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ONTOGENETIC VARIATIONS IN CUTICLE MORPHOLOGY IN THE BLUE CRAB *CALLINECTES SAPIDUS* RATHBUN, 1896

David A. Waugh, Rodney M. Feldmann, Jennifer L. Burrell, Angela L. Hull, Kristie Hein, and Carrie E. Schweitzer

(DAW, RMF, JLB, ALH, KH) Department of Geology, Kent State University, Kent, Ohio 44242, U.S.A.
(Corresponding Author (DAW): (dwaugh@kent.edu)
(CES) Department of Geology, Kent State University Stark Campus, 6000 Frank Ave. NW, North Canton, Ohio 44720, U.S.A.

ABSTRACT

In an effort to use aspects of the cuticle as taxonomic characters in phylogenies of fossil and extant decapods, variation due to gender, growth, sample location on the carapace, and molt cycle must be understood so that taxonomically important characters can be identified. In this study, effects of sample location on the carapace and carapace size were examined. A series of male *Callinectes sapidus* Rathbun, 1896, specimens from 2-6 cm in length were collected on the Rhode River of the Chesapeake Bay, MD, USA. To study the effects of sample location and carapace size on parameters of the cuticle, the cuticle was examined in thin section and on the surface of the dorsal carapace. The distributional density of setal pits and nodes and node size were measured on the surface. In thin section, thickness of the cuticle and construction of the nodes and setal pits was examined. Thickness of the cuticle, node size, and setal pit density increased during growth of the crab. Node density decreased with growth. Construction of nodes and setal pits remained constant in all specimens and sample locations. Morphometric parameters of the cuticle were consistent with previously reported growth rates of the carapace in *C. sapidus*. Differences in the rate of change for the cuticle metrics studied occurred at carapace sizes that are attained upon reaching sexual maturity. Growth rates of cuticular features provide context for comparison with similar data in other species. In addition, the change in growth rates of these features, if recognized in fossil crab populations, may allow the determination of population age structures and size at maturity.

KEY WORDS: *Callinectes sapidus*, cuticle structure, Decapoda, ontogeny, Portunidae

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INTRODUCTION

In an effort to use aspects of the cuticle as taxonomic characters in fossil and extant decapods, variation in the cuticle due to gender, growth, sample location on the carapace, and molt cycle must be understood so that taxonomically important characters can be identified (Waugh and Feldmann, 2003). In addition to its relevance to taxonomic work, an understanding of cuticular changes that are a result of the molt cycle, gender, and ontogeny may eventually be applied to fossilized cuticle in an effort to understand population age structures as well as other non-taxonomic factors in fossilized crabs. However, significant characters must first be recognized in the cuticle of extant taxa and correlated to the molt cycle, gender, and ontogeny before either taxonomic or ecologic conclusions can be drawn from fossil cuticle. The purpose of this paper is to examine variations within the cuticle of male *Callinectes sapidus* Rathbun, 1896, as a function of growth and sample location (Fig. 1). Effects of the molt cycle on cuticle within the ecdysial sutures is also examined within a few specimens; effects of gender will be addressed in future works.

Microstructure is preserved in fossil cuticle and has been studied primarily in a descriptive, functional, or taphonomic context (Neville and Berg, 1971; Feldmann and Tshudy, 1987; Vega et al., 1994, 1998, 2005; Feldmann and Gaździcki, 1998; Briggs et al., 1998; Haj and Feldmann, 2002; Guinot and Breton, 2006; Waugh et al., 2004, 2006). A better understanding of cuticular architecture in extant decapods is needed to further exploit this growing data set of fossilized cuticle. Therefore, this study focuses on aspects of modern cuticle, with emphasis on factors that may be preserved within fossil cuticle.

We use the terminology of Drach (1939) to divide layers of the cuticle into an epicuticle, exocuticle, endocuticle, and membranous layer (Fig. 2A-D). These subdivisions of the cuticle are recognized on the basis of timing of development, appearance, and staining characteristics. The epicuticle and exocuticle are secreted prior to molting, and the endocuticle and membranous layer form just after the molt occurs (Drach, 1939; Skinner, 1962; Green and Neff, 1972).

Due to the abundance and economic importance of *Callinectes sapidus*, the cuticle of this species has received more attention than other decapods. Vigh and Dendinger (1982) examined calcium, magnesium, chitin, and proteins within the cuticle. Marlowe et al. (1994) examined changes in proteins in the cuticle during the molt cycle. Elliott and Dillaman (1999) observed the construction of the branchiostegal cuticle. Williams et al. (2003) focused on the arthrodial membrane. Priester et al. (2005) studied the cuticle of the ecdysial sutures. Dillaman et al. (2005) detailed the initial stages of calcification and its progression within the exocuticle. Thus, we used this well-researched species to examine the effects of growth and sample location on cuticle morphology.

MATERIALS AND METHODS

Specimens of *Callinectes sapidus* were collected at the Smithsonian Environmental Research Center in Edgewater, Maryland, located on the

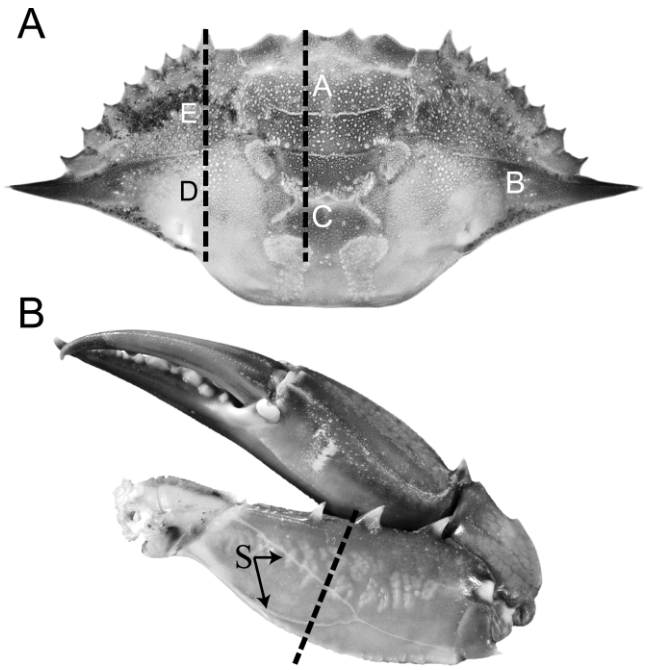

Fig. 1. A, sample locations on the carapace of *Callinectes sapidus*. Dashed lines indicate location of thin sections. Letters indicate specific sites of the observations on the thin section or of the surface. Observations of the cuticle surface were taken from the right side of the carapace at the same locations indicated by letters on the left. Location B was only observed on the surface and was not thin sectioned; B, location of thin section taken across the merus to examine the ecdysial sutures (S).

Rhode River of the Chesapeake Bay, during August of 2006. A total of 41 specimens, including 13 immature females and 28 mature and immature males, were collected in shallow water using seine nets. The crabs were anesthetized by slow cooling and subsequently stored in 70 percent ethyl alcohol. Maximum carapace width was measured between the tips of the last anterolateral spines, and the carapace length was measured from the maximum projection of the frontal spines to the junction of the carapace with the first abdominal somite along the midline. With assistance from the SERC staff, molt stage was assessed in the field by examining tissue of the paddle-shaped propodus and dactylus of the fifth pereiopod. Examination of the thin cuticle on these elements allows viewing of the epidermis and its relation with the cuticle, thus allowing identification of the molt stage (Drach, 1939). Insufficient numbers of mature females were collected to fully examine their growth, and for this study, we selected only the intermolt males. A few premolt males were also studied to examine the cuticle of the ecdysial suture in preparation for the molt.

Five sample locations on the dorsal carapace were selected for examination of the cuticle surface and cross section (Fig. 1A). Sections of cuticle were excised from the dorsal carapace using either dissecting scissors or a small rotary diamond saw, depending on the thickness of the cuticle. One centimeter wide sections were cut from the carapace so that the desired line of section was in the center of the excised sample. Samples were removed from the merus (Fig. 1B) in a similar manner. Sections were then rinsed in fresh alcohol and dried at 40°C in a cabinet to remove moisture before they were mounted in epoxy blocks. The epoxy blocks were then ground and polished to the desired plane of section and glued to glass slides using a UV light curing resin. Remaining material was cut from the slide with a diamond saw, and the sections were ground and polished to the desired thickness of about 40 microns. The final surface was polished with 1200 grit abrasive paper and a coverslip attached with emersion oil. To retain cuticle that had newly formed underneath the existing cuticle in sections cut across the merus (Fig. 1B), samples were freeze dried to stabilize soft tissues before they were embedded in epoxy.

Thin sections were examined with a polarizing microscope using both plain polarized light (PPL) and cross polarized light (XPL). Surface views of the carapace were studied with a reflected light microscope at the same locations that were thin sectioned, but on opposite sides of the bilaterally symmetrical carapace. To obtain sufficient contrast on the surface views, the dried surface was coated with vaporized ammonium chloride to create a uniform white surface. The surfaces were then illuminated at low angles and photographed.

Measurements of the cuticle in thin section and on surface views were taken on calibrated photomicrographs using NIH Image for the Macintosh. Nodes, or elevated projections of the cuticle surface, were measured by outlining the node with the freehand selection tool in NIH Image; the program then fit an ellipse to the selected area, and from this, the program generated minimum and maximum lengths for the nodes. Maximum length of the nodes was used in the final analysis, although tests with the minimum lengths showed little difference in the results other than slight changes in magnitude.

Node and setal pit distribution density was measured by drawing a square of known area over the surface and counting the number of features contained within it. Measurements of cuticle thickness were taken by measuring the length of lines drawn perpendicular to the cuticle surface. Measurements were taken of the total thickness (not including the membranous layer), thickness of the endocuticle, and the thickness of the membranous layer only. Exocuticle thickness was calculated by subtracting the endocuticle thickness from the total thickness.

Changes in density of cuticle features and cuticle thickness were individually compared to carapace dimensions using the allometry equation (Raup and Stanley, 1978) which allows quantification of the rate of growth of one part of the organism with that of another:

$$Y = bX^a$$

Y, the independent variable, is one of the attributes of the cuticle; *X*, the dependent variable, is carapace size. Values for the constants *a* and *b* were found by fitting power curves to the dimensions using the PC program SPSS (ver. 14) which also calculated R^2 and significance levels for the growth curve (see Table 1 for values and explanations of the variables). The constant *b* is known as the initial value, and *a* is the allometric growth coefficient (Raup and Stanley, 1978).

Results

Cuticle Layers

The uppermost layer of the cuticle, or epicuticle, is only distinguishable from the exocuticle at high magnifications (Fig. 2C); consequently, throughout the remainder of the paper epicuticle is not explicitly differentiated from the exocuticle. The exocuticle is differentiated from the endocuticle based on the finer spacing of the exocuticular laminations compared to the endocuticle and the presence of interprismatic septa (Fig. 2C) in the exocuticle (Travis, 1963; Green and Neff, 1972; Dillaman et al., 2005). In addition, the upper exocuticle has a light amber color in PPL (Fig. 2C). The appearance of the exocuticle did not vary significantly between sample locations on the carapace or between specimens of different sizes. Thickness of the constituent layers and the extent of calcification is variable and is discussed in sections to follow. The membranous layer (Fig. 2A, D) is discussed more thoroughly because of its absence in some sections, the implications it has for recognition of stages in the decapod molt cycle, and its variation in appearance between decapod taxa (Skinner et al., 1992).

The membranous layer is the last to be deposited, and its presence within the cuticle is considered indicative of the crab entering the intermolt or C_4 stage of the molt cycle (Drach, 1939; Travis, 1955, 1960; Drach and Tchernigovtzeff, 1967; Skinner et al., 1992; Promwikorn et al., 2005). Timing of membranous layer formation (postmolt) and resorption (premolt) appears to vary between taxa and with sample location (Skinner et al., 1992). Our results support this observation in *Callinectes sapidus*.

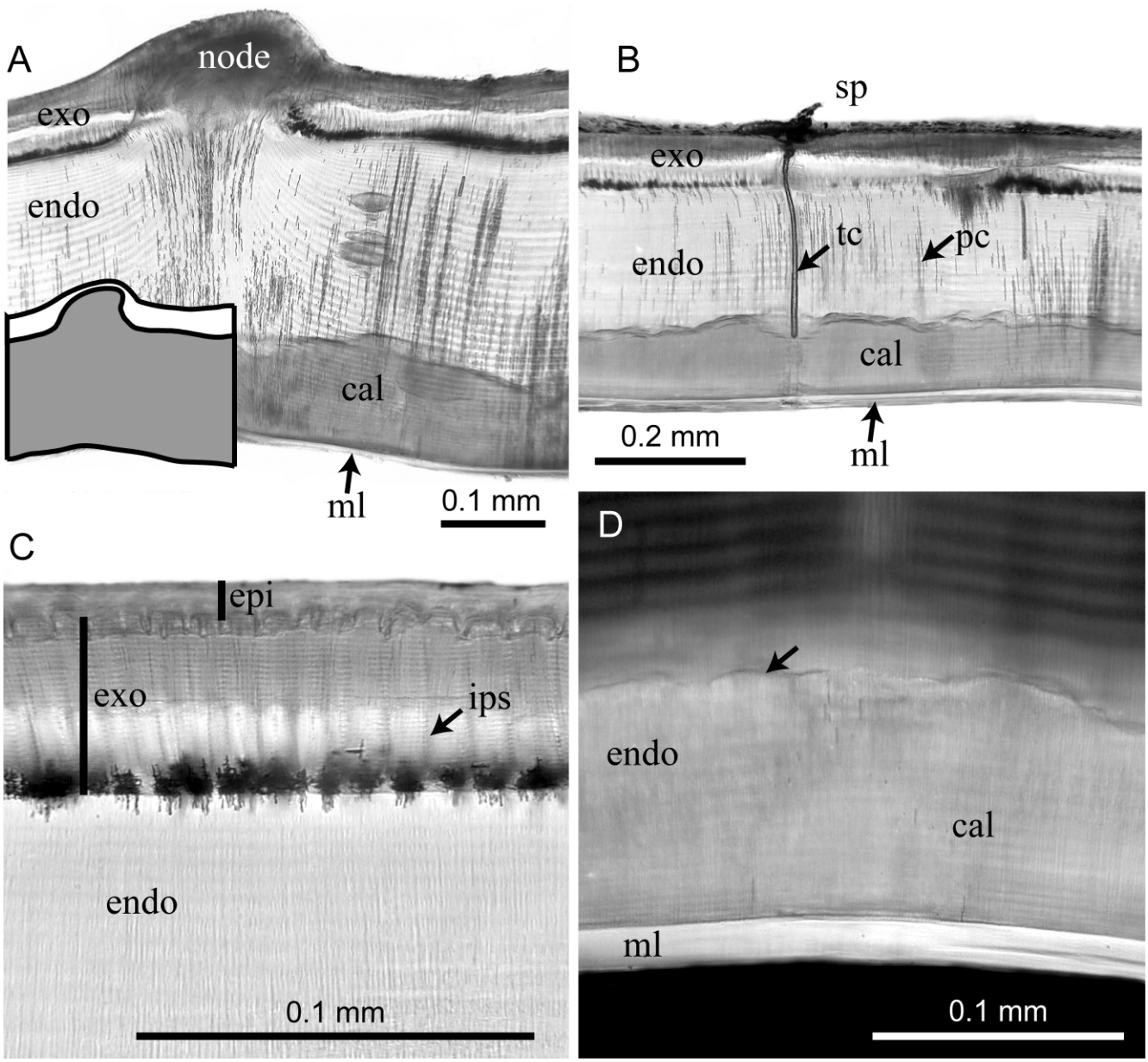

Fig. 2. Cuticle of *Callinectes sapidus* in thin section; epi, epicuticle; exo, exocuticle; endo, endocuticle; ml, membranous layer; pc, pore canal; sp, setal pit; tc, tegumental canal; ips, interprismatic septa; cal, calcified region; PPL, plain polarized light; XPL, cross polarized light. A, node construction, PPL, inset shows endocuticle in grey, exocuticle in white; B, setal pit and hair, PPL; C, epicuticle and exocuticle, note darker color (amber) of the epicuticle and upper half of the exocuticle, PPL; D, lower endocuticle and membranous layer, XPL, photographed with cuticle sample oriented 45 degrees to planes of polarization.

The membranous layer in *Callinectes sapidus* is similar in appearance to that illustrated in Compère et al. (1998, fig. 21), but differs from the membranous layer in tiger shrimp illustrated by Promwikorn et al. (2005, 2007) whose membranous layer is thicker and maintains a diffuse boundary with the overlying endocuticle (see Skinner et al., 1992, for a discussion of the interspecific variation and possible homologies of the membranous layer among arthropods). This interspecific variation in appearance may explain the different terminology used for the membranous layer, for example, the "uncalcified zone" of Dennell (1947).

The membranous layer was distinguished based on its location at the base of the endocuticle (Fig. 2D). In *Callinectes sapidus*, the membranous layer is thin and maintains a distinct margin with the overlying endocuticle (Fig. 2D). The membranous layer is constructed of fine laminations (Travis, 1960, 1963), although at the magnifications used we were unable to consistently resolve them (Fig. 2D). Some sections did exhibit delamination within the membranous layer, which supports a laminar construction. Recognition of the membranous layer is also aided by its pattern of birefringence under XPL (Figs. 2D, 3E, 3I). Rotation of cuticle thin sections under XPL caused

Table 1. Regression results based on the allometry equation $Y = bX^a$. Y is the independent variable; X is the dependant variable; b is initial value, and a is the allometric growth coefficient; $N_{density}$, node density; N_{size}, node size; T_{endo}, thickness of endocuticle; T_{total}, total cuticle thickness; L, carapace length; W, carapace width. Data and units from Appendix 1, which includes male *Callinectes sapidus* specimens between 2 and 6 cm in length. Loc, sample location of Y measurements based on Fig. 1. n, number of data points used to fit curve. * data from Newcombe et al. (1949) for male *Callinectes* 1.79 to 7.7 cm in length.

Y	Loc	X	*n*	*a*	*b*	R^2	Sig.
$N_{density}$	A	L	5	−1.633	21.517	0.893	0.015
$N_{density}$	B	L	5	−2.124	357.427	0.946	0.005
$N_{density}$	D	L	4	−1.583	59.022	0.981	0.010
$N_{density}$	E	L	4	−1.317	10.695	0.987	0.007
N_{size}	A	L	5	0.587	0.132	0.961	0.003
N_{size}	D	L	4	0.447	0.132	0.887	0.058
N_{size}	E	L	4	0.386	0.144	0.977	0.012
T_{endo}	A	L	7	1.089	0.059	0.962	0.000
T_{exo}	A	L	7	0.789	0.024	0.981	0.000
T_{total}	A	L	7	1.023	0.082	0.971	0.000
T_{total}	C	L	7	0.925	0.109	0.926	0.001
T_{total}	D	L	9	0.690	0.142	0.794	0.001
T_{total}	E	L	9	0.899	0.102	0.937	0.000
W	—	L	7	1.163	1.847	0.994	0.000
W	*	L	8	1.087	1.900	0.999	0.000
Molt#	*	L	8	.279	11.26	0.997	0.000
E-S	*	L	8	1.163	0.670	0.999	0.000
L	*	W	8	0.919	0.555	0.999	0.018
Molt #	*	W	8	0.257	9.553	0.993	0.013
E-S	*	W	8	1.070	0.337	1	0.000

simultaneous extinction of the entire membranous layer for every 90 degrees of sample rotation. The birefringence of the membranous layer is differentiated from that of the calcified regions of the cuticle, which exhibits patchy extinction and higher order birefringence colors. Birefringence within calcified regions of the cuticle is lost when sections are treated with acid. Birefringence observed in both the membranous layer and laminations of endocuticle are acid stable.

In general, morphological indicators used to determine molt stage in decapods do not necessarily occur simultaneously throughout the cuticle and integument (Skinner et al., 1992; Cheng and Chang, 1991; Waddy et al., 1995; Elliott and Dillaman, 1999; Williams et al., 2003). The membranous layer, which defines the beginning of the intermolt, appears to be no exception. In the lateral portion of the dorsal carapace of the fiddler crab, the membranous layer was recognized one day post-molt after all other layers of the cuticle had been deposited, while at the same time the other layers were still thickening (Green and Neff, 1972), suggesting that the membranous layer may appear before the other cuticle layers have finished developing. The membranous layer is not present in the cuticle of the branchiostegal cuticle of *C. sapidus* (Elliot and Dillaman, 1999).

The membranous layer observed in the prepared samples of *Callinectes sapidus* was not consistent in either its presence or appearance. Thicker and continuous sections of the membranous layer were observed in sections containing the largest percentages of calcified cuticle. In sections that displayed minimal calcification, the membranous layer tended to be absent or ragged and discontinuous. Samples that lacked the membranous layer were generally less calcified, suggesting that they were not fully within the intermolt stage, during which the membranous layer should be present (Drach, 1939), or were actively resorbing cuticular components. The samples from the three crabs that were in the process of forming a new cuticle, indicating they had entered the premolt phase, did not possess a membranous layer. This is consistent with published accounts of resorption at this stage. Cuticle sampled on the merus of premolt crabs, in which new cuticle secretion had begun, did retain the membranous layer, indicating that the relationship of the membranous layer to molt stage is somewhat complex. Some samples with fully calcified cuticles also lacked the membranous layer. In these cases it is unclear if it was mechanically lost during preparation of the thin section or if the layer was resorbed in preparation for formation of a new cuticle (Drach, 1939; Travis, 1960; Skinner, 1962; Skinner et al., 1992; Promwikorn et al., 2005). The correlation of presence of membranous layer with more completely calcified cuticle suggests that the crabs were in the intermolt condition. Its presence in crabs that were actively forming a new cuticle seems to indicate the membranous layer does not necessarily get resorbed.

Lack of calcite within the membranous layer (Travis, 1963; Green and Neff, 1972) makes preservation and subsequent identification of this layer in fossil material unlikely. Vega et al. (1994), in a study of *Costacopluma mexicana* Vega and Perrilliat, 1989, described and illustrated a homogenous layer at the base of the cuticle that may represent the membranous layer, suggesting that its preservation is possible under some conditions. Regardless of preservation potential, presence or absence of the membranous layer appears to be of limited value in determining molt stage or the differentiation of exuvia from corpses in fossil material because of the apparent variation in timing of formation and possible sensitivity to sample location.

Ecdysial Sutures

At ecdysis, the cuticle ruptures along the ecdysial sutures allowing the organism to withdraw from the old exoskeleton. These sutures have been examined previously on the carapace of *Callinectes sapidus* (Priester et al., 2005). We examined the ecdysial sutures present on the merus (Fig. 1B) to observe their morphology and the extent that cuticle may be resorbed during ecdysis.

Ecdysial sutures on the merus of three intermolt crabs and nine premolt crabs were thin sectioned; three of the nine premolt crabs sectioned were in the process of forming a new cuticle beneath the existing one in preparation for the next molt (Fig. 4A). Sutures in all samples, regardless of molt stage, were visible on the exterior surface of the cuticle before preparation of the thin section. In thin section, a slight depression in the upper margin of the cuticle was visible in all cross-sections marking the suture location visible on the surface (Fig. 4A-C). Basal margins of the suture were variable to the extent that cuticle was deflected upward toward the exocuticle. Localized thinning of the cuticle at the suture zone should not be confused with a broader change of cuticle thickness that extends well beyond the suture zone such that cuticle on one side of the suture is thinner than the other (Fig. 4A-C); the thinnest

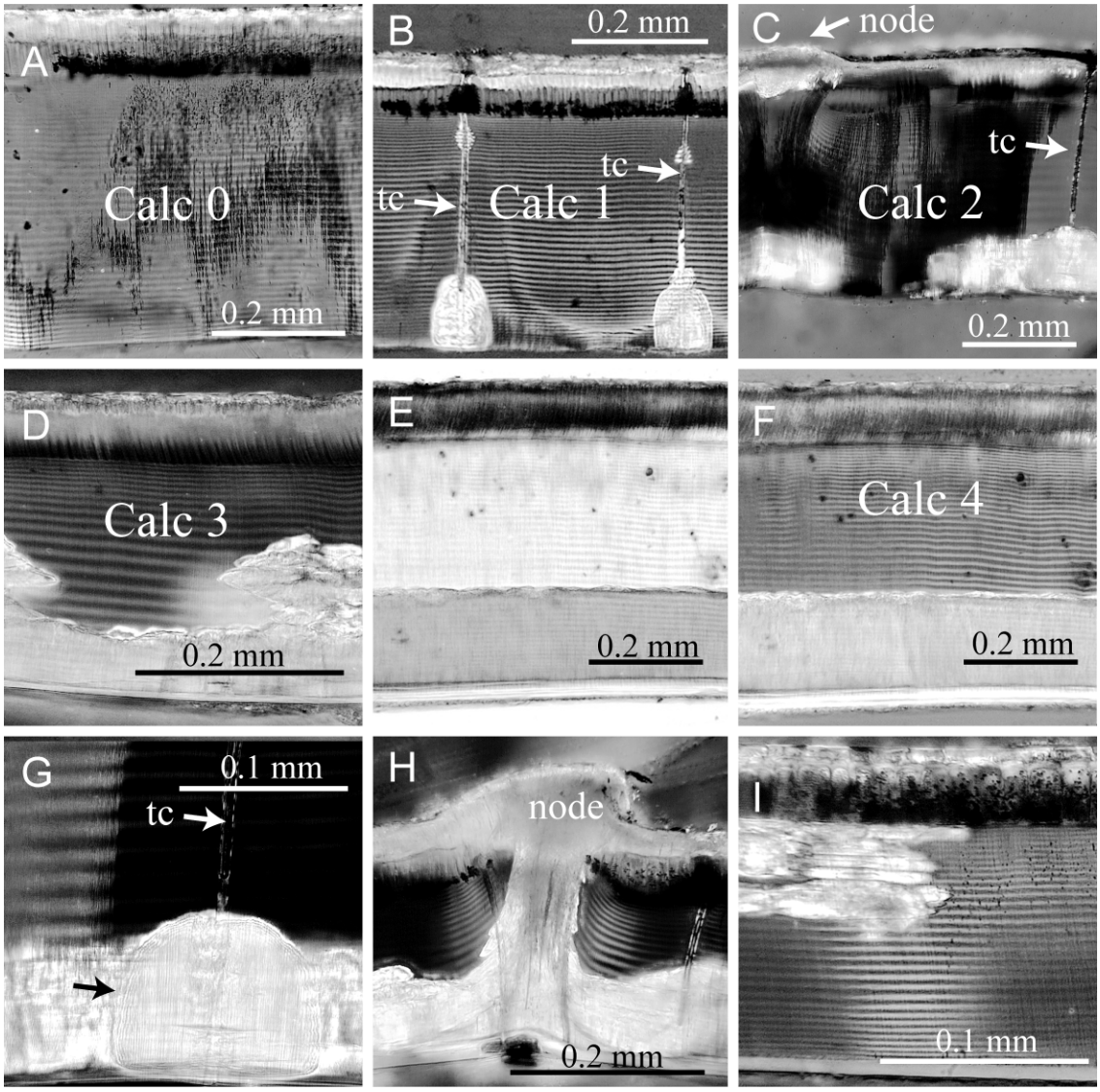

Fig. 3. Photomicrographs showing the distribution of calcite within the cuticle of *Callinectes sapidus*, all photomicrographs in XPL except for 3E, membranous layer present in 3.C, E, F, H, I. A, KSU D40, calcification pattern "0"; B, KSU D36, calcification pattern "1", upper exocuticle calcified, note calcite pillows at the base of the canals; C, KSU D30, calcification pattern "2", partial calcification of lower endocuticle, node at upper left, tegumental canal at right (tc); D, KSU D32, calcification pattern "3", calcification of lower endocuticle with uneven upper margin; E, KSU D39, PPL, same view as 3.6, note calcite in lower endocuticle is darker; F, KSU D39, calcification pattern "4", calcified lower endocuticle with horizontal upper margin; G, KSU D30, calcified lower endocuticle, distinct calcified region surrounding base of tegumental canal; H, KSU D32, node on upper surface with calcified core and lower endocuticle, composite photomicrograph from multiple planes of focus; I, KSU D40, note chitin birefringence in laminations and membranous layer and isolated calcified region of upper exocuticle.

part of the cuticle both marks this transition and the suture location.

Endocuticle within the suture zone was generally not calcified (Fig. 4A, C). At the lateral limits of the suture zone is a calcified region that extended throughout the entire thickness of the endocuticle, thus differentiating the suture margin from the remaining endocuticle (Fig. 4A, C). Only the upper portions of the exocuticle within the suture were calcified. The lower exocuticle and interprismatic septa remained uncalcified in contrast to adjacent exocuticle that was more completely calcified (Fig. 4C). Lower levels of calcification within the exocuticle of the suture zone may have allowed some deformation of the exocuticle during the drying and embedding process accentuating the concavity observed on the upper margin.

Premolt crabs that were actively secreting a new cuticle exhibited increased levels of cuticular thinning at the suture locations. This thinning of cuticle within the suture zone appears to correspond to the progression of new cuticle development. Crabs nearing ecdysis were not successfully sectioned; the sutures fractured during the drying and embedding process. Although these very weak sutures could not be examined in thin section, cuticle in the suture zone was clearly subject to further degradation.

In each thin section cut perpendicular to the long axis of the merus, two sutures were intersected. These sections

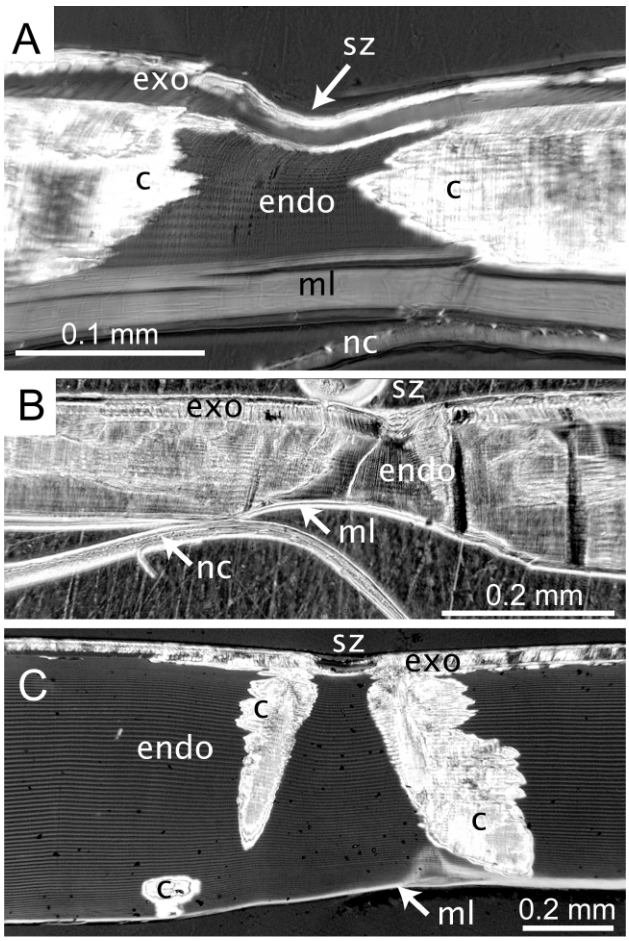

Fig. 4. A-B, two sutures on the merus of a single premolt *Callinectes sapidus*, 7.7 cm in length; A, suture under XPL, note layers of the endocuticle breaking away and merging into the membranous layer; B, Second suture from the same thin section as Fig. 4A, new cuticle (nc) under the existing cuticle, loss of lamination across the suture zone (sz) and retention of a thin membranous layer (ml), photographed under phase contrast illumination; C, Suture on the merus of a specimen field-identified as premolt, XPL, note lack of apparent thinning of cuticle within the suture, endocuticle marginal to the suture zone is calcified (c), depression of the exocuticle within the suture, and lower levels of calcification within the exocuticle.

show that thinning of the endocuticle within the suture zone is caused by reduction in lamination thickness across the suture (Fig. 4A), and in some cases, lamination loss (Fig. 4B). In one section, laminations separate from the endocuticle and appear to merge with the membranous layer (Fig. 4A). The membranous layer adjacent to the suture is thinner than within the suture region, suggesting that the membranous layer is locally increasing in thickness as laminations of the endocuticle become thinner and take on the appearance of the membranous layer. The second suture in the same specimen appears to have had the basal laminations of the endocuticle resorbed across the suture zone (Fig. 4B). This same suture exhibits a thin membranous layer, suggesting that endocuticular laminations were not necessarily incorporated into the membranous layer as observed in the previously described suture. It is hypothesized that either the missing laminations were subsumed by the membranous layer that was then partially resorbed or the laminations were simply resorbed without total loss of the underlying membranous layer. A larger number of samples and finer control of the molt-cycle are needed to refine the exact nature of cuticular thinning and degradation along ecdysial sutures. Our results suggest that a general thinning and weakening of the suture approaching the molt is observable, although our techniques did not provide a mechanism or quantification of the process. Our results, although not as detailed, are similar to the findings of Priester et al. (2005). Our data differ in that we have concentrated on the laminations and thickness, which have a greater preservation potential, than on the nature of proteins and other chemical changes that occur within, or immediately surrounding, the suture.

Node Construction

Nodes on the dorsal carapace are defined as isolated elevations of the surface, and in most cases they appear to be broader than high (Figs. 5, 2A). Nodes cover the entire dorsal carapace of *Callinectes sapidus* with the exception of the grooves separating raised regions of the carapace. Node tops generally slope toward the posterior, forming a subtle, anterior facing escarpment, most notably in sample location A (Fig. 5A, E, I). However, this form also occurs on other raised regions of the carapace. Observed in thin section, the exocuticle of the node tops is elevated and thinner than adjacent exocuticle (Fig. 2A). The endocuticular laminations are folded upward, entering the level of the cuticle that would normally contain the exocuticle (Fig. 2A). The height of the folded endocuticle forming the core of the node is higher than the actual expression of the node on the surface. Endocuticular folding is reduced basally so that laminations toward the inner surface of the cuticle are scarcely folded. Construction of the nodes does not vary across the sampled locations or during ontogeny. Size and density of node distribution does vary; this is discussed in following sections.

Setal Pits

Setal pits (Fig. 2B, E) are distributed over the entire carapace, including the carapace grooves. In thin sections that intersect pit centers, large tegumental canals can be seen extending from the setal pit to the base of the cuticle (Figs. 2B, 3B). These tegumental canals appear hollow in thin section, but they lose definition (optically) when intersecting calcified regions of the cuticle (Figs. 2B, 3G).

Calcification

Use of cross polarized light reveals only the crystallized carbonate phases within the cuticle. Therefore, the patterns of calcite distribution described herein are only of the crystallized portions that occur at a resolvable scale. Amorphous $CaCO_2$ is considered the precursor to crystallized calcite in the cuticle. This amorphous phase is then secondarily crystallized when a nucleation agent is introduced or a crystallization inhibitor is denatured (Roer and Dillaman, 1984; Coblentz et al., 1998; Tweedie et al., 2004; Dillaman et al., 2005). Distribution of calcite within the cuticle is variable both within and between specimens. Our results correspond with findings of Hegdahl et al. (1977) that the endocuticle does not show a homogenous distribution of

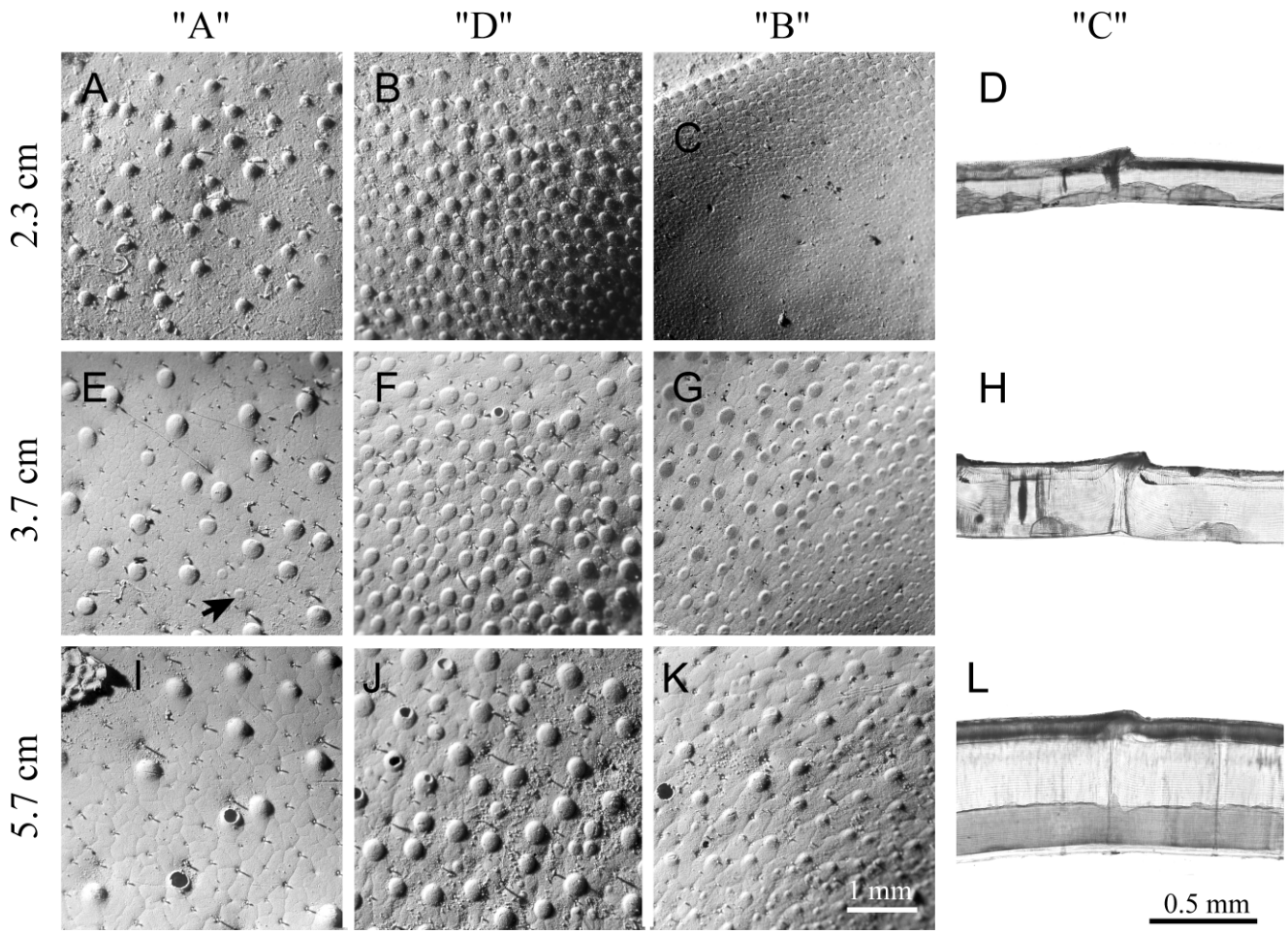

Fig. 5. Photomicrographic matrix of *Callinectes sapidus* surface features and thin sections. Rows represent individual sampled crab with length indicated at left. Columns represent sample locations (Fig. 1), with locations indicated at the top of each column. First row (A-D), KSU D23, 2.3 cm in length; second row (E-H), KSU D30, 3.7 cm in length, arrow indicates small node; third row (I-L), KSU D39, 5.7 cm in length. Thin section photomicrographs (D, H, L) taken under PPL. Scale for all surfaces shown in Fig. 5K; scale for all thin sections shown in Fig. 5L.

calcite. The extent and pattern of calcification at the sample locations was qualitatively scored by creating models of the distribution pattern constructed with observed variations in all the sections. These patterns of calcification are identified with numbers in Appendix 1 and are illustrated in Figure 3. Individual samples could then be matched to the model most closely characterizing the pattern observed in a single specimen. One pattern of calcification was found within the exocuticle, and five patterns were discernable within the endocuticle. In the exocuticle, calcification consisted of a continuous layer in the upper exocuticle (Fig. 3A-D). This layer in the exocuticle ranged from one-quarter to three-quarters of the upper exocuticle thickness. No samples contained a completely uncalcified exocuticle.

The endocuticle displays more diversity than the exocuticle in calcification patterns, ranging from complete calcification to near absence. The most typical form is a calcified layer that appears within the lower portion of the endocuticle, extending from the top of the membranous layer or base of the endocuticle in cases where the membranous layer is absent (Fig. 3F). The upper boundary of the calcified band is typically parallel to the surface of the cuticle and follows internal laminations. An additional pattern of calcification involves the same band of calcite within the lower endocuticle, but with isolated projections and irregularities of the upper limit (Fig. 3D). Calcite may also appear as isolated ''pillows'' at the base of the endocuticle (Fig. 3B). These pillows, when present, are typically observed with the same tegumental canals associated with the setal pits (Fig. 3B, D).

Calcified cuticle was also found in laminations that are folded to form the nodes (Fig. 3H). Recognition of calcite in the core of the node is dependent on the thin section bisecting the node axially. Since the orientation of the thin section relative to the node axis cannot be controlled finely enough to transect each node, spatial trends were not discernable.

Although the cuticle and structures of the dorsal carapace were the main focus of this study, some of the prepared sections also contained subdorsal portions of the carapace. Calcification in these regions differed from patterns observed in the dorsal carapace. These subdorsal portions of the carapace were the only regions that contained a fully calcified endocuticle. However, the distribution of calcite

was generally irregular. Limited sections retained the subdorsal portions of the carapace so the variation in these regions cannot be fully documented.

It appears that complete calcification of the upper exocuticle and lower endocuticle represent the maximum degree of calcification of the dorsal carapace in male *Callinectes sapidus* up to 6 cm in length. The exocuticle and the epicuticle are the first layers of the cuticle to calcify (Dillaman et al., 2005), and that condition apparently remains stable throughout ontogeny. Based on sections that contain less calcified regions (Fig. 3A-C, I), judging that they represent intermediate stages of calcification, a few hypotheses regarding the progression of mineralization within the endocuticle can be made. In one model, crystallization would originate in the endocuticle radiating from the base of the tegumental canals. As the calcification front spreads, nucleation points (Fig. 3B) would coalesce horizontally to form a discontinuous layer (Fig. 3C) and then finally a continuous layer of calcite within the endocuticle (Fig. 3F). Another possibility is that two different sources account for the calcification; one that originates from the tegumental canals and another originating from the pore canals. Fluids originating from the pore canals that appear at a much higher density than the tegumental canals, would account for the broad horizontal sheets that follow laminations of the endocuticle. A two-source model is supported by the seemingly different morphology of calcite surrounding the tegumental canals.

Calcification of the exocuticle in *Callinectes sapidus* proceeds simultaneously from both the top and bottom of the layer containing the inter-prismatic septa (Dillaman et al., 2005), making the presence of two sources in the endocuticle plausible. Both pore (Travis, 1957, 1963; Green and Neff, 1972) and tegumental canals would allow fluid exchange between the epidermis and the cuticle. Calcified cuticle within the lower endocuticle often maintains a horizontal transition with the upper non-calcified cuticle (Fig. 3F). The calcite surrounding tegumental canals can still be differentiated from the surrounding calcified cuticle when present, based on crystal orientations observed under cross-polarized light (Fig. 3G). The distinction between the smooth and jagged upper boundary of the calcite within the endocuticle may be somewhat artificial and may only represent an intermediate stage of calcification or intrinsic properties of the cuticle as it was formed. The largest crab studied that had a significant amount of calcite exhibited the most planar transition between calcified and uncalcified endocuticle. Larger crabs also have longer intermolt periods (Churchill, 1919; Vega-Villasante et al., 2007), allowing more time for mineralization and increasing the likelihood that they would be collected during the intermolt phase when the cuticle is fully formed and calcified. At this time it has not been possible to determine if the calcite surrounding nucleation points simply coalesces to form the planar layer of calcified cuticle in the lower endocuticle, or if two vectors exist for the distribution of $CaCO_3$ or fluids that initiate crystallization that result in two calcification patterns. Radiation from both point and linear sources fits the observed pattern seen at the base of the tegumental canals and suggests that, regardless of when the calcite is incorporated into the cuticle, the crystallization is secondary to the secretion of the bulk of the endocuticle. Secondary mineralization in the endocuticle of *Callinectes sapidus* has been observed by Roer and Dillaman (1993). This process must therefore take place after formation of the cuticle and is not compatible with the idea that the endocuticle is calcified at the same time that it is being formed (Drach, 1939; Travis, 1957, 1963). It is important to note that the crystallization does not necessarily take place at the same time that the calcium is incorporated into the cuticle, so the results from studies that use methods that detect calcium may differ from those using methods that only detect crystalline phases.

Morphometrics

Overview.—Morphometric analysis of decapod dimensions has been used extensively to study taxonomic, ontogenetic, and ecologic questions (Finney and Abele, 1981; Olmi and Bishop, 1983; Restrepo, 1989; Josileen and Menon, 2005; Guimarães and Negreiros-Fransozo, 2005; Rufino et al., 2006). Species of commercial value such as *Callinectes sapidus* have been studied more intensively in an effort to establish age and growth models used in assessing fishery conditions (Miller and Smith, 2003). Morphometric studies concerning parameters of the cuticle have been less common, although thickness of the cuticle in comparison to body volume has been studied in terms of its effect on the specific gravity of crustaceans (Pütz and Buchholz, 1991; Amato et al., 2008). Cuticle thickness is often a component in studies of the molt cycle used to monitor secretion of the new cuticle and resorption of the old cuticle; in these studies, effects of ontogeny are typically not incorporated (Pratoomchat et al., 2002). Our data cover many aspects of the cuticle including total thickness, exocuticle thickness, endocuticle thickness, node size, and the distribution density of nodes and pits on the carapace surface. When compared to carapace dimensions such as width and length, it is clear that these metrics are correlated with growth, although the absolute values and rates of change may vary with sample location on the carapace.

Pits and Nodes.—The node distribution density varies with both sample location and carapace size (Figs. 5, 6A). For nodes from a given sample location, density decreases with carapace size (Fig. 6A). Densities were highest in sample location B and lowest in E. The change in density shows positive allometry compared to carapace length for all sample locations. Although the growth constant is a negative value, its absolute value is greater than one (Table 1). Smaller nodes are apparently introduced between the larger nodes during growth (Fig. 5E). The effect on density is unclear, but introduction of smaller nodes must occur at a slower rate than the internodal space increases as shown by the continued decline in density. In carapaces approximately 4 cm in length there appears to be a subtle change in the rate of decline of the node density (Fig. 6A). This change may be a result of fluctuation in growth rates associated with reaching maturity.

Average node diameter is positively correlated with carapace dimension (Fig. 6B). The largest sizes appear in sample location A and smallest in location B. Average node

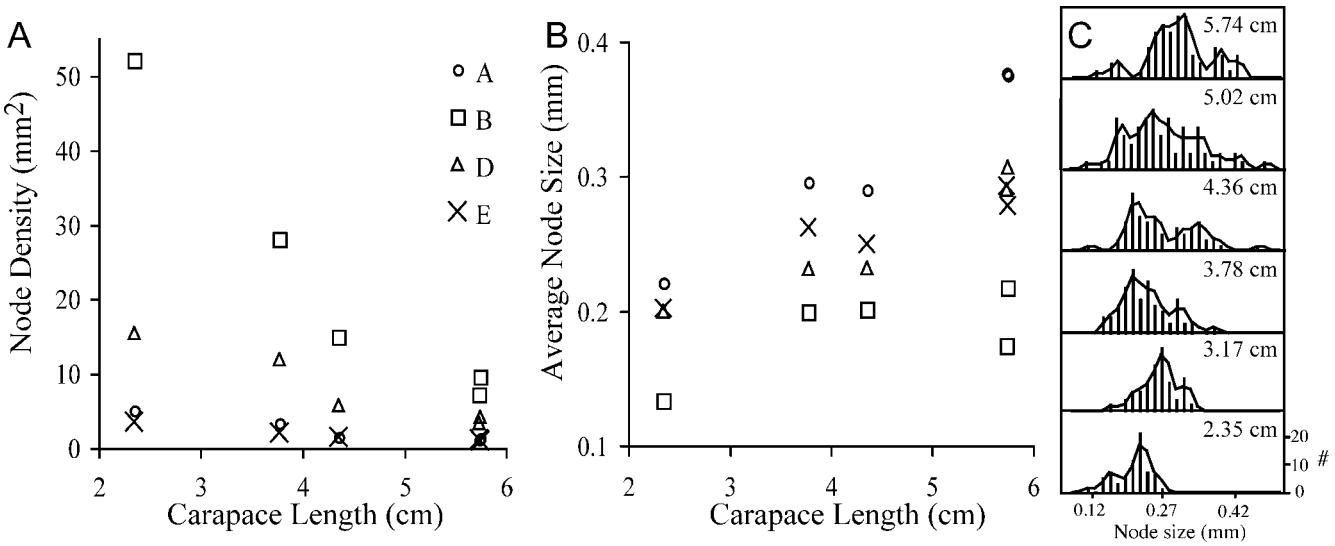

Fig. 6. Density and size of nodes distributed on the carapace of *Callinectes sapidus*; sample locations correspond to those shown in Fig. 1A, node distribution density plotted against maximum carapace length; B, average node size plotted against maximum carapace length; graph shares legend with Fig. 6A; C, histograms of node size distributions from the D sample location, carapace length indicated in upper right of each histogram; axes for all histograms on bottom histogram; # = Indicates number of values for a given size on the horizontal axis.

sizes from all sample locations have an allometric growth constant less than one and therefore have a negative allometry compared to carapace length (Table 1). Histograms of individual measurements made on the sample locations D show that the node size distribution is multimodal (Fig. 6C), suggesting continued growth of each node population introduced during successive molts. A stasis in node size expansion occurs around carapace lengths of 4 cm and may be caused by an increased rate in the introduction of smaller nodes, or, as will be discussed in following sections, may correspond to reaching sexual maturity. Histograms of node sizes indicate that there is a continual increase in the largest node size fraction (Fig. 6C). This increase is partially masked when comparing average values of the node sizes (Fig. 6B). The incremental nature of arthropod growth appears to be imprinted on the node size distribution and indicates that simple metrics such as average node size may mask some complexities.

An increase in node size and a decrease in node density with growth suggest that the epidermis does not secrete cuticle with nodes of a fixed density and size at each molt; rather, features are preserved and enlarged through successive instars. This can be seen in the continued increase of the largest node size fraction, but with retention of size classes in the histograms (Fig. 6C). The model of pure expansion of existing features described above is only one component of cuticle growth. The apparent insertion of new smaller nodes indicates that the epidermis does add new features at each instar.

The density distribution of setal pits, although variable between sample locations, shows an increase in density corresponding to carapace size, with an abrupt density decline in the largest specimens in all but location A and location E in one specimen (Fig. 7). The initial increase in pit density, in contrast to the continual decline of node density, indicates that setal pits are introduced at a rate fast enough to prevent the predicted decline that would occur on an expanding surface with a fixed number of pits. The small size of the pits in relation to the nodes resulted in difficulty counting pits at the scales used in the photomicrographs, especially when the surface was abraded. This problem reduces our confidence in this data, although the general trend of increasing density, followed by decline or stasis, does seem apparent.

The trend in pit density appears to correspond to changes in the percent increase in carapace dimensions that occur with each successive molt (Fig. 8). Although carapace size always increases at each molt, the magnitude and the percentage of that increase do not (Newcombe et al., 1949).

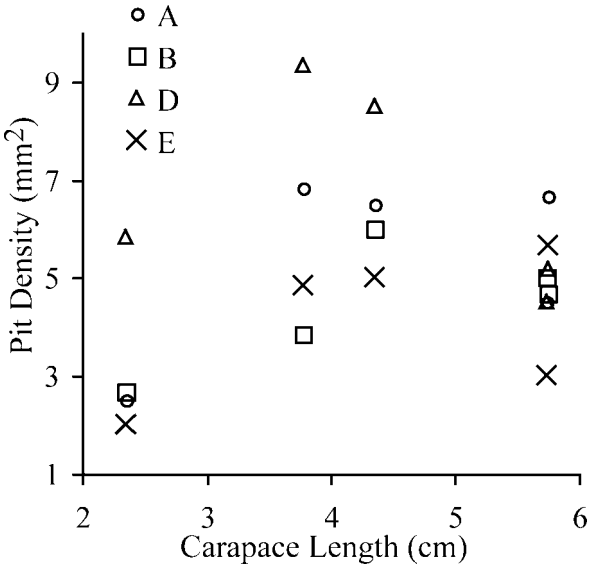

Fig. 7. Pit distribution density plotted against carapace length.

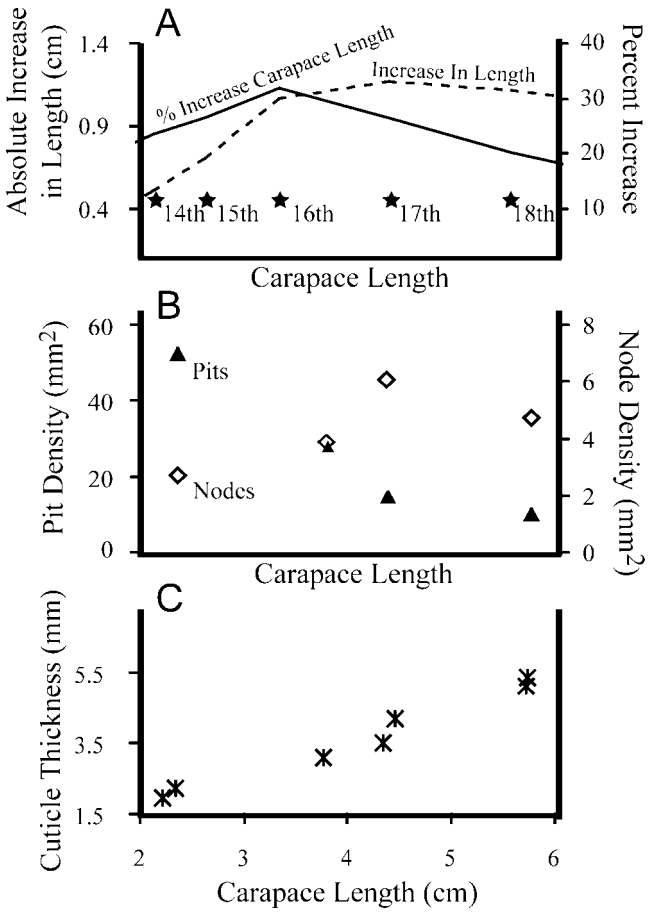

Fig. 8. Stacked graphs comparing previously collected data on increase in length from the last instar, and new data on the cuticle, all plotted against carapace length. A, all data from Newcombe et al. (1949), percent increase in carapace length from last instar for male *Callinectes sapidus* and the absolute increase in carapace length from the last molt, solid stars indicate average size at each instar, instar numbers indicated next to star; B, pit density and node density against carapace length; C, cuticle thickness.

Without more data it would be premature to directly attribute changes in pit densities to the percent increase of carapace dimensions at each molt, but it is apparent that the setal pit density is influenced by factors differing from those affecting node density and that the percentage that a cuticle must expand at each molt should influence the density of any cuticular surface feature.

Cuticle Thickness.—The total thickness of the cuticle sampled on the dorsal carapace is positively correlated ($R = 0.946$) with carapace size (Fig. 9A, B). Additionally, the cuticle thickness varies with sample location, even on the dorsal carapace of a single specimen (Fig. 9A, B). Total thickness of the cuticle, excluding the membranous layer, shows near isometric growth in sample locations A and C and negative allometry in the D and E sample locations (Table 1). The exocuticle and epicuticle, taken together, increase in thickness at a slower rate than the rate of increase of the endocuticle, resulting in a decrease in the percentage of the exocuticle contribution to the total thickness (Fig. 10), although the endocuticle and exocuticle both continue to thicken (Fig. 9C, D). The allometric equation for growth of the exocuticle in region D does not support the general trend of decreasing exocuticle growth rate seen in the other sample locations. Two of the specimens included in the D and E thickness measurements were in the process of forming a new cuticle and their inclusion may have both altered the allometry constants and accounted for the poor fit of the allometry curve ($R^2 = 0.794$) compared to the higher R^2 values for other regions.

The rate of increasing total thickness is fairly consistent between the sampled regions except for the largest samples, where the increase in thickness slows and the variability of thickness between sample locations is reduced (Fig. 9A, B). In the two smallest specimens, sample location D is the thickest; sample location C becomes the thickest in the mid-size carapaces. The variability in growth rates could be an artifact caused by differential inflation of carapace regions, mechanics of sampling a curved surface, or slight deviation in the selection of sample locations from specimen to specimen. Measurements of total cuticle thickness taken on an entire transect of one cuticle section show the high variability of thickness across the carapace and show the variation between sampled locations and possible errors induced by slight deviations from the intended sample locations (Fig. 11A). Variability along this transect shows that carapace cuticle thickness cannot be reduced to a single measurement and that cuticle thickness should be reported along with sample location.

Supporting the observation of increasing endocuticle thickness on the dorsal carapace, measurements taken of the endocuticle thickness on sections of the merus display a similar trend of increasing thickness (Fig. 11B). The endocuticle of the merus is initially thinner than the endocuticle of the dorsal carapace in crabs of smaller size. For carapaces around 5 cm in length the trend of endocuticle thickness in the merus appears to cross and become greater than the cuticle thickness of the carapace (Fig. 11B). The two points that deviate from this trend were two samples that came from the left merus, which is the appendage containing the smaller claw. This thinner endocuticle associated with the smaller of the two crabs suggests that the cuticle of the merus is also a function of claw asymmetry in addition to ontogeny.

Pütz and Buchholz (1991) studied cuticle thickness in an array of taxa and found that when compared to body volume, pelagic species did not continue to significantly thicken their cuticle during growth, while benthic and nekto-benthic species displayed continued increase in cuticle thickness. They attributed these different growth rates to selection pressure for increasing buoyancy by the reduction of heavy skeletal components such as the cuticle. It is difficult to compare data on cuticle thickness in relation to body volume because of the effect that body shape has on the rate volume increases with growth. Use of volume makes sense when density is concerned, as studied by Pütz and Buchholz (1991), but our data concerning the rate of size increase to cuticle thickness cannot be compared directly to their data. The well known swimming abilities of *Callinectes sapidus* should be aided by a decrease in density; therefore, there should be some selection pressure on maintaining a cuticle that is as thin and weakly calcified

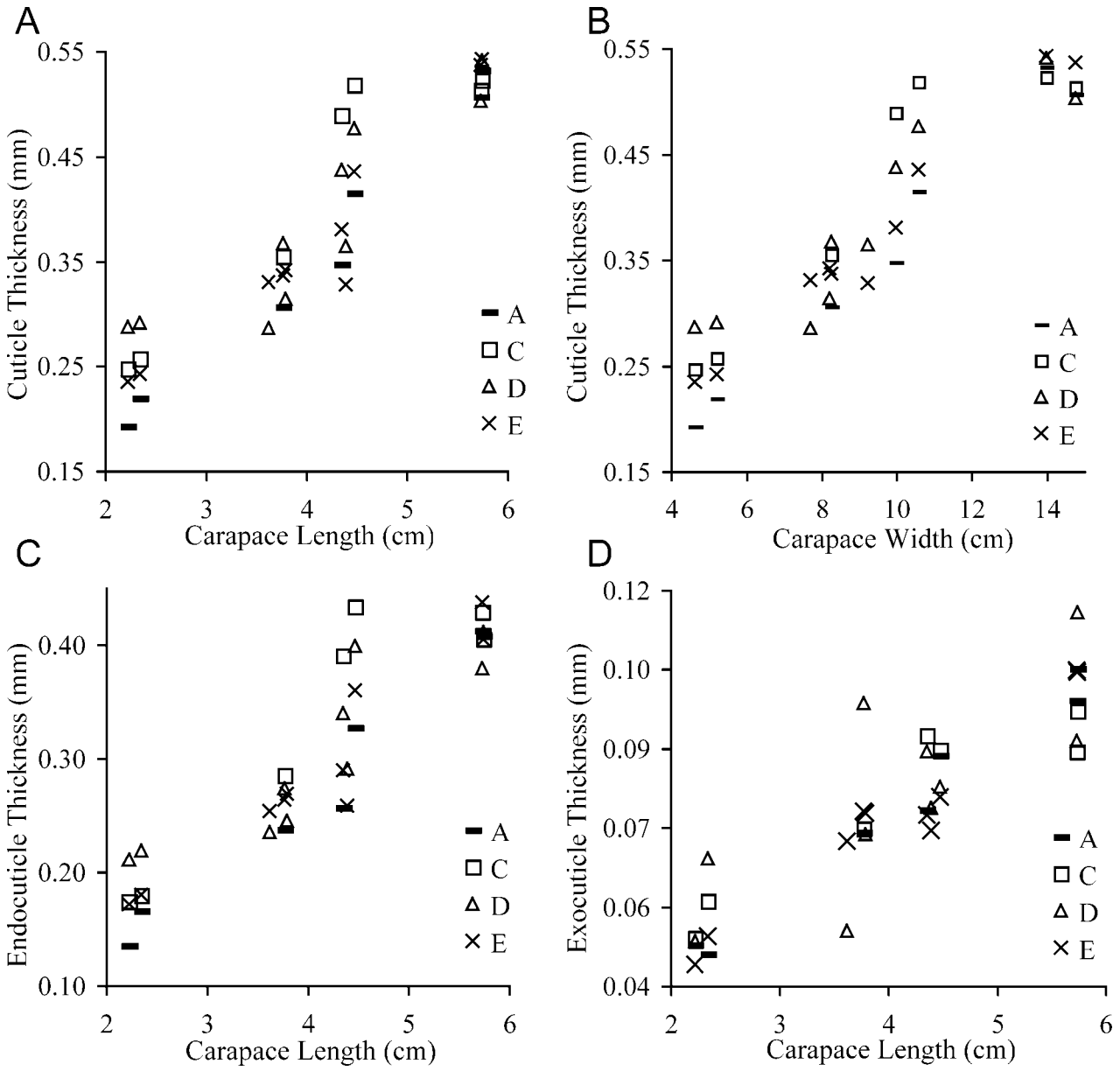

Fig. 9. Thickness measurements taken at site locations A, C, D, and E (Fig. 1), excluding the membranous layer, of the cuticle in *Callinectes sapidus*. A, total thickness plotted against carapace length; B, total thickness plotted against width; C, endocuticle thickness plotted against carapace length; D, exocuticle thickness, including epicuticle, plotted against carapace length.

as possible while maintaining enough strength to support and protect the body.

Discussion

Callinectes sapidus Cuticle

Although significant changes can be observed in the size and density distribution of nodes, density of setal pits, thickness of the cuticle, variations in the degree of calcification, and presence or absence of the membranous layer, the general construction of the cuticle and morphology of the nodes and setal pits do not vary significantly with carapace size. This is especially important in analysis of fossil material where only limited sample sizes may exist. Qualitative features of construction of nodes and setal pits appear to retain enough stability during ontogeny to be used in a taxonomic analysis without regard to carapace size. The quantifiable variation such as thickness of the cuticle, node and pit density, and node size can be measured and compared to other taxa, but carapace size must be included in the analysis. Although the non-intermolt specimens of *Callinectes sapidus* collected have not yet been fully examined, variation observed in the membranous layer and the degree of calcification, both factors known to be controlled by the molt cycle, was not great enough to alter other characters such as the morphology of pits and nodes significantly. Construction, size, and distribution of nodes and pits would remain unaffected by preparation for a molt.

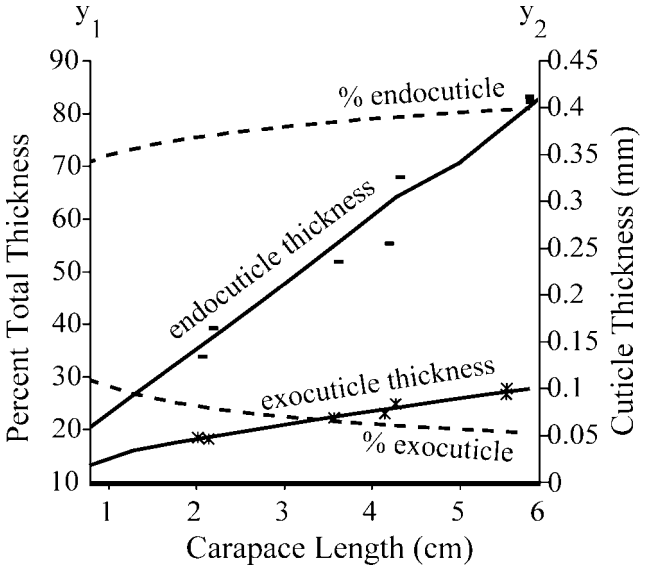

Fig. 10. Percent contribution of the endocuticle, axis y_1, upper dashed line, and exocuticle, lower dashed line, to the total cuticle thickness plotted against carapace length. Upper black line is the growth curve for endocuticle thickness, axis y_2, and lower black line is the curve for exocuticle thickness. Actual data points for thickness are also plotted and appear next to their respective growth curves.

The changes in calcification are significant even in samples of extant crabs judged to be in the intermolt stage, suggesting that distribution of calcite within the cuticle should be used with caution in taxonomic work involving fossils unless enough individuals of a population can be studied to ascertain the full range of variation and rule out diagenetic alteration in fossil specimens. The inconsistency in the presence or absence of the membranous layer, compounded by issues of preservation, make paleontologic use of the membranous layer problematic. In addition to the taxonomic implications, the thicknesses of the cuticle and the amount of calcite have adaptive implications such as protection and buoyancy regulation (Pütz and Buchholz, 1991; Amato et al., 2008).

Ontogenetic Implications

Decapods do not grow continuously, but rather grow incrementally at each molt. This incremental growth may vary in both the magnitude of expansion of a particular morphometric parameter and in the time interval between successive molts. Change of instar duration and rate of growth may correspond to events in the life cycle such as reaching the juvenile stage or attainment of sexual maturity (Haefner and Van Engel, 1975; Somerton, 1980; Hartnoll, 1978; Donaldson, 1981; Restrepo, 1989; Josileen and Menon, 2005; Guimarães and Negreiros-Fransozo, 2005). These growth parameters can be determined by observing laboratory-raised populations, or by carefully tagging and recollecting natural populations. The growth rate, or a change in growth rate, is determined by the comparison of two morphometric measurements in cases where age is unknown. Percent increase in growth at each molt and molt number are known in a few extant species, including *Callinectes sapidus*. In *C. sapidus*, shifts in intermolt duration and magnitude of growth can be seen in the seventh and sixteenth instars, corresponding to the end of prepuberty and sexual maturity respectively (Newcombe et al., 1949). Our data on the cuticle span the fourteenth and eighteenth instars in which sexual maturity is reached (Newcombe et al., 1949). These growth rate changes are apparent even in the limited data collected on the node and setal pit density, node size, and cuticle thickness in *C. sapidus* plotted with data from Newcombe et al. (1949) (Fig. 8). Populations of fossil decapods should exhibit similar growth rate changes, and the possibility exists that such

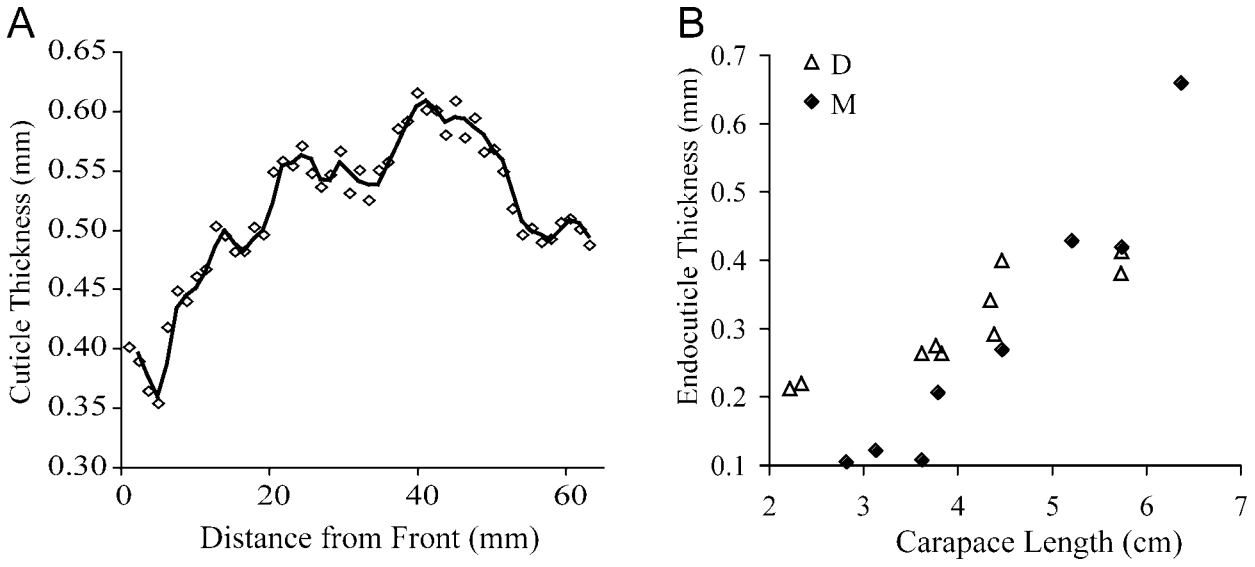

Fig. 11. A, Thickness measurements across the entire length of a thin section that contained sample locations E and D for a crab with a carapace length of 5.75 cm plotted against distance of the measurement from the anterior of the carapace, length on graph exceeds carapace length due to curvature of the dorsal carapace; B, Graph of endocuticle thickness of the merus (M) and sample location D on the dorsal carapace (Fig. 1A) plotted against carapace length.

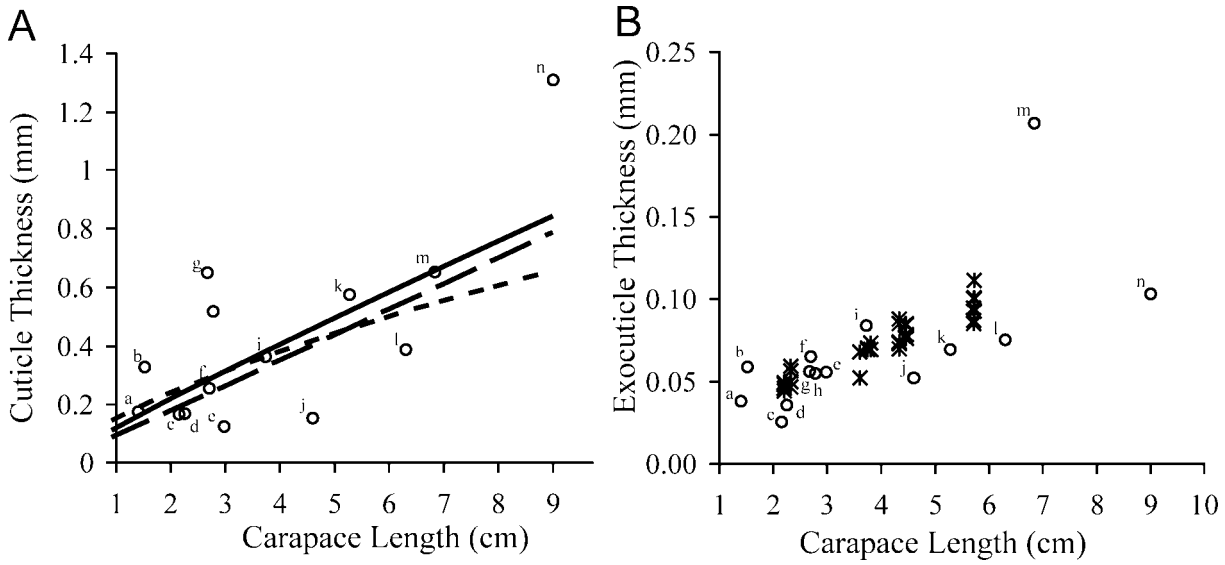

Fig. 12. Graphs showing cuticle morphometrics of *Callinectes sapidus* compared to other decapod taxa; A, Cuticle thickness plotted against carapace length; trend lines are allometric growth curves for cuticle thickness from the three sample locations to show the range in thicknesses for the different sample locations; open circles are single measurements taken on an array of different taxa; a, *Leptodius floridanus* (Gibbes, 1850); b, *Teratomaia richardsoni* (Dell, 1960); c, *Emerita talpoida* (Say, 1817); d, *Munida quadrispina* (Benedict, 1902); e, *Penaeus* sp.; f, *Lyreidus nitidus* de Haan, 1841; g, *Cycloes bairdi* Stimpson, 1860; h, *Calappa augusta* A. Milne-Edwards, 1880; i, *Callinectes sapidus*; j, *Hemigrapsus nudus* (Dana, 1851); k, *Hepatus* sp.; l, *Ovalipes catharis* (White, 1843); m, *M. novaezelandiae* (Jacquinot *in* Jacquinot and Lucas, 1853); n, *Carpilius maculatus* (Linnaeus, 1758); B, exocuticle thickness plotted against carapace length; crosses, *Callinectes sapidus* exocuticle thickness taken from all sample locations; open circles are the same taxa and labels as 12A.

metrics could be applied to fossil populations to infer their age structures and distinguish juveniles from adults if the species under examination shows a significant change in growth rate. Inclusion of a large number of measurements increases the likelihood of finding these rate changes because a change need not be apparent in all growth parameters. Identification of a change in the rate of growth is more likely to be recognized than is the distance between clusters representing the incremental growth at each instar, especially within small sample populations. Surface feature density is an attractive metric because the measurement is nondestructive and even cuticle which is damaged may retain remnants of surface sculpture. Further work will test the hypothesis that these rate changes can be recognized in fossil populations.

Comparison with Other Species

Working with a growth series of *Callinectes sapidus* allows morphometric data from cuticle to be examined in the context of ontogeny. If morphometric data of the cuticle are to be used in a phylogenetic context, the data must be demonstrated to be unique within a taxon and recognizably different between taxa.

Twelve taxa were compared to *Callinectes sapidus* in terms of total cuticle thickness (Fig. 12A) and exocuticle thickness (Fig. 12B), versus carapace length. Half of the taxa plotted within the maximum cuticle thickness range of *C. sapidus* constructed using the minimum and maximum growth curves for all samples of *C. sapidus* (Fig. 12B). The remaining taxa plotted outside the range, some to a significant degree. The intracarapace variation observed in *C. sapidus* becomes relatively small when compared to cuticle thicknesses in taxa of diverse taxonomic placement as seen in Figure 12A. Data collected from other species comes from individual specimens, so cuticle growth trends cannot be compared with those of *C. sapidus*.

A comparison of exocuticle thickness did not permit distinguishing between *Callinectes sapidus* and almost all the other taxa (Fig. 11B). *Metacarcinus novaezelandiae* (Jacquinot in Jacquinot and Lucas, 1853) (Fig. 12A, m) which has a thick exocuticle, was the exception. In all crabs, the exocuticle and epicuticle are formed premolt, which might explain the apparent lack of variation in exocuticle thickness, as increasing the thickness of premolt layers might hinder withdrawal of the body from the old cuticle at the time of ecdysis. Thus, the mechanics of withdrawing from the old cuticle may provide a physiological barrier to increase in exocuticle thickness. The endocuticle, because it is formed postmolt, appears to be the layer that varies to control the total thickness and strength of the cuticle, which in turn, alters strength and buoyancy of the carapace (Pütz and Buchholz, 1991).

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Appendix 1

Tabulated measurements taken on the dorsal carapace and on thin sections of *Callinectes sapidus*. SN, specimen number; Loc, cuticle sample location (Fig 1); Ctotal, total thickness of the cuticle (mm); Texo, thickness of the exocuticle (mm); Cendo, thickness of the endocuticle (mm); Cml, thickness of the membranous layer (mm), zero if absent; Nd, node density per mm^2; Pd, pit density per mm^2; Ns, average node size; W, carapace width (cm); L, carapace length (cm); Calc, calcification pattern as illustrated in Fig. 3.

SN	Loc	Ctotal	Texo	Cendo	Cml	Nd	Pd	Ns	W	L	Calc
12	D	0.364	0.074	0.290	0.000	—	—	—	9.24	4.36	3
12	E	0.327	0.069	0.258	0.000	—	—	—	9.24	4.36	3
15	D	0.289	0.052	0.237	0.000	—	—	—	7.71	3.63	3
15	E	0.320	0.067	0.253	0.010	—	—	—	7.71	3.63	3
19	D	0.313	0.069	0.245	0.000	—	—	—	8.22	3.84	3
19	E	0.341	0.073	0.268	0.000	—	—	—	8.22	3.84	4
23	A	0.211	0.046	0.165	0.008	5.00	2.50	0.221	5.21	2.35	3
23	C	0.235	0.056	0.179	0.022	—	—	—	5.21	2.35	3
23	D	0.278	0.059	0.220	0.008	15.33	5.83	0.199	5.21	2.35	3
23	E	0.229	0.049	0.180	0.012	3.50	2.00	0.202	5.21	2.35	3
23	B	—	0.000	—	—	52.00	2.67	—	5.21	2.35	—
26	A	0.183	0.048	0.135	0.009	—	—	—	4.63	2.23	4
26	C	0.223	0.049	0.174	0.024	—	—	—	4.63	2.23	4
26	D	0.257	0.046	0.212	0.027	—	—	—	4.63	2.23	4
26	E	0.215	0.044	0.171	0.019	—	—	—	4.63	2.23	4
30	A	0.306	0.069	0.237	0.000	3.33	6.83	0.295	8.27	3.78	1
30	C	0.354	0.070	0.285	0.000	—	—	—	8.27	3.78	1
30	B	—	0.000	—	—	28.00	3.83		8.27	3.78	—
32	A	0.329	0.073	0.256	0.018	1.50	6.50	0.290	10.00	4.36	4
32	C	0.478	0.087	0.390	0.011	—	—	—	10.00	4.36	4
32	D	0.424	0.084	0.340	0.013	5.67	8.50	0.232	10.00	4.36	4
32	E	0.361	0.072	0.289	0.018	1.50	5.00	0.249	10.00	4.36	4
32	B	—	0.000	—	—	14.83	6.00	—	10.00	4.36	—
36	A	0.410	0.084	0.327	0.004	—	—	—	10.60	4.48	1
36	C	0.518	0.085	0.433	0.000	—	—	—	10.60	4.48	3
36	D	0.476	0.078	0.398	0.000	—	—	—	10.60	4.48	1
36	E	0.435	0.076	0.359	0.000	—	—	—	10.60	4.48	0
39	A	0.507	0.100	0.407	0.025	1.33	6.67	0.375	14.00	5.75	4
39	C	0.497	0.092	0.405	0.026	—	—	—	14.00	5.75	4
39	D	0.522	0.111	0.411	0.019	4.17	5.17	0.306	14.00	5.75	4
39	E	0.504	0.099	0.404	0.038	1.00	5.67	0.278	14.00	5.75	4
39	B	—	0.000	—	—	9.50	4.67	—	14.00	5.75	—
40	A	0.506	0.094	0.412	0.000	1.17	4.50	0.376	14.78	5.74	4
40	C	0.513	0.084	0.428	0.000	—	—	—	14.78	5.74	0
40	D	0.465	0.086	0.379	0.037	3.33	4.50	0.290	14.78	5.74	4
40	E	0.536	0.100	0.437	0.000	1.17	3.00	0.292	14.78	5.74	0
40	B	—	—	—	—	7.17	5.00	—	14.78	5.74	—