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Insect Biochemistry and Molecular Biology 60 (2015) 7–12

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Elucidation of the serosal cuticle machinery in the beetle *Tribolium* by RNA sequencing and functional analysis of *Knickkopf1*, *Retroactive* and *Laccase2*

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ARTICLE INFO

Article history:
Received 23 October 2014
Received in revised form
22 February 2015
Accepted 23 February 2015
Available online 4 March 2015

Keywords:
Tribolium castaneum
Cuticle
Knickkopf
Retroactive
Laccase
Desiccation

ABSTRACT

Insects have been extraordinary successful in colonizing terrestrial habitats and this success is partly due to a protective cuticle that mainly contains chitin and proteins. The cuticle has been well studied in larvae and adults, but little attention has been paid to the cuticle of the egg. This cuticle is secreted by the serosa, an extraembryonic epithelium that surrounds the yolk and embryo in all insect eggs, but was lost in the Schizophoran flies to which *Drosophila* belongs. We therefore set out to investigate serosal cuticle formation and function in a beetle (*Tribolium castaneum*) using RNAi-mediated knockdown of three candidate genes known to structure chitin in the adult cuticle, and we aimed to identify other serosal cuticle genes using RNA sequencing. Knockdown of *Knickkopf* (*TcKnk-1*) or *Retroactive* (*TcRtv*) affects the laminar structure of the serosal cuticle, as revealed by Transmission Electron Microscopy in knockdown eggs. In the absence of this laminar structure, significantly fewer eggs survive at low humidity compared to wild-type eggs. Survival in dry conditions is also adversely affected when cross-linking among proteins and chitin is prevented by *Laccase2* (*TcLac-2*) RNAi. Finally, we compare the transcriptomes of wild-type eggs to serosa-less eggs and find serosa-biased expression of 21 cuticle-related genes including structural components, chitin deacetylases and chitinases. Our data indicate that the serosal cuticle utilizes the same machinery for structuring the cuticle as adults. We demonstrate that the structure of the cuticle is crucial for desiccation resistance, and we put forward the serosal cuticle of *Tribolium* as an excellent model to study the ecological properties of the insect cuticle.

1. Introduction

Insects are among the earliest land animals and their cuticle facilitated their spectacular terrestrial success (Grimaldi and Engel, 2005). This cuticle is an apical extracellular matrix that is produced by the epidermis and mainly consists of chitin complexed with proteins (Merzendorfer, 2006; Moussian, 2010). The two main groups of structural cuticle proteins are CPAPs (Cuticular Proteins Analogous to Peritrophins) containing the chitin binding Peritrophin-A motif (Jasrapuria et al., 2010), and CPRs (Cuticular Proteins that contain the Rebers and Riddiford chitin binding domain) (Andersen et al., 1997; Ioannidou et al., 2014; Rebers and Riddiford, 1988). The cuticle further contains metabolites that are involved in tanning, and lipids that are mainly present in the outer layer of the cuticle, the epicuticle (Merzendorfer, 2006; Moussian, 2010).

The production of cuticle has received much attention, and many genes involved in the process have been identified including *Chitin synthase, Retroactive* and *Laccase* (Charles, 2010; Moussian, 2010, 2013; Ostrowski et al., 2002). Chitin synthase produces chitin that is subsequently secreted to the extracellular space through pores in the cell membrane (Arakane et al., 2004, 2005b, 2008; Merzendorfer, 2006; Moussian, 2010). There, it is organized into lamellae by Knickkopf proteins, a three membered family of which Knickkopf1 has been most extensively studied (Chaudhari et al., 2011; Moussian et al., 2006). Retroactive is involved in the trafficking of Knickkopf to the cuticle (Chaudhari et al., 2013; Moussian et al., 2005). To stabilize this extracellular structure, the cuticle of *Tribolium castaneum* is sclerotized and pigmented by the phenoloxidase Laccase2 (Arakane et al., 2005a). This sclerotization

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http://dx.doi.org/10.1016/j.ibmb.2015.02.014
0965-1748/

includes the cross-linking of proteins (Andersen, 2010; Kramer et al., 2001), but cross-linking between chitin and proteins is also thought to occur (Schaefer et al., 1987). Chitin can be digested by chitinases, required for molting (Zhu et al., 2008).

Although much is known about the genetic pathways involved in the production of the larval and adult cuticle, there is little information on the serosal cuticle in the insect egg. In insect eggs, a serosal cuticle is formed by the extraembryonic serosa (Furneaux et al., 1969; Goltsev et al., 2009; Hinton, 1981; Jacobs et al., 2013; Lamer and Dorn, 2001; Rezende et al., 2008; Vargas et al., 2014). This serosa envelops both the embryo and the yolk, and is formed early during development (Panfilio, 2008; van der Zee et al., 2005) (Fig. 1). Although lacking in *Drosophila*, the serosa is prevalent across Insecta (Roth, 2004) and protects the embryo from desiccation and infection (Gorman et al., 2004; Jacobs et al., 2013, 2014). In the moth *Manduca sexta*, secretion of the serosal cuticle takes place until 44 h after egg laying, i.e. until 36% of total embryonic development (Lamer and Dorn, 2001). In the cricket *Acheta domesticus*, a decrease in thickness of the serosal cuticle is observed after 108 h, i.e. after 50% of total embryonic development, presumably to prepare for hatching (Furneaux et al., 1969).

In *Acheta, Manduca*, and *Tribolium*, the serosal cuticle shows a laminar organization which is similar to the adult cuticle (Chaudhari et al., 2011, 2013; Furneaux et al., 1969; Jacobs et al., 2013; Lamer and Dorn, 2001). In *Tribolium,* parental RNAi for *chitin synthase 1* (*TcCHS-1*; also known as *CHS-A* (Arakane et al., 2008)) leads to a depletion of chitin in the serosal cuticle (Jacobs et al., 2013). This phenotype is similar to the one found in adults (Arakane et al., 2005b, 2008). Furthermore, experiments using microarrays in the mosquito *Anopheles gambiae* indicated that the same genes are utilized for the production of both the adult and serosal cuticle (Goltsev et al., 2009). This suggests that a similar machinery might be utilized by the serosa for cuticle production; however, functional data on serosal cuticle synthesis are still missing.

Here, we chose three genes known from larval and adult cuticle formation (*Knickkopf1*, *Retroactive* and *Laccase2),* and set out to investigate their role in production of the serosal cuticle in the red flour beetle *T. castaneum*. We first show the effect of the knockdown of these genes on cuticle structure by Transmission Electron Microscopy (TEM). As knockdown still allowed larvae to hatch, this provided a unique opportunity to assess the effect of cuticle structure on the ability of eggs to survive dry circumstances. To identify other genes that might play a role in the serosal cuticle, we compared the transcriptomes of wild-type eggs to serosa-less eggs generated by *TcZen-1* (*Zerknüllt-1*) RNAi (Jacobs et al., 2013; van der Zee et al., 2005). *TcZen-1* specifies the serosa during early development, and its knockdown transforms the entire blastoderm into germ rudiment deleting the serosa (van der Zee et al., 2005). Our data confirm that the same machinery is utilized to structure the serosal cuticle and that structure and cross-linking are important for the water-proofing abilities of the cuticle.

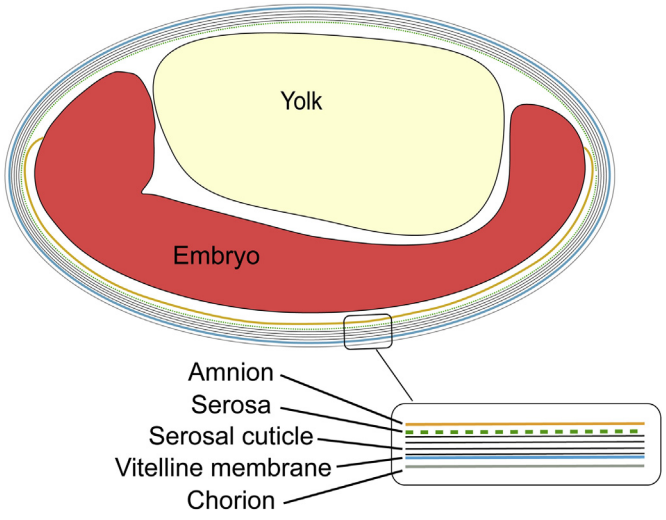

Fig. 1. Schematic overview of the *Tribolium castaneum* egg. The *Tribolium castaneum* egg is surrounded by two maternal layers, the chorion and the vitelline membrane. Beneath these, the extraembryonic serosa secretes a chitinous cuticle (serosal cuticle). The serosa and serosal cuticle envelop both the embryo and the yolk.

2. Materials and methods

2.1. Insect rearing

The *T. castaneum* wild-type strain San Bernardino was used for all experiments. Beetles were kept as in van der Zee et al. (2005). The eggs of *T. castaneum* take approximately 85 h to develop at 30 °C (Howe, 1956) and the serosa develops between 8 and 14 h after laying (Handel et al., 2000). The embryonic cuticle develops late, at least not before 65 h after laying (Jacobs et al., 2013).

2.2. Molecular cloning and parental RNAi

Gene fragments were amplified from embryonic cDNA using the primers described in Chaudhari et al. (2011) for *TcKnk-1,* and in Chaudhari et al. (2013) for *TcRtv.* For *TcLac-2* we used the primers fw '5-CCAGCTCAGTTGACGATTCA-3' and rv '5-TCAACTTTGTGGGTGCACAT-3' giving a sequence that targets both of the *TcLac-2* splice variants (Arakane et al., 2005a). These fragments were cloned into the pCRII-TOPO vector (Invitrogen) which was subsequently linearized with BamHI or XhoI for in vitro transcription using SP6 or T7 polymerases (MEGAscript kit, Ambion). Although injection of *TcKnk-1*, *TcRtv* or *TcLac-2* dsRNA into pupae of the Georgia strain is lethal (Arakane et al., 2005a, 2005b; Chaudhari et al., 2011; Chaudhari et al., 2013), injection of 0.2 μl of a 0.5–1.0 μg/μl dsRNA solution into the abdomen of female pupae from the San Bernardino strain resulted in viable adult females from which eggs were collected after non-injected males were added. As the parental RNAi phenotype in eggs has been reported to be the strongest in the first week of egg laying (Bucher et al., 2002), we only used the eggs laid in this first week.

2.3. RNA extraction and qRT-PCR

Total RNA of approximately 300 eggs that were 16–24 h old was extracted using TRIzol (Invitrogen), DNA digested, and purified using the RNeasy kit (Qiagen). The quality of RNA preparation was confirmed by electrophoresis on an agarose gel and by photospectrometry. cDNA was synthesized from 1 μg of total RNA using the Cloned AMV First Strand Synthesis kit (Invitrogen). qPCR reactions of 25 μl were assembled using the qPCR kit for SYBR Green I (Eurogentec) and contained 12.5 ng of cDNA. 45 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s were run on a CFX96 thermocycler (Biorad), followed by a ramp from 65 °C to 95 °C with a read every 0.5 °C for dissociation analysis. The qPCR primers for *TcKnk-1* were '5- CCTACAAGGGCGAGACCATC-3' and '5-GGTGGTGTTCGTGCGGAATA-3'; for *TcRtv* '5-TTTGTATGCCGATCTCGGGG-3' and '5-GGAACTGCCTCTACGCCTTT-3'; and for *TcLac-2* '5-TACAACAGACATTTAGTTGCACCA-3' and rv '5-AGGTGGGGCCATGTAGGAAA-3'. *RPL13a* was used as reference gene (Lord et al., 2010), and relative quantification of mRNA was done using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Two biological replicates (independent RNA isolations) were collected and each sample was measured by qRT-PCR twice (technical replication). T-tests with

correction for multiple testing were performed on the ΔC_T −values to identify significant expression differences.

2.4. Transmission Electron Microscopy

For electron microscopy, 24–39 h old wild-type, and *TcKnk-1*, *TcRtv,* and *TcLac-2* knockdown eggs were collected, dechorionated in 4% hypochlorite (w/v), and fixed for 1 h in 5 ml heptane and 5 ml of a 2.5% gluteraldehyde (v/v) and 2% paraformaldehyde (w/v) solution in 0.1 M cacodylate buffer (pH 7.4). Subsequently, eggs were removed from this solution, washed with 70% ethanol to remove the heptane, and fixed for another hour in the same fixative without heptane. After fixation, specimens were washed (3 × 10 min) in cacodylate buffer and placed for 1 h in 1% osmium tetraoxide (w/v). Then, specimens were dehydrated with increasing concentrations of ethanol and embedded in Agar100. Sections of about 70 nm thickness were contrasted with uranyl acetate and lead citrate, and studied with a JEOL 1010 transmission microscope coupled to an Olympus MegaView camera.

2.5. Generation of serosa-less eggs

Serosa-less eggs were generated using *TcZen-1* RNAi (van der Zee et al., 2005). This method prevents development of the serosa and is well-established to analyze its function (Jacobs et al., 2013, 2014; Jacobs and van der Zee, 2013).

2.6. Hatching rate assays

Eggs were collected overnight and put individually in a well of a 96-well plate, and incubated at 5, 50 or 90% relative humidity (RH) at 35 °C. Hatching rates were assayed after 4 days, providing ample time for hatching which takes 2.7 days at 35 °C (Howe, 1956). The experiments at each humidity were repeated four to nine times, giving rise to standard errors as in Fig. 3. Five per cent RH was obtained using silica gel; 50 and 90% RH were obtained in climate chambers. Temperature and humidity were constantly monitored using a MicroDAQ EL-USB-2 datalogger.

2.7. Analysis of RNA sequencing data

To obtain a list of genes with serosa-biased expression, we reanalyzed sequencing data from wild-type and *TcZen-1* RNAi eggs that were 30–46 h old (Jacobs et al., 2014). These data contain 3 biological replicates, and we used the DEseq package in R (Anders and Huber, 2010) to identify significantly differentially expressed genes (adjusted p-value < 0.01). Gene Ontology (GO) terms were assigned to *Tribolium* genes (TCnumbers from assembly 3.0 at BeetleBase) by performing BLASTX against *Drosophila melanogaster* proteins and taking the *Drosophila* GO terms of the best hit (E value < 1.0E-10). The sequence data have been deposited in NCBI's Gene Expression Omnibus (Barrett et al., 2013) and are accessible through GEO Series accession numbers GSM1305910, GSM1305911, GSM1305912. GSM1305931, GSM1305932, GSM1305933 (http://www.ncbi.nlm.nih.gov/geo).

2.8. Statistical analyses of hatching data

As the hatching data deviated significantly from the normal distribution, we performed a square root transformation to obtain a normal Gaussian distribution. We analyzed the hatching rates by ANOVA and assessed differences between humidities by a post-hoc Tukey HSD test. All analyses were performed in R (R Development Core Team, 2009).

3. Results and discussion

3.1. TcKnk-1 and TcRtv RNAi affect the laminar structure of the serosal cuticle

To assess the efficiency of the parental RNAi, we verified gene knockdown in 16–24 h old eggs using qPCR. This time interval begins soon after the serosa has been completely formed (after 14 h, Handel et al., 2000), and very likely includes the initial period of cuticle deposition. Knockdown of *Knickkopf* (*TcKnk-1*), *Retroactive* (*TcRtv*), and *Laccase* (*TcLac-2*) was effective, as transcript levels were reduced at least ten times by the respective RNAi treatment when compared to the wild-type (Table 1). The RNAi treatments appear to cause a slight increase in the expression of the two other non-targeted cuticle genes analyzed, but this increase is not significant (Table 1). Thus, although not formally excluded, there are no indications for off-target effects.

Next, we studied the effect of the RNAi on cuticle structure using TEM on 24–39 h old *Tribolium* eggs. This time window was chosen as late as possible to ensure a fully developed cuticle, but before 50% of total embryonic development (which takes 85 h at 30 °C) when a decrease in thickness of the serosal cuticle has been reported for *Acheta* (Furneaux et al., 1969). A cuticle with a clear laminar structure is formed in these wild-type *Tribolium* eggs (Fig. 2a–c). In contrast, knockdown of *TcKnk-1* or *TcRtv* completely eliminates the laminar organization of the serosal cuticle (Fig. 2d, e), like in the adults (Chaudhari et al., 2011, 2013). The same phenotype of *TcKnk-1* and *TcRtv* knockdown is to be expected because *TcRtv* is essential for the trafficking of *TcKnk-1* to the cuticle (Chaudhari et al., 2013).

Contrary to the reported effect of *MaLac-2* RNAi on the larval and adult cuticles of the pine sawyer beetle *Monochamus alternatus* (Niu et al., 2008), our TEM micrographs did not reveal any disturbance of the serosal cuticle upon *TcLac-2* RNAi in *Tribolium* (Fig. 2f). However, it is entirely possible that a role of Lac2 in sclerotization of the serosal cuticle (as has been shown in the larval and adult cuticle (Arakane et al., 2005a; Lomakin et al., 2011)) is not visible in TEM. In support of this, we do find a significant decrease in hatching rates of TcLac2 depleted eggs at low humidity (see below). Sclerotization is thought to increase the waterproofing abilities of the cuticle (Furneaux and McFarlane, 1965; Hinton, 1981). A role for Lac2 in pigmentation of the serosal cuticle is unlikely, as the serosal cuticle is colorless. However, more detailed TEM analyses and other assays are required to analyze the function of TcLac2 in the serosal cuticle.

Taken together, our TEM analysis suggests that two genes, *TcKnk-1* and *TcRtv*, known from larval and adult cuticle formation, also exert their function in the serosal cuticle, and this is not excluded for *TcLac-2*.

3.2. The structure of the serosal cuticle affects survival in dry conditions

To discover whether the structure of the cuticle influences survival in dry circumstances, we assayed hatching rates of untreated wild-type eggs and the RNAi eggs at different humidities. Previous experiments have shown that RNAi itself does not influence the hatching rates of eggs, as hatching rates of eggs from mothers that were injected with a non-*Tribolium* dsRNA sequence did not differ from wild-type hatching rates (Jacobs et al., 2013). Wild-type eggs display a consistent hatching rate of approximately 90% at all relative humidities (Fig. 3). When the laminar structure was absent after *TcKnk-1* knockdown, survival at 90% RH was almost as high as for wild-type eggs. However, at 50% RH survival was significantly lower than at 90% RH (Tukey HSD, $p < 0.001$) and was even lower at 5% RH (Tukey HSD, $p < 0.01$). Also for *TcRtv* RNAi

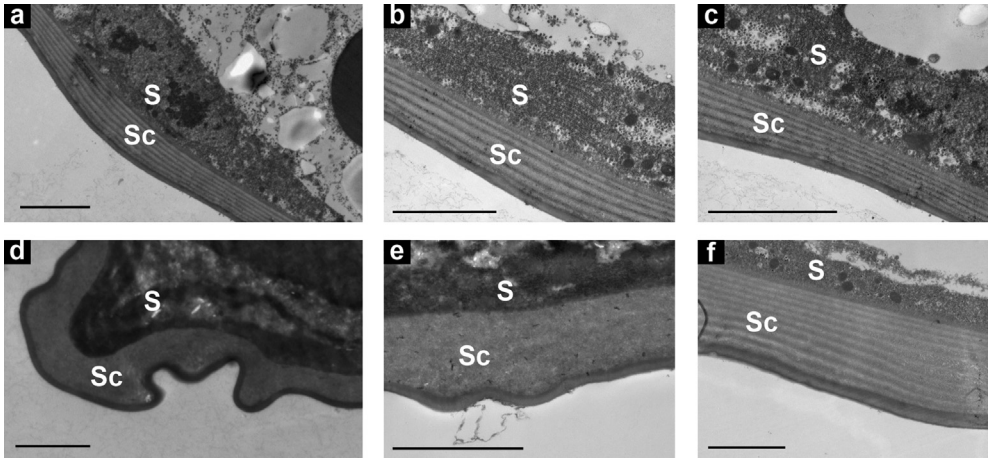

Fig. 2. TEM of the serosal cuticle. The serosal cuticle of wild-type eggs shows a clearly laminated structure (a–c). After knockdown of both *TcKnk-1* (d) and *TcRtv* (e), this laminar organization is completely lost. Knockdown of *TcLac-2* (f) however, shows no structural difference compared to wild-type eggs. S = serosa; Sc = Serosal cuticle; scale bars represent 2.5 μm.

eggs, significantly lower survival was found at 5% RH when compared to 50% RH (Tukey HSD, $p < 0.05$), and these hatching rates were significantly lower than wild-type hatching rates. However, survival decreased again at 90% RH. The low survival at high humidities suggests that *TcRtv* has another function besides trafficking *TcKnk-1* to the serosal cuticle. Overall, our data show that the laminar structure of the cuticle is essential for protection against desiccation.

Although TEM could not reveal structural differences in the serosal cuticle after *TcLac-2* knockdown, we investigated the effect of *TcLac-2* knockdown on survival at low humidities, since sclerotization might still be affected upon *TcLac-2* RNAi. The effect of *TcLac-2* RNAi on survival is as strong as the effect of *TcKnk-1* (Fig. 3). *TcLac-2* RNAi eggs survive almost as well as wild-type eggs at 90% RH but hatching rates decreased already significantly at 50% RH (Tukey HSD, $p < 0.001$). The survival at 5% RH is even significantly lower than at 50% RH (Tukey HSD, $p < 0.001$). In adult *T. castaneum*, *TcLac-2* is known to be involved in tanning of the cuticle (Arakane et al., 2005a; Lomakin et al., 2011), but lethality during the pupal stage prevented the assessment of its role in survival in dry conditions. Our data provide the first experimental indication that sclerotization of the serosal cuticle plays a considerable role in desiccation resistance of the egg.

Taken together, *TcKnk-1*, *TcRtv* or *TcLac-2* RNAi causes a significant decrease in egg hatching rates at low humidities. These decreased hatching rates could be directly caused by the disturbed structure of the serosal cuticle, but also more indirectly by a change in cuticular lipid composition, for instance. For all knockdowns, the reduced survival at low humidity is significant, but still not as low as previously found for *TcCHS-1* RNAi (Jacobs et al., 2013). This is possibly due to the remaining chitin which, although not properly structured, still reduces water evaporation to some extent. Nevertheless, our data indicate that sclerotization and structure of the serosal cuticle is crucial for the desiccation resistance of the *Tribolium* egg.

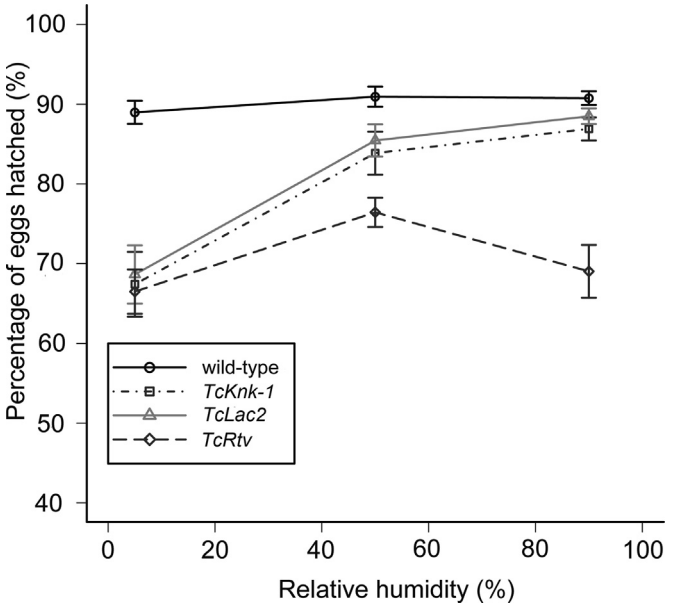

Fig. 3. Survival at different humidities of *T. castaneum* eggs. Eggs with an altered serosal cuticle become desiccation-susceptible. Hatching rates of wild-type (circles), *TcKnk-1* RNAi (squares), *TcLac-2* RNAi (triangles), and *TcRtv* RNAi (diamonds) eggs at 35 °C. Error bars indicate standard error among 4–9 replicates of 96 eggs.

3.3. Many cuticle genes are specifically expressed in the serosa

To identify other genes involved in the serosal cuticle, we reanalyzed sequencing data from wild-type and *TcZen-1* RNAi eggs that were 30–46 h old (Jacobs et al., 2014). As this time window overlaps the midpoint of embryonic development, we do not only expect to find genes involved in the formation and maintenance of the serosal cuticle, but also genes involved in degradation of this cuticle, since a decrease in thickness of the serosal cuticle has been reported for the cricket *Acheta domestica* after 50% of embryonic

Table 1
Efficiency and specificity of *TcKnk1*, *TcLac-2* and *TcRtv* RNAi.

	TcKnk-1 RNAi	*TcLac-2* RNAi	*TcRtv* RNAi
Expression			
TcKnk-1	0.07 ± 0.01 ($p = 0.02$)**	1.92 ± 0.47 ($p = 0.58$)	1.27 ± 0.20 ($p = 0.75$)
TcLac-2	1.66 ± 0.12 ($p = 0.31$)	0.10 ± 0.05 ($p = 0.003$)***	1.41 ± 0.14 ($p = 0.48$)
TcRtv	1.70 ± 0.53 ($p = 0.46$)	1.91 ± 0.41 ($p = 0.43$)	0.07 ± 0.01 ($p = 0.004$)***

Fold change in gene expression as measured by qRT-PCR, compared to wild-type (±s.e.). p-Values indicate the significance of the fold change (T-tests corrected for multiple testing).

development (Furneaux et al., 1969). The expression of 280 genes is significantly higher in wild-type eggs than in serosa-less eggs (Supplementary file 1). Six of these genes have the GO term "structural constituent of cuticle" (Table 2), making this a significantly overrepresented category considering its size (Supplementary Table 1). 14 genes have the GO term "chitin metabolic process" (these genes also have the GO term "chitin binding"; Table 2), making these two categories the two most significantly overrepresented categories of all differentially expressed genes (Supplementary Table 1). Many other significantly overrepresented categories, like "carbohydrate metabolic process", "oxidation-reduction process" or "glycerol-3-phosphate metabolic process" could also be associated with processes in the cuticle (Supplementary Table 1). These data indicate the importance of the serosa in the chemistry of the cuticle.

The genes identified by the GO-term chitin metabolic process have been previously characterized by bioinformatics in *T. castaneum* (Dixit et al., 2008; Jasrapuria et al., 2010). Of these genes, the CPAP families have been screened by RNAi (Jasrapuria et al., 2012), but no role in development of the embryo was found for the five CPAP genes with serosa-specific expression. This could well be the case if these genes are only involved in formation of the serosal cuticle. It would be interesting to investigate the knockdown phenotype of these genes at low humidity. Of the six genes with the GO-term "Structural Constituent of the Cuticle", two (TC008767 and TC015720) have not been annotated as cuticle genes in the *Tribolium* genome. They likely are cuticular proteins as they contain the Chitin-Bind-4 motif, identified using the Pfam database (Punta et al., 2012).

Surprisingly, *TcKnk-1*, *TcRtv* and *TcLac-2* are not among the 280 differentially regulated genes. It could be that expression of these enzymes is much lower than of the structural components, preventing detection of significant differences. This could be the case for the lowly expressed *TcRtv* and *TcLac-2*. It could also be that the genes are not only expressed in the serosa, but also in other tissue, thus preventing detecting different expression levels in serosa-less eggs. This could be the case for *TcKnk-1* that is also substantially expressed in serosa-less eggs. The most likely explanation, however, is timing. It could be that *TcKnk-1*, *TcRtv* and *TcLac-2* show highest expression during the early production of the serosal cuticle. The transcriptome data were collected at a later stage when the serosal cuticle reaches its maximal thickness. The expression of chitinases, needed to prepare the egg for hatching (Zhu et al., 2008), indicates that synthesis of the cuticle was declining at the time of sequencing.

Finally, we analyzed the 280 serosa-biased genes using CutProtFam-Pred (Ioannidou et al., 2014). This software uses Hidden Markov Models to predict cuticular proteins and has been successfully used in several arthropod families (Ioannidou et al., 2014). This approach identified 12 cuticular proteins, of which only TC013671, a low-complexity cuticular protein with conserved glycines, was not found using the GO term analysis (Supplementary Table 2). This gene shows the largest difference in expression between eggs with and without serosa (3519 fold difference), supporting that this gene might be important for the serosal cuticle. The CutProtFam-Pred software furthermore established that the chitin-binding domain of the un-annotated genes TC008767 and TC015720 is a Rebers-and-Riddiford2 motif (Andersen et al., 1997; Ioannidou et al., 2014; Rebers and Riddiford, 1988). Thus, in total we found 21 cuticle-related genes that show serosa-dependent expression, of which three have not been previously annotated as cuticle genes. These are valuable candidate genes for future study.

We put forward the serosal cuticle of *Tribolium* as an excellent model for cuticle development. The laminar structure of the serosal cuticle is similar to the adult cuticle (Chaudhari et al., 2011, 2013; Jacobs et al., 2013; Lamer and Dorn, 2001) and RNAi can be used to study serosal cuticle production, as absence of the serosal cuticle does not lead to mortality. It is surprising that we could use parental RNAi in this case, as *TcKnk-1*, *TcRtv* or *TcLac-2* RNAi in pupae of the Georgia strain prevents eclosion and is lethal (Arakane et al., 2005a; Chaudhari et al., 2011, 2013). However, strain-specific genetic backgrounds could underlie these differences (Kitzmann et al., 2013). The serosal cuticle provides a unique opportunity to study the effect of modifications on the physiological and ecological properties of the cuticle. Our data suggest that structural differences or degree of cross-linking can cause differences in resistance to desiccation, and could underlie, for instance, the recently reported differences in drought resistance of three mosquito species (Vargas et al., 2014).

3.4. Conclusions

Taken together, the serosa expresses many cuticle-related genes. We have shown that *TcKnk-1* and *TcRtv* RNAi severely affect the structure of the serosal cuticle causing increased mortality at low humidities. RNAi for the sclerotization gene *TcLac-2* also leads to

Table 2
Cuticle genes differentially expressed in the serosa.

Gene ID	GO-term	Annotation	Best hit *Drosophila*
TC008767	Structural constituent of cuticle	uncharacterized	GC6469
TC008768	Structural constituent of cuticle	Cuticular protein 1	GC5879
TC015720	Structural constituent of cuticle	uncharacterized	GC10112
TC010054	Structural constituent of cuticle	calphotin	GC6240
TC010057	Structural constituent of cuticle	Cuticle protein-like	GC6240
TC008770	Structural constituent of cuticle	Larval cuticle protein A2B-like	GC6240
TC003876	Chitin metabolic process/chitin binding	Chitinase 6	GC2989
TC002107	Chitin metabolic process/chitin binding	Chitin binding Peritrophin-A domain containing	GC17698
TC012734	Chitin metabolic process/chitin binding	Chitinase 10	GC18140
TC011142	Chitin metabolic process/chitin binding	Cpap3-d1 cuticular protein analogous to peritrophins 3-D1	GC17058
TC014101	Chitin metabolic process/chitin binding	Chitin deacetylase 2	GC8756
TC003877	Chitin metabolic process/chitin binding	Chitin binding Peritrophin-A domain containing	GC15313
TC007635	Chitin metabolic process/chitin binding	Cda4 chitin deacetylase 4	GC17905
TC011140	Chitin metabolic process/chitin binding	Cpap3-a1 cuticular protein analogous to peritrophins 3-A1	GC17052
TC011139	Chitin metabolic process/chitin binding	Cpap3-b cuticular protein analogous to peritrophins 3-B	GC4778
TC006846	Chitin metabolic process/chitin binding	Chitin deacetylase 5	GC2761
TC011141	Chitin metabolic process/chitin binding	Cpap3-a2 cuticular protein analogous to peritrophins 3-A2	GC17052
TC015481	Chitin metabolic process/chitin binding	Chitinase 7	GC1869
TC009894	Chitin metabolic process/chitin binding	Cpap1-h cuticular protein analogous to peritrophins 1-H	GC13676
TC014100	Chitin metabolic process/chitin binding	Cda1 chitin deacetylase 1	GC8748

reduced hatching rates at low humidities. Our data suggest that the same genes structure the adult and serosal cuticle, and that this structuring is crucial for desiccation resistance.

Acknowledgments

We thank Kees Koops for maintaining the beetle stock.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.02.014.

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