Surviving embryogenesis

The extraembryonic serosa protects the insect egg against desiccation and infection

Chris G.C. Jacobs

Jacobs, C.G.C.

Surviving embryogenesis
The extraembryonic serosa protects the insect egg against desiccation and infection

PhD thesis, Faculty of Science, Leiden University, 2014
In English, with summary in Dutch

ISBN: 978-94-9101-433-8

Cover design by Chris Jacobs

Surviving embryogenesis

The extraembryonic serosa protects the insect egg against desiccation and infection

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 4 december 2014
klokke 12.30 uur

door

Chris Gerardus Cornelus Jacobs

geboren te Asten, Nederland
in 1986

Promotiecommissie

Promotor
Prof. Dr. Herman P. Spaink (Universiteit Leiden)

Co-promotor
Dr. Maurijn van der Zee (Universiteit Leiden)

Overige leden
Prof. Dr. Carel J. ten Cate (Universiteit Leiden)
Dr. Annemarie H. Meijer (Universiteit Leiden)
Prof. Dr. Menno Schilthuizen (Naturalis)
Dr. Daniel E. Rozen (Universiteit Leiden)
Prof. Dr. Siegfried Roth (University of Cologne)

Table of Contents

Chapter 1

General introduction

Insect diversity

Life on earth is incredibly diverse. It can be found in almost every corner of the planet. The number of species that is currently described is around 1.5 million, approximately 58% of which are insects (Figure 1-1). Insects are by far the most diverse animal group on this planet and many have not yet been described. Estimates of the total diversity of insect species vary from 2.5 to 10 million species (Grimaldi & Engel, 2005). Their enormous diversity, biomass, and ecological impact show that studying insects should provide profound insight into evolution. But how did insects become so successful?

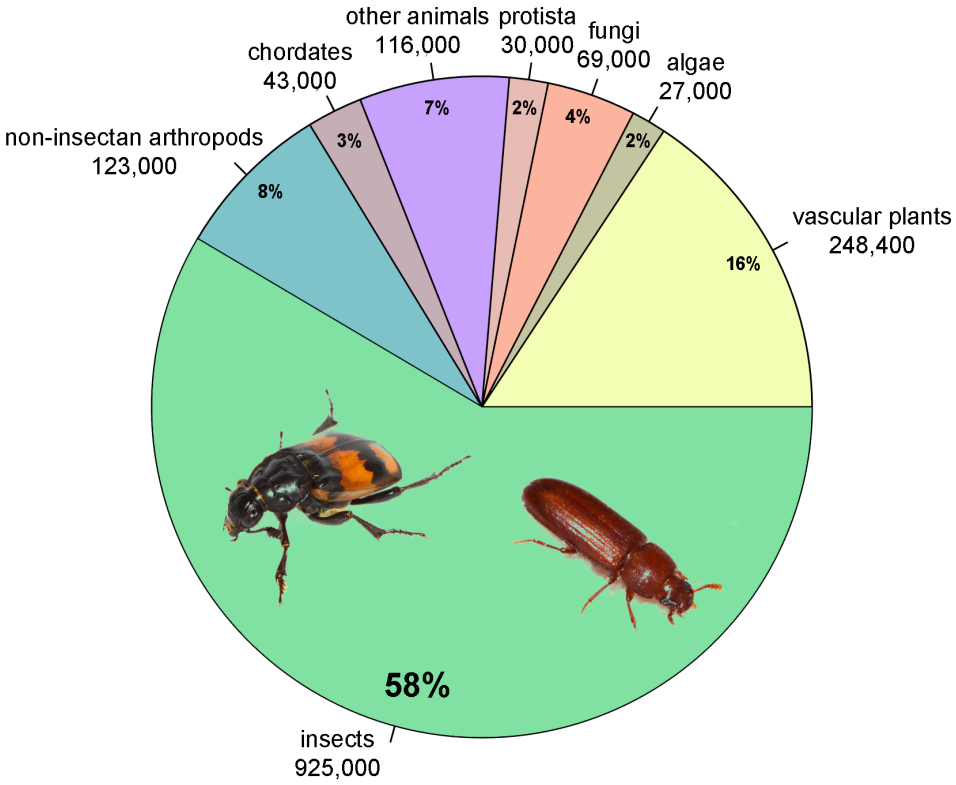


Figure 1-1: Insects are the most diverse group of animals on earth, of the 1.5 million species, 58% are insects (adapted from Grimaldi, 2005).

Traits that influenced insect diversity

There are many hypotheses about why insects have become so diverse, the most prominent include evolutionary novelties known as key innovations (Mayhew, 2007). The 4 most widely recognized innovations are (1) the insect "bauplan", (2) wings, (3) folded wings and (4) complete metamorphosis (Mayhew, 2007). Although there is some discussion on the relative contribution of these factors to insect diversity, a recent analysis confirmed the importance of wings and complete metamorphosis for insect diversity (Nicholson et al., 2014). Further factors that influenced insect diversity are their small size and their co-evolution with plants (Gillott, 2005; Grimaldi & Engel, 2005). All these factors have undoubtedly contributed to insect diversity. However, all these factors concern adult insects. In 1989, Zeh et al. proposed that the egg might have played an important part in the success of insects (Zeh et al., 1989). A suite of egg-stage characters would reduce constraints on suitable sites for egg deposition, enabling insects to diversify in previously unavailable niches. This self-sufficient egg might also explain the low incidence of parental care in insects compared to other terrestrial arthropods (Zeh et al., 1989). Much is known about insect eggs, their development, structures and protection (Hilker & Meiners, 2002; Hinton, 1981). Despite the plethora of knowledge concerning insect eggs, many people see insect eggs as vulnerable and dependent on their parents for protection. Indeed, insect eggs are covered by a maternal eggshell (Figure 1-2), and some are protected by diverse mechanisms of maternal input like chemical protection and the selection of suitable egg laying sites (Hilker & Meiners, 2002). However, there is increasing evidence that many insect eggs are not as helpless as is generally thought. This self-protection is reached by the development of an zygotic extraembryonic membrane, called the serosa.

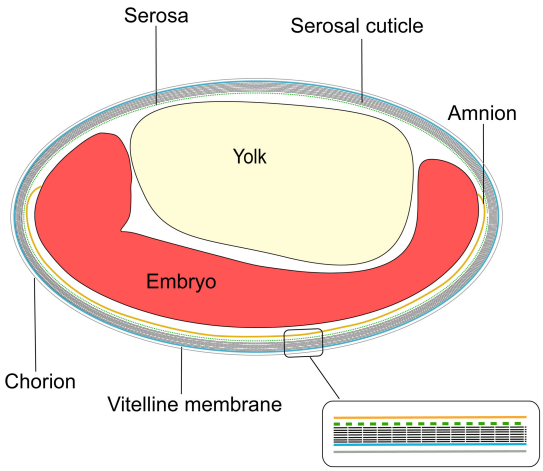


Figure 1-2: Schematic overview of the insect egg. The insect egg is surrounded by two maternal layers, the chorion and the vitelline membrane. Beneath these, the extraembryonic serosa secretes a chitinous cuticle (serosal cuticle). The serosa and serosal cuticle envelope both the embryo and the yolk. The extraembryonic amnion covers the ventral side of the embryo.

Extraembryonic development

The fertilized egg will start from a single nucleus and develop into a fully formed larva by the end of embryonic development. However, a larva is not the only thing which is formed during development. Insect eggs also develop two extraembryonic membranes, the amnion and the serosa, which will not become part of the larva (Panfilio, 2008; Schmidt-Ott et al., 2010). These membranes are found in almost all insects (Roth, 2004). Only the higher diptera, to which *Drosophila melanogaster* belongs, do not develop a serosa but instead develop a single dorsal extraembryonic membrane called the amnioserosa (Rafiqi et al., 2008; Schmidt-Ott, 2000). The single nucleus in the fertilized egg divides multiple times in the yolk. Subsequently, these nuclei migrate to the periphery of the insect egg after which the first cell membranes will form around the nuclei, forming the first cell layer. This first cell layer in the insect egg is called the blastoderm, and a large part of it is used in insect eggs to form the extraembryonic serosal epithelium (Figure 1-3)(Machida, 2006; Machida & Ando, 1998; Roth, 2004; Schwalm, 1988).

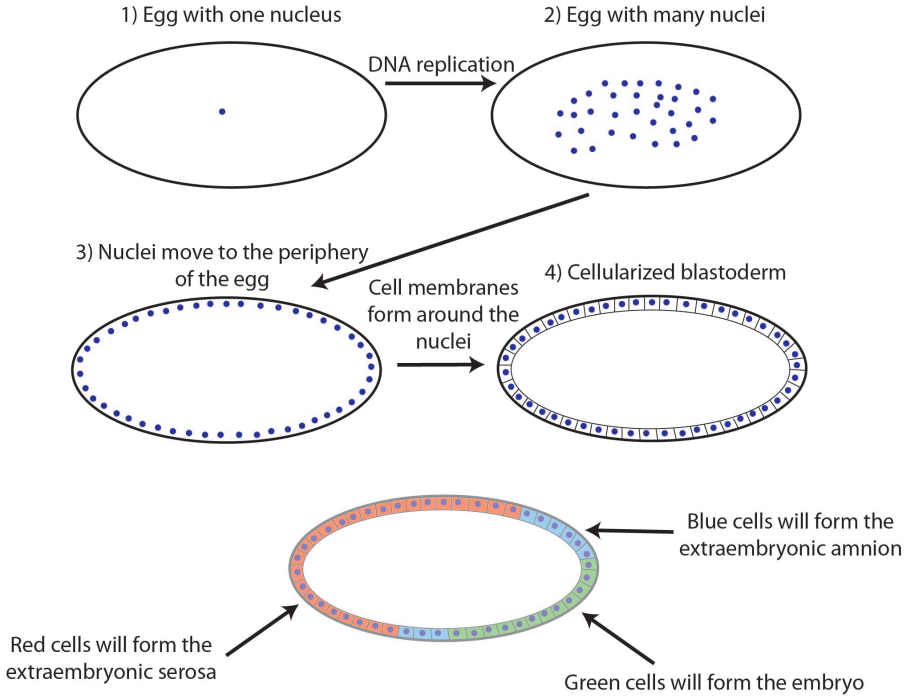


Figure 1-3: The formation of the blastoderm in insect eggs. Indicated are parts of the blastoderm that will form the embryo (green), the extraembryonic amnion (blue) and the extraembryonic serosa (red).

During gastrulation, the embryo will fold into the yolk and the serosa will completely surround the embryo and the yolk, forming a barrier between the embryo and the outside world (Figure 1-4). Due to the fact that the serosa separates the embryo and yolk from the outside world, it is a prime candidate for protection from outside hazards.

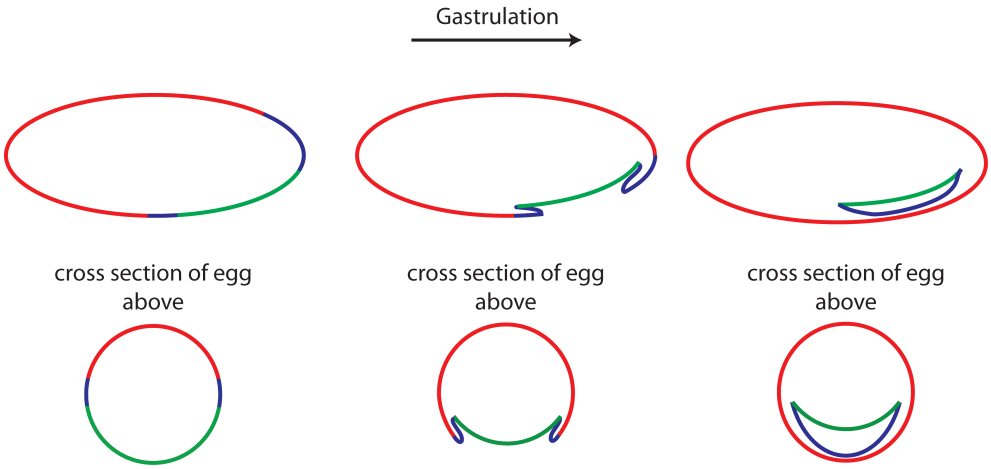


Figure 1-4: During gastrulation, the developing embryo will fold into the yolk and the serosa will envelop both the yolk and the embryo. Embryo = green ; amnion = blue ; serosa = red

The serosa as barrier epithelium

The serosa has been suggested to function as a protective layer around the insect egg. One of the protective functions that has been suggested is the protection against desiccation. The serosa secretes a chitinized cuticle, the serosal cuticle, underneath the maternal eggshell (Hinton, 1981; Lamer & Dorn, 2001). In mosquitos, desiccation resistance coincides with the time of serosal cuticle secretion (Goltsev et al., 2009; Rezende et al., 2008; Vargas et al., 2014). This indicates that the serosa might protect the insect egg against desiccation by secreting a serosal cuticle.

A second protective function for the serosa has been proposed, namely against infection. The NF-κB transcription factor Dorsal is highly expressed in the serosa of the red flour beetle *Tribolium castaneum*, and translocates to the nucleus upon injury (Chen et al., 2000). NF-κB factors are well known for their involvement in the innate immune response (Lemaitre & Hoffmann, 2007), so it might be possible that the serosa indeed protects against microbes by inducing immune genes upon infection. Supporting this notion, it has been found that in the tobacco hornworm (*Manduca sexta*), immune genes are induced upon infection in the extraembryonic tissues of the egg (Gorman et al., 2004).

The serosa has long been thought to protect the insect egg, however, no experimental proof exists. This is because it is impossible to physically remove the zygotic serosa without affecting the overlying maternal eggshell, which consists of an exochorion, endochorion and vitelline membrane (Furneaux et al., 1969). In the red flour beetle (*Tribolium castaneum*), it is possible to prevent the development of the serosa without affecting the maternal eggshell using 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. This single dorsal membrane is similar to the reduced extraembryonic amnioserosa in *Drosophila melanogaster* (Figure 1-5). This provides us with the unique opportunity to experimentally assess the function of the serosa.

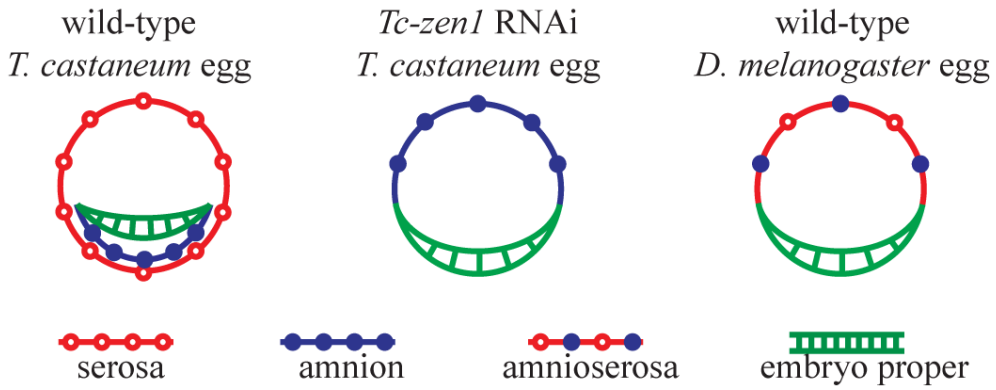


Figure 1-5: During normal development in *T. castaneum*, the serosa will envelop both the embryo and the yolk. However, after *Tc-zen1* RNAi no serosa will be formed and the amnion will cover the dorsal side of the egg. This resembles normal development in the *D. melanogaster* egg, where the amnioserosa only covers the dorsal side of the egg.

Aim and outline of this thesis

The general aim of this thesis is to experimentally test the protective functions of the serosa in the red flour beetle (*Tribolium castaneum*). An additional goal is to uncover some of the ecological aspects that might have contributed to the evolution of this protective layer.

The first part of this thesis (**CHAPTER 2 AND 3**) deals with the question of whether the serosa protects against desiccation. In **CHAPTER 2** I first assess whether the development of the serosa is an evolutionary novelty of insects. I then test whether the serosa protects against desiccation by using the above mentioned method of preventing serosal development by *Tc-zen1* RNAi. I furthermore show the presence of a serosal cuticle in the *Tribolium* egg and separate the protective function of the serosal epithelium itself and the cuticle it secretes. In **CHAPTER 3** I take a closer look at the function of the serosal cuticle. I assess whether the same genes are utilized to produce the serosal cuticle as are used to produce the adult cuticle. I then look at how cuticle structure influences desiccation resistance. I furthermore analyze transcriptome data of serosa-less eggs and wild-type eggs to identify cuticular genes which are specifically expressed by the serosa.

The second part of this thesis (**CHAPTER 4-6**) focusses on whether the serosa protects against infection. In **CHAPTER 4** I study the immune response of eggs and adults of both *T. castaneum* and *D. melanogaster*. Next, I study the immune response of *T. castaneum* eggs with and without serosa. I then compare the immune response of eggs with adults. In **CHAPTER 5** I look at the immune response of the *Tribolium* egg more extensively. I first study the proliferation of bacteria in eggs with and without serosa. Next, I extensively characterize the immune response of wild-type, control RNAi and serosa-less eggs by RNA sequencing. Finally, I show where immune genes are expressed, both constitutively and induced, by *in situ* hybridization. In **CHAPTER 6** I look at the protective function of the serosa in another beetle species, the burying beetle (*Nicrophorus vespilloides*). This species has a very different ecology than *Tribolium*, it shows extensive

parental care and lives on ephemeral food sources. I first show the effects of exposure to microorganisms on the survival of eggs. Then I study whether a serosa is present in this species. Next, I measure the immune response of both eggs and larvae. I furthermore test whether these eggs are able to survive dry circumstances.

Finally, in **CHAPTER 7** of this thesis, the findings are summarized. The differences between species are discussed and how these differences could have arisen due to their different life histories. I furthermore discuss exciting new directions for future research.

References

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(23), 5145-5156.

Furneaux, P. J. S., James, C. R., & Potter, S. A. (1969). The Egg Shell of the House Cricket (Acheta Domesticus): An Electron-Microscope Study. Journal of Cell Science, 5(1), 227-249.

Gillott, C. (2005). Entomology (3rd ed.). Dordrecht: Springer.

Goltsev, Y., Rezende, G. L., Vranizan, K., Lanzaro, G., Valle, D., & Levine, M. (2009). Developmental and evolutionary basis for drought tolerance of the Anopheles gambiae embryo. Developmental Biology, 330(2), 462-470. doi: 10.1016/j.ydbio.2009.02.038

Gorman, M. J., Kankanala, P., & Kanost, M. R. (2004). Bacterial challenge stimulates innate immune responses in extra-embryonic tissues of tobacco hornworm eggs. Insect Molecular Biology, 13(1), 19-24.

Grimaldi, D. A., & Engel, M. S. (2005). Evolution of the Insects: Cambridge University Press.

Hilker, M., & Meiners, T. (2002). Chemoecology of Insect Eggs and Egg Deposition. Berlin: Blackwell Publishing.

Hinton, H. E. (1981). The Biology of Insect Eggs. (1st ed.). Oxford: Pergamon Press.

Lamer, A., & Dorn, A. (2001). The serosa of Manduca sexta (Insecta, Lepidoptera): ontogeny, secretory activity, structural changes, and functional considerations. Tissue & Cell, 33(6), 580-595. doi: 10.1054/tice.2001.0213

Lemaitre, B., & Hoffmann, J. (2007). The Host Defense of Drosophila melanogaster. Annual Review of Immunology, 25(1), 697-743. doi: doi:10.1146/annurev.immunol.25.022106.141615

Machida, R. (2006). Evidence from embryology for reconstructing the relationships of hexapod basal clades. Arthropod Systematics & Phylogeny, 64(1), 95-104.

Machida, R., & Ando, H. (1998). Evolutionary Changes in Developmental Potentials of the Embryo Proper and Embryonic Membranes along with the Derivative Structures in Atelocerata, with Special Reference to Hexapoda (Arthropoda). Proc. Arthropod. Embryol. Soc. Jpn, 33, 1-13.

Mayhew, P. J. (2007). Why are there so many insect species? Perspectives from fossils and phylogenies. Biological Reviews, 82(3), 425-454. doi: 10.1111/j.1469-185X.2007.00018.x

Nicholson, D. B., Ross, A. J., & Mayhew, P. J. (2014). Fossil evidence for key innovations in the evolution of insect diversity. Proceedings of the Royal Society B: Biological Sciences, 281(1793). doi: 10.1098/rspb.2014.1823

Panfilio, K. A. (2008). Extraembryonic development in insects and the acrobatics of blastokinesis. Developmental Biology, 313(2), 471-491. doi: 10.1016/j.ydbio.2007.11.004

Rafiqi, A. M., Lemke, S., Ferguson, S., Stauber, M., & Schmidt-Ott, U. (2008). Evolutionary origin of the amnioserosa in cyclorrhaphan flies correlates with spatial and temporal expression changes of zen. Proceedings of the National Academy of Sciences of the United States of America, 105(1), 234-239. doi: 10.1073/pnas.0709145105

Rezende, G. L., Martins, A. J., Gentile, C., Farnesi, L. C., Pelajo-Machado, M., Peixoto, A. A., & Valle, D. (2008). Embryonic desiccation resistance in Aedes aegypti: presumptive role of the chitinized Serosal Cuticle. Bmc Developmental Biology, 8(82). doi: 10.1186/1471-213x-8-82

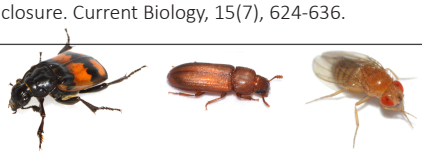
Roth, S. (2004). Gastrulation in other insects. Gastrulation: From Cells to Embryos (pp. 105-121). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Schmidt-Ott, U. (2000). The amnioserosa is an apomorphic character of cyclorrhaphan flies. Development Genes and Evolution, 210(7), 373-376.

Schmidt-Ott, U., Rafiqi, A., & Lemke, S. (2010). Hox3/zen and the Evolution of Extraembryonic Epithelia in Insects Hox Genes: Studies from the 20th to the 21st Century (Vol. 689, pp. 133-144).

Schwalm, F. E. (1988). Insect Morphogenesis. Basel: S. Karger AG.

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(7), 624-636.



Vargas, H. C. M., Farnesi, L. C., Martins, A. J., Valle, D., & Rezende, G. L. (2014). Serosal cuticle formation and distinct degrees of desiccation resistance in embryos of the mosquito vectors Aedes aegypti, Anopheles aquasalis and Culex quinquefasciatus. Journal of Insect Physiology, 62(0), 54-60. doi: http://dx.doi.org/10.1016/j.jinsphys.2014.02.001

Zeh, D. W., Zeh, J. A., & Smith, R. L. (1989). Ovipositors, Amnions and eggshell architecture in the diversification of terrestrial arthropods. Quarterly Review of Biology, 64(2), 147-168.

Chapter 2

The extraembryonic serosa protects the insect egg against desiccation

Chris G.C. Jacobs, Gustavo L. Rezende, Gerda E. M. Lamers and Maurijn van der Zee

Published in Proceedings of the Royal Society: Biological Sciences (2013) 280,
doi: 10.1098/rspb.2013.1082

Insects have been extraordinarily successful in occupying terrestrial habitats, in contrast to their mostly aquatic sister group, the crustaceans. This success is typically attributed to adult traits such as flight, whereas little attention has been paid to adaptation of the egg. An evolutionary novelty of insect eggs is the serosa, an extraembryonic membrane that enfolds the embryo and secretes a cuticle. To experimentally test the protective function of the serosa, we exploit an exceptional possibility to eliminate this membrane by *zerknüllt1* RNAi in the beetle *Tribolium castaneum*. We analyze hatching rates of eggs under a range of humidities and find dramatically decreasing hatching rates with decreasing humidities for serosa-less eggs, but not for control eggs. Furthermore, we show serosal expression of *Tc-chitin-synthase1* and demonstrate that its knockdown leads to absence of the serosal cuticle and a reduction in hatching rates at low humidities. These developmental genetic techniques in combination with ecological testing provide experimental evidence for a crucial role of the serosa in desiccation resistance. We propose that the origin of this extraembryonic membrane facilitated the spectacular radiation of insects on land, as did the origin of amniote egg in the terrestrial invasion of vertebrates.

Key words: desiccation resistance, cuticle, Tribolium castaneum, chs (chitin synthase), zen (zerknüllt).

Introduction

Insects comprise three quarters of all described animal species and their diversification represents an unparalleled episode in the course of evolution (Grimaldi & Engel, 2005; Zeh et al., 1989). Insects are among the earliest land animals and their exoskeleton preadapted them for terrestrial life. However, without the ability to oviposit on land, insects would have never been able to attain this incredible diversity (Zeh et al., 1989). In insect eggs, an extraembryonic membrane, the serosa, envelops the embryo and yolk (Schwalm, 1988). To investigate the possibility that the serosa is an evolutionary novelty in insects that protects the embryo against desiccation, we first explore the literature to examine the correlation between the humidity of the habitat and the presence of a serosa in the major arthropod groups: the chelicerates, the myriapods, the crustaceans and the hexapods (comprising entognaths and insects).

All major arthropod groups have colonized land to some extent. Chelicerates have been quite successful in terrestrial environments, because they evolved maternal adaptations to support terrestrial egg development (Beament, 1951; Foelix, 1996; Gillott, 2005; Grimaldi & Engel, 2005; Lees, 1948; Witaliñski, 1993): spiders wrap their eggs in silk, scorpions are viviparous, and mites develop elaborate maternal eggshells and wax layers. Eggs of the mite *Halotydeus destructor* have even been reported to develop inside the maternal body, which serves as a protective envelope after death (Norris, 1950). Chelicerates do not have an extraembryonic membrane that envelops the embryo (Figure 2-1a) (Anderson, 1973) (see Dearden et al. 2002, Mitmann and Wolff 2012, and Wolff and Hilbrant 2011 for more recent descriptions), although scorpion embryos have been reported to be covered by two extraembryonic membranes (Korschelt & Heider, 1936; Laurie, 1890; Rafiqi, 2008) (Figure 2-1a).

In contrast to the chelicerates, crustaceans are largely aquatic, myriapods mainly occur in tropical soil or leaf litter, and the Entognatha are restricted to humid conditions (Gillott, 2005; Grimaldi & Engel, 2005; Larink & Bilinski, 1989). It is striking that all these arthropod groups have a single extraembryonic membrane that covers the yolk dorsally, but never envelopes the embryo (Anderson, 1973) (see e.g. Brena and Akam 2012, Browne et al. 2005, Extavour, 2005, for more recent descriptions; Figure 2-1a). In insects, however, a serosa is present that completely enfolds the embryo (Machida, 2006; Machida & Ando, 1998; Rafiqi, 2008; Roth, 2004; Schwalm, 1988) (Figure 2-1a). Insects occupy all terrestrial habitats and have radiated into more than a million species (Grimaldi & Engel, 2005; Zeh et al., 1989). In some basal (apterygote) insects, the serosa does not close completely beneath the embryo (Jura, 1972). Consistent with this, variation exists in their desiccation resistance: most apterygotes live in leaf litter, under bark and other places with high humidity, but some are extremely resistant to desiccation (Chapter 5 in Gillott, 2005). In the pterygotes, the serosa completely envelopes the embryo. More than two thirds of all blastodermal cells will give rise to the serosa in most insects, suggesting an important role for this membrane (Roth, 2004).

Exceptionally, in a single group of higher flies (Schizophora), including *Drosophila*, a secondary reduction of the extraembryonic membranes to a single dorsal amnioserosa took place (Rafiqi et al., 2008; Schmidt-Ott, 2000) (Figure 2-1a). Eggs of these flies are generally deposited in rotting vegetable matter or moist soil, or plant or animal tissues (Colless & McAlpine, 1970; Ferrar, 1987; McAlpine, 1989). There are drosophilids that occur in dry habitats such as the Sonoran desert, but their eggs develop in necrotic tissue of cacti where humidity is higher than in the surrounding air at day and reaches over 90% relative humidity (RH) at night (Gibbs et al., 2003). *Drosophila melanogaster* eggs do not survive RH below 80 per cent (Al-Saffar et al., 1995). Overall, we find an intriguing correlation between the capacity of arthropod eggs to develop under dry conditions and the presence of a serosa that completely enfolds the embryo. Interestingly, the serosa secretes a chitinized cuticle underneath the maternal eggshell (Hinton, 1981). Since eggs of the mosquitoes *Aedes aegypti* and *Anopheles gambiae* gain

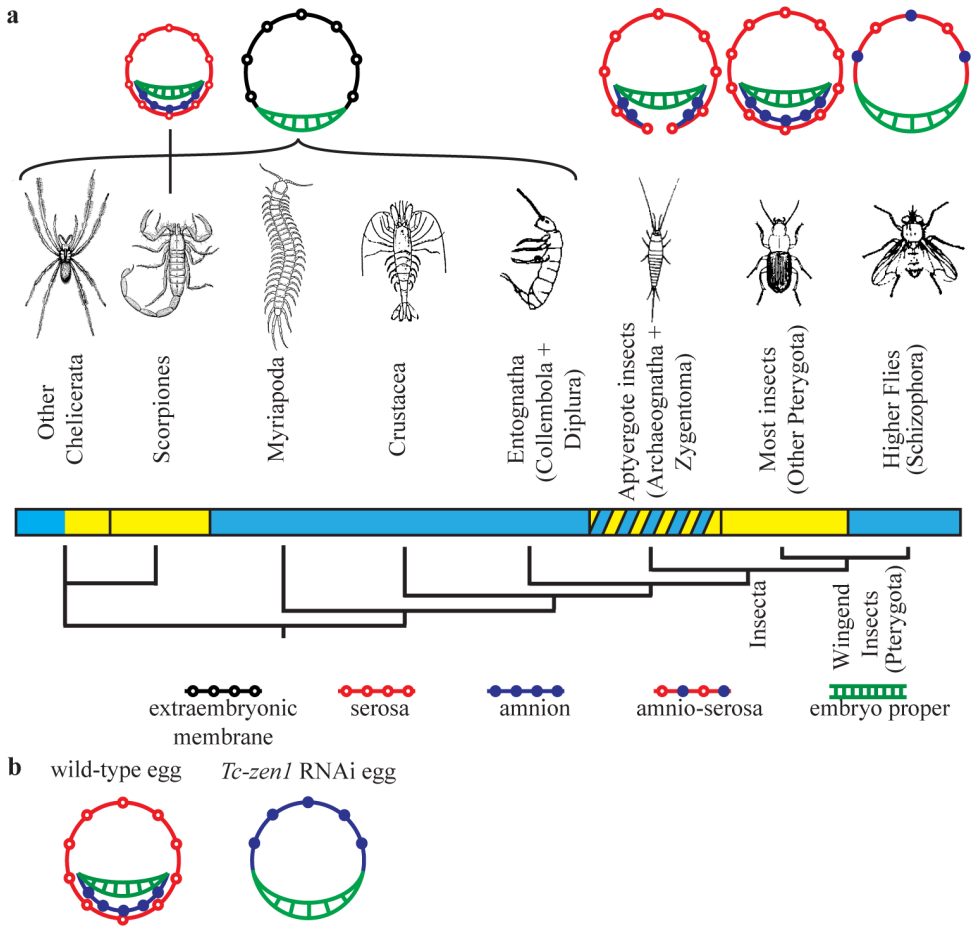


Figure 2-1: The serosa is an evolutionary novelty of insects. (a) Phylogeny of the main arthropod groups. The bar under the groups indicates whether species in this class are generally aquatic or restricted to humid environments for reproduction (blue), or terrestrial (yellow), or if species of this class live in very different environments concerning humidity (yellow diagonal stripes). Above the groups, schematic cross-section drawings of the embryo (green) and extraembryonic membranes are shown (open black circles, extraembryonic membrane; red open circles, serosa; blue closed circles, amnion). Jura (1972), Machida (2006) and Machida & Ando (1998) call the extraembryonic membrane in Entognatha a serosa. We, however, adopted the terminology of Anderson (1973). Although parallel evolution of two extraembryonic membranes took place in the scorpions, a serosa completely enveloping the embryo and secreting a cuticle is an evolutionary novelty of the insects. In the Schizophoran flies, a secondary reduction took place. (b) Schematic drawing of *Tribolium* wild-type and *Tc-zen1* RNAi development. In wild-type eggs, the serosa completely envelops yolk and embryo. After *Tc-zen1* RNAi, an amnion covers the yolk dorsally; the serosa is absent.

desiccation resistance at the time of serosal cuticle secretion, the serosal cuticle has been suggested to protect the developing embryo against desiccation (Goltsev et al., 2009; Rezende et al., 2008). However, no experimental evidence exists because it is impossible to physically remove the zygotic serosa or serosal cuticle in insects without affecting the overlying maternal eggshell, which consists of an exochorion, endochorion and vitelline membrane (Furneaux et al., 1969).

To investigate the hypothesis that the serosa protects the embryo against desiccation, we use RNAi in *Tribolium castaneum*. In this beetle, it is possible to prevent the development of the serosa without affecting the maternal eggshell using parental *Tc-zerknüllt1* (*Tc-zen1*) RNAi (van der Zee et al., 2005). In *Tc-zen1* RNAi eggs, a single amnion (the inner extraembryonic membrane that normally covers the embryo ventrally) covers the yolk dorsally and does not envelop the embryo (Figure 2-1b). This is similar to the reduced extraembryonic membrane in *Drosophila* (Figure 2-1a). *Zen* RNAi or mutations lead to lethal alterations of late morphogenetic movements in other insects, as does *Tc-zen2* RNAi in Tribolium (Panfilio, 2008; Panfilio et al., 2006; van der Zee et al., 2005; Wakimoto et al., 1984). *Tribolium Tc-zen1* RNAi eggs, however, can hatch under normal laboratory conditions (van der Zee et al., 2005). Thus, *Tribolium* provides a unique system to experimentally test our hypothesis. Finally, using *in situ* hybridization and RNAi, we investigate whether *Tc-chs1*, a key enzyme in cuticle synthesis (Arakane et al., 2004; Arakane et al., 2005; Arakane et al., 2008), is involved in serosal cuticle secretion and desiccation resistance of the egg.

Our results unravel a dual role for the serosa; first, a role in desiccation resistance mediated by the secreted serosal cuticle; and second, mediated by the serosal cells themselves, a role in dorsal closure, the process during which the lateral halves of the embryo meet dorsally and enclose the yolk (Panfilio, 2008).

Materials and methods

Molecular cloning and RNAi

The plasmid containing a 850 bp *Tc-zen1* fragment was obtained from Falciani et al. (1996). The 333 bp *Tc-chs1* fragment was cloned according to Arakane et al. (Arakane et al., 2008). Its dsRNA targets both splice variants of *Tc-chs1* (Arakane et al., 2008). Non-targeting control dsRNA was synthesized from a 500 bp vector sequence (pCRII, Invitrogen) cloned with the forward primer 5'-TGCCGGATCAAGAGCTACCAA-3' and the reverse primer 5'-TGTGAGCAAAAGGCCAGCAA-3'. dsRNA was synthesized using the MEGAscript RNAi kit (Ambion), and about 0.2 µl of a 0.5µg /µl dsRNA solution was injected into pupae of the San Bernardino strain, according to Bucher et al. (Bucher et al., 2002).

Hatching rate assays

Eggs were collected at 18-24 h old and put individually in a well of a 96 well plate, and incubated at 5, 20, 50, 65, 75 or 90 per cent RH and at 25°C, 30°C or 35°C (5° below, exactly at, and 5° above the temperature at which our laboratory stock is kept, respectively). Hatching rates were assayed after 4 days for 35°C, 5 days
for 30°C and 8 days for 25°C. These data points were repeated three to ten times, giving rise to standard errors such as in Figure 2-2a. Heat maps correlating hatching rates to humidity and temperature were generated using bivariate interpolation (Akima, 1978) in R v. 2.13.1 (R Development Core Team, 2009). Five per cent RH was obtained using silica gel; 20 per cent RH was obtained adjusting a KOH solution at the bottom of a glass desiccator (Winston & Bates, 1960). Relative humidities of 50, 65, 75 and 90 per cent were obtained in climate chambers. We excluded higher humidities to avoid condensation of liquid water. For the hatching rate assays, eggs were put into a 96 well plate with their chorion and adhering flour. To investigate the role of the exochorion, however, we removed

the exochorion by washing the eggs in 50 per cent bleach for 1 min, after which they were rinsed in water, dried at room temperature and incubated at 30°C at the different humidities for 5 days. All temperatures and humidities were constantly monitored using a MicroDAQ datalogger.

Embryo fixation, *in situ* hybridizations and immunohistochemistry

The *Tc-chs1* fragment used for *in situ* hybridization was cloned with the forward primer 5'-AACGACTTCATCTCGCACCAACACG-3' and the reverse primer 5'-AAATTGGCAGTTCCATGAGCCGG-3'. Embryo fixation and *in situ* hybridization were performed according to Shippy et al. (2008). Fluorescein isothiocyanate (FITC)-coupled wheat germ agglutinin (WGA) stainings were performed according to Rezende et al. (2008), where WGA is a lectin highly specific for N-acetylglucosamine polymers, therefore specifically labeling chitinous structures. For egg size measurements, 8 per cent formaldehyde in phosphate-buffered saline (PBS) was used for fixation, and the methanol shock was omitted to prevent shape changes.

Transmission Electron Microscopy

For electron microscopical preparations, both wild-type and *Tc-chs1* RNAi eggs were collected at 37 and 63 hours after egg lay (AEL) and fixed for 1 h at 30°C. Eggs were dechorionated and fixed in 5 ml heptane and 5 ml of a solution of 2.5 per cent gluteraldehyde, 2 per cent paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). After this initial fixation, eggs were removed from the solution, washed with 70 per cent ethanol to remove the heptane and fixed for another hour in 5 ml of 2.5 per cent gluteraldehyde, 2 per cent paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). After fixation, specimens were washed (3x 10 min) in cacodylate buffer and placed for 1 h in 1 per cent osmium tetroxide. 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.

Results

Serosa-less eggs are prone to desiccation

To experimentally test the hypothesis that the serosa protects against desiccation, we assayed hatching rates of serosa-less (*Tc-zen1* RNAi) eggs at various humidities and three temperatures (25°C, 30°C and 35°C). Since *Tc-zen1* is solely expressed in the serosa, we expect only serosa-related effects. The penetrance of the serosa-less phenotype (van der Zee et al., 2005) is higher than 95 per cent and this was constantly monitored in fixed material in parallel to the experiments. As a control, a non-targeting dsRNA was used (See Methods). In total, we analyzed over 40 000 eggs. At 35°C, wild-type eggs and control eggs show hatching rates of

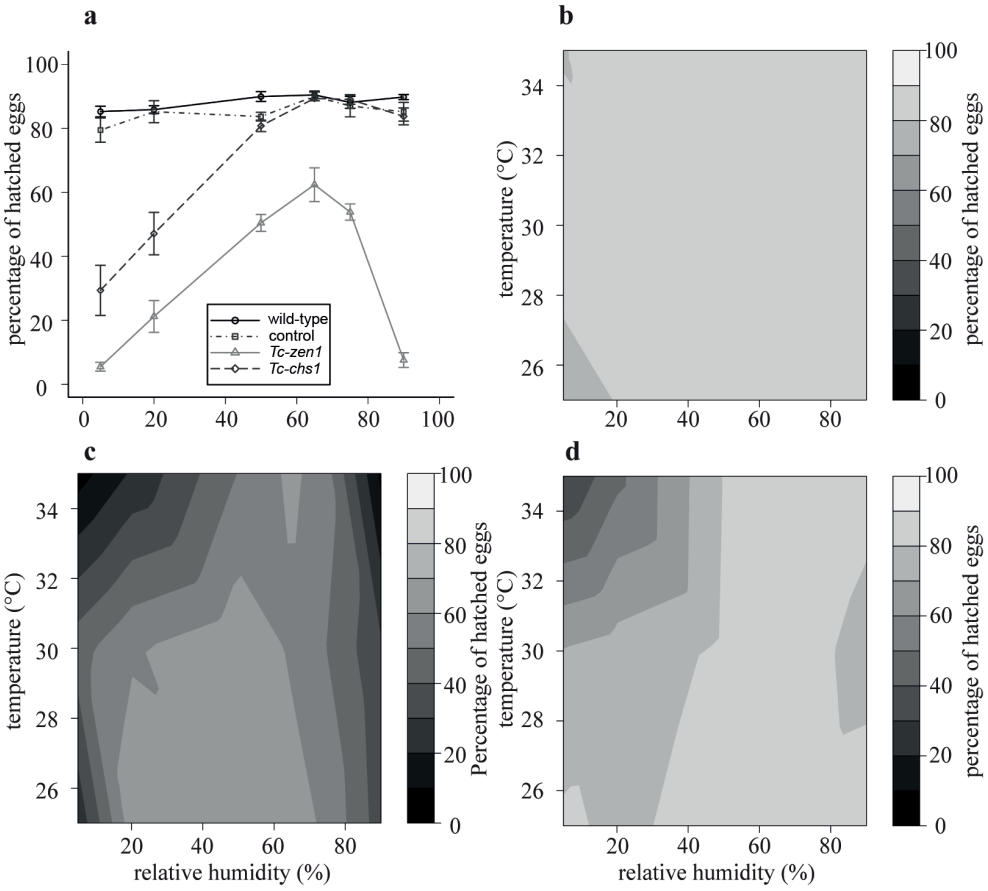


Figure 2-2: Eggs without a serosa or a serosal cuticle become desiccation-susceptible. (a) Hatching rates of control (grey squares), wild-type (black circles), *Tc-zen1* RNAi (grey triangles) and *Tc-chs1* RNAi (black diamonds) eggs at 35°C. Error bars indicate standard error among three to ten replicates of 96 eggs. (b–d) Heat maps summarizing hatching rates of (b) control eggs, (c) *Tc-zen1* RNAi eggs and (d) *Tc-chs1* RNAi eggs.

about 80 per cent in all humidities (Figure 2-2a). At this temperature, serosa-less eggs display lower hatching rates, with a peak average hatching rate of 62.4 per cent at 65 per cent RH (Figure 2-2a). In contrast to the control and wild-type eggs, hatching rates of the serosa-less eggs decrease dramatically to 5.5 per cent at 5 per cent RH. We display the hatching rates at all three temperatures and their interpolation in heat maps (Figure 2-2b,c). Hatching rates of control eggs and wild-type eggs are above 75 per cent for all conditions (Figure 2-2b and Figure S2-1). In strong contrast to control eggs, serosa-less eggs show dramatically decreased hatching rates in low humidities at all temperatures (Figure 2-2c). The effect is most pronounced at higher temperatures, when evaporation is maximal (Figure 2-2c). These data provide strong evidence that the serosa is crucial for desiccation resistance.

To verify that only the zygotic serosa, but not the maternal eggshell mediates desiccation resistance, we performed a hatching rate assay on eggs from which the

exochorion was removed by a bleach treatment. We found no significant differences in hatching rates between eggs with and without the exochorion (Figure S2-2). These data suggest that it is not the maternal eggshell that is required for desiccation resistance.

The serosa secretes a cuticle that protects against desiccation

To differentiate the effect of the serosal cells themselves and the secreted serosal cuticle, we investigated serosal cuticle formation in *Tribolium* using transmission electron microscopy (TEM). In wild-type eggs, a serosal cuticle with clear chitinous layers is present, as in the lepidopteran *Manduca sexta* (Lamer & Dorn, 2001) (Figure 2-3a). Next, we cloned *Tc-chitin-synthase1* (*Tc-chs1*, also called *TcCHS-A*), a key enzyme involved in *Tribolium* cuticle production (Arakane et al., 2004; Arakane et al., 2005; Arakane et al., 2008). During early development, we detected mRNA of this gene in the serosa, but not in the embryo (Figure 2-3c-f). After injection of 0.5 µg/µl *Tc-chs1* dsRNA in female pupae of the San Bernardino strain, we obtained eggs. In these eggs, the serosal cuticle was severely affected (Figure 2-3b). Amniotic and serosal cells were found, but the chitinous layers of the cuticle were absent. Thus, *Tc-chs1* pRNAi is an efficient method to disrupt serosal cuticle formation. We subjected *Tc-chs1* RNAi eggs to the same hatching rate assay as *Tc-zen1* RNAi eggs. In eggs without a serosal cuticle, hatching rates decreased at low humidities, similar to serosa-less eggs (Figure 2-2a,d). We conclude that it is mainly the cuticle secreted by the serosal cells that protects the egg against desiccation.

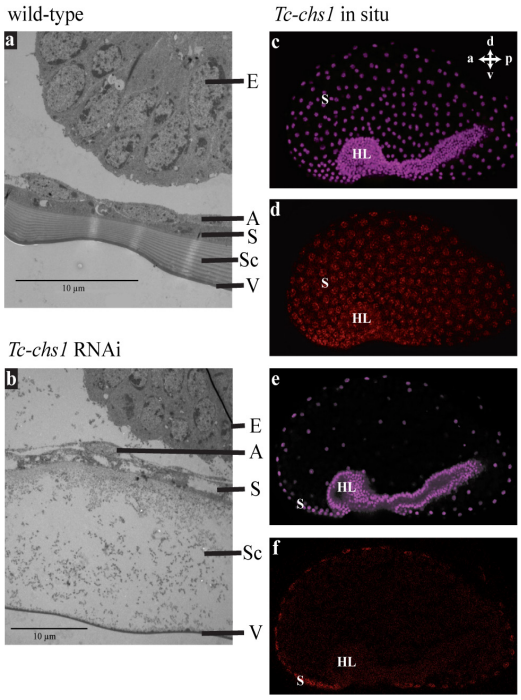


Figure 2-3: *Tribolium castaneum* produces a chitinized serosal cuticle. (a,b) TEM pictures of an approximately 37 h old (a) wild-type and (b) *Tc-chs-1* RNAi egg. Note the absence of the chitinous layers in the Sc after *Tc-chs1* RNAi. The vitelline membrane no longer sticks to the cuticle. The serosal cells might have a slightly aberrant appearance because they lost contact with the extracellular matrix, similar to the chitin-secreting epidermal cells in *Drosophila chs1 (kkv)* mutants (Moussian et al. 2005). (c) Nuclear DRAQ5 stain (purple) of the egg shown in (d). The serosal nuclei are widely spaced. Ventrally, the dense nuclei of the embryo proper (headlobes to the left) are prominently visible. (d) *Tc-chs1 in situ* hybridization during gastrulation. *Tc-chs1* mRNA (red) is detected around the serosal nuclei and not in the embryo. (e,f) A single confocal plane showing an optical cross-section of the embryo shown in (c) and (d). a, anterior; p, posterior; d, dorsal; v, ventral. HL, head lobes; E, embryo; A, amnion; S, serosa; Sc, serosal cuticle; V, vitelline membrane.

Arakane et al. (Arakane et al., 2008) report involvement of *Tc-chs1* in epidermal cuticle formation. We cannot exclude that the epidermal cuticle contributes to the desiccation resistance found in Figure 2-2d. However, TEM analysis shows that an epidermal cuticle is still not present when embryos are at least 65 h old (Figure 2-4h,i). The

larvae hatch when they are 82 h old. That means that the contribution of the epidermal cuticle to desiccation resistance must be relatively small. Furthermore, lactic acid digestions of *Tc-chs1* RNAi eggs suggest that larval cuticle secretion is hardly affected in our RNAi treatments. Insect cuticles resist digestion by lactic acid (Wieschaus & Nusslein-Volhard, 1986) and we find larval cuticles of normal appearance after lactic acid digestions of *Tc-chs1* RNAi eggs (Figure S2-4u). Together with the fact that our *Tc-chs1* RNAi eggs can hatch, this could mean that injection of 0.5 µg/µl *Tc-chs1* dsRNA in pupae of the San Bernardino strain affects the epidermal cuticle less than injection of 1 µg/µl in pupae of the Georgia strain (Arakane et al., 2008). Finally, chitin can be detected using WGA (Rezende et al., 2008; Wright, 1984). Staining of eggshells using WGA-FITC and digestions of eggs by lactic acid reveal the serosal cuticle in wild-type eggs, and confirm its absence after *Tc-chs1* and *Tc-zen1* RNAi (Figure S2-3, S2-4).

The serosa facilitates proper dorsal closure

Unexpectedly, hatching rates of serosa-less eggs not only decrease at low humidities, but also at relative humidities higher than 75 per cent (Figure 2-2a,c). A process important at these high humidities might be the uptake of water by osmosis. To assess water uptake, we measured the volume of eggs lacking a serosa or serosal cuticle using microscopy, and weighed single eggs and groups of eggs on an ultrafine balance that could measure one-tenth of a microgram. However, we could not detect any consistent increase (or slower decrease) in volume or weight for *Tc-zen1* or *Tc-chs1* RNAi eggs, compared with control eggs. Nevertheless, by measuring width and length of eggs at 65-75 h AEL, we could detect a consistent shape change at 90 per cent RH towards a more rounded shape for both *Tc-zen1* RNAi and *Tc-chs1* RNAi eggs (Figure 2-4a-d). At this time point, no epidermal cuticle is present yet (Figure 2-4h,i). The shape change at high humidity suggests an increase in internal pressure, and thus water uptake. This would mean that our weight measurements are not sensitive enough, and that both *Tc-zen1* RNAi and *Tc-chs1* RNAi eggs do take up water at high humidities.

We wondered whether this shape change affects dorsal closure. During this process, which occurs at the end of embryonic development, the extraembryonic membrane actively pulls the sides of the embryo over the yolk to close dorsally (Panfilio, 2008; Solon et al., 2009; van der Zee et al., 2005) (Figure S2-5). In order to assay dorsal closure, we analyzed fixed eggs a few hours before hatching. In *Tc-zen1* RNAi eggs, 24 per cent and 26 per cent of the embryos did not complete dorsal closure (n=93 and n=65) at 5 per cent and 50 per cent RH respectively (Figure S2-6). These proportions are not significantly different from each other (proportion test, $p > 0.05$ (Newcombe, 1998)). Importantly, the visible defects in dorsal closure (dorsal open phenotypes) for serosa-less eggs double to 52 per cent (n=54) at 90 per cent RH (Figure 2-4f, Figure S2-6). The occurrence of dorsal closure failure at 90 per cent RH was significantly higher than in 5 per cent RH (proportion test, $p < 0.05$) and than in 50 per cent RH (proportion test, $p < 0.01$).

We cannot exclude a severe delay in dorsal closure in *Tc-zen1* RNAi eggs. However, the number of embryos that do not close dorsally matches well the

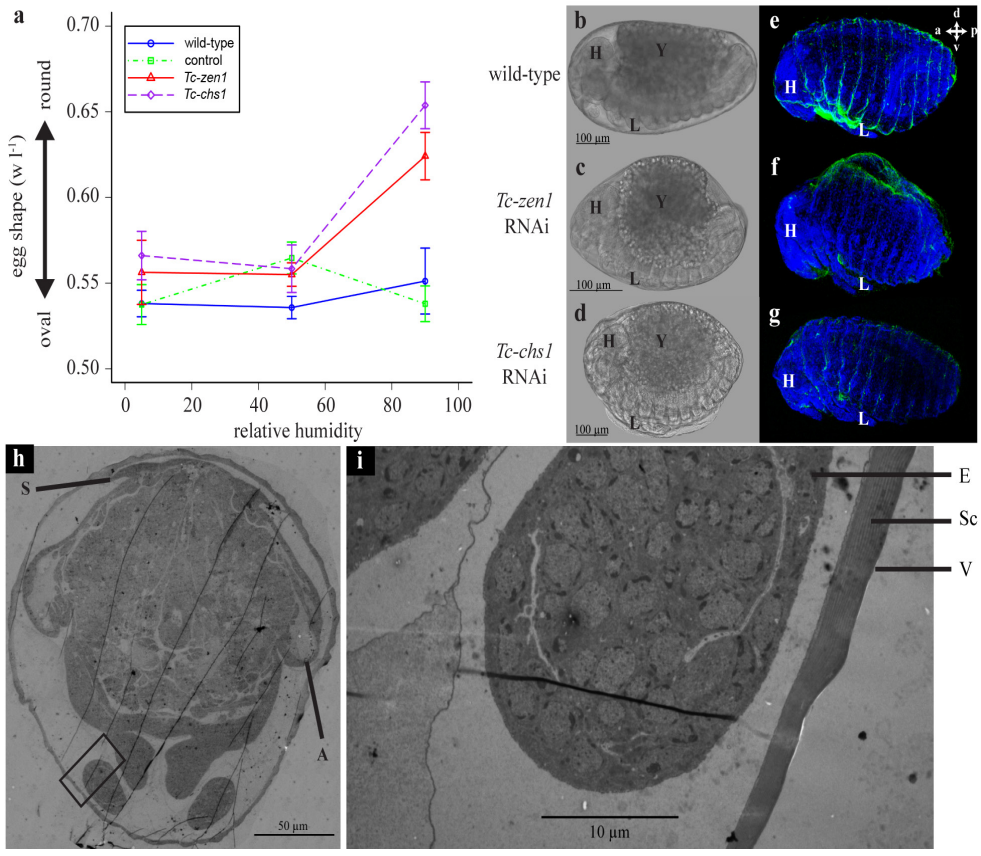


Figure 2-4: *Tc-zen1* RNAi embryos display defects in dorsal closure at high humidity. (a) Width/length ratio (1 = perfect sphere) of control (green squares), wild-type (blue circles), *Tc-zen1* RNAi (red triangles) and *Tc-chs1* RNAi (purple diamonds) eggs measured in their vitelline membrane using IMAGEJ (Schneider et al. 2012). (b–d) Phase contrast images of a 65–75 h old (b) wild-type, (c) *Tc-zen1* RNAi and (d) *Tc-chs1* RNAi embryo at 90% RH. (e–g) DAPI (blue) label cell nuclei and WGA-FITC (green) label chitin of the larval cuticle in (e) a dorsally closed wild-type embryo, (f) a *Tc-zen1* RNAi embryo that has not completed dorsal closure, and (g) a dorsally closed *Tc-chs1* RNAi embryo. a, anterior; p, posterior; d, dorsal; v, ventral. In (e), a small piece of vitelline membrane is stuck between the legs. (h) Overview TEM pictures of 65–75 h old wild-type egg during dorsal closure with dorsally condensed serosa. (i) Magnification of area boxed in (h). A layered serosal cuticle can be detected (Sc), but the serosa has condensed dorsally. No epidermal cuticle is found 65 h AEL. H, head; Y, yolk; L, legs; E, embryo; Sc, serosal cuticle; V, vitelline membrane; S, serosa; A, amnion.

number of embryos that do not hatch (Figure 2-2a), suggesting that dorsal closure fails and is not simply delayed at these humidities. We conclude that the decreased hatching rates of serosa-less eggs at high humidity are largely caused by an increased incidence of dorsal closure defects. The amnion that is present at the dorsal side of *Tc-zen1* RNAi eggs instead of the serosa (Figure 2-1b, Figure S2-5) might not apply sufficient pulling force for dorsal closure, especially in rounded eggs. Despite rounding up at high humidities, *Tc-chs1* RNAi eggs do not show defects in dorsal closure and hatch at the same rates as control eggs (Figure 2-2a,d and 2-4e,g). This means that the serosal cuticle normally prevents shape changes at higher humidities, possibly because of its rigidity, but that this function is not required for dorsal closure. Rounded eggs can perform dorsal closure provided that serosal

cells are present, which is the case in *Tc-chs1* RNAi eggs.

Discussion

We provided experimental evidence for a role for the serosa in desiccation resistance. *Tc-zen1* and *Tc-chs1* pRNAi provided an excellent approach to reveal this function. It should be noted, however, that an amniotic cavity (the space between embryo and amnion) is eliminated in *Tc-zen1* RNAi eggs, because of the ectopic dorsal amnion. We cannot exclude formally that the lack of an amniotic cavity causes increased desiccation sensitivity in these eggs. However, we do not think that this is the case since *Tc-chs1* RNAi eggs that do possess an amniotic cavity are prone to desiccation too. It should be noted that apart from chitin, the serosal cuticle is also comprises proteins, tyrosine-derived quinones responsible for sclerotization and lipids (Furneaux & McFarlane, 1965a, 1965b; Goltsev et al., 2009; McFarlane, 1960). Thus, in *Tc-chs1* RNAi eggs, the other components of the serosal cuticle are still produced. These other cuticular components in combination with intact serosal cells might protect slightly against desiccation and could provide an explanation for the higher viability of *Tc-chs1* RNAi eggs when compared to *Tc-zen1* RNAi eggs at low relative humidities (Figure 2-2a).

It is interesting that desiccation resistance is a zygotic investment. Although protective maternal eggshells are known from Chelicerata, the present work in *Tribolium* shows that, in insects, it is not the maternal eggshell that protects against desiccation, but the cuticle secreted by the zygotic serosa, as suggested before for mosquitoes (Rezende et al., 2008).

We also showed that dorsal closure is more robust in presence of serosal cells. In the context of dorsal closure, the serosa compacts into a condensed structure, the dorsal organ (Anderson, 1973; Panfilio, 2008) (Figure S2-5e). This quick condensation is probably required for proper dorsal closure in humid conditions. The actual final dorsal closure (that is, the joining of the two lateral sides of the embryo) takes place after degeneration of the serosa and must be mediated by the amnion. It could well be that the role of an extraembryonic membrane in dorsal closure was ancestral in arthropods (Machida, 2006; Machida & Ando, 1998). This membrane would then have differentiated into the amnion that mediates final dorsal closure, and into the serosa that was recruited to fold around the embryo and secrete a cuticle to mediate desiccation resistance.

Overall, non-insect terrestrial arthropods are generally restricted to cryptozoic environments and have undergone limited speciation (Zeh et al., 1989). Desiccation-resistant eggs must have been crucial for insect habitat expansion, as were the amniote egg and the seed for vertebrates and plants, respectively (Reisz, 1997; Stewart, 1983). Using *T. castaneum* as a model, we have demonstrated a critical role for the insect serosa in desiccation resistance. We propose that the origin of the serosa opened up a whole new range of terrestrial oviposition sites and facilitated the spectacular radiation of insects on land.

Acknowledgements

We thank Siegfried Roth and Bas Zwaan for ideas, Rodrigo Fonseca and Jessica Cande for technical advice, Agnieszka Doroszuk, Daniel Rozen, Julia Hunn, Michael Richardson, Paul Brakefield, Hans Slabbekoorn and Siegfried Roth for critically reading the manuscript, and Kees Koops for practical assistance. G.L.R. thanks Mike Levine for the opportunity to be introduced to *T. castaneum*. Part of this work was funded by the EU Network of Excellence LifeSpan (FP6 036894). G.L.R was supported by Faperj and CNPq; M.v.d.Z by NWO VENI grant no 863.09.014.

References

Akima, H. (1978). Bivariate Interpolation and Smooth Surface Fitting for Irregularly Distributed Data Points. ACM Transactions on Mathematical Software, 4(2), 160-164.

Al-Saffar, Z. Y., Grainger, J. N. R., & 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(5), 389-397.

Anderson, D. T. (1973). Embryology and phylogeny in annelids and arthropods. Oxford Pergamon Press.

Arakane, Y., Hogenkamp, D. G., Zhu, Y. C., Kramer, K. J., Specht, C. A., Beeman, R. W., . . . Muthukrishnan, S. (2004). Characterization of two chitin synthase genes of the red flour beetle, Tribolium castaneum, and alternate exon usage in one of the genes during development. Insect Biochemistry and Molecular Biology, 34(3), 291-304. doi: 10.1016/j.ibmb.2003.11.004

Arakane, Y., Muthukrishnan, S., Kramer, K. J., Specht, C. A., Tomoyasu, Y., Lorenzen, M. D., . . . Beeman, R. W. (2005). The Tribolium chitin synthase genes TcCHS1 and TcCHS2 are specialized for synthesis of epidermal cuticle and midgut peritrophic matrix. Insect Molecular Biology, 14(5), 453-463. doi: 10.1111/j.1365-2583.2005.00576.x

Arakane, Y., Specht, C. A., Kramer, K. J., Muthukrishnan, S., & Beeman, R. W. (2008). Chitin synthases are required for survival, fecundity and egg hatch in the red flour beetle, Tribolium castaneum. Insect Biochemistry and Molecular Biology, 38(10), 959-962. doi: 10.1016/j.ibmb.2008.07.006

Beament, J. W. L. (1951). The structure and formation of the egg of the fruit tree red spider mite, Metatetranychus ulmi Koch. Annals of Applied Biology, 38(1), 1-24. doi: 10.1111/j.1744-7348.1951.tb07787.x

Bucher, G., Scholten, J., & Klingler, M. (2002). Parental RNAi in Tribolium (Coleoptera). Current Biology, 12(3), R85-R86.

Colless, D. H., & McAlpine, D. K. (1970). Diptera. In D. F. Waterhouse (Ed.), The insects of Australia (pp. 656-740): Melbourne University Press.

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(16), 8479-8484.

Ferrar, P. (1987). A guide to the breeding habits and immature stages of Diptera Cyclorrhapha. Leiden: Brill u.a.

Foelix, R. F. (1996). Biology of Spiders (2nd ed.). Oxford: Oxford University Press.

Furneaux, P. J. S., James, C. R., & Potter, S. A. (1969). The Egg Shell of the House Cricket (Acheta Domesticus): An Electron-Microscope Study. Journal of Cell Science, 5(1), 227-249.

Furneaux, P. J. S., & McFarlane, J. E. (1965a). Identification, estimation, and localization of catecholamines in eggs of the house cricket, Acheta domesticus (L.). Journal of Insect Physiology, 11(5), 591-600. doi: 10.1016/0022-1910(65)90141-1

Furneaux, P. J. S., & McFarlane, J. E. (1965b). A possible relationship between the occurrence of catecholamines and water absorption in insect eggs. Journal of Insect Physiology, 11(5), 631-635. doi: 10.1016/0022-1910(65)90145-9

Gibbs, A. G., Perkins, M. C., & Markow, T. A. (2003). No place to hide: microclimates of Sonoran Desert Drosophila. Journal of Thermal Biology, 28(5), 353-362. doi: 10.1016/S0306-4565(03)00011-1

Gillott, C. (2005). Entomology (3rd ed.). Dordrecht: Springer.

Goltsev, Y., Rezende, G. L., Vranizan, K., Lanzaro, G., Valle, D., & Levine, M. (2009). Developmental and evolutionary basis for drought tolerance of the Anopheles gambiae embryo. Developmental Biology, 330(2), 462-470. doi: 10.1016/j.ydbio.2009.02.038

Grimaldi, D. A., & Engel, M. S. (2005). Evolution of the Insects: Cambridge University Press.

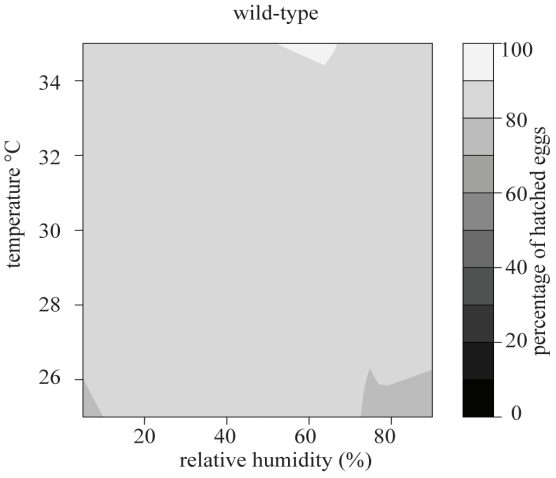
Hinton, H. E. (1981). The Biology of Insect Eggs. (1st ed.). Oxford: Pergamon Press.

Jura, C. (1972). Development of Apterygote Insects. In S. J. Counce & C. H. Waddington (Eds.), Developmental

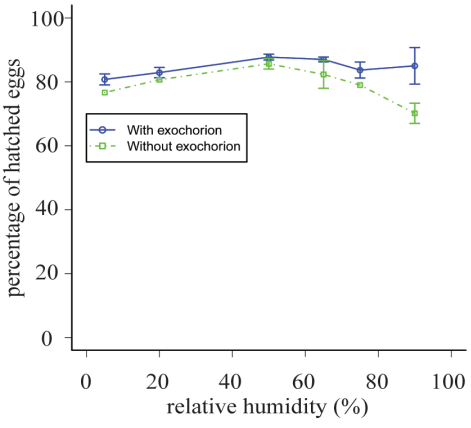
Systems: Insects (pp. 49-94). London: Academic Press Inc.
Korschelt, E., & Heider, K. (1936). Vergleichende Entwicklungsgeschichte der Tiere. New York: Fischer.
Lamer, A., & Dorn, A. (2001). The serosa of Manduca sexta (Insecta, Lepidoptera): ontogeny, secretory activity, structural changes, and functional considerations. Tissue & Cell, 33(6), 580-595. doi: 10.1054/tice.2001.0213
Larink, O., & Bilinski, S. M. (1989). Fine structure of the egg envelopes of one proturan and two Collembolan genera (apterygota). International Journal of Insect Morphology & Embryology, 18(1), 39-45. doi: 10.1016/0020-7322(89)90034-2
Laurie, M. (1890). The Embryology of a Scorpion (Euscorpius italicus). Quarterly Journal of Microscopical Science, 31(2), 105-142.
Lees, A. D. (1948). Passive and active water exchange through the cuticle of ticks. Discussions of the Faraday Society, 3, 187-192.
Machida, R. (2006). Evidence from embryology for reconstructing the relationships of hexapod basal clades. Arthropod Systematics & Phylogeny, 64(1), 95-104.
Machida, R., & Ando, H. (1998). Evolutionary Changes in Developmental Potentials of the Embryo Proper and Embryonic Membranes along with the Derivative Structures in Atelocerata, with Special Reference to Hexapoda (Arthropoda). Proc. Arthropod. Embryol. Soc. Jpn, 33, 1-13.
McAlpine, J. F. (1989). Manual of Nearctic Diptera: Research Branch, Agriculture Canada.
McFarlane, J. E. (1960). Structure and function of the egg shell as related to water absorption by the eggs of Acheta domesticus (L.). Canadian Journal of Zoology, 38(2), 231-241. doi: 10.1139/z60-029
Newcombe, R. G. (1998). Two-sided confidence intervals for the single proportion: comparison of seven methods. Stat Med, 17(8), 857-872.
Norris, K. R. (1950). The aestivating eggs of the Red-legged earth mite, Halotydeus destructor (Tucker). Bull Commonw sci indust Res Aust, No. 253, 1-26.
Panfilio, K. A. (2008). Extraembryonic development in insects and the acrobatics of blastokinesis. Developmental Biology, 313(2), 471-491. doi: 10.1016/j.ydbio.2007.11.004
Panfilio, K. A., Liu, P. Z., Akam, M., & Kaufman, T. C. (2006). Oncopeltus fasciatus zen is essential for serosal tissue function in katatrepsis. Developmental Biology, 292(1), 226-243. doi: 10.1016/j.ydbio.2005.12.028
R Development Core Team. (2009). R: A language and environment for statistical computing (Version 2.9.0). Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org
Rafiqi, A. M. (2008). Morphological transitions and the genetic basis of the evolution of extraembryonic tissues in flies. (Proefschrift Wageningen), s.n.], [S.l. Retrieved from http://edepot.wur.nl/122052
Rafiqi, A. M., Lemke, S., Ferguson, S., Stauber, M., & Schmidt-Ott, U. (2008). Evolutionary origin of the amnioserosa in cyclorrhaphan flies correlates with spatial and temporal expression changes of zen. Proceedings of the National Academy of Sciences of the United States of America, 105(1), 234-239. doi: 10.1073/pnas.0709145105
Reisz, R. R. (1997). The origin and early evolutionary history of amniotes. Trends in Ecology and Evolution, 12(6), 218-222. doi: 10.1016/s0169-5347(97)01060-4
Rezende, G. L., Martins, A. J., Gentile, C., Farnesi, L. C., Pelajo-Machado, M., Peixoto, A. A., & Valle, D. (2008). Embryonic desiccation resistance in Aedes aegypti: presumptive role of the chitinized Serosal Cuticle. Bmc Developmental Biology, 8(82). doi: 10.1186/1471-213x-8-82
Roth, S. (2004). Gastrulation in other insects. Gastrulation: From Cells to Embryos (pp. 105-121). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
Schmidt-Ott, U. (2000). The amnioserosa is an apomorphic character of cyclorrhaphan flies. Development Genes and Evolution, 210(7), 373-376.
Schwalm, F. E. (1988). Insect Morphogenesis. Basel: S. Karger AG.
Shippy, T. D., Ronshaugen, M., Cande, J., He, J. P., Beeman, R. W., Levine, M., . . . Denell, R. E. (2008). Analysis of the Tribolium homeotic complex: insights into mechanisms constraining insect Hox clusters. Development Genes and Evolution, 218(3-4), 127-139. doi: 10.1007/s00427-008-0213-4
Solon, J., Kaya-Çopur, A., Colombelli, J., & Brunner, D. (2009). Pulsed Forces Timed by a Ratchet-like Mechanism Drive Directed Tissue Movement during Dorsal Closure. Cell, 137(7), 1331-1342.
Stewart, W. N. (1983). Paleobotany and the Evolution of Plants. New York: Cambridge University Press.
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(7), 624-636.
Wakimoto, B. T., Turner, F. R., & Kaufman, T. C. (1984). Defects in embryogenesis in mutants associated with the antennapedia gene-complex of Drosophila melanogaster. Developmental Biology, 102(1), 147-172.
Wieschaus, E., & Nusslein-Volhard, C. (1986). Looking at embryos. In D. B. Roberts (Ed.), Drosophila: A Practical

Approach (pp. 199-227). Oxford: IRL Press.
Winston, P. W., & Bates, D. H. (1960). Saturated solutions for the control of humidity in biological research. Ecology, 41(1), 232-237.
Witaliński, W. (1993). Egg shells in mites: vitelline envelope and chorion in Acaridida (Acari). Experimental and Applied Acarology, 17(5), 321-344. doi: 10.1007/bf00058596
Wright, C. S. (1984). Structural comparison of the two distinct sugar binding sites in wheat germ agglutinin isolectin II. Journal of Molecular Biology, 178(1), 91-104. doi: 10.1016/0022-2836(84)90232-8
Zeh, D. W., Zeh, J. A., & Smith, R. L. (1989). Ovipositors, Amnions and eggshell architecture in the diversification of terrestrial arthropods. Quarterly Review of Biology, 64(2), 147-168.

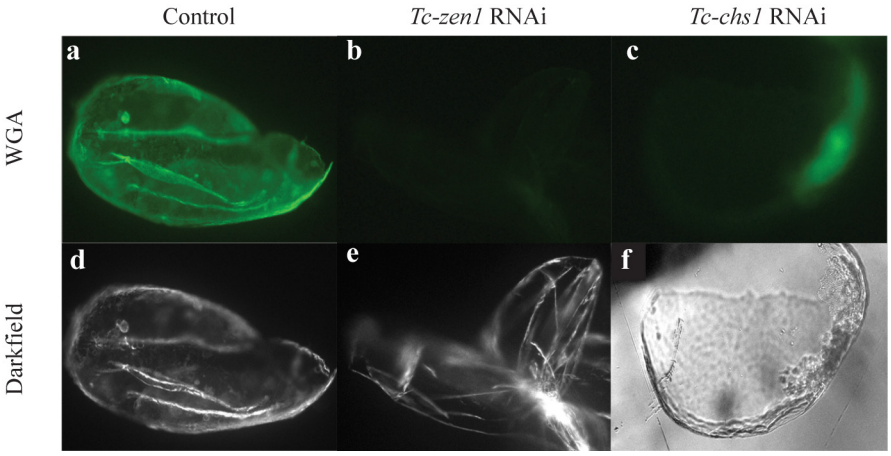
Supplementary information



Supplementary Figure 2-1: Wild-type hatching rates, heat map correlating hatching rates to temperature and humidity. Hatching rates are above 75% at all conditions.

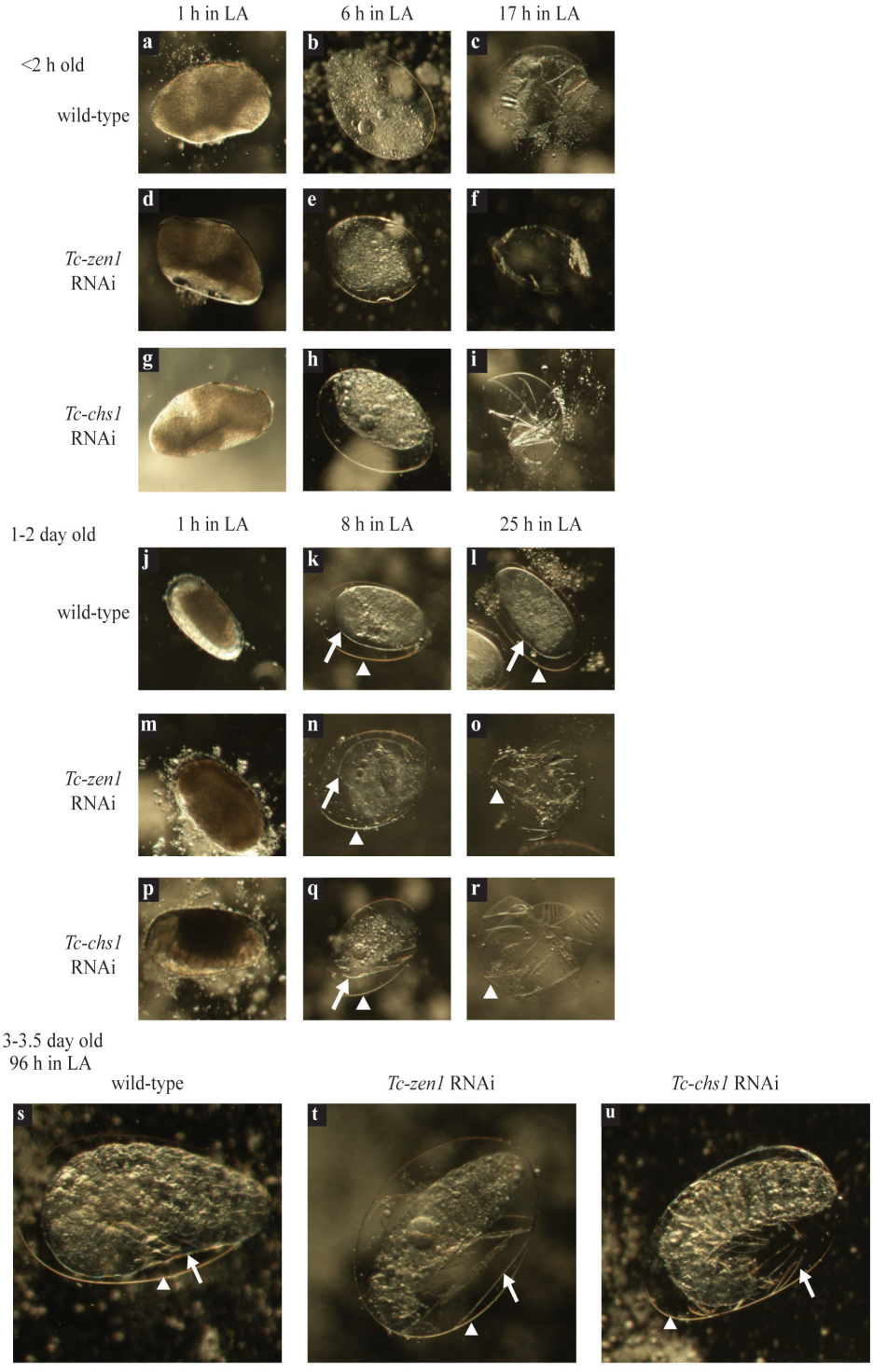


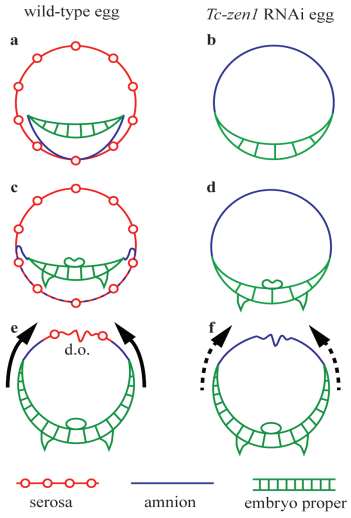
Supplementary Figure 2-2: The effect of the maternal exochorion. Plot of means showing the hatching rates of eggs with and without exochorion, error bars indicate standard error among 3-5 replicates of batches of 50 eggs. Eggs with 0.5-1 day old were washed for 1 minute with 50% household bleach. This treatment promptly removes the external exochorion (also named 'chorion' within the *Drosophila* field) while maintains the inner endochorion (named 'vitelline membrane' within the *Drosophila* field) intact (Furneaux and McFarlane, 1965).



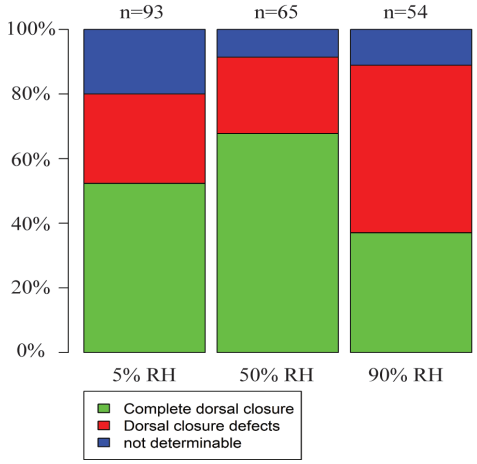
Supplementary Figure 2-3: WGA stain of wild-type, *Tc-zen1* RNAi and *Tc-chs1* eggs. (a-c) 2 day old eggshells stained with the FITC associated lectin WGA (green) detecting chitin [34]. (a) In control eggshells, a serosal cuticle is present. (b) After *Tc-zen1* RNAi and (c) after *Tc-chs1* RNAi, the serosal cuticle is absent. (d-e) darkfield images of the same eggshells as in a-c. (f) Phase contrast image of the same eggshell as in e revealing the imprints of the serosal cells that did not secrete chitin. Some aspecific staining is present along the margin in (c).

Supplementary Figure 2-4: (see next page) Lactic acid digestion of wild-type, *Tc-zen1* RNAi and *Tc-chs1* RNAi eggs. (a-i) 0-2h old eggs are digested completely after 17 h in lactic acid, because no serosal cuticle is present yet. (c,f,i) The outer chorion remains visible as a transparent membrane. (j-l) 1-2 day old wild-type eggs in lactic acid. (k,l) The inner parts of the egg are digested, but the outer chorion (arrow head) and the inner serosal cuticle secreted against the vitelline membrane (arrow) remain undigested. (m-o) *Tc-zen1* RNAi eggs in lactic acid. (n) The inner parts of the egg are digested, but the outer chorion (arrow head) and a soft inner vitelline membrane (arrow) are undigested after 8h in lactic acid. (o) Only the outer chorion (arrow head) remains undigested after 25h in lactic acid. No serosal cuticle is observed. (p-q) *Tc-chs1* RNAi eggs (q) The inner parts of the egg are digested, but the outer chorion (arrow head) and a soft inner vitelline membrane (arrow) are undigested after 8h in lactic acid. (r) Only the outer chorion (arrow head) remains undigested after 25h in lactic acid. No serosal cuticle is observed. (s) In 3 day old wild-type eggs, the outer chorion (arrow head), the inner vitelline membrane with serosal cuticle (arrow) and the embryonic cuticle remain undigested after 25h in lactic acid. (t) In 3 day old *Tc-zen1* RNAi eggs, the outer chorion (arrow head), and a soft inner vitelline membrane (arrow) remain undigested after 25h in lactic acid. An embryonic cuticle is visible. (u) In 3 day old *Tc-chs1* RNAi eggs, the outer chorion (arrow head), and a soft inner vitelline membrane (arrow) remain undigested after 25h in lactic acid. Importantly, following *Tc-chs1* RNAi, we confirmed absence of the serosal cuticle (r, arrowhead), but confirmed the presence of a normal embryonic cuticle (u, arrow).


1 h in LA
6 h in LA
17 h in LA
<2 h old
wild-type
Tc-zen1 RNAi
Tc-chs1 RNAi
1-2 day old
1 h in LA
8 h in LA
25 h in LA
wild-type
Tc-zen1 RNAi
Tc-chs1 RNAi
3-3.5 day old
96 h in LA
wild-type
Tc-zen1 RNAi
Tc-chs1 RNAi



Supplementary Figure 2-5: Schematic drawings of dorsal closure. Red open circles represent the serosa. Blue lines represent amnion. Green represents the embryo proper. (a, c, e) wild-type eggs. (b, d, f) *Tc-zen1* RNAi eggs. (a, c) The amnion and serosa start to intercalate. This intercalation proceeds towards the dorsal side. (b, d) The amnion covers the yolk at the dorsal side. (e) The intercalated amnion and serosa disappear. The remaining of the serosa forms the dorsal organ and pulls the sides of the embryo proper over the yolk (arrows). This leads to dorsal closure. (f) The amnion allows dorsal closure at medium and low humidity, but possibly does not apply pulling force to the sides of the embryo (dashed arrows). After Van der Zee et al., Curr. Biol. 15, 624-636 (2005). d.o. = dorsal organ



Supplementary Figure 2-6: Dorsal closure defects of *Tc-zen1* RNAi eggs at different humidities. Green represents complete dorsal closure. Red represents dorsal closure defects. Blue represents the undeterminable fraction, i.e. unfertilized eggs or severe earlier phenotypes. First bar: 24% of the embryos show dorsal closure defects at 5% RH. Second bar: 26 % of the embryos show dorsal closure defects at 50% RH. Third bar: 52% of the embryos show dorsal closure defects at 90% RH. The proportion of dorsal closure phenotypes between 5% RH and 50% RH is not significantly different (Proportion test, $p>0.05$) but the proportion of dorsal closure phenotypes in 90% RH is significantly higher than in 5% RH (Proportion test, $p<0.05$) and in 50% RH (Proportion test, $p<0.01$).

Chapter 3

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

Chris G.C. Jacobs, Nora Braak, Gerda E.M. Lamers, Maurijn van der Zee

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.

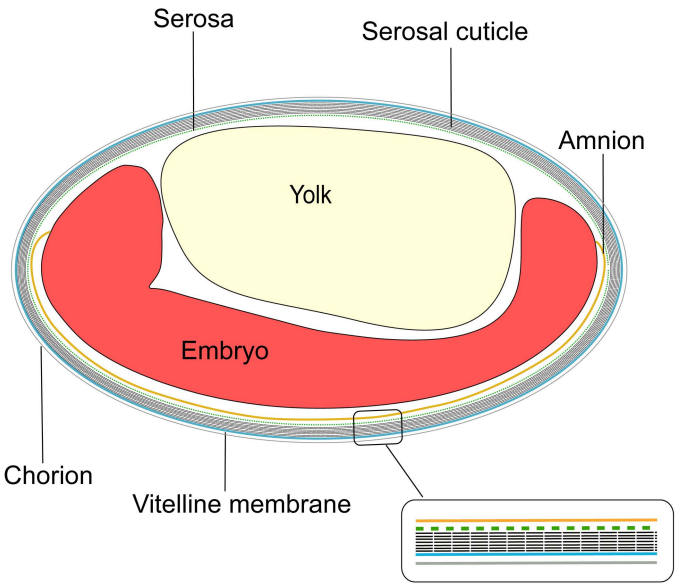


Figure 3-1: Schematic overview of the *Tribolium castaneum* egg. The *Tribolium castaneum* egg is surrounded by two maternal layers, the chorion and the vitelline membrane. Beneath these, the extraembryonic serosa secretes a chitinous cuticle (serosal cuticle). The serosa and serosal cuticle envelope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

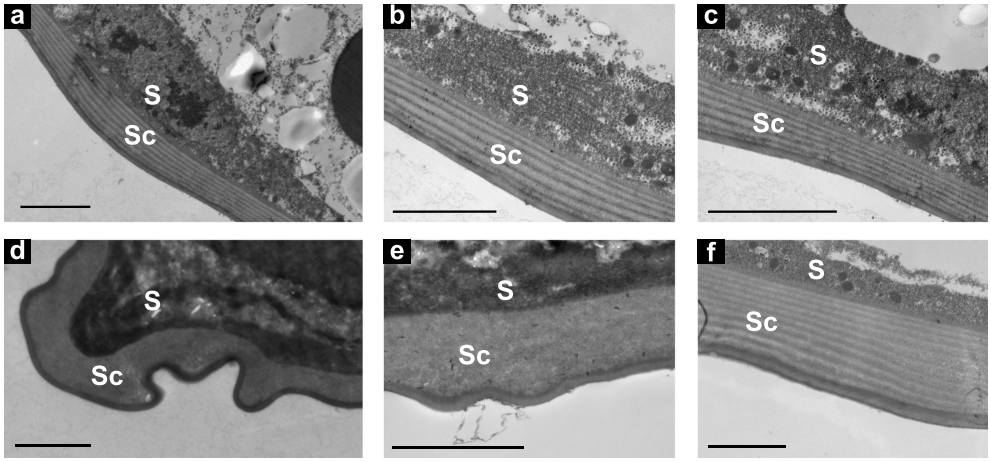


Figure 3-2: Transmission Electron Microscopy of the serosal cuticle. The serosal cuticle of wild-type eggs shows a clearly laminated structure (a-c). After knockdown of both *Tc-knk1* (d) and *Tc-rtv* (e), this laminar organization is completely lost. Knockdown of *Tc-lac2* (f) however, shows no structural 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.

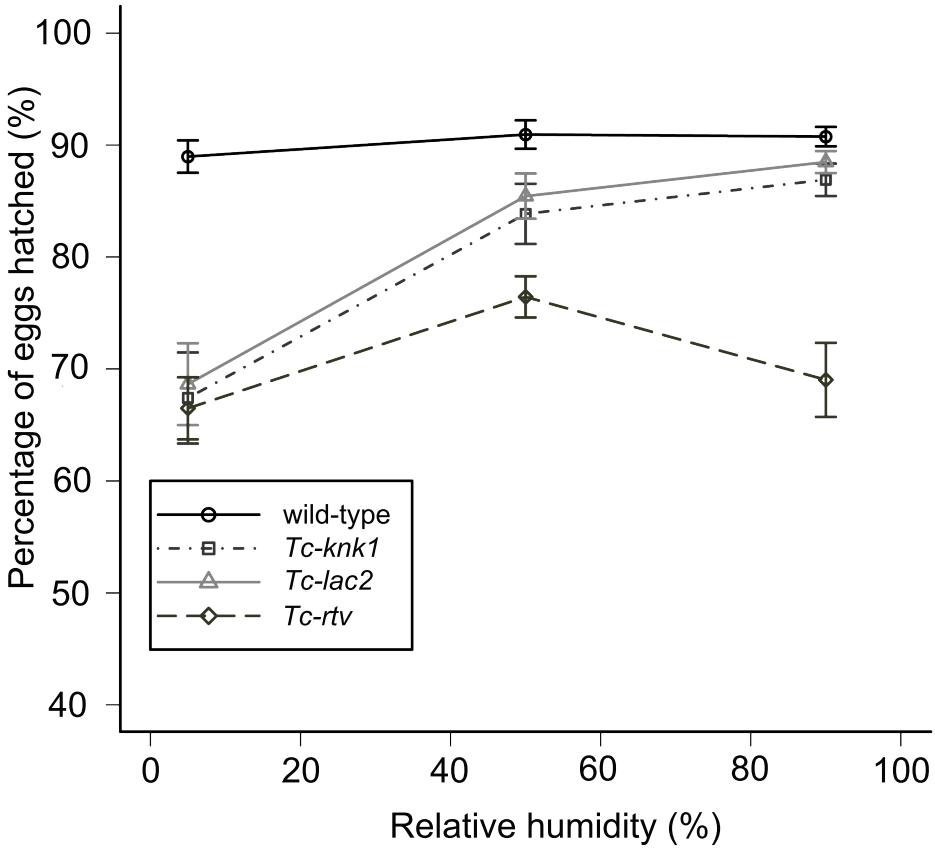


Figure 3-3: Survival at different humidities of the *T. castaneum* egg. Eggs with an altered serosal cuticle become desiccation-susceptible. Hatching rates of wild-type (circles), *Tc-knk1* RNAi (squares), *Tc-lac2* RNAi (triangles), and *Tc-rtv* RNAi (diamonds) eggs at 35°C. Error bars indicate standard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

References

Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. Genome Biology, 11(10), R106.

Arakane, Y., Hogenkamp, D. G., Zhu, Y. C., Kramer, K. J., Specht, C. A., Beeman, R. W., . . . Muthukrishnan, S. (2004). Characterization of two chitin synthase genes of the red flour beetle, Tribolium castaneum, and alternate exon usage in one of the genes during development. Insect Biochemistry and Molecular Biology, 34(3), 291-304. doi: 10.1016/j.ibmb.2003.11.004

Arakane, Y., Muthukrishnan, S., Beeman, R. W., Kanost, M. R., & Kramer, K. J. (2005a). Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. Proceedings of the National Academy of Sciences of the United States of America, 102(32), 11337-11342. doi: 10.1073/pnas.0504982102

Arakane, Y., Muthukrishnan, S., Kramer, K. J., Specht, C. A., Tomoyasu, Y., Lorenzen, M. D., . . . Beeman, R. W. (2005b). The Tribolium chitin synthase genes TcCHS1 and TcCHS2 are specialized for synthesis of epidermal cuticle and midgut peritrophic matrix. Insect Molecular Biology, 14(5), 453-463. doi: 10.1111/j.1365-2583.2005.00576.x

Arakane, Y., Specht, C. A., Kramer, K. J., Muthukrishnan, S., & Beeman, R. W. (2008). Chitin synthases are required for survival, fecundity and egg hatch in the red flour beetle, Tribolium castaneum. Insect Biochemistry and Molecular Biology, 38(10), 959-962. doi: 10.1016/j.ibmb.2008.07.006

Barrett, T., Wilhite, S. E., Ledoux, P., Evangelista, C., Kim, I. F., Tomashevsky, M., . . . Soboleva, A. (2013). NCBI GEO: archive for functional genomics data sets—update. Nucleic Acids Research, 41(D1), D991-D995. doi: 10.1093/nar/gks1193

Bucher, G., Scholten, J., & Klingler, M. (2002). Parental RNAi in Tribolium (Coleoptera). Current Biology, 12(3), R85-R86.

Charles, J. P. (2010). The regulation of expression of insect cuticle protein genes. Insect Biochemistry and Molecular Biology, 40(3), 205-213. doi: 10.1016/j.ibmb.2009.12.005

Chaudhari, S. S., Arakane, Y., Specht, C. A., Moussian, B., Boyle, D. L., Park, Y., . . . Muthukrishnan, S. (2011). Knickkopf protein protects and organizes chitin in the newly synthesized insect exoskeleton. Proceedings of the National Academy of Sciences. doi: 10.1073/pnas.1112288108

Chaudhari, S. S., Arakane, Y., Specht, C. A., Moussian, B., Kramer, K. J., Muthukrishnan, S., & Beeman, R. W. (2013). Retroactive Maintains Cuticle Integrity by Promoting the Trafficking of Knickkopf into the Procuticle of Tribolium castaneum. PLoS Genet, 9(1), e1003268. doi: 10.1371/journal.pgen.1003268

Chaudhari, S. S., Moussian, B., Specht, C. A., Arakane, Y., Kramer, K. J., Beeman, R. W., & Muthukrishnan, S. (2014). Functional Specialization Among Members Of Knickkopf Family Of Proteins In Insect Cuticle Organization. PLoS Genet, 10(8), e1004537. doi: 10.1371/journal.pgen.1004537

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., & Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics, 21(18), 3674-3676. doi: 10.1093/bioinformatics/bti610

Dixit, R., Arakane, Y., Specht, C. A., Richard, C., Kramer, K. J., Beeman, R. W., & Muthukrishnan, S. (2008). Domain organization and phylogenetic analysis of proteins from the chitin deacetylase gene family of Tribolium castaneum and three other species of insects. Insect Biochemistry and Molecular Biology, 38(4), 440-451. doi: 10.1016/j.ibmb.2007.12.002

Furneaux, P. J. S., & McFarlane, J. E. (1965a). Identification, estimation, and localization of catecholamines in eggs of the house cricket, Acheta domesticus (L.). Journal of Insect Physiology, 11(5), 591-600. doi: 10.1016/0022-1910(65)90141-1

Furneaux, P. J. S., & McFarlane, J. E. (1965b). A possible relationship between the occurrence of catecholamines and water absorption in insect eggs. Journal of Insect Physiology, 11(5), 631-635. doi: 10.1016/0022-1910(65)90145-9

Goltsev, Y., Rezende, G. L., Vranizan, K., Lanzaro, G., Valle, D., & Levine, M. (2009). Developmental and evolutionary basis for drought tolerance of the Anopheles gambiae embryo. Developmental Biology, 330(2), 462-470. doi: 10.1016/j.ydbio.2009.02.038

Hinton, H. E. (1981). The Biology of Insect Eggs. (1st ed.). Oxford: Pergamon Press.

Ioannidou, Z. S., Theodoropoulou, M. C., Papandreou, N. C., Willis, J. H., & Hamodrakas, S. J. (2014). CutProtFam-Pred: Detection and classification of putative structural cuticular proteins from sequence alone, based on profile Hidden Markov Models. Insect Biochemistry and Molecular Biology, 52(0), 51-59. doi: http://dx.doi.org/10.1016/j.ibmb.2014.06.004

Jacobs, C. G. C., Rezende, G. L., Lamers, G. E. M., & van der Zee, M. (2013). The extraembryonic serosa protects the insect egg against desiccation. Proceedings of the Royal Society B: Biological Sciences, 280(1764). doi: 10.1098/rspb.2013.1082

Jacobs, C. G. C., & van der Zee, M. (2013). Immune competence in insect eggs depends on the extraembryonic serosa. Developmental & Comparative Immunology, 41(2), 263-269. doi: http://dx.doi.org/10.1016/j.dci.2013.05.017

Jasrapuria, S., Arakane, Y., Osman, G., Kramer, K. J., Beeman, R. W., & Muthukrishnan, S. (2010). Genes encoding proteins with peritrophin A-type chitin-binding domains in Tribolium castaneum are grouped into three distinct families based on phylogeny, expression and function. Insect Biochem Mol Biol, 40(3), 214-227. doi: 10.1016/j.ibmb.2010.01.011

Jasrapuria, S., Specht, C. A., Kramer, K. J., Beeman, R. W., & Muthukrishnan, S. (2012). Gene Families of Cuticular Proteins Analogous to Peritrophins (CPAPs) in Tribolium castaneum Have Diverse Functions. PLoS ONE, 7(11), 1-15.

Lamer, A., & Dorn, A. (2001). The serosa of Manduca sexta (Insecta, Lepidoptera): ontogeny, secretory activity, structural changes, and functional considerations. Tissue & Cell, 33(6), 580-595. doi: 10.1054/tice.2001.0213

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25(4), 402-408. doi: 10.1006/meth.2001.1262

Lord, J. C., 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(2), 219-221. doi: http://dx.doi.org/10.1016/j.mimet.2009.12.007

McFarlane, J. E. (1960). Structure and function of the egg shell as related to water absorption by the eggs of Acheta domesticus (L.). Canadian Journal of Zoology, 38(2), 231-241. doi: 10.1139/z60-029

Merzendorfer, H. (2006). Insect chitin synthases: a review. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 176(1), 1-15. doi: 10.1007/s00360-005-0005-3

Moussian, B. (2010). Recent advances in understanding mechanisms of insect cuticle differentiation. Insect Biochemistry and Molecular Biology, 40(5), 363-375. doi: 10.1016/j.ibmb.2010.03.003

Moussian, B. (2013). The apical plasma membrane of chitin-synthesizing epithelia. Insect Science, 20(2), 139-146. doi: 10.1111/j.1744-7917.2012.01549.x

Moussian, B., Soding, J., Schwarz, H., & Nusslein-Volhard, C. (2005). Retroactive, a membrane-anchored extracellular protein related to vertebrate snake neurotoxin-like proteins, is required for cuticle organization in the larva of Drosophila melanogaster. Developmental Dynamics, 233(3), 1056-1063. doi: 10.1002/dvdy.20389

Moussian, B., Tang, E., Tonning, A., Helms, S., Schwarz, H., Nusslein-Volhard, C., & Uv, A. E. (2006). Drosophila Knickkopf and Retroactive are needed for epithelial tube growth and cuticle differentiation through their specific requirement for chitin filament organization. Development, 133(1), 163-171. doi: 10.1242/dev.02177

Panfilio, K. A. (2008). Extraembryonic development in insects and the acrobatics of blastokinesis. Developmental Biology, 313(2), 471-491. doi: 10.1016/j.ydbio.2007.11.004

R Development Core Team. (2009). R: A language and environment for statistical computing (Version 2.9.0). Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org

Rezende, G. L., Martins, A. J., Gentile, C., Farnesi, L. C., Pelajo-Machado, M., Peixoto, A. A., & Valle, D. (2008). Embryonic desiccation resistance in Aedes aegypti: presumptive role of the chitinized Serosal Cuticle. Bmc Developmental Biology, 8(82). doi: 10.1186/1471-213x-8-82

Roth, S. (2004). Gastrulation in other insects. Gastrulation: From Cells to Embryos (pp. 105-121). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

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(7), 624-636.

Vargas, H. C. M., Farnesi, L. C., Martins, A. J., Valle, D., & Rezende, G. L. (2014). Serosal cuticle formation and

distinct degrees of desiccation resistance in embryos of the mosquito vectors Aedes aegypti, Anopheles aquasalis and Culex quinquefasciatus. Journal of Insect Physiology, 62(0), 54-60. doi: http://dx.doi.org/10.1016/j.jinsphys.2014.02.001

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics, 13, 134. doi: 10.1186/1471-2105-13-134

Zhu, Q., Arakane, Y., Beeman, R. W., Kramer, K. J., & Muthukrishnan, S. (2008). Functional specialization among insect chitinase family genes revealed by RNA interference. Proceedings of the National Academy of Sciences, 105(18), 6650-6655. doi: 10.1073/pnas.0800739105

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*

Chapter 4

Immune competence in insect eggs depends on the extraembryonic serosa

Chris G.C. Jacobs, Maurijn van der Zee

Published in Developmental & Comparative Immunology (2013) 41 (2) 263-269,
doi: 10.1016/j.dci.2013.05.017

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).

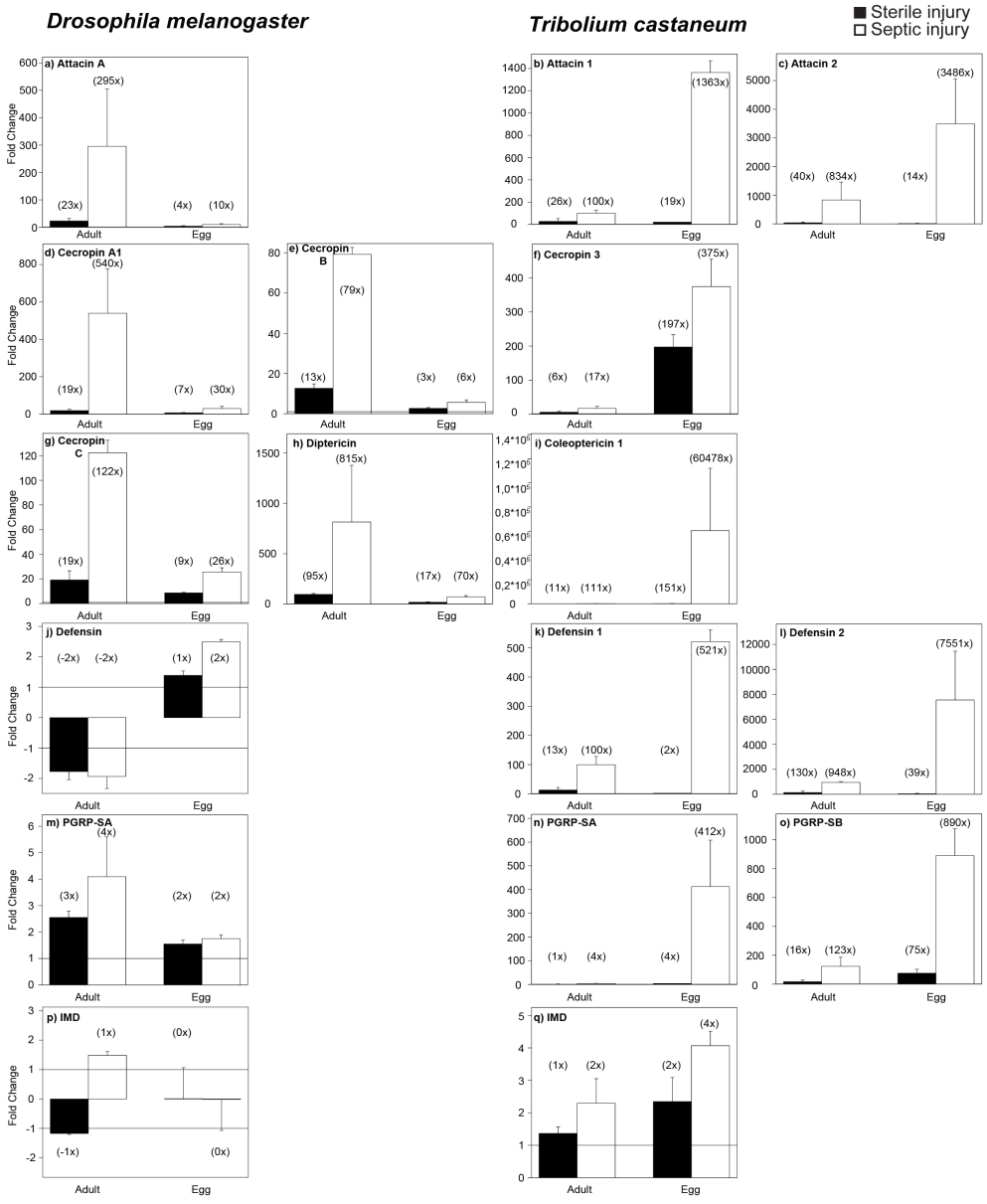


Figure 4-1: The expression of immune genes in adults and eggs of *D. melanogaster* and *T. castaneum*. Plotted is the mean fold change (in parenthesis) plus standard error based on two biological replicates (each replicate is the mean of two technical replicates). Clear induction of immune genes is visible 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.

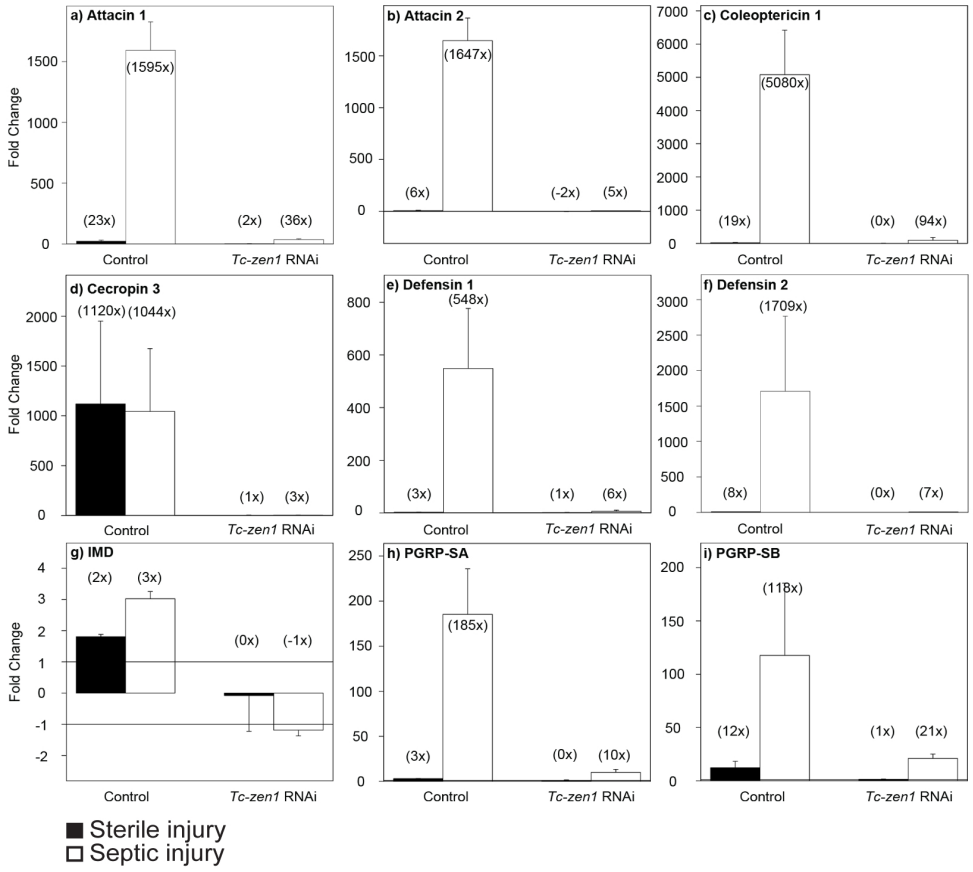


Figure 4-2: The expression of immune genes in the *T. castaneum* egg depends on the serosa. Plotted is the mean fold change (in parenthesis) plus standard error based on two biological replicates (each replicate is the mean of two technical replicates). When control eggs are infected a clear upregulation 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

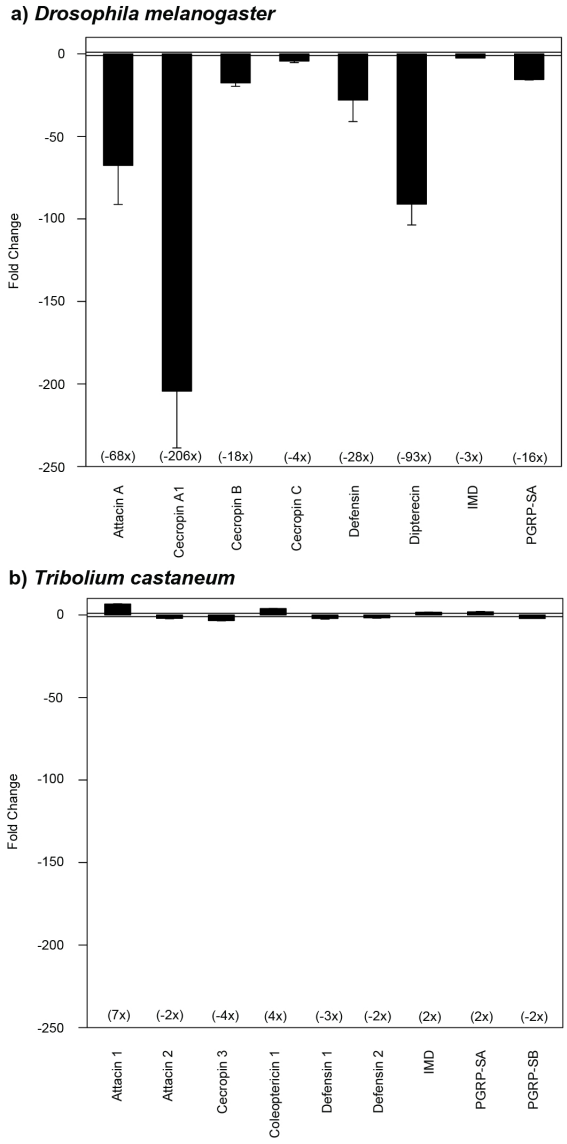


Figure 4-3: The expression of immune genes after septic injury in *D. melanogaster* and *T. castaneum* eggs as compared to adult expression. Plotted is the mean fold change (in parenthesis) plus standard error based on two biological replicates (each replicate is the mean of two technical replicates). (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.

Acknowledgements

We thank Peter Steenbergen, Arjan Kemp and Ruben Vijverberg for their help with the qRT-PCR experiment, Dominique Ferrandon for providing *E. coli* and *M. luteus*. We also thank Jelle Zandveld and Bas Zwaan for providing the *D. melanogaster* stock.

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.

Chapter 5

The extraembryonic serosa is a frontier epithelium providing the insect egg with a full-range innate immune response

Chris G.C. Jacobs, Herman P. Spaink and Maurijn van der Zee

eLife - accepted for publication

Drosophila larvae and adults possess a potent innate immune response, but the response of _Drosophila_ eggs is poor. In contrast to _Drosophila_, eggs of the beetle _Tribolium_ are protected by a serosa, an extraembryonic epithelium that is present in all insects except higher flies. Here, we test a possible immune function of this frontier epithelium using _Tc-zen1_ RNAi-mediated deletion. First, we show that bacteria propagate twice as fast in serosa-less eggs. Then, we compare the complete transcriptomes of wild-type, control RNAi and _Tc-zen1_ RNAi eggs before and after sterile or septic injury. Infection induces genes involved in Toll and IMD-signaling, melanisation, production of reactive oxygen species and antimicrobial peptides in wild-type eggs, but not in serosa-less eggs. Finally, we demonstrate constitutive and induced immune gene expression in the serosal epithelium using _in situ_ hybridization. We conclude that the serosa provides insect eggs with a full-range innate immune response.

Keywords: RNA sequencing; Tribolium castaneum; innate immunity; serosa; NF-kappaB; Toll

Introduction

To combat infection, insects rely on humoral and local immune responses. The humoral immune response is characterized by the massive secretion of antimicrobial peptides into the hemolymph and is mainly exerted by the fat body. Epithelia and hemocytes play the main role in local immune defenses that comprise melanisation, local AMP production, phagocytosis and encapsulation (Davis & Engstrom, 2012; Ferrandon, 2013; Ganesan et al., 2011; Lemaitre & Hoffmann, 2007; Ligoxygakis, 2013; Wang et al., 2014). The mechanisms regulating these innate immune responses have largely been uncovered with the aid of genetic and molecular studies in the fruit fly *Drosophila melanogaster.* When microbes invade the fly, their released peptidoglycans are sensed by peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) leading to the activation of the main immune signaling pathways. The mesodiaminopimelic acid-type (DAP-type) peptidoglycans of Gram-negative bacteria activate the IMD pathway, whereas the Lys-type peptidoglycans of Gram-positive bacteria activate the Toll pathway. The activation of the Toll-pathway is mediated by a proteolytic cascade of serine proteases leading to the cleavage of the cytokine Spätzle, the ligand of the transmembrane receptor Toll. Activation

of the immune signaling pathways leads to nuclear localization of the NF-kappaB factors Dorsal, Dif or Relish that induce antimicrobial peptides (AMPs). Other upregulated genes are prophenoloxidases (proPOs which mediate melanisation) and dual oxidase (DUOX which produces reactive oxygen species).

Drosophila has been extremely helpful uncovering those mechanisms, but research in other insects, such as the mealworm beetle *Tenebrio molitor* has also generated insightful results (see Park et al., 2010). The biochemical details of pathway activation, for instance, have mainly been unraveled using this beetle (see Park et al., 2010 for review). With the availability of tools such as RNAseq and RNAi, more insect species are being established as model organism for innate immunity research (Altincicek & Vilcinskas, 2007; Gerardo et al., 2010; Johnston et al., 2013; Johnston & Rolff, 2013; Waterhouse et al., 2007; Zhu et al., 2013). In particular the red flour beetle (*Tribolium castaneum*), has received much attention in innate immune studies (Altincicek et al., 2013; Altincicek et al., 2008; Behrens et al., 2014; Contreras et al., 2013; Milutinović et al., 2013; Roth et al., 2010; Zhong et al., 2013; Zou et al., 2007). Comparative genome analysis has revealed that components of intracellular immune signaling pathways (Toll, IMD and JAK/STAT) in *Drosophila* are 1:1 conserved in *Tribolium* (Zou et al., 2007). The RNAi knockdown technology has shown that the IMD and Toll pathway are largely functionally conserved (Shrestha & Kim, 2010; Yokoi et al., 2012a; Yokoi et al., 2012b). Their activity does, however, not strictly depend on either Gram-negative or Gram-positive bacteria (Yokoi et al., 2012a; Yokoi et al., 2012b), but this distinction is also not completely black and white in *Drosophila* (Leone et al., 2008; Leulier et al., 2003). Nevertheless, species-specific family expansion and sequence divergence in the PGRP and AMP families indicate species-specific differences, possibly required for effective recognition and elimination of evolving pathogens (Altincicek et al., 2008; Christophides et al., 2002; Park et al., 2010; Zou et al., 2007).

Not only larvae and adults, but also insect eggs are constantly threatened by pathogens (see Blum & Hilker, 2008; Kellner, 2008 for review). *Serratia* bacteria, for instance, have been found inside eggs of corn earworms and corn borers (Bell, 1969; Lynch et al., 1976) and can infect eggs in the laboratory (Sikorowski et al., 2001). We have also shown that *Serratia* infection leads to reduced egg survival in the burying beetle *Nicrophorus vespilloides* (Jacobs et al., 2014). Maternal investments have been proposed to counter microbial infections. Female medflies, for example, cover their eggs with antimicrobial secretions (Marchini et al., 1997) and in the absence of maternal care, eggs of earwigs die of fungal infection (Boos et al., 2014). Two studies focusing on transgenerational immune priming, however, have shown that the antimicrobial activity of eggs is of internal origin (Sadd & Schmid-Hempel, 2007; Zanchi et al., 2012). This is often implicitly interpreted as maternal loading of antimicrobials into the egg (Moreau et al., 2012), but maternal transfer of bacteria to the eggs also leaves zygotic investment as possibility (Freitak et al., 2014; Trauer & Hilker, 2013). Overall, it is ecologically relevant to gain a better understanding of the immune system in insect eggs.

The zygotic response in *Drosophila* eggs, however, seems poor. It is not until late stage 15, (one of the latest stages in development when ectoderm and trachea have differentiated) that eggs show up to 25-fold upregulation of antimicrobial peptides (Tan et al., 2014). This is incomparable to the upregulation in adult flies that is at least an order of magnitude larger. Except for Cecropin (Tingvall et al., 2001), stage 11 embryos do not show

any induction of antimicrobial peptides, and cannot contain an infection of non-pathogenic bacteria, leading to reduced survival (Tan et al., 2014). In strong contrast, we have shown that eggs of *Tribolium* which were not even half way during development could upregulate several AMPs to levels comparable to the adult (Jacobs & van der Zee, 2013). This upregulation depends on the serosa, an extraembryonic epithelium that envelopes yolk and embryo (Jacobs & van der Zee, 2013). This membrane is present in all insects, but was lost in a small group of higher Diptera (the Schizophora) to which *Drosophila* belongs (Rafiqi et al., 2008; Schmidt-Ott, 2000). Although two maternal extracellular coverings, the chorion and the vitelline membrane envelop the insect egg, the serosa is the first cellular epithelium surrounding the egg at the interface between the microbe rich external milieu on the one side and the yolk and embryo at the other side. Thus, the serosa could function as an immune competent barrier epithelium. This has been suggested before, as the NF-kappaB factor Dorsal is highly expressed in the presumptive serosa (Chen et al., 2000). The absence of the serosa might account for the poor immune response in *Drosophila* eggs.

To gain deeper insights into the role of the serosa, we chose *Tribolium castaneum*, a beetle that possesses a serosa, like all non-Schizophoran insects. In this beetle, we can prevent development of the serosa by parental RNA interference with *Tc-zerknüllt1* (*Tc-zen1*). This technique generates *Tribolium* eggs with an amnion at the dorsal side, but without a serosa (van der Zee et al., 2005). At the relative humidity of the air of the laboratory, normal larvae hatch from these eggs (Jacobs et al., 2013). As *Tc-zen1* is only expressed in the early serosa (van der Zee et al., 2005) and is not expressed anymore by the time the experiments are performed (see discussion), we expect only to find effects that are a consequence of the absence of the serosa. We investigated growth of bacteria in serosa-less and wild-type eggs, sequenced the whole transcriptome of naive and immune-challenged eggs with and without a serosal epithelium and confirmed constitutive and induced gene expression in the serosa by *in situ* hybridization. We conclude that the serosa is a frontier epithelium that provides immune competence to the insect egg.

Results

Bacteria propagate twice as fast in serosa-less eggs

To examine the influence of the serosa on bacterial growth in infected eggs, and to standardize our infection method, we counted colony forming units (cfu's) directly after infection (t=0) and 6 hours later (t=6) (Figure 5-1). We pricked 24-40h old eggs (i.e. up to half-way during development) with a tungsten needle dipped in a concentrated mix of *Escherichia coli* and *Micrococcus luteus* cultures (see Materials and Methods). To determine cfu's, we shortly treated eggs with 0.5% hypochlorite to sterilize the outside. Untreated eggs did hardly contain bacteria that grow on LB agar plates (on average 3 cfu's were found). Sterile injury did not increase this number (Figure 5-1, lower lines). In contrast, septic injury introduced on average 53 bacteria into wild-type eggs and 49 into serosa-less eggs. These numbers increased on average to 747 cfu's in wild-type eggs and to 7260 cfu's in serosa-less eggs. When we use the formula $N_{(t)} = N_{(0)} * e^{kt}$, the specific bacterial growth rate k in wild-type eggs is 0.44 h-1, whereas k = 0.83 h-1 in serosa-less eggs. This means that bacteria grow twice as fast in serosa-less eggs and suggests that the serosa exerts an immune function.

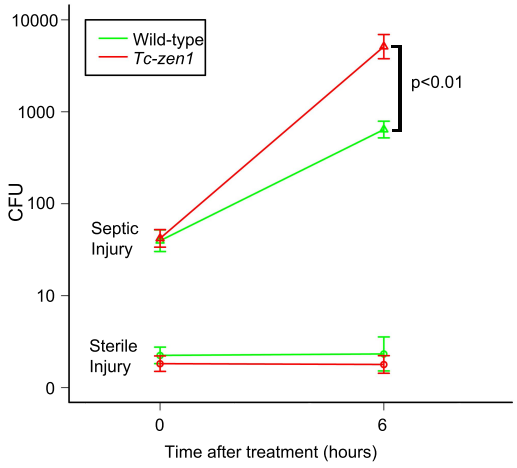


Figure 5-1: Counts of colony forming units (cfu's) after sterile and septic injury. Green lines represent bacterial growth in wild-type eggs. Red lines represent bacterial growth in *Tc-zen1* RNAi (serosa-less) eggs. Sterile injury did not introduce bacteria (lower lines: average of 2 cfu's found at t=0, and an average of 5 cfu's found at t=6). Septic injury introduced on average 53 bacteria into wild-type eggs and 49 into serosa-less eggs. These numbers increased to 747 +/- 106 cfu's in wild-type eggs (green upper line) and to 7260 +/- 1698 cfu's in serosa-less eggs (red upper line) at t=6. This means that bacteria propagate twice as fast in serosa-less eggs ($p<0.01$, as determined by a Pearson's chi-square test). Suspensions of 10 eggs were used per LB agar plate (see Materials and Methods), and 10 plates were analyzed per treatment and time point, giving rise to the error bars presented in the graph (standard error).

RNAseq reveals a full-range immune response in *Tribolium* eggs

To characterize this immune function, we sequenced the whole transcriptome of wild-type eggs, *Tc-zen1* RNAi (serosa-less) eggs and control RNAi eggs without injury, after sterile injury and after septic injury (Figure 5-2). The control RNAi consists of a injection of a 500bp dsRNA derived from a vector sequence without target in the *Tribolium castaneum* genome. For these 9 different treatments, three biological replicates were carried out (independent RNAi, independent injury) giving a total of 27 samples (Figure 5-2). Illumina next generation sequencing resulted in over 970 million cDNA reads with over 49 billion bp sequence information. Approximately 72% of the reads could be mapped to *Tribolium* gene models built on the 3.0 genome assembly (Richards et al., 2008) (Supplementary Table 5-1). We found expression of 14,903 of the total of 16,541 predicted genes, of which 13,464 genes were expressed in wild-type, control and *Tc-zen1* eggs and 1440 genes were expressed in a subset of these treatments.

Table 5-1: Number of differentially expressed immune genes in *Tribolium castaneum* eggs (Green = induction ; Red = repression)

	Wild-type Sterile injury		**Wild-type** Septic injury		**Control** Sterile injury		**Control** Septic injury		***Tc-zen1*** Sterile injury		***Tc-zen1*** Septic injury	
Microbial recognition	4	1	7	2	6	0	8	3	1	0	0	0
Extracellular signal transduction and modulation	27	6	32	10	33	4	34	10	4	5	2	0
Intracellular transduction pathways (Toll/IMD/JNK/JAK-STAT)	2	1	3	2	2	2	6	3	3	2	2	1
Execution / Stress	12	0	20	2	16	4	24	7	5	2	3	1
Total	**45**	**8**	**62**	**16**	**57**	**10**	**72**	**23**	**13**	**9**	**7**	**2**
	up	**down**	**up**	**down**	**up**	**down**	**up**	**down**	**up**	**down**	**up**	**down**

a

b

	Naive eggs	Sterile Injury	Septic Injury
Wild-type	3x	3x	3x
Control RNAi	3x	3x	3x
Tc-zen1 RNAi	3x	3x	3x

Figure 5-2: Experimental setup a) We collected eggs from wild-type, control RNAi and *Tc-zen1* RNAi beetles overnight. These eggs were incubated for 24h at 30°C to ensure development of the serosa. Eggs are then maximally 40h old, while total developmental time is close to 85h at 30°C. Eggs were pricked with a sterile needle (sterile injury), pricked with a mix of *E. coli* and *M. luteus* (septic injury), or remained untreated (naive). They were incubated for another 6 hours at 30°C before total RNA was extracted for RNAseq. To analyze the immune response, the transcriptomes of sterilely injured eggs and of septically injured eggs were compared to naive eggs. This was done for wild-type, control and *Tc-zen1* RNAi eggs. b) We collected 3 biological samples for each combination of eggtype (wild-type, Control RNAi or *Tc-zen1* RNAi) and treatment (naive, sterile injury or septic injury) giving a total of 27 biological samples.

First, we identified the immune-responsive genes by determining differential expression of genes between naive eggs on the one hand and sterilely injured eggs or septically injured eggs on the other hand. We only considered genes with at least a 2-fold change in expression and an adjusted p-value smaller than 0,01. This gave a total of 415 differentially expressed genes in sterilely injured eggs compared to the naive eggs, and a total of 538 differentially regulated genes in septically injured eggs compared to naive eggs. This shows that *Tribolium* eggs possess an extensive transcriptional response upon infection.

To obtain a global impression of the kind of genes differentially regulated upon infection in wild-type and control RNAi eggs, we assigned gene ontology terms (GO-terms) to all *Tribolium* genes. As no GO-term annotation is available for *Tribolium*, we blasted *Tribolium* genes against *Drosophila* and used the *Drosophila* GO-terms of the best hit. Using the Wallenius approximation (Young et al., 2010), we found several highly overrepresented GO-term categories with a p-value below 0.001 in both wild-type eggs (Figure 5-3a) and control RNAi eggs (Figure 5-3b). The overrepresented categories are mostly immune related. This indicates that our approach does not depend on artefacts generated by pricking eggs (e.g. delayed development), but mainly identifies genes involved in the innate immune response.

To obtain a more detailed analysis of the immune response in wild-type and control eggs, we focused on 368 genes that have been annotated as immune genes (Altincicek et al., 2013; Zou et al., 2007) (Supplementary files 4-9, available online). Of these genes, 78 were differentially regulated in wild-type eggs upon septic injury (Table 5-1 and Supplementary Table 5-3 and file 5), while 95 immune genes were differentially regulated in control RNAi eggs (Table 5-1 and Supplementary Table 5-3 and file 7). This

indicates that RNAi itself leads to an increased number of differentially regulated genes upon bacterial challenge, but, more importantly, shows that *Tribolium* eggs possess an elaborate immune response. In the following sections, we take a closer look at the exact genes involved in this extensive immune response.

Recognition of microbes and extracellular signal transduction

Of the 7 predicted peptidoglycan recognition proteins (PGRPs) in *Tribolium* we found significant induction of *PGRP-LA*, *LC*, *SA* and *SB* (Supplementary Table 5-2). Of these PGRPs, *PGRP-SA* and *SB* were induced over 200-fold (Figure 4a, Supplementary Table 5-2). Thus, it could be that these PGRPs rather function as effectors digesting Gram-positive bacteria, as shown for human *PGRP-S* (Dziarski et al., 2003). At least *PGRP-SB* shows all the amino acid residues characteristic for catalytic PGRPs (Kim et al., 2003). No induction was found for *PGRP-LE* and *LD*. These findings strongly resemble the response of *Tribolium* adults, in which the same PGRPs responded to infection (Altincicek et al., 2013). Of the Gram-negative binding proteins (GNBP), we found induction of *GNBP2* and *GNBP3* (Supplementary Table 5-2). In *Tribolium* adults and in *Drosophila*, however, only *GNBP3* is immune-inducible (Altincicek et al., 2013; Lemaitre & Hoffmann, 2007), whereas *GNBP1* and *GNBP3* are immune-inducible in *Tenebrio* (Johnston et al., 2013).

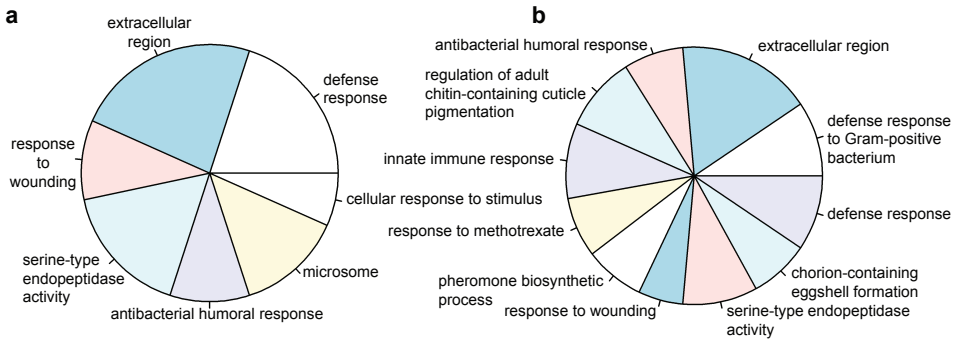


Figure 5-3: Types of genes that are differentially regulated a) Significantly over-represented GO-terms in the genes induced in wild-type eggs after septic injury ($p<0.001$). b) Significantly over-represented GO-terms in the genes induced in control RNAi eggs after septic injury ($p<0.001$). These categories indicate that the detected differential regulation does not result from artefacts induced by treatments (such as death or delayed development) and show that *Tribolium* eggs display an elaborate immune response.

Thioester-containing proteins (TEPs) have also been suggested to function as pattern recognition proteins, possibly targeting microbes for phagocytosis (Stroschein-Stevenson et al., 2005; Wang & Wang, 2013). We did not find induction of thioester-containing proteins (TEPs) but rather repression, for instance of *TEP-D* (Supplementary Table 5-2). This is surprising, since TEPs are upregulated in *Tribolium* larvae and adults (Altincicek et al., 2013; Behrens et al., 2014); and *Drosophila* (Stroschein-Stevenson et al., 2005; Wang & Wang, 2013). Similar to *Drosophila*, however, we did find induction of a putative TEP/complement-binding receptor-like protein (*LpR2*). We also found induction of *C-type lectin 6* and repression of *C-type lectin 1* and *13*. These lectins are thought to be involved in microbial recognition but no induction or repression has been found in *Drosophila* or *Tribolium* adults (Altincicek et al., 2013; De Gregorio et al., 2001).

The serine proteases and serpins have significantly expanded in number in *Tribolium* (Zou et al., 2007), similar to *Anopheles* (Christophides et al., 2002). Interestingly, most of them seem to be functional in the immune response as we found induction of 36 serine proteases and serpins, and repression of another 10 upon infection (Supplementary Table 5-2). This number is much higher than previously reported for adults (Altincicek et al., 2013). Of the Spaetzle ligands, we found induction of *spz1* and *spz2* and repression of *spz4* and *5* (Supplementary Table 5-2). In larvae and adults, however, different Spaetzles were induced or repressed, indicating specific use at different stages of the life cycle (Altincicek et al., 2013; Behrens et al., 2014).

In total, 51 of the 78 immune genes that are differentially regulated in wild-type eggs are involved in bacterial recognition and extracellular signal transduction, showing the prominence of these extracellular processes in the modulation of the immune response of the *Tribolium* egg.

Transmembrane and intracellular signal transduction

We found induction of several intracellular signaling components of the Toll, IMD and JNK pathways (Figure 5-4a, Supplementary Table 5-2) upon immune-challenge of *Tribolium* eggs. This suggests that these pathways are largely functionally conserved between *Drosophila* and *Tribolium*, although we could hardly detect expression of *dredd*, the endoprotease that cleaves Relish for nuclear translocation. Similar to larvae and adults (Altincicek et al., 2013, Behrens et al., 2014), JAK-STAT pathway components were not differentially regulated. Interestingly, we found significant upregulation of the *toll3* receptor upon infection. This was also found in larvae and adults (Altincicek et al., 2013; Behrens et al., 2014) and suggests that it is not *toll1*, but *toll3* that plays a major role in the innate immune response of *Tribolium*.

Execution mechanisms

As expected, we found the highest induction amongst the antimicrobial peptides. We detected generally more than 500-fold upregulation of defensins, attacins, coleoptericins, cecropins and thaumatin (Figure 5-4a, Supplementary Table 5-2). This means that *Tribolium* eggs can induce AMPs to comparable levels as larvae and adults (Altincicek et al., 2013; Behrens et al., 2014). We also found upregulation of *prophenoloxidase1* (*proPO1*), a gene involved in melanisation, and of *heme peroxidase 11*, a dual oxidase (*DUOX*) ortholog involved in the production of reactive oxygen species (Supplementary Table 5-2). This shows that *Tribolium* eggs are indeed able to respond with the full complement of immune defense mechanisms.

Currently, 19 AMPs are recognized in *Tribolium*, based on homology with known AMPs. However, due to the presence of species-specific AMPs and extreme sequence diversity of these molecules, homology based searches have probably missed several AMPs (Yang et al., 2011; Zou et al., 2007). AMPs are generally small (less than 30 kDa), cationic, hydrophobic and possibly have high glycine and/or proline content (Bulet & Stöcklin, 2005; Philippe Bulet et al., 2004). Based on the antimicrobial peptide database (Wang et al., 2009), many proteins encoded in the *Tribolium* genome fulfil those criteria and are identified as candidate antimicrobial peptides. Using our RNA sequencing data, however, we could select those candidate proteins that exhibit at least a two-fold induction upon infection. Based on these criteria, we found 20 potential new AMPs

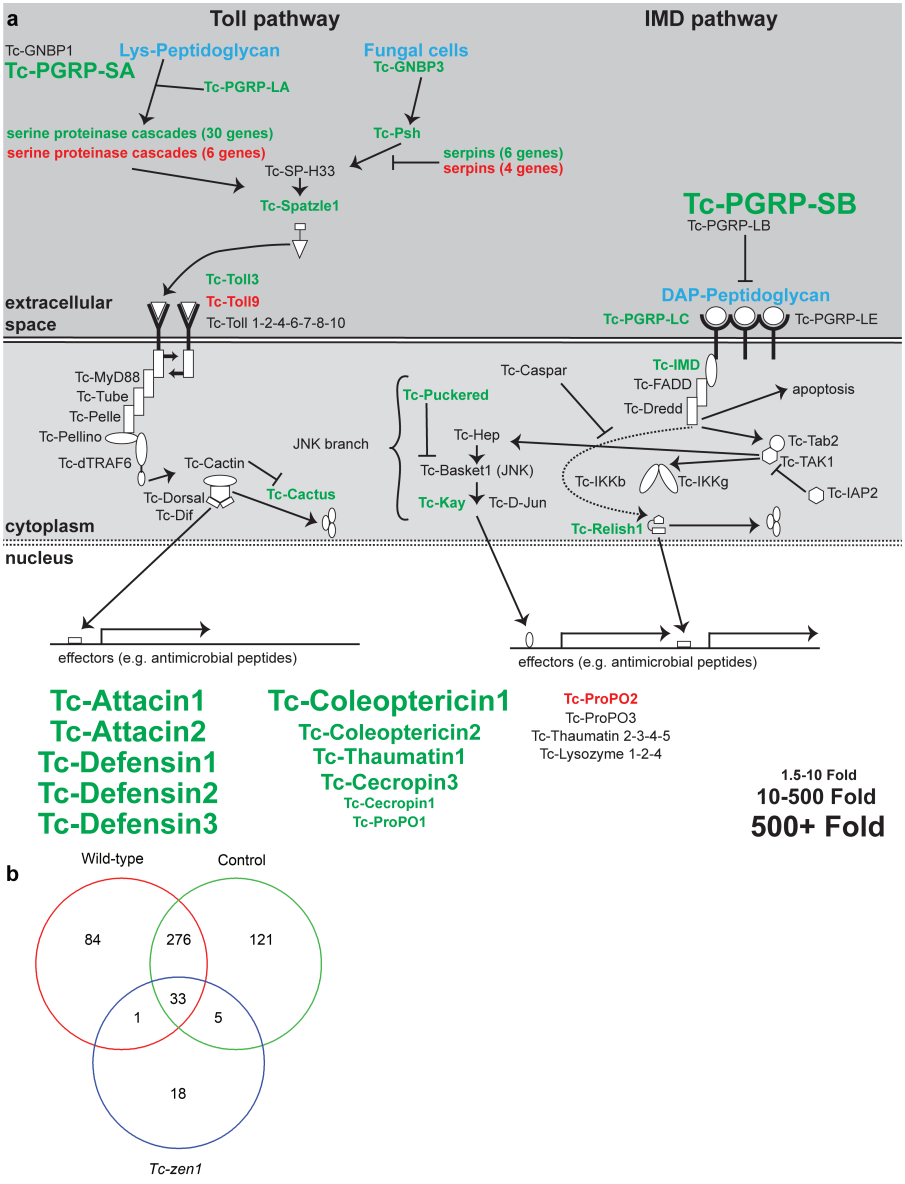


Figure 5-4: Immune-responsive genes in wild-type, control and *Tc-zen1* RNAi eggs. a) Schematic representation of the immune signaling pathways in *Tribolium* as described in (Zou et al., 2007). Significantly induced genes after septic injury in wild-type or control RNAi eggs are indicated in green; significantly repressed genes after septic injury in wild-type or control RNAi eggs are indicated in red. Genes not differentially expressed are black. The size of the gene names represents the fold change (small = 1.5-10 fold, medium = 10-500 fold, large = 500+ fold expression). b) Venn-diagram showing the number of differentially expressed genes in septically injured eggs as compared to naive eggs (FDR<0.01). In total, 538 genes are differentially expressed upon infection, of which 394 in wild-type eggs, 435 in control RNAi eggs and only 57 in *Tc-zen1* RNAi eggs. This means that *Tribolium* eggs display an extensive transcriptional response upon infection and that this response is largely abolished in eggs without a serosa.

Table 5-2: Antimicrobial properties of known and potential new antimicrobial peptides in *Tribolium castaneum*. In the table are known antimicrobial peptides and those proteins that show at least a two-fold induction upon infection, are smaller than 200 amino acids and are not negatively charged. *TC009336* was included because of the high glycine content.

Gene ID	Molecular weight (kDa)	Peptide length (AA)	Hydrophobic ratio	Net charge	Glycine content	Proline content	Foldchange Wild-type	Foldchange Control
Cecropin1 / TC000499	3.67	31	58%	+5	6%	0%	Inf	Inf
Cecropin3 / TC000500	9.80	90	43%	+2	6%	13%	Inf	49x
attacin2 / TC007738	15.80	145	37%	+7	12%	4%	3098x	2190x
Coleoptericin1 / TC005093	15.99	141	30%	-1	9%	7%	2392x	18067x
Defensin2 / TC010517	8.73	79	50%	+6	6%	1%	1183x	Inf
Defensin3 / TC012469	9.42	83	50%	+7	3%	1%	908x	Inf
attacin1 / TC007737	17.49	165	28%	+9	18%	3%	869x	3696x
TC007858	20.14	182	35%	0	11%	3%	484x	54x
Defensin1 / TC006250	14.91	132	46%	+11	4%	3%	187x	1551x
TC011036	12.89	109	39%	+13	2%	6%	138x	11x
Coleoptericin2 / TC005096	15.96	141	30%	-1	9%	7%	91x	227x
TC015479	13.00	120	42%	+6	5%	1%	80x	26x
TC007763	16.87	158	37%	+4	6%	17%	47x	67x
TC004646	15.04	135	34%	+2	7%	7%	40x	29x
TC008806	15.83	142	33%	+2	10%	2%	31x	37x
TC009336	13.50	137	30%	-4	39%	2%	15x	7x
TC014565	20.73	176	38%	+17	2%	2%	14x	9x
TC001030	14.62	137	29%	+9	10%	12%	9x	16x
TC001784	13.54	150	27%	+7	43%	2%	8x	6x
TC005478	13.70	122	45%	+10	4%	1%	6x	7x
TC015612	20.32	182	36%	+7	6%	6%	6x	2x
TC007901	7.25	64	25%	+5	7%	10%	6x	5x
TC015304	19.30	180	38%	+2	6%	9%	5x	no hit
TC011733	11.89	106	46%	+3	2%	0%	5x	5x
TC003374	12.22	124	61%	+3	1%	9%	2x	9x
TC008557	17.82	172	31%	+2	18%	0%	3x	5x
TC015754	15.69	140	34%	+5	4%	7%	2x	2x
TC000435	11.84	105	37%	+5	5%	0%	2x	2x
TC009096	12.84	111	16%	+16	9%	6%	2x	2x

(Table 5-2, we included the properties of several known AMPs as a reference). Although the antimicrobial properties of these peptides still have to be experimentally verified, this shows the strength of unbiased approaches to find novel immune genes.

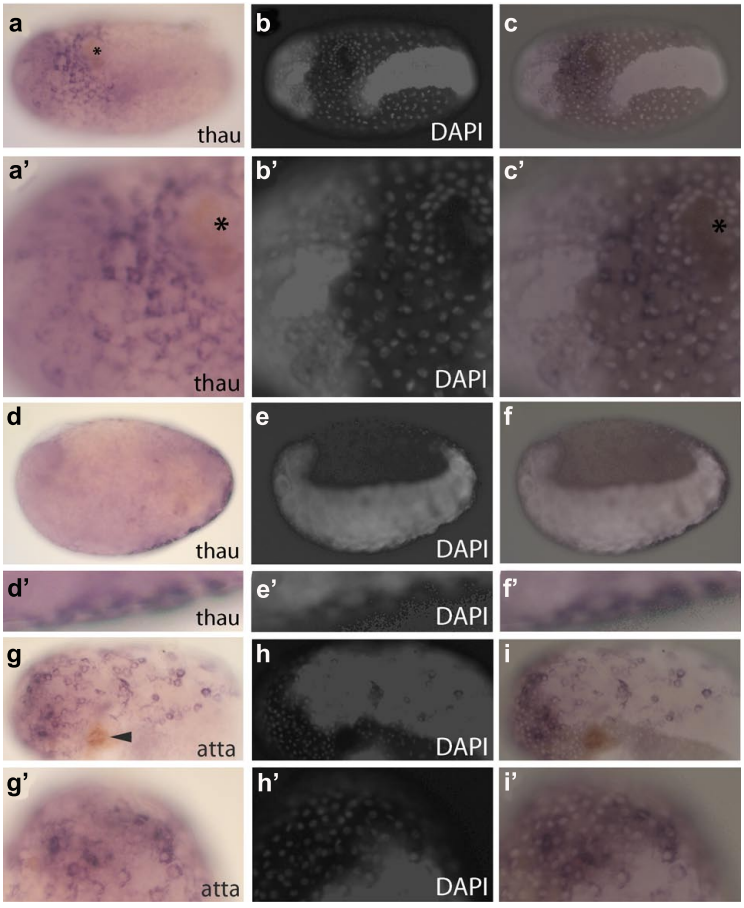


Figure 5-5: *In situ* hybridization showing expression of AMP genes in the serosa upon septic injury. a-f *thaumatin1 in situ* hybridization. a) Superficial view. *thaumatin1* is expressed around the site of injury (asterix). Brown melanisation is observed around the site of injury. a') Magnification of the expression area shown in a. Asterix marks the site of injury. b) DAPI counterstaining of the same egg as in a. The large polyploid serosal nuclei can be distinguished from the oversaturated DAPI signal from the germ band. Head lobes to the left. b') magnification of b. c) Overlay of the *in situ* hybridization shown in a and the DAPI staining shown in b. The *thaumatin1* expression associates with the large polyploid serosal nuclei and is not found in the embryo proper. c') magnification of the expression area shown in c. d) Focal plane through the egg. *Thaumatin1* is expressed in a thin outer layer at the surface of the egg. d') Magnification of the expression area shown in d. e) DAPI staining of the same egg shown in d. The embryo is brightly visible. Head to the left. e') Magnification of e. f) Overlay of the *in situ* hybridization shown in d and the DAPI staining shown in e. f') Magnification of the expression area. g) *attacin1 in situ* hybridization. Brown melanisation is visible around the site of injury (arrowhead). g') Magnification of the anterior region of the egg shown in g. h) DAPI staining of the same egg shown in g. The germband is brightly stained (head to the left) and the separate large serosal nuclei are visible. h') Magnification of the anterior of the egg shown in h. i) Overlay of the *in situ* hybridization shown in g and the DAPI staining shown in h. *Attacin1* is expressed in the large serosal cells covering the germband and is not expressed in the dense cells of the germ band. i') Magnification of the anterior of the egg shown in i. The *attacin1* staining associates with the large serosal nuclei.

The immune response is dependent on the extraembryonic serosa

To investigate the role of the serosa in the immune response, we compared the transcriptional response of wild-type and control eggs to the response of serosa-less eggs. Of all 538 genes differentially regulated upon bacterial challenge, 481 genes are only responsive in eggs with a serosa. The vast majority, 276 genes, are differentially regulated in both wild-type and control eggs, but not in serosa-less eggs (Figure 5-4b). In the serosa-less *Tc-zen1* RNAi eggs merely 57 genes are differentially regulated upon microbial challenge, despite our finding that RNAi rather increases the number of immune responsive genes. Of all 368 *Tribolium* genes that are annotated as immune genes (Altincicek et al., 2013; Zou et al., 2007), only 9 were differentially regulated upon infection in serosa-less eggs (Table 5-1 and Supplementary Table 5-2). Except for *serpin24*, all of the other 8 genes were also differentially regulated in response to sterile injury, indicating that they do not respond to infection but to wounding. Notably, none of the AMPs is induced upon infection in serosa-less eggs, neither *proPO1*, nor the DUOX ortholog *Hpx11* (Supplementary Table 5-2). Thus, the serosa is essential for the early immune response of the *Tribolium* egg.

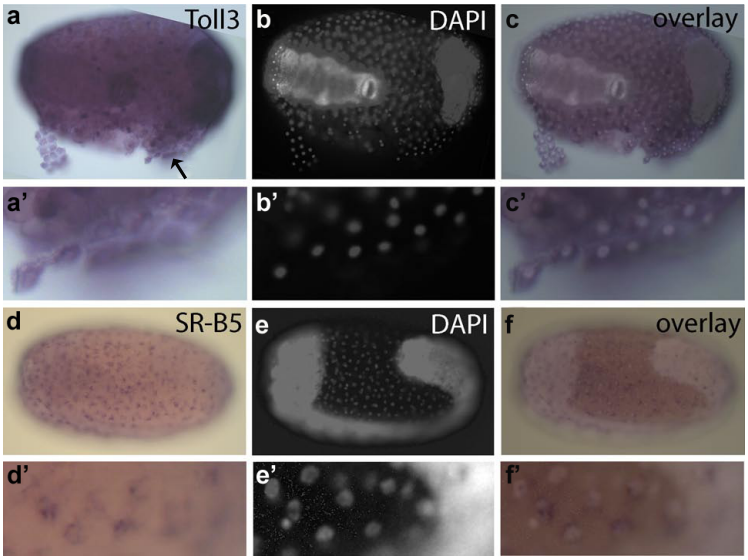


Figure 5-6: Constitutive expression of immune genes in the serosa. a-c *toll3 in situ* hybridization. a) *toll3* is expressed in the flat and thin serosal cells (partly detached from the egg), but also in the germ rudiment (head lobes to the right). a') Magnification of the area indicated with an arrow in a. b) DAPI staining of the same egg shown in a. The bright staining of the germ band can be distinguished from the large nuclei of the serosa. c) Overlay of the *in situ* hybridization shown in a and the DAPI staining shown in b. c') Magnification of c. *Toll3* is expressed in cells of the serosa. d-f *scavenger receptor B5 in situ* hybridization. d) *scavenger receptor B5* shows expression in every serosal cell at the surface. d') Magnification of d. e) DAPI staining of the same egg shown in d. The germband is brightly stained (head to the left) and the staining of the serosal nuclei is clearly visible when not overwhelmed by staining of the dense nuclei of the germ band. e') Magnification of e. The serosal nuclei are visible. Bright staining of the germband to the right. f) Overlay of the *in situ* hybridization shown in d and the DAPI staining shown in e. *Scavenger receptor B5* expression follows the serosal nuclei, and is not detected in the germ band. f') Magnification of f. *Scavenger receptor B5* mRNA is detected around the large polyploid serosal nuclei, and not around the dense nuclei of the germ rudiment.

These data corroborate our previous qPCR study showing that AMP and PGRP upregulation upon infection is abolished in serosa-less eggs (Jacobs & van der Zee, 2013). To see if we could also independently confirm serosa-dependent induction of some of our newly identified candidates, we performed qPCR on the transmembrane recognition protein of the IMD pathway *PGRP-LC*, the serine proteases *cS-P8*, *SPH-H57*, *SPH-H70*, the serine protease inhibitors *serpin24* and *serpin26*, the Toll receptor *toll3* and the novel potential AMPs *TC004646*, *TC007763*, *TC007857*, *TC008806* and *TC015479* (Supplementary Figure 5-1). The fold-changes detected by qPCR after sterile and septic injury of wild-type eggs match the values found in the RNAseq data. The largest deviation was found for the potential AMP *TC007858* that is upregulated 156 times upon septic injury in our qPCRs, but 484 times according to the RNAseq data (Supplementary Figure 5-1j). Most importantly, all qPCRs convincingly showed absence of induction in *Tc-zen1* RNAi eggs, thus providing independent support for our conclusion that the serosa is required for the immune response in *Tribolium* eggs.

To investigate if it is the serosal epithelium itself that expresses the identified immune genes and to exclude indirect effects, we performed *in situ* hybridization on two AMPs (*thaumatin1* and *attacin1*) of which mRNA length permitted *in situ* detection. In naive eggs, we could not detect *thaumatin1* or *attacin1* expression. In contrast, expression was obvious in challenged eggs (Figure 5-5). In these eggs, brown melanisation was found at the site of injury (asterix in Figure 5-5a and a' and arrowhead in Figure 5-5g) and the individual nuclei of the serosa can be distinguished from the oversaturated DAPI signal marking the germband (Figure 5-5b, e, h) (Handel et al., 2000). The *thaumatin1* expression clearly associates with the large polyploid serosal nuclei and not with the dense cells of the germ band (overlay in Figure 5-5c and c'). A deeper focal plane of a different egg demonstrates exclusive expression in the overlying serosa on the outer surface (Figure 5-5d, d'), and not in the underlying embryo proper (Figure 5-5e, f). Also *attacin1* expression consistently associated with the large polyploid serosal nuclei (Figure 5-5g-i').

Thus, it is the serosal epithelium itself that expresses these AMPs upon infection. Although we cannot exclude an indirect role of the serosa in the expression of the other identified immune genes, we propose that the serosa itself expresses these genes and thus regulates the described immune response involving melanisation, the generation of reactive oxygen species and the massive production of AMPs.

The serosa constitutively expresses some immune genes

To discover immune genes that are constitutively expressed in the serosa, we compared the transcriptomes of naive *Tc-zen1* eggs to naive wild-type eggs. We found 44 immune genes that have serosa-dependent expression
(Table 5-3). Of these genes, more than 75% is involved in the recognition of microbes and extracellular signal transduction (Table 5-3) such as *PGRP-LA*, many serine proteases and *Spz4* and *Spz5*. In contrast, most of the genes of the intracellular signal transduction were present in *Tc-zen1* eggs at similar levels as in wild-type eggs. Notably, the transmembrane receptor *toll3* exhibits higher expression in unchallenged eggs with a serosa than in eggs without a serosa. These data indicate that the serosa is an immune competent epithelium that expresses many genes involved in bacterial recognition and transduction of this recognition to receptor activation.

To confirm constitutive expression of these identified genes, we performed *in*

situ hybridization on naive eggs. We chose the receptor *toll3* that shows 2 times higher expression in eggs with a serosa, and the *scavenger receptor B5* that shows 30 times higher expression in eggs with a serosa (Table 5-3). We found ubiquitous expression of *toll3* in the egg (Figure 5-6a). Although *toll3* was clearly expressed in the serosa (partly detached from the egg Figure 5-6a'), we detected also expression in the embryo. As *in situ* hybridization is not a quantitative technique, and because the serosal cells are flat and thin, it is possible that we could not detect the two-fold higher expression in the serosa. For *scavenger receptor B5* that has a 30-fold higher expression in eggs with a serosa, we did find clear expression in the serosal epithelium (Figure 5-6d), whereas the underlying germ band was not stained (Figure 5-6f and f'). We propose that all genes listed in Table 5-3 are constitutively expressed in the serosa and thus make the serosa an immune-competent frontier epithelium.

Taken together, we have shown that eggs of the beetle *Tribolium castaneum* display an extensive transcriptional immune response. This response is entirely dependent on the serosa, an extraembryonic epithelium that envelops yolk and embryo. This immune competent frontier epithelium constitutively expresses some immune genes and can induce massive amounts of AMPs.

Discussion

We have provided the first characterization of the complete transcriptional immune response in an insect egg. We identified 538 immune responsive genes in the *Tribolium* egg, of which 481 are only found in eggs with a serosal epithelium. The number of immune-responsive genes found in the *Tribolium* egg is comparable to the number found in larvae (Behrens et al., 2014) and higher than what was found in adults (Altincicek et al., 2013), but this might be due to differences in sequence coverage. We cannot exclude that some expression differences we found might be due to somewhat delayed development after pricking the eggs. However, the GO-categories of the differentially regulated genes (shown in Figure 5-3) are mainly immune-related, suggesting that an effect of delayed development is negligible.

The induction of several genes from both the Toll- and IMD-pathway indicates that both pathways are utilized in the immune response of the *Tribolium* egg. It is striking that Toll signaling seems to be involved in innate immunity in the egg, because Toll signaling at this stage has only been associated with developmental functions until now (Leulier & Lemaitre, 2008; Nunes da Fonseca et al., 2008). In *Drosophila*, Toll1 has been described as the essential immune-related Toll receptor (Leulier & Lemaitre, 2008). Other Tolls are not essential for the immune response, except for an antiviral function of Toll7 (Nakamoto et al., 2012). Interestingly, *toll3* is significantly upregulated upon infection of the egg, and not *toll1*. *Toll3* is also upregulated in infected adults and larvae (Altincicek et al., 2013; Behrens et al., 2014), suggesting a novel role for *toll3* in *Tribolium* innate immunity. It should be noted that *toll1-4* in *Tribolium* are all closely related to *Drosophila toll1*, and more distantly to *Drosophila toll3* (Nunes da Fonseca et al., 2008; Zou et al., 2007). Thus, subfunctionalization into developmental and immune-related functions might have occurred among the *Tribolium toll1-4* paralogs.

Table 5-3: Differentially regulated immune genes in naive wild-type eggs compared to naive *Tc-zen1* eggs. SP = Serine protease ; SPH = Non catalytic serine protease ; cSP = Clip-domain serine protease

Gene ID	Description	Fold change	FDR adjusted p-value	Gene ID	Description	Fold change	FDR adjusted p-value
Extracellular signal transduction and modulation							
TC000247	cSPH-H2	2.70	<0.01	TC005754	serpin22	5.26	<0.01
TC000248	cSPH-H3	4.11	<0.01	TC006255	serpin24	0.69	0.03
TC000249	cSPH-H4	5.16	<0.01	TC011718	serpin27	1.62	<0.01
TC000740	SPH-H17	9.28	<0.01	TC006726	Spz4	3.00	<0.01
TC000829	SPH-H18	8.26	<0.01	TC013304	Spz5	122.56	<0.01
TC007026	cSPH-H78	29.79	<0.01	**Microbial recognition**			
TC012390	SPH-H129	1.60	<0.01	TC002789	PGRP-LA	3.95	0.02
TC000495	cSP-P8	6.57	<0.01	TC014664	TEP-B	2.90	0.02
TC000497	cSP-P10	4.50	<0.01	TC005976	PSH	3.43	<0.01
TC000547	SP-P13	2.41	<0.01	TC006978	C-type lectin1	14.52	<0.01
TC000635	SP-P16	2.54	<0.01	TC013911	C-type lectin 13	18.21	<0.01
TC004160	cSP-P44	9.79	<0.01	**Toll-signalling pathway**			
TC004624	cSP-P52	0.52	<0.01	TC004438	Toll3	2.28	<0.01
TC004635	cSP-P53	51.56	<0.01	**IMD-signalling pathway**			
TC005230	cSP-P61	250.00	<0.01	TC014708	NFAT	2.01	<0.01
TC006033	SP-P68	1.54	<0.01	**Execution mechanisms**			
TC009090	cSP-P91	2.80	<0.01	TC005375	hexamerin2	0.38	<0.01
TC009092	cSP-P93	3.00	<0.01	TC005493	Heme peroxidase 1	3.84	<0.01
TC009093	cSP-P94	27.76	<0.01	TC015234	Heme peroxidase 2	6.30	<0.01
TC013277	cSP-P136	3.04	<0.01	TC010356	Scavenger receptor-B13	0.60	0.03
TC013415	SP-P141	11.85	<0.01	TC015854	Scavenger receptor-B2	1.91	<0.01
TC000760	serpin1	5.29	<0.01	TC014946	Scavenger receptor-B5	29.29	<0.01
TC005750	serpin18	1.92	<0.01	TC000948	Scavenger receptor-B6	163.90	<0.01
TC005752	serpin20	2.30	<0.01	TC014954	Scavenger receptor-B9	1.96	<0.01

In *Tribolium*, only 19 AMPs have been identified (Zou et al., 2007). This is in strong contrast to another beetle species, *Harmonia axyridis*, in which more than 50 putative AMPs have been recognized (Vilcinskas et al., 2013). We were able to identify 20 new potential AMPs based on the antimicrobial properties of known AMPs (Bulet et al., 1999; Bulet & Stöcklin, 2005). Additional AMPs might still be discovered, as we have not investigated peptides longer than 200 amino acids. Some of these long peptides, for instance the Thaumatins, are known to have antimicrobial properties (Altincicek et al., 2013; Altincicek et al., 2008). We might also have missed AMPs because some might be specifically expressed at other stages, for instance in larvae or adults. Although activity assays against bacteria and fungi are needed to verify antimicrobial properties, the discovery of 20 new potential AMPs shows the power of our experimental strategy for getting an unbiased understanding of insect immunity.

The most important conclusion of our study is that the immune response in *Tribolium* eggs depends on the extraembryonic serosa. To delete the serosa, we used parental *Tc-zen1* RNAi (van der Zee et al., 2005). Formally, it is possible that the lack of the immune response we reported is not caused by absence of the serosa, but by a more direct effect of *Tc-zen1* RNAi, for instance if the transcription factor Zen would directly regulate immune genes in the embryo. This is highly unlikely, as *Tc-zen1* is only expressed in the early serosa (van der Zee et al., 2005) and is not expressed anymore by the time

we performed infection. Indeed, we found only three RNAseq reads that map to *Tc-zen1*, confirming that *Tc-zen1* is practically not expressed at the time we performed experiments. Thus, we are confident to conclude that the lack of the full-range immune response after *Tc-zen1* RNAi is exclusively due to the absence of the serosa.

Eggs with a serosa express crucial bacterial recognition genes, such as *PGRP-LA*, and many extracellular signaling components, such as serine proteases, at higher levels than serosa-less eggs, indicating constitutive expression in the serosa. It could be that these components activate receptors elsewhere in the egg, for instance the *toll3* receptor that is more ubiquitously expressed. However, our *in situ* hybridizations unambiguously demonstrate that it is the serosal epithelium itself that expresses AMPs upon infection, indicating that it is the serosal epithelium itself that harbors the functional immune response reducing bacterial propagation in infected eggs (Figure 5-1).

Overall, bacterial infection of *Tribolium* eggs induces genes involved in melanisation, the acute-phase oxidative response and AMP production, and differentially regulates many other immune genes. This response is completely abolished in eggs without a serosa, the extraembryonic epithelium that envelopes yolk and embryo at the interface with the microbe-rich external milieu. Barrier epithelia like the midgut have recently been highlighted as key players in the local immune defenses in insects (Davis & Engstrom, 2012; Ferrandon, 2013). We conclude that the serosa is a frontier epithelium that provides the insect egg with a full-range immune response.

Interestingly, the separation of the serosal cells from the germ rudiment is the first morphological distinction that can be made in the blastoderm of the developing egg (Handel et al., 2000). The serosal cells will have enveloped the complete embryo before the ectoderm starts to differentiate. These serosal cells can provide the insect egg with an innate immune response long before the embryonic ectoderm or trachea are immune responsive. In addition, the polyploid nuclei allow the serosal cells to quickly synthesize large amounts of proteins providing protection for the vulnerable developing embryo. Thus, the serosa is well suited to provide early immune protection to the egg. *Drosophila* eggs do not develop a serosa, as this extraembryonic membrane was lost in the Schizophoran flies (Rafiqi et al., 2008; Schmidt-Ott, 2000). A trade-off with developmental speed might have driven the loss of the serosa in these flies living on ephemeral food sources (Jacobs et al., 2014). We suggest that the absence of the serosa in the Schizophora accounts for the poor immune response of *Drosophila* eggs. Since all other insects possess a serosa, we propose that early immune competence is a general property of insect eggs.

Conclusions

Tribolium casteneum eggs can mount a full-range innate immune response involving antimicrobial peptides, melanisation and the production of reactive oxygen species. This response depends entirely on the extraembryonic serosa, an immune competent frontier epithelium that is absent in *Drosophila*.

Materials and methods

Beetles and *Tc-zen1* RNAi

The *Tribolium* stock used for this study was the *T. castaneum* wild-type strain San Bernardino. Stock keeping and *Tc-zen1* RNAi was performed as described in (van der Zee

et al., 2005). The control dsRNA was synthesized from a 500bp vector sequence cloned from the pCRII vector (Invitrogen) using the primers 5'-TGCCGGATCAAGAGCTACCAA-3' and 5'-TGTGAGCAAAAGGCCAGCAA-3' and has no targets in the *Tribolium castaneum* genome (see also Jacobs et al., 2013; Jacobs & van der Zee, 2013).

Infection

Infection experiments were performed as described in (Jacobs & van der Zee, 2013). 24-40h old eggs (total developmental time is close to 85h) were pricked with a sterile tungsten needle or with a tungsten needle dipped in a concentrated mix of *E. coli* and *M. luteus* cultures (bacteria provided by D. Ferrandon, Strasbourg) or were not pricked at all. To allow comparison to the extensive body of work in *Drosophila*, we have used the same strains of *E. coli* and *M. luteus* as are traditionally used in *Drosophila* (Ferrandon et al., 2007). 6h later, eggs were used for RNA isolation or *in situ* hybridization.

Cfu counts

Cfu's were determined directly after infection (t=0) or 6h after infection (t=6). Eggs were shortly washed for 15 seconds in a 0.5% hypochlorite solution to sterilize the outside, and rinsed with water. 10 eggs were pooled and homogenized in 100 µl water with a sterile pestle. For t=0, 25 µl of this suspension was directly plated on LB agar plates; for t=6 these 100 microliters were either diluted 50 times in 50 µl water (for wild-type eggs), or 500 times in 50 µl water (for *Tc-zen1* eggs). Of these dilutions 25 µl was plated on LB agar plates. Colonies were counted after an overnight incubation at 37 °C, and average numbers of cfu´s were calculated per egg. For each combination of time and treatment, the cfu´s were measured 10 times. Statistical significance was determined by performing a Pearson's chi-square test. Bacterial load of wild-type eggs increased to on average 32,975 cfu's after 24h, but at this time point comparisons to *Tc-zen1* RNAi eggs were unreliable as bacteria might have reached a maximum. At t=6, bacteria were still in their exponential growth phase and the formula $N_{(t)} = N_{(0)} * e^{kt}$ could be used to calculate the specific growth rate.

Sample collection for transcriptional analysis

For RNAseq and qPCR, 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). We collected 3 biological samples for each of the 9 treatments, giving a total of 27 biological samples (Figure 5-2). cDNA library synthesis and sequencing was performed by the ZF-screens (Leiden, the Netherlands) sequencing company on an Illumina HiSeq2500 sequencer.

Data analysis and bioinformatics

Sequencing reads were mapped with CLC genomics workbench 6 using the first 51 bp with highest sequencing quality and score values over 20, allowing 2 mismatches to the reference sequence of the *Tribolium* genome 3.0 which was obtained from Ensemble (Flicek et al., 2013). The mismatch cost was set to 2, the insertion cost to 3, the deletion cost to 3, the length fraction to 0.5 and the similarity fraction was set at 0.8. To calculate statistical differences of the expression levels of genes between treatments we utilized the DESeq package (Anders & Huber, 2010) in Bioconductor (Gentleman et al., 2004) in R (R Development Core Team, 2009). The P values were adjusted for multiple testing with the

Benjamini-Hochberg procedure, which determines false discovery rate (FDR). We trimmed the data to only contain genes that are induced more than 2 fold or repressed more than 2 fold. To minimize false discovery rate we set the cut-off value for significant genes to an FDR of <0.01. DESeq was used to normalize the count data, calculate mean values, fold changes, size factors, variance and P values (raw and adjusted) of a test for differential gene expression based on generalized linear models using negative binomial distribution errors.

Sequence annotation

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 web server were performed remotely by utilizing BLAST2GO.

qPCR

RNA was collected as described under "Sample collection for transcriptional analysis". The quality of RNA preparation was confirmed spectro-photometrically and on gel. 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 (25 µl) 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 (Bio-rad). 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 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* mRNA amount for *Tribolium* (primers as in Lord et al., 2010). Total RNA for each treatment was isolated two times (biological replication) and each sample was measured by qRT-PCR twice (technical replication). The primers used for qPCR are in Supplementary Table 5-3.

***In situ* hybridizations**

In situ hybridizations involving alkaline phosphatase-based visualization of DIG-labelled probes were essentially performed as described in (Tautz & Pfeifle, 1989), but without the proteinaseK step. Eggs were fixed for 20 minutes in a 1:1 mix of heptane and 3,7% formaldehyde in PBST. As the serosa tightly associates with the vitelline membrane, we used *Tc-CHS1* RNAi eggs (Jacobs et al., 2013), making it possible to manually dissect eggs containing the serosa from the vitelline membrane. The following primers were used to amplify 500bp fragments of *thaumatin1*, *attacin1*, *toll3* and *scavenger receptor B5*.
Thaumatin1 FW 5'-CTAAGCGAAGGGGGTTTCGT-3' RV 5'-TTTGTGGTCATCGTAGGCGT-3'
Attacin1 FW 5'-ATCGTCCAAGACCAGCAAGG-3' RV 5'-GAAGCGGTGGCTAAACTGGA-3'
Toll3 FW 5'-AACTGGGAGGTTTTGCACAC-3' RV 5'-AACTCCATTTTCCCCCAAAC-3'
SR-B5 FW 5'-AGCCAGGGAGTTCATGTTCG-3' RV 5'-TGATTTGGTAACGGACGGCA-3'
PCR fragments were cloned into the TOPO II vector (Invitrogen) according to the

manufacturer's protocol. From these plasmids, templates for probe synthesis were amplified using M13 primers. DIG-labelled probes were synthesized using the MEGAscript kit (Ambion) according to the manufacturer's protocol, but with Roche RNA-labelling mix (Roche).

Data access

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Barrett et al., 2013) and are accessible through GEO Series accession number GSE54018 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54018).

Acknowledgments

We thank Nora Braak, Romée de Blois and O.L. van de Peppel for help with the *in situ* hybridizations. Heiko Vogel from the Max Planck Institute for Chemical Ecology, Jena, Germany for critically reading the manuscript. MvdZ was funded by NWO VENI grant 863.09.014.

References

Altincicek, B., Elashry, A., Guz, N., Grundler, F. M., Vilcinskas, A., & Dehne, H. W. (2013). Next generation sequencing based transcriptome analysis of septic-injury responsive genes in the beetle Tribolium castaneum. PLoS ONE, 8(1), 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(5), 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

Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. Genome Biology, 11(10), R106.

Barrett, T., Wilhite, S. E., Ledoux, P., Evangelista, C., Kim, I. F., Tomashevsky, M., . . . Soboleva, A. (2013). NCBI GEO: archive for functional genomics data sets—update. Nucleic Acids Research, 41(D1), D991-D995. doi: 10.1093/nar/gks1193

Behrens, S., PeuSZ, R., Milutinovi, B., Eggert, H., Esser, D., Rosenstiel, P., . . . Kurtz, J. (2014). Infection routes matter in population-specific responses of the red flour beetle to the entomopathogen Bacillus thuringiensis. BMC Genomics, 15(1), 445.

Bell, J. V. (1969). Serratia marcescens found in eggs of Heliothis zea: Tests against Trichoplusia ni. Journal of Invertebrate Pathology, 13(1), 151-152.

Blum, M. S., & Hilker, M. (2008). Chemical Protection of Insect Eggs. In M. Hilker & T. Meiners (Eds.), Chemoecology of Insect Eggs and Egg Deposition (pp. 61-90): Blackwell Publishing Ltd.

Boos, S., Meunier, J., Pichon, S., & Kölliker, M. (2014). Maternal care provides antifungal protection to eggs in the European earwig. Behavioral Ecology, 25(4), 754-761. doi: 10.1093/beheco/aru046

Bulet, P., Hetru, C., Dimarcq, J. L., & Hoffmann, D. (1999). Antimicrobial peptides in insects; structure and function. Developmental and Comparative Immunology, 23(4-5), 329-344.

Bulet, P., & Stöcklin, R. (2005). Insect Antimicrobial Peptides: Structures, Properties and Gene Regulation. Protein and Peptide Letters, 12, 3-11.

Bulet, P., Stöcklin, R., & Menin, L. (2004). Anti-microbial peptides: from invertebrates to vertebrates. Immunological Reviews, 198(1), 169-184. doi: 10.1111/j.0105-2896.2004.0124.x

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(23), 5145-5156.

Christophides, G. K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., . . . Kafatos, F. C. (2002). Immunity-Related Genes and Gene Families in Anopheles gambiae. Science, 298(5591), 159-165. doi: 10.1126/science.1077136

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., & Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics, 21(18), 3674-3676. doi: 10.1093/bioinformatics/bti610

Contreras, E., Rausell, C., & Real, M. D. (2013). Proteome Response of Tribolium castaneum Larvae to Bacillus thuringiensis Toxin Producing Strains. PLoS ONE, 8(1), e55330. doi: 10.1371/journal.pone.0055330

Davis, M. M., & Engstrom, Y. (2012). Immune response in the barrier epithelia: lessons from the fruit fly Drosophila melanogaster. J Innate Immun, 4(3), 273-283. doi: 10.1159/000332947

De Gregorio, E., Spellman, P. T., Rubin, G. M., & Lemaitre, B. (2001). Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. Proceedings of the National Academy of Sciences, 98(22), 12590-12595. doi: 10.1073/pnas.221458698

Dziarski, R., Platt, K. A., Gelius, E., Steiner, H., & Gupta, D. (2003). Defect in neutrophil killing and increased susceptibility to infection with nonpathogenic gram-positive bacteria in peptidoglycan recognition protein-S (PGRP-S)-deficient mice. Blood, 102(2), 689-697. doi: 10.1182/blood-2002-12-3853

Ferrandon, D. (2013). The complementary facets of epithelial host defenses in the genetic model organism Drosophila melanogaster: from resistance to resilience. Current Opinion in Immunology, 25(1), 59-70. doi: http://dx.doi.org/10.1016/j.coi.2012.11.008

Ferrandon, D., Imler, J. L., Hetru, C., & Hoffmann, J. A. (2007). The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. Nature Reviews Immunology, 7(11), 862-874. doi: 10.1038/nri2194

Flicek, P., Ahmed, I., Amode, M. R., Barrell, D., Beal, K., Brent, S., . . . Searle, S. M. J. (2013). Ensembl 2013. Nucleic Acids Research, 41(D1), D48-D55. doi: 10.1093/nar/gks1236

Freitak, D., Schmidtberg, H., Dickel, F., Lochnit, G., Vogel, H., & Vilcinskas, A. (2014). The maternal transfer of bacteria can mediate trans-generational immune priming in insects. Virulence, 5(4), 547-554.

Ganesan, S., Aggarwal, K., Paquette, N., & Silverman, N. (2011). NF-kappaB/Rel proteins and the humoral immune responses of Drosophila melanogaster. Curr Top Microbiol Immunol, 349, 25-60. doi: 10.1007/82_2010_107

Gentleman, R., Carey, V., Bates, D., Bolstad, B., Dettling, M., Dudoit, S., . . . Zhang, J. (2004). Bioconductor: open software development for computational biology and bioinformatics. Genome Biology, 5(10), R80.

Gerardo, N. M., Altincicek, B., Anselme, C., Atamian, H., Barribeau, S. M., de Vos, M., . . . Vilcinskas, A. (2010). Immunity and other defenses in pea aphids, Acyrthosiphon pisum. Genome Biology, 11(2), R21. doi: 10.1186/gb-2010-11-2-r21

Handel, K., Grunfelder, C. G., Roth, S., & Sander, K. (2000). Tribolium embryogenesis: a SEM study of cell shapes and movements from blastoderm to serosal closure. Development Genes and Evolution, 210(4), 167-179.

Jacobs, C. G. C., Rezende, G. L., Lamers, G. E. M., & van der Zee, M. (2013). The extraembryonic serosa protects the insect egg against desiccation. Proceedings of the Royal Society B: Biological Sciences, 280(1764). doi: 10.1098/rspb.2013.1082

Jacobs, C. G. C., & van der Zee, M. (2013). Immune competence in insect eggs depends on the extraembryonic serosa. Developmental & Comparative Immunology, 41(2), 263-269. doi: http://dx.doi.org/10.1016/j.dci.2013.05.017

Jacobs, C. G. C., Wang, Y., Vogel, H., Vilcinskas, A., Van der Zee, M., & Rozen, D. E. (2014). Egg survival is reduced by grave-soil microbes in the carrion beetle, Nicrophorus vespilloides. BMC Evolutionary Biology, in press.

Johnston, P. R., Makarova, O., & Rolff, J. (2013). Inducible Defenses Stay Up Late: Temporal Patterns of Immune Gene Expression in Tenebrio molitor. G3 (Bethesda). doi: 10.1534/g3.113.008516

Johnston, P. R., & Rolff, J. (2013). Immune- and wound-dependent differential gene expression in an ancient insect. Developmental & Comparative Immunology, 40(3-4), 320-324. doi: 10.1016/j.dci.2013.01.012

Kellner, R. L. L. (2008). The Role of Microorganisms for Eggs and Progeny. In M. Hilker & T. Meiners (Eds.), Chemoecology of Insect Eggs and Egg Deposition (pp. 149-167): Blackwell Publishing Ltd.

Kim, M.-S., Byun, M., & Oh, B.-H. (2003). Crystal structure of peptidoglycan recognition protein LB from Drosophila melanogaster. Nature Immunology, 4(8), 787-793.

Lemaitre, B., & Hoffmann, J. (2007). The Host Defense of Drosophila melanogaster. Annual Review of Immunology, 25(1), 697-743. doi: doi:10.1146/annurev.immunol.25.022106.141615

Leone, P., Bischoff, V., Kellenberger, C., Hetru, C., Royet, J., & Roussel, A. (2008). Crystal structure of Drosophila PGRP-SD suggests binding to DAP-type but not lysine-type peptidoglycan. Mol Immunol, 45(9), 2521-2530. doi: 10.1016/j.molimm.2008.01.015

Leulier, F., & Lemaitre, B. (2008). Toll-like receptors- taking an evolutionary approach. Nature Reviews Genetics, 9(3), 165-178.

Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J. H., Caroff, M., Lee, W. J., . . . Lemaitre, B. (2003). The Drosophila

immune system detects bacteria through specific peptidoglycan recognition. Nature Immunology, 4(5), 478-484. doi: 10.1038/ni922
Ligoxygakis, P. (2013). Genetics of immune recognition and response in Drosophila host defense. Adv Genet, 83, 71-97. doi: 10.1016/b978-0-12-407675-4.00002-x
Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25(4), 402-408. doi: 10.1006/meth.2001.1262
Lord, J. C., 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(2), 219-221. doi: http://dx.doi.org/10.1016/j.mimet.2009.12.007
Lynch, R. E., Lewis, L. C., & Brindley, T. A. (1976). Bacteria associated with eggs and first-instar larvae of the European corn borer: Identification and frequency of occurrence. Journal of Invertebrate Pathology, 27(2), 229-237. doi: http://dx.doi.org/10.1016/0022-2011(76)90150-6
Marchini, D., Marri, L., Rosetto, M., Manetti, A. G. O., & Dallai, R. (1997). Presence of Antibacterial Peptides on the Laid Egg Chorion of the Medfly Ceratitis capitata. Biochemical and Biophysical Research Communications, 240(3), 657-663. doi: http://dx.doi.org/10.1006/bbrc.1997.7694
Milutinović, B., Stolpe, C., Peuβ, R., Armitage, S. A. O., & Kurtz, J. (2013). The Red Flour Beetle as a Model for Bacterial Oral Infections. PLoS ONE, 8(5), e64638. doi: 10.1371/journal.pone.0064638
Moreau, J., Martinaud, G., Troussard, J.-P., Zanchi, C., & Moret, Y. (2012). Trans-generational immune priming is constrained by the maternal immune response in an insect. Oikos, 121(11), 1828-1832. doi: 10.1111/j.1600-0706.2011.19933.x
Nakamoto, M., Moy, R. H., Xu, J., Bambina, S., Yasunaga, A., Shelly, S. S., . . . Cherry, S. (2012). Virus recognition by Toll-7 activates antiviral autophagy in Drosophila. Immunity, 36(4), 658-667. doi: 10.1016/j.immuni.2012.03.003
Nunes da Fonseca, R., von Levetzow, C., Kalscheuer, P., Basal, A., van der Zee, M., & Roth, S. (2008). Self-Regulatory Circuits in Dorsoventral Axis Formation of the Short-Germ Beetle Tribolium castaneum. Developmental cell, 14(4), 605-615.
Park, J. W., Kim, C. H., Rui, J., Park, K. H., Ryu, K. H., Chai, J. H., . . . Lee, B. L. (2010). Beetle Immunity. In K. Söderhall (Ed.), Invertebrate Immunity (Vol. 708, pp. 163-179): Landes Bioscience and Springer Science+Business Media.
R Development Core Team. (2009). R: A language and environment for statistical computing (Version 2.9.0). Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org
Rafiqi, A. M., Lemke, S., Ferguson, S., Stauber, M., & Schmidt-Ott, U. (2008). Evolutionary origin of the amnioserosa in cyclorrhaphan flies correlates with spatial and temporal expression changes of zen. Proceedings of the National Academy of Sciences of the United States of America, 105(1), 234-239. doi: 10.1073/pnas.0709145105
Richards, S., Gibbs, R. A., Weinstock, G. M., Brown, S. J., Denell, R., Beeman, R. W., . . . Zdobnov, E. M. (2008). The genome of the model beetle and pest Tribolium castaneum. Nature, 452(7190), 949-955.
Roth, O., Joop, G., Eggert, H., Hilbert, J., Daniel, J., Schmid-Hempel, P., & Kurtz, J. (2010). Paternally derived immune priming for offspring in the red flour beetle, Tribolium castaneum. Journal of Animal Ecology, 79(2), 403-413. doi: 10.1111/j.1365-2656.2009.01617.x
Sadd, B. M., & Schmid-Hempel, P. (2007). Facultative but persistent transgenerational immunity via the mother's eggs in bumblebees. Current Biology, 17(24), R1046-R1047. doi: 10.1016/j.cub.2007.11.007
Schmidt-Ott, U. (2000). The amnioserosa is an apomorphic character of cyclorrhaphan flies. Development Genes and Evolution, 210(7), 373-376.
Shrestha, S., & Kim, Y. (2010). Activation of immune-associated phospholipase A2 is functionally linked to Toll/Imd signal pathways in the red flour beetle, Tribolium castaneum. Developmental and Comparative Immunology, 34(5), 530-537. doi: 10.1016/j.dci.2009.12.013
Sikorowski, P. P., Lawrence, A. M., & Inglis, G. D. (2001). Effects of Serratia marcescens on Rearing of the Tobacco Budworm (Lepidoptera: Noctuidae). American entomologist, 47(1), 51-60.
Stroschein-Stevenson, S. L., Foley, E., O'Farrell, P. H., & Johnson, A. D. (2005). Identification of Drosophila Gene Products Required for Phagocytosis of Candida albicans. PLoS Biology, 4(1), e4. doi: 10.1371/journal.pbio.0040004
Tan, Kiri L., Vlisidou, I., & Wood, W. (2014). Ecdysone Mediates the Development of Immunity in the Drosophila Embryo. Current Biology, 24(10), 1145-1152. doi: http://dx.doi.org/10.1016/j.cub.2014.03.062
Tautz, D., & Pfeifle, C. (1989). A non-radioactive insitu hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. Chromosoma, 98(2), 81-85. doi: 10.1007/bf00291041

Tingvall, T. O., 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 of the United States of America, 98(7), 3884-3888. doi: 10.1073/pnas.061230198

Trauer, U., & Hilker, M. (2013). Parental Legacy in Insects: Variation of Transgenerational Immune Priming during Offspring Development. PLoS ONE, 8(5), e63392. doi: 10.1371/journal.pone.0063392

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(7), 624-636.

Vilcinskas, A., Mukherjee, K., & Vogel, H. (2013). Expansion of the antimicrobial peptide repertoire in the invasive ladybird Harmonia axyridis. Proceedings of the Royal Society B: Biological Sciences, 280(1750), 20122113. doi: 10.1098/rspb.2012.2113

Wang, G., Li, X., & Wang, Z. (2009). APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Research, 37(suppl 1), D933-D937. doi: 10.1093/nar/gkn823

Wang, L., Kounatidis, I., & Ligoxygakis, P. (2014). Drosophila as a model to study the role of blood cells in inflammation, innate immunity and cancer. Front Cell Infect Microbiol, 3, 113. doi: 10.3389/fcimb.2013.00113

Wang, X.-W., & Wang, J.-X. (2013). Pattern recognition receptors acting in innate immune system of shrimp against pathogen infections. Fish & Shellfish Immunology, 34(4), 981-989. doi: http://dx.doi.org/10.1016/j.fsi.2012.08.008

Waterhouse, R. M., Kriventseva, E. V., Meister, S., Xi, Z., Alvarez, K. S., Bartholomay, L. C., . . . Christophides, G. K. (2007). Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. Science, 316(5832), 1738-1743. doi: 10.1126/science.1139862

Yang, W., Cheng, T., Ye, M., Deng, X., Yi, H., Huang, Y., . . . Xia, Q. (2011). Functional Divergence among Silkworm Antimicrobial Peptide Paralogs by the Activities of Recombinant Proteins and the Induced Expression Profiles. PLoS ONE, 6(3), e18109. doi: 10.1371/journal.pone.0018109

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(2), 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(0), 72-82. doi: 10.1016/j.rinim.2012.03.002

Young, M., Wakefield, M., Smyth, G., & Oshlack, A. (2010). Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biology, 11(2), R14.

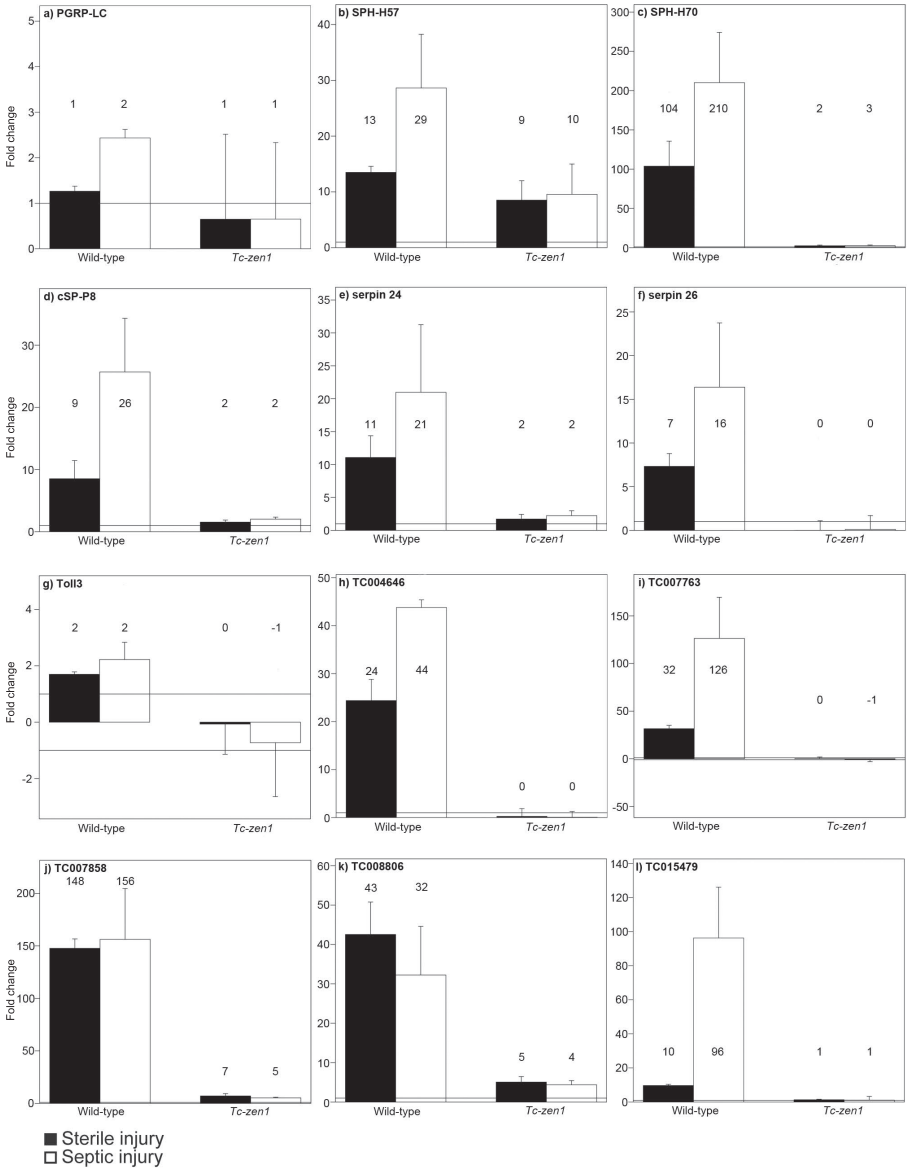
Zanchi, C., Troussard, J. P., Moreau, J., & Moret, Y. (2012). Relationship between maternal transfer of immunity and mother fecundity in an insect. Proceedings of the Royal Society B: Biological Sciences, 279(1741), 3223-3230. doi: 10.1098/rspb.2012.0493

Zhong, D., Wang, M. H., Pai, A., & Yan, G. (2013). Transcription profiling of immune genes during parasite infection in susceptible and resistant strains of the flour beetles (Tribolium castaneum). Exp Parasitol. doi: 10.1016/j.exppara.2013.01.014

Zhu, J.-Y., Yang, P., Zhang, Z., Wu, G.-X., & Yang, B. (2013). Transcriptomic Immune Response of Tenebrio molitor Pupae to Parasitization by Scleroderma guani. PLoS ONE, 8(1), e54411. doi: 10.1371/journal.pone.0054411

Zou, Z., Evans, J. D., Lu, Z. Q., Zhao, P. C., Williams, M., Sumathipala, N., . . . Jiang, H. B. (2007). Comparative genomic analysis of the Tribolium immune system. Genome Biology, 8(8). doi: 10.1186/gb-2007-8-8-r177

Supplementary information



Supplementary Figure 5-1: RT-qPCR verification of immune gene expression. The expression levels of several immune genes was verified by RT-qPCR. Expression shown relative to the expression in naive eggs, the mean fold change of the biological replicates (based on 2 technical replicates) is plotted and error bars show the standard error. Black bars represent expression after sterile injury, white bars represent expression after septic injury. Expression levels measured by RT-qPCR show very similar results as the expression levels measured by RNAseq (see Table S1). A) *PGRP-LC*, B) *SPH-H57*, C) *SPH-H70*, D) *cSP-P8*, E) *serpin 24*, F) *serpin 26*, G) *toll3*, H) *TC004646*, I) *TC007763*, J) *TC007858*, K) *TC008806*, L) *TC015479*. See Methods for experimental details.

Supplementary Table 5-1: Significant differently expressed immune genes in *Tribolium castaneum* eggs. (part 1/3)
Green = induction ; Red = repression ; Black = not significant
SP = Serine protease ; SPH = Non catalytic serine protease ; cSP = Clip-domain serine protease

Gene ID	Description	Wildtype eggs				Control eggs				*Tc-zen1* eggs			
		Sterile injury		Septic injury		Sterile injury		Septic injury		Sterile injury		Septic injury	
		Fold change	FDR adjusted p-value	Fold change	FDR adjusted p-value	Fold change	FDR adjusted p-value	Fold change	FDR adjusted p-value	Fold change	FDR adjusted p-value	Fold change	FDR adjusted p-value
Microbial recognition													
TC002789	PGRP-LA	1.63	0.31	3.12	<0.01	2.53	<0.01	3.33	<0.01	1.12	1.00	1.67	1.00
TC002790	PGRP-LC	1.28	1.00	1.65	0.09	1.88	<0.01	2.43	<0.01	1.40	0.57	1.29	1.00
TC010611	PGRP-SA	4.53	0.96	241.06	<0.01	3.40	0.49	217.03	<0.01	1.11	1.00	9.38	0.89
TC013620	PGRP-SB	119.59	<0.01	937.86	<0.01	Inf	<0.01	Inf	<0.01	1.92	0.99	34.74	0.31
TC011529	βGRP2	2.65	<0.01	4.94	<0.01	2.53	<0.01	5.03	<0.01	1.13	1.00	1.29	1.00
TC003991	βGRP3	4.38	<0.01	4.90	<0.01	4.22	<0.01	5.47	<0.01	2.12	1.00	1.02	1.00
TC000808	TEP-D	1.29	1.00	1.18	1.00	0.64	0.19	0.57	0.05	0.71	0.80	0.70	1.00
TC001981	LpR2	1.29	0.44	1.47	<0.01	1.26	0.06	1.40	<0.01	1.40	0.04	1.41	0.21
TC006978	C-type lectin1	0.83	1.00	0.40	<0.01	0.70	0.08	0.41	<0.01	0.84	1.00	0.72	1.00
TC003708	C-type lectin6	2.16	<0.01	2.38	<0.01	2.86	<0.01	2.99	<0.01	1.20	0.38	1.16	0.56
TC013911	C-type lectin13	0.22	<0.01	0.21	<0.01	0.51	0.07	0.38	<0.01	1.52	1.00	1.64	1.00
Extracellular signal transduction and modulation													
TC000248	cSPH-H3	0.75	0.54	0.48	<0.01	0.81	0.41	0.61	<0.01	0.78	0.65	0.84	1.00
TC000249	cSPH-H4	9.50	<0.01	16.54	<0.01	7.38	<0.01	12.31	<0.01	1.13	1.00	2.00	1.00
TC000252	cSPH-H6	1.20	0.81	1.23	0.16	1.11	0.83	1.16	0.41	1.37	0.02	1.31	0.31
TC000740	SPH-H17	0.58	<0.01	0.36	<0.01	0.60	0.04	0.52	<0.01	0.60	0.68	0.86	1.00
TC000829	SPH-H18	2.87	<0.01	3.92	<0.01	2.38	<0.01	3.02	<0.01	0.62	1.00	0.90	1.00
TC002150	cSPH-H34	1.09	1.00	2.40	0.60	4.84	0.13	5.91	0.04	2.91	0.33	3.01	0.80
TC004622	cSPH-H51	1.02	1.00	1.06	1.00	0.66	0.03	0.60	<0.01	0.84	0.61	0.98	1.00
TC004900	SPH-H57	6.42	0.02	22.34	<0.01	10.36	<0.01	14.76	<0.01	4.06	0.02	3.68	0.23
TC005908	SPH-H64	20.64	<0.01	16.37	<0.01	6.54	<0.01	7.57	<0.01	3.21	1.00	1.44	1.00
TC006246	SPH-H70	252.58	<0.01	495.16	<0.01	21.54	<0.01	59.55	<0.01	3.48	1.00	5.22	1.00
TC006247	SPH-H71	Inf	1.00	Inf	0.13	Inf	0.14	Inf	<0.01	0.00	1.00	0.00	1.00
TC007026	cSPH-H78	1.16	1.00	0.81	1.00	1.59	<0.01	1.13	0.78	1.05	1.00	0.95	1.00
TC011067	cSPH-H125	1.09	1.00	1.84	0.14	2.48	<0.01	2.26	0.02	2.00	0.04	1.94	0.23
TC000495	cSP-P8	7.51	<0.01	26.66	<0.01	10.81	<0.01	40.80	<0.01	1.43	1.00	1.48	1.00
TC000496	SP-P9	36.96	<0.01	110.13	<0.01	7.17	<0.01	13.49	<0.01	0.88	1.00	1.03	1.00
TC000497	cSP-P10	4.39	<0.01	7.19	<0.01	8.02	<0.01	12.89	<0.01	1.21	1.00	1.46	1.00
TC000547	SP-P13	0.67	<0.01	0.51	<0.01	0.68	<0.01	0.51	<0.01	0.72	0.02	0.75	0.23
TC002659	SP-P36	5.44	<0.01	5.06	<0.01	4.73	<0.01	4.15	<0.01	2.16	0.93	4.42	0.89
TC004160	cSP-P44	1,43	<0.01	1.97	<0.01	1.78	<0.01	2.24	<0.01	0.99	1.00	1.04	1.00
TC004523	SP-P46	4.69	<0.01	7.19	<0.01	5.38	<0.01	7.50	<0.01	1.26	0.92	1.51	0.47
TC004535	SP-P50	3.26	<0.01	4.40	<0.01	3.69	<0.01	4.90	<0.01	0.79	0.97	0.69	0.94
TC005976	cSP-PSH	2.08	<0.01	2.96	<0.01	2.54	<0.01	3.08	<0.01	1.60	0.41	1.64	0.80
TC006033	SP-P68	1.57	<0.01	1.74	<0.01	1.37	<0.01	1.48	<0.01	1.09	1.00	1.02	1.00
TC006034	SP-P69	1.38	<0.01	2.19	<0.01	1.57	<0.01	2.57	<0.01	1.52	<0.01	1.53	0.02
TC008653	cSP-P83	20.28	<0.01	17.73	<0.01	40.65	<0.01	33.53	<0.01	2.15	1.00	1.41	1.00
TC008657	cSP-P84	12.81	0.18	15.19	0.02	5.11	0.24	3.06	0.79	3.13	1.00	3.97	1.00
TC009089	cSP-P90	1.84	0.47	2.24	<0.01	1.37	0.74	2.14	<0.01	1.03	1.00	0.95	1.00
TC009090	cSP-P91	2.04	<0.01	4.23	<0.01	2.12	<0.01	5.62	<0.01	1.29	0.95	1.39	1.00
TC009092	cSP-P93	3.31	<0.01	7.32	<0.01	2.92	<0.01	7.04	<0.01	1.19	0.99	1.06	1.00
TC009093	cSP-P94	0.82	1.00	0.49	<0.01	1.16	1.00	0.72	0.11	0.72	1.00	0.68	1.00
TC009094	cSP-P95	1.50	0.08	1.36	0.11	2.35	<0.01	2.00	<0.01	1.55	0.39	1.55	0.87
TC011078	cSP-P126	7.69	<0.01	8.28	<0.01	4.11	<0.01	5.81	<0.01	0.92	1.00	1.37	1.00
TC013277	cSP-P136	4.38	<0.01	5.84	<0.01	6.27	<0.01	7.46	<0.01	1.37	0.61	1.59	0.37
TC013326	cSP-P140	1.34	0.07	1.50	<0.01	1.79	<0.01	1.82	<0.01	0.93	0.93	1.05	1.00
TC015110	SP-P153/SR-A4	1.15	1.00	1.20	0.81	1.68	0.04	1.38	0.32	0.67	0.53	0.74	1.00
TC015295	SP-P156	1.66	1.00	12.17	<0.01	2.45	<0.01	12.49	<0.01	1.66	0.66	1.54	0.85
TC015297	SP-P157	0.75	0.98	0.62	0.03	0.82	0.54	0.66	<0.01	0.48	<0.01	0.64	0.07
TC002085	serpin2	0.63	0.29	0.75	0.50	0.64	0.19	0.61	0.01	0.82	0.77	0.86	1.00
TC005750	serpin18	0.57	<0.01	0.51	<0.01	0.56	<0.01	0.56	<0.01	0.45	<0.01	0.71	0.21
TC005751	serpin19	0.76	0.97	0.97	1.00	0.73	0.61	0.94	1.00	0.32	0.04	0.43	0.33
TC005752	serpin20	0.53	<0.01	0.62	0.03	0.74	0.36	0.74	0.49	0.57	0.11	0.69	0.86
TC005753	serpin21	0.67	0.82	0.44	<0.01	0.57	0.13	0.50	0.01	0.40	<0.01	0.41	0.14
TC005754	serpin22	3.40	<0.01	4.09	<0.01	3.64	<0.01	4.32	<0.01	0.87	1.00	0.87	1.00
TC006255	serpin24	7.70	<0.01	13.68	<0.01	8.81	<0.01	13.71	<0.01	1.39	0.06	1.60	<0.01

Supplementary Table 5-1: Significant differently expressed immune genes in *Tribolium castaneum* eggs. (part 2/3)
Green = induction ; Red = repression ; Black = not significant
SP = Serine protease ; SPH = Non catalytic serine protease ; cSP = Clip-domain serine protease

TC007869	serpin26	6.38	<0.01	14.76	<0.01	7.56	<0.01	17.97	<0.01	1.09	1.00	1.33	1.00
TC011718	serpin27	3.00	<0.01	5.38	<0.01	3.15	<0.01	5.23	<0.01	1.03	1.00	0.90	1.00
TC013310	serpin28	2.93	<0.01	4.44	<0.01	2.82	<0.01	3.80	<0.01	1.04	1.00	1.08	1.00
TC014237	serpin30	8.42	<0.01	7.51	<0.01	5.64	<0.01	5.47	<0.01	1.04	1.00	1.22	1.00
TC000520	Spz1	1.41	0.42	1.76	<0.01	1.43	0.17	1.45	0.08	1.08	1.00	1.09	1.00
TC001054	Spz2	10.86	0.01	58.65	<0.01	12.16	<0.01	42.30	<0.01	2.64	1.00	2.32	1.00
TC006726	Spz4	0.56	0.02	0.42	<0.01	0.76	0.30	0.51	<0.01	0.71	0.60	0.81	1.00
TC013304	Spz5	0.42	<0.01	0.23	<0.01	0.61	0.14	0.35	<0.01	0.56	0.69	0.55	0.91
Toll-signalling pathway													
TC004438	Toll3	1.58	<0.01	1.96	<0.01	2.01	<0.01	2.49	<0.01	0.91	0.97	0.95	1.00
TC000625	Toll9	1.34	1.00	1.19	1.00	0.56	0.12	0.50	0.03	0.67	0.43	0.67	0.77
TC008202	ML1	0.53	0.46	0.43	0.02	0.68	<0.01	0.59	<0.01	0.64	0.12	0.90	1.00
TC002003	Cactus	1.18	1.00	1.52	0.06	1.27	0.07	1.28	0.03	1.08	1.00	1.09	1.00
IMD-signalling pathway													
TC010851	IMD	2.02	<0.01	3.31	<0.01	1.78	<0.01	3.39	<0.01	0.92	1.00	0.94	1.00
TC014026	casps4	0.77	0.70	0.74	0.21	0.90	0.98	0.88	0.79	0.71	0.02	0.76	0.13
TC011191	REL1	1.04	1.00	2.08	<0.01	1.06	1.00	2.39	<0.01	1.29	0.03	1.38	0.04
TC014708	NFAT	0.39	<0.01	0.31	<0.01	0.39	<0.01	0.30	<0.01	0.32	<0.01	0.42	<0.01
JNK-signalling pathway													
TC010766	Puckered	1.28	1.00	1.77	0.06	1.22	0.63	1.46	<0.01	1.54	0.04	1.46	0.40
TC011870	Kay	1.27	1.00	1.93	0.08	1.23	0.28	1.52	<0.01	1.43	<0.01	1.49	0.05
Execution mechanisms													
TC007737	Attacin1	16.69	0.10	869.41	<0.01	56.07	<0.01	3696.08	<0.01	4.02	0.70	44.43	0.18
TC007738	Attacin2	12.25	0.60	3098.01	<0.01	6.62	0.21	2190.29	<0.01	0.47	1.00	5.28	1.00
TC006250	Defensin1	1.29	1.00	187.05	0.07	5.93	1.00	1551.17	<0.01	0.51	1.00	6.30	1.00
TC010517	Defensin2	22.67	0.78	1183.49	<0.01	Inf	0.07	Inf	<0.01	0.60	1.00	2.56	1.00
TC012469	Defensin3	16.53	0.24	907.69	<0.01	Inf	0.10	Inf	<0.01	1.84	1.00	8.26	1.00
TC000517	Thaumatin1	56.16	<0.01	89.30	<0.01	71.76	<0.01	103.03	<0.01	2.17	0.28	1.58	1.00
TC000499	Cecropin1	no hit	no hit	Inf	0.65	no hit	no hit	Inf	0.01	no hit	no hit	no hit	no hit
TC000500	Cecropin3	Inf	<0.01	Inf	<0.01	21.26	<0.01	48.59	<0.01	no hit	no hit	no hit	no hit
TC005093	Coleoptericin1	7.61	0.60	2392.39	<0.01	24.39	0.02	18067.24	<0.01	0.46	1.00	12.60	1.00
TC005096	Coleoptericin2	10.09	1.00	91.36	0.38	32.45	0.18	227.32	<0.01	1.04	1.00	7.30	0.91
TC000325	ProPO1	1.58	0.34	1.80	0.03	1.38	0.36	1.52	0.03	0.93	0.95	0.96	1.00
TC014907	ProPO2	0.84	1.00	0.72	0.59	0.74	<0.01	0.73	<0.01	0.91	0.63	0.88	0.71
TC005377	hexamerin4	13.49	<0.01	13.73	<0.01	16.05	<0.01	10.85	<0.01	22.95	<0.01	23.87	<0.01
TC011090	catalase2	0.96	1.00	0.88	0.99	0.54	<0.01	0.59	0.01	0.73	0.65	0.68	0.96
TC010362	Glutathione oxidase 1	1.47	0.16	1.79	<0.01	1.44	0.41	1.64	0.02	1.50	0.17	1.38	0.78
TC005493	Heme peroxidase 1	1.03	1.00	1.31	0.30	1.33	0.89	1.89	0.02	1.21	1.00	1.19	1.00
TC004551	Heme peroxidase 5	2.01	<0.01	2.79	<0.01	2.03	<0.01	2.74	<0.01	2.66	<0.01	2.32	<0.01
TC000175	Heme peroxidase 7	2.14	<0.01	2.25	<0.01	2.17	0.02	2.08	0.01	1.06	1.00	1.28	1.00
TC001556	Heme peroxidase 9	0.96	1.00	1.02	1.00	0.62	<0.01	0.61	<0.01	0.62	<0.01	0.62	<0.01
TC004592	Heme peroxidase 11	7.36	<0.01	12.07	<0.01	5.57	<0.01	8.83	<0.01	1.65	0.51	1.41	1.00
TC011676	Superoxide dismutase 2	1.31	0.03	1.20	0.21	1.24	0.24	1.09	0.80	0.92	1.00	0.92	1.00
TC011675	Superoxide dismutase 4	1.20	1.00	1.57	0.67	2.84	0.05	2.59	0.08	2.21	0.96	1.65	1.00
TC010356	Savenger receptor B13	3.10	<0.01	4.80	<0.01	3.33	<0.01	4.68	<0.01	6.06	<0.01	4.95	<0.01
TC012758	Savenger receptor B16	0.85	1.00	0.68	0.11	0.84	0.68	0.71	0.03	0.85	0.51	1.02	1.00
TC015854	Savenger receptor B2	1.32	0.16	1.60	<0.01	1.50	<0.01	1.86	<0.01	0.72	0.10	0.68	0.32
TC008210	Savenger receptor B3	9.30	<0.01	17.12	<0.01	24.52	<0.01	38.10	<0.01	0.66	1.00	1.13	1.00
TC014946	Savenger receptor B5	1.97	<0.01	2.77	<0.01	2.91	<0.01	3.51	<0.01	1.38	1.00	1.50	0.95
TC000948	Savenger receptor B6	1.15	1.00	0.51	0.21	0.94	0.98	0.46	<0.01	1.13	1.00	0.34	1.00
TC014954	Savenger receptor B9	0.72	0.17	0.67	0.02	0.80	0.12	0.72	<0.01	1.08	1.00	1.13	0.86
TC015640	Savenger receptor C	1.11	1.00	1.39	0.04	1.27	0.79	1.369	0.37	1.63	<0.01	1.50	0.15
TC011427	Nimrod A	0.70	1.00	0.45	0.03	0.48	<0.01	0.32	<0.01	0.49	0.02	0.55	0.34

Supplementary Table 5-1: Significant differently expressed immune genes in *Tribolium castaneum* eggs. (part 3/3)
Green = induction ; Red = repression ; Black = not significant
SP = Serine protease ; SPH = Non catalytic serine protease ; cSP = Clip-domain serine protease

Stress-related immune-responsive genes												
TC015563	apoD	7.50	<0.01	15.03	<0.01	6.39	<0.01	12.51	<0.01	1.11	1.00	1.15	1.00
TC010172	Hsp68	6.06	<0.01	9.53	<0.01	4.86	<0.01	7.53	<0.01	2.25	0.23	1.84	0.86
TC005338	Hsp27	29.05	0.37	66.54	0.07	28.20	<0.01	67.72	<0.01	7.53	<0.01	5.02	0.11
Total number of immune genes		**368**		**368**		**368**		**368**		**368**		**368**	
Total upregulated		**45**		**62**		**57**		**72**		**13**		**7**	
Total downregulated		**8**		**16**		**10**		**23**		**9**		**2**	

Supplementary Table 5-3: Primers used for qRT-PCR.

Gene	Forward primer	Reverse primer
Tribolium castaneum		
PGRP-LC	5'-GAAGGAACGCTCGCTAACCC-3'	5'-CGACAGCACCATCACACTCA-3'
SPH-H57	5'-ACCCATCCACAATATGCGGG-3'	5'-CGCGGGATTATTTTGGTCTCC-3'
SPH-H70	5'-GACAATTTCCGTGGCAGGTG-3'	5'-ACACCCACAGAATCCATTTCAT-3'
cSP-P8	5'-CCGAATGTGGAGTCCAGGAAG-3'	5'-AATGAGAGTTCCGCCACAGG-3'
Serpin 24	5'-TGCCACTGCTGTCATTTTCC-3'	5'-AAACCTCGGGCGAAACAACT-3'
Serpin 26	5'-GGGTGTCTGAACGGACCAAA-3'	5'-AAGGGACGTCTTTTCGTGCT-3'
Toll3	5'-AACCAGATTACGGGCAACTACA-3'	5'-TGCAGCTCTTGTAAGCCTATGA-3'
TC004646	5'-GCTGATCCTCGCACTGTGTA-3'	5'-GCGAAAACGACGAAGAATTTCATTT-3'
TC007763	5'-AGTCTTTTGTATGCGTAGCACTC-3'	5'-GTGCTGGTAGACGGGAACTG-3'
TC007858	5'-GCACATGGCGAGCCAGATTA-3'	5'-GTCTCTCCCACCCACAATGG-3'
TC008806	5'-ACGCTAGTGACTGTGTGGTC-3'	5'-CCAAAACTTTCCCGTTGCCT-3'
TC015479	5'-GACCCTCATCCTGTGCTGTC-3'	5'-GTTGTTGCACTCCGCTTCTT-3'

Chapter 6

Egg survival is reduced by grave-soil microbes in the carrion beetle, *Nicrophorus vespilloides*

Chris G.C. Jacobs*, Yin Wang*, Heiko Vogel, Andreas Vilcinskas, Maurijn van der Zee, Daniel E. Rozen
*Authors contributed equally to this work.

Published in BMC Evolutionary Biology (2014) 14 (1) 208-215,
doi: 10.1186/s12862-014-0208-x

Nicrophorus vespilloides **eggs are deposited into the soil in close proximity to the decomposing vertebrate carcasses that these insects use as an obligate resource to rear their offspring. Eggs in this environment potentially face significant risks from the bacteria that proliferate in the grave-soil environment following nutrient influx from the decomposing carcass. Our aims in this paper are twofold: first, to examine the fitness effects of grave-soil bacteria to eggs, and second, to quantify egg immunocompetence as a defence against these bacteria. Our results provide strong evidence that grave-soil microbes significantly reduce the survival of *Nicrophorus* eggs. Females provided with microbe rich carcasses to rear broods laid fewer eggs that were less likely to hatch than females given uncontaminated carcasses. Furthermore, we show that egg hatch success is significantly reduced by bacterial exposure. Using a split-brood design, which controlled for intrinsic differences in eggs produced by different females, we found that eggs washed free of surface-associated bacteria show increased survival compared to unwashed eggs. By contrast, eggs exposed to the entomopathogen *Serratia marcescens* show decreased survival compared to unexposed eggs. We next tested the immune competence of eggs under challenge from bacterial infection, and found that eggs lacked endogenous production of antimicrobial peptides, despite well-developed responses in larvae. Finally, we found that despite lacking immunity, *N. vespilloides* eggs produce an extraembryonic serosa, indicating that the serosa has lost its immune inducing capacity in this species. The dependency on ephemeral resources might strongly select for fast developing animals. Our results suggest that *Nicrophorus* carrion beetles, and other species developing on ephemeral resources, face a fundamental trade-off between egg immunity and development time.**

Key words: Trade-off; Burying beetle; egg immunity; developmental speed

Introduction

Exposure to harmful microbes poses numerous and diverse threats to developing animals (Brock et al., 2014). For animals with internal development, microbial pathogens that can directly harm the embryo can be controlled by the surveillance of maternal adaptive and innate immunity (Delves et al., 2011; King et al., 2007). By contrast, microbial defence in animals that develop externally is provided by barrier protection from the egg surface,

from maternally provided antimicrobials or through intrinsic immunity coordinated by the developing embryo (Rolff & Reynolds, 2009). These modes of protection have been extensively examined in vertebrates (Hasselquist & Nilsson, 2009). For example, avian egg shells provide direct physical protection against external microbial challenge, while mothers provision eggs prior to laying with a suite of general and specific antimicrobials, such as lysozyme, avidin and ovotransferrin (D'Alba et al., 2010), which provide crucial protection to the embryo prior to the maturation of the embryonic immune response. In invertebrates, parents can similarly invest in offspring defence via trans-generational immunity that provides diverse defences against pathogens and parasites that parents have encountered and which may pose specific threats to offspring (Hathaway et al., 2010). This can occur via deposition of antimicrobials onto the insect egg surface, or maternal provisioning of antimicrobials into the egg itself (Freitak et al., 2014; Hernández López et al., 2014; Moreau et al., 2012; Roth et al., 2010; Trauer & Hilker, 2013; Zanchi et al., 2012). In addition, embryos in some invertebrate species can also mount endogenous defences against pathogen challenge by producing antimicrobial peptides within eggs (Freitak et al., 2014; Gorman et al., 2004; Jacobs & van der Zee, 2013). However, this response is not universal and is notably absent in the well-studied model species *Drosophila* (Jacobs & van der Zee, 2013).

Here we examine the role of egg immunity in the burying beetle *Nicrophorus vespilloides*. This species is particularly suited for this investigation because eggs of this species face considerable challenge from the bacteria they encounter during development (Rozen et al., 2008). *Nicrophorus* species reproduce on small vertebrate cadavers which they bury in the soil after they are located through volatiles emitted from the carcass. Burying beetle eggs are laid into the soil adjacent to vertebrate carcasses (Scott, 1998). After a two-three day incubation, eggs hatch and larvae migrate to the carcass where they are communally reared by one or both parents (Smiseth et al., 2006). Caring parents regurgitate food to their developing larvae and also provide protection against insect competitors and predators (Lock et al., 2004; Scott, 1998). In addition, parents protect offspring against bacterial competitors growing on the decomposing carcass by depositing antimicrobial secretions, e.g. lysozyme, on the carcass surface (Arce et al., 2012; Cotter & Kilner, 2010; Hall et al., 2011; Reavey et al., 2014; Rozen et al., 2008). Parental lysozyme secretion peaks during brood rearing and significantly increases larval survival (Arce et al., 2012). Larvae also contribute to brood social immunity by secreting antimicrobials that inhibit bacterial growth (Arce et al., 2013; Reavey et al., 2014). They also show a progressive increase in humoral and cellular immunity through development (Urbański et al., 2014). Although different life stages of the burying beetle show both behavioural and immunological responses to reduce the negative effects of microbial challenge, studies of these responses to date have focussed on post-hatch behaviours and reductions in fitness (Cotter et al., 2010; Rozen et al., 2008; Steiger et al., 2011). However, pre-hatch reductions in fitness as a consequence of microbial exposure have not been studied; therefore, it remains unknown how or if eggs respond to the adverse environment in which they are laid.

In this study we investigated both the impact of soil-borne bacteria on egg development and the ability of the eggs to mount immune responses. We first measured the consequences of microbial challenge on pre-hatch fitness by assessing egg survival across contrasting environmental conditions. Next, we tested whether antimicrobial

peptide genes are expressed in burying beetle eggs in response to infection (Vogel et al., 2011). Briefly, we show that eggs are significantly harmed by exposure to microbes in grave soil and that eggs lack endogenous immunity. We discuss this lack of an immune response in the light of a trade-off with developmental speed.

Methods

General procedures

Experimental animals were taken from an outbred laboratory population derived from wild-caught *N. vespilloides* individuals trapped in Warmond near Leiden in The Netherlands, between May and June 2013. Beetles were maintained in the laboratory at 20°C with a 15:9 hour light:dark cycle. All adults were fed fresh chicken liver twice weekly. To collect eggs, non-sibling pairs of beetles were allowed to mate for 24 hours, after which the female was removed and provided with either a Fresh or Aged mouse carcass weighing 24-26g in a 15 cm x 10 cm plastic box filled with approximately 1-2 cm of soil. The state of found carcasses in the field across the breeding season remains unclear. Accordingly our treatments are meant to represent different extremes of the potential continuum of carcass decay. Following (Rozen et al., 2008), Fresh carcasses are defined as mice that were thawed after removal from the freezer and provided directly to mated females, while Aged carcasses were allowed to age for 7 days on top of commercial peat soil before mated females were added.

Egg survival

Mated females were provided with either a Fresh (n = 35) or Aged (n = 35) carcass in order to quantify the role of carcass age on egg number and survival. Commencing the morning following set-up, boxes with mice and females were visually inspected every 12 hours to determine the timing of egg appearance. 48 hours later, eggs were removed from the soil and allowed to hatch in petri plates at 20°C containing 1.5% water agar. Egg hatch was monitored every 3 hours until no further hatching was observed.

To examine the role of soil-borne microbes on egg hatch we carried out two different experiments using a split-brood design. In the first experiment, eggs were collected from the soil from females provided with a Fresh carcass (n = 32). Each brood with a minimum of 20 total eggs (n = 30) was split into two treatment groups. Half of each brood was gently rinsed in sterile water and then allowed to hatch on sterile 1% water agar. The other half of each brood was rinsed in a solution containing the entomopathogenic bacterium *Serratia marcescens* at a density of 10^8/ml, after which eggs were placed to hatch onto sterile water agar. The split-brood design allowed us to control for intrinsic differences in the hatch rate of broods from different females.

In the second experiment, eggs were collected from females provided with an Aged carcass. Using a split-brood design and with the same minimum threshold for inclusion of 20 eggs (n = 29), broods were divided into two treatment groups. A control group of washed eggs from each family was transferred to sterile water agar. The other half of each brood was first surface sterilized in an antimicrobial solution of hen egg-white lysozyme (1 mg/ml), streptomycin (500 µg/ml) and ampicillin (100 µg/ml), and then placed onto water agar plates to hatch. Previous experiments have shown that eggs thus treated are free of bacteria (Arce et al., 2013).

To assess the ability of *N. vespilloides* eggs to withstand desiccation we collected eggs from soil 15 hours after females were given a carcass. This cut-off was used to ensure that eggs were roughly of the same age. Eggs were placed onto 1% sterile water agar plates and incubated for 24 hours at 20 °C. Next, eggs were transferred to glass petri dishes and allowed to hatch at 20 °C with either 75% or 90% relative humidity (RH) in an environmental chamber. A separate set of eggs was retained on water agar as a control. The proportion of hatched eggs was scored after 3 days.

Experimental infection of *N. vespilloides* eggs and larva

To examine the capacity for eggs to mount an immune response against microbial challenge, eggs were experimentally infected with a concentrated solution of *Escherichia coli* and *Micrococcus luteus*. Eggs were collected 15 hours after females were provided with a fresh carcass and then kept at 20 °C for 24 hours on 1% sterile water agar. Next, eggs were pricked with a sterile 1 micron tip tungsten needle (Fine Science Tools) dipped into bacterial solution (septic injury) or with a sterile needle alone (sterile injury). After infection/sterile injury, eggs were incubated for 6 hours at 20 °C before RNA extraction. For larval infection we allowed eggs to hatch on 1% water agar. Larvae between 0-24 h old were then pricked with either a sterile needle or, with a needle previously dipped into the same bacterial solution as above. Larvae were incubated for 6 hours at 20°C before RNA extraction.

RNA extraction and real-time quantitative RT-PCR (qRT-PCR)

Total RNA of 5-10 eggs or larvae was extracted using TRIzol (Invitrogen) after which the RNA was purified and DNA digested on column with the RNeasy kit (Qiagen). The quality of the 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 (25 µl) contained 2.5 ng of cDNA, and the real-time detection and analyses were done using SYBR green dye chemistry with 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 15s, 60 °C for 30s, 72 °C for 30s. This was followed by 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 RPL7 mRNA amount. Total RNA for each treatment was isolated twice (biological replication) and each sample was measured by qRT-PCR twice (technical replication). Comparisons between treatments (untreated, sterile injury and septic injury) were performed within one brood.

Immune-related genes and primers used for qRT-PCR

Real-time PCR oligonucleotide primers were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) by applying the rules of highest maximum efficiency and sensitivity to avoid the formation of dimers, hairpins and other artefacts. The following immune-related genes were examined: Attacin 2, Defensin 1, Defensin 2, Coleoptericin 1, Coleoptericin 2, Coleoptericin 3 and the normalizer of qRT-PCR ribosomal protein 7 (RPL7). Sequences of immune-related genes were derived from (Vogel et al., 2011), and primer pairs of respective target genes were designed for qRT-PCR (Table 6-1).

Table 6-1: Primers for immune sequences of *Nicrophorus vespilloides*.

Gene	Forward primer	Reverse primer
Attacin2	5'-ACGTCACAGGAGAAGAGCTGA-3'	5'-TCGGAAGGCCTGTGTGTGTA-3'
Defensin1	5'-GTCGATACGCCCATCGGTTC-3'	5'-GCAATTGCAGACTCCGTCGA-3'
Defensin2	5'-AGAGGTGCATGCGATCTGTT-3'	5'-TGTGCCTTTGGTGTATCCGT-3'
Coleoptericin1	5'-CGAAACGGTGGTGAACAGGT-3'	5'-TGCATTGGTTGTACCGTCGG-3'
Coleoptericin2	5'-TGGTCTCCGCCGAATCCTAA-3'	5'-GCACCTGGTCTTTCGTGCTT-3'
Coleoptericin3	5'-ACTTTGGCGCGAGTCGATTT-3'	5'-TTGATCGCCCAACTCGCTTC-3'
RPL7	5'-TGCCATCAAGAAGCGCTCTG-3'	5'-GCGCTCTTGGCTTGATGGAT-3'

Embryo fixation and microscopy

The extraembryonic serosa in *Tribolium castaneum* is known to be involved in both desiccation resistance (Jacobs et al., 2013) and endogenous immune competence of the eggs (Jacobs & van der Zee, 2013). All insect species studied to date, with the exception of one group of higher flies (Rafiqi et al., 2008; Schmidt-Ott, 2000), develop a serosa (Roth, 2004). Embryonic development of *N. vespilloides* however, has not been studied. To examine the development of the serosa in *N. vespilloides*, fixed eggs were visualized under the confocal microscope (5x magnification). Eggs were placed onto 1% water agar plates at 20 °C and left for 24 hours to ensure that enough time had passed to develop the serosa. Next, eggs were fixed for 18 hours at room temperature in a solution of 4 ml phosphate buffered saline (PBS), 1 ml 37% formaldehyde and 5 ml of heptane. They were removed from the fixative and cut in half with a scalpel. The cut eggs were washed 3 times in PBS-Tween and then stained with DAPI for 2 hours at room temperature. After staining, the eggs were washed 3 times with PBS-T and embedded in glycerol on a glass bottom petri dish. Samples were studied with a Zeiss Cell Observer.

Results

Egg number and survival is reduced in the presence of an Aged carcass

Females that were provided with an Aged carcass laid significantly fewer eggs than females that were provided with a Fresh carcass (two-tailed Mann-Whitney U Test, P = 0.012, Figure 6-1a). In addition, the survival of eggs laid by females provided with an Aged carcass was significantly lower than the survival of eggs laid near a Fresh carcass (two-tailed Mann-Whitney U Test, P = 0.011, Figure 6-1a). Combining these to obtain an overall estimate of brood size, by taking the product of egg number and hatch proportion, we find that broods laid near to Fresh carcasses are significantly larger than those laid near to Old carcasses (Fresh: 32.57 ± 3.01 vs Old: 23.11 ± 2.61; two-tailed Mann-Whitney U Test, P = 0.005). Together these data show that pre-hatch fitness is reduced by the presence of an Aged carcass.

To test the idea that bacteria in the soil cause this reduction in survival, we split broods laid near an Aged carcass and surface-sterilized one half with an antimicrobial

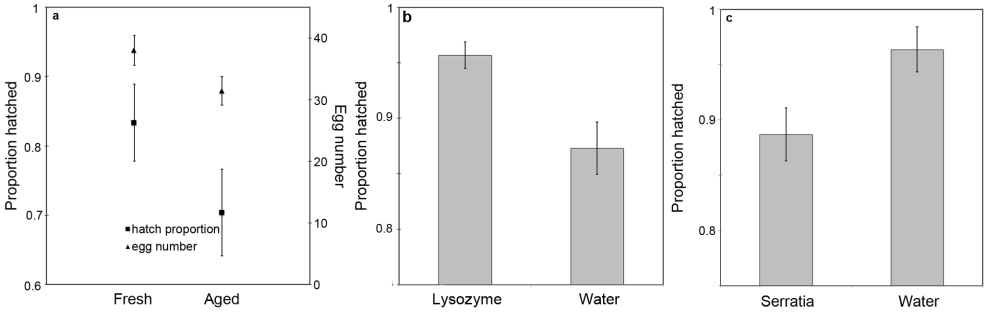


Figure 6-1: Egg survival and number under different treatments. a) Both egg number and egg survival are significantly lower when in the presence of an Aged carcass. b) Eggs collected from an Aged carcass show increased survival when sterilized, indicating the negative effect of high bacterial numbers surrounding Aged carcasses. c) Eggs collected from a Fresh carcass show decreased survival when experimentally exposed to the entomopathogenic bacterium *S. marcescens*.

solution while leaving the other half unsterilized. As predicted, if bacteria on the surface of eggs contributed to the failure of eggs to hatch, sterilizing eggs significantly increased egg survival when compared to washing eggs with water (paired t-test, df = 29, $p < 0.001$, Figure 6-1b). To further examine the idea that exposure to high bacterial numbers decreases pre-hatch fitness, we again used a split-brood design and experimentally exposed eggs laid near a Fresh carcass to the soil borne entomopathogen *S. marcescens* and compared these to eggs washed in water. Exposure to *S. marcescens* had a pronounced negative effect on pre-hatch fitness (paired t-test, df = 28, $p < 0.001$, Figure 6-1c). Notably, the reduction in survival following experimental infection, and the increase in survival following surface sterilization are roughly equivalent. Furthermore, these differences are similar to the differences first observed in untreated eggs laid near Aged and Fresh carcasses. Together, these data strongly indicate that harmful bacteria in the environment of Aged carcasses significantly reduce pre-hatch fitness.

Antimicrobial peptide expression in response to infection

Although survival of *N. vespilloides* eggs is reduced in the presence of an Aged carcass, overall egg viability is still quite high; approximately 70% of the eggs still survive even under these challenging conditions (Figure 6-1a). As we have previously shown that the eggs of *Tribolium castaneum* can induce antimicrobial peptide genes upon infection (Jacobs & van der Zee, 2013), induction of antimicrobial peptides might also increase survival in adverse conditions for the eggs of *N. vespilloides*. We measured gene expression of several antimicrobial peptides after both sterile injury and septic injury in *N. vespilloides* eggs and larva. Surprisingly, in eggs we found marginal, if any, upregulation of antimicrobial peptide genes after infection (Figure 6-2). Only one gene (Coleoptericin 2, Figure 6-2e) was induced over 10 fold after infection. By contrast, freshly emerged larvae show clear induction of all antimicrobial peptide genes tested (Figure 6-2). To verify that mRNA levels are lower in the eggs, we compared infected eggs with infected larvae. As expected, transcript levels are higher in larvae (Figure S6-1). These data show that although freshly emerged larvae can induce immune genes upon infection, eggs of *N. vespilloides* show very limited AMP inducing capacities.

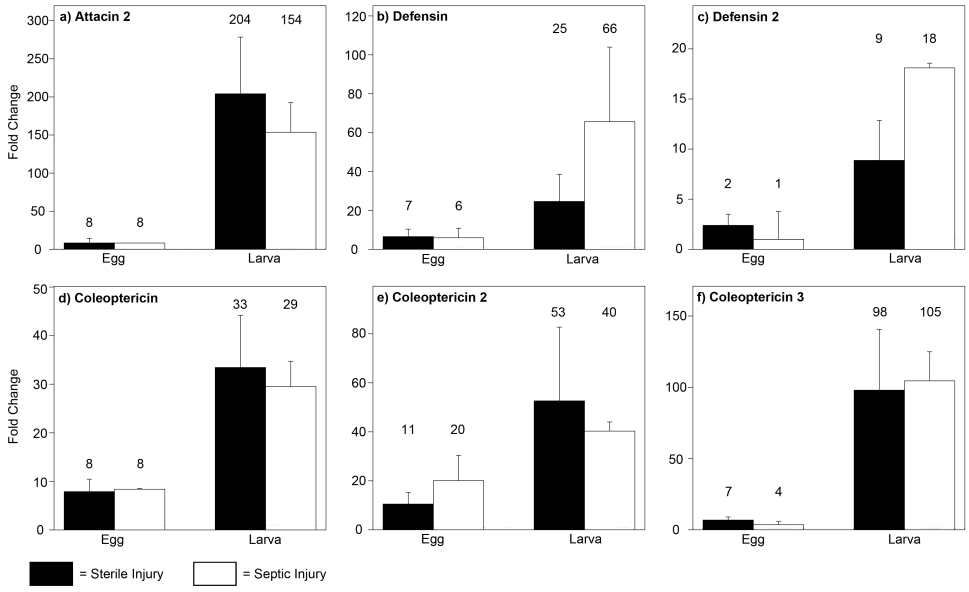


Figure 6-2: Expression of antimicrobial peptide genes in response to sterile injury (black bars) and septic injury (white bars). Whereas larvae show clear induction of all genes tested, eggs show hardly any induction of antimicrobial peptides at all. a) *Attacin 2* b) *Defensin 1* c) *Defensin 2* d) *Coleoptericin 1* e) *Coleoptericin 2* f) *Coleoptericin 3*

Eggs develop an extraembryonic serosa

The immune response of *Tribolium castaneum* eggs depends on the presence of an extraembryonic epithelium called the serosa (Jacobs & van der Zee, 2013). By contrast, the immune response is poor in eggs of the fruit fly *Drosophila melanogaster*, which lack this epithelium. Given the apparent absence of endogenous egg immunity in *N. vespilloides*, we hypothesized that this species, like *Drosophila*, would lack a serosal epithelium. We tested this idea in two ways, first by measuring desiccation tolerance of eggs, as the serosa imparts drought resistance in *T. castaneum* (Jacobs et al., 2013), and second by directly examining DAPI stained eggs via confocal microscopy. *N. vespilloides* eggs are highly susceptible to desiccation; egg survival dropped from 92% at 90% RH to 0% at 75% RH (chi-square test, $p < 0.001$). Although this result, together with the absence of endogenous immunity is consistent with the absence of a serosal epithelium, DAPI-stained confocal microscopy clearly revealed an epithelium around the egg (Figure 6-3a). This epithelium could easily be distinguished from the amnion in optical sections (Figure 6-3b) and was identified as serosa.

Discussion

Nicrophorus eggs are deposited into the soil in close proximity to vertebrate carcasses (Scott, 1998). Eggs in this grave-soil environment are exposed to increased nutrient fluxes from carcass decay that increases the biomass of endogenous bacteria and of bacteria that migrate to the soil from the perforated carcass (Carter et al., 2007). Several previous studies have documented the diverse and persistent negative effects of this flora on the

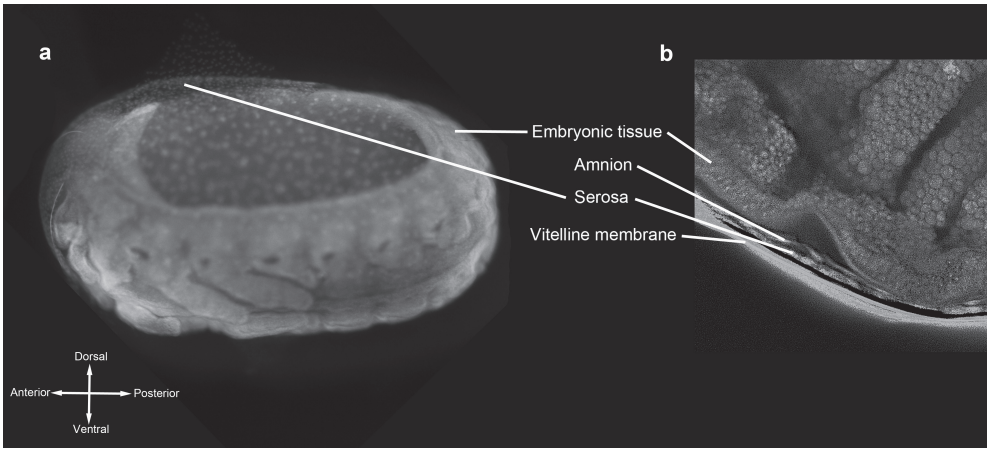


Figure 6-3: The eggs of *N. vespilloides* develop an extraembryonic serosa. a) Overview of a complete embryo, the developing head is visible at the anterior. The serosal epithelium can be clearly seen just above the head. b) Optical section of an *N. vespilloides* egg. The embryo, serosa, amnion and vitelline membrane can be clearly distinguished.

survival and growth of developing larvae (Cotter et al., 2010; McLean et al., 2014; Rozen et al., 2008; Steiger et al., 2011). Here we extend these findings by showing that carcass associated bacteria also significantly reduce the survival of *Nicrophorus* eggs. We found that females provided with an Aged carcass laid fewer eggs that were less likely to hatch than their female counterparts provided with a Fresh carcass (Figure 6-1a). In addition, we show that egg hatching success is a direct function of bacterial exposure; eggs washed free of surface-associated bacteria show increased survival compared to unwashed eggs (Figure 6-1b) while eggs washed in a bacterial solution show decreased survival compared to unexposed eggs (Figure 6-1c). The overall consequence of this exposure is an approximate 30% decline in potential brood size. This cost, in addition to those already identified at later stages of beetle development, clarify the risks to *Nicrophorus* of rearing young on microbe-rich contaminated carcasses.

The carcasses that *Nicrophorus* larvae rely upon are classical bonanza resources that are unpredictable in time and space. Parents modify the carcass in numerous ways that increase larval growth and survival. The carcass is buried, stripped of fur and coated with both antibacterial and antifungal compounds (Arce et al., 2012; Cotter & Kilner, 2010; Cotter et al., 2010; Degenkolb et al., 2011; Hall et al., 2011). In addition, parents defend the carcass before and following the arrival of larvae from insect competitors like flies or other carrion beetles (Rozen et al., 2008; Scott, 1998; Wilson & Fudge, 1984). In contrast to these elaborate behaviours used to defend larvae, there is surprisingly little direct evidence for parental defence of eggs. Earlier research failed to find any lysozyme-like activity inside or on the *N. vespilloides* egg (Arce et al., 2013), suggesting an absence of direct antimicrobial provisioning. And although antiseptic volatiles secreted by parents into the soil surrounding the carcass may provide an indirect benefit to eggs, this is as yet untested (Degenkolb et al., 2011).

Why is egg defence apparently missing in this species? One possible explanation is that explicit care of eggs trades-off with carcass maintenance and defence. Thus rather than investing in individual eggs, parents instead invest in preserving the resource that will provide an aggregate benefit to any larvae that survive the egg-stage and eventually

migrate to the carcass. Consistent with this idea, egg production in *Nicrophorus* does not appear to engender significant costs (Ward et al., 2009), the number of eggs observed in experimental *Nicrophorus* broods typically exceeds the number of larvae found on the carcass and infanticidal culling is common (Trumbo, 1990; Trumbo & Fernandez, 1995). It is likely that there is further mortality in the field where eggs face additional predation risks that are not present in the lab. Finally, by excreting antimicrobials on the carcass surface, parents can maintain the carcass in a suitable state for extended time periods, assuming it is found prior to significant decomposition. Also, because parents prefer a Fresh over an Aged carcass (Rozen et al., 2008), eggs may not have been selected to be able to cope with high levels of associated bacteria on extensively decomposed carrion.

A second possibility is that explicit defence is prohibitively expensive, especially when, even in its absence, egg survival is quite high (Figure 6-1c). This contrasts markedly with other species, like earwigs, where untended eggs challenged with mold infection show far more dramatic declines in hatch success (Boos et al., 2014). Although we do not know the cause for high rates of intrinsic survival, it is possible that this is facilitated by the barrier defence provided by the embryonic serosa (Figure 6-3). If so, this would be consistent with an immune-related function for the *Nicrophorus* serosa, even if the serosa in this species appears not to extensively regulate endogenous AMP production as it does for eggs of *Tribolium castaneum*. A challenge for future studies is to explicitly test this hypothesis using RNAi based targeted knock-outs of the developmental genes that regulate the production of this extraembryonic tissue.

Even in the absence of parental protection, eggs of some insects retain the capacity to generate an endogenous immune response against pathogen challenge (Freitak et al., 2014; Gorman et al., 2004; Jacobs & van der Zee, 2013); this is thought to be one important cause for the low incidence of parental care in insects (Royle et al., 2012; Zeh et al., 1989). Yet this endogenous response is absent in *N. vespilloides*. In that respect, there are striking similarities in development beween *N. vespilloides* and *D. melanogaster*. Both species lack inducible egg immunity and develop on ephemeral resources that favour rapid development times (Abasa, 1983; Jacobs & van der Zee, 2013; Scott, 1998), and specifically rapid embryonic development. Embryonic development in *Nicrophorus* is approximately 3-6 times faster than *Tribolium* and *Manduca* (Howe, 1956; Kingsolver & Nagle, 2007), and about 20 hours faster than *Aedes*, which are known to go into diapause, meaning they have to survive for a long time until the conditions favour hatching (Urbanski et al., 2010). By contrast, *Nicrophorus* develop in the presence of a highly valuable and decaying resource; individuals need to hatch, feed and disperse before the carcass is either claimed by another animal or becomes unsuitable for development. This strong selection for fast development might be reflected by a trade-off between a well-protected but slow developing egg and a fast-developing but less protected egg. Similar trade-offs between growth and immune competence are known from plants (Lozano-Durán et al., 2013), birds (Brommer, 2004) and insects (Diamond & Kingsolver, 2011; Siva-Jothy et al., 2005). Although additional experiments are needed to confirm the relation between rapid development and the lack of immune competence in insect eggs, the high survival and poor immune competence of both *N. vespilloides* and *D. melanogaster* eggs under normal conditions suggests that fast development is obtained at the expense of immune competence.

Conclusions

Our work builds upon previous studies demonstrating the profound costs to *N. vespilloides* from rearing their offspring in the presence of microbial competitors or pathogens in the soil environment. Although parental care in this species can serve to mitigate some of these risks, our data suggest that at least direct care does not extend to eggs. The indirect effects of fumigation with volatiles of the surrounding microhabitat might be important, however this conjecture requires further testing. The lack of direct parental provisioning of eggs may result from a trade-off between egg protection and carcass maintenance. Similarly, the lack of immune competence may be caused by a trade-off between immunity and the need for rapid growth on a rich and ephemeral resource. Although similar life-history trade-offs are known in a broad range of species, we are unaware of results showing this trade-off for eggs. This result therefore has broad implications owing to the obvious importance of egg survival for lifetime reproductive success, and suggests the need to investigate the development of immune competence more broadly as a function of developmental timing.

Acknowledgements

This work was supported by start-up funds from the University of Leiden to DER. YW was supported by a graduate fellowship from the China Scientific Council (CSC).

References

Abasa, R. O. (1983). Effects of temperature, relative humidity, lipid and water content on post-oviposition development of eggs of Stomoxys calcitrans. Entomologia Experimentalis Et Applicata, 33(3), 259-262.

Arce, A. N., Johnston, P. R., Smiseth, P. T., & Rozen, D. E. (2012). Mechanisms and fitness effects of antibacterial defences in a carrion beetle. Journal of Evolutionary Biology, 25(5), 930-937. doi: 10.1111/j.1420-9101.2012.02486.x

Arce, A. N., Smiseth, P. T., & Rozen, D. E. (2013). Antimicrobial secretions and social immunity in larval burying beetles, Nicrophorus vespilloides. Animal Behaviour, 86(4), 741-745. doi: http://dx.doi.org/10.1016/j.anbehav.2013.07.008

Boos, S., Meunier, J., Pichon, S., & Kölliker, M. (2014). Maternal care provides antifungal protection to eggs in the European earwig. Behavioral Ecology, 25(4), 754-761. doi: 10.1093/beheco/aru046

Brock, P. M., Murdock, C. C., & Martin, L. B. (2014). The History of Ecoimmunology and Its Integration with Disease Ecology. Integr Comp Biol. doi: 10.1093/icb/icu046

Brommer, J. E. (2004). Immunocompetence and its costs during development: an experimental study in blue tit nestlings. Proceedings of the Royal Society B-Biological Sciences, 271, S110-S113. doi: 10.1098/rsbl.2003.0103

Carter, D. O., Yellowlees, D., & Tibbett, M. (2007). Cadaver decomposition in terrestrial ecosystems. Naturwissenschaften, 94(1), 12-24. doi: 10.1007/s00114-006-0159-1

Cotter, S. C., & Kilner, R. M. (2010). Sexual division of antibacterial resource defence in breeding burying beetles, Nicrophorus vespilloides. Journal of Animal Ecology, 79(1), 35-43. doi: 10.1111/j.1365-2656.2009.01593.x

Cotter, S. C., Topham, E., Price, A. J. P., & Kilner, R. M. (2010). Fitness costs associated with mounting a social immune response. Ecology Letters, 13(9), 1114-1123. doi: 10.1111/j.1461-0248.2010.01500.x

D'Alba, L., Shawkey, M., Korsten, P., Vedder, O., Kingma, S., Komdeur, J., & Beissinger, S. (2010). Differential deposition of antimicrobial proteins in blue tit (Cyanistes caeruleus) clutches by laying order and male attractiveness. Behavioral Ecology and Sociobiology, 64(6), 1037-1045. doi: 10.1007/s00265-010-0919-y

Degenkolb, T., During, R. A., & Vilcinskas, A. (2011). Secondary metabolites released by the burying beetle Nicrophorus vespilloides: chemical analyses and possible ecological functions. J Chem Ecol, 37(7), 724-735. doi: 10.1007/s10886-011-9978-4

Delves, P. J., Martin, S. J., & Burton, D. R. (2011). Essentials : Roitt's Essential Immunology (12th Edition). Hoboken, NJ, USA: Wiley-Blackwell.

Diamond, S. E., & Kingsolver, J. G. (2011). Host plant quality, selection history and trade-offs shape the immune responses of Manduca sexta. Proceedings of the Royal Society B: Biological Sciences, 278(1703), 289-297. doi: 10.1098/rspb.2010.1137

Freitak, D., Schmidtberg, H., Dickel, F., Lochnit, G., Vogel, H., & Vilcinskas, A. (2014). The maternal transfer of bacteria can mediate trans-generational immune priming in insects. Virulence, 5(4), 547-554.

Gorman, M. J., Kankanala, P., & Kanost, M. R. (2004). Bacterial challenge stimulates innate immune responses in extra-embryonic tissues of tobacco hornworm eggs. Insect Molecular Biology, 13(1), 19-24.

Hall, C. L., Wadsworth, N. K., Howard, D. R., Jennings, E. M., Farrell, L. D., Magnuson, T. S., & Smith, R. J. (2011). Inhibition of Microorganisms on a Carrion Breeding Resource: The Antimicrobial Peptide Activity of Burying Beetle (Coleoptera: Silphidae) Oral and Anal Secretions. Environmental Entomology, 40(3), 669-678. doi: 10.1603/EN10137

Hasselquist, D., & Nilsson, J.-Å. (2009). Maternal transfer of antibodies in vertebrates: trans-generational effects on offspring immunity. Philosophical Transactions of the Royal Society B: Biological Sciences, 364(1513), 51-60. doi: 10.1098/rstb.2008.0137

Hathaway, J. J., Adema, C. M., Stout, B. A., Mobarak, C. D., & Loker, E. S. (2010). Identification of protein components of egg masses indicates parental investment in immunoprotection of offspring by Biomphalaria glabrata (gastropoda, mollusca). Dev Comp Immunol, 34(4), 425-435. doi: 10.1016/j.dci.2009.12.001

Hernández López, J., Schuehly, W., Crailsheim, K., & Riessberger-Gallé, U. (2014). Trans-generational immune priming in honeybees. Proceedings of the Royal Society B: Biological Sciences, 281(1785). doi: 10.1098/rspb.2014.0454

Howe, R. W. (1956). The effect of temperature and humidity on the rate of development and mortality of Tribolium Castaneum (Herbst) (Coleoptera, Tenebrionidae). Annals of Applied Biology, 44(2), 356-368.

Jacobs, C. G. C., Rezende, G. L., Lamers, G. E. M., & van der Zee, M. (2013). The extraembryonic serosa protects the insect egg against desiccation. Proceedings of the Royal Society B: Biological Sciences, 280(1764). doi: 10.1098/rspb.2013.1082

Jacobs, C. G. C., & van der Zee, M. (2013). Immune competence in insect eggs depends on the extraembryonic serosa. Developmental & Comparative Immunology, 41(2), 263-269. doi: http://dx.doi.org/10.1016/j.dci.2013.05.017

King, A. E., Paltoo, A., Kelly, R. W., Sallenave, J. M., Bocking, A. D., & Challis, J. R. G. (2007). Expression of Natural Antimicrobials by Human Placenta and Fetal Membranes. Placenta, 28(2–3), 161-169. doi: http://dx.doi.org/10.1016/j.placenta.2006.01.006

Kingsolver, J. G., & Nagle, A. (2007). Evolutionary divergence in thermal sensitivity and diapause of field and laboratory populations of manduca sexta. Physiol Biochem Zool, 80(5), 473-479. doi: 10.1086/519962

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25(4), 402-408. doi: 10.1006/meth.2001.1262

Lock, J. E., Smiseth, P. T., & Moore, A. J. (2004). Selection, Inheritance, and the Evolution of Parent-Offspring Interactions. The American Naturalist, 164(1), 13-24. doi: 10.1086/421444

Lozano-Durán, R., Macho, A. P., Boutrot, F., Segonzac, C., Somssich, I. E., Zipfel, C., & Nürnberger, T. (2013). The transcriptional regulator BZR1 mediates trade-off between plant innate immunity and growth. eLife, 2. doi: 10.7554/eLife.00983

McLean, A. H. C., Arce, A. N., Smiseth, P. T., & Rozen, D. E. (2014). Late-life and intergenerational effects of larval exposure to microbial competitors in the burying beetle Nicrophorus vespilloides. Journal of Evolutionary Biology, 27(6), 1205-1216. doi: 10.1111/jeb.12394

Moreau, J., Martinaud, G., Troussard, J.-P., Zanchi, C., & Moret, Y. (2012). Trans-generational immune priming is constrained by the maternal immune response in an insect. Oikos, 121(11), 1828-1832. doi: 10.1111/j.1600-0706.2011.19933.x

Rafiqi, A. M., Lemke, S., Ferguson, S., Stauber, M., & Schmidt-Ott, U. (2008). Evolutionary origin of the amnioserosa in cyclorrhaphan flies correlates with spatial and temporal expression changes of zen. Proceedings of the National Academy of Sciences of the United States of America, 105(1), 234-239. doi: 10.1073/pnas.0709145105

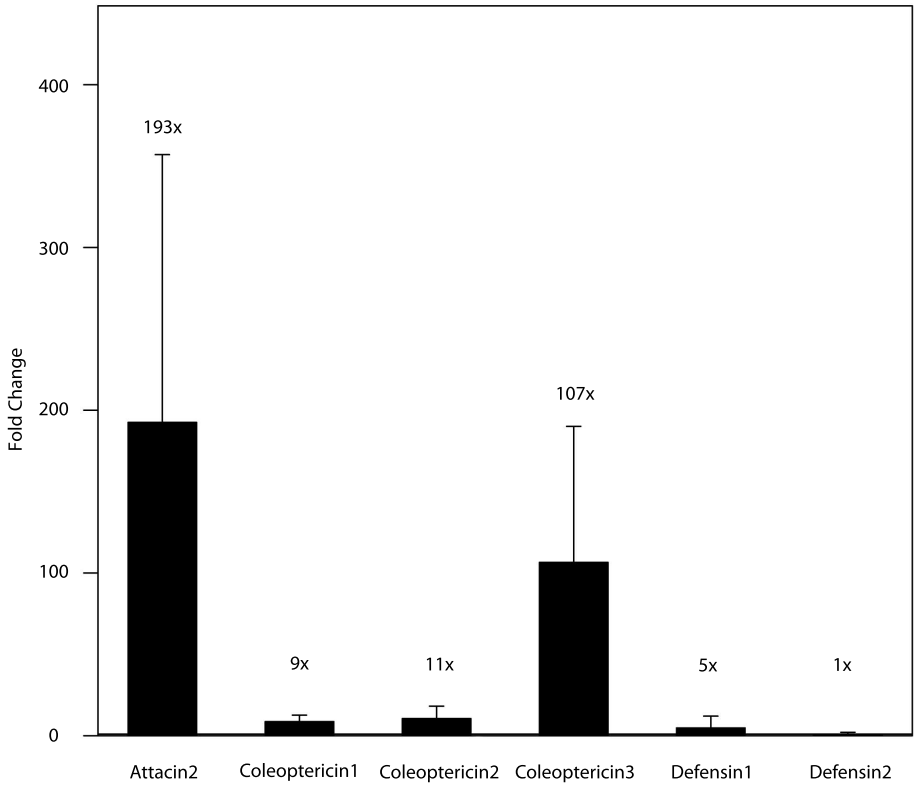
Reavey, Catherine E., Warnock, Neil D., Vogel, Heiko, & Cotter, S. C. (2014). Trade-offs between personal immunity and reproduction in the burying beetle, Nicrophorus vespilloides. Behavioral Ecology. doi: 10.1093/beheco/art127

Reavey, C. E., Beare, L., & Cotter, S. C. (2014). Parental care influences social immunity in burying beetle larvae. Ecological Entomology, 39(3), 395-398. doi: 10.1111/een.12099

Rolff, J., & Reynolds, S. E. (2009). Insect Infection and Immunity; Evolution, Ecology and Mechanisms (J. Rolff & S.

E. Reynolds Eds.): Oxford University Press.
Roth, O., Joop, G., Eggert, H., Hilbert, J., Daniel, J., Schmid-Hempel, P., & Kurtz, J. (2010). Paternally derived immune priming for offspring in the red flour beetle, Tribolium castaneum. Journal of Animal Ecology, 79(2), 403-413. doi: 10.1111/j.1365-2656.2009.01617.x
Roth, S. (2004). Gastrulation in other insects. Gastrulation: From Cells to Embryos (pp. 105-121). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
Royle, N. J., Smiseth, P. T., & Kölliker, M. (2012). The Evolution of Parental Care. Oxford: Oxford University Press.
Rozen, D. E., Engelmoer, D. J. P., & Smiseth, P. T. (2008). Antimicrobial strategies in burying beetles breeding on carrion. Proceedings of the National Academy of Sciences, 105(46), 17890-17895. doi: 10.1073/pnas.0805403105
Schmidt-Ott, U. (2000). The amnioserosa is an apomorphic character of cyclorrhaphan flies. Development Genes and Evolution, 210(7), 373-376.
Scott, M. P. (1998). THE ECOLOGY AND BEHAVIOR OF BURYING BEETLES. Annual Review of Entomology, 43(1), 595-618. doi: doi:10.1146/annurev.ento.43.1.595
Siva-Jothy, M. T., Moret, Y., & Rolff, J. (2005). Insect Immunity: An Evolutionary Ecology Perspective. In S. J. Simpson (Ed.), Advances in Insect Physiology (Vol. Volume 32, pp. 1-48): Academic Press.
Smiseth, P. T., Ward, R. J. S., & Moore, A. J. (2006). Asynchronous hatching in Nicrophorus vespilloides, an insect in which parents provide food for their offspring. Functional Ecology, 20(1), 151-156. doi: 10.1111/j.1365-2435.2006.01072.x
Steiger, S., Gershman, S. N., Pettinger, A. M., Eggert, A.-K., & Sakaluk, S. K. (2011). Sex differences in immunity and rapid upregulation of immune defence during parental care in the burying beetle, Nicrophorus orbicollis. Functional Ecology, 25(6), 1368-1378. doi: 10.1111/j.1365-2435.2011.01895.x
Trauer, U., & Hilker, M. (2013). Parental Legacy in Insects: Variation of Transgenerational Immune Priming during Offspring Development. PLoS ONE, 8(5), e63392. doi: 10.1371/journal.pone.0063392
Trumbo, S. T. (1990). REGULATION OF BROOD SIZE IN A BURYING BEETLE, NICROPHORUS-TOMENTOSUS (SILPHIDAE). Journal of Insect Behavior, 3(4), 491-500. doi: 10.1007/bf01052013
Trumbo, S. T., & Fernandez, A. G. (1995). Regulation of brood size by male parents and cues employed to assess resource size by burying beetles. Ethology Ecology & Evolution, 7(4), 313-322.
Urbański, A., Czarniewska, E., Baraniak, E., & Rosiński, G. (2014). Developmental changes in cellular and humoral responses of the burying beetle Nicrophorus vespilloides (Coleoptera, Silphidae). Journal of Insect Physiology, 60(0), 98-103. doi: http://dx.doi.org/10.1016/j.jinsphys.2013.11.009
Urbanski, J. M., Benoit, J. B., Michaud, M. R., Denlinger, D. L., & Armbruster, P. (2010). The molecular physiology of increased egg desiccation resistance during diapause in the invasive mosquito, Aedes albopictus. Proceedings of the Royal Society B: Biological Sciences, 277(1694), 2683-2692. doi: 10.1098/rspb.2010.0362
Vogel, H., Badapanda, C., & Vilcinskas, A. (2011). Identification of immunity-related genes in the burying beetle Nicrophorus vespilloides by suppression subtractive hybridization. Insect Molecular Biology, 20(6), 787-800. doi: 10.1111/j.1365-2583.2011.01109.x
Ward, R. J. S., Cotter, S. C., & Kilner, R. M. (2009). Current brood size and residual reproductive value predict offspring desertion in the burying beetle Nicrophorus vespilloides. Behavioral Ecology, 20(6), 1274-1281. doi: 10.1093/beheco/arp132
Wilson, D. S., & Fudge, J. (1984). Burying beetles: intraspecific interactions and reproductive success in the field. Ecological Entomology, 9(2), 195-203. doi: 10.1111/j.1365-2311.1984.tb00715.x
Zanchi, C., Troussard, J. P., Moreau, J., & Moret, Y. (2012). Relationship between maternal transfer of immunity and mother fecundity in an insect. Proc Biol Sci, 279(1741), 3223-3230. doi: 10.1098/rspb.2012.0493
Zeh, D. W., Zeh, J. A., & Smith, R. L. (1989). Ovipositors, Amnions and eggshell architecture in the diversification of terrestrial arthropods. Quarterly Review of Biology, 64(2), 147-168.

Supplementary information



Supplementary Figure 6-1: Expression differences between infected eggs and infected larvae. Expression of antimicrobial peptides in general is significantly higher in larvae than in eggs. Only the difference in *Defensin 2* was small, which is expected as it is the least induced antimicrobial peptide in this study.

Chapter 7

Summary, discussion and perspective

Summary

Insect eggs have likely played an important part in the origins of insect diversity. A common perception is that insect eggs are vulnerable and in need of maternal protection (discussed in Chapter 1). However, most insect eggs develop an extraembryonic membrane called the serosa (Roth, 2004). This membrane has been hypothesized to protect the egg against desiccation and infection (Chen et al., 2000; Goltsev et al., 2009; Rezende et al., 2008; Vargas et al., 2014). The impossibility to remove the zygotic serosa without removing the maternal eggshell, has so far prevented experimental assessment of these protective functions. Exploiting the unique possibility to prevent serosal development by *Tc-zen1* RNAi in *T. castaneum* (van der Zee et al., 2005), I was able to test the protective value of the serosa and show that it protects against desiccation and infection.

The serosa as protective layer against desiccation

In **CHAPTER 2**, I addressed the protective value of the serosa and the cuticle it secretes against desiccation (Jacobs et al., 2013). By the use of Transmission Electron Microscopy (TEM), I show the presence of a serosal cuticle in *T. castaneum*. I furthermore show that, when absent, eggs are less likely to survive in dry conditions. By knocking down *chitin synthase 1* (*Tc-chs1*), which is crucial for the synthesis of the major cuticle component chitin (Arakane et al., 2004), I was able to test the protective value of the serosal cuticle without removing the serosa itself. I showed a crucial role of the serosal cuticle in the protection against dehydration.

Next, in **CHAPTER 3**, I assessed whether the same genes are used to build both the adult and serosal cuticle. In adult insects, cuticle structure is affected by the proteins Knickkopf, Retroactive and Laccase2 (Arakane et al., 2005; Chaudhari et al., 2011; Chaudhari et al., 2013). Using RNAi of these cuticle genes and studying serosal cuticle structure with Transmission Electron Microscopy, I show that Knickkopf and Retroactive fulfill the same function in eggs as they do in the adult cuticle. I furthermore show that when any of these three genes is knocked down, survival of eggs in dry circumstances decreases. I furthermore analyzed transcriptome data (from Chapter 5) and detect several cuticle genes that are specifically expressed in the serosa. Together, these data show that the serosal cuticle utilizes the same genetic machinery as the adult cuticle. I provide experimental evidence that cuticle structure is important for its waterproofing ability.

The serosa as protective layer against infection

In **CHAPTER 4**, I investigated the immune response of eggs and adults of *Tribolium castaneum* and *Drosophila melanogaster* (Jacobs & van der Zee, 2013). Contrary to *T. castaneum* eggs, *D. melanogaster* eggs do not develop a serosa (Rafiqi et al., 2008;

Schmidt-Ott, 2000). If induction of immune genes is specific for the serosal epithelium, no induction would be found in the *D. melanogaster* egg. I show that indeed both adults and eggs of *Tribolium castaneum* can induce immune genes upon infection. However, only *Drosophila melanogaster* adults are able to respond to infection. When serosal development in *T. castaneum* is prevented by *Tc-zen1* RNAi, eggs completely lose immune responsiveness. These data provide strong support for an immune function of the serosa.

In **CHAPTER 5**, I characterize the *T. castaneum* egg immune response more extensively. First I show that bacteria propagate twice as fast in eggs without a serosa than in eggs with serosa. Next, by RNA sequencing, I show that *T. castaneum* eggs are capable of inducing the full complement of immune genes. Furthermore, this response is completely lacking in eggs without a serosa. By comparing the transcriptome of unchallenged wild-type eggs with unchallenged serosa-less eggs, I show that this lack of response is most likely due to the absence of genes involved in the recognition of bacteria. Finally, by *in situ* hybridization I show that immune genes are expressed in the serosal epithelium. I confirm both constitutive and induced expression of immune genes in the serosa, showing that it is the serosa itself that expresses immune genes.

In **CHAPTER 6**, I put my data in evolutionary perspective. A well protected egg is likely costly and there are clear trade-offs of growth with immunity (Diamond & Kingsolver, 2011; Siva-Jothy et al., 2005). As eggs of *Drosophila melanogaster* develop extremely rapidly (Al-Saffar et al., 1995), it is plausible that *Drosophila melanogaster* sacrificed immune competence in the egg for fast growth. To assess whether life-history traits could influence the protective value of the serosa, I tested immune gene induction in eggs of the burying beetle (*Nicrophorus vespilloides*). This beetle has a similar life history as *D. melanogaster*; both depend on ephemeral resources for reproduction and develop extremely quickly (Smiseth et al., 2006). First, I confirm that a serosa is indeed present in this species. Then I show that survival of eggs is negatively influenced by high bacterial numbers in the environment. I could both counteract this negative influence by sterilizing the eggs and reenact this effect by experimentally exposing eggs to bacteria. I also found that, much like in *Drosophila*, immune gene expression is absent in the eggs. Furthermore, eggs are unable to survive in conditions dryer than 90% RH. I conclude that the lack of immune responsiveness of the serosa in this species is likely the results of a trade-off between fast development and the immune response. Selection for fast development might have selected against immune responsiveness in *N. vespilloides* and might have caused the loss of the serosa in *D. melanogaster*.

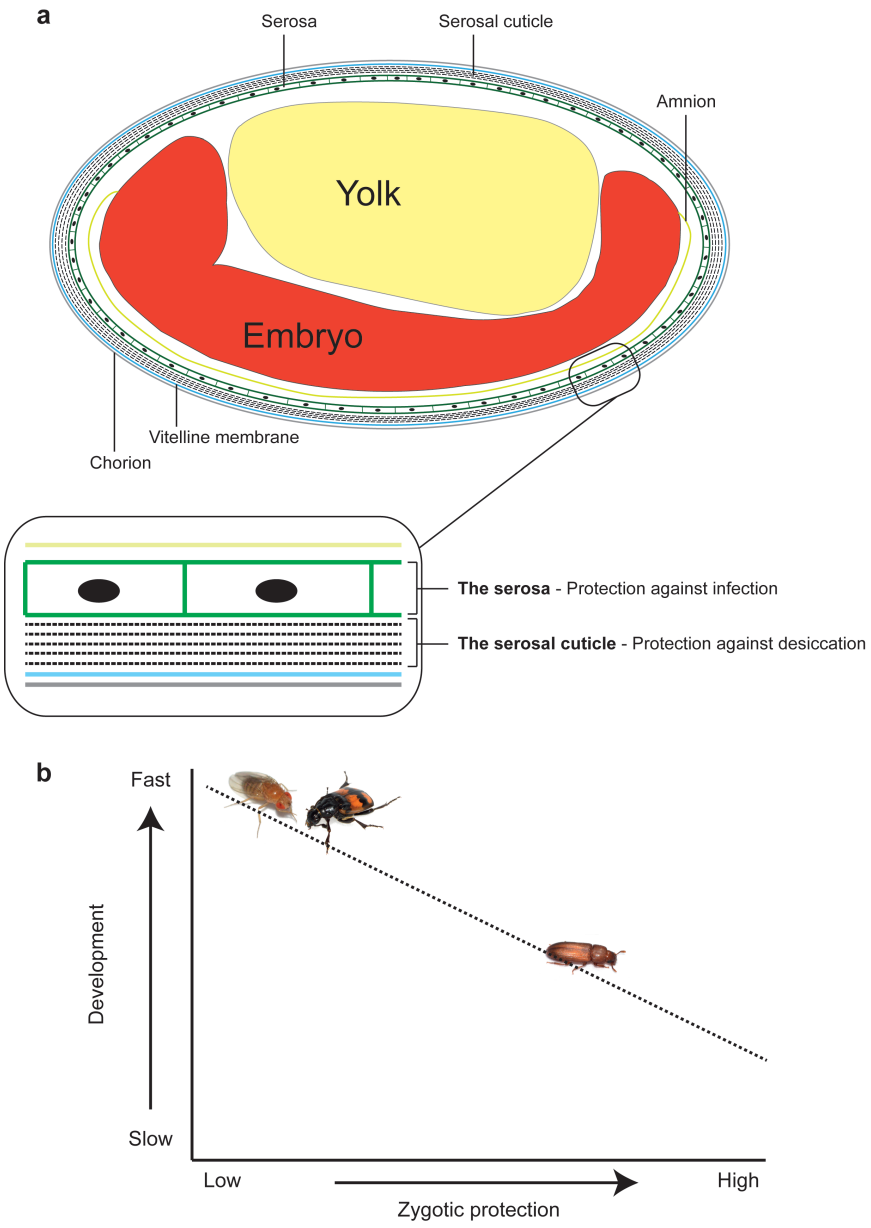


Figure 7-1: Summary of the main findings in this thesis. a) In this thesis I have shown that the serosal cuticle in *Tribolium castaneum* protects against dehydration (Chapter 3 and 4). I have also shown that the serosa itself protects the embryo against infection (Chapter 5 and 6). b) In Chapter 6 I have shown that not all insect eggs are well protected by the serosa and propose that there might be a trade-off between developmental speed and a well protected egg.

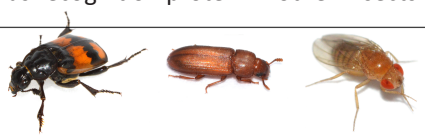
Discussion and Perspective

In this thesis, I have shown that insect eggs are far from helpless. The serosa, which is found in almost all insects, protects against both desiccation and infection. The serosal cuticle protects the egg from desiccation and is similar to the adult cuticle (Chapter 2 and 3). Although we find a clear negative effect of the lack of a serosal cuticle, this effect might be much larger for different insect species. While *T. castaneum* develops in approximately 3 days at 35 °C (Howe, 1956), many insect eggs take much longer to complete embryogenesis (Howe, 1967). Longer development means a longer period in which they must survive dry conditions. Future experiments testing the desiccation resistance conferred by the serosal cuticle in slower developing insects are needed to confirm this hypothesis. Preventing serosal development by *zen*-knockdown has proven lethal in other insects (Panfilio, 2009), however knockdown of *chitin synthase* to test the function of the serosal cuticle directly might prove fruitful.

The structure of the cuticle plays a less severe but significant role in the waterproofing ability of the insect egg (Chapter 3). Knockdown of *Tc-knk1* or *Tc-rtv* which effects cuticle structure, also influences chitin levels in the cuticle (Chaudhari et al., 2011; Chaudhari et al., 2013; Chaudhari et al., 2014). The decrease in chitin levels are caused by chitinase (Chaudhari et al., 2011; Chaudhari et al., 2013; Chaudhari et al., 2014; Zhu et al., 2008). The decreased survival of eggs at low humidity might be due to this decreased chitin level rather than the loss of cuticle structure. For future studies, it will be useful to perform double knockdowns of *Tc-knk1* and *chitinase* to check whether the loss of structure influences its waterproofing ability or the reduced chitin content. I identified three *chitinases* that have a serosa-specific expression (6, 7 and 10, Table 3-2). Of these *chitinases*, *chitinase 10* is be the most promising candidate while it was important for survival in all developmental stages (Zhu et al., 2008). The possibility to knockdown genes by RNAi and experimentally assess the effect of cuticle structure on survival make *T. castaneum* an exciting model system for the functional analysis of cuticle structure.

In Chapters 4 and 5, I show that the serosa protects the insect egg against infection. The expression of immune genes is localized in the serosa. However, all the genes involved in the intracellular signal transduction are also expressed in the embryo itself. Future experiments are needed to uncover the exact mechanism that prevents the embryo from mounting an immune response while inducing this response in the serosa. A likely mechanisms is the lack of recognition proteins in the embryo as described in Chapter 5. Localization experiments of the recognition proteins will clarify whether these are translocated to the perivitelline space (the space between the serosa and the vitelline membrane). Localization of these proteins to the perivitelline space would prevent the embryo from responding to infection while the serosa does respond.

Many immune genes have been functionally tested in *Drosophila* (Ferrandon, 2013; Ferrandon et al., 2007; Hoffmann, 2003; Lemaitre & Hoffmann, 2007; Lemaitre et al., 1997; Leulier & Lemaitre, 2008; Ligoxygakis, 2013). Most knowledge about immune genes in *Tribolium* comes from sequence similarity (Zou et al., 2007). Several aspects of the immune response in *Tribolium* seem different from *Drosophila*. The almost undetectable levels of PGRP-SA in uninfected state and the high upregulation after infection, indicate that it might be an effector rather than a recognition protein (Chapter 5). PGRP-SA does function as recognition protein in other insects (Kim et al., 2008; Royet et



al., 2011). So PGRP-SA warrants further study to discover its role in the *Tribolium* immune response. Another surprise in *Tribolium* is the immune responsiveness of *toll3* (Chapter 5, Altincicek et al., 2013; Behrens et al., 2014). Toll1 is the main immune receptor for bacterial infections in Drosophila (Leulier & Lemaitre, 2008) and is also involved in dorsal-ventral patterning (Anderson et al., 1985). My data suggests that in *Tribolium*, it is *toll3* that regulates the immune response. However, future functional experiments are needed to confirm this hypothesis. The differences mentioned here are just a few examples. Extensive examination of the *Tribolium* immune response is necessary to assess how applicable data collected in *Drosophila* are for insects in general.

In Chapter 6 I show that immune responsiveness of the serosa might not be true for all insects. One of the factors that might play a role is the fact that *Nicrophorus vespilloides* shows extensive parental care (Scott, 1998). Much focus has been put on parental investment in protecting insect eggs (Hilker & Meiners, 2002). Parental investment might indeed reduce the need for a self-sufficient egg that is able to mount an immune response itself. However, parental care is uncommon in insects (Zeh et al., 1989). Although *Nicrophorus vespilloides* eggs do not show an serosa-induced immune response, this might be an exception. Screening for an endogenous immune response in insect eggs from diverse phyla will teach us whether the self-sufficient egg is common throughout the Insecta.

Another explanation for the lack of an immune response in *Nicrophorus vespilloides* might be its fast development. Living on ephemeral resources that are available only for short periods puts high selective pressure on quick development. Immune responses are costly and likely trade-off with developmental speed. A correlation between immune responsiveness and developmental time could be made when enough species have been screened. However, to show that it is the pressure to develop quickly which trades-off with immune responsiveness, selection experiments for quick development have to be performed.

Conclusions

In conclusion, I have shown that the insect egg is not as vulnerable as is generally thought. My data are likely to spark many new experiments by a wide array of scientists currently focusing on the parental defense of the insect egg. Much remains to be discovered mechanistically, ecologically as well as evolutionary. Future studies will certainly teach us much more on how insects are able to survive almost anywhere.

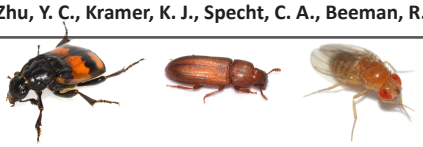
References

Al-Saffar, Z. Y., Grainger, J. N. R., & 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(5), 389-397.

Altincicek, B., Elashry, A., Guz, N., Grundler, F. M., Vilcinskas, A., & Dehne, H. W. (2013). Next generation sequencing based transcriptome analysis of septic-injury responsive genes in the beetle Tribolium castaneum. PLoS ONE, 8(1), e52004. doi: 10.1371/journal.pone.0052004

Anderson, K. V., Jürgens, G., & Nüsslein-Volhard, C. (1985). Establishment of dorsal-ventral polarity in the Drosophila embryo: Genetic studies on the role of the Toll gene product. Cell, 42(3), 779-789. doi: http://dx.doi.org/10.1016/0092-8674(85)90274-0

Arakane, Y., Hogenkamp, D. G., Zhu, Y. C., Kramer, K. J., Specht, C. A., Beeman, R. W., . . . Muthukrishnan, S.



(2004). Characterization of two chitin synthase genes of the red flour beetle, Tribolium castaneum, and alternate exon usage in one of the genes during development. Insect Biochemistry and Molecular Biology, 34(3), 291-304. doi: 10.1016/j.ibmb.2003.11.004

Arakane, Y., Muthukrishnan, S., Beeman, R. W., Kanost, M. R., & Kramer, K. J. (2005). Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. Proceedings of the National Academy of Sciences of the United States of America, 102(32), 11337-11342. doi: 10.1073/pnas.0504982102

Behrens, S., PeuSZ, R., Milutinovi, B., Eggert, H., Esser, D., Rosenstiel, P., . . . Kurtz, J. (2014). Infection routes matter in population-specific responses of the red flour beetle to the entomopathogen Bacillus thuringiensis. BMC Genomics, 15(1), 445.

Chaudhari, S. S., Arakane, Y., Specht, C. A., Moussian, B., Boyle, D. L., Park, Y., . . . Muthukrishnan, S. (2011). Knickkopf protein protects and organizes chitin in the newly synthesized insect exoskeleton. Proceedings of the National Academy of Sciences. doi: 10.1073/pnas.1112288108

Chaudhari, S. S., Arakane, Y., Specht, C. A., Moussian, B., Kramer, K. J., Muthukrishnan, S., & Beeman, R. W. (2013). Retroactive Maintains Cuticle Integrity by Promoting the Trafficking of Knickkopf into the Procuticle of Tribolium castaneum. PLoS Genet, 9(1), e1003268. doi: 10.1371/journal.pgen.1003268

Chaudhari, S. S., Moussian, B., Specht, C. A., Arakane, Y., Kramer, K. J., Beeman, R. W., & Muthukrishnan, S. (2014). Functional Specialization Among Members Of Knickkopf Family Of Proteins In Insect Cuticle Organization. PLoS Genet, 10(8), e1004537. doi: 10.1371/journal.pgen.1004537

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(23), 5145-5156.

Diamond, S. E., & Kingsolver, J. G. (2011). Host plant quality, selection history and trade-offs shape the immune responses of Manduca sexta. Proceedings of the Royal Society B: Biological Sciences, 278(1703), 289-297. doi: 10.1098/rspb.2010.1137

Ferrandon, D. (2013). The complementary facets of epithelial host defenses in the genetic model organism Drosophila melanogaster: from resistance to resilience. Current Opinion in Immunology, 25(1), 59-70. doi: http://dx.doi.org/10.1016/j.coi.2012.11.008

Ferrandon, D., Imler, J. L., Hetru, C., & Hoffmann, J. A. (2007). The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. Nature Reviews Immunology, 7(11), 862-874. doi: 10.1038/nri2194

Goltsev, Y., Rezende, G. L., Vranizan, K., Lanzaro, G., Valle, D., & Levine, M. (2009). Developmental and evolutionary basis for drought tolerance of the Anopheles gambiae embryo. Developmental Biology, 330(2), 462-470. doi: 10.1016/j.ydbio.2009.02.038

Hilker, M., & Meiners, T. (2002). Chemoecology of Insect Eggs and Egg Deposition. Berlin: Blackwell Publishing.

Hoffmann, J. A. (2003). The immune response of Drosophila. Nature, 426(6962), 33-38. doi: 10.1038/nature02021

Howe, R. W. (1956). The effect of temperature and humidity on the rate of development and mortality of Tribolium Castaneum (Herbst) (Coleoptera, Tenebrionidae). Annals of Applied Biology, 44(2), 356-368.

Howe, R. W. (1967). TEMPERATURE EFFECTS ON EMBRYONIC DEVELOPMENT IN INSECTS. Annual Review of Entomology, 12, 15-&.

Jacobs, C. G. C., Rezende, G. L., Lamers, G. E. M., & van der Zee, M. (2013). The extraembryonic serosa protects the insect egg against desiccation. Proceedings of the Royal Society B: Biological Sciences, 280(1764). doi: 10.1098/rspb.2013.1082

Jacobs, C. G. C., & van der Zee, M. (2013). Immune competence in insect eggs depends on the extraembryonic serosa. Developmental & Comparative Immunology, 41(2), 263-269. doi: http://dx.doi.org/10.1016/j.dci.2013.05.017

Kim, C.-H., Kim, S.-J., Kan, H., Kwon, H.-M., Roh, K.-B., Jiang, R., . . . Lee, B. L. (2008). A Three-step Proteolytic Cascade Mediates the Activation of the Peptidoglycan-induced Toll Pathway in an Insect. Journal of Biological Chemistry, 283(12), 7599-7607. doi: 10.1074/jbc.M710216200

Lemaitre, B., & Hoffmann, J. (2007). The Host Defense of Drosophila melanogaster. Annual Review of Immunology, 25(1), 697-743. doi: doi:10.1146/annurev.immunol.25.022106.141615

Lemaitre, B., Reichhart, J.-M., & Hoffmann, J. A. (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.

Leulier, F., & Lemaitre, B. (2008). Toll-like receptors- taking an evolutionary approach. Nature Reviews Genetics, 9(3), 165-178.

Ligoxygakis, P. (2013). Genetics of immune recognition and response in Drosophila host defense. Adv Genet, 83, 71-97. doi: 10.1016/b978-0-12-407675-4.00002-x

Panfilio, K. A. (2009). Late extraembryonic morphogenesis and its zen(RNAi)-induced failure in the milkweed bug

Oncopeltus fasciatus. Developmental Biology, 333(2), 297-311. doi: 10.1016/j.ydbio.2009.06.036
Rafiqi, A. M., Lemke, S., Ferguson, S., Stauber, M., & Schmidt-Ott, U. (2008). Evolutionary origin of the amnioserosa in cyclorrhaphan flies correlates with spatial and temporal expression changes of zen. Proceedings of the National Academy of Sciences of the United States of America, 105(1), 234-239. doi: 10.1073/pnas.0709145105
Rezende, G. L., Martins, A. J., Gentile, C., Farnesi, L. C., Pelajo-Machado, M., Peixoto, A. A., & Valle, D. (2008). Embryonic desiccation resistance in Aedes aegypti: presumptive role of the chitinized Serosal Cuticle. Bmc Developmental Biology, 8(82). doi: 10.1186/1471-213x-8-82
Roth, S. (2004). Gastrulation in other insects. Gastrulation: From Cells to Embryos (pp. 105-121). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
Royet, J., Gupta, D., & Dziarski, R. (2011). Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. Nat Rev Immunol, 11(12), 837-851.
Schmidt-Ott, U. (2000). The amnioserosa is an apomorphic character of cyclorrhaphan flies. Development Genes and Evolution, 210(7), 373-376.
Scott, M. P. (1998). THE ECOLOGY AND BEHAVIOR OF BURYING BEETLES. Annual Review of Entomology, 43(1), 595-618. doi: doi:10.1146/annurev.ento.43.1.595
Siva-Jothy, M. T., Moret, Y., & Rolff, J. (2005). Insect Immunity: An Evolutionary Ecology Perspective. In S. J. Simpson (Ed.), Advances in Insect Physiology (Vol. Volume 32, pp. 1-48): Academic Press.
Smiseth, P. T., Ward, R. J. S., & Moore, A. J. (2006). Asynchronous hatching in Nicrophorus vespilloides, an insect in which parents provide food for their offspring. Functional Ecology, 20(1), 151-156. doi: 10.1111/j.1365-2435.2006.01072.x
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(7), 624-636.
Vargas, H. C. M., Farnesi, L. C., Martins, A. J., Valle, D., & Rezende, G. L. (2014). Serosal cuticle formation and distinct degrees of desiccation resistance in embryos of the mosquito vectors Aedes aegypti, Anopheles aquasalis and Culex quinquefasciatus. Journal of Insect Physiology, 62(0), 54-60. doi: http://dx.doi.org/10.1016/j.jinsphys.2014.02.001
Zeh, D. W., Zeh, J. A., & Smith, R. L. (1989). Ovipositors, Amnions and eggshell architecture in the diversification of terrestrial arthropods. Quarterly Review of Biology, 64(2), 147-168.
Zhu, Q., Arakane, Y., Beeman, R. W., Kramer, K. J., & Muthukrishnan, S. (2008). Functional specialization among insect chitinase family genes revealed by RNA interference. Proceedings of the National Academy of Sciences, 105(18), 6650-6655. doi: 10.1073/pnas.0800739105
Zou, Z., Evans, J. D., Lu, Z. Q., Zhao, P. C., Williams, M., Sumathipala, N., . . . Jiang, H. B. (2007). Comparative genomic analysis of the Tribolium immune system. Genome Biology, 8(8). doi: 10.1186/gb-2007-8-8-r177

Chapter 8

Nederlandse samenvatting - Summary in Dutch

De meest soortenrijke groep op aarde zijn de insecten. Ongeveer driekwart van de ongeveer anderhalf miljoen beschreven soorten zijn insecten (Figuur 1-1). Er zijn veel ideeën over waarom insecten zo ontzettend succesvol zijn. Zo kunnen ze bijvoorbeeld heel makkelijk op nieuwe plekken komen doordat ze kunnen vliegen, maar ook co-evolutie met de planten zou een grote rol hebben gespeeld. Zo zijn er nog veel meer aspecten van insecten die in meer of mindere mate hebben bijgedragen aan de enorme soortenrijkdom van de insecten. De rol van de eieren van insecten in hun succes heeft echter weinig aandacht gekregen. Dit komt waarschijnlijk omdat het insectenei doorgaans gezien wordt als een weerloos stadium van ontwikkeling. Zonder de mogelijkheid om zichzelf te beschermen zijn ze afhankelijk van de verscheidene mechanismes van de moeder om het ei te beschermen. Dit kan door de productie van een stevige eischaal door de moeder, door de uitscheiding van beschermende stofjes op het ei door de moeder en ook door de actieve bescherming van de eieren door de ouders. Veel onderzoekers hebben zich verdiept in deze manieren van bescherming. In dit proefschrift laat ik zien dat hoewel sommige insecteneieren behoorlijk weerloos zijn, zijn andere insecteneieren lang niet zo weerloos zijn als gedacht.

De serosa

Bijna alle insecteneieren ontwikkelen een cellaag rondom het ei, genaamd de serosa (Figuur 1-2). Deze laag wordt door het ei zelf gemaakt en niet door de moeder en bevindt zich onder de eierschaal die gemaakt is door de moeder. In veel insecten wordt meer dan de helft van de allereerste cellen in het ei gebruikt om de serosa te maken (Figuur 1-3). Dat zoveel van deze eerste cellen gebruikt worden om de serosa te maken i.p.v. het embryo zelf, geeft aan dat deze laag een belangrijke rol in de ontwikkeling moet vervullen. In sommige soorten is het bekend dat de serosa een harde chitinelaag (waar het exoskelet van insecten van gemaakt wordt) uitscheidt. Hierdoor werd het al langer gedacht dat de serosa een beschermende functie vervult, het is echter nog niet eerder gelukt om dit experimenteel te testen. In de kastanjebruine rijstmeelkever (*Tribolium castaneum*) is het mogelijk om de ontwikkeling van de serosa te voorkomen door een gen (*Tc-zen1*) uit te schakelen. Dit geeft ons de perfecte mogelijkheid om te testen of de serosa daadwerkelijk een beschermende functie heeft.

De kastanjebruine rijstmeelkever- *Tribolium castaneum*

De serosa als bescherming tegen uitdroging

Eén van de gevaren die insecten eieren lopen is uitdroging. Anders dan eieren van de kreeftachtigen (*Crustacea*), die in het water worden gelegd, worden insecteneieren vaak op droge plekken gelegd. In hoofdstuk 2 laat ik zien dat de serosa in de meelkever ook een chitinelaag uitscheidt. De eieren van deze meelkever kunnen zowel in extreem droge als erg natte omgevingen overleven. Echter wanneer we de ontwikkeling van de serosa voorkomen, overleven eieren het een stuk minder goed in droge omgevingen. Om er zeker van te zijn dat het de chitinelaag is die deze bescherming biedt, heb ik ook het eiwit dat chitine maakt (*chitine synthase*) uitgeschakeld. Wanneer de serosa er wel is, maar de chitinelaag niet, overleven eieren droge omstandigheden ook niet. In hoofdstuk 3 laat ik vervolgens zien dat de structuur van deze chitinelaag ook belangrijk is voor de overleving van eieren in droge omgevingen. Wanneer deze structuur verloren is, overleven eieren slechter als het droog is. Deze data laten zien dat de serosa inderdaad bescherming biedt tegen uitdroging. Deze beschermende functie was waarschijnlijk erg belangrijk toen insecten op het land gingen leven.

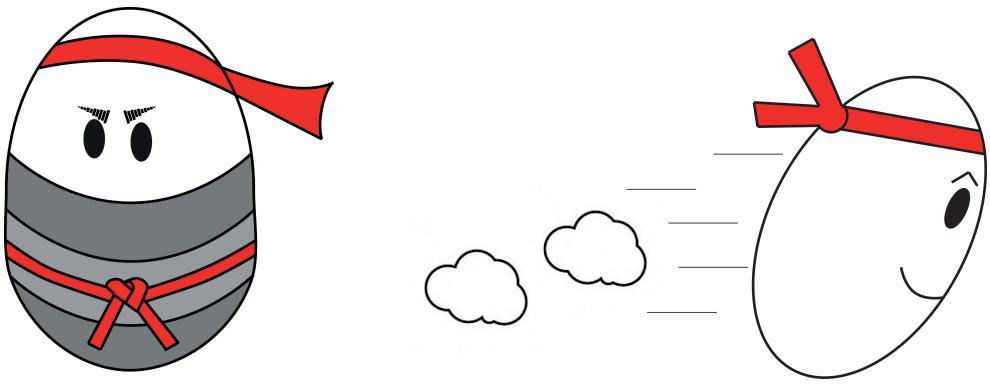
De serosa als bescherming tegen infectie

Uitdroging is niet het enige gevaar dat insecteneieren lopen. Bacteriën zijn overal en insecteneieren zitten vol met voedingsstoffen. Volwassen insecten hebben een uitgebreid afweersysteem om zichzelf te beschermen tegen infecties. Een heel scala van antimicrobiële stofjes voorkomt dat insecten ten onder gaan aan infecties. Van insecteneieren was echter niet bekend of ze zichzelf konden beschermen of dat ze voor bescherming ook afhankelijk zijn van de moeder. Sommige insecteneieren worden wel bedekt met antimicrobiële stofjes door de moeder, maar dit geldt niet voor alle soorten. Door eieren te prikken met een naald met bacteriën laat ik in hoofdstuk 4 zien dat de eieren van de meelkever zeker in staat zijn om op infectie te reageren. De genen die coderen voor antimicrobiële stofjes verhogen de activiteit tot wel duizend maal het niveau zonder infectie. De eieren van de fruitvlieg (*Drosophila melanogaster*) ontwikkelen geen serosa. Wanneer ik deze eieren infecteer met bacteriën reageren de genen voor antimicrobiële stofjes niet. Door weer de ontwikkeling van de serosa in de meelkever te voorkomen ontdekte ik dat deze immuunreactie in het ei volledig afhankelijk is van de serosa. Genen die in gewone eieren wel reageren doen weinig in eieren zonder serosa.

In hoofdstuk 5 ga ik dieper in op de immuunreactie van het meelkever ei. Eerst keek ik naar de groei van bacteriën in het ei. Bacteriën in eieren zonder serosa groeien twee keer zo snel als bacteriën in normale eieren met serosa. Door te kijken naar alle genen tegelijk kwam ik erachter dat het ei net zo'n uitgebreid scala aan immuungenen gebruikt als de volwassen kever. Verder ontdekte ik dat deze reactie volledig verdween wanneer de serosa er niet was. Vervolgens kon ik laten zien dat de immuungenen ook daadwerkelijk in de cellen van de serosa actief waren. Al met al laat dit onderzoek zien dat de eieren van de meelkever dankzij de serosa alles behalve weerloos zijn tegen infectie.

Eieren van de doosgraver zijn wel weerloos

De meelkever is niet het enige insect dat bestaat, dus heb ik in hoofdstuk 6 gekeken naar de eieren van een andere keversoort, de doodsgraver (*Nicrophorus vespilloides*). Deze kevers brengen de larven groot op een lijkje van een muis of ander klein dier. Anders dan veel andere insecten vertonen deze kevers broedzorg. Ze begraven het lijkje, houden het schoon en verzorgen de larven. De moeder legt de eieren in de aarde rondom het lijkje. Eieren in de buurt van een lijkje staan bloot aan veel bacteriën, dus verwachtte ik dat deze eieren een goede immuunreactie nodig hadden. De waarheid was echter heel anders. Ik vond dat eieren in de buurt van een rottend lijkje inderdaad minder goed overleefden en dat dit kwam door de aanwezigheid van bacteriën. Echter, de eieren vertonen geen immuunreactie na infectie met bacteriën. Verder kunnen ze ook bijzonder slecht tegen uitdroging. Toch ontwikkelen deze eieren wel een serosa. Dit verrassend resultaat is logisch als we kijken naar de manier van leven van dit insect. Wanneer moeder een lijkje vindt gaat ze deze begraven en eieren leggen. Het lijkje blijft echter niet eeuwig daar liggen. De eieren moeten uitkomen en zich vol eten in het lijkje voordat het lijkje weg is. De eieren van deze soort ontwikkelen dan ook bijzonder snel; slechts 2.5 dag hebben ze nodig. Zodra larfjes uit de eieren komen, kunnen ze zichzelf wel beschermen tegen infectie. Ik denk dan ook dat deze eieren geen energie investeren om zichzelf als ei te beschermen, maar deze energie gebruiken om zo snel mogelijk te ontwikkelen zodat ze naar het lijkje kunnen. Een kort eistadium geeft waarschijnlijk ook een minder grote kans op infectie. Er valt nog veel te leren over de relatie tussen snel ontwikkelen en een goed beschermd ei. De toekomst zal uitwijzen hoe sterk deze relatie is.



Een zelf verdedigend ei of een supersnel ei?

Conclusie

Lang werd gedacht dat insecteneieren weerloos waren. In dit proefschrift laat ik zien dat dat niet altijd opgaat. Eieren van de meelkever kunnen zichzelf heel goed beschermen tegen zowel uitdroging als infectie met behulp van de serosa. Niet alle eieren zijn echter zo goed in zelfverdediging, sommige (zoals die van de fruitvlieg en de doodsgraver) zijn liever snel dan veilig. Toekomstig onderzoek zal uitwijzen hoe algemeen een goed beschermd ei is in de insecten wereld.

Addendum

List of Publications

1. **Jacobs, C.G.C., Spaink, H.P., van der Zee, M.** (Accepted). The extraembryonic serosa is a frontier epithelium providing the insect egg with a full-range innate immune response. eLife
2. **Jacobs, C.G.C.*, Wang, Y.*, Vogel, H., Vilcinskas, A., van der Zee, M., Rozen, D.E.** (2014). Egg survival is reduced by grave-soil microbes in the carrion beetle, *Nicrophorus vespilloides*. BMC evolutionary biology. 14 (1), 208-215. doi: 10.1186/s12862-014-0208-x
3. **Lee-Yaw, J.A., Jacobs, C.G.C., Irwin, D.E.,** (2014). Individual performance in relation to cytonuclear discordance in a northern contact zone between long-toed salamander (*Ambystoma macrodactylum*) lineages. Molecular Ecology. 23(18), 4590-4602. doi: 10.1111/mec.12878
4. **Jacobs, C.G.C., van Overveld, T., Careau, V., Matthysen, E., Adriaensen, F., Slabbekoorn, H.** (2014). Personality-dependent response to field playback in great tits: slow explorers can be strong responders. Animal Behaviour, 90, 65-71. http://dx.doi.org/10.1016/j.anbehav.2014.01.016
5. **Jacobs, C.G.C. and van der Zee, M.** (2013). Immune competence in insect eggs depends on the extraembryonic serosa. Developmental and Comparative Immunology, 41, 263-269. http://dx.doi.org/10.1016/j.dci.2013.05.017
6. **Jacobs, C.G.C., Rezende, G.L., Lamers, G.E.M. and van der Zee, M.** (2013). The extraembryonic serosa protects the insect egg against desiccation. Proceedings of the Royal Society B: Biological Sciences, 280(1764). http://dx.doi.org/10.1098/rspb.2013.1082

- **Van der Zee M, Benton M.A., Jacobs C.G.C., Vazquez Faci, T. and Rabouille C.** (In review). The gap junction protein Innexin7a is essential for basal cell closure during cellularization of the *Tribolium castaneum* blastoderm.
- **Jacobs, C.G.C., Braak, N., Lamers, G.E.M., van der Zee, M.** (In review). The role of *knickkopf1*, *retroactive* and *laccase2* in serosal cuticle production and desiccation resistance of the *Tribolium* egg.

Curriculum Vitae

Personal information

Family name, First name:	Jacobs, Chris
Researcher unique identifier(s):	ORCID: 0000-0002-3128-2288
Date of birth:	20-07-1986
Place of Birth	Asten, the Netherlands
URL for web site:	http://www.science-explained.com

Chris Jacobs - Picture by Joris van Alphen, published with permission.

Education

11/2010 – 11/2014	PhD Institute of Biology, Leiden University, the Netherlands Supervisors: Prof. H.P. Spaink and Dr. M. van der Zee.
02/2009 – 09/2010	Master – Evolutionary and Ecological Sciences Institute of Biology, Leiden University, the Netherlands
09/2005 – 02/2009	Bachelor – Biology Institute of Biology, Leiden University, the Netherlands

Current positions

11/2014 – 05/2015	Postdoctoral Research Associate Institute of Biology, Leiden University, the Netherlands Supervisors: Prof. H.P. Spaink and Dr. M. van der Zee.
11/2010 – 11/2014	PhD Institute of Biology, Leiden University, the Netherlands Supervisors: Prof. H.P. Spaink and Dr. M. van der Zee.

Fellowships and awards

2013	Researcher of the year of the Institute of Biology (2013), Leiden University, the Netherlands.
2010	Scholarship, Outbound Study Grant, Leiden University. Leiden, the Netherlands.
2010	Scholarship, LUF Internationaal Studiefonds (LISF), Leiden, the Netherlands.

Supervision

10/2011 – 11/2014	Supervision of in total 5 Master and 6 Bachelor Students Institute of Biology, Leiden University, the Netherlands

Teaching activities

05/2013	Teaching position – Assisting in the field course: "Behavioural Biology", Institute of Biology, Leiden University, Leiden, the Netherlands.
05/2012	Teaching position – Assisting in the field course: "Behavioural Biology", Institute of Biology, Leiden University, Leiden, the Netherlands.
05/2009	Teaching position – Assisting in the field course: "Behavioural Biology", Institute of Biology, Leiden University, Leiden, the Netherlands.

Invited presentations

01/2014	Surviving embryogenesis: the extraembryonic serosa protects against desiccation and infection. Department of Earth Sciences, Uppsala University, Uppsala, Sweden.
07/2013	Surviving embryogenesis: the extraembryonic serosa protects against desiccation and infection. Michael Akam lab, Cambridge University, Cambridge, England.
07/2012	Invited speaker at the 4th meeting of the European Society for Evolutionary Developmental Biology (EED), Lisbon, Portugal.

Conference presentations / attendance

2013	Poster presentation on the 25th Annual Entomology Meeting, Ede, the Netherlands.
2013	Speaker at the iBeetle symposium "New horizons in molecular Zoology", Göttingen, Germany.
2012	Attended the 24th Annual Entomology Meeting, Ede, the Netherlands.
2011	Speaker at the 23rd Annual Entomology Meeting, Ede, the Netherlands
2011	Attended the Sixt International Symposium on Molecular Insect Science, Amsterdam, the Netherlands.
2011	Poster presentation at the 5th Annual Arthropod Genomics Symposium, Kansas, USA.
2011	Speaker at the International Tribolium meeting, Kansas, USA.
2010	Speaker at the Annual Meeting of the Netherlands Society for Behavioural Biology, Soesterberg, the Netherlands.
2009	Speaker at the Biannual Regional Tribolium Meeting, Cologne, Germany.

Commissions of trust

07/2014 – present	Reviewer for Animal Behaviour
02/2011 – 02/2012	Vice-chair and treasurer of the Leiden PhD Community (LEO), Leiden, the Netherlands.
09/2008 – 09/2009	Vice-chair of the student association Leids Heren Dispuut Cobra, Leiden, the Netherlands.
09/2007 – 09/2008	Treasurer of the student association Leids Heren Dispuut Cobra, Leiden, the Netherlands.

Courses

2013	Summer school in Evolutionary Developmental Biology: From Gene Networks to Organismal Systems, Venice, Italy.
2013	Writing an excellent research grant proposal, Leiden, the Netherlands.
2013	Confocal Light Microscopy: Fundamentals, Advanced techniques and Biological Applications. Amsterdam, the Netherlands.
2012	Scientific Integrity. Leiden, the Netherlands.
2012	Effective Communication, Leiden, the Netherlands.
2011	Time Management, Leiden, the Netherlands.
2011	Communication in Science, Leiden, the Netherlands.

International Experience

09/2011	Research visit to learn infection protocols in the lab. of Prof. Jules Hoffman, Strasbourg University, Strasbourg, France.
03/2010 – 08/2010	Master research project on speciation between subspecies of the long-toed salamander (*Ambystoma macrodactylum*). University of British Columbia, Vancouver, Canada.
04/2008 – 05/2008	Bachelor research project on personality related field playback response, Antwerp University, Antwerp, Belgium.

Public engagement

2012 - present	Website about Science www.science-explained.com
2014	The Science Explained PhD Game www.science-explained.com/science-explained-phd-game/
2014	Speaker at the science communication conference, Amsterdam, the Netherlands.
2013	Movie explaining how a desiccation proof insect egg helped insects conquer terrestrial habitats. https://www.youtube.com/watch?v=BqlDBReWDak

My work in the media

2014	Ganzenbord voor wetenschappers. Quest. http://www.quest.nl/artikel/speel-ganzenbord-als-een-echte-wetenschapper
2014	Interview about my website on kijkopkennis.nl http://www.kijkopkennis.nl/wp/2014/04/ik-wil-mensen-inzicht-geven-in-hoe-wetenschap-werkt/
2013	Waterproof eggs let insects conquer dry land. Nature News. doi:10.1038/nature.2013.13217
2013	Membraan om ei was grote troef insecten. NRC handelsblad. 20 Juni 2013
2013	Insecteneitjes. Mare. 20 Juni 2013
2013	Insecten op land dankzij nieuw ei. Bionieuws. 22 Juni 2013
2013	Hoe insecten te land kwamen. Leidsch Dagblad. 26 Juni 2013
2013	Evolutie als tupperwareparty. Volkskrant. 12 Oktober 2013

Acknowledgements

Four years pass so quickly and so many people have helped me in some way in getting to this point of my career. From little things as a pat on the back after a talk on a conference, to major things as being there to guide me through the entire four years. Thank you everyone I have met in the past four years in relation to science.

I would like to thank my parents, who have supported my choice of going to the university. This even though my grades were so bad, that any well-thinking human being would advise otherwise. I would also like to thank the rest of my family (including in-laws) for their continued support during my PhD. Special thanks to my fiancée Judith, who has showed unconditional support for my scientific endeavours. For accepting that I had to do "just a little work" in the weekends, evenings and so on. For protecting me from embarrassing spelling errors on my website and for being there for me when I got rejected (papers, summer school, funding proposal). I would like to thank all my friends who have made me feel at home during my stay in Leiden, but also my friends back home for their support. Specifically Michiel Fokkelman has helped me during my studies by kicking me out of bed before college and our discussions about science and other stuff during coffee have definitely helped me become a better scientist.

All of this wouldn't have happened if it wasn't for Paul Brakefield, who introduced me to Maurijn van der Zee for my master project, so thank you Paul. I would also like to thank all the other people from the evolutionary biology group for helpful discussions. Special thanks to Kees Koops, who has kept the beetles alive for all this time. Furthermore, thanks to all the other people in the IBL. Thank you Hans Slabbekoorn for always trying to get me to go the extra mile and keeping tabs on me well after I finished my Bachelor work with you. Thank you Gerda Lamers for your enthusiasm and support during all my microscopical endeavours. Thanks to all the other technicians that have prevented the institute from falling apart so I had a place to work. And let's not forget the students, Joana, Ruben, Arjan, Nora, Rens, Romee, Ellen, Yanell and Maaike. Thanks for your help! Nora, thanks for Figure 3-1. Thanks to Daniel Rozen and Yin Wang for introducing me to *Nicrophorus*. Thank you Menno Schilthuizen for motivating me to get into science communication.

Last but not least, I would like to thank my supervisors Herman and Maurijn. Herman's enthusiasm is absolutely contagious and after talking to him, doing science sounds as easy as boiling an egg (it is not). Thank you for your support and enthusiasm during this project! Maurijn has been the best supervisor I could wish for. He was always there for me when I needed advice, feedback, or just someone to organize my thoughts. He has always given me the freedom to pursue my own ideas, which has been very motivational! The reason I got the chapters published that I did, is because he was always very quick with looking at my drafts. Maurijn, thank you!