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SYMPOSIUM

Exoskeletons across the Pancrustacea: Comparative Morphology, Physiology, Biochemistry and Genetics

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Synopsis The exoskeletons of pancrustaceans, as typified by decapod crustaceans and insects, demonstrate a high degree of similarity with respect to histology, ultrastructure, function, and composition. The cuticular envelope in insects and the outer epicuticle in crustaceans both serve as the primary barrier to permeability of the exoskeleton, preventing loss of water and ions to the external medium. Prior to and following ecdysis, there is a sequence of expression and synthesis of different proteins by the cuticular epithelium for incorporation into the pre-exuvial and post-exuvial procuticle of insects and the exocuticle and endocuticle of crustaceans. Both exhibit regional differences in cuticular composition, e.g., the articular (intersegmental) membranes of insects and the arthrodial (joint) membranes of crustaceans. The primary difference between these cuticles is the ability to mineralize. Crustaceans' cuticles express a unique suite of proteins that provide for the nucleation and deposition of calcium carbonate. Orthologs of genes discussed in the present review were mined from a recently completed cuticular transcriptome of the crayfish, *Cherax quadricarinatus*, providing new insights into the nature of these proteins.

Introduction

One of the defining characteristics of the Arthropoda is the possession of a rigid exoskeleton comprised of chitin and protein as its principal organic components. Being enclosed in such a rigid exoskeleton requires that all arthropods must undergo a molt or ecdysis in order to metamorphose and grow. Thus, many of the features of the exoskeletons of the Pancrustacea are common across modern taxa and presumably predate the divergence of the Malacostraca and Hexapoda. Others, such as calcification, are nearly ubiquitous in the non-hexapod Pancrustacea and extremely rare in the Hexapoda. The similarities are reflected in the morphology of the cuticular layers, their deposition and sclerotization, and common motifs in their structural proteins. Differences are apparent in proteins involved in mineralization and, perhaps, in the timing of biochemical changes in the cuticle following ecdysis. In this review the above similarities and differences found in the literature are further discussed in light of a case study of a molt-related transcriptomic library recently established for a crayfish (Abehsera et al. 2015). This library originates from different exoskeletal-forming epithelia at four distinctive molt stages thus is representative of the heavily calcified crustacean cuticle as opposed to the non-mineralized insect cuticle.

Morphology

While the morphologies of the decapod and hexapod exoskeletons are very similar at the level of the light microscope (Fig. 1) and electron microscope (Fig. 2), the nomenclatures that describe them, and which are now well-accepted, differ. Both possess an outer layer comprised of two distinct regions. These are designated as the outer and inner epicuticles in the decapods (Compère 1995; Dillaman et al. 2013) and the envelope and epicuticle in the hexapods (Locke 2001). In the decapods, the outer epicuticle is

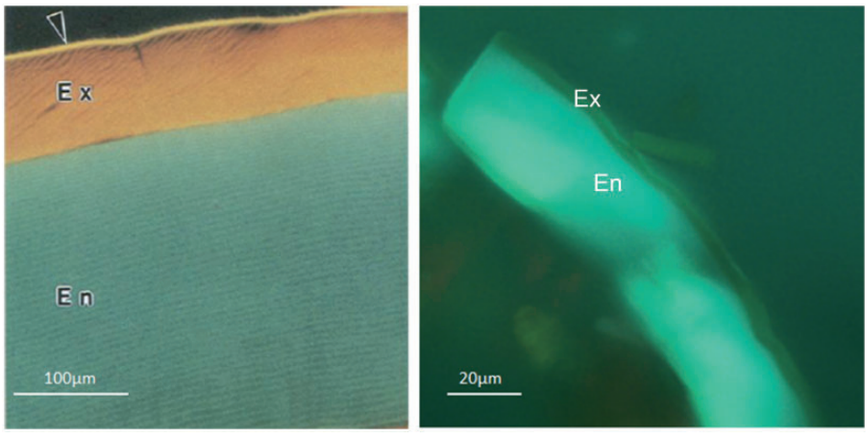

Fig. 1 Epifluorescent light micrographs of the exoskeletons of the dorsal carapace of the blue crab, *Callinectes sapidus* (left), and the pronotum of the field cricket, *Gryllus pennsylvanicus* (right). The cuticle was fixed in alcoholic formalin and stained with acridine orange. Note that in both tissues, the acridine orange differentiates the exocuticle (Ex) from the endocuticle (En). The epicuticle (visible in the crab's exoskeleton, arrowhead) is autofluorescent. The image of the blue crab's cuticle modified from Marlowe and Dillaman (1995) with permission from the publisher.

bilaminate, displaying an outer surface coat and an inner cuticulin layer that has five distinguishable sublayers. The hexapod envelope is trilaminate in appearance (Locke 2001). In both taxa, the outer regions lack chitin and are composed largely of lipid and protein. These outer regions are important as barriers to permeability (see Hadley [1994] for review), and as protection from abrasion and infection. The establishment of the permeability barrier relative to the molt cycle is discussed below.

The exocuticle and endocuticle of the decapods are collectively designated as the procuticle in hexapods (Fig. 3) (Roer and Dillaman 1984; Locke 2001). In both taxa they have an organic matrix that is principally formed from chitin and protein microfibers that are laid down in parallel sheets. Each sheet is slightly offset in its orientation from the one above it, forming a helicoidal arrangement first described by Bouligand (1965, 1972) for the decapod exoskeleton. In cross-section, this arrangement gives rise to the lamellate appearance of these cuticular layers, such that each lamella represents a rotation of 180° in the orientation of the fibers (Fig. 4). The exocuticle of decapods and the outer portion of the procuticle in hexapods (along with the epicuticles and envelope) are deposited prior to ecdysis and are, therefore, called pre-exuvial layers. Subsequent to ecdysis, both the pre-exuvial exocuticle and the procuticle are hardened by quinone crosslinking or sclerotization. This process entails the active secretion of acyldopamines into the cuticle from the underlying epithelium and the conversion of these compounds to quinones by phenyloxidase (Andersen 2010). The secretion and the initiation of sclerotization appear to be under the control of the hormone bursicon in both taxa (Luo et al. 2005; Wilcockson and Webster 2008); however, the mechanism of transport of compounds into the pre-exuvial layers is unknown.

Unlike most hexapods, decapods commonly impregnate the largest part of their epicuticles, exocuticles, and endocuticles with calcium carbonate, either in the form of calcite or amorphous calcium carbonate (Dillaman et al. [2013] for review). Even in the heavily mineralized decapods, certain cuticularized regions remain uncalcified, notably the arthrodial membranes at the joints of appendages, the lining of the branchial cavity, the gills, and portions of the foregut and hindgut. It is also vital that the pre-exuvial layers that are destined to mineralize do not do so until after ecdysis. The control over which areas mineralize and when that occurs resides in the composition of the cuticular proteins (CPs) and glycoproteins, and in their postecdysial alterations, as is discussed below (Roer and Dillaman 1984, 1993; Shafer et al. 2006; Dillaman et al. [2013] for review).

There are a few examples of hexapods in which regions of the cuticle mineralize, at least during some developmental stages. While not strictly an example of calcification, the tips of ovipositors of the parasitic wasps *Gabunia* sp. (Ichneumonidae: Cryptinae) from Uganda and the cosmopolitan *Heterospilus prosopidis* (Braconidae: Doryctinae) display high concentrations both of calcium and of manganese in their cuticles (Quicke et al. 2004). The face fly, *Musca autumnalis*

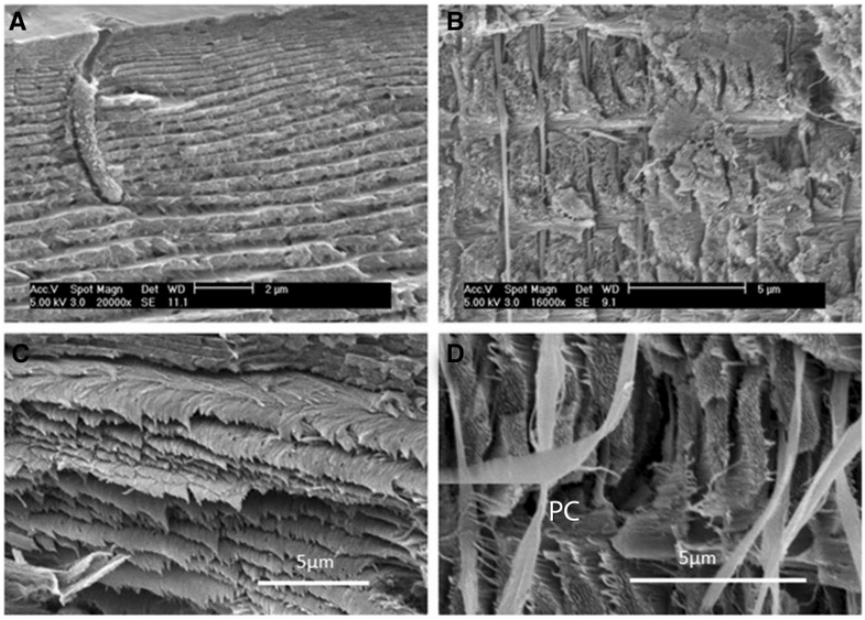

Fig. 2 Scanning electron micrographs of the exoskeletons of the pronotum of the field cricket, *Gryllus pennsylvanicus* (**A**, **C**) and the dorsal carapace of the blue crab, *Callinectes sapidus* (**B**, **D**). Note that both tissues display lamellae (A, B) that are comprised of parallel sheets of chitin–protein fibrils whose orientation changes between layers (C, D). Pore canals (PC) are evident throughout the exocuticles and endocuticles in the crab (D), but are not apparent in those of the insect (C).

and house fly, *M. domestica* (Diptera: Muscidae), produce a calcified puparium (Fraenkel and Hsiao 1967; Gilby and McKellar 1976; Grodowitz et al. 1987). The closest homolog to the mineralized decapod exoskeleton is found in certain members of the Coleoptera. Within the family Tenebrionidae, the subfamily Phrenapetinae contains a number of genera in which the cuticle is impregnated with calcium carbonate (Leschen and Cutler 1994) (Fig. 5). Although some analysis of the amino-acid composition of the organic matrix of the puparium was performed (Bodnaryk 1972), no comparison of the proteins expressed in the mineralized hexapod cuticle to those of the decapods has been made.

Beneath the decapod endocuticle lies the membranous layer. This layer resembles the endocuticle in its lamellate structure, but remains uncalcified. There is no similar structure described in the literature on hexapods. It may be that the function of the membranous layer is to limit the extent of mineralization of the innermost endocuticle. As such, it would not be expected to be deposited in most hexapods. An investigation of whether or not a membranous layer is present in the calcified cuticles of beetles would provide further evidence of its potential role in this regard.

The epithelium that underlies the cuticle bears numerous microvilli that extend up through the exocuticle and into the inner epicuticle in most regions of the decapod exoskeleton, thereby forming pore canals. In the dorsal carapace of the green crab, *Carcinus maenas*, the diameter of the typical pore canal is approximately 0.4 µm and there are nearly 950,000 pore canals/mm^2 of epithelial surface (Roer 1980). Pore canals are not universally present in the cuticle of decapods; they are conspicuously absent in the cuticle of gills, for example (Dickson et al. 1991). While pore canals do occur in hexapods, they are not always apparent, even in the pronotum (Fig. 2), a region that would correspond to the dorsal carapace of decapods (Fig. 2). Decapods' pore canals are likely involved in the post-ecdysial mineralization of the pre-exuvial layers (Roer 1980), and probably contribute to the transport of enzymes and aclydopamines as well. Their role in the hexapods and the mode of transport in regions that lack pore canals needs to be investigated.

Resorption and deposition during the molt cycle

The dynamics of cuticular resorption and deposition in relation to the molt cycle of decapods and hexapods have been extensively reviewed (Roer and Dillaman 1993; Moussian 2010). There are a number of phenomena that emerge when comparing these two taxa, however, that bear further scrutiny and future research.

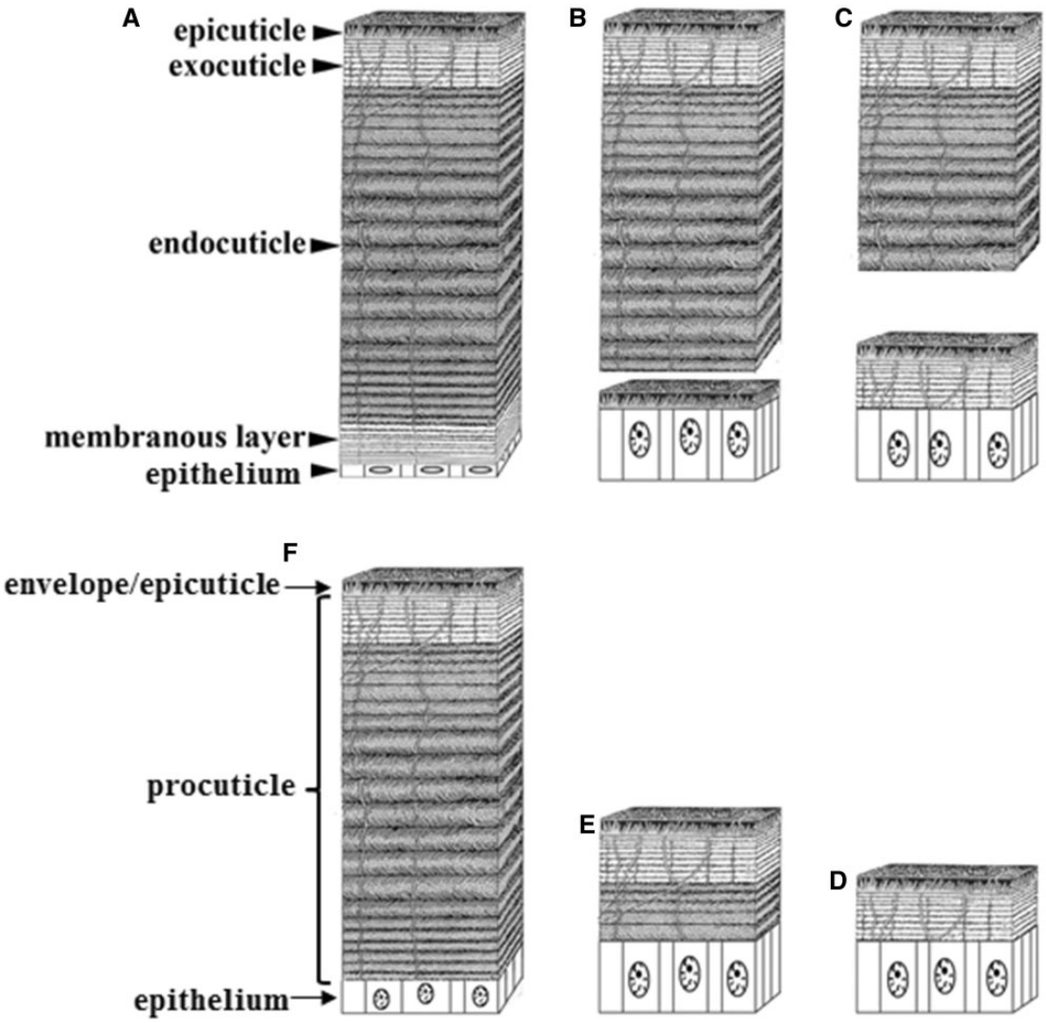

Fig. 3 Schematic representation of the pancrustacean exoskeletal structure and its changes throughout the molt cycle. The nomenclature for the intermolt cuticle of decapods is shown in **A**, while that for hexapods is shown in **F**. The outer epicuticle of the decapod corresponds to the envelope in the hexapods, and the decapod's inner epicuticle is called the epicuticle in the hexapods. The decapods' exocuticle and endocuticle are the procuticle in the hexapods. The onset of pre-molt in both taxa is marked by the separation of the epithelial layer from the overlying old cuticle (**B**). During late pre-molt, the pre-exuvial layers (the new epicuticle and exocuticle in decapods; the envelope, epicuticle, and pre-exuvial procuticle in hexapods) are deposited beneath the old cuticle (**C**). Ecdysis (**D**) and post-molt deposition of the endocuticle (decapods) and post-exuvial procuticle (hexapods) continues (**E**, F) until the exoskeleton is fully formed in intermolt (A). Modified from Dillaman et al. (2013).

During premolt, there is the simultaneous enzymatic digestion of the old exoskeleton (or puparium in hexapods) and deposition of the pre-exuvial layers of the new exoskeleton (Fig. 3). Degradation of the old cuticle is accomplished by the secretion of chitinases, chitobiases, and proteases into the molting space. This poses a potential problem since the components of the new and old cuticle are chemically identical and, therefore, the newly synthesized cuticle might be susceptible to degradation by the enzymes digesting the old exoskeleton.

It has been proposed that the new cuticle in hexapods is protected by the envelope. Locke (2001) suggested that the envelope becomes impermeable as soon as it is fully synthesized by the epithelium. If that is the case, the envelope would isolate the newly synthesized chitin–protein matrix from the molting space and the enzymes that act on the old exoskeleton. However, an impermeable envelope would prevent the resorption of the products of the breakdown of the old cuticle through the new cuticle and epithelium into the hemolymph. Indeed, Cornell and Pan (1983) and Yarema et al. (2000) provided evidence that the molting fluid containing breakdown products (e.g., glutamate) are not absorbed across the integument, but passed either

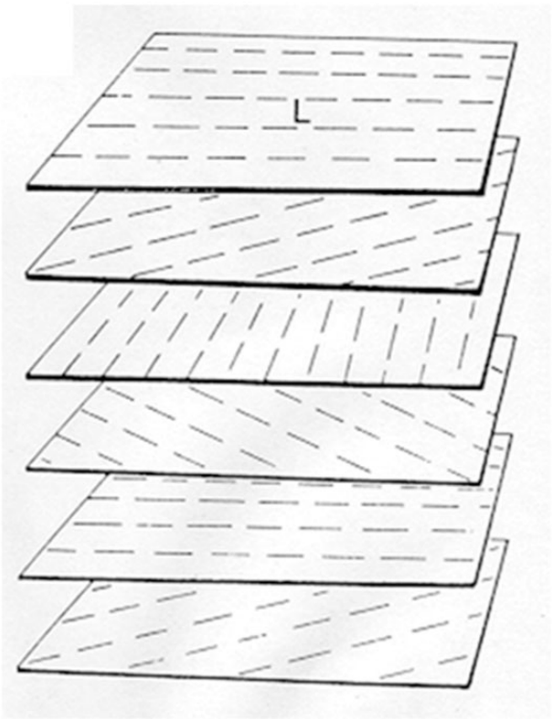

Fig. 4 The changing orientation of chitin–protein fibrils in the cuticle. A 180° rotation in the orientation of the layers (L) produces a lamella within the cuticle. From Bouligand (1972) with permission from the publisher.

anteriorly or posteriorly and ingested via the mouth or anus. However, Lensky et al. (1970), using Buffalo Black dye, demonstrated that molting fluid in the abdomen of *Cecropia* was resorbed through pits in the underlying new envelope. The pits were associated with tonofibrillar attachments and became impermeable to dye after the moth emerged from the puparium.

In the decapods, it is clear that the pre-exuvial cuticle is permeable and is the site of resorption, at least of Ca^{2+} that is released from the old cuticle (Roer 1980). Williams et al. (2009) demonstrated that the pre-exuvial cuticle of the blue crab, *Callinectes sapidus*, remained permeable to water and p-nitrophenylphosphate (used as a tracer) until just after molting (Fig. 6). Permeability decreased markedly within the first 15 min postmolt, and was entirely impermeable by 1 h after ecdysis. The change in permeability was associated with an alteration in the structure of the outer epicuticle (Fig. 7), and transmission electron microscopy, using La^{3+} as a marker, showed that the outer epicuticle was indeed the barrier to permeability. The change in permeability precedes the initiation of postmolt mineralization in the epicuticle. The first evidence of mineral deposition in *Callinectes* does not appear before 2 h postmolt (Dillaman et al. 2005).

Thus, in the decapods and in at least some regions of the hexapods, the new cuticle may be exposed to the enzymes that are actively digesting the old cuticle. Data from the literature on hexapods suggest that protection of the new cuticular chitin–protein fibrils may be afforded by a highly conserved protein (Knickkopf) that is incorporated into the newly synthesized cuticle by the underlying epithelium (Chaudhari et al. 2011). The Knickkopf protein was initially implicated in cuticular synthesis in *Drosophila melanogaster*, where *knk* deletion mutants exhibited cuticular defects (Ostrowski et al. 2002). First, Chaudhari et al. (2011) established that, in fact, chitinases co-located with chitin in the newly deposited cuticle of the red flour beetle, *Tribolium castaneum*. This observation suggested that the envelope may not provide protection to the new cuticle from the degradative enzymes in the molting fluid/space. They then identified a *T. castaneum* ortholog (*TcKnk*) of the *D. melanogaster* gene, and determined that dsRNA-mediated knockdown of *TcKnk* resulted in lethal defects in molting in all stages of larval, pupal, and adult development. Quantitative chemical analyses and immunohistochemistry showed that RNAi of *TcKnk* resulted in near-total loss of cuticular chitin in the newly secreted cuticle. The loss of chitin was due to the action of secreted chitinases, as simultaneous knockdown of two chitinases genes (*TcCht-5* and *TcCht-10*) restored the chitin to control levels (Chaudhari et al. 2011). Finally, TcKnk colocalizes with chitin in the newly synthesized cuticle, but is absent from the old cuticle that is being degraded.

Knickkopf protein orthologs were subsequently found to be present in most taxa with chitin in their extracellular matrices, except fungi, and including one non-hexapod pancrustacean, *Daphnia pulex*. Here we report the first evidence of a knickkopf protein ortholog in the Malacostraca. In the transcriptomic library of the crayfish (*Cherax quadricarinatus*) that we studied, a knickkopf ortholog showing a high similarity to known hexapod knickkopf proteins was found, and named Cq knickkopf protein (KR025533). It is interesting to note that the expression pattern throughout the molt cycle of this knickkopf ortholog (Fig. 8) is similar to the previously described expression pattern of genes related to chitin metabolism (Abehsera et al. 2015). In the cuticle-forming epithelium the expression pattern is molt-independent, being expressed through the entire molt cycle with no differences. In the gastrolith-forming epithelium the expression pattern

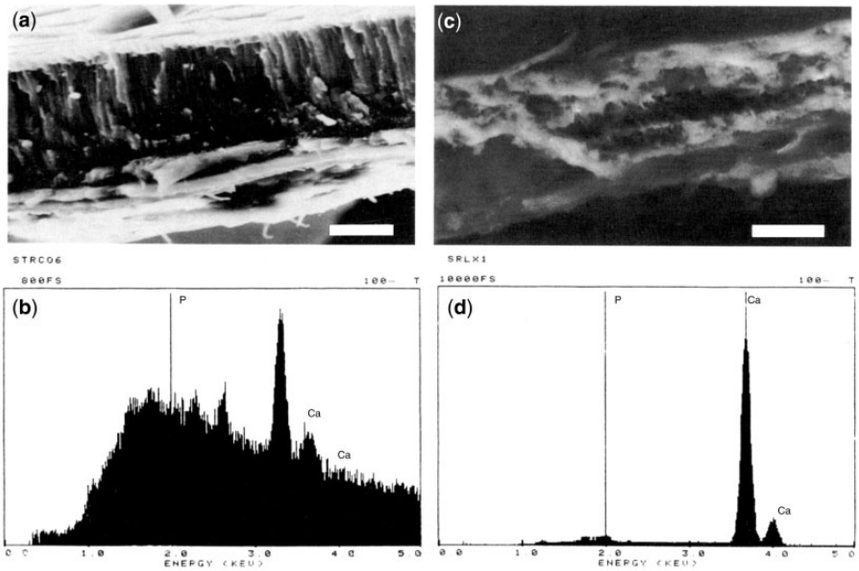

Fig. 5 Scanning electron micrographs and energy-dispersive analysis of X-ray (EDAX) spectra of the cuticles of two species of tenebrionid beetles. The cuticle of *Tribolium confusum* (**A**) shows no evidence of mineralization, confirmed by a lack of a Ca peak in the EDAX spectrum (**B**, full scale = 800 counts). The cuticle of *Zypoetes epieroides* (**C**) shows a dense cross-section characteristic of mineralized cuticle, confirmed by a pronounced Ca peak in the EDAX spectrum (**D**, full scale = 10,000 counts). From Leschen and Cutler (1994); the labels for the Ca_{Ka} peaks have been enhanced.

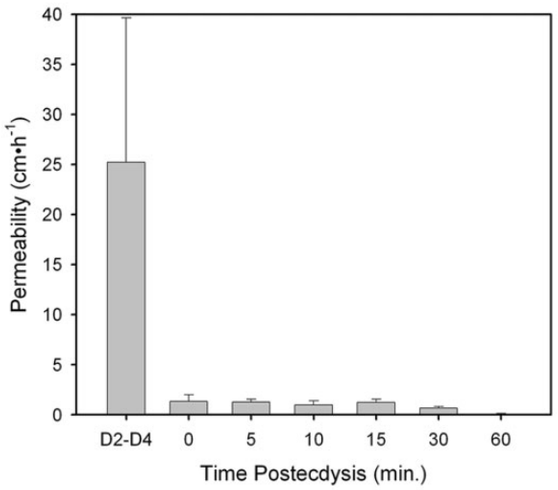

Fig. 6 Change in cuticular permeability between late pre-molt (stages D_2–D_4) and times immediately following ecdysis in the blue crab, *Callinectes sapidus*. Permeability was assessed *in vitro* using p-nitrophenol as a marker. Note the pronounced decrease in cuticular permeability upon ecdysis. From Williams et al. (2009) with permission from the publisher.

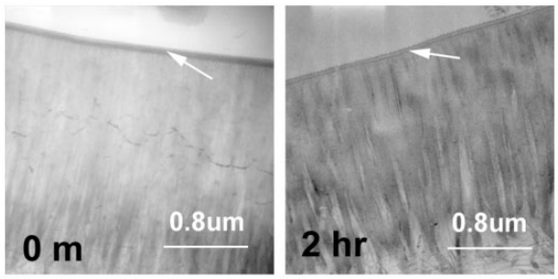

Fig. 7 Transmission electron micrographs of the epicuticle of the dorsal carapace of the blue crab, *Callinectes sapidus*. Note the change in the appearance of the outer epicuticle (arrows) between a newly molted crab (0 m) and one 2 h after ecdysis (2 h). The outer epicuticle changes from an amorphous morphology to a trilaminate structure. The changes correspond to the formation of a permeability barrier in this layer. Modified from Williams (2000).

is molt-related, being highly expressed during premolt. The search for knickkopf orthologs among other taxa of the Pancrustacea should be a focus of future investigation.

Deposition of the new cuticle during premolt follows the separation of the epithelium from the old exoskeleton, referred to as apolysis. It is generally agreed that the formation of the new envelope and epicuticle in the hexapods and the epicuticle in the decapods occurs by self-assembly after components are secreted from vesicles of the underlying epithelium (Leopold et al. 1992; Compère 1995; Locke 1998). While some question remains regarding the role of epithelial plaques, electron-dense regions at the apex of epithelial microvilli, in the organization of the epicuticular layers. The absence of plaques in regions of

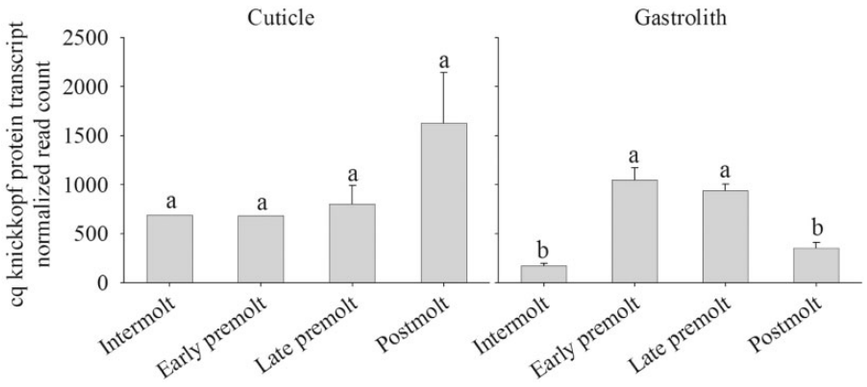

Fig. 8 Normalized read count of cq knickkopf protein transcript. Normalized read count from the cuticle-forming epithelium (left) and the gastrolith-forming epithelium (right). The *X*-axis represents the four molt stages: inter-molt, early pre-molt, late pre-molt, and post-molt. Letters represent statistical groups that are significantly different ($P < 0.05$). Error bars represent standard error.

the cuticle in the green crab, *C. maenas*, and the fact that full assembly of the outer epicuticle occurs at some distance from the epithelium preclude direct organization of the layers by the epithelial microvilli in decapods (Compère 1995).

Following the formation of the epicuticle, the epithelial cells begin to deposit the pre-exuvial layers of the procuticle (hexapods) and exocuticle (decapods). In both cases, the components of the chitin–protein fibrils are secreted at the surface of epithelial microvilli and are associated with the microvillar plaques (Leopold et al. 1992; Locke 1998; Dillaman et al. 2013) (Fig. 9). In *Drosophila*, Moussian (2012) showed that these plaques are located on the apical ridges of epithelial undulae (rather than on microvilli). The plaques are associated with membrane-bound chitin synthase, and he hypothesized that the chitin fibrils are secreted from the peaks of the undulae, while the CPs are secreted in the valleys (Moussian 2012). Moussian also ascribed a role of the undulae in orienting the fibrils. Locke (1998) also hypothesized that the apical microvilli controlled the orientation of the chitin–protein fibrils as they were being deposited. However, micrographs both from decapods (*Callinectes*, Greenaway et al. 1995; Dillaman et al. 2013) and hexapods (*Anthonomus*, Leopold et al. 1992) show a pronounced assembly or polymerization zone in which the secreted material is clearly unorganized at a distance from the apical surface of the epithelial cells (Fig. 9). These data suggest that the chitin–protein fibrils self-assemble and self-orient. Data from a number of researchers using material from insect cuticle have demonstrated that chitin fibrils will self-assemble into a helicoidal arrangement (see Neville [1998] for review). This is an important area for future research. The ability of complex macromolecules to form a highly ordered assembly in an extracellular environment could have significant fundamental biological, as well as commercial, implications.

Both in decapods and hexapods, a surface layer is deposited outside of the envelope or outer epicuticle. This is the wax layer in insects (Locke 1998) and is referred to as a surface coat in decapods (Compère 1995). Both appear to be formed by secretions of specialized cells or glands that communicate with the epicuticle via ducts or canals. While the wax layer is secreted postmolt, the surface coat of *Carcinus* is formed prior to ecdysis, during late stage D_2, after the pre-exuvial exocuticle has been completed (Compère 1995). The role of the wax layer in waterproofing and protection is well-established (Hadley 1994), but the function of decapods' surface coat is as yet unknown.

Comparison of cuticular structural proteins

Numerous studies have identified and characterized CPs both from hexapods (Andersen 1988b, 2000; He et al. 2007; Charles 2010; Dittmer et al. 2012; Willis et al. 2012; Noh et al. 2014) and from decapods (Andersen 1988a, 1999; Endo et al. 2000, 2004; Watanabe et al. 2000, 2006; Inoue et al. 2001, 2003, 2004; Wynn and Shafer 2005; Shafer et al. 2006; Faircloth and Shafer 2007; Kuballa and Elizur 2008; Kuballa et al. 2011; Ma et al. 2013; Suzuki et al. 2013; Tom et al. 2014; including a mini-review of known gastrolith proteins, Glazer and Sagi 2012). However, only a few of these have determined the expression patterns of these proteins relative to the molt (Togawa et al. 2008) and to the type of cuticle in which they are expressed. In a recently studied transcriptomic library of cuticular

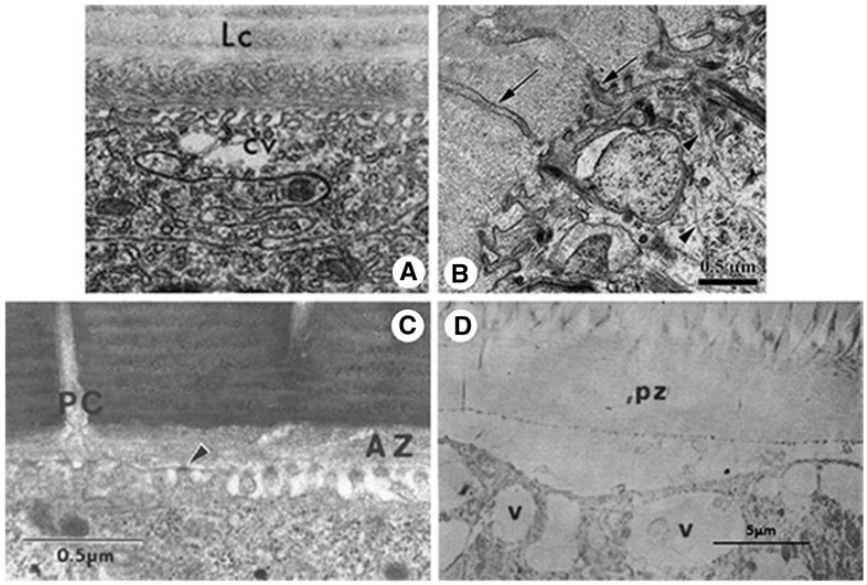

Fig. 9 Transmission electron micrographs of the epithelial–cuticular interfaces of insects (**A**, **C**) and crabs (**B**, **D**) during cuticular deposition. (A) Pre-exuvial deposition of the fifth larval procuticle of *Calpodes ethlius* showing apical microvilli depositing elements of the lamellar cuticle (Lc) presumably supplied by coated vesicles (cv). From Locke (1998) with permission from the publisher. (B) Pre-exuvial deposition of the exocuticle of *Callinectes sapidus* showing similar apical microvilli at the site of deposition along with cytoplasmic extensions (pore canals, arrows) and abundant microtubules within the epithelial cells (arrowheads). From Dillaman et al. (2013). (C) Higher magnification of a pre-exuvial adult insect (*Anthonomus grandis*) showing apical microvilli secreting cuticular components (arrowhead) into the assembly zone (AZ). Cytoplasmic extensions (pore canals, PC) are also evident. From Leopold et al. (1992) with permission from the publisher. (**D**) Post-molt deposition of the endocuticle in *Callinectes sapidus* showing the polymerization (=assembly) zone (pz) above the apical epithelial membrane and associated vesicles (v). From Greenaway et al. (1995) with permission from the publisher.

elements of the crayfish *C. quadricarinatus*, the expression pattern through the molt cycle of each of the above genes can be visualized, thereby providing a resourceful tool (Abehsera et al. 2015) that is used throughout the present review to identify and evaluate crustaceans' orthologs.

In order to fully understand the structural and functional similarities and differences between hexapod and decapod CPs, it is important to know if they are expressed prior to ecdysis and, therefore, incorporated into the pre-exuvial exoskeleton, or post-ecdysis and thus be components of the post-exuvial procuticle of insects or the endocuticle of malacostracans. It is also important to know if the proteins are expressed in hard (heavily-sclerotized and/or mineralized) or soft (flexible) cuticle. While a number of studies satisfy a subset of these criteria (e.g., Togawa et al. 2008; Charles 2010; Tom et al. 2014), this section focuses on those CPs for which these expression patterns are known, and for which there is enough sequence known to give an indication of function.

The proteins have been sorted into eight bins, four each for hexapods and decapods: hard versus soft cuticle and pre-molt versus post-molt expression (Table 1). There are no proteins that have been identified for insects that are exclusively expressed during post-molt in soft cuticle. However, three proteins are expressed in pre-molt hard cuticle and eight in soft cuticle; 20 proteins are expressed post-molt in hard cuticle. For the decapods, seven proteins are expressed prior to ecdysis, four in arthrodial cuticle, and three in calcifying cuticle. Post-molt, seven are expressed in arthrodial cuticle and nine in calcifying cuticle. Some of these (as discussed further below) are expressed both pre-molt and post-molt, and one both in arthrodial and calcifying cuticle. In addition, orthologs of each protein were searched in the recently established molt-related transcriptomic library originating from the hard exoskeleton-forming epithelia of a decapod (Abehsera et al. 2015). The criteria utilized for ortholog discovery are listed in Supplementary Table S1. The temporal expression was examined for each of the orthologs found. A higher degree of conservation was found among proteins originating from the soft cuticle both for hexapods and decapods compared with our studied decapod (Table 1). These findings suggest that the

Table 1 Expression of CP mRNA by species and molt stage among the Hexapoda and Decapoda

	Species	Premolt			Postmolt			Citation	*Cherax quadricarinatus* transcriptome (Abehsera et al. 2015)		
		Protein	Function	Accession number	Protein	Function	Accession number		Homolog existence	Homolog expression	Degree of homology
Hexapoda											
Hard cuticle	*Tribolium castaneum*	TcCPR18—RR-2	Chitin-binding structural	XP_967633				Noh et al. (2014)	None		
	(red flour beetle)	TcCPR27—RR-2	Chitin-binding structural	XP_971678				Noh et al. (2014)	None		
					TcasCPR33—RR-2	Chitin-binding structural	GLEAN 13 987	Dittmer et al. (2012)	None		
					TcasCPR34—RR-2	Chitin-binding structural	GLEAN 13 988	Dittmer et al. (2012)	None		
					Glu, Arg, His rich	Unknown—low complexity	GLEAN 14 462	Dittmer et al. (2012)	None		
					Similar to TmACP17	Unknown—Glycine-rich	GLEAN 08228	Dittmer et al. (2012)	None		
					TcasCPR101—RR-2	Chitin-binding structural	GLEAN 11 338	Dittmer et al. (2012)	None		
					TcasCPR87—RR-2	Chitin-binding structural	GLEAN 15 908	Dittmer et al. (2012)	None		
	Blaberus craniifer				BcNCP3.8		P82122	Andersen (2000)	None		
	(cockroach)				BcNCP14.6—Postmolt-18		P82118	Andersen (2000)	None		
					BcNCP14.9—RR-3, Postmolt-18	Chitin-binding structural	P82119	Andersen (2000)	Exists	Postmolt	Low
					BcNCP15.0—RR-3, Postmolt-18	Chitin-binding structural	P82120	Andersen (2000)	Exists	Postmolt	Low
					BcNCP21.1—RR-2	Chitin-binding structural	P82121	Andersen (2000)	None		
	Locusta migratoria				LmNCP4.9		P82168	Andersen (2000)	None		
	(locust)				LmNCP5.1		P82169	Andersen (2000)	None		
					LmNCP6.4		P82170	Andersen (2000)	None		
					LmNCP9.5		P82171	Andersen (2000)	None		
					LmNCP18.7—Postmolt-18		P82165	Andersen (2000)	None		
					LmNCP19.8—RR-2	Chitin-binding structural	P82166	Andersen (2000)	None		
					LmNCP21.3		P82167	Andersen (2000)	None		

(continued)

Table 1 Continued

	Species	Premolt			Postmolt			Citation	*Cherax quadricarinatus* transcriptome (Abehsera et al. 2015)		
		Protein	Function	Accession number	Protein	Function	Accession number		Homolog existence	Homolog expression	Degree of homology
	Anopheles gambiae	AgamCPF3	Epicuticular structural	VectorBase: AGAP004690	AgamCPLCG3	Structural/insecticide resistance	VectorBase: AGAP008446	Vannini et al. (2014)	None		
	(mosquito)				AgamCPLCG4	Structural/insecticide resistance	VectorBase: AGAP008447	Vannini et al. (2014)	None		
Soft Cuticle	*Tribolium castaneum*	TcasCPR17—RR-2	Chitin-binding structural	GLEAN 03363				Dittmer et al. (2012)	Exists	Premolt	Low
	(red flour beetle)	Tcas CPR81—RR-1	Chitin-binding structural	GLEAN 13127				Dittmer et al. (2012)	Exists	Premolt	Low
		TcasCPR22—RR-1	Chitin-binding structural	GLEAN 03830				Dittmer et al. (2012)	Exists	Premolt	Low
		TcasCPR23—RR-1	Chitin-binding structural	GLEAN 03831				Dittmer et al. (2012)	Exists	Premolt	Medium
		TcasCPR103—RR-2	Pro-resilin	GLEAN 03362				Dittmer et al. (2012)	None		
		TcasCPAP3-E—CPAP type 3	Cuticular protein analogous to peritrophin	GLEAN 11349				Dittmer et al. (2012)	Exists	Premolt and Postmolt	High
		TcasCPAP1-C—CPAP type 1	Cuticular protein analogous to peritrophin	GLEAN 00316				Dittmer et al. (2012)	Exists	Premolt	Low
		TcasCPR69—RR-1	Chitin-binding structural	GLEAN 13137				Dittmer et al. (2012)	Exists	Premolt	Low
Decapoda											
Hard cuticle	*Procambarus clarkii*				CAP-1—RR	Calcification-associated peptide	AB103035	Inoue et al. (2003)	Exists	Premolt	High
	(crayfish)				CAP-2—RR	Calcification-associated peptide	AB167814	Inoue et al. (2004)	Exists	Premolt	Low
	Penaeus japonicus				DD4—RR-like (Crustocalcin)	Calcification-associated protein	AB114444/ AB114445	Endo et al. (2004)	Exists	Postmolt	Low
	(shrimp)				DD5	Calcification-associated protein	AB049147	Ikeya et al. (2001)	Exists	Postmolt	High
					DD1	Unknown function—endocuticular	AB194409	Watanabe et al. (2006)	None		

(continued)

Table 1 Continued

	Species	Premolt			Postmolt			Citation	*Cherax quadricarinatus* transcriptome (Abehsera et al. 2015)		
		Protein	Function	Accession number	Protein	Function	Accession number		Homolog existence	Homolog expression	Degree of homology
	Callinectes sapidus	CsCP14.1—RR-1	Pre-exuvial calcification inhibitor	DQ288151				Faircloth and Shafer (2007)	Exists	Premolt	High
	(blue crab)				CsCP6.1—Partial RR	Calcification promoter	DQ288153	Faircloth and Shafer (2007)	None		
		CsCP15.0—Postmolt-18	Chitin/protein-binding structural	DQ288154	CsCP15.0—Postmolt-18	Chitin/protein-binding structural	DQ288154	Faircloth and Shafer (2007)	Exists	Postmolt	Medium
					CsCP19.0—Postmolt-18	Chitin/protein-binding structural	DQ288149/ DQ228150	Faircloth and Shafer (2007)	Exists	Postmolt	Medium
		CsAMP/CP13.7—RR-3	Chitin-binding structural		CsAMP/CP13.7—RR-3	Chitin-binding structural		Shafer et al. (2006)	No data		
Soft cuticle	*Marsupenaeus japonicus*				DD9A—RR-1	Chitin-binding structural	AB031223	Watanabe et al. (2000)	Exists	Premolt and Postmolt	Medium
	(shrimp)				DD9B—RR-1	Chitin-binding structural	AB031224	Watanabe et al. (2000)	Exists	Premolt and Postmolt	Medium
	Callinectes sapidus				CsAMP16.3—RR-1,RGD	Anchoring cells to chitin	DQ310582	Faircloth and Shafer (2007)	Exists	Premolt and Postmolt	High
	(blue crab)	CsAMP16.5—RR-1	Chitin-binding structural	DQ288152	CsAMP16.5—RR-1	Chitin-binding structural	DQ288152	Faircloth and Shafer (2007)	Exists	Premolt and Postmolt	High
		CsAMP13.4—RR-1	Chitin-binding structural	DQ288147	CsAMP13.4—RR-1	Chitin-binding structural	DQ288147	Faircloth and Shafer (2007)	Exists	Premolt and Postmolt	Medium
		CsAMP9.3—RR-1	Regulatory protein	DQ288148					Exists	Premolt	Medium
		CsAMP/CP13.7—RR-3	Chitin-binding structural		CsAMP/CP13.7—RR-3	Chitin-binding structural		Shafer et al. (2006)	No data		
					CsAMP23.7			Shafer et al. (2006)	No data		

formation of the soft cuticle is more conserved across the pancrustaceans. This might be due to the fact that components serving as scaffold, such as chitin, are similar in the soft and hard cuticle and both in hexapods and decapods. The timing of expression was not similar in most cases; these differences may be attributed to the comparison between calcified and non-calcified cuticles or to differences in research methods.

A total of 242 additional sequences in the recently established crayfish molt-related transcriptomic library (Abehsera et al. 2015) were found to have homology to different proteins related to the arthropod cuticle, mostly from hexapod origin (Supplementary Table S1). This is probably due to the fact that hexapods are more studied and thus comprise most of the available arthropod gene sequences in the global gene bank. The transcripts found are related to several aspects of cuticular formation. Many are homologs to the known family of arthropods' structural CPs (Willis 2010; see Supplementary Table S1). Other proteins are related to the metabolism of chitin, such as chitin synthase and chitinase (Merzendorfer and Zimoch 2003; Supplementary Table S1), or involved in sclerotization (enzymes from the family of peroxidases) (Hasson and Sugumaran 1987). Some are decapods' proteins related to the mineralization of the exoskeleton, such as GAP65 (Shechter et al. 2008). Many of these proteins were found to be expressed differentially through the molt cycle. Several sequences found to be related to the arthropod cuticle are uncharacterized (e.g., uncharacterized protein loc100881531) or might have an indirect relation (e.g., DNA-binding protein elf-1) (Bray and Kafatos 1991) or could be false positives. Such results are due to the fact that the bioinformatics tools used in the present article take into account a large quantity of information with different degrees of relevance that might include some degree of inaccuracies (Hawkins and Kihara 2007; Klimke et al. 2011). Other sequences that were found to be uniquely expressed in the exoskeletal-forming epithelia and having a strong induction in certain molt stages were not annotated. These sequences are likely to be yet-unknown CPs that should be the focus of further research. The large number of known and unknown probable cuticular-proteins found in the crayfish molt-related transcriptome (Abehsera et al. 2015) demonstrates the importance of these proteins as stated by Willis (2010). A recently developed on-line tool for the identification of arthropods' CPs should aid in further probing this and other transcriptomes (Ioannidou et al. 2014).

Certain motifs are common to many of these CPs. In both hexapod and decapod proteins, the most common is the Rebers–Riddiford (RR) consensus sequence. First described by Rebers and Riddiford (1988) and characterized by Rebers and Willis (2001), the RR motif has been identified as a chitin-binding site. An additional chitin-binding domain is the cysteine-rich chitin-binding domain (cys-CBD), which includes two types, 1 and 2. Type 1 is reported only from a fungus (Wright et al. 1991) and thus not relevant to our review, while type 2 is found in arthropods (cys-CBD2) (Jasrapuria et al. 2010). The latter domain is also found in other taxa such as mammals and plants (Suetake et al. 2000; Tjoelker et al. 2000). The occurrence of these domains in the decapod exoskeleton is well demonstrated in the recently established molt-related crayfish transcriptomic library (Abehsera et al. 2015). A total of 86 sequences of the library were found to share the RR chitin-binding domain (Table 2). A total of 74 sequences of the library were found to share the cysteine-rich, chitin-binding domain (Table 3). There are two well-defined variants of the Rebers–Riddiford sequence, RR-1 ($Gx_8Gx_6YxAxExGYx_7Px_2P$) and RR-2 ($Gx_8Gx_6YxAx_4GFNAVV$). RR-1 is typically found in flexible cuticle of hexapods, whereas RR-2 is generally found in the heavily-sclerotized cuticle of insects. Additionally, RR-2 is usually expressed in pre-exuvial cuticle, while RR-1 expression is often post-exuvial (Andersen 2000; Togawa et al. 2008). RR-1 is also typical of arthrodial CPs in decapods. RR-2 had not previously been identified in any CPs of decapods (Faircloth and Shafer 2007), but the RR-2 motif was recently found in several sequences in the present crayfish library (Abehsera et al. 2015) and may be common to calcified cuticle. However, one protein from the calcified cuticle of *Callinectes* that is expressed during pre-molt does have the RR-1 motif (Faircloth and Shafer 2007). Phylogenetic analysis shows that the RR-1 sequences from hexapods and malacostracans form separate clades (Shafer et al. 2006). One exception to this is a CP of *Manduca sexta* (MsAAL90881.1) (Suderman et al. 2003) which is basal to both clades and may represent an ancestral CP. A less-defined RR sequence has been termed RR-3 (Andersen 2000) and is found in a few CPs both from pre-molt and post-molt calcified and arthrodial decapodan cuticle, and a few post-molt proteins from hard and soft cuticles of hexapods. In the crayfish transcriptomic library (Abehsera et al. 2015), sequences found to have an RR domain possessed either both RR-1 and RR-2 domains, or the RR-2 domain exclusively. These results

Table 2 Sequences bearing a R&R chitin-binding domain in a molt-related transcriptome of a crayfish

Contig number	Description according to most significant BLAST results	Domain accession number		Number of RR domains
		PS51155 (PROSITE) RR-2	PS00233 (PROSITE) RR-1	
4	Endocuticle structural glycoprotein bd-8-like	+	+	1
11	Cuticle protein	+		1
28	Cuticular protein RR-1 motif 8 precursor	+	+	1
32	Pupal cuticle protein	+	+	1
45	Cuticular protein 49aa cg30045-pb	+	+	1
50	Endocuticle structural glycoprotein bd-8-like	+		1
54	Cuticle precursor	+	+	1
64	Cuticular protein RR-1 family member 39 precursor	+	+	1
73	Cuticular protein 50cb cg6305-pa	+	+	1
112	Structural constituent of a cuticle	+	+	1
116	Cuticular protein 49aa cg30045-pb	+	+	1
136	Larval cuticle protein lcp-17-like	+		1
138	Larval cuticle protein lcp-17-like	+	+	1
159	Larval cuticle protein lcp-17-like	+	+	1
160	RNA-directed DNA polymerase from mobile element jockey-like	+	+	1
163	RNA-directed DNA polymerase from mobile element jockey-like	+	+	1
194	Exosporium glycoprotein	+		1
199	Cuticular protein RR-1 family member 39 precursor	+	+	1
214	Cuticle protein	+	+	1
270	RR-1 cuticle protein 7 precursor	+		1
287	NADH dehydrogenase subunit 5	+	+	1
307	Exosporium glycoprotein	+	+	1
321	Endocuticle structural glycoprotein bd-2-like	+	+	1
414	Endocuticle structural glycoprotein bd-8-like	+		1
474	Calcium-activated chloride channel regulator 1	+	+	1
828	Pupal cuticle protein	+	+	1
865	Pupal cuticle protein	+	+	1
1038	Cuticular protein 49aa cg30045-pb	+	+	1
1050	Cuticular protein 49aa cg30045-pb	+	+	3
1076	Pupal cuticle protein	+	+	1
1092	Cuticle protein	+	+	1
1276	Pupal cuticle protein	+	+	1
1366	Uncharacterized protein loc100881531	+	+	1
1495	Endocuticle structural glycoprotein bd-1-like	+	+	1
1848	Cuticle protein	+	+	1
2547	Uncharacterized protein loc100829785	+	+	1
2595	Endocuticle structural glycoprotein bd-1-like	+	+	1
4422	Cuticular protein RR-1 motif 10 precursor	+		1
5022	Uncharacterized protein loc100379437	+		1
5263	RR-1 cuticle protein 11 precursor	+	+	6
5615	Cuticular protein 19 precursor	+	+	1
5974	Larval cuticle protein lcp-17-like	+	+	1
7163	Endocuticle structural glycoprotein bd-1-like	+		1
8496	Cuticular protein RR-1 motif 44 precursor	+		1

(continued)

Table 2 Continued

Contig number	Description according to most significant BLAST results	Domain accession number		Number of RR domains
		PS51155 (PROSITE) RR-2	PS00233 (PROSITE) RR-1	
9169	Cuticular protein RR-1 motif 47 precursor	+	+	1
9551	Endocuticle structural glycoprotein bd-1-like	+	+	1
9753	Uncharacterized protein loc101456472	+	+	1
9778	Cuticular protein 49aa cg30045-pb	+	+	1
10155	Cuticular protein RR-1 family (agap000344-pa)	+		1
10513	Endocuticle structural glycoprotein bd-2-like	+	+	1
10566	Larval cuticle protein lcp-17-like	+	+	1
10829	Isoform a	+	+	1
12304	Uncharacterized protein loc101456651	+	+	1
12692	Uncharacterized protein loc100900032	+	+	1
12806	Cuticular protein RR-2 family member 23 precursor	+	+	1
12930	Pupal cuticle	+		1
13317	Pupal cuticle protein	+	+	1
13575	Endocuticle structural glycoprotein bd-1-like	+		1
13616	nfx1-type zinc finger-containing protein 1	+		1
13933	Cuticle protein precursor	+		1
16099	Cuticular protein hypothetical 5 precursor	+		1
17298	Cuticular protein 50cb cg6305-pa	+	+	1
17574	Uncharacterized protein loc101741297 isoform x2	+		1
18006	Cuticular protein precursor	+	+	1
21231	Uncharacterized protein loc101457732	+	+	1
21934	Cuticle protein	+		1
22337	Cuticular protein RR-1 family (agap005456-pa)	+		1
22364	DNA-directed RNA polymerase II largest subunit-like	+		1
22935	cg1136 cg1136-pa	+	+	1
23215	Cuticular protein RR-2 family (agap012466-pa) partial	+	+	1
24363	Cuticular protein 50cb cg6305-pa	+	+	1
24450	Cuticular protein rr-2 motif 131 precursor	+	+	1
25389	Cuticular protein precursor	+		1
25899	Collagen precursor	+		1
26962	Isoform a	+	+	1
31715	Cuticular protein 50cb cg6305-pa	+		1
33180	Structural constituent of	+	+	1
35345	cuticular protein 50cb cg6305-pa	+	+	1
35594	Larval cuticle protein lcp-17-like	+	+	1
36815	Flexible cuticle protein 12-like	+	+	1
39296	Cuticular protein 50cb cg6305-pa	+	+	1
47852	Adult cuticle	+	+	1
48948	Larval cuticle protein lcp-17-like	+	+	1
51299	Cuticular protein 62bc cg1919-pa	+	+	1
54053	Cuticle protein	+	+	1
56504	Cuticular protein isoform b	+	+	1

Notes: Contig number according to the transcriptome is shown (left). For each contig, the description according to most significant BLAST result (middle) and the accession number according to used-database (right) is shown.

Table 3 Sequences bearing a cysteine-rich chitin-binding domain in a molt-related transcriptome of a crayfish

Contig number	Description according to most significant BLAST results	Domain accession number	Number of cys-CBD2
1	Cuticular protein analogous to peritrophins 3-a1 precursor	SM00494 (SMART) cys-CBD2	3
6	Uncharacterized protein loc101462416 isoform x2	SM00494 (SMART) cys-CBD2	1
110	Acidic mammalian chitinase	SM00494 (SMART) cys-CBD2	2
120	Uncharacterized protein loc101462122 isoform x1	PS50940 (PROSITE) cys-CBD2	1
161	Cytokine receptor-like factor 1-like	SM00494 (SMART) cys-CBD2	1
182	Uncharacterized protein loc100872047	SM00494 (SMART) cys-CBD2	1
301	Uncharacterized protein loc100901383	SM00494 (SMART) cys-CBD2	1
385	Cuticular protein analogous to peritrophins 3-b precursor	SM00494 (SMART) cys-CBD2	3
515	Cuticular protein analogous to peritrophins 3-b precursor	SM00494 (SMART) cys-CBD2	3
532	Chitin binding peritrophin-	SM00494 (SMART) cys-CBD2	3
571	Cuticular protein analogous to peritrophins 3-c precursor	SM00494 (SMART) cys-CBD2	3
606	Probable chitinase 3-like	SM00494 (SMART) cys-CBD2	1
714	Uncharacterized protein loc100906622	SM00494 (SMART) cys-CBD2	3
756	Uncharacterized protein loc100901383	SM00494 (SMART) cys-CBD2	1
957	Probable chitinase 3-like	SM00494 (SMART) cys-CBD2	2
996	Uncharacterized protein loc101740538	SM00494 (SMART) cys-CBD2	1
1065	Uncharacterized protein loc100877019	PS50940 (PROSITE) cys-CBD2	1
1113	Uncharacterized protein loc100904950	SM00494 (SMART) cys-CBD2	1
1173	Cuticular protein analogous to peritrophins 1-h precursor	SM00494 (SMART) cys-CBD2	1
1212	Cuticular protein analogous to peritrophins 3-a1 precursor	SM00494 (SMART) cys-CBD2	3
1258	c-Type lectin domain family 4 member f-like	SM00494 (SMART) cys-CBD2	1
1585	Uncharacterized protein loc100904950	SM00494 (SMART) cys-CBD2	1
1624	Chitin binding peritrophin-	SM00494 (SMART) cys-CBD2	8
1867	Cuticular protein analogous to peritrophins 3-c precursor	SM00494 (SMART) cys-CBD2	1
2164	Isoform c	SM00494 (SMART) cys-CBD2	3
2210	Hemolectin cg7002-pa	SM00494 (SMART) cys-CBD2	1
2301	Isoform b	SM00494 (SMART) cys-CBD2	3
2697	Chitin binding peritrophin-	SM00494 (SMART) cys-CBD2	1
2755	Uncharacterized protein loc101740538	PS50940 (PROSITE) cys-CBD2	1
2908	Isoform c	SM00494 (SMART) cys-CBD2	2
3645	Uncharacterized protein loc100868462	SM00494 (SMART) cys-CBD2	1
3724	Uncharacterized protein loc100865193	SM00494 (SMART) cys-CBD2	1
3751	Serine-rich adhesin for platelets-like	SM00494 (SMART) cys-CBD2	2
4172	Tyrosine kinase	SM00494 (SMART) cys-CBD2	1
4512	Uncharacterized protein loc100865193	SM00494 (SMART) cys-CBD2	1
5213	Chitin binding peritrophin-	SM00494 (SMART) cys-CBD2	14
5488	agap009480-partial	SM00494 (SMART) cys-CBD2	1
5919	Brain chitinase and chia	SM00494 (SMART) cys-CBD2	1
6530	Uncharacterized protein ddb_g0271606-partial	PS50940 (PROSITE) cys-CBD2	1
7251	Uncharacterized protein loc100901383	SM00494 (SMART) cys-CBD2	1
8662	Uncharacterized protein loc100879380	SM00494 (SMART) cys-CBD2	2
8757	Cuticular protein analogous to peritrophins 1-f precursor	SM00494 (SMART) cys-CBD2	1
10016	Serine-rich adhesin for platelets-like	SM00494 (SMART) cys-CBD2	2
11239	Uncharacterized protein loc100879380	SM00494 (SMART) cys-CBD2	2
12674	Probable chitinase 3-like	SM00494 (SMART) cys-CBD2	1

(continued)

Table 3 Continued

Contig number	Description according to most significant BLAST results	Domain accession number	Number of cys-CBD2
13510	Cuticular	SM00494 (SMART) cys-CBD2	1
13894	Mucin-related isoform b	PS50940 (PROSITE) cys-CBD2	1
17678	Uncharacterized protein loc101458687	SM00494 (SMART) cys-CBD2	1
19091	Isoform b	PS50940 (PROSITE) cys-CBD2	1
19563	Uncharacterized protein loc100865751	PS50940 (PROSITE) cys-CBD2	1
28384	Tryptophan-rich antigen (pv-fam-a)	SM00494 (SMART) cys-CBD2	3
30096	Uncharacterized protein loc100877019	PS50940 (PROSITE) cys-CBD2	1
31283	Uncharacterized protein loc100901383	PS50940 (PROSITE) cys-CBD2	1
31969	Uncharacterized protein loc100904950	SM00494 (SMART) cys-CBD2	1
32588	Isoform b	SM00494 (SMART) cys-CBD2	1
32829	Chitin binding peritrophin-partial	PS50940 (PROSITE) cys-CBD2	1
34783	Uncharacterized protein	PS50940 (PROSITE) cys-CBD2	1
39430	Uncharacterized protein loc100869463	SM00494 (SMART) cys-CBD2	1
41127	Chondroitin proteoglycan-2-like	SM00494 (SMART) cys-CBD2	1
41428	Uncharacterized protein loc100899344	SM00494 (SMART) cys-CBD2	1
44368	Collagen alpha-1 chain-like	PS50940 (PROSITE) cys-CBD2	1
50056	agap012133-partial	PS50940 (PROSITE) cys-CBD2	1
58677	Mucin 68 d	PS50940 (PROSITE) cys-CBD2	1
58975	Fibrous sheath CABYR-binding	PS50940 (PROSITE) cys-CBD2	1
61273	Acidic mammalian chitinase-like	SM00494 (SMART) cys-CBD2	1
61615	Endochitinase-like	SM00494 (SMART) cys-CBD2	1
62176	agap011416-partial	SM00494 (SMART) cys-CBD2	1
62909	Cell surface glycoprotein 1-like	SM00494 (SMART) cys-CBD2	1
62932	Low-density lipoprotein receptor	SM00494 (SMART) cys-CBD2	1
62950	agap011617-partial	SM00494 (SMART) cys-CBD2	3
62983	Probable chitinase 3-like	SM00494 (SMART) cys-CBD2	1
63036	Uncharacterized protein loc101462315	SM00494 (SMART) cys-CBD2	4
63053	agap010360-partial	SM00494 (SMART) cys-CBD2	1

Notes: Contig number according to the transcriptome is shown (left). For each contig, the description according to most significant BLAST result, the accession number according to used-database, and the number chitin-binding domains are shown.

can be explained by the fact that the domains were found *in silico* and the existence of a certain RR domain might be a false positive or bearing a different RR domain that shares a high similarity with another. The 74 sequences bearing a cysCBD were found to have a cysCBD2 domain (Table 2), the common cysCBD of invertebrates.

Another common motif is VxDTPEVAAAKAA FxAAY, termed postmolt-18 (Faircloth and Shafer 2007). As the name implies, it is found predominantly in proteins from hexapods (Andersen 2000) and decapods that are isolated from hard cuticle and that are expressed post-molt. One exception to this is CsCP15.0, a protein from the calcified cuticle of *Callinectes* that is expressed both pre-molt and post-molt (Faircloth and Shafer 2007). This domain was found in the crayfish molt-related transcriptome (Abehsera et al. 2015). The transcript, named cq post-molt protein 1(KP984531), was found to be highly expressed at post-molt in the cuticle (Fig. 10 left) and during intermolt in the gastrolith (Fig. 10 right). A final sequence is found only in proteins from calcified cuticle of decapods and designated as the crust-18 motif: x[L/V][I/V]GPSGIV[T/S]x[D/N] GxN[I/V]Q[V/L]. This domain was also found in the crayfish molt-related transcriptome (Abehsera et al. 2015). Two transcripts, named cq crustacean CP 1(KP984532) and cq crustacean CP 2 (KP984533), were found to bear this domain. Both proteins had a pattern of expression related to post-molt in the cuticle, during which calcification occurs (Fig. 11A, B left). In the gastrolith, neither was highly expressed,

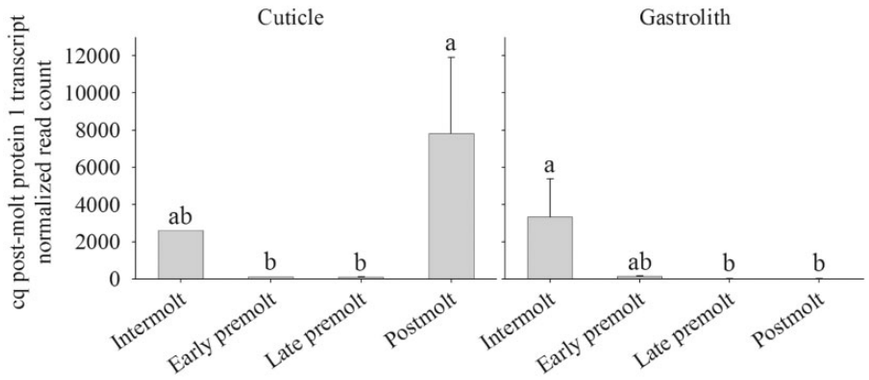

Fig. 10 Normalized read count of cq Post-molt protein 1 transcript. Normalized read count from the cuticle-forming epithelium (left) and the gastrolith-forming epithelium (right). The *X*-axis represents the four molt stages: inter-molt, early pre-molt, late pre-molt, and post-molt. Letters represent statistical groups that are significantly different ($P < 0.05$). Error bars represent standard error.

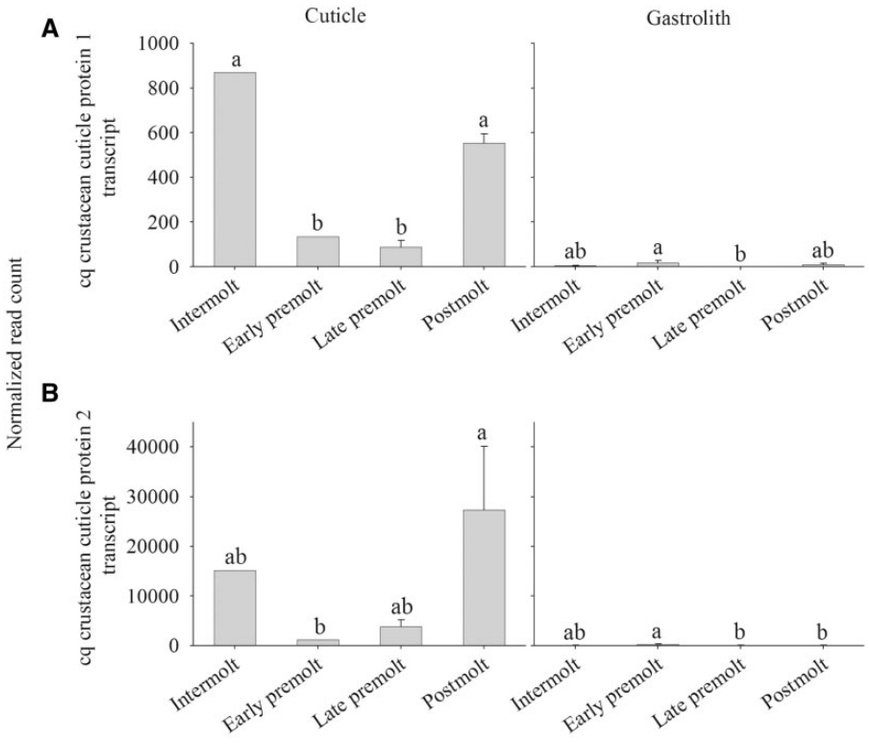

Fig. 11 Normalized read count of cq crustacean cuticle proteins transcript. Normalized read count of two proteins, cq crustacean cuticle protein 1 (**A**) and cq crustacean cuticle protein 2 (**B**), from the cuticle-forming epithelium (left) and the gastrolith-forming epithelium (right). The *X*-axis represents the four molt stages: inter-molt, early pre-molt, late pre-molt, and post-molt. Letters represent statistical groups that are significantly different ($P < 0.05$). Error bars represent standard error.

although a rise during early pre-molt seems to occur for both proteins (Fig. 11A, B right).

Two proteins from *Callinectes*, CsAMP/CP13.7 and CsAMP23.7 (Shafer et al. 2006), are of particular interest. CsAMP/CP13.7 is unusual in that it is universally expressed in both arthrodial and calcified cuticle and in both pre-molt and post-molt. It is, therefore, likely to be a fundamental CP. It bears a RR-3-like motif similar to that found in some insects' CPs (Andersen 2000). Both it and CsAMP23.7 show sequences similar to those of a number of proteins from insects and other arthropods. Their phylogenetic relationship is very different from that of the RR-1 bearing proteins, which segregate into separate decapodan and hexapodan clades. Instead, these proteins and those with similar

sequences do not form separate clades, suggesting that their divergence may have occurred within the Pancrustacea prior to the split between the Malacostraca and the Hexapoda (Shafer et al. 2006). The sequences of these proteins are not available in the literature or GeneBank and therefore were not mined from our molt-related transcriptomic library (Abehsera et al. 2015).

The importance of some of these proteins to the integrity of the cuticle has been demonstrated by knocking down their expression using RNAi. Arakane et al. (2012) created dsRNA for two proteins expressed in the pharate (pre-exuvial/eclosion) cuticle of adult *Tribolium*, TcCPR18 and TcCPR27. Larvae were injected with *dsTcCPR18*, *dsTcCPR27* or both, with *dsTcVer* (RNA coding for Vermillion—a pigment protein) serving as a control. The controls had the normal pharate cuticle of adults while those in which *TcCPR18* and/or *TcCPR27* had been knocked down displayed grossly abnormal exoskeletons. TEM examination of the abnormal cuticles showed defects in organization of the chitin–protein lamellae, fibers of the vertical pore canals, and the envelope and epicuticle (Noh et al. 2014). Orthologs of these proteins were not found in our molt-related transcriptomic library (Abehsera et al. 2015).

RNAi approaches are becoming more and more popular in research on crustaceans (Sagi et al. 2013). One study of RNAi has demonstrated the role of GAP-65, a cysCBD2 domain-bearing protein with a polysaccharide deacetylase domain, from the gastrolith of the crayfish, *C. quadricarinatus* (Shechter et al. 2008). Knockdown of the expression of GAP-65 inhibits the mineralization of amorphous calcium carbonate in the gastrolith. Silencing of another gastrolith protein, GAP10, was shown to cause a delay in the duration of pre-molt and cause surface irregularities in the gastrolith's structure (Glazer et al. 2010). To date, no experiments on RNAi have been performed that elucidate the roles of the numerous decapodan CPs in the structure and mineralization of the exoskeleton.

The role of glycosylation of CPs has been a subject of intense study in the Decapoda (Marlowe et al. 1994; Shafer et al. 1994, 1995; Coblentz et al. 1998; Roer et al. 2001; Tweedie et al. 2004; Roer and Towle 2004; Kuballa and Elizur 2008). There is a dramatic change in the nature and extent of glycosylation of CPs in *Callinectes* within 0.5–2 h post-molt (Marlowe et al. 1994; Shafer et al. 1994, 1995). These changes coincide with the onset of mineralization in the pre-exuvially deposited exocuticle. At least some of the alterations are due to the activity of an N-acetylhexosaminidase that is expressed by the epithelium immediately after the molt (Roer et al. 2001; Roer and Towle 2004). Treating pre-molt exoskeleton with an exogenous N-acetylhexosaminidase renders it capable of mineralizing *in vitro* (Pierce et al. 2001). Tweedie et al. (2004) documented the removal of a glycoprotein from the newly molted exocuticle that is coincident, both spatially and temporally, with the onset of mineralization.

Kuballa and Elizur (2008) also have demonstrated a role of glycoproteins in calcification of the post-molt cuticle of the crab *Portunus pelagicus*. A C-type lectin receptor is expressed during pre-molt deposition of the cuticle. Following the molt a mannose-binding protein is upregulated, implicating both the receptor and binding protein in the control of mineralization.

There are also reports of glycosylation of insects' CPs (Willis et al. [2012] for review). Lectin-binding studies of *Hyalophora cecropia* indicate the presence of N-acetylgalactosamine and mannose residues, along with some limited binding to N-acetylglucosamine, galactose, and fucose. Most of the glycosylation sites appear to be O-glycosylated threonines. The role of glycosylation in hexapodan cuticle has not been elucidated (Willis et al. 2012).

Future directions

The comparisons above have revealed a number of aspects of decapods' and hexapods' structure and function that are ripe for further research:

- Are chitin–protein fibers self-assembled into lamellae or organized by epithelial microvilli? Are there fundamental differences in this regard between hexapods and decapods?
- How does Knickkopf protect chitin from digestion? Is that protein common in malacostracans? If not, how do malacostracans protect the pre-exuvial cuticle?
- Does the permeability of insects' cuticle change just after ecdysis? Studies of permeability are needed.
- How is the pre-exuvial cuticle modified after ecdysis to promote sclerotization and mineralization? What is the role of pore canals, especially in the hexapods?
- Are the CPs of the few insects that display mineralization similar to those in malacostracans?
- What is the role of glycosylation in insects' CPs?
- Can RNAi knockout experiments be conducted using malacostracan CPs, similar to those performed with insects?

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Supplementary data

Supplementary data available at *ICB* online.

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