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CRUSTACEAN GENOMICS

The search for proteins involved in the formation of crustacean cuticular structures

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Abstract Crustacean cuticular structures are key features formed during a molt cycle. These structures are complex biomaterials comprising chitin and different mineral forms in distinct scaffold organizations. The formation of these complex biomaterials is controlled by the organic extracellular matrix including structural proteins. Since cuticular structures are formed *de novo* during each molt cycle, the spatial and temporal expression patterns of structural proteins are tightly linked to molt cycle events. As a model scenario, we demonstrate the molt-related pattern of expression of the gene encoding GAP65, a core structural protein involved in the formation of the cuticular structures of *Cherax quadricarinatus*. Based on this typical pattern of expression and using a binary-patterning approach, which is a specialized tool for the study of molt-related proteins, we revealed and characterized additional candidate proteins involved in the formation of crustacean cuticular structures. We propose that our approach be applied as a framework in the search for proteins involved in the formation of the crustacean cuticle. To stimulate research on this important aspect of structural biology, we put forward a schematic representation of the extracellular matrix and its proteins in three cuticular structures of *C. quadricarinatus*, the gastroliths, the mandibles, and the mineralized cuticle.

Keywords Chitinous scaffold · Crustacean cuticle · Extracellular matrix · Molt cycle · Structural proteins · Transcript binary patterning

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Introduction

Crustaceans—like all arthropods—are characterized by a rigid exoskeleton (Roer et al., 2015), which is a composite bio-material made up of a chitinous scaffold in which minerals are deposited (Roer & Dillaman, 1984). The formation and mineralization of the skeletal structure is controlled by an organic matrix that includes specialized structural proteins (Weiner & Dove, 2003). Many of the known cuticular proteins in crustaceans have been reported to be associated with the processes in which the skeletal structures are

formed, such as proteins related to chitin metabolism (Buchholz, 1989; Tellam et al., 2000; Merzendorfer & Zimoch, 2003; Abehsera et al., 2015). There are also reports of a small number of proteins that are involved in the mineralization processes; for example, in the red swamp crawfish, *Procambarus clarkii*, a soluble matrix protein was found to have an inhibitory effect on calcium carbonate precipitation in the exoskeleton (Inoue et al., 2008). In our study organism, the decapod crustacean, *Cherax quadricarinatus*, yet other proteins have been found to be associated with the formation of other cuticular structures such as the mandible cuticle (Tynyakov et al., 2015a; Abehsera et al., 2017) and the gastroliths. Our group has shown that among the proteins found in the gastroliths of *C. quadricarinatus*, some are associated with the calcification process (Glazer & Sagi, 2012), and one, designated GAP65, is involved in both the stabilization of amorphous calcium carbonate (ACC) and in the formation of the chitinous scaffold (Shechter et al., 2008b). We have further suggested that GAP65—a glycoprotein possessing a cysteine-rich type chitin-binding domain, a low-density lipoprotein receptor class A domain, and a polysaccharide deacetylase domain—acts as a key protein in the gastroliths of *C. quadricarinatus*, since it forms protein complexes with other structural proteins and has been found in all protein complexes studied to date in the gastroliths (Glazer et al., 2015). GAP65 may therefore be regarded as a core structural protein with a central role in the formation of the gastrolith matrix.

Since crustacean skeletal structures are formed de novo in every molt cycle, the expression of genes encoding the proteins involved in the formation of the skeletal structures is tightly related to molt cycle events (Roer et al., 2015). This linkage has been exploited by our group for the development of a specialized study tool—binary patterning—aimed to facilitate the mining of proteins involved in the formation of cuticular structures (Abehsera et al., 2015). In this approach, a binary code with four positions represents the four molt stages sampled for our transcriptomic library (the first position represents inter-molt; the second position, early pre-molt; the third, late pre-molt; and the fourth, post-molt), where each position is accorded either the number 1 for high expression or the number 0 for low expression at that position. For example, the binary code 1001 represents high expression at inter-molt and post-molt and low expression at the two pre-molt stages. The strength of this approach lies in its ability to cluster genes according to pre-defined binary patterns of expression during the molt cycle. It therefore enables the selection of candidate protein genes exhibiting a certain pattern of expression that is related to a particular molt event, as recently demonstrated for the MARS protein family during the formation of the mandible (Abehsera et al., 2017).

As mentioned above, there are three major mineralized cuticular structures in *C. quadricarinatus*, namely, the exoskeleton, the gastroliths, and the mandibles, the latter two showing unique timing of mineralization and having unique mineral forms. The bulk exoskeleton is composed of three distinct layers—endocuticle, exocuticle, and epicuticle. The endo- and exo-cuticles undergo mineralization with ACC and the crystalline form of calcium carbonate, calcite, at post-molt (Roer & Dillaman, 1984; Shechter et al., 2008a). The two gastroliths, which are specialized skeletal structures serving for transient calcium storage (Shechter et al., 2008a), are located on the stomach wall. They are made up of a simple layered chitinous scaffold mineralized mostly by ACC (Habraken et al., 2015; Luquet et al., 2016). The gastroliths are formed and mineralized at pre-molt, and during post-molt they collapse into the stomach, where they are digested, providing calcium for the mineralization of the hardening cuticle (Travis 1963; Ueno 1980; Ueno & Mizuhira, 1984; Shechter et al., 2008a). The mandibles, composed of a molar teeth and an incisor teeth, differ from the other two types of cuticular structures in a number of ways: the molar teeth are already partially mineralized during the pre-molt stage (Tynyakov et al., 2015a; Abehsera et al., 2017); they contain apatite, a crystalline phosphate mineral, in a form similar to that found in vertebrate skeletons (Bentov et al., 2012); and both the molar and the incisor appear to have unique vertical elements that are believed to support their load-bearing role (Bentov et al., 2012; Huber et al., 2014; Abehsera et al., 2017). To further clarify the fascinating and complex picture that underlies the formation of crustacean cuticular structures, we investigated the different temporal and structural elements of mineralization that coexist in three cuticular structures of the crayfish *C. quadricarinatus* (Table 1).

Here, we sought, in particular, to demonstrate the complex gene expression patterns for proteins

Table 1 Features of the different cuticular elements of *C. quadricarinatus*

Cuticular element	Mineral forms	Mineralization stage	Special features	Proteins discovered	References
Cuticle	Calcite Amorphous calcium carbonate Amorphous calcium phosphate	Post-molt	Three distinct layers	DD4 CAP-1	Endo et al. (2000) and Inoue et al. (2008)
Gastrolith	Amorphous calcium carbonate Amorphous calcium phosphate	Pre-molt	Single layer	GAP10 GAP65	Glazer et al. (2010), Habraken et al. (2015), Luquet et al. (2016) and Shechter et al. (2008a, b, c)
Mandible	Apatite Calcite Amorphous calcium phosphate Amorphous calcium carbonate	Pre-molt Post-molt	Vertical elements	M15 M13 MARS	Abehsera et al. (2017) and Tynyakov et al. (2015a, b)

involved in the formation of the above three exoskeletal elements with the aim of further elucidating the molecular and biochemical processes occurring during the formation of these cuticular structures. The key structural protein, GAP65, is used as a model protein to demonstrate the nature of a typical expression pattern of such proteins. Based on this typical pattern of expression, new putative structural proteins were mined and characterized in silico. Using previous knowledge on known structural proteins in crustaceans and several newly found putative proteins, we present a schematic representation of the presence and possible involvement of proteins in different exoskeletal elements. Thus, as a result of our efforts to delineate a general path for the study of the proteinaceous basis for cuticle formation in crustaceans, we are now in a position to suggest a general approach to be employed when studying structural proteins in crustaceans, as is described below.

Materials and methods

Animal and molt induction

Cherax quadricarinatus crayfish were grown in artificial ponds at Ben-Gurion University of the Negev (BGU), Beer-Sheva, Israel. Food comprising shrimp pellets (Rangen Inc., Buhl, ID, USA, 30% protein) were supplied ad libitum three times a week. The temperature was kept at 27 ± 2°C, and a photoperiod of 14 h light and 10 h dark was applied. Water quality was assured by circulating the entire volume of water through a bio-filter. For all molt-induction experiments, inter-molt crayfish were held in individual cages and endocrinologically induced to enter pre-molt through daily α-ecdysone injections, as previously described (Abehsera et al., 2017). The progression of the molt cycle was monitored daily by measuring the gastrolith molt mineralization index (MMI), which correlates molt stages with hormonal titers (Shechter et al., 2007, 2008a). MMI values used for molt staging were: inter-molt, 0; early pre-molt, 0.02–0.04; and late pre-molt 0.1–0.2. Post-molt

animals were harvested on the day following ecdysis. For all dissection procedures, crayfish were placed on ice for 10–15 min until they became anesthetized.

In-silico mining for candidate structural proteins

Mining for candidate structural proteins was conducted on the basis of our molt-related transcriptomic library (Abehsera et al., 2015). In brief, a reference *C. quadricarinatus* transcriptome was constructed from next generation sequencing (NGS) of samples from the mandible cuticle-forming and gastrolith-forming epithelia. For each of the two types of epithelium, animals were sampled at four different molt stages: inter-molt (one pool of three animals, i.e., $n = 1$), early pre-molt (one pool of three animals, i.e., $n = 1$), late pre-molt (two single animals and one pool of two animals, i.e., $n = 3$), and post-molt (two single animals, i.e., $n = 2$). Filtering for candidate structural proteins was performed using the binary-patterning mining approach described above (Abehsera et al., 2015). With this approach, transcripts having a binary pattern that is related to one of the four major molt cycle stages—inter-molt, early pre-molt, late pre-molt, and post-molt—were grouped and viewed as potential candidates, thereby producing a list of candidate transcripts. The candidate transcripts were computationally translated to proteins using the translate tool of the ExPASy Proteomics Server (Gasteiger et al., 2005) (http://ca.expasy.org/tools/dna.html), and the longest open reading frame was selected as the putative protein sequence. This list was then submitted to the following in-silico tests: SignalP 4.0 (Petersen et al., 2011) to test for the presence of a signal peptide; the ProtParam tool by ExPASy (Gasteiger et al., 2005) for various physical and chemical parameters, such as the predicted pI and amino acid content; and IUPRED (Dosztanyi et al., 2005) for the presence of predicted intrinsically disordered regions.

In vitro expression pattern of GAP65 using qPCR

Cherax quadricarinatus males were molt induced (as described above) and then dissected at the four major molt stages ($n = 5$ for each molt stage). RNA was extracted from the gastrolith, mandible cuticle, and carapace cuticle-forming epithelia by using an EZ-RNA Total RNA Isolation Kit (Biological Industries, Beit Haemek, Israel), according to the manufacturer's protocol. First-strand cDNA was synthesized by reverse transcription using the qScript cDNA Kit (Quanta BioSciences, Gaithersburg, MD) with 1 μg of total RNA. In addition, RNA was extracted from the muscle tissue of an inter-molt animal for normalization. Relative quantification of transcript levels was performed using Roche Diagnostics FastStart Universal Probe Master Mix (Basel, Switzerland) and Roche Universal Probe Library probes. The following primers and probe were used: q*GAP65* F and q*GAP65* R, and Probe #68. *Cherax quadricarinatus 18S*, which served as a normalizing gene, was also quantified by means of real-time RT PCR using the primers, qcq*18S* F and qcq*18S* R, and Probe #22. All primer sequences are shown in Table S1. Reactions were performed in the ABI Prism7300 Sequence Detection System, Applied Biosystems (Foster City, CA). Statistical analyses for relative transcript levels between the molt stages were performed using the non-parametric Kruskal–Wallis rank sum test, followed by multiple pair-wise comparisons using the Wilcoxon rank sum test; $P < 0.05$ was considered statistically significant.

Protein purification

To validate the presence of GAP65 in the investigated cuticular structures, we obtained late pre-molt gastroliths and exuviae (for cuticle and mandible that includes the molar and incisor teeth) which for sure do not contain any cellular components while most of the cuticular content is in place. All samples were washed in de-ionized distilled water, frozen in liquid nitrogen, ground to powder, and then incubated with the solvents as described below. The powder was dissolved at a concentration of 1 g per 20 ml 0.02 M ammonium acetate, pH 5.0, containing 0.5 M EGTA, with continuous stirring at 4°C overnight. The insoluble residue was precipitated by centrifugation ($1{,}500 \times g$ for 20 min at 4°C), and the supernatant was separated off. The supernatant was dialyzed twice, using a Cellu Sep dialysis bag, with a 6,000–8,000 Da cut-off (MFPI, Seguin, TX, USA)—first against 5 l of 200 mM ammonium acetate, pH 7.0, and then against 5 l of 20 mM ammonium acetate, pH 7.0, at 4°C overnight. The samples were concentrated using Vivaspin 20 (MWCO 7,000; Vivaproducts, Inc. Littleton MA, USA). Protein concentration in each sample was determined by the Bradford method using Bio-Rad reagent (Bio-Rad, Berkeley,

CA, USA). The protein profiles of the soluble fractions from the three cuticular structures were separated on an SDS-PAGE 4–12% Tris–glycine gel (Genscript, Piscataway, NJ, USA) using MES buffer, pH 5. The resulting bands were visualized by Coomassie brilliant blue staining, excised from the gel, and analyzed by tandem mass spectrometry, as described below.

Mass spectrometry

Extraction of the bands from the gel, mass spectrometry, and data analysis were performed according to Shechter et al. (2008c). The reduction, alkylation, and trypsinization steps were carried out as previously described (Roth et al., 2010). The tryptic digest was separated on a column constructed in-house (15 cm long, 75 μm internal diameter fused silica) that was packed with Jupiter C-18, 300 Å, 5-μm beads (Phenomenex, Torrance, CA, USA) and connected to an Eksigent nano-LC system (Eksigent, Dublin, CA). The peptides were eluted with the following solutions: buffer A was composed of 2% acetonitrile, 0.1% formic acid, and buffer B was composed of 80% acetonitrile in 0.1% formic acid, in nano-pure water. A linear gradient of 20–65% of buffer B was created over 45 min. MS peptide analysis and tandem MS fragmentation was performed using an LTQ-Orbitrap (Thermo Fisher Scientific, San Jose, CA, USA). The mass spectrometer was operated in the data-dependent mode to switch between MS and collision-induced dissociation tandem MS of the top six ions. The collision-induced dissociation fragmentation was performed at 35% collision energy and 30 ms activation time. Proteins were identified and validated against an internal database containing the predicted best translation of all the contigs from the molt-related transcriptomic library described above (Abehsera et al., 2015); for this purpose the Sequest algorithm operated under Proteome Discoverer 1.2 software (Thermo Fisher Scientific) was used. The following search parameters were used: enzyme specificity-trypsin, maximum two missed cleavage sites, cysteine carbamidomethylation, methionine oxidation, and a maximum of 10 ppm or 0.8 Da error tolerances for full scan and MS/MS analysis, respectively. Protein identification criteria were defined as: a minimal score of > 100, a minimum of two peptides, and a false discovery rate (FDR) with a P-value < 0.01.

Results

Characterization of GAP65 in the different cuticular structures

As the first step in investigating the typical patterns of structural proteins in cuticular structures, a representative structural protein that is known to play a central role in the formation of the gastrolith matrix, GAP65, was characterized in the three distinct epithelium-forming cuticular structures investigated in this study. To this end, qPCR was performed to quantify the expression of GAP65 at the different stages of the molt cycle in these cuticular structures. The expression patterns of *GAP65* in the mandible cuticle- and gastrolith-forming epithelia were compared to the in-silico findings based on our existing molt-related transcriptomic library (Fig. 1a). The pre-molt-related expression pattern in the gastrolith-forming epithelium and the molt-independent pattern of expression in the mandible cuticle-forming epithelium were verified in vitro (Fig. 1a). qPCR was also used to provide information on the expression pattern of *GAP65* in the carapace cuticle-forming epithelium. In the carapace cuticle-forming epithelium, the molt-related pattern of expression found in vitro showed high expression at post-molt and a significant reduction in GAP65 expression at early pre-molt (Fig. 1a). The presence of GAP65 in the extracellular matrix was revealed by protein purification from the exuviae of the mandible and the cuticle followed by MS/MS (showing a molecular mass of 65 kD, as expected) and validated in the gastrolith, which served as a positive control. As can be seen in Fig. 1b, the relative representation of GAP65 differs in the three distinct exoskeletal elements: it appears as a prominent band in the gastrolith, a less prominent band in the cuticle, and an almost insignificant band in the mandible.

Mining for matrix proteins

Based on the expression patterns of *GAP65*, new matrix proteins were mined from the transcriptomic library of the mandible and gastrolith using the binary-patterning approach. The results revealed both typical matrix proteins and new candidate matrix proteins. In the gastrolith-forming epithelium, the molt-independent 1111 pattern indicated mainly housekeeping genes, such as ribosomal proteins, and no putative

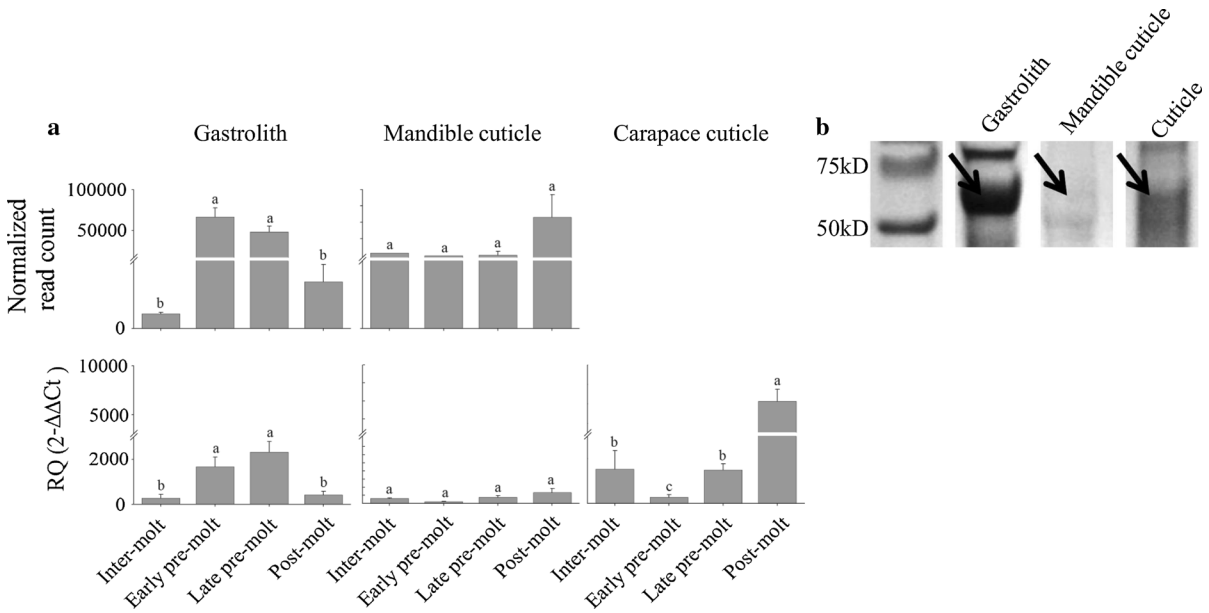

Fig. 1 Characteristics of GAP65, a typical structural protein of crustaceans. **a** Read count of *GAP65* in the molt-related transcriptomic library originating from the gastrolith- and mandible-forming epithelia (top). Relative levels of *GAP65* in the gastrolith-, mandible- and carapace cuticle-forming epithelia at four molt stages, as determined by qPCR. For all molt stages $n = 5$ (bottom). Different letters represent groups that are significantly different ($P < 0.05$); error bars represent standard error. **b** Electrophoresis of proteins extracted from the gastrolith, mandible and cuticle. Arrows point to the band in which GAP65 was detected

matrix proteins were revealed (Fig. 2, left). The pre-molt-related 0110 pattern, similar to *GAP65* expression in this exoskeletal element, showed the presence of known matrix proteins, such as GAP10 (Glazer et al., 2010), and newly discovered putative matrix proteins. Examples for that can be seen in acidic protein 1 and fructose-bisphosphate aldolase (Fig. 2, right).

In the mandible-forming epithelium, the molt-independent 1111 pattern, similar to *GAP65* expression in this exoskeletal element, revealed housekeeping genes, such as ribosomal proteins, and additional putative matrix proteins, such as chitin-binding protein 2 and cuticular protein 1 (Fig. 3, left). Unlike GAP65, the pre-molt-related binary pattern 0110 showed the presence of known matrix proteins such as M15 (Tynyakov et al., 2015c) and newly discovered putative matrix proteins. Examples for that can be seen in glycine-rich protein 1 and cuticular protein 3 (Fig. 3, right).

Characterization of newly found putative matrix proteins

The newly found putative matrix proteins were characterized in terms of the domains and amino acid sequences typical of known matrix proteins from crustaceans and other taxa. In the gastrolith and the mandible -forming epithelia a total of six putative chitin-binding proteins were found, namely, chitin-binding proteins 1 and 2 and cuticular proteins 1, 2, 3, and 4. These proteins had either a cysteine-rich chitin-binding domain (Fig. 4a) or a Rebers–Riddiford (R&R)-type chitin-binding domain (Fig. 4b). All the chitin-binding proteins were found to have regions predicted to be disordered and a signal peptide (Fig. 4) and were acidic in nature (Table S2). Two glycine-rich proteins—glycine-rich proteins 1 and 2—were also found. These proteins did not contain any known domains, but they did have a signal peptide. Their sequences were comprised mainly of multiple poly-glycine repeats (44.5 and 44.6% for glycine-rich proteins 1 and 2, respectively), and each had a lysine residue in its N′ terminal (Fig. 5). In addition, two proteins predicted to have an acidic nature were

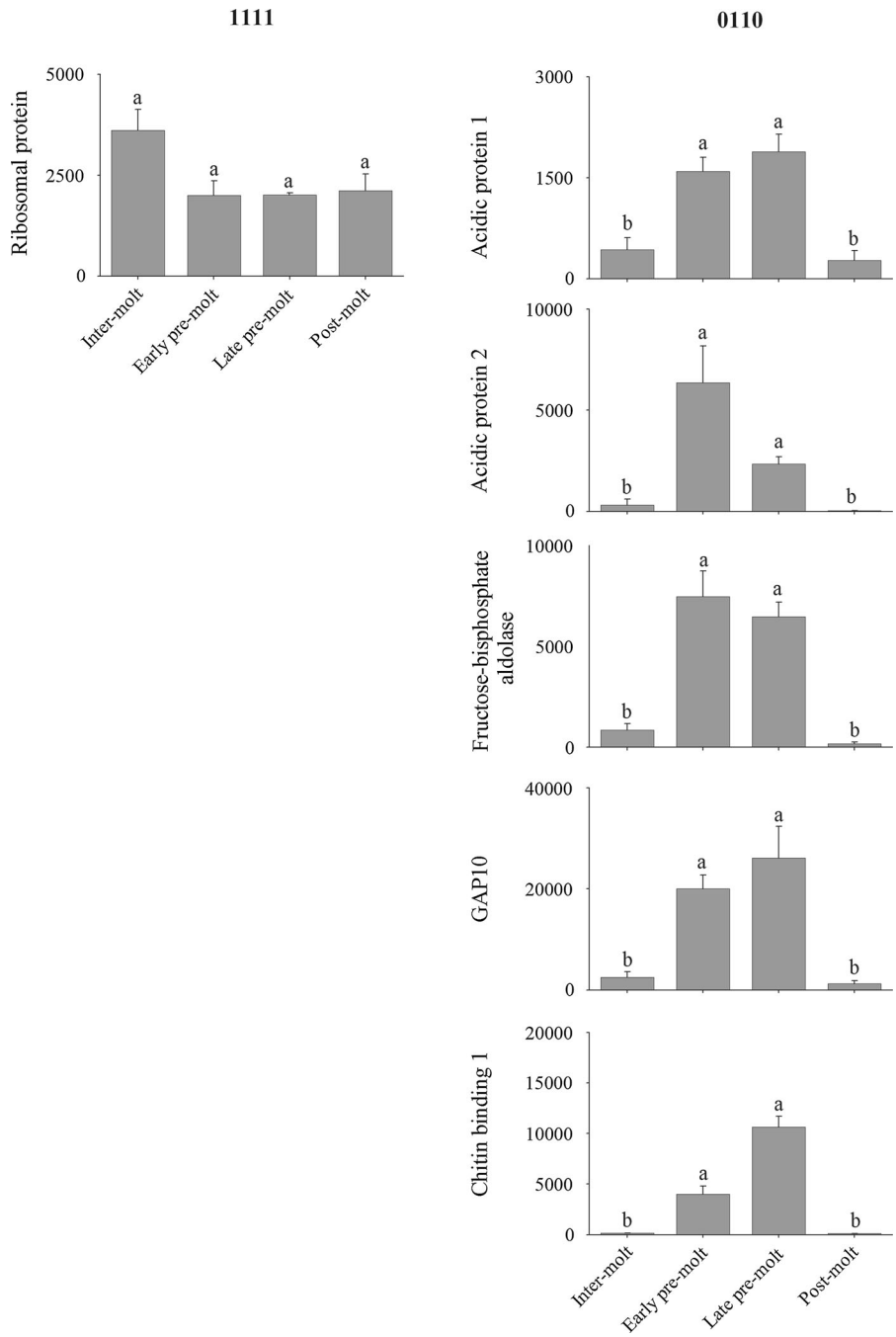

Fig. 2 Transcripts from the gastrolith that were found using the binary-patterning approach. *In-silico* temporal expression based on normalized read count of different transcripts found in the molt-related transcriptomic library of *Cherax quadricarinatus* from the gastrolith-forming epithelium having a 1111 binary pattern (left) and a 0110 binary pattern (right). Letters represent statistical groups that are significantly different (P-value < 0.05); error bars represent standard error

revealed (Table S2). These proteins—acidic proteins 1 and 2—also did not contain any known domains, but they did have disordered regions and a signal peptide (Fig. 6). Forty nine negatively charged residues (out of 308 residues) were found in the sequence of acidic protein 1, and 17 negatively charged residues were found in the sequence of acidic protein 2 (out of 93 residues). Both acidic proteins contained predicted phosphorylation sites—30 sites for acidic protein 1 and four sites for acidic protein 2.

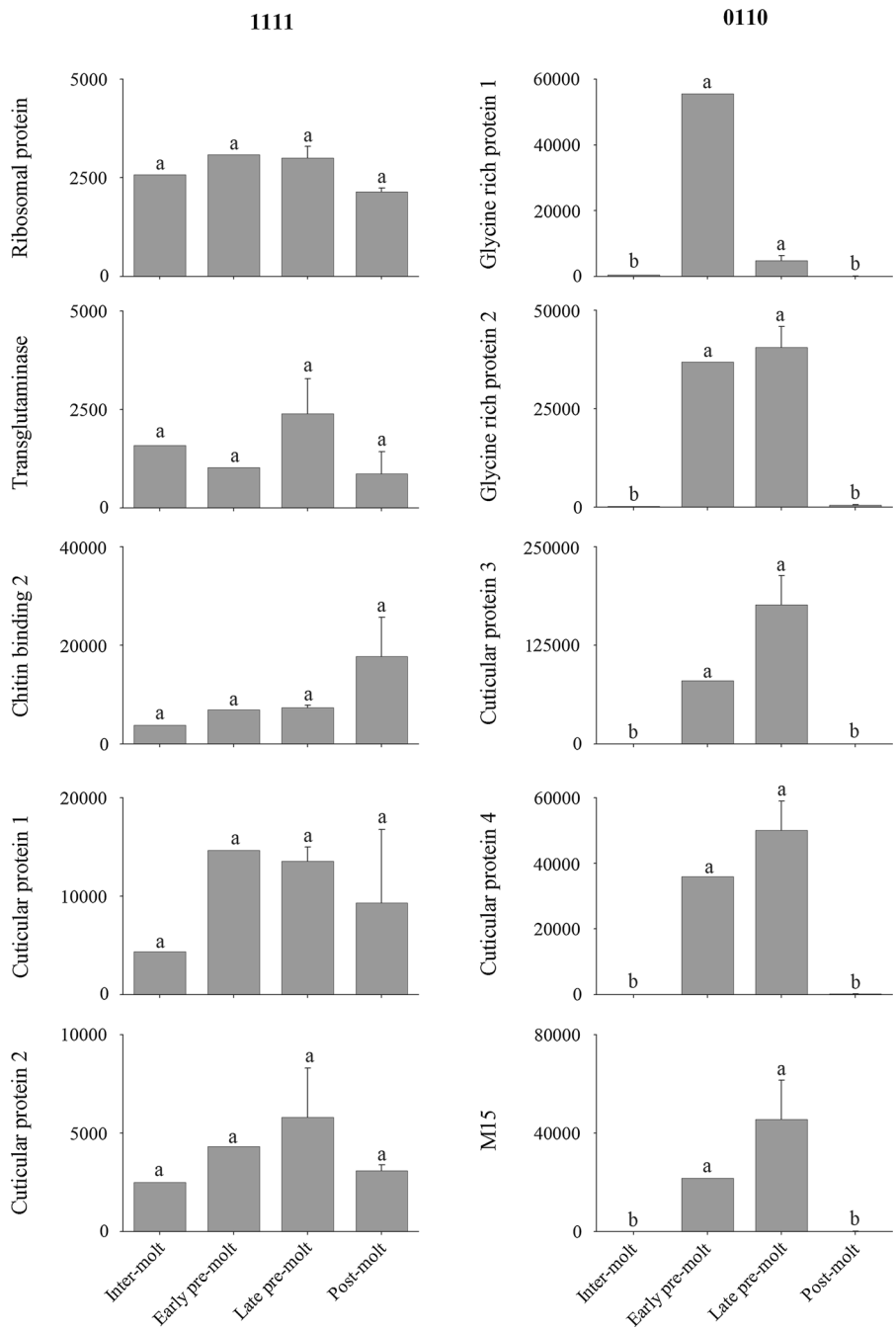

Fig. 3 Transcripts from the mandible cuticle that were found using the binary-patterning approach. *In-silico* temporal expression based on normalized read count of different transcripts found in the molt-related transcriptomic library of *Cherax quadricarinatus* from the mandible cuticle-forming epithelium having a 1111 binary pattern (left) and a 0110 binary pattern (right). Letters represent statistical groups that are significantly different (P-value < 0.05); error bars represent standard error

Discussion

GAP65, a crustacean-cuticle core protein

In this article a comprehensive approach, based on genomic techniques, for the study of structural proteins in crustaceans is demonstrated. The expression pattern of the gene encoding for GAP65, a key structural protein in *C. quadricarinatus* (Shechter et al., 2008c), is used to demonstrate a typical pattern of expression of a structural protein in the different forming epithelia of a crustacean. It is important to note that this typical pattern is used as a reference in our study, but it is neither needed in every

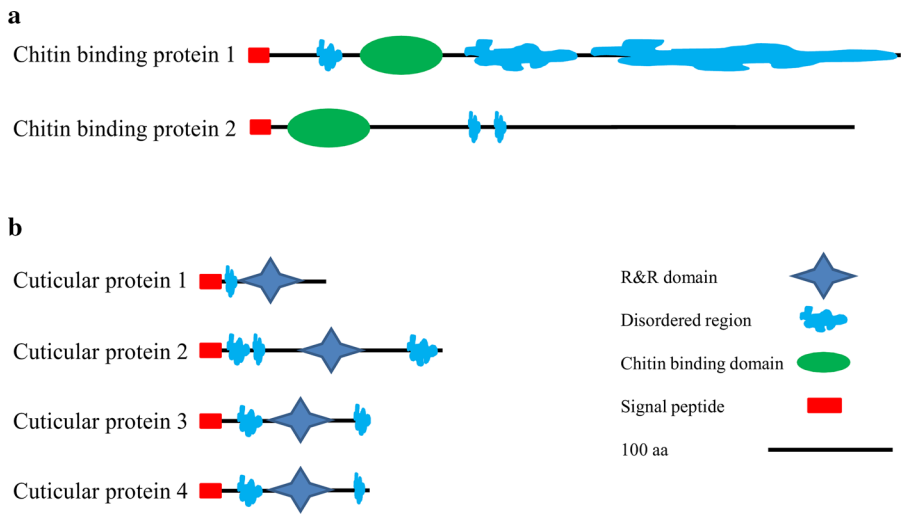

Fig. 4 Domain organization of newly found putative structural proteins. Schematic representation of newly found putative structural-proteins and their domain organization with **a** cysteine-rich type chitin-binding domain and **b** R&R type chitin-binding domain

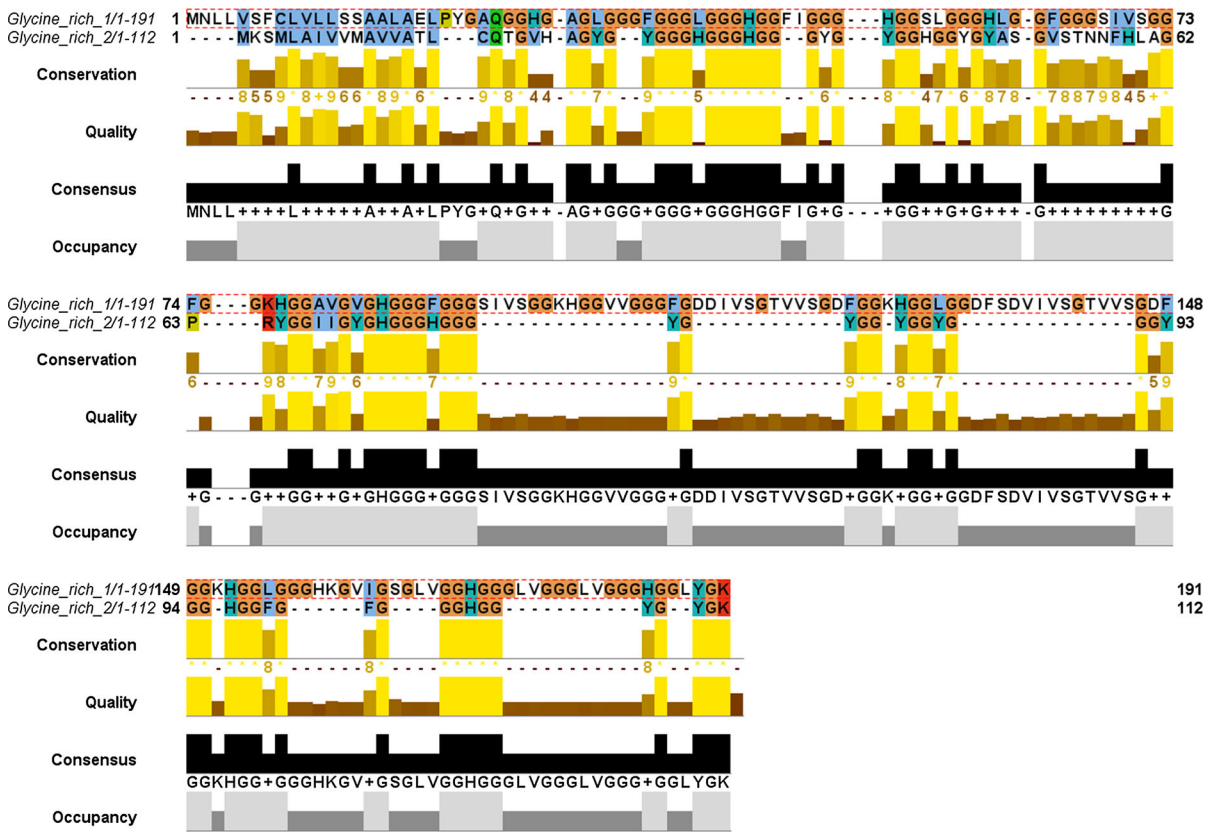

Fig. 5 Multiple sequence alignment of newly found glycine-rich proteins. Multiple sequence alignment of the glycine-rich proteins found in *Cherax quadricarinatus*. For each position in the sequence alignment, the conservation degree, quality, consensus sequence and occupancy are shown below

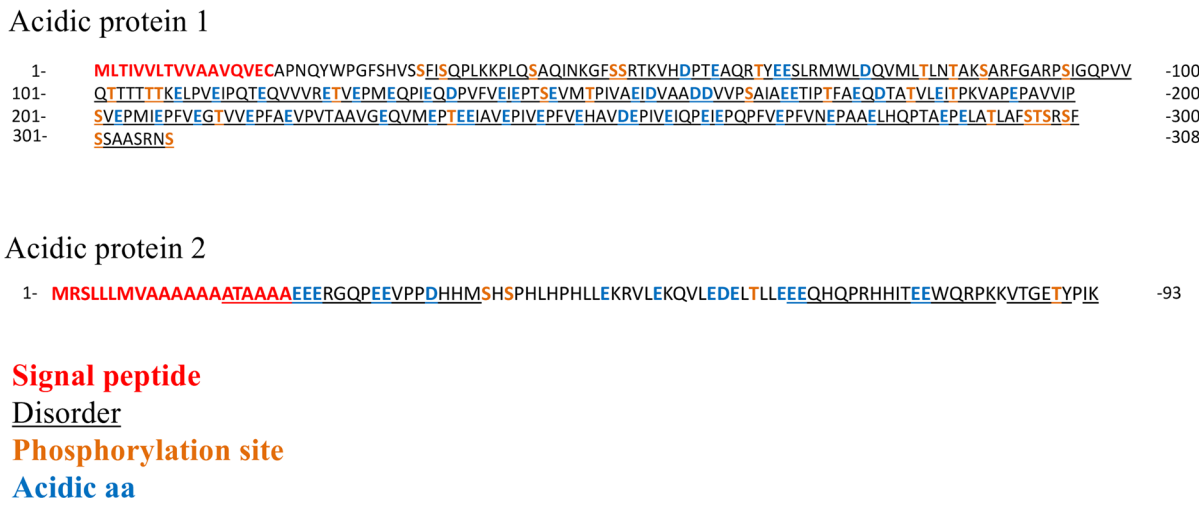

Fig. 6 Sequence characteristics of newly found acidic proteins. Sequence characteristics of the acidic proteins found in *Cherax quadricarinatus*. Predicted phosphorylation sites, acidic residues and predicted disordered regions are shown

study on structural proteins in crustaceans nor it is representative of the expression patterns of all the structural proteins of crustaceans. In the gastrolith-forming epithelium, the *GAP65* transcript was highly expressed during pre-molt concomitant with the rapid buildup of the gastroliths (Shechter et al., 2008a). Therefore, increased synthesis of structural proteins, such as GAP65, was to be expected in the gastroliths at pre-molt. In contrast, in the mandible's cuticle-forming epithelium, *GAP65* was highly expressed during the entire molt cycle. The most likely explanation for this constancy of the expression pattern is that during pre-molt and post-molt the protein is involved in the formation of the mandible (Bentov et al., 2012; Tynyakov et al., 2015a; Abehsera et al., 2017). The expression at inter-molt might be attributed to an unknown function of GAP65 which is not related to the cuticular structures. It might be attributed to maintenance processes of the teeth that undergo abrasion constantly which possibly leads to the expression of structural proteins in the mandible-forming epithelium during inter-molt. A similar expression pattern (high throughout the entire molt-cycle) has previously been reported in the mandibles of *C. quadricarinatus* for genes related to chitin metabolism (Abehsera et al., 2015). In the cuticle-forming epithelium, the expression of *GAP65* seems to follow the formation of the different cuticular layers. It is probable that the reduction in *GAP65* expression during early pre-molt, when the outer non-chitinous epicuticle layer is formed, is related to the lack of GAP65 involvement in the formation of this layer (Roer & Dillaman, 1984), which requires a different set of structural proteins that are not related to the chitinous scaffold. GAP65's role in the formation of chitinous matrices is reflected in the rise in *GAP65* expression during late pre-molt, when a chitinous layer, the exocuticle, is partially formed (Roer & Dillaman, 1984). The significant post-molt rise in *GAP65* expression may be attributed to the rapid formation of the chitinous endocuticle and the completion of the exocuticle formation (Roer & Dillaman, 1984). Here, too, as suggested above for the mandible, the high *GAP65* expression detected during inter-molt might be related to maintenance. These findings strengthen the notion that GAP65 is a core protein involved in the formation of the cuticular structures in crustaceans, as it is present in all three different cuticular structures of *C. quadricarinatus* and in all the protein complexes found in the gastrolith (Glazer et al., 2015). Since *GAP65* has two binary patterns (1111 in the mandible cuticle and 0110 in the gastrolith), these two binary patterns were chosen as representative examples to demonstrate the utility of our approach for mining for putative structural proteins: additional molt-related transcripts were indeed found in the present study to have a binary pattern of expression. To summarize our findings and to place them in the context of the body of knowledge about cuticular proteins, we present a schematic

representation of the presence and possible involvement of proteins in the different cuticular structures of *C. quadricarinatus* (Fig. 7), which is discussed in detail in the final section of this "Discussion".

The final point that emerges from our study of GAP65 is the necessity to study crustacean structural protein transcripts not only spatially in different tissues but also temporally at different stages of the molt cycle. When GAP65 was first reported, it was believed to be specific to the gastrolith-forming epithelium (Shechter et al., 2008c). This premise of gastrolith specificity was based on the expression of

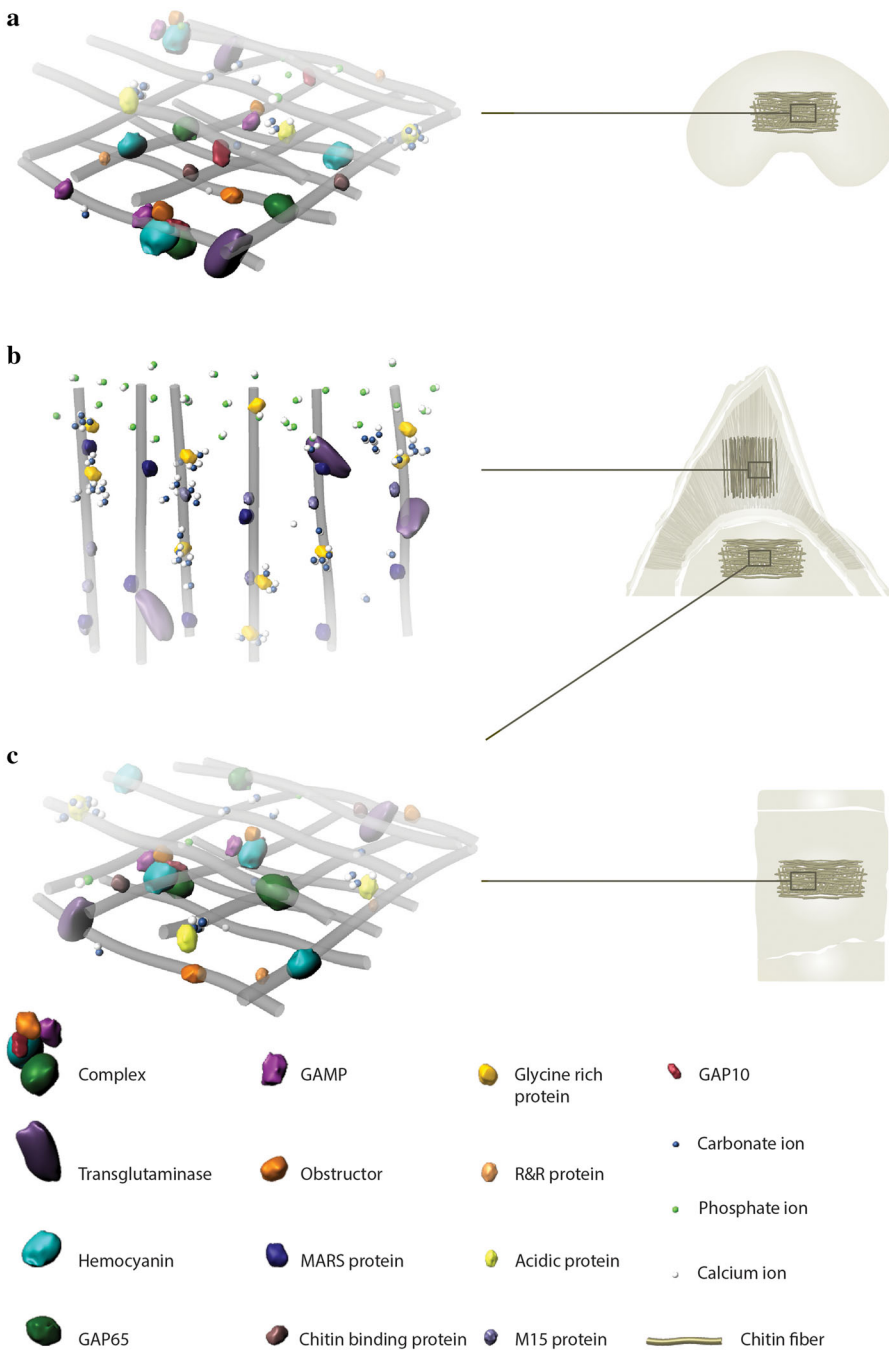

Fig. 7 Schematic representation of the presence and putative involvement of proteins in *Cherax quadricarinatus* cuticular structures. **a** gastrolith, **b** mandible, including the top vertical layer and the bottom horizontal layer, and **c** general exoskeletal cuticle. The bottom horizontal layer of the mandible and the bulk cuticle are presumed to be similar. A legend describing the different proteins and ions present in the schematic representation is given at the bottom of the figure

GAP65 at a single time point that probably corresponded to early pre-molt, during which the expression of *GAP65* is high in the gastrolith but low in the cuticle (Shechter et al., 2008c). However, the findings of this study demonstrate that *GAP65* is systematically expressed in all three different types of tissue/organ-forming epithelia of *C. quadricarinatus*. The initial misapprehension regarding the expression of *GAP65* thus serves to stress the need to study crustacean structural protein transcripts both in different tissues and in the context of the entire molt cycle and not at specific stages/time points.

Expression patterns of proteins involved in the formation of the gastroliths

As mentioned above, gastroliths are transient calcium storage organs composed of a simple chitinous scaffold mineralized mostly by ACC (Shechter et al., 2008a). The molt-independent 1111 binary pattern for the gastrolith-forming epithelium demonstrates the sole presence of housekeeping gene-like transcripts, probably due to the fact that gastrolith buildup occurs at pre-molt rather than at post-molt and inter-molt. Many molt-related transcripts were found to have a pre-molt-related binary pattern (0110) in the gastrolith-forming epithelium, similar to *GAP65*. Among these molt-related transcripts, *GAP10*, which encodes a structural protein found to play a role in the mineralization of the gastroliths (Glazer et al., 2010), was identified in this study and is included in our schematic representation (Fig. 7). Expression of yet another transcript probably related to the mineralization of the gastrolith is that of *fructose bisphosphate aldolase*, which catalyzes the formation of the triose phosphates, dihydroxyacetone phosphate (DHAP), and glyceraldehyde 3-phosphate (G3P) (Marsh & Lebherz, 1992). The assumption that the *fructose bisphosphate aldolase* transcript plays a role in the mineralization of the gastrolith is based on findings of Sato et al., (2011) that glycolytic intermediates are involved in the stabilization of ACC in the gastroliths of *P. clarkii* and findings regarding the involvement of glycolytic intermediates in the stabilization of ACC in the gastroliths of *C. quadricarinatus* (Akiva-Tal et al., 2011). A rise in glycolysis to meet the higher energy needs in the gastroliths during pre-molt might be another explanation for these results. Other transcripts predicted to be involved in the mineralization of the gastroliths are genes encoding acidic proteins, which were found to have a pre-molt-related 0110 binary pattern. These proteins are predicted to play a role in the mineralization of the gastrolith ACC, as previously suggested regarding acidic proteins by Addadi et al. (2003).

Other putative proteins identified in the gastrolith were the chitin-binding proteins. These proteins probably play a role in the formation of the chitinous scaffold that is mediated through the formation of a protein complex, as is indicated by the presence of predicted intrinsically disordered regions (IDR). In other IDR-bearing proteins, the structure of the IDRs is achieved only upon binding to a substrate in a disorder-to-order transition (Dyson & Wright, 2002; Vuzman & Levy, 2012). In the extracellular matrix of *C. quadricarinatus*, such a substrate is most likely to be a structural protein having an appropriate domain composition, such as GAP65, and known to form protein complexes as described above (Shechter et al., 2008b; Glazer et al., 2015). In summary, the expression pattern of proteins involved in the formation of the gastroliths is related to pre-molt, and since the gastroliths have a rather simple structure, these proteins are mostly related to the formation of the chitinous scaffold and to the stabilization of ACC directly or through different molecules such as carbohydrates.

Expression patterns of proteins involved in the formation of the mandible

The expression of the protein transcripts involved in the formation of the mandible-forming epithelium comprised two central binary patterns, the molt-independent binary pattern 1111, and the pre-molt-related binary pattern 0110. First, as found for the *GAP65* transcript in the mandible-forming epithelium, the 1111 binary pattern in the gastrolith revealed putative molt-related proteins. Additionally, as expected, housekeeping-gene-like transcripts were also found. Among the molt-related proteins having the 1111 binary pattern, we found transglutaminase. This protein is known to catalyze crosslinking through an isopeptide bond. This function is involved in hardening of the cuticle of different arthropods such as insects and horseshoe crab (Iijima et al., 2005; Shibata et al., 2010) and probably also of the crustacean cuticle, as shown in our schematic representation

(Fig. 7). Other transcripts exhibiting the 1111 pattern are chitin-binding proteins (having a cysteine-rich type chitin-binding domain), whose role could be related to the formation of the chitinous scaffold. Since they have an acidic nature, these proteins may also be involved in the mineralization processes, as is known for many acidic proteins (Addadi & Weiner, 1985). The expression of these proteins during intermolt is puzzling. It might be attributed to the same reasons as mentioned above for GAP65's expression, i.e., maintenance, or a different yet unknown role.

The pre-molt-related 0110 binary pattern in the mandible-forming epithelium is in keeping with mineral deposition and the formation of chitinous vertical elements during this stage (Bentov et al., 2012; Tynyakov et al., 2015a; Abehsera et al., 2017). The glycine-rich proteins having a 0110 expression pattern are predicted to be involved in the reinforcement of vertical elements, similarly to the MARS protein family, as depicted in our schematic representation (Fig. 7). In many taxa, glycine-rich proteins are known to reinforce extracellular matrices (Ringli et al., 2001; Patino et al., 2002; Ding et al., 2014; Guerette et al., 2014). In *C. quadricarinatus*, such proteins are possibly involved in the formation of all the cuticular elements, as found in insects (Charles et al., 1992; Zhong et al., 2006), while in the mandible the reinforcement of the vertical elements probably necessitates higher expression of such glycine-rich proteins (Fig. 7). Cuticular proteins having an R&R chitin-binding domain (a domain that is found uniquely in arthropod cuticular proteins) (Rebers & Riddiford, 1988; Rebers & Willis, 2001) were found to have a 0110 binary pattern in the mandible. Among these proteins, M15 has previously been suggested to be involved in the mineralization of the mandible (Tynyakov et al., 2015b). Additional cuticular proteins having an R&R domain and an acidic nature are most probably also involved in the mineralization of the mandible (Fig. 7). The complexity of the proteinaceous basis for mandible formation—including the involvement of an extensive set of proteins having different expression patterns—is probably related to structural variations in the mandible, which has different layers and includes a variety of mineral polymorphs (Fig. 7) (Bentov et al., 2012; Abehsera et al., 2017).

Expression patterns of proteins involved in the formation of the cuticle

The cuticle-forming epithelium was not sampled in the transcriptomic library (Abehsera et al., 2015) on which our study is based. Therefore, we can only deduce the involvement of structural proteins in the formation of the cuticle from the pattern of expression of *GAP65* and previous data in a variety of publications. As stated above, *GAP65* expression in the cuticle seems to follow the formation of the different chitinous cuticular layers. Other putative chitin scaffold related proteins, such as chitin-binding proteins, found in this study are predicted to have similar expression patterns in the cuticle-forming epithelium. The expression of structural proteins involved in the mineralization of the cuticle is probably specific to the post-molt stage in which the cuticle undergoes rapid hardening through mineralization (Roer & Dillaman, 1984). An example for such a pattern of expression in the cuticle is the expression pattern of the acidic protein DD4 found in the prawn *Penaeus japonicas* having a calcium-binding ability and highly expressed at post-molt (Endo et al., 2000). Another possible scenario for an expression pattern for mineralization-related proteins in the cuticle comprises expression of the proteins at pre-molt, followed by their incorporation into the newly forming cuticle, so that at post-molt they can incorporate calcium immediately. Other examples for proteins involved in the formation of the crustacean cuticle are proteins having the known chitin-binding R&R domain (Wynn & Shafer, 2005) and glycoproteins (Compere et al., 2002; Tweedie et al., 2004). The formation of the epicuticle during early pre-molt probably involves a set of structural proteins that differs from that in the chitinous layers of the cuticle, since the former layer is composed only of proteins and lipids (Welinder, 1974; Roer & Dillaman, 1984).

In general, in light of the importance of the cuticle, the proteinaceous basis for cuticle formation in crustaceans has not been investigated extensively with a small number of studies regarding that subject (Kragh et al., 1997; Faircloth & Shafer, 2007; Kuballa et al., 2007). This lack of information regarding the proteinaceous basis for the formation of the crustacean cuticle is surprising. A major reason for that might lie in the fact that the crustacean cuticle is a highly complex bio-material that includes different minerals

such as calcium carbonate, calcium phosphate, and magnesium calcite, and these minerals are found in different polymorphs including stabilized amorphous phases (Becker et al., 2005; Hild et al., 2008; Al-Sawalmih et al., 2009; Bentov et al., 2012, 2016; Vatcher et al., 2015). Therefore, the field of biomineralization and structural biology should benefit greatly from further research on the proteinaceous basis for cuticle formation in crustaceans.

A schematic representation for the proteinaceous basis for the formation of cuticular structures in crustaceans

Here we suggest a schematic representation for the proteinaceous basis of the cuticular structures of crustaceans. In this schematic representation, three cuticular structures are presented with their chitinous scaffolds and known and newly found structural proteins (Fig. 7). It is assumed that three proteins are present in all the different structures—GAP65 (the core protein), hemocyanin, and transglutaminase (involved in hardening through sclerotization)—since these proteins are commonly held to play key roles in the formation of the chitinous scaffold and hence to be part of the basic toolkit of crustacean cuticular structures. Proteins found so far only in one cuticular structure, such as GAMP (Ishii et al., 1996) and GAP10 (Glazer et al., 2010) in the gastrolith, or the MARS family found only in the mandible (Abehsera et al., 2017) are shown in those particular cuticular structures (Fig. 7). Such proteins might be involved in the formation of other cuticular structures but have not been found due to technical reasons or they might be part of a specialized toolkit for each structure providing that structure with its unique features.

Chitin-binding proteins are common in the crustacean cuticle and indeed in arthropods in general. Their ability to bind to chitinous scaffolds provides them with the potential to be multifunctional. An example presented in our schematic representation is M15, which is known to bind to chitin through an R&R domain and is predicted to play a role in mineralization (Tynyakov et al., 2015c). Another example shown in our schematic representation is the Obstructor protein family, first identified in *Drosophila melanogaster* by Behr & Hoch (2005). This family of cuticular proteins is characterized by an N-terminal signaling peptide and three cysteine-rich chitin-binding domains (CBDs) (Behr & Hoch, 2005). These proteins have the dual ability to bind to the chitinous scaffold and to form protein complexes (Pesch et al., 2015). Such a dual role is predicted for all the different chitin-binding proteins, mainly because anchoring to the chitinous scaffold does not seem to have a purpose in itself in the formation of the cuticle. In general, multifunctionality of proteins is known to exist in several biomineralization processes such as in mollusk shell matrix proteins (Marin et al., 2007; Yan et al., 2007) and vertebrates bone proteins (Fisher et al., 2001; Cho et al., 2009; Vincent & Durrant, 2013).

The final group of proteins that are included in our schematic representation comprises the acidic proteins (including the chitin-binding acidic proteins), which are believed to control the complex mineral morphism (Welinder, 1974; Addadi & Weiner, 1985; Lowenstam & Weiner, 1989; Simkiss & Wilbur, 2012) that is unique to the crustacean cuticle (Bentov et al., 2012; Luquet 2012; Huber et al., 2014). As may be seen in our schematic representation the proteinaceous basis for cuticle formation in crustaceans involves many different types of proteins, the necessity for such a wide set of proteins probably being related to the structural complexity of the crustacean cuticle. Thus far, this representation is based only on a partial picture and more research is needed to complete the picture.

In conclusion

In this study, we established the framework for a comprehensive approach for the study of cuticular proteins in crustaceans. The sparsity of information regarding such proteins strengthens the need to develop such an approach. Importantly, we demonstrate that the expression of cuticular transcripts must be tested both temporally during the molt cycle and spatially in the different cuticular structures. The use of filtering research methods, such as the binary-patterning approach, that are based on spatial and temporal expression is therefore essential for any study of this nature. We also demonstrate that in such studies it is necessary to take into account the characteristics of the cuticular proteins, including: their acidic nature, their ability to bind chitin, their functions related to cuticle formation, such as sclerotization, and the presence of repetitive motifs. Since

cuticle formation in crustaceans is an important issue in fields such as structural biology (Lowenstam & Weiner, 1989) and bio-mimetics, employing our framework could greatly contribute to progress in these fields.

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