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*.

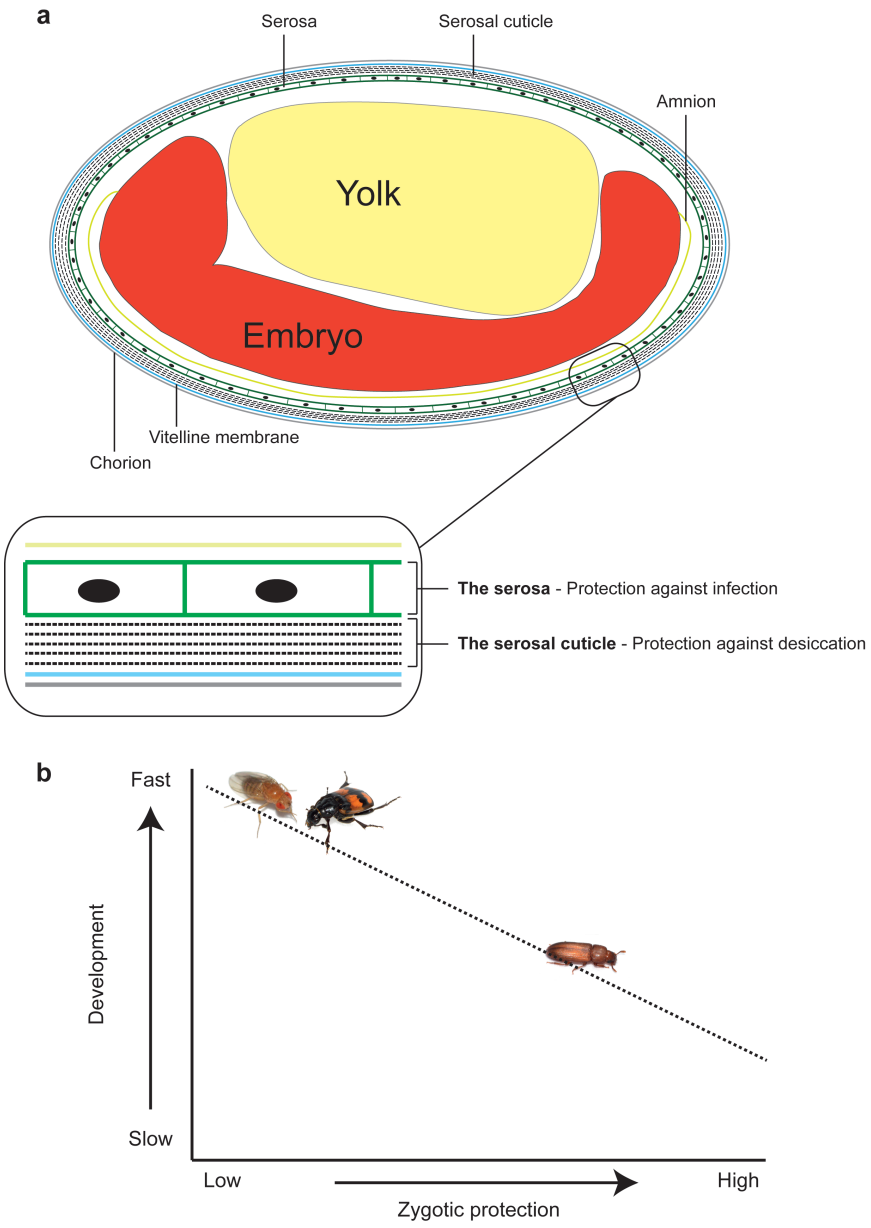


Figure 7-1: Summary of the main findings in this thesis. a) In this thesis I have shown that the serosal cuticle in *Tribolium castaneum* protects against dehydration (Chapter 3 and 4). I have also shown that the serosa itself protects the embryo against infection (Chapter 5 and 6). b) In Chapter 6 I 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.

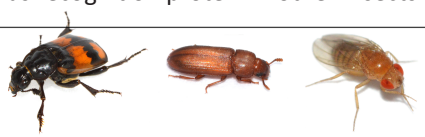
Discussion and Perspective

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.

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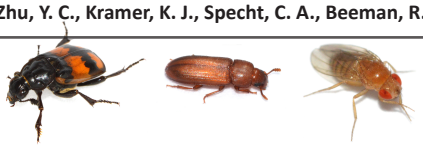

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