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Chapter 3

The role of *knickkopf1*, *retroactive* and *laccase2* in serosal cuticle production and desiccation resistance of the *Tribolium* egg

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Insects have been extraordinary successful in colonizing terrestrial habitats and this success is partly due to their protective cuticle. The cuticle has been well studied in larvae and adults, but little attention has been paid to the cuticle of the egg, the serosal cuticle. 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. Here, we show that in *Tribolium castaneum*, three genes that are crucial for adult cuticle formation are just as important for the development of the serosal cuticle. Both *knickkopf* (*Tc-knk1*) and *retroactive* (*Tc-rtv*) affect the laminar structure of the serosal cuticle, as revealed by Transmission Electron Microscopy in RNAi knockdown eggs. In the absence of the laminar structure, significantly fewer eggs survive at low humidity than at high humidity. Survival in dry conditions is also negatively influenced when cross-linking is prevented by *laccase* (*Tc-lac2*) 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 cuticle production 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.

Keywords: Tribolium castaneum; cuticle; knickkopf; retroactive; laccase; desiccation

Introduction

Insects are among the earliest land animals and their chitinized exoskeleton preadapted them for terrestrial life. This insect cuticle is an apical extracellular matrix which is produced by the epidermis and contains mainly chitin (Merzendorfer, 2006; Moussian, 2010), but also other proteins, tyrosine-derived quinones responsible for sclerotization and lipids (Furneaux & McFarlane, 1965a, 1965b; Goltsev et al., 2009; McFarlane, 1960).

The molecular pathways involved in cuticle synthesis and their effect on cuticle structure have received much attention (Charles, 2010; Moussian, 2010, 2013). Chitin is produced by chitin synthase (Arakane et al., 2004; Arakane et al., 2005b; Arakane et al., 2008; Merzendorfer, 2006; Moussian, 2010) and is secreted to the extracellular space through pores in the cell membrane (Merzendorfer, 2006). There, it is organized into lamellae by the protein Knickkopf (Chaudhari et al., 2011; Chaudhari et al., 2014; Moussian

et al., 2006), which itself is transported to the cuticle by Retroactive (Chaudhari et al., 2013; Moussian et al., 2005). To stabilize this proteinaceous extracellular structure, the cuticle is sclerotized by the phenoloxidase Laccase 2 (Arakane et al., 2005a). Although much is known about the genetic pathways involved in the production of the larval and adult cuticle, there is little information about the formation of the serosal cuticle in the insect egg.

In insect eggs, a serosal cuticle is formed by the extraembryonic serosa (Goltsev et al., 2009; Hinton, 1981; Jacobs et al., 2013; Lamer & 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)(Figure 3-1). It is prevalent across Insecta (Roth, 2004) and protects the embryo from desiccation and infection (Jacobs et al., 2013; Jacobs & van der Zee, 2013). The serosal cuticle shows a similar morphology to the adult cuticle (Chaudhari et al., 2011; Chaudhari et al., 2013; Lamer & Dorn, 2001). Furthermore, in *T. castaneum*, parental RNAi of *chitin synthase 1* (*Tc-chs1*) 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; Arakane et al., 2008). Earlier experiments using microarrays in the mosquito *A. gambiae* indicated that the same genes are utilized for the production of both the adult and serosal cuticle (Goltsev et al., 2009). This indicates that a similar machinery might be utilized by the serosa for cuticle production, however structural and functional data on serosal cuticle synthesis are still missing.

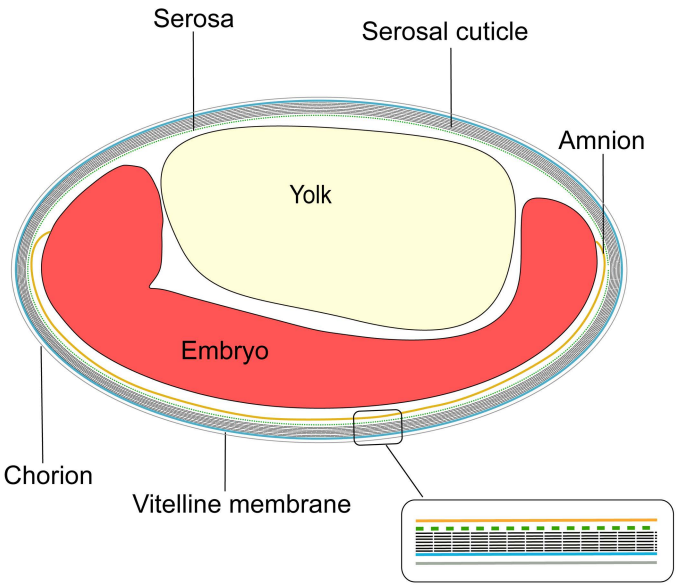

Figure 3-1: Schematic overview of the *Tribolium castaneum* egg. The *Tribolium castaneum* egg is surrounded by two maternal layers, the chorion and the vitelline membrane. Beneath these, the extraembryonic serosa secretes a chitinous cuticle (serosal cuticle). The serosa and serosal cuticle envelope both the embryo and the yolk.

Here, we set out to investigate the role of *knickkopf* (*Tc-knk1*), *retroactive* (*Tc-rtv)* and *laccase2* (*Tc-lac2*) in serosal cuticle production of the red flour beetle *Tribolium 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 us with the unique opportunity to assess the effect of cuticle structure on the ability of eggs to survive dry circumstances. Finally, we compare the transcriptomes of wild-type eggs to serosa-less eggs to identify cuticle-related genes that are specifically expressed in the serosa. Our data confirm that the same machinery is utilized for the production of the serosal cuticle and that structure and cross-linking are important for the water-proofing abilities of the cuticle.

Materials and Methods

Insect rearing

The *Tribolium castaneum* wild-type strain San Bernardino was used for all experiments. Beetles were kept as in (van der Zee et al., 2005).

Molecular cloning and RNAi

The genes were amplified from a cDNA library using primers from Chaudhari et al. (Chaudhari et al., 2011) for *Tc-knk1*, from Chaudhari et al. (Chaudhari et al., 2013) for *Tc-rtv*, and from Arakane et al. (Arakane et al., 2005a) for *Tc-lac2*. These were cloned into the pCRII-TOPO vector (Invitrogen) using the manufacturers protocol. dsRNA was synthesized using the MEGAscript RNAi kit (Ambion), and about 0,2 µg of a 0.5-1 µg/µl dsRNA solution was injected into pupae according to Bucher et al. (Bucher et al., 2002).

RNA extraction and qRT-PCR

Total RNA of approximately 300 eggs was extracted using TRIzol extraction (Invitrogen), after which the RNA was purified and DNA digested on column with the RNeasy kit (Qiagen). The quality of RNA preparation was confirmed on gel and spectrophotometrically. One microgram of total RNA was used for cDNA synthesis. First strand cDNA was made using the Cloned AMV First Strand Synthesis kit (Invitrogen). Each qRT-PCR mixture (25µl) contained 12.5 ng of cDNA, and the real-time detection and analyses were done based on SYBR green dye chemistry using the qPCR kit for SYBR Green I (Eurogentec) and a CFX96 thermocycler (Biorad). Thermal cycling conditions used were 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s; this was followed by a dissociation analysis of a ramp from 65 to 95 °C with a read every 0.5 °C. Relative quantification for each mRNA was done using the Livak-method (Livak & Schmittgen, 2001). The values obtained for each mRNA were normalized by *RPL13a* amount. Total RNA for each treatment was isolated twice (biological replication) and each sample was measured by qRT-PCR twice (technical replication). The primers for *RPL13a* were as in Lord et al. (Lord et al., 2010). For *Tc-knk1* (TC010653), *Tc-rtv* (TC007384) and *Tc-lac2* (TC010489) sequences were retrieved from beetlebase (www.beetlebase.org) and primers were designed using primer blast (Ye et al., 2012). The primers for *Tc-knk1* were fw '3- CCTACAAGGGCGAGACCATC-5' and rv '3-GGTGGTGTTCGTGCGGAATA-5', for *Tc-rtv* '3-GGCGAGAGTCCACGTAAACA-5' and rv '3-GTCTTGCTGCTCTCCTTCGT-5', and for *Tc-lac2* '3-TACAACAGACATTTAGTTGCACCA-5' and rv '3-AGGTGGGGCCATGTAGGAAA-5'.

Transmission Electron Microscopy
For electron microscopical preparations, both wild-type, *Tc-knk1*, *Tc-rtv* and *Tc-lac2* eggs aged between 24-39 h were collected, dechorionated and fixed for 1 h in 5 ml heptane and 5 ml of a solution of 2.5% gluteraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). After this initial fixation, eggs were removed from the solution, washed with 70% ethanol to remove the heptane and fixed for another h in 5 ml of a solution of 2.5% gluteraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). After fixation, specimens were washed (3 x 10 min) in cacodylate buffer and placed for 1 h in 1% osmium tetraoxide. 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.

Generation of serosa-less eggs
Serosa-less eggs were generated using *Tc-zen1* RNAi (van der Zee et al,. 2005). This method prevents development of the serosa and is a well-established to analyze the function of the serosa (Jacobs et al., 2013; Jacobs & van der Zee, 2013, Chapter 5).

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. The combination of this temperature and these humidities was chosen because differences between survival were most likely to be detected in these conditions (Jacobs et al., 2013). Hatching rates were assayed after 4 days. The experiments at each humidity were repeated four to nine times, giving rise to standard errors as in Figure 3-3. Five per cent humidity was obtained using silica gel, 50 and 90% were obtained in climate chambers. We excluded higher humidities to avoid condensation of liquid water. Temperature and humidity was constantly monitored using a MicroDAQ datalogger.

Analysis of RNA sequencing data
To obtain a list of serosa specific expressed genes we reanalyzed sequencing data we previously obtained (Chapter 5). In short, we obtained serosa-less eggs by *Tc-zen1* RNAi and extracted RNA from both wild-type and serosa-less eggs. Approximately 300 eggs of 30-46h old were used for each extraction. We collected 3 biological replicates for both wild-type and serosa-less eggs and compared gene expression using the DEseq package in R (Anders & Huber, 2010). To identify significantly differentially expressed genes we used an adjusted p-value of 0.01 as cut-off value. The sequence data has been deposited in NCBI's Gene Expression Omnibus (Barrett et al., 2013) and are accessible through GEO Series accession numbers GSM1305910 - GSM1305912 and GSM1305931 - GSM1305933 (http://www.ncbi.nlm.nih.gov/geo). Sequence homology searches of predicted reference gene sequences and subsequent functional annotation by gene ontology terms (GO) and InterPro terms (InterProScan, EBI) were determined using the BLAST2GO software suite v2.6.6 (Conesa et al., 2005). First, homology searches were performed through BLASTX against sequences of the *Drosophila* protein database with a cut-off value of 1.0E-10. Subsequently, GO classification annotations were created after which InterPro searches on the InterProEBI webserver were performed remotely by utilizing BLAST2GO.

Statistical analyses
We performed a square root transformation to obtain normality in the hatching data. 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).

Results and Discussion

Serosal cuticle structure is effected by the same genes as the adult cuticle
To assess the effect of the RNAi against cuticle genes we first verified their knockdown. Knockdown of *Tc-knk1* and *Tc-lac2* were specific; expression after knockdown was reduced 74 to 90% compared to wild-type expression, whereas the other genes were not affected (Table 1). Knockdown of *Tc-rtv* led to a 27% reduction in expression, whereas the other genes were not affected (Table 1). This is much less efficient than the knockdown of *Tc-knk1* and *Tc-lac2*. This might be while the expression of *Tc-rtv* is extremely low (4-6 cycli later than *Tc-lac2* and *Tc-knk1*). Although the expression if very low, knockdown of *Tc-rtv* shows a clear phenotype in cuticle structure, indicating efficient knockdown.

Table 3-1: Specificity of knockdown, expression compared to wild-type is shown.

	***Tc-knk1* RNAi**	***Tc-lac2* RNAi**	***Tc-rtv* RNAi**
Expression			
Tc-knk1	0.10	0.91	1.02
Tc-lac2	1.39	0.26	0.94
Tc-rtv	1.40	0.96	0.73

We then studied the effect of the knockdown on cuticle structure using TEM. In wild-type eggs, a normal cuticle is formed by the serosa (Figure 3-2a-c). The laminar structure is clearly visible. This is consistent with our previous findings (Jacobs et al., 2013), and with the structure found in the serosal cuticle of *Manduca sexta* (Lamer & Dorn, 2001). The serosal cuticle resembles the adult cuticle of *T. castaneum* (Chaudhari et al., 2011; Chaudhari et al., 2013). In contrast, knockdown of *Tc-knk1* or *Tc-rtv* completely eliminates the laminar organization of the serosal cuticle (Figure 3-2d, e), like in the adults (Chaudhari et al., 2011; Chaudhari et al., 2013). The same phenotype of *Tc-knk1* and *Tc-rtv* is to be expected because *Tc-rtv* is essential for the trafficking of *Tc-knk1* to the cuticle (Chaudhari et al., 2013). We also assessed the effect of the knockdown of *Tc-lac2*. Contrary to the effects of *Tc-knk1* and *Tc-rtv,* no structural differences could be found by TEM in the *Tc-lac2* knockdown (Figure 3-2f). The lack of clearly distinguishable structural effects is not surprising, as this gene is involved in the sclerotization of the adult cuticle, not in organizing its structure (Arakane et al., 2005a). Although cuticle structure is not visually affected after *Tc-lac2* knockdown, our hatching data show that *Tc-lac2* does play a key role in the waterproofing ability of the serosal cuticle (see next paragraph). Taken together, our data suggest that both concerning morphology and concerning the genes involved, the

adult and the serosal cuticle are similar.

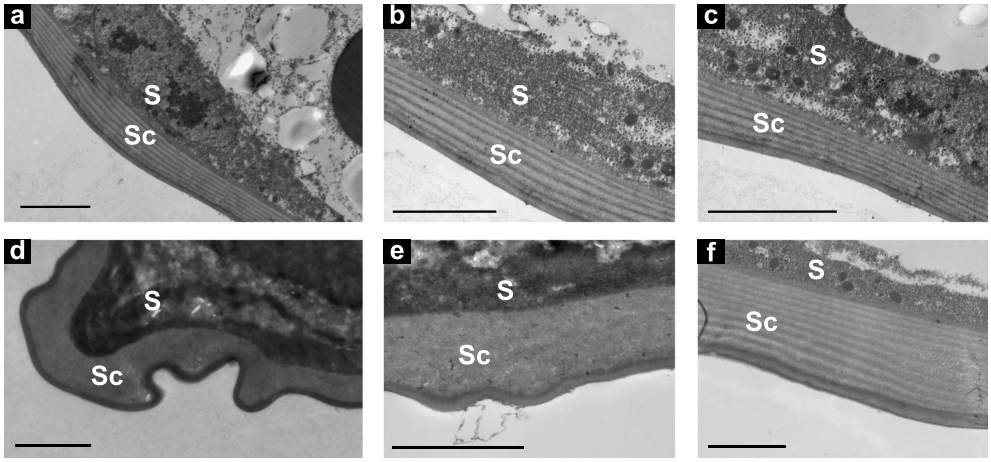

Figure 3-2: Transmission Electron Microscopy of the serosal cuticle. The serosal cuticle of wild-type eggs shows a clearly laminated structure (a-c). After knockdown of both *Tc-knk1* (d) and *Tc-rtv* (e), this laminar organization is completely lost. Knockdown of *Tc-lac2* (f) however, shows no structural difference compared to wild-type eggs. S = serosa ; Sc = Serosal cuticle ; error bars represent 2.5 µm.

Cuticle structure influences survival in dry conditions

To discover whether cuticle structure influences survival in dry circumstances we scored hatching rates at different humidities. Whereas wild-type eggs have a consistent hatching rate of approximately 90% at all relative humidities, knockdown of any cuticle gene affected survival in dry circumstances (Figure 3-3). When the laminar structure was absent after *Tc-knk1* 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 at 5% RH survival was even lower than at 50% RH (Tukey HSD, $p < 0.01$). Similar results were obtained after knockdown of *Tc-rtv*. Significantly lower survival of eggs was found at 5% RH when compared to 50% RH (Tukey HSD, $p < 0.05$). However, no significant difference was found between 5% RH and 90% RH. The low survival at high humidities after knockdown of *Tc-rtv* suggests that it has another function besides trafficking *Tc-knk1* to the cuticle.

For all knockdowns, the reduced survival at low humidity is significant, but still not as low as previously found for *Tc-chs1* RNAi (Jacobs et al., 2013). This is likely due to the remaining chitin which, although not properly structured, still provides a certain amount of protection against dehydration. Overall, our data show that the structure of the serosal cuticle is important for desiccation resistance of the egg.

Next, we studied the effect of *Tc-lac2* knockdown on survival at low humidity. Although no structural differences can be seen with TEM in the serosal cuticle, the effect on survival is as strong as the effect of *Tc-knk1* (Figure 3-3). 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, $p < 0.001$). These data show a strong relation between the ability to sclerotize the serosal cuticle and the ability to survive dry circumstances. *Tc-lac2* is known to be involved in cross-linking proteins in the cuticle in adult *T. castaneum* (Arakane et al.,

2005a), but lethality during the pupal stage prevented the assessment of its impact on survival in dry conditions. Sclerotization of the serosal cuticle has also been proposed to play an important role in survival of mosquito eggs under dry conditions (Goltsev et al., 2009). Our data provide the first experimental evidence that sclerotization of the serosal cuticle plays a considerable role in desiccation resistance of the egg.

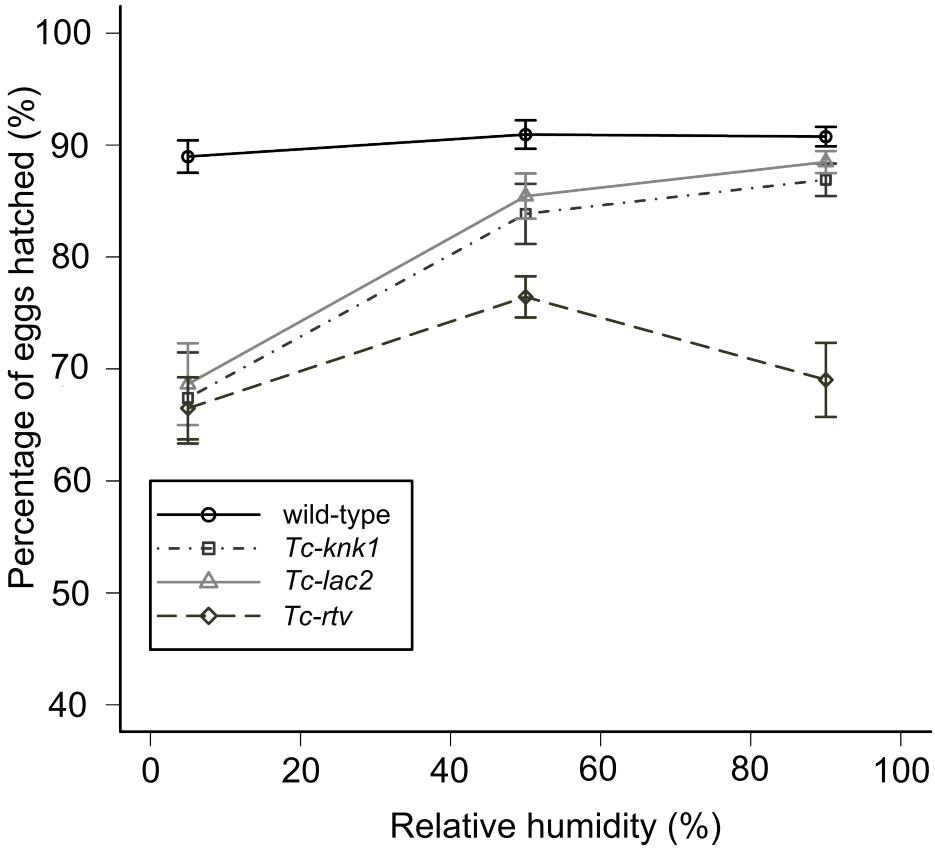

Figure 3-3: Survival at different humidities of the *T. castaneum* egg. Eggs with an altered serosal cuticle become desiccation-susceptible. Hatching rates of wild-type (circles), *Tc-knk1* RNAi (squares), *Tc-lac2* RNAi (triangles), and *Tc-rtv* RNAi (diamonds) eggs at 35°C. Error bars indicate standard error among 4-9 replicates of 96 eggs.

Table 3-2: Predicted cuticle genes identified from the RNAseq data.

Gene ID	GO-term	Annotation
TC008767	Structural constituent of cuticle	uncharacterized
TC008768	Structural constituent of cuticle	Cuticular protein 1
TC015720	Structural constituent of cuticle	uncharacterized
TC010054	Structural constituent of cuticle	calphotin
TC010057	Structural constituent of cuticle	Cuticle protein-like
TC008770	Structural constituent of cuticle	Larval cuticle protein A2B-like
TC003876	Chitin metabolic process / chitin binding	Chitinase6
TC002107	Chitin metabolic process / chitin binding	Chitin binding Peritrophin-A domain containing
TC012734	Chitin metabolic process / chitin binding	Chitinase10
TC011142	Chitin metabolic process / chitin binding	Cpap3-d1 cuticular protein analogous to peritrophins 3-D1
TC014101	Chitin metabolic process / chitin binding	Chitin deacetylase 2
TC003877	Chitin metabolic process / chitin binding	Chitin binding Peritrophin-A domain containing
TC007635	Chitin metabolic process / chitin binding	Cda4 chitin deacetylase 4
TC011140	Chitin metabolic process / chitin binding	Cpap3-a1 cuticular protein analogous to peritrophins 3-A1
TC011139	Chitin metabolic process / chitin binding	Cpap3-b cuticular protein analogous to peritrophins 3-B
TC006846	Chitin metabolic process / chitin binding	Chitin deacetylase 5
TC011141	Chitin metabolic process / chitin binding	Cpap3-a2 cuticular protein analogous to peritrophins 3-A2
TC015481	Chitin metabolic process / chitin binding	Chitinase 7
TC009894	Chitin metabolic process / chitin binding	Cpap1-h cuticular protein analogous to peritrophins 1-H
TC014100	Chitin metabolic process / chitin binding	Cda1 chitin deacetylase 1

Many cuticle genes are specifically expressed in the serosa

To identify serosa-specific expression, we compared available transcriptome data of serosa-less eggs to wild-type eggs (Chapter 5). The expression of 251 genes is significantly higher in wild-type eggs than in serosa-less eggs. 6 of these genes have the GO term "structural constituent of cuticle" (Table 3-2), making this a significantly overrepresented category. 14 genes have the GO term "chitin metabolic process" (these genes also have the GO term "chitin binding"), making these two categories the most significantly

overrepresented categories of all differentially expressed genes (Supplementary Table 3-1). The genes identified by the GO-term chitin metabolic process have been previously identified by bioinformatics in *T. castaneum* (Dixit et al., 2008; Jasrapuria et al., 2010). All genes of the CPAP families have been screened by RNAi (Jasrapuria et al., 2012), however no defects in embryonic development for the 5 CPAP genes with serosa-specific expression was found. The serosa-specific expression and the lack of defects in embryonic development indicate that these genes are indeed involved in the production of the serosal cuticle. The expression of many different cuticle genes shows the importance of the serosa in cuticle production.

Surprisingly, *Tc-knk1*, *Tc-rtv* and *Tc-lac2* are not among the 251 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 *Tc-rtv* and *Tc-lac2*. 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 *Tc-knk1* that is also substantially expressed in serosa-less eggs. Another likely explanation is timing. It could be that *Tc-knk1*, *Tc-rtv* and *Tc-lac2* show highest expression during the early production of the serosal cuticle. The transcriptome data were collected at a later stage when the serosal cuticle has completely formed. The expression of chitinases, needed to prepare the egg for hatching (Zhu et al., 2008), indicates that cuticle synthesis was declining at the time of sequencing.

Finally, we analyzed the 251 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 3-2). Interestingly, this gene shows the largest difference in expression between eggs with and without serosa (3519 fold difference). The second largest difference between eggs with and without serosa (TC008767; 1351 fold difference) is also uncharacterized, indicating that these genes might be of great importance for the synthesis of the serosal cuticle. Thus, in total we found 21 cuticle-related genes that show serosa-dependent expression. These are valuable candidate genes for future study.

We put forward the serosal cuticle of *Tribolium* as an excellent model for cuticle development. Its structure is similar to the adult cuticle (Chaudhari et al., 2011; Chaudhari et al., 2013; Jacobs et al., 2013; Lamer & Dorn, 2001) and RNAi can be used to study cuticle production. As absence of the serosal cuticle does not lead to mortality, this provides a unique opportunity to study the effect of modifications on the physiological and ecological properties of the cuticle. Recently, eggs of three species of mosquitos were shown to differ considerably in their ability to survive dry circumstances (Vargas et al., 2014). Our data suggest that structural changes or degree of cross-linking in the serosal cuticle might underlie these differences.

Conclusions

Taken together, we have shown that *Tc-knk1* and *Tc-rtv* RNAi severely affect the structure of the serosal cuticle causing increased mortality at low humidities. *Tc-Lac2* RNAi, involved in protein cross-linking, also leads to reduced hatching rates at low humidities. Our data suggest that the same genes are involved in adult and serosal cuticle formation, and that cuticular structure and cross-linking are important for desiccation resistance. This provides further insights into how insects are able to cope with dry conditions.

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Supplementary information

Supplementary Table 3-1: GO-term analysis

GO-term category	Over represented p-value	Under represented p-value	Number of genes in category	Category size	Description
GO:0006030	5,01E-12	1	14	56	chitin metabolic process
GO:0008061	5,01E-12	1	14	56	chitin binding
GO:0004252	1,46E-10	1	21	187	serine-type endopeptidase activity
GO:0003824	6,07E-10	1	43	696	catalytic activity
GO:0008233	5,62E-09	1	23	262	peptidase activity
GO:0005576	2,10E-08	1	17	163	extracellular region
GO:0008236	6,34E-08	1	13	95	serine-type peptidase activity
GO:0006508	1,53E-07	1	29	443	proteolysis
GO:0080019	3,62E-05	0,999997	6	27	fatty-acyl-CoA reductase (alcohol-forming) activity
GO:0008483	6,11E-05	0,999998	4	10	transaminase activity
GO:0005975	0,00010182	0,999976	13	159	carbohydrate metabolic process
GO:0016884	0,000303567	0,999994	3	6	carbon-nitrogen ligase activity, with glutamine as amido-N-donor
GO:0016787	0,000414139	0,999831	25	524	hydrolase activity
GO:0008272	0,001311322	0,999949	3	9	sulfate transport
GO:0015116	0,001311322	0,999949	3	9	sulfate transmembrane transporter activity
GO:0004867	0,001903428	0,999757	5	39	serine-type endopeptidase inhibitor activity
GO:0055114	0,001966289	0,999113	24	566	oxidation-reduction process
GO:0016810	0,002022423	0,999818	4	23	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
GO:0030170	0,003141399	0,999546	5	43	pyridoxal phosphate binding
GO:0008152	0,004160391	0,998146	20	459	metabolic process
GO:0042302	0,005152303	0,998943	6	113	structural constituent of cuticle
GO:0016620	0,006435438	0,999493	3	16	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor
GO:0020037	0,006783899	0,997795	10	166	heme binding
GO:0004030	0,008070281	0,999742	2	6	aldehyde dehydrogenase [NAD(P)+] activity
GO:0006081	0,008070281	0,999742	2	6	cellular aldehyde metabolic process
GO:0006072	0,008890004	0,999701	2	6	glycerol-3-phosphate metabolic process
GO:0008241	0,009207014	0,999684	2	6	peptidyl-dipeptidase activity

Supplementary Table 3-2: Predicted cuticle proteins by CutProtFam-Pred.

Gene ID	SCP Family	E-Value	Score	Foldchange
TC013671	*CPLCG*	*1.4^{e}-08*	*23.9*	*3519*
TC008767	*CPR_RR-2*	*7.2^{e}-14*	*44.7*	*1351*
TC008768	*CPR_RR-2*	*1.1^{e}-34*	*113.8*	*45*
TC015720	*CPR_RR-2*	*9.6^{e}-08*	*24.3*	*181*
TC011142	*CPAP3*	*7.1^{e}-71*	*227.4*	*55*
TC010054	*CPR_RR-2*	*4.2^{e}-33*	*108.6*	*368*
TC010057	*CPR_RR-2*	*3.2^{e}-33*	*109.0*	*706*
TC011140	*CPAP3*	*1.2^{e}-75*	*243.0*	*9*
TC011139	*CPAP3*	*2.0^{e}-74*	*239.0*	*9*
TC011141	*CPAP3*	*7.3^{e}-64*	*204.5*	*4*
TC008770	*CPR_RR-2*	*7.1^{e}-33*	*107.8*	*Inf*
TC009894	*CPAP1*	*8.2^{e}-22*	*66.7*	*3*