5 μm (high resolution) isotropic voxel resolution, 40 kV x-ray voltage, 222 μA x-ray current and 0.25 mm Aluminum filter. The raw acquisitions were collected every 0.2 rotational degree with a 1900 ms exposure time for each image. Sample M2C was scanned at 0.5 μm (highest resolution) isotropic voxel resolution, 40 kV x-ray voltage, 222 μA x-ray current and 0.25 mm Aluminum filter. The raw acquisitions were collected every 0.1 rotational degree with a 3800 ms exposure time for each image. The raw acquisitions were reconstructed to axial cross-sectional slices using Nrecon (ver 1.6.9.8) using standard parameters to generate 3Dimensional grayscale images. All datasets were further reoriented and cropped to smaller regions using DataViewer (ver 1.5.1.2). Since creating outlines of linear structures is difficult when they are not orthogonal to a current axis, it was of particular use to rotate long structures such as canals so that their new orientation was perpendicular to a new voxel plane before extracting their contours.

2.4. Software

Several software packages were essential to this study and were non-proprietary or freely available. Data from the microCT data processed by Nrecon exists as individual 2D slices in typical lossless BMP, TIF formats. A lossless storage of pixel values is necessary for quantitative measurements on the data. DataViewer (available at http://bruker-microct.com/products/downloads.htm) is essential for rotating and subsetting the huge number of voxels in the available 2D sliced files into smaller prismatic assemblages of voxels as a smaller or reoriented set of 2D slices that contain a structural object of interest. These subset slices can be accessed by ImageJ (the