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

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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?

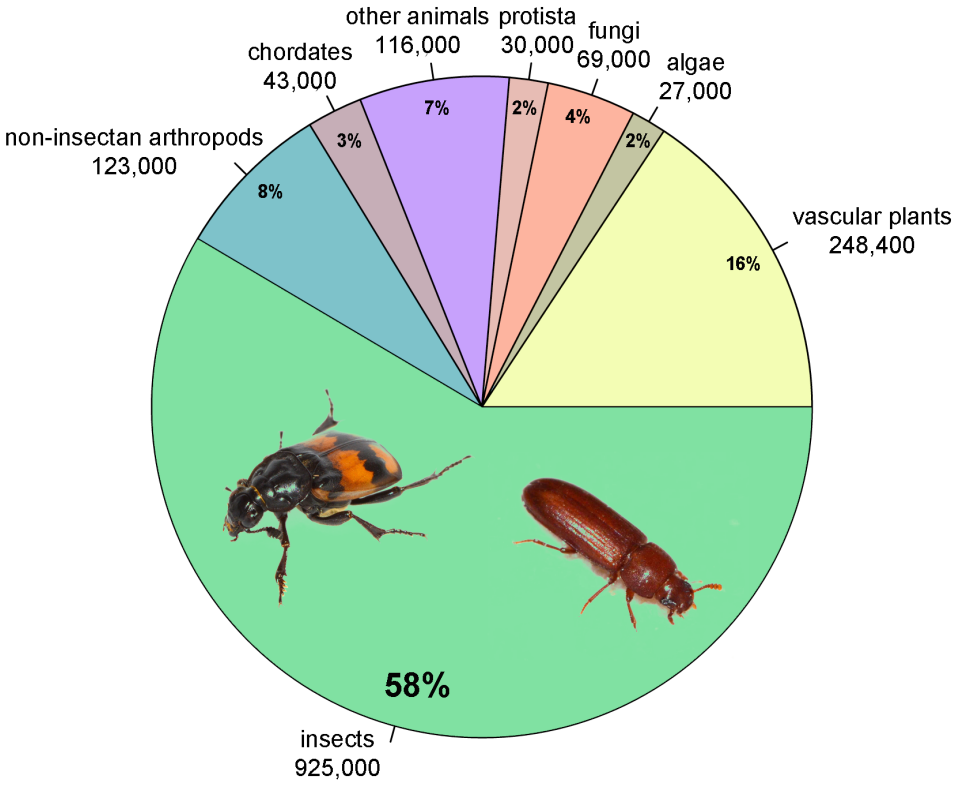

Figure 1-1: Insects are the most diverse group of animals on earth, of the 1.5 million species, 58% are insects (adapted from Grimaldi, 2005).

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.

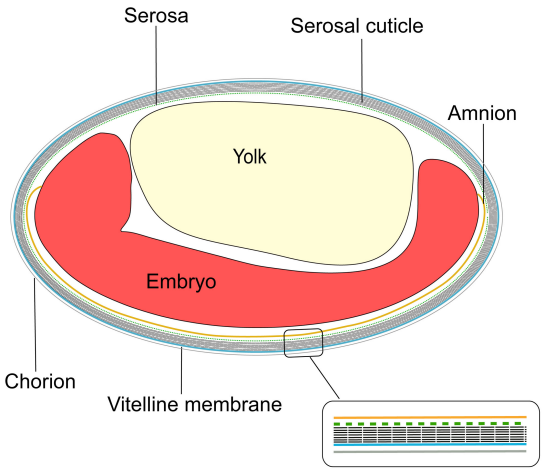

Figure 1-2: Schematic overview of the insect egg. The insect egg is surrounded by two maternal layers, the chorion and the vitelline membrane. Beneath these, the extraembryonic serosa secretes a chitinous cuticle (serosal cuticle). The serosa and serosal cuticle envelope both the embryo and the yolk.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

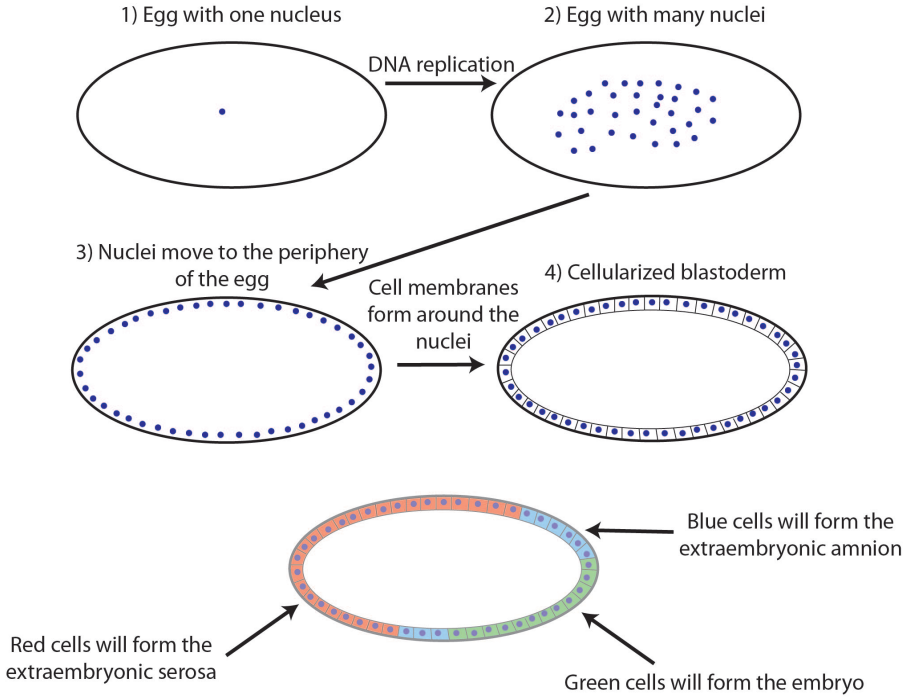

Figure 1-3: The formation of the blastoderm in insect eggs. Indicated are parts of the blastoderm that will form the embryo (green), the extraembryonic amnion (blue) and the extraembryonic serosa (red).

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.

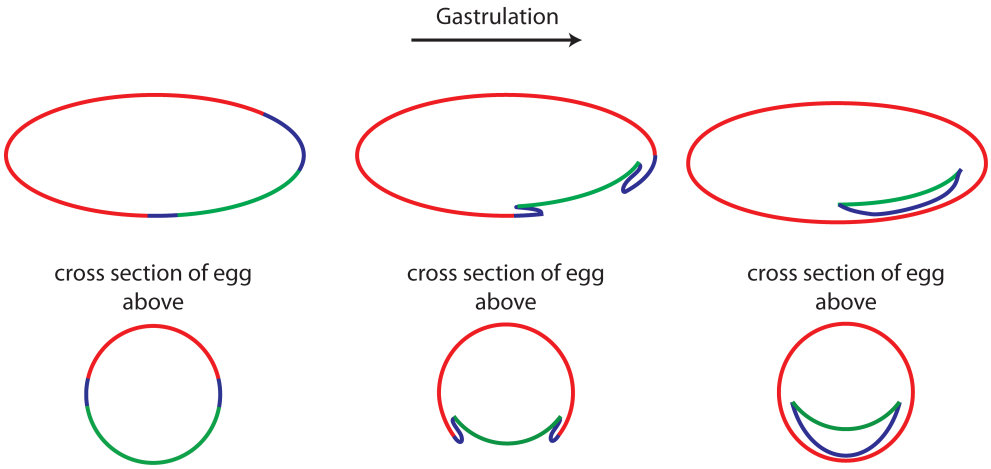

Figure 1-4: During gastrulation, the developing embryo will fold into the yolk and the serosa will envelop both the yolk and the embryo. Embryo = green ; amnion = blue ; serosa = red

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.

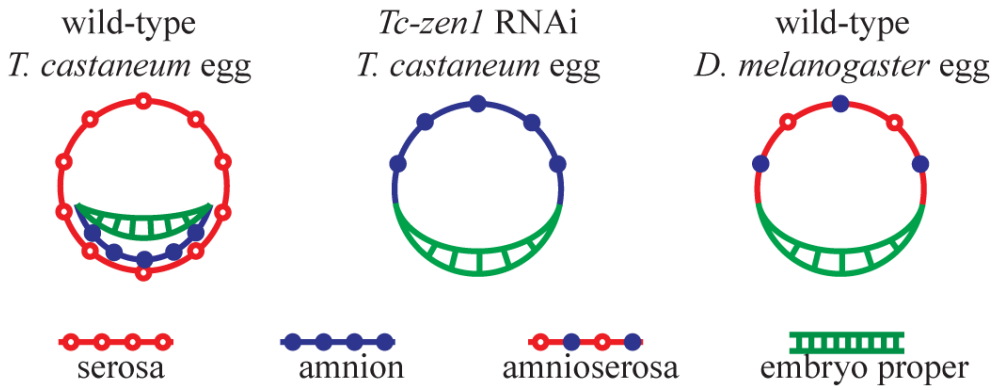

Figure 1-5: During normal development in *T. castaneum*, the serosa will envelop both the embryo and the yolk. However, after *Tc-zen1* RNAi no serosa will be formed and the amnion will cover the dorsal side of the egg. This resembles normal development in the *D. melanogaster* egg, where the amnioserosa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

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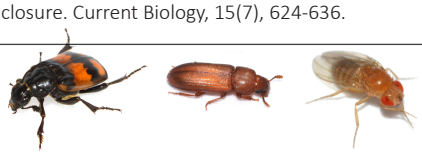

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