Chapter 4

Immune competence in insect eggs depends on the extraembryonic serosa

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Innate immunity is common to all metazoans and serves as a first line of defense against pathogens. Although the immune response of adult and larval insects has been well characterized, it remains unknown whether the insect egg is able to mount an immune response. Contrary to *Drosophila*, *Tribolium* eggs develop an extraembryonic epithelium, the serosa. Epithelia are well known for their ability to fight infection, so the serosa has the potential to protect the embryo against pathogens. To test this hypothesis we created serosa-less eggs by *Tc-zen1* parental RNAi. We found that the *Tribolium* egg upregulates several immune genes to comparable levels as adults in response to infection. *Drosophila* eggs and serosa-less *Tribolium* eggs, however, have little to no upregulation of any of the tested immune genes. We conclude that the extraembryonic serosa is crucial for the early immune competence of the *Tribolium* egg.

Key words: T. castaneum, D. melanogaster, antimicrobial peptides (AMPs), innate immunity, Tc-zen1

Introduction

To combat infection, insects rely on both physical barriers as well as local and systemic immune responses. This systemic response is mainly exerted by the fat body while epithelia provide local protection against microbial infection through the expression of AMPs (Lemaitre and Hoffmann, 2007; Tzou et al., 2000). In response to microbial infection, insects synthesize massive amounts of antimicrobial peptides (AMPs) (Lemaitre and Hoffmann, 2007). The mechanisms regulating these immune responses have been largely uncovered with the aid of genetic and molecular studies in *Drosophila*, but with the increasing number of insect genomes available, other insect species are being established as model organisms for immunity research (Altincicek and Vilcinskas, 2007; Gerardo et al., 2010; Waterhouse et al., 2007; Zou et al., 2007).

The red flour beetle (*Tribolium castaneum*) has received much attention and the immune response shares many similarities with *Drosophila* (Altincicek et al., 2013; Altincicek et al., 2008; Yokoi et al., 2012a; Yokoi et al., 2012b; Zhong et al., 2013; Zou et al., 2007). Comparative genome analysis shows 1:1 orthology of intracellular immune signaling pathways (Toll, IMD and JAK/STAT) with *Drosophila*. In contrast, species specific family expansion and sequence divergence in the PGRP and AMP families indicate importance for the specific recognition and effective elimination of evolving pathogens (Altincicek et

al., 2008; Zou et al., 2007). The IMD and Toll pathway have been shown to be conserved between *Drosophila* and *Tribolium*, but more promiscuous activation and usage of the two pathways may occur in *T. castaneum* (Yokoi et al., 2012a; Yokoi et al., 2012b), when compared to the more specific activation by either Gram-negative or Gram-positive bacteria and fungi in *Drosophila*.

Although our understanding of adult immune responses has increased greatly in recent years, evidence for immune competence in insect eggs is scarce. Two AMPs, CecropinA1 and Diptericin, have been found in the yolk and in the embryonic epidermis of *Drosophila* (Esfahani and Engstrom, 2011; Tingvall et al., 2001). Several immune-related genes were detected in the extraembryonic tissues of the tobacco hornworm (*Manduca sexta*), although this response could not be specifically attributed to the extraembryonic epithelia (Gorman et al., 2004). Another suggestion for an immune function of extraembryonic epithelia comes from *Tribolium castaneum*, in which the NF-κB transcription factor Dorsal is highly expressed in the serosa, and translocates to the nucleus upon injury (Chen et al., 2000).

The distinction between serosa and germ rudiment is the earliest cell differentiation event in an insect embryo, taking place at blastoderm stage before gastrulation starts. The serosa envelopes the yolk and the embryo (Schwalm, 1988) and most insects invest a substantial fraction of their blastoderm into the serosa (Roth, 2004). The early development of this epithelium and the fact that it surrounds both the embryo and the yolk make it a prime candidate to provide early protection against infection. However, in the lineage that gave rise to *Drosophila*, the serosa has been dramatically reduced and does not envelop the embryo (Schmidt-Ott, 2000, 2005). In the red flour beetle *Tribolium castaneum*, it is possible to prevent the development of the serosa by parental *Tc-zerknüllt1* (*Tc-zen1*) RNAi (van der Zee et al., 2005). In *Tc-zen1* RNAi eggs, a single amnion covers the yolk dorsally and does not envelop the embryo, similar to the reduced extraembryonic membrane in *Drosophila*. Furthermore, *Tribolium Tc-zen1* RNAi eggs can hatch under normal laboratory conditions (van der Zee et al., 2005), providing us with the unique opportunity to investigate the immune competence of the serosal epithelium.

Here we quantify the expression of immune genes (AMPs, PGRPs and IMD) in response to infection with both Gram-positive and Gram-negative bacteria by qRT-PCR in adults and eggs of both *Drosophila melanogaster* and *Tribolium castaneum*. We furthermore quantify the expression of immune genes in *Tribolium castaneum* eggs with and without a serosal epithelium. Our results show that the serosal epithelium plays a crucial role in the early immune response of *Tribolium castaneum* eggs.

Materials and Methods

Insect rearing

Beetle stocks were kept as in van der Zee et al. (van der Zee et al., 2005). Fly stocks were reared on standard corn meal agar medium at 25°C.

Infection experiments

All infections were performed with a tungsten needle with a 1 micron tip (Fine Science Tools). After treatment, eggs and adults were incubated for 6 hours before RNA extraction.

Infection of adults
Adult flies and beetles were pricked with a sterile needle (sterile injury) or, with a needle previously dipped in a concentrated mixed culture of *Escherichia coli* and *Micrococcus luteus* (septic injury).

Infection of T. castaneum eggs
T. castaneum eggs were collected overnight and kept at 30°C for another 24 hours before they were pricked with either a sterile needle (sterile injury) or with a needle dipped in a concentrated mixed culture of *Escherichia coli* and *Micrococcus luteus* (septic injury).

Infection of D. melanogaster eggs
D. melanogaster eggs were collected on apple juice-agar plates for 2 hours and discarded to prevent the collection of aged eggs. Immediately after these two hours, eggs were collected on a fresh apple juice-agar plate for another 2 hours. These eggs were kept at 20°C for 13 hours after which they were pricked with either a sterile needle or a needle dipped in a concentrated mixed culture of *Escherichia coli* and *Micrococcus luteus*.

Sequences of immune-related genes and primers used for real-time quantitative RT-PCR (qRT-PCR)
The immune-related genes of *T. castaneum* in this study are: Attacin1 (TC007737), Attacin2 (TC007738), Cecropin3 (TC000500), Coleoptericin1 (TC005093), Defensin1 (TC006250), Defensin2 (TC010517), PGRP-SA (TC010611), PGRP-SB (TC013620), IMD (TC010851) and the normalizer of qRT-PCR ribosomal protein 13a (RPL13a) (TC013477). The primers for Def1, Col1, Cec3, PGRP-SA, PGRP-SB and IMD were as in Zou et al. (2007), the primers for RPL13a as in Lord et al. (Lord et al., 2010). The other sequences were retrieved from the Beetlebase (http://www.beetlebase.org), and primer pairs of respective target genes designed for qRT-PCR (Table 1).

The immune-related genes of *D. melanogaster* in this study are: AttacinA (CG10146), CecropinA1 (CG1365), CecropinB (CG1878), CecropinC (CG1373), Diptericin (CG12763), Defensin (CG1385), PGRP-SA (CG11709), IMD (CG5576) and the normalizer of qRT-PCR ribosomal protein 32 (RPL32) (CG7939). The primers for RPL32 were as in Haghayeghi et al. (Haghayeghi et al., 2010), for Def as in DiMarcq et al. (Dimarcq et al., 1994), for Dip as in Costa et al. (Costa et al., 2009) and PGRP-SA as in Bischoff et al. (Bischoff et al., 2006). The other sequences were retrieved from Flybase (http://www.flybase.org), and primer pairs of respective target genes were designed for qRT-PCR (Table 1).

Table 4-1. Primers for immune sequences of *Tribolium castaneum* and *Drosophila melanogaster*.

Gene	Forward primer	Reverse primer
T. castaneum		
Attacin1	5'-TTTTGCCTCCAAACAATTCC-3'	5'-CACCGACGTTTAGGTTCGAT-3'
Attacin2	5'-CCCGGAATCCTCAAACTACA-3'	5'-GGGGCATCTTTATTGACGAA-3'
Defensin2	5'-TCACTTGTGACGTCCTCAGC-3'	5'-CGCGTTTCTTCAAAAAGAGG-3'
D. melanogaster		
AttacinA	5'-ATCCTAATCGTGGCCCTGGT-3'	5'-GCGGGATTGGAGGTTAAGGA-3'
CecropinA1	5'-CTCAGACCTCACTGCAATATCA-3'	5'-TGTTTTATTTACAGGGAGCAACA-3'
CecropinB	5'-CAGCCTCTGAGTTTCCCAGG-3'	5'-CGCACAGTTCTCACTGCAAC-3'
CecropinC	5'-AAGCCGGTTGGCTGAAGAAA-3'	5'-GCGCAATTCCCAGTCCTTGA-3'
IMD	5'-TTGTCTTGCGCTTCTCCAGT-3'	5'-GGGATCTTGGCATGTCGGAA-3'

RNA extraction and qRT-PCR

Total RNA of 5 adults or 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 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 2.5 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 50 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s; this was followed by dissociation analysis of a ramp from 65°C to 95°C with a read every 0.5°C. Relative quantification for each mRNA was done using the Livak-method (Livak and Schmittgen, 2001). The values obtained for each mRNA were normalized by RPL32 mRNA amount for *D. melanogaster* and RPL13a for *T. castaneum*. Total RNA for each treatment was isolated twice (biological replication) and each sample was measured by qRT-PCR twice (technical replication).

Molecular cloning and parental RNAi

The *Tc-zen1* plasmid was obtained from Falciani et al. (Falciani et al., 1996). Non-targeting control dsRNA was synthesized from a 500 bp vector sequence (pCRII, Invitrogen) cloned with the primers 5'-TGCCGGATCAAGAGCTACCAA-3' forward and 5'-TGTGAGCAAAAGGCCAGCAA-3' reverse. dsRNA was synthesized using the MEGAscript RNAi kit (Ambion), and parental RNAi was performed according to Bucher et al. (Bucher et al., 2002).

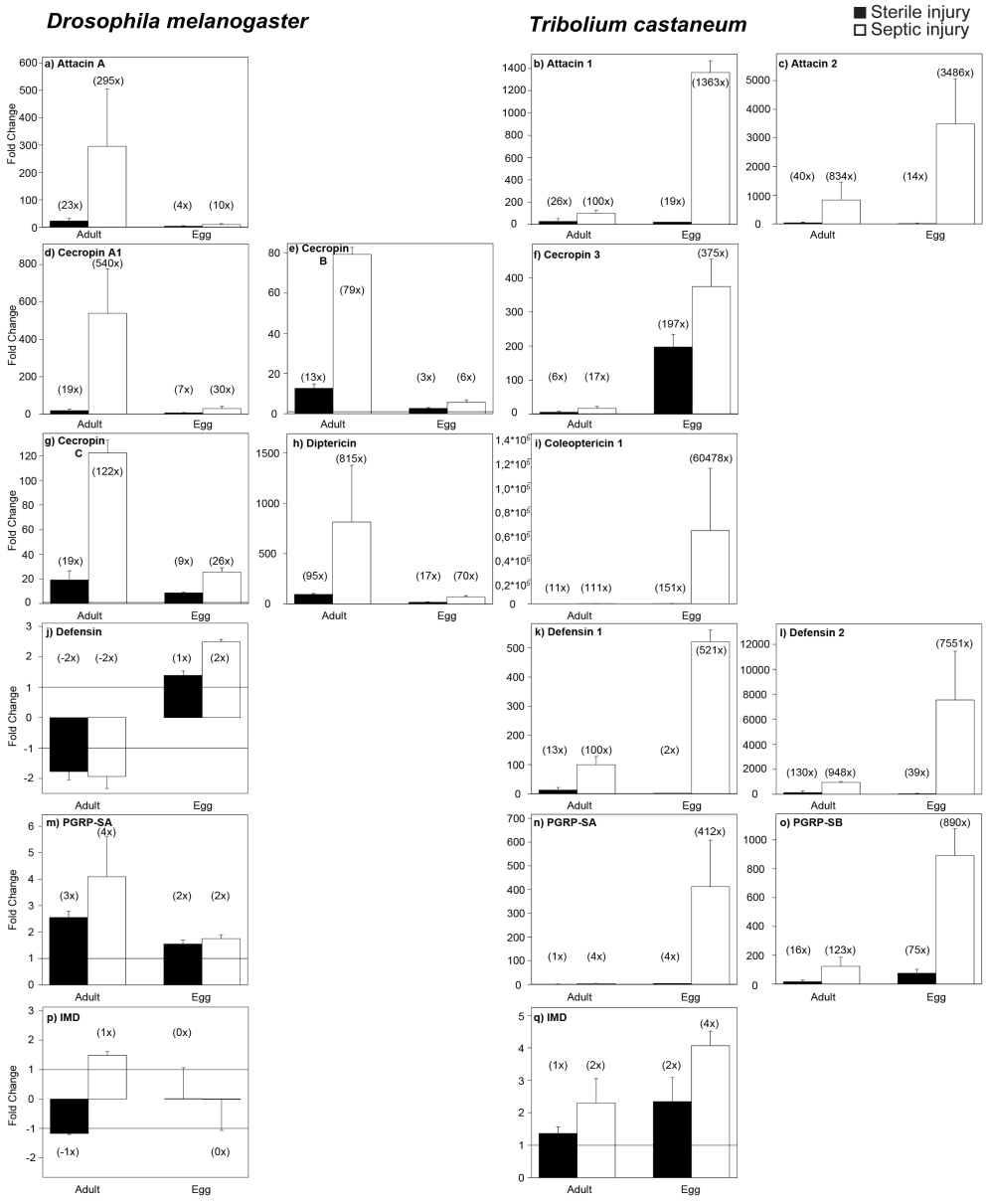

Figure 4-1: The expression of immune genes in adults and eggs of *D. melanogaster* and *T. castaneum*. Plotted is the mean fold change (in parenthesis) plus standard error based on two biological replicates (each replicate is the mean of two technical replicates). Clear induction of immune genes is visible in both *D. melanogaster* and *T. castaneum* adults. The induction of immune genes in *T. castaneum* eggs is also clear, however, almost no induction of immune genes is found in *D. melanogaster* eggs. (a) *Dm-AttacinA* (b) *Tc-Attacin1* (c) *Tc-Attacin2* (d) *Dm-CecropinA1* (e) *Dm-CecropinB* (f) *Tc-Cecropin3* (g) *Dm-CecropinC* (h) *Dm-Diptericin* (i) *Tc-Coleoptericin1* (j) *Dm-Defensin* (k) *Tc-Defensin1* (l) *Tc-Defensin2* (m) *Dm-PGRP-SA* (n) *Tc-PGRP-SA* (o) *Tc-PGRP-SB* (p) *Dm-IMD* (q) *Tc-IMD*. For details see results.

Results

Induction of immune genes in *Drosophila* and *Tribolium* adults and eggs
To quantify the immune response of eggs and adults of *Drosophila* and *Tribolium* we either pricked them with a sterile needle (sterile injury) or infected them with a mix of live *E. coli* and *M. luteus* (septic injury) and compared the expression to the expression of non-injured eggs and adults.

Attacins
In *Drosophila* adults, we found a weak upregulation of AttacinA upon sterile injury but a strong upregulation after septic injury (Figure 4-1a). In *Drosophila* eggs, we found a weak upregulation of AttacinA after both sterile and septic injury (Figure 4-1a). Also in *Tribolium* adults, upregulation of both Attacin1 and Attacin2 was higher after septic injury than after sterile injury (Figure 4-1b,c). In contrast to the *Drosophila* egg, the *Tribolium* egg has a strong upregulation of both attacins after septic injury (Figure 4-1b,c).

Cecropins
In *Drosophila* adults, we found a weak upregulation of CecropinA1, CecropinB and CecropinC upon sterile injury but all show a strong upregulation upon septic injury. *Drosophila* eggs, however, show weak upregulation upon both septic and sterile injury (Figure 4-1d,e,g). In *Tribolium* adults, Cecropin3 shows a weak upregulation to both sterile and septic injury. *Tribolium* eggs however, show a strong upregulation to both sterile and septic injury (Figure 4-1f).

Diptericin and Coleoptericin
Diptericin is upregulated upon sterile injury in *Drosophila* adults but we found a stronger upregulation after septic injury. There is clear upregulation of Diptericin in *Drosophila* eggs, albeit less strong than in adults (Figure 4-1h). In *Tribolium* adults, Coleoptericin1 is weakly upregulated after sterile injury but we found a strong upregulation after septic injury. We found an even stronger upregulation in *Tribolium* eggs, both after septic and sterile injury (Figure 4-1i).

Defensins
For *Drosophila*, we found no induction of Defensin in response to sterile and septic injury for both adults and eggs in our study (Figure 4-1j). In contrast, in *Tribolium* Defensin1 and Defensin2 both showed strong upregulation after septic injury in adults and even stronger upregulation in eggs (Figure 4-1k,l). Defensin2 was induced more strongly than Defensin1 in both adults and eggs.

PGRP
PGRP-SA showed very weak upregulation in *Drosophila* adults in response to injury and even less in eggs (Figure 4-1m). The upregulation of PGRP-SA in *Tribolium* is very similar to *Drosophila* in adults, but PGRP-SA it is strongly expressed in the *Tribolium* egg upon septic injury (Figure 4-1n). PGRP-SB is expressed strongly in adults after septic injury and is even expressed stronger in *Tribolium* eggs (Figure 4-1o).

IMD

In *Drosophila* we found no upregulation of IMD in both adults and eggs (Figure 4-1p). The expression of IMD in *Tribolium* adults is similar to the adults of *Drosophila* with no clear upregulation. We found a weak upregulation of IMD in the eggs of *Tribolium* (Figure 4-1q).

Taken together, the antimicrobial peptides show strong upregulation in both *Drosophila* and *Tribolium* adults. PGRP-SA and IMD show less strong responses in adults, although PGRP-SB was upregulated quite strongly in *Tribolium* adults. The investigated immune genes showed only weak expression in *Drosophila* eggs, in contrast to the strong upregulation found in *Tribolium* eggs.

The involvement of the serosal epithelium in the immune defense

In order to test whether the immune response in the *Tribolium* egg is restricted to the serosal epithelium we created serosa-less eggs by *Tc-zen1* RNAi and as a control a non-targeting dsRNA was used (see methods). We quantified the expression of immune genes in serosa-less eggs and control eggs after sterile injury and septic injury and compared it to non-injured eggs (Figure 4-2). Both Attacin1 and Attacin2 showed clear upregulation after septic injury in control eggs but lacked this upregulation in serosa-less eggs (Figure 4-2a,b). Coleoptericin1 is also upregulated in response to septic injury in control eggs and shows weak upregulation in serosa-less eggs (Figure 4-2c). Cecropin3 is upregulated in response to both sterile and septic injury in control eggs. This upregulation is absent in serosa-less eggs (Figure 4-2d). Defensin1 and Defensin2 are both strongly upregulated after septic injury in control eggs but serosa-less eggs do not upregulate defensins (Figure 4-2e,f). IMD shows only weak expression after injury in control eggs and we see no significant change in expression in serosa-less eggs (Figure 4-2g). Both PGRP-SA and PGRP-SB are strongly expressed after septic injury in control eggs but are only weakly expressed in serosa-less eggs (Figure 4-2h,i). In summary, all the immune genes tested show weak to no expression in serosa-less eggs, these results are comparable to the expression of immune genes in *Drosophila* eggs.

The expression of immune genes in adults compared to eggs

The expression of most immune genes was significantly higher in *Tribolium* eggs than in *Tribolium* adults (Figure 4-1). This could mean that either the adults had a higher amount of transcripts before injury or that eggs produced more transcript in response to injury. To differentiate between these two hypotheses, we compared the expression of immune genes in *Tribolium* after septic injury between eggs and adults. To verify that in *Drosophila* adults have higher amounts of transcript, we also compared the expression of immune genes in *Drosophila* after septic injury between eggs and adults. As expected from the results above, the expression in *Drosophila* eggs was lower than in adults for all genes tested, as indicated by the negative values in Figure 4-3a. The expression was much lower for AttacinA, CecropinA1 and Diptericin (Figure 4-3a). Contrary to *Drosophila*, the expression of immune genes after septic injury does not differ between adults and eggs of *Tribolium* (Figure 4-3b). This indicates that *Tribolium* eggs have much lower transcript levels in untreated condition than adults, but reach similar levels after infection. *Drosophila* eggs on the other hand do not reach the same amount of transcript as adults do.

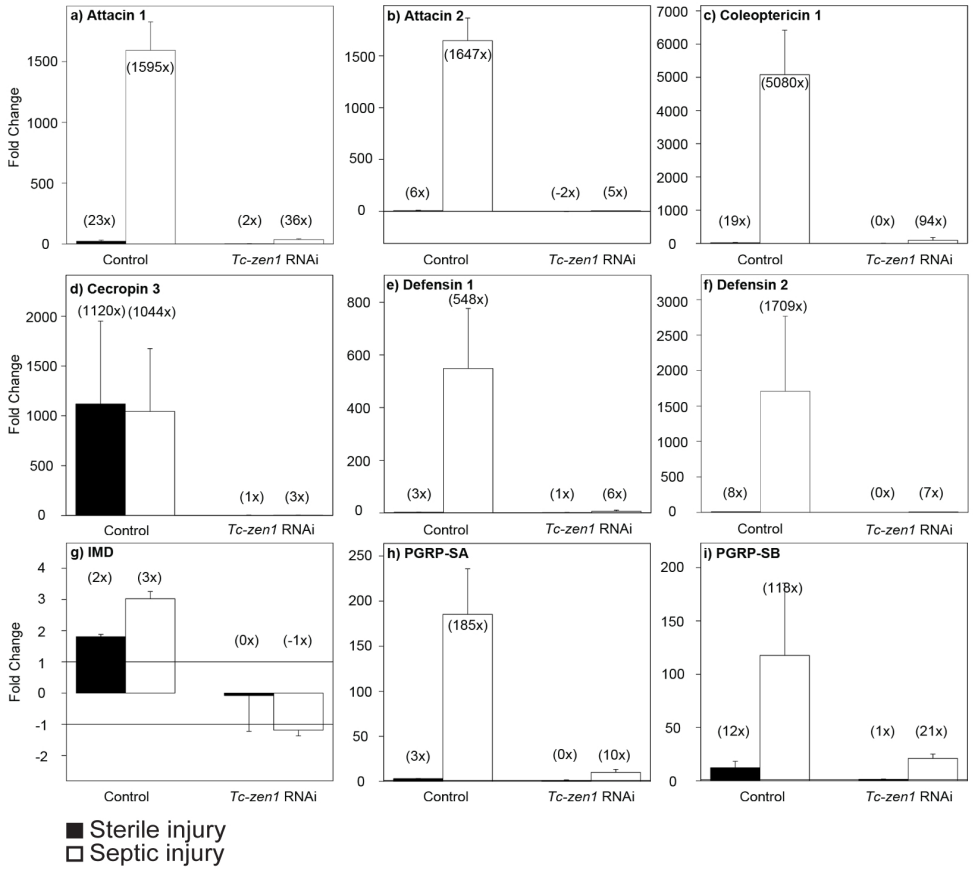

Figure 4-2: The expression of immune genes in the *T. castaneum* egg depends on the serosa. Plotted is the mean fold change (in parenthesis) plus standard error based on two biological replicates (each replicate is the mean of two technical replicates). When control eggs are infected a clear upregulation of immune genes is visible, however, if we prevent the development of the serosa with *Tc-zen1* RNAi almost no induction of immune genes is visible. (a) *Tc-Attacin1* (b) *Tc-Attacin2* (c) *Tc-Coleoptericin1* (d) *Tc-Cecropin3* (e) *Tc-Defensin1* (f) *Tc-Defensin2* (g) *Tc-IMD* (h) *Tc-PGRP-SA* (i) *Tc-PGRP-SB*. For details see results.

Discussion

In this study, we investigated the induction of immune gene expression in the eggs and adults of *Drosophila* and *Tribolium*. We found strong induction of immune gene expression in adults of both *Drosophila* and *Tribolium*. Furthermore, we found strong induction of immune gene expression in *Tribolium* eggs but not in *Drosophila* eggs. We show that the induction of immune gene expression in the *Tribolium* egg is comparable to adults and is dependent on the serosal epithelium.

The expression levels found for *Drosophila* adults in this study are comparable to the levels reported previously (Lemaitre et al., 1997), albeit the upregulation in our study is higher. This might be because we infected simultaneously with *E. coli* and *M. luteus* whereas Lemaitre et al. (1997) performed the infection separately. The upregulation of immune genes in the *Drosophila* egg has not been quantified earlier, but our finding of

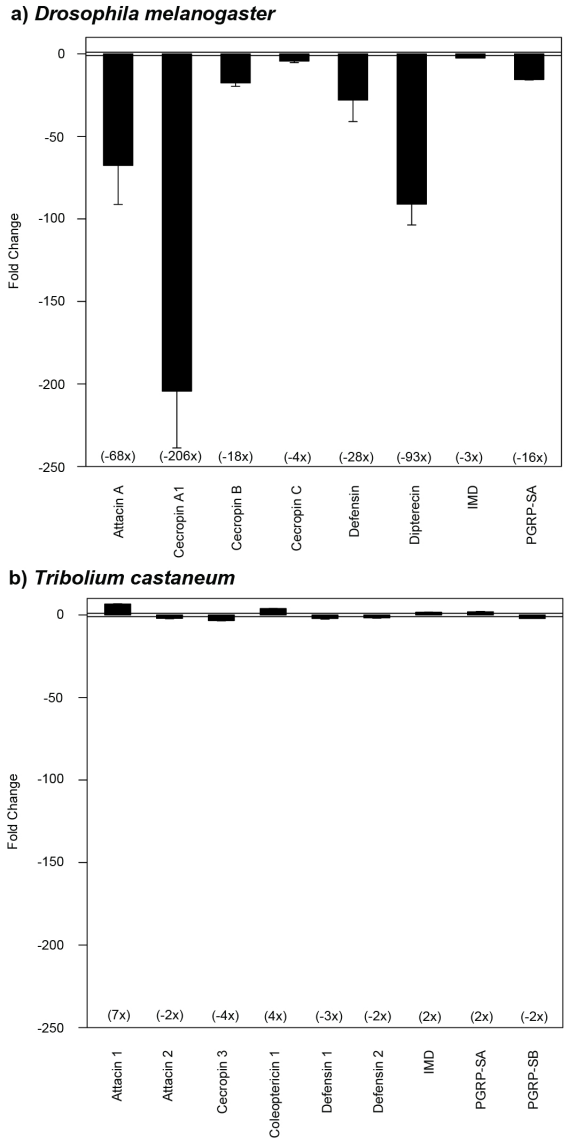

Figure 4-3: The expression of immune genes after septic injury in *D. melanogaster* and *T. castaneum* eggs as compared to adult expression. Plotted is the mean fold change (in parenthesis) plus standard error based on two biological replicates (each replicate is the mean of two technical replicates). (a) *D. melanogaster* egg expression as compared to adult expression. For most genes tested there is clearly less expression in the egg than in the adult. (b) *T. castaneum* egg expression as compared to adult expression. For all the genes tested the expression is as high in the egg as it is in the adult. For details see results.

the relatively weak upregulation of CecropinA1 and Diptericin is in accordance with previous semi-quantitative measurements in *Drosophila* embryo's (Esfahani and Engstrom, 2011; Tingvall et al., 2001). We furthermore found weak upregulation upon infection of AttacinA, CecropinB and CecropinC.

The expression levels of immune genes in adult *Tribolium* generally corresponded well with the levels that are reported in the literature (Altincicek et al., 2008; Yokoi et al., 2012a; Yokoi et al., 2012b; Zou et al., 2007), although Yokoi et al. (2012b) found no expression of Defensin1 in pupae while we found a clear upregulation of this gene in adults. This could either be because a different stage was used (pupae vs adults) or because a different strain of *Tribolium* was used by Yokoi et al. (2012b).

We found a very strong upregulation of all of the genes tested except for IMD, which shows that the *Tribolium* egg is able to mount a potent immune defense upon infection with bacteria. Surprisingly, both in our study as in the study by Yokoi et al. (2012b), Cecropin3 was hardly upregulated upon infection. The egg, however, exhibits a clear upregulation of this gene after both sterile and septic injury, and reaches a comparable amount of transcript as adults. These results show that, although Cecropin3 is not upregulated much in adults, the transcript is already present at high levels in untreated adults, suggesting that it might be used in adults to prevent rather

than fight infection. The same is true for PGRP-SA, although this is less surprising as PGRP-SA is a receptor and functions in the recognition of pathogens and should therefore be available at any time (Lemaitre and Hoffmann, 2007; Zou et al., 2007).

In *Drosophila*, the expression pattern of adults is clearly distinguishable from the expression pattern in the egg, mainly due to the lower upregulation in the egg. This indicates that the *Drosophila* egg is not able to mount an immune defense comparable to the adult. Contrary to *Drosophila*, the expression levels of *Tribolium* eggs and adults are indistinguishable; this suggests that the *Tribolium* egg can mount an equally potent immune defense as adults.

As the *Drosophila* egg does not develop a serosal epithelium (Schmidt-Ott, 2000, 2005) the expression of immune genes is localized to either the yolk or the embryonic epithelium. However, the response in the yolk diminishes around midembryogenesis (Tingvall et al., 2001). We infected eggs approximately 14 hours after egglay (AEL) at 20 degrees Celsius, which corresponds to midembryogenesis (Al-Saffar et al., 1995). This suggests that the weak upregulation found in *Drosophila* eggs is caused by the expression of immune genes in the early embryonic epithelium.

In *Tribolium*, the expression of these immune genes in the egg depends on the presence of the serosal epithelium. In the absence of the serosal epithelium in *Tc-zen1* RNAi eggs, almost all expression of the genes tested was lost. The expression profile in serosa-less *Tribolium* eggs strongly resembles the expression pattern we see in naturally serosa-less *Drosophila* eggs.

In this study, we presented evidence that the *Tribolium* egg is able to mount a potent immune response. Contrary to *Tribolium* eggs, the *Drosophila* egg only exhibits weak expression of immune genes. In addition, when we prevent the development of the serosa in *Tribolium* eggs by *Tc-zen1* RNAi, we can find similar expression levels of immune genes as in the *Drosophila* eggs. Our results strongly suggest a crucial role of the serosa in the early immune defense of insect eggs.

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References

Al-Saffar ZY, Grainger JNR, Aldrich J, (1995). Influence of constant and changing temperature and humidity on the development and survival of the eggs and pupae of Drosophila melanogaster (Meigen). Journal of Thermal Biology 20:389-397.

Altincicek B, Elashry A, Guz N, Grundler FM, Vilcinskas A, Dehne HW, (2013). Next generation sequencing based transcriptome analysis of septic-injury responsive genes in the beetle Tribolium castaneum. PLoS One 8:e52004. doi: 10.1371/journal.pone.0052004.

Altincicek B, Knorr E, Vilcinskas A, 2008. Beetle immunity: Identification of immune-inducible genes from the model insect Tribolium castaneum. Developmental and Comparative Immunology 32:585-595. doi: 10.1016/j.dci.2007.09.005.

Altincicek B, Vilcinskas A, (2007). Analysis of the immune-inducible transcriptome from microbial stress resistant, rat-tailed maggots of the drone fly Eristalis tenax. BMC genomics 8:326. doi: 10.1186/1471-2164-8-326.

Bischoff V, Vignal C, Duvic B, Boneca IG, Hoffmann JA, Royet J, (2006). Downregulation of the Drosophila immune response by peptidoglycan-recognition proteins SC1 and SC2. Plos Pathogens 2:139-147. doi: 10.1371/

journal.ppat.0020014.
Bucher G, Scholten J, Klingler M, (2002). Parental RNAi in Tribolium (Coleoptera). Current Biology 12:R85-R86.
Chen G, Handel K, Roth S, (2000). The maternal NF-kappa B/Dorsal gradient of Tribolium castaneum: dynamics of early dorsoventral patterning in a short-germ beetle. Development 127:5145-5156.
Costa A, Jan E, Sarnow P, Schneider D, (2009). The Imd pathway is involved in antiviral immune responses in Drosophila. PLoS One 4:e7436. doi: 10.1371/journal.pone.0007436.
Dimarcq JL, Hoffmann D, Meister M, Bulet P, Lanot R, Reichhart JM, Hoffmann JA, (1994). Characterization and transcriptional profiles of a Drosophila gene encoding an insect defensin- a study in insect immunity. European Journal of Biochemistry 221:201-209. doi: 10.1111/j.1432-1033.1994.tb18730.x.
Esfahani SS, Engstrom Y, (2011). Activation of an innate immune response in large numbers of permeabilized Drosophila embryos. Developmental and Comparative Immunology 35:263-266. doi: 10.1016/j.dci.2010.11.002.
Falciani F, Hausdorf B, Schroder R, Akam M, Tautz D, Denell R, Brown S, (1996). Class 3 Hox genes in insects and the origin of zen. Proceedings of the National Academy of Sciences of the United States of America 93:8479-8484.
Gerardo NM, Altincicek B, Anselme C, Atamian H, Barribeau SM, de Vos M, Duncan EJ, Evans JD, Gabaldon T, Ghanim M, Heddi A, Kaloshian I, Latorre A, Moya A, Nakabachi A, Parker BJ, Perez-Brocal V, Pignatelli M, Rahbe Y, Ramsey JS, Spragg CJ, Tamames J, Tamarit D, Tamborindeguy C, Vincent-Monegat C, Vilcinskas A, (2010). Immunity and other defenses in pea aphids, Acyrthosiphon pisum. Genome Biology 11:R21. doi: 10.1186/gb-2010-11-2-r21.
Gorman MJ, Kankanala P, Kanost MR, (2004). Bacterial challenge stimulates innate immune responses in extra-embryonic tissues of tobacco hornworm eggs. Insect Molecular Biology 13:19-24.
Haghayeghi A, Sarac A, Czerniecki S, Grosshans J, Schock F, (2010). Pellino enhances innate immunity in Drosophila. Mech Dev 127:301-307. doi: 10.1016/j.mod.2010.01.004.
Lemaitre B, Hoffmann J, (2007). The Host Defense of Drosophila melanogaster. Annual Review of Immunology 25:697-743. doi: doi:10.1146/annurev.immunol.25.022106.141615.
Lemaitre B, Reichhart J-M, Hoffmann JA, (1997). Drosophila host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proceedings of the National Academy of Sciences 94:14614-14619.
Livak KJ, Schmittgen TD, (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408. doi: 10.1006/meth.2001.1262.
Lord JC, Hartzer K, Toutges M, Oppert B, (2010). Evaluation of quantitative PCR reference genes for gene expression studies in Tribolium castaneum after fungal challenge. Journal of Microbiological Methods 80:219-221. doi: http://dx.doi.org/10.1016/j.mimet.2009.12.007.
Roth S, (2004). Gastrulation in other insects. Gastrulation: From Cells to Embryos Cold Spring Harbor: Cold Spring Harbor Laboratory Press. p. 105-121.
Schmidt-Ott U, (2000). The amnioserosa is an apomorphic character of cyclorrhaphan flies. Development Genes and Evolution 210:373-376.
Schmidt-Ott U, (2005). Insect serosa: A head line in comparative developmental genetics. Current Biology 15:R245-R247. doi: 10.1016/j.cub.2005.03.022.
Schwalm FE, (1988). Insect Morphogenesis. Basel: S. Karger AG.
Tingvall TO, Roos E, Engstrom Y, (2001). The GATA factor Serpent is required for the onset of the humoral immune response in Drosophila embryos. Proceedings of the National Academy of Sciences 98:3884-3888. doi: 10.1073/pnas.061230198.
Tzou P, Ohresser S, Ferrandon D, Capovilla M, Reichhart J-M, Lemaitre B, Hoffmann JA, Imler JL, (2000). Tissue-Specific Inducible Expression of Antimicrobial Peptide Genes in Drosophila Surface Epithelia. Immunity 13:737-748.
van der Zee M, Berns N, Roth S, (2005). Distinct functions of the Tribolium zerknullt genes in serosa specification and dorsal closure. Current Biology 15:624-636.
Waterhouse RM, Kriventseva EV, Meister S, Xi Z, Alvarez KS, Bartholomay LC, Barillas-Mury C, Bian G, Blandin S, Christensen BM, Dong Y, Jiang H, Kanost MR, Koutsos AC, Levashina EA, Li J, Ligoxygakis P, Maccallum RM, Mayhew GF, Mendes A, Michel K, Osta MA, Paskewitz S, Shin SW, Vlachou D, Wang L, Wei W, Zheng L, Zou Z, Severson DW, Raikhel AS, Kafatos FC, Dimopoulos G, Zdobnov EM, Christophides GK, (2007). Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. Science 316:1738-1743. doi: 10.1126/science.1139862.
Yokoi K, Koyama H, Ito W, Minakuchi C, Tanaka T, Miura K, (2012a). Involvement of NF-κB transcription factors in antimicrobial peptide gene induction in the red flour beetle, Tribolium castaneum. Developmental and

Comparative Immunology 38:342-351. doi: 10.1016/j.dci.2012.06.008.
Yokoi K, Koyama H, Minakuchi C, Tanaka T, Miura K, (2012b). Antimicrobial peptide gene induction, involvement of Toll and IMD pathways and defense against bacteria in the red flour beetle, Tribolium castaneum. Results in Immunology 2:72-82. doi: 10.1016/j.rinim.2012.03.002.
Zhong D, Wang MH, Pai A, Yan G, (2013). Transcription profiling of immune genes during parasite infection in susceptible and resistant strains of the flour beetles (Tribolium castaneum). Experimental parasitology. doi: 10.1016/j.exppara.2013.01.014.
Zou Z, Evans JD, Lu ZQ, Zhao PC, Williams M, Sumathipala N, Hetru C, Hultmark D, Jiang HB, (2007). Comparative genomic analysis of the Tribolium immune system. Genome Biology 8. doi: 10.1186/gb-2007-8-8-r177.