How does a butterfly embryo cope with environmental stress?

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Publications

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Abstract

Butterfly abundance and species diversity have been in decline and several anthropogenic factors have been implicated in the observed downwards trend. This thesis focusses on environmental stress directed at butterfly eggs, using the model systems *Pararge aegeria* and *Bicyclus anynana.* Eggs are a vulnerable life stage in which environmental stressors can have a major impact on both survival and development. The majority of winged insects, including butterflies, develop a protective sheet of cells surrounding the embryo during early embryogenesis. This sheet of epithelial cells is called the serosa and appears to protect the embryo against toxins, desiccation and pathogens. I first developed a morphological staging including a description of the extra-embryonic serosa for *P. aegeria*. Such a description will aid in understanding the developmental perturbations environmental stressors may cause. One of the stressors butterflies are (inadvertently) exposed to are insecticides, which is reviewed in full here. A research programme is proposed to incorporate the developmental genetic mechanisms underlying the pesticide response in a conservation context. The effects of two hormone analogs (i.e. of juvenile hormone and 20E) to study their effect on endocrine disruption during embryogenesis; both a maternal effect and through direct exposure. Eggs displayed increased sensitivity early in development before serosa formation and were in general more significantly affected as a result of a maternal effect. Finally, using transcriptomics and *in-situ* hybridisations, the effects of a natural stressor in *B. anynana* eggs was investigated; bacterial exposure in combination with wounding. The serosa, rather than the embryo, was able to mount an immune response, evidenced by significant upregulation of immune-related genes, such as *hemolin*, and genes in the Toll, IMD, Mapk-Jnk-P38 and JAK-STAT pathways. In conclusion, the butterfly embryo copes with environmental stress, such as toxins and immune challenges, with the help of the serosa.

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Chapter 1- Introduction

1.1 Background

Butterfly habitats, like those of many other insects, are increasingly changing; not only in terms of availability (including both quantity and quality), but also in prevailing climatic conditions experienced (Gibbs *et al.*, 2011, 2012; Hill *et al.*, 2001; Pateman *et al.*, 2016). As a result, declines in both numbers and diversity have been observed (Bonebrake *et al.*, 2016; Eskildsen *et al.*, 2015; Thomas, 2005, 2016). It is clear that an understanding of how various (environmental) factors interact with each other and affect butterflies is needed, so that we might better mitigate the impact of any future environmental changes (Gibbs *et al.*, 2011, 2012; Klockmann and Fischer, 2017; Pardikes *et al.*, 2017; Sambhu *et al.*, 2017; Seymoure, 2018). Currently, studies on the impact of environmental change on butterflies are largely confined to population dynamics, species trends, molecular ecology (e.g. in metapopulation context) and more theoretical studies on the adaptive capabilities in general, of butterfly populations (Gilburn *et al.*, 2015; Hanski and Thomas, 1994; McDermott Long *et al.*, 2016; Oliver *et al.*, 2015). Something that has not been adequately considered is how butterflies respond to environmental change from a more mechanistic perspective; the traits involved, and the genes underpinning the development and function of these traits. Such genes will be candidates for selection, both their sequence and expression profiles, and should be prime targets for studies on the evolvability of relevant traits under various environmental change scenarios. In this thesis, I address how butterfly eggs, one of the most vulnerable life stages, cope with environmental stress (Seymoure, 2018). Specifically, I focus on one specific trait, the extra-embryonic serosa (Fig. 1.1), and the genes involved in its function when confronted with environmental stressors.

There has been a global decline in insects, with Hallmann *et al.* (2017) estimating a decline in flying insect biomass of up to 75% over the last 27 years. A decrease in the amount and diversity of insects will affect ecosystem functioning, as insects play an important role in a

large variety of processes. This includes herbivory and detrivory (Mattson and Addy, 1975; Yang and Gratton, 2014), nutrient cycling (Yang and Gratton, 2014) and of course pollination (Ollerton *et al.*, 2011; Potts *et al.*, 2010, 2016). Additionally, they provide a food source for higher trophic levels such as birds, mammals and amphibians (Benton *et al.*, 2002; Hallmann *et al.*, 2014).The monetary value of the vital ecological roles insects perform have been estimated at $57 billion in the US (Losey and Vaughan, 2006), up to a £700 million in the UK (Vanbergen *et al.*, 2014), and their role in pollination alone can be valued at over $200 billion/year worldwide (Lebuhn *et al.*, 2013). Figures quoted are approximations, and in all likelihood an underestimation, particularly considering that not all pollinators are taken into account and long-term effects are poorly understood. Therefore, investigating the underlying causes for insect declines and associated declines in pollination services, as well as ecosystem services in general, are of the utmost importance (Vanbergen *et al.*, 2014).

Butterflies play an important role in ecosystems as plant pollinators (Feber *et al.*, 1997; Potts *et al.*, 2016) and as prey for other organisms (Pinheiro and Cintra, 2017). Butterflies are somewhat iconic, hold widespread appeal to amateurs and professionals alike, and as such also hold a great intrinsic value (Fox *et al.*, 2015). In a number of countries (including the UK) butterflies have been the subject of long-term large-scale monitoring schemes, and an increasing number of countries are in the process of setting up similar schemes (Brereton *et al.*, 2017; Dennis *et al.*, 2017a, b; Pollard and Yates, 1994; Schmucki *et al.*, 2016; Taron and Ries, 2015). Butterflies are also recognised and used as indicators of environmental health (Kremen, 1992; van Swaay *et al.*, 2013; Whitworth *et al.*, 2018). Comparatively their ecology, range and abundance are much better known than any other invertebrate taxa (Dennis and Whiteley, 1992; New, 1997; Thomas, 2005). This allows the possibility to investigate impacts of environmental factors across a large ecological range and time scale (Fontaine *et al.*, 2016).

A variety of factors, most related to anthropogenic disturbance, have been suggested to contribute to the observed decline in butterfly abundance and species diversity, both in the UK and elsewhere in the world (Bonebrake *et al.*, 2016; Chen *et al.*, 2011; Gallou *et al.*, 2017; Gilburn *et al.*, 2015; Malcolm, 2018; Stenoien *et al.*, 2018; Wilson *et al.*, 2007). These factors include land-use change, the use of chemicals (e.g. pesticides) and climate change. There is often an interaction between these factors. Land-use change encompasses a wide variety of factors such as habitat loss, fragmentation, degradation and a lack of resource diversity (Fischer and Lindenmayer, 2007; Hendrickx *et al.*, 2007; Potts *et al.*, 2010). A pertinent example is the current situation in South-Sweden, where 44% of the resident butterfly species have gone extinct in the last century as a direct result of land-use change (Nilsson *et al.*, 2008). This high extinction rate has been associated with the disappearance of flower-rich habitats in woodland and open farmland (Nilsson *et al.*, 2008). Other examples include the UK where urbanisation negatively impacts butterfly abundance of several species (Dennis *et al.*, 2017b) and the Netherlands where intense land-use led to a severe decline in distribution and abundance of over half of the common widespread butterflies species (Van Dyck *et al.*, 2009). The influence of land-use changes have also been seen at the species-specific level and across shorter time scales. For *Danaus plexippus* (Monarch butterfly) it is known that breeding season habitat loss drives population decline (Flockhart *et al.*, 2015). The gradual loss of Monarch breeding season habitat is for the most part driven by the loss of the milkweed host plant. Host plant loss is primarily due to modern agricultural practices, specifically increased adoption of genetically modified crops, land cover change and herbicide use (Flockhart *et al.*, 2015; Stenoien *et al.*, 2018).

The use of pesticides is closely related to habitat degradation and other factors associated with land-use change. The European Union has recently banned the use of certain insecticides in the class of neonicotinoids which have been implicated as a major contributor to the decline of bee populations in various parts of the world (Blacquière *et al.*, 2012;

European-Commission, 2013, 2018a, b, c; Goulson, 2013; Wood and Goulson, 2017). Furthermore, neonicotinoid insecticides are not only harmful for bees as declines in aquatic insects (Van Dijk *et al.*, 2013) and butterflies (Forister *et al.*, 2016; Gilburn *et al.*, 2015) have also been associated with neonicotinoid use. Of course, neonicotinoids are not the only class of insecticides, and other insecticides and pesticides in general have been shown to negatively impact invertebrates (Desneux *et al.*, 2007). One such class of insecticides that is presently under close scrutiny, and which I investigated in Chapter 4, are the endocrine disruptors (Casassus, 2018; Lyssimachou and Muilerman, 2016; Matthiessen *et al.*, 2018). Although it is immediately obvious that endocrine disruptors will affect the development, reproduction and physiology of animals, precise quantification of such effects is sparse, in particular in relation to field-realistic doses as well as the long-term effects (Casassus, 2018; Kabat, 2017; Lyssimachou and Muilerman, 2016). Furthermore, we do not fully understand how animals may protect themselves against perturbations of their endocrine system (Orth *et al.*, 2003). Endocrine disruptors can also remain potent for long periods of time in the environment, thereby affecting non-target animals (Casassus, 2018; Lyssimachou and Muilerman, 2016; Matthiessen *et al.*, 2018). Ultimately, such non-target effects of pesticides are likely to affect the ecosystem balance (Frampton Geoff *et al.*, 2009), e.g. by killing natural enemies of pest species (Theiling and Croft, 1988). Most butterfly species are not a direct target for pesticides but it is highly likely they are often adversely affected through non-target effects of pesticide use (Braak *et al.*, 2018). In Chapter 3 of this thesis, I will review current knowledge of non-target effects on butterflies and make recommendations for future avenues of research on this topic, as well as advocating firmly integrating pesticide research into studies on the effects of environmental change on butterflies. I propose an integrated approach to investigate the complex interaction effects of pesticide exposure with other environmental factors and its influence on butterflies from molecular to meta-population level.

The third factor responsible for the decline in insect populations is climate change (Chown *et al.*, 2010; Parmesan and Yohe, 2003; Sunday *et al.*, 2012). This encompasses a number of elements; such as an increase in extreme weather events, higher temperatures, elevated CO_2 and O_3 concentrations, increased UV radiation and changes in rainfall (Stott, 2016). The effects of climate change will increase as years go by, and there is a lot of work being done to predict future changes and the interaction with aforementioned environmental factors. Climate change has been predicted to influence butterflies in a variety of ways, in Roy and Sparks (2000), it was suggested that the rise in temperature could mean that butterfly abundance and range could increase in the UK by a northwards habitat shift. However, they also predicted that this effect could be nullified by factors such as drought and extreme weather events. This is supported by studies on *Pararge aegeria* (Linnaeus, 1758), where butterflies raised on drought stressed plants take longer to develop, have reduced survival to adulthood and reduced fecundity (Gibbs *et al.*, 2012). In a similar vein, Forister *et al.* (2010) investigated the effect of climate change and habitat disturbance on 159 butterfly species in California. Low elevation sites showed declines in species richness and abundance, when compared to high-elevation sites; apart from specialist alpine species. The clear upward shift in elevation ranges is consistent with the levels of global warming (Forister *et al.*, 2010). A similar shift has been observed for butterflies in central Spain and terrestrial organisms in the UK (Chen *et al.*, 2011; Wilson *et al.*, 2007). Other climate change factors, including drought, are also predicted to affect butterfly species richness and abundance, especially when coupled with habitat loss and fragmentation (Oliver *et al.*, 2015).

It is becoming increasingly clear that butterflies are exposed and affected by a large range of interacting environmental factors, both natural and man-made, which can result in complex effects. These factors can become stressors when their levels display rapid and/or unpredictable changes from the norm (Chown *et al.*, 2010; Fischer *et al.*, 2010; Whitman and Ananthakrishnan, 2009). The response of butterflies to environmental stressors is poorly

understood at the molecular level [see review in Chapter 3]. Elucidation of these responses particularly when coupled with the development and functioning of relevant traits (e.g. serosa, see below and Fig. 1.1) will generate valuable insights into their ability to adapt to a changing environment and will allow for a more profound understanding of observed ecological trends.

In recent years, a number of butterfly species have been sequenced (Dasmahapatra *et al.*, 2012; Ferguson *et al.*, 2014; Nowell *et al.*, 2017; Shen *et al.*, 2016; Triant *et al.*, 2018). This availability of genomic data for an ever-increasing number of butterfly species opens up a new world of possibilities to understand the underlying molecular mechanisms responsible for the effects observed under environmental stress (Challis *et al.*, 2016). A large number of tools have been developed to study the evolution of relevant genes, as well as elucidating their expression patterns and functionality. This has greatly facilitated the progress made in the field of butterfly evolutionary ecology and development, in particular for *P. aegeria* and *Bicyclus anynana* (Butler, 1879*)*, the two butterfly model systems that I will use in this thesis (Beldade and Peralta, 2017; Carter *et al.*, 2013, 2015; Ferguson *et al.*, 2014; Livraghi *et al.*, 2018b; Mazo-Vargas *et al.*, 2017; Zhang *et al.*, 2017). These tools include transcriptomics, *In situ* hybridisations and most recently CRISPR/Cas9 (Carter *et al.*, 2013; Ferguson *et al.*, 2014; Livraghi *et al.*, 2018a; Mazo-Vargas *et al.*, 2017).

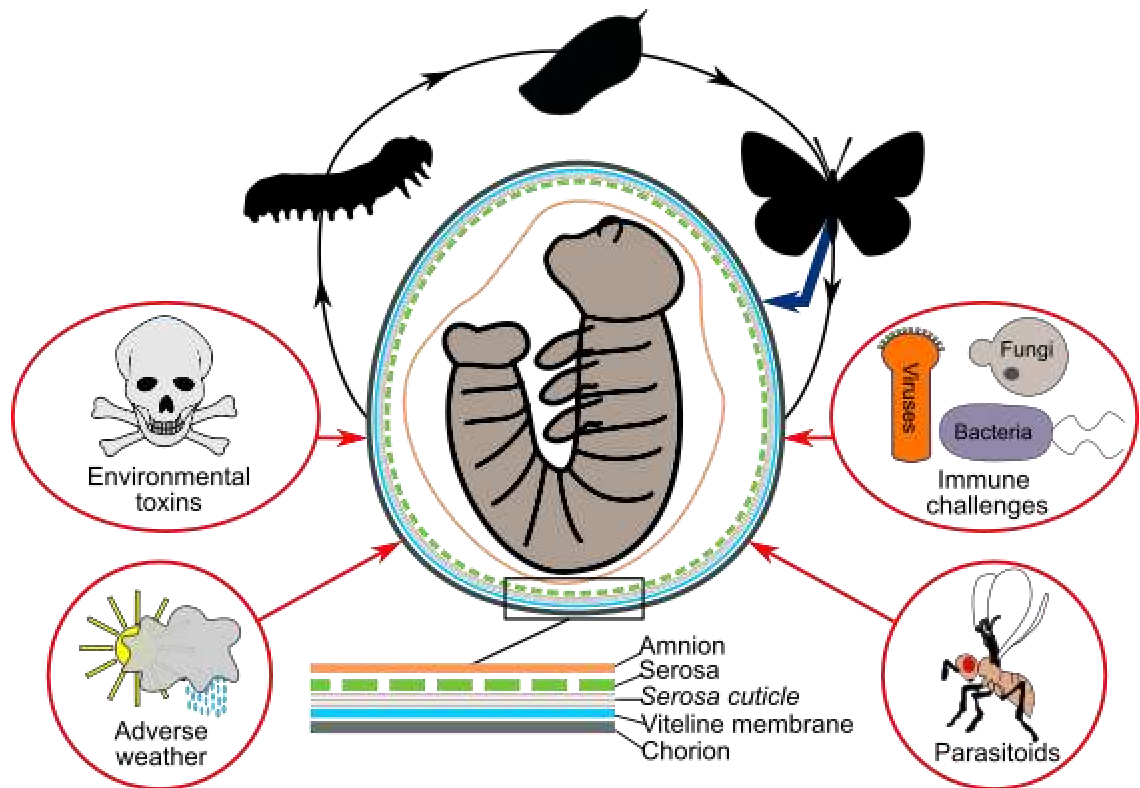

Figure 1.1: A butterfly embryo meeting environmental challenges. Throughout its life-cycle a butterfly (holometabolous: egg, larval instars (i.e. caterpillar stages), pupa and adult) is faced with environmental challenges. When a female lays an egg she provides her developing offspring with yolk (not depicted here, but it surrounds the embryo in early stages), and she provides the instructions for its initial development (Carter *et al.*, 2013, 2015). This maternal input is indicated by the blue arrow. As reviewed here, an extra-embryonic serosa forms outside the embryo inside the egg. The egg depicted above shows that the embryo is surrounded by the chorion, vitelline membrane, the serosal cuticle, the serosa and the lastly the amnion (based on data from Chapter 2, Panfilio, 2008 and Lamer and Dorn, 2001). The environmental challenges have been specifically indicated here for the egg; the range of environmental stressors depicted by the red circles. For a detailed overview refer to the text (1.2), for more information on the effect of environmental toxins on butterflies and their eggs see Chapter 3 and 4 and for the effect of immune challenges refer to Chapter 5.

1.2 Environmental perturbations of the egg-stage

Butterflies are affected by (adverse) environmental factors in all stages of their life cycle, from egg to adult (Chown and Nicolson, 2004) (Fig. 1.1). The larval and adult stage are generally the best-studied; for example in relation to pesticide exposure, where the caterpillar consumes

contaminated plants, whilst the adult females lay eggs and may feed on such plants (i.e. nectar and water) (Braak *et al.*, 2018). However, the eggs themselves are poorly studied in respect to the responses to environmental perturbations (Fig. 1.1) (Seymoure, 2018).

In this thesis, the emphasis is on the egg, with particular focus on the serosa (e.g. Chapters 2, 4 and 5) (Fig. 1.1). Eggs are deposited at a site chosen by their mother, after which they are exposed to the elements without the option to relocate. The egg sites are therefore carefully chosen for their quality by the female butterflies, with most laying their eggs on the plants on which their larvae will later feed (Jaumann and Snell-Rood, 2017; Wiklund, 1984). Female butterflies can make different resource investments to their eggs; laying a large number of small eggs or less bigger eggs (i.e. egg size – number trade-off) (Fischer and Fiedler, 2001; Wiklund and Persson, 1983). In *P. aegeria*, the larger well provisioned eggs are usually laid on high quality host plants (Gibbs *et al.*, 2005). In *P. aegeria* it has been shown that egg size significantly affects various aspects of offspring development; in an experiment in which egg production and subsequent development, were investigated over the life of females with different investments in flight, larger eggs were found to have shorter development times, and higher survival (Gibbs *et al.*, 2010a). Although the quality of the oviposition site is a key driver for location choice, ovipositing at the most optimal site is not always possible. For example, *P. aegeria* females lay smaller eggs at less optimal sites when harassed by males (Gibbs *et al.*, 2005). Egg production in female butterflies in general is strongly environmentally dependent, thus displaying significant reproductive plasticity. Environmental effects may be transgenerational, whereby the conditions a female herself experienced during her life have an effect on offspring development above and beyond the effects of the environmental conditions experienced by the offspring themselves (see for an in-depth review e.g. Woestmann and Saastamoinen, 2016). The mechanisms underpinning such transgenerational effects consist of egg provisioning, as well the inclusion of proteins and mRNA of genes with key roles in development (growth and patterning), physiology, immune defence and

metabolism (e.g. Carter *et al.,* 2013). It has been observed that temperature during oogenesis affects how the resultant embryos themselves develop under a range of temperatures in both my models *B. anynana* and *P. aegeria* (Fischer *et al.,* 2003b; Gibbs *et al.,* 2010c; Gibbs and Breuker, in prep). In *B. anynana* conditions experienced earlier in the life-cycle of females impact the type of egg they later produce. For example, higher quality food consumed when they were caterpillars, results in higher quality and larger eggs produced (Bauerfeind *et al.*, 2007; Geister *et al.*, 2008a; Karl *et al.*, 2007) while low quality food decrease egg mass and size (Bauerfeind and Fischer, 2005; Saastamoinen *et al.*, 2010). As a female ages, the number, size and quality of eggs produced declines (Bauerfeind *et al.*, 2007; Gibbs *et al.*, 2010a, c; Wiklund and Persson, 1983). Imposed resource trade-offs, for example, increased investment in flight as a result of habitat fragmentation at the time of egg production, are also detrimental to egg size, number and quality (Gibbs and Van Dyck, 2009; Gibbs *et al.*, 2010a, b, c). It is clear that maternal effects play a significant role in the subsequent life of their offspring, in terms of their development, physiology, metabolism and (immune) defence, and thus how they cope with environmental stress.

The environmental stresses encountered by the embryo can be both general and egg specific (Fig. 1.1). First, the egg's presence can induce a plant defence response that can harm the eggs directly and/or alert parasitoids like wasps to the presence of host eggs (for a comprehensive review see Hilker and Fatouros*.,* 2015). Direct egg killing plant defences range from formation of plant neoplasms, to loosen the eggs from the plant, (Doss *et al.*, 2000), egg crushing plant tissues, (Desurmont and Weston, 2011), ovicidal substances (Seino *et al.*, 1996), and leaf necrosis which could lead to desiccation of the eggs (Griese *et al.*, 2017). The plants might also have a role in attracting parasitoids, parasitic wasps can use the chemical signals of the plant to locate the freshly laid eggs, by both herbivore and oviposition induced plant volatiles and even un-induced plant cues (Fatouros *et al.*, 2008; Hilker and Fatouros, 2015; Meiners and Hilker, 2000).

In addition to plant mediated stressors the egg can also be exposed to other more general stressors such as climatic variation, including plant micro-climatic variation affected by the macroclimate (Jones, 1993). The macroclimate includes the general weather and extreme weather events like drought or torrential rain (De Frenne *et al.*, 2013). These types of events are increasingly common; this increase in frequency can be attributed to climate change (Stott, 2016), eggs can also fall prey to predators and parasitoids. Most of what we know of egg predators is from the study of natural predators of pest species like *Pieris rapae* (small cabbage white) (Schmaedick *et al.*, 1990). Oviposition-induced plant cues can help attract parasitoids, although this is not a universal mechanism (Fatouros *et al.*, 2008; Romeis *et al.*, 2005). Lepidopteran eggs are often parasitised by the minute polyphagous wasps from the genera *Trichogramma*, who lay their eggs in their host species' eggs (Flanders and Quednau, 1960), and for *P. aegeria* (Gibbs et al., 2004). The parasitoids complete their development in the host egg, killing the host in the process. Female parasitic wasps use multiple strategies to locate the freshly laid eggs, including sensing chemical signals like kairomones and sex pheromones or even catching a ride on the adult host (for a review see Fatouros *et al.,* 2008). The infection rate upon exposure to these parasitoids can be very high. Parker (1970) introduced *Trichogramma evanescens* to an area where *P. rapae* was present and the parasitism ranged from 20-75% on the plots where the parasitoid was released. Parasitoids are not the only species that attack butterfly eggs. For example, the eggs of *P. rapae* are found to be predated upon by beetles (*Coleomegilla maculate lengi* and *Stenolophus comma*), harvestman *(Phalangium opilio)* and the tarnished plant bug (*Lygus lineolaris*) (Schmaedick *et al.*, 1990).

Eggs can be exposed to a range of environmental toxins both naturally occurring and man-made. Some plants excrete toxins especially designed to harm or kill eggs (Hilker and Meiners, 2011; Hilker and Fatouros, 2015). Additionally, eggs can be exposed to man-made toxins like pesticides. Eggs of insects are often not the primary target of the pesticides, as they do not bite or feed, but can be affected if they are laid in or near pesticide treated areas,

especially if they are seen as a pest species after hatching. Campbell *et al.* (2016) makes an argument for the more targeted approach of using pesticides against eggs as that would kill the pest-insect before it does any damage, if adopted this approach could lead to more dangerous non-target effects for eggs of other species. The last type of stressor the egg can be exposed to during development are harmful microbes such as viruses, bacteria and fungi (Kellner, 2002). These can be from the oviposition location or even deposited on (or in) the egg by the mother (for a review see Kellner, 2002). Several entomopathogenic fungi have been found to penetrate the egg and have a pathogenic effect (Marta *et al.*, 2006). Wounding of the egg by failed predation or infestation also leaves the egg vulnerable to penetration by micro-organism, and successful parasitism can introduce pathogenic microbes into the egg (Tanada and Kaya, 2012). For instance, *Serratia* bacteria have been found inside eggs of corn earworms and corn bores (Bell, 1969; Lynch *et al.*, 1976) and can infect eggs in the laboratory (Sikorowski *et al.*, 2001).

Thus far egg quality and survival rates have been mostly considered from the perspective of transgenerational effects, oviposition site choice, and maternal defences deposited into/on the eggs (Hilker and Fatouros, 2015; Woestmann and Saastamoinen, 2016). Although these factors to a large extent explain variation in the development and survival of eggs, the eggs themselves might play an important and active role in its protection against environmental stressors (Jacobs *et al.*, 2013; Jacobs and van der Zee, 2013).

1.3 Egg morphology

The eggs of butterflies and insects in general, are complex structures; the embryo and the yolk are surrounded by a number of membranes, cuticles and various extra-embryonic tissue layers (Fig. 1.1). In general, the embryo is surrounded (starting from eggshell-side) by the chorion, vitelline membrane, serosal cuticle(s), and the extra-embryonic tissue layers (i.e. serosa and amnion) (Fig. 1.1). The two most external of these layers, the chorion and vitelline membrane

are of maternal origin. Both the vitelline membrane and the chorion are produced within the female ovarioles during oogenesis by the follicular epithelium (Campbell *et al.*, 2016). Insect egg shells are structurally very diverse but in the simplest form there are three maternal extra-embryonic layers; the exochorion, endochorion and vitelline membrane (Hinton, 1981). A more in-depth breakdown of these maternal extra-embryonic layers in Lepidoptera can be found in Chapter 2. The chorion has a number of structures such as aeropyles for respiration, the presence of which interestingly greatly varies in Lepidoptera, and micropyles through which the egg is fertilised (García-Barros and Martín, 1995; Hinton, 1981; Omelina *et al.*, 2013; Wigglesworth and Beament, 1950). The eggshell layers and the elaborate respiratory systems of insect eggs seem to be adapted to conserve water. For example, terrestrial insects that lay their eggs on land have a pillar system for embryonic gas exchange, where an aeropyle connects to an inner air-filled space which moves oxygen from the outer egg shell to the inner embryo (i Monteys *et al.*, 2005; Woods *et al.*, 2005; Woods, 2010). Many insects have reduced their number of aeropyles to reduce the possibility for water evaporation out of the egg, and some butterflies like *P. aegeria* have lost them completely (Campbell *et al.*, 2016; García-Barros and Martín, 1995; Hinton, 1981). Apart from the internal variation of the maternally produced eggshell there is a lot of external morphological variation. There is homology in insect egg shell characters, which can be used for systematics and has been used for phylogenetic analysis of butterflies (García-Barros and Martín, 1995). This homology might be explained by both the crucial biological roles of the eggshell and the maternal ovarial phenotype (García-Barros and Martín, 1995).

Besides the protective layers deposited around the egg by the mother, there are also a number of layers of extra-embryonic layers of embryonic origin. The majority of winged insects, including butterflies, develop a protective sheet of cells surrounding the embryo during embryogenesis (Ferguson *et al.*, 2014; Jacobs *et al.*, 2013; Panfilio, 2008). This sheet of epithelial cells is called the serosa. In insects, the development of the extra-embryonic layers

starts at the blastoderm stage. Soon after the first cellularisation, when a single cell layer forms around the yolk, the identity of future serosal cells and germ cells can be distinguished morphologically. At this stage a substantial proportion of the cells, often as much as two-thirds, are specified to become the serosa (Davis and Patel, 2002). Soon after the serosa envelops the embryo it starts secreting a cuticle. The serosal cuticle consists of laminated layers of chitin, and in some species, it stays in place until hatching (Jacobs *et al.*, 2015; Lamer and Dorn, 2001).

1.4 The evolution of the extra-embryonic membranes of embryonic origin

In most pterygotes (winged insects), the serosa is the outermost cellular epithelium enclosing all other embryonic material. The serosa is the first layer that can actively adapt to a changing and invading environment; all other layers offer a more passive protection. Therefore, the serosa has been argued to be a key adaptation that allowed insects to exploit a plethora of ecological niches (Jacobs *et al.,* 2013). This is, in part, due to the serosa's protective function, that buffers the embryo against external environmental perturbations and invasive assaults (Horn *et al.*, 2015), all of which will be discussed in detail in the next section. It is interesting to note that the evolution of the serosa seems to, in large parts, coincide with the terrestrialisation of arthropods. This hypothesis is supported by the fact, that there seems to be a correlation of the capacity of arthropods to develop under dry conditions and the presence of a serosa that completely enfolds the embryo (Jacobs *et al.*, 2013).

The evolution of the extra-embryonic membranes of embryonic origin is tightly linked to the evolution of *Hox3 (i.e. zerknüllt, zen)*, encoding a Q50 homeodomain-containing protein (Lynch and Desplan, 2003). Hox genes are a highly conserved set of homeobox genes that are involved in specification of segment-identity along the anterior-posterior body axis during embryonic development, as well as various other instances of patterning during embryogenesis (McGinnis and Krumlauf, 1992). Hox genes are generally highly conserved (in

particular the homeobox), as well as their chromosomal arrangement (i.e. gene order), generated by tandem gene duplication early in animal evolution (De Rosa *et al.*, 1999; Kappen *et al.*, 1989). There are few recorded cases of tandem duplication within the Hox cluster, with the exception of the Hox3 paralogy group in insects (Hughes and Kaufman, 2002).

Comparative expression data across different arthropod lineages shows that the ancestral *Hox3* might have lost its function in specifying segment identity along the anterior-posterior axis and gained a role in the specification of extra-embryonic membranes during the radiation of Pterygota (Hughes *et al.*, 2004; Schmidt-Ott *et al.*, 2010). Following this radiation, the *Hox3* locus has been shown to have duplicated independently in a variety of insects and orders (Falciani *et al.*, 1996; Ferguson *et al.*, 2014; Rafiqi *et al.*, 2008; Stauber *et al.*, 1999) (Fig. 1.2). After the radiation of the Pterygota the *Hox3* gene is referred to as *zerknüllt*, the German for crumpled-up, after the phenotype in *Drosophila* (Schmidt-Ott *et al.*, 2010; Wakimoto *et al.*, 1984). *Tribolium castaneum* has two paralogs named *zen-1* and *zen-2* (Brown *et al.*, 2002; Falciani *et al.*, 1996), *Episyrphus balteatus* (hoverfly) has seven *zen* paralogs (Rafiqi *et al.*, 2008), and the fruit fly *Drosophila melanogaster* has two *zen* paralogs and the highly divergent *bcd*, encoding a K50 homeodomain protein (Stauber *et al.*, 1999, 2002) (Fig.1.2). During the radiation of the Ditrysia, a clade of the Lepidoptera containing around 98% of the described Lepidopteran species, *Hox3* duplications resulted in five *Hox3* paralogs; *zen* and the highly divergent *Special homeobox genes* (*ShxA-D*) (Ferguson *et al.*, 2014). Hox3 paralogs display both sub- and neofunctionalisation (Hughes and Kaufman, 2002; Schmidt-Ott *et al.*, 2010; Stauber *et al.*, 1999) (Fig. 1.3).

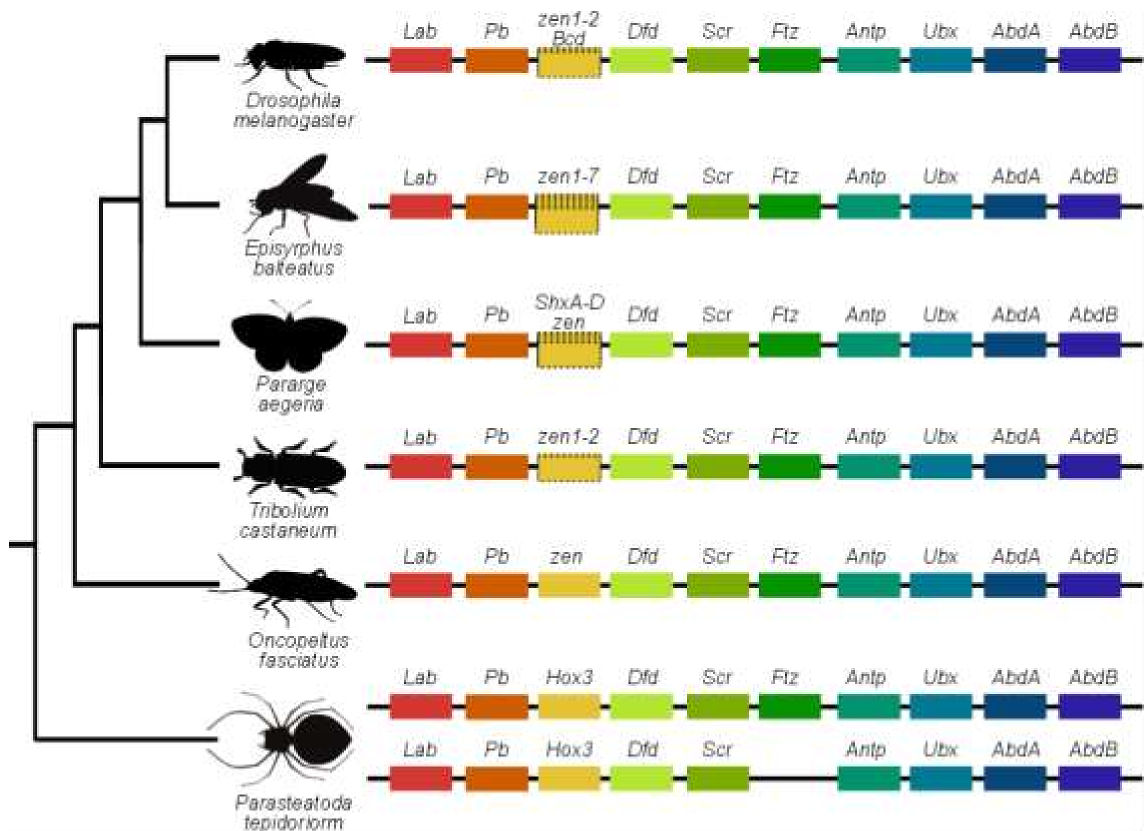

Figure 1.2: Hox cluster expansion in arthropods *Hox*-genes and their chromosomal organisation appear highly conserved. *Hox3* is a gene that unlike the others can tandem duplicate in insects. During the radiation of the pterygota the *Hox3* gene lost its ancestral canonical Hox-function and became involved in the formation of extra-embryonic membrane, whereafter it is named *zen. Zen* has undergone multiple independent duplications in various insect lineages, giving rise to 2 copies of *zen* in *T. castaneum* (Falciani *et al.*, 1996), 1 copy of *zen* plus 4 *Shx*-genes in *P. aegeria* (Ferguson *et al.*, 2014), 7 copies in *E. balteatus* (Rafiqi *et al.*, 2008) and 2 copies of *zen* and the highly divergent *bcd* in *D. melanogaster* (Stauber *et al.*, 1999). The chelicerate duplication of the *Hox*-genes, as seen here in *P. tepidoriorm,* is the result of whole genome duplication (Schwager *et al.*, 2017). (Figure adapted from Livraghi, 2017)

The role change of the *Hox3* ancestral patterning to *zen* extra-embryonic membrane function and the subsequent diversification of the function of the *Hox3* paralogs are caused by both cis and trans regulatory changes. In ancestral arthropods, *Hox3* is expressed as a canonical *Hox*-gene and is involved in morphological diversification of segment identity as is shown in spiders, centipede and the crustacean *Daphnia pulex* (Hughes *et al.*, 2004; Panfilio and Akam, 2007;

Papillon and Telford, 2007) (Fig. 1.3). Interestingly, the *Hox3* ortholog in the basal apterygote insect *Thermobia domestica* has an intermediate expression pattern; it is expressed in both the extra-embryonic tissues and in the mouth bearing parts (Hughes *et al.*, 2004). The changes from canonical *Hox*-gene expression to extra-embryonic is accompanied by several protein structure rearrangements. These changes can be summarised by the loss of the hexapeptide (YPWM) motif which enables interaction of Hox-proteins with cofactor Exd/Pbx, which is essential to confer DNA-binding specificity of Hox proteins (Mann and Chan, 1996). Additionally, *zen* gained a conserved "A-box motif" and the homeodomain moved towards the amino terminal (Panfilio and Akam, 2007). Z*en* paralogs may display subfunctionalisation; in *T. castaneum* both *zen-1* and *zen-2* are expressed in the serosa tissue but only *zen-2* is expressed in the developing amnion. Knock-down of *zen-1* by maternal RNAi abolish the serosa so that *T. castaneum* only develops the amnion, but the embryo otherwise develops normally (van der Zee *et al.*, 2005). Whereas knockdown of *zen-2* abolishes the fusion of the amnion and serosa, which prevents amniotic rupture during katatrepsis and dorsal closure which can lead to an inverted larva (Hilbrant *et al.*, 2016; Horn *et al.*, 2015; van der Zee *et al.*, 2005). The role of the serosa and amnion in dorsal closure and katatrepsis are discussed in detail in the following section (1.5).

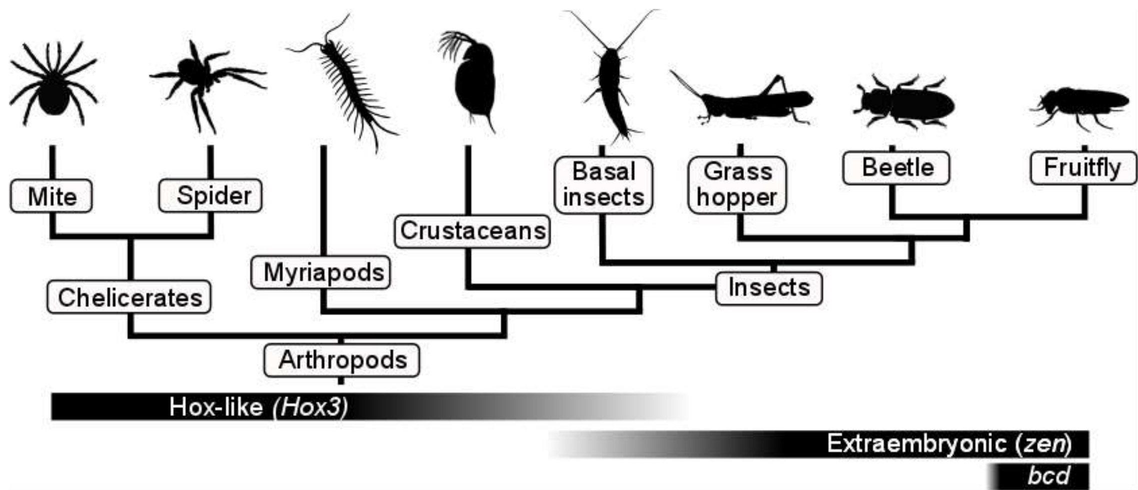

Figure 1.3: *Hox3* functional diversification in arthropods. In the chelicerates, myriapods and crustaceans *Hox3* expression patterns during embryonic development are indicative of a canonical *Hox*-function. The basal wingless insect *Thermobia* shows both embryonic and extra-embryonic expression of *Hox3* which is considered a transition stage between canonical *Hox3* and insect (extra-embryonic) *zen.* The grasshopper and beetle show only extra-embryonic expression of *zen* and in *Drosophila* the expression of z*en* is in the extra-embryonic membranes, while the highly divergent *bcd* displays embryonic expression again (adapted from Hughes and Kaufman, 2002).

At the base of the cyclorraphan Dipterans, *zen* duplicated independently giving rise to both an extra copy of *zen* and the highly derived *bcd* (Stauber *et al.*, 1999, 2002). *Zen* is zygotically expressed in the extra-embryonic membrane in flies, the amnio- serosa. The Bcd protein acts as a morphogen in specifying the anterior of the embryo (Driever and Nüsslein-Volhard, 1988). It is maternally expressed and in its role as a maternally provided anterior determinant it has functionally replaced *orthodenticle* (*otd*) in the higher flies (Lynch and Desplan, 2003; Schröder, 2003). Both Otd and Bcd are functionally characterised by a K50 homeodomain, whereas Hox3 (i.e. Zen) is characterised by a Q50 (Lynch and Desplan, 2003). Rather interestingly, the hoverfly (*E. balteatus)* has seven *zen*-like genes but none similar are to *bcd, s*uggesting that *E. balteatus* split from the other Cyclorrhapha before the origin of *bcd* or has secondarily lost the gene (Rafiqi *et al.*, 2008). The *E. balteatus zen*-paralogues show no

maternal expression, four are expressed during early development; two of which contribute to the specialisation of the serosa (Rafiqi *et al.*, 2008).

Ditrysia (i.e. derived Lepidoptera) possess four highly divergent *Shx (ShxA-D*) paralogs alongside *zen* (Ferguson *et al.*, 2014). The expression of these five *Hox3* genes has been closely studied in the *P. aegeria,* with localised expression patterns of these genes before and after serosa formation over the first 48 hours of embryonic development (Ferguson *et al.*, 2014). Maternal transcripts of *ShxC, ShxD* and *zen* are included into *P. aegeria* eggs (Ferguson *et al.*, 2014). The localisation of maternal *ShxC* transcripts in a freshly laid egg shows a distinctive hour-glass localisation pattern and can be seen to emerge during oogenesis (Carter *et al.*, 2015; Ferguson *et al.*, 2014). The region where these maternal transcripts are localised is fated to become the presumptive serosa. *Zen* transcripts are detected in the follicle cells around the oocyte, while *ShxD* transcripts are present in the whole developing oocyte (Ferguson *et al.*, 2014). Given the role of ShxC, there is a strong maternal effect in Ditrysia in the formation of the serosa, and thus indirectly there is a maternal determination of the protection of the embryo against environmental stressors. What we do not know at present is whether maternal effects, and thus possibly transgenerational effects, also pertain to the functioning of the serosa (Chapter 6). We only recently started to elucidate the functioning of the serosa, and at present this is still only in the context of direct immune challenges on the developing embryo (Jacobs and van der Zee, 2013; Jacobs *et al.,* 2014a; Chapter 5).

Around the time of the switch from maternal to zygotic transcription (~eight hours after egg laying), all four *Shx* genes are detected in the presumptive serosa domain in the celluralised blastoderm. Thus a clear boundary between future embryonic and extra-embryonic tissues is visible (Ferguson *et al.*, 2014). In contrast, *zen* transcripts are weakly localised throughout the blastoderm, it seems to have lost its normal Pterygote role in the extra-embryonic tissues. Not only do the four Shx paralogs display a striking sequence

divergence from each other and between species (Ferguson *et al.*, 2014), but also within a species these genes have been shown to evolve rapidly, possibly under selection (Livraghi *et al.*, 2018b). This divergence in sequence and expression of the *Hox3* genes, in general and in Lepidoptera specifically, leads to questions about the divergence in the functions of the extra-embryonic tissues. Not least as the serosa remains in place throughout development (Chapter 2), and is maternally specified by means of a highly divergent, and rapidly evolving, hox3 paralog (i.e. ShxC). Furthermore, it still remains to be investigated whether *Shx* genes play a role in the functioning of the serosa, similar to *zen* in other insect orders (van der Zee *et al.,* 2005; see also Chapter 5).

1.5 The functions of the extra-embryonic membranes

One of the main (and most widely studied) roles of the extra-embryonic membranes is the role in morphogenetic events. The first event is the movement involved in serosa formation, anatrepsis, where the serosa folds over the embryo. Second, is the role of the serosa in katatrepsis and dorsal closure. The serosa is involved in dorsal closure for most hemimetabolous and some holometabolous insects, before it is absorbed into the yolk (Horn *et al.*, 2015; Panfilio, 2008; Panfilio *et al.*, 2013; Schmidt-Ott and Kwan, 2016).
The process of the serosa folding over the embryo varies significantly between different orders (Fig. 1.4). For example, in the Hemiptera (true bugs) *Oncopelthus fasciatus* serosa formation occurs by invagination of the posterior blastoderm while in the Coleoptera (beetles) such as *T. castaneum* it closes over the ventral blastoderm (Schmidt-Ott *et al.*, 2010). In both cases the enveloped gastrulated blastoderm pinches of from the serosa. While in Lepidoptera (moths and butterflies), the serosa detaches from the germ-band which sinks slightly into the yolk where after the serosa slides over the germ-band and closes around the embryo and the yolk (Ferguson *et al.,* 2014; Panfilio, 2008; Chapter 2).

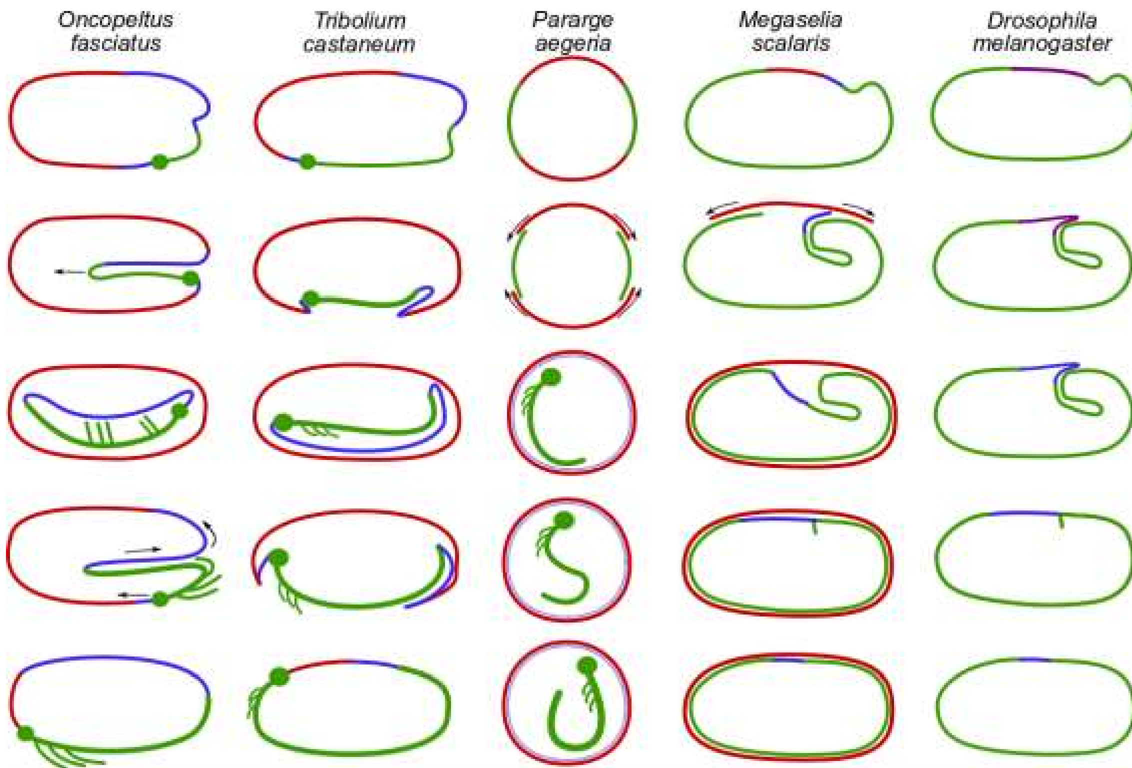

Figure 1.4: Extra-embryonic developmental trajectories. Embryonic (green), amniotic (blue), serosa tissue (red) and amnioserosa (purple) are indicated at consecutive developmental stages from the differentiation of serosal tissue until after katatrepsis (where applicable). Sketches are based on *Oncopeltus fasciatus, Tribolium castaneum, Pararge aegeria, Megaselia scalaris* and *Drosophila melanogaster.* The developmental stages of the species are roughly matched. A filled circle indicates the head and the dotted amnion line in the *Pararge* embryos indicates where the amnion is expected but which has not been confirmed by studies to date (Adapted from Schmidt-Ott *et al.*, 2010).

The role of the serosa in dorsal closure and katatrepsis is equally diverse. When the serosa is involved in these processes, it generally starts with fusion of the serosa and amnion to generate a continuous serosa-amnion epithelium which retracts towards the anterior or dorsal side of the egg. This process ruptures the amniotic cavity and creates the serosal window. The site of serosal rupture appears to be triggered by the amnion (Schmidt-Ott and Lynch, 2016). This takes place while the embryo closes around the dorsal midline (dorsal closure), after retraction the extra-embryonic membranes are absorbed. In hemimetabolous insects (lower Pterygota) this serosa-amnion fusion seems to be widely conserved and it is required for katatrepsis where the embryo turns around in the egg to align with the anterior-

posterior axis of the egg (Schmidt-Ott and Kwan, 2016). In holometabolous insects, serosa-amnion fusion has been found as well, but there is major variation within orders. For example, Coleoptera like *T. castaneum* seem to show the classical serosa-amnion fusion while in Lepidoptera like *P. aegeria* the serosa doesn't seem to be involved in the process of dorsal closure and katatrepsis at all (Chapter 2 and Panfilio, 2008). As a consequence of these differences in morphological movements, the serosa stays in place for varying lengths of time. Where the serosa is absorbed by the yolk after katatrepsis in some orders, it can stay in place until the end of development or until the moment of hatching in others. In other orders, it might not be the yolk that is involved in serosa uptake and degradation, but the serosa itself that influences yolk degradation, as has been observed in the moth *Manduca sexta* (Lamer and Dorn, 2001). This variation in morphogenetic functioning and the time the serosa surrounds the embryo during development raises questions about potential variation in the other, protective, functions of the serosa.

The serosa has been implicated in a number of protective functions. The first is that the serosa plays an integral role in preventing eggs from drying out. In *T. castaneum* the formation of the serosa can be prevented through maternal RNAi targeting the *Hox3*-gene *zen-1* (van der Zee *et al.*, 2005). If this gene is knocked-down the serosa does not develop but the embryo is otherwise viable and has a normal hatching phenotype. This system was used to investigate whether the serosa is involved in drought resistance, by comparing the hatching rates of serosa-less to wildtype eggs at different relative humidity's (Jacobs *et al.*, 2013). Indeed the eggs with a serosa develop at significantly higher rates at low relative humidity's than serosa-less eggs (Jacobs *et al.*, 2013). However, it was then suggested that it is not the serosa itself but the serosal cuticle it secretes that may be important for protection against drought. For example, in mosquitos the eggs only become drought resistant after the excretion of the serosal cuticle (Vargas *et al.*, 2014). To test this hypothesis, Jacobs *et al.* (2013) knocked-down *chitin-synthase 1* (*chs1),* the main component of the serosal cuticle, through maternal

RNAi. They found that the drought tolerance was significantly reduced in serosal cuticle-less eggs compared to the controls, suggesting that the serosal cuticle is indeed essential for drought resistance in *T. castaneum* (Jacobs *et al.*, 2013). Taking that one step further, they looked into whether the structure of the chitin in the organised layers of the serosal cuticle is necessary for its drought resistance function or if the chitin just provides an extra physical boundary (Jacobs *et al.*, 2015). Knocking-down a number of genes responsible for chitin-organisation and tanning through maternal RNAi, they found that all though the presence of chitin might offer some protection against drought, the specific organisation and tanning of the cuticle has an effect on the efficiency of drought protection (Jacobs *et al.*, 2015).

The serosa has also been implicated in protecting embryos against environmental toxins (Berger-Twelbeck *et al.,* 2003; Orth *et al.,* 2003; Chapter 4). Eggs of the moth *M. sexta* were exposed to the ovicide Ov.165049 (Berger-Twelbeck *et al.*, 2003). The ovicide treatment induced fine structural changes in the cells of the serosa, such as the enlargement of the mitochondria. It has been suggested that this is a sign that the ovicide disturbs the normal metabolic processes of the serosa which leads to its disintegration. Only after the serosa ruptures does the embryo show any damage. This study suggests an important role of the serosa in metabolism and excretory processes (Berger-Twelbeck *et al.*, 2003). The serosa of *M.sexta* also expresses juvenile hormone binding proteins, which might be able to protect the embryo from juvenile hormone analogs which are often used as pesticides in the endocrine disruptor class (Orth *et al.*, 2003). For butterflies specifically, there is little information available on the susceptibility of eggs to environmental toxins (Braak *et al.*, 2018).

Lastly, the serosa has been implicated in mounting an immune response. Several immune related genes were detected in the extra-embryonic tissue of the *M. sexta* but this response could not be specifically attributed to the extra-embryonic epithelia (Gorman *et al.*, 2004). Since then, using the *T. castaneum* system described above, where a serosa-less

embryo is created by maternal RNAi of *zen-1*, the immune competence of eggs with and without a serosa was examined (Jacobs and van der Zee, 2013). This work showed that the serosa is needed for the expression of immune genes in the *T. castaneum* embryo and the level of this immune gene response is comparable to that of an adult beetle (Jacobs and van der Zee, 2013). The immune response of the serosa significantly reduces bacterial growth; bacteria propagate twice as fast in serosa-less eggs (Jacobs *et al.*, 2014a). This provides solid evidence for the role of the serosa in embryonic immune competence. However not all insects or even beetles with a serosa are able to mount an immune response as has been demonstrated in the carrion beetle (*Nicrophorus vespilloides*) (Jacobs *et al.*, 2014b). Therefore, having a serosa does not necessarily assure the presence of an embryonic immune response. Additionally, although we know that certain genes are upregulated in *T. castaneum* eggs with a serosa compared to serosa-less eggs, we don't know what the serosa gene expression looks like. In Chapter 5, I will discuss the immune competence of *B. anynana* eggs and investigate the serosa specific gene expression upon septic injury.

1.6 Study species

In my thesis I have used two model butterfly species to investigate the effects of environmental stress on butterfly embryo's; the emerging eco-evo-devo model Speckled Wood butterfly *P. aegeria* (e.g. Schmidt-Ott and Lynch, 2016, Chapter 2 and 4) and the established model species *B. anynana* (Brakefield *et al.*, 2009b, Chapter 5) (Figure 1.5). Both *B. anynana* and *P. aegeria* are grass-feeding Satyrids, and their degree of phylogenetic relatedness as well as diet, facilitates cross-species comparisons (Peña *et al.*, 2006; Wahlberg *et al.*, 2009).

Pararge aegeria is a temperate zone woodland species that has a widespread distribution throughout predominantly Europe and Northern Africa (Livraghi et al 2018, and references therein). *Pararge aegeria* is a very suitable model species for monitoring the response to environmental change; for example, it displays a relatively rapid (although population-specific)

rate of recovery after drought episodes (Oliver *et al.*, 2015 and references therein). It has northwards range-expansion, tracking climate change (Hill *et al.*, 1999; Pateman *et al.*, 2016; Tison *et al.*, 2014), while other species may not be able to do the same, such as the closest relative of *P. aegeria* in the UK; the Wall Brown (*Lasiommata meger*a) (Palmer *et al.*, 2015; Van Dyck *et al.*, 2014; Weingartner *et al.*, 2006). The phylogeography and patterns of gene flow in this species have been characterised at great depth (Habel *et al.*, 2013; Livraghi *et al.*, 2018b; Tison *et al.*, 2014; Weingartner *et al.*, 2006). *Parage aegeria* is of particular interest to answer the question of how butterfly embryos protect themselves against environmental stressors as it is in *P. aegeria* that it was shown, for first time in Lepidoptera, how the extraembryonic serosa is specified by the mother, and that the expression patterns of all *Shx* genes is involved in patterning the serosa (Ferguson *et al.*, 2014). More information on the model species *P. aegeria* especially regarding egg formation and embryonic development can be found in Chapter 2.

Bicyclus anynana is a well-established model organism for the research on seasonal polyphenism (i.e. developmental plasticity) and evolution thereof in wing morphology and patterning, size, behaviour and reproductive strategies (Bhardwaj *et al.*, 2018; Brakefield and Larsen, 1984; Brakefield *et al.*, 2009b; Fischer *et al.*, 2010; Franke *et al.*, 2014; Geister *et al.*, 2008a, b; Monteiro, 2015; Özsu *et al.*, 2017). A more detailed overview of this model species can be found in Chapter 5.

As briefly mentioned before in Chapter 1.1, there are many tools available for my two model species to help to elucidate how a butterfly embryo protects itself against environmental stressors. These include, genomic resources which are available on LepBase (Challis *et al.*, 2016), transcriptomics and custom bioinformatic pipelines (Chapter 5 and Carter *et al.*, 2016), *In situ* hybridisation and most recently CRISPR/Cas9 (Beldade and Peralta, 2017; Carter *et al.*, 2013, 2015; Ferguson *et al.*, 2014; Livraghi *et al.*, 2018b; Mazo-Vargas *et al.*, 2017; Zhang *et al.*,

2017). In this thesis, I will add a embryonic morphological time series for *P. aegeria* (Chapter 2) to the available resources.

Figure 1.5: The two butterfly species; *Pararge aegeria* and *Bicyclus anynana,* used in this thesis (**A**) *P. aegeria* adult, dorsal view; (**B**) *P. aegeria* egg close to hatching; (**C**) Two *B. anynana* adults (Wet-season morphs), ventral view; (**D**) A *B. anynana* egg close to hatching (Copyright indicated).

1.7 Aims

In this thesis I will aim to investigate how a butterfly embryo copes and reacts to environmental stress. Given its hypothesised role in responding to environmental stressors such as drought, pesticides, and immune attacks, the serosa is a candidate trait for how butterflies may cope with environmental change. I will investigate the functional role of the serosa under environmental stress. Furthermore, although we already have some knowledge about the genetic basis of its development, we do not know the genes involved in its functioning (under stress). Elucidation of both the function and genes involved is of paramount

importance to better understand how eggs respond to environmental stressors and allow us to identify candidate genes that could be under selection.

1.7.1 Research objective 1- *Pararge aegeria* egg morphology and embryogenesis

Chapter two details the embryonic development of *Pararge aegeria*, one of my two main model systems. The main aim was a clear and detailed characterisation of the egg morphology throughout embryonic development; both of the embryo and the serosa. It is very likely that the embryo and its serosa respond differently to environmental stressors depending on the developmental stage (Lamer and Dorn, 2001). To aid this understanding a detailed overview of the morphology is necessary.

1.7.2 Research objective 2- The effects of insecticides on butterflies

Chapter 3 reviews current knowledge of the effects of insecticides on butterflies, and outlines suggestions for future studies investigating insecticide effects on butterflies from the molecular level up to a landscape-scale level. In particular, it highlights the need to understand the molecular basis, including the genes involved, to fully gauge how butterflies may adapt to an environment that becomes increasingly stressful, not least through inadvertent contact with pesticides.

1.7.3 Research objective 3- The direct and indirect effects of hormone analogs on *Pararge aegeria* egg production and embryonic development

Pyriproxyfen (a juvenile hormone, JH analog) and commercially available ecdysteroid 20E analogs are often used as pesticides and classified as endocrine disruptors. Given that they are analogs of the two key insect hormones that are involved in insect development, reproduction and physiology, their effects may be very significant when they enter the insect systems. Butterfly embryos can come into contact with such analogs in a variety of ways; directly (i.e. hormone analog covers the eggshell), or indirectly (i.e. transgenerational via the mother; hormone analog affects egg production and may get incorporated into the egg and/or affect

hormonal regulation in the developing offspring (Carter *et al.*, 2013; Dorn and Buhlmann, 1982; Riddiford and Williams, 1967)). In Chapter 4, these direct and indirect effects of JH and 20E analogs on embryonic development and survival will be investigated. For the indirect effects, I will not only investigate the immediate effects on the developing embryo but also assess whether hormonal regulation is affected in later development, by investigating wing size and shape, known to be affected by hormonal levels (Gokhale *et al.*, 2016).

1.7.4 Research objective 4- The role of the extra-embryonic serosa in mediating an (immune) response in *Bicyclus anynana*

Chapter 5 details the functional role of the serosa in mediating an (immune) response to a bacterial challenge, and the genes involved therein. One of the natural environmental challenges developing eggs can be exposed to are microbes. The egg has multiple physical protections, but this Chapter investigates the response of the butterfly egg when microbes manage to penetrate these layers. Using an artificial infection protocol in which I pricked eggs with lyophilised Gram-positive and negative bacteria I will establish whether *B. anynana* embryos are able to mount an immune response. More specifically, I aim to establish whether the extra-embryonic serosa plays an instrumental role in this immune response, using *in situ* hybridisations and RNA-sequencing.

Chapter 2- Embryonic development of the Speckled Wood butterfly (*Pararge aegeria)*

2.1 Introduction

Insects comprise a very diverse group of species displaying tremendous morphological variability, including many lineage-specific evolutionary novelties (Busey *et al.*, 2016; Linz and Tomoyasu, 2018). Although *Drosophila melanogaster* has proven to be an excellent model system for investigating insect development in general (Casas-Vila *et al.*, 2017; Venken *et al.*, 2016), there is a need to investigate a much wider range of species to gauge how differences in developmental programs generate diversity in insect body plans (Schmidt-Ott and Lynch, 2016). In a broader context, understanding the variability in developmental mechanisms will aid understanding the developmental changes that underlie adaptive evolution within and between species in an ecological context (Abouheif *et al.*, 2014; Carroll, 2008). Recent advances in affordable high-throughput sequencing, and functional genomic techniques such as CRISPR/Cas9 (Livraghi *et al.*, 2018a), have opened up the study of comparative developmental genetics, and seen the advent of a new generation of insect model organisms, specifically in the context of embryogenesis (Schmidt-Ott and Lynch, 2016). Furthermore, it has become apparent that not only embryos themselves display developmental diversity, but also their extra-embryonic tissues, for example the serosa (Carter *et al.*, 2015; Ferguson *et al.*, 2014; Panfilio, 2008; Schmidt-Ott and Kwan, 2016). The insect serosa in itself can be considered an evolutionary novelty (Jacobs *et al.*, 2013) and is hypothesised to be associated with ecological diversification, although we are only just beginning to gather evidence in support of this hypothesis (Chapter 5; Jacobs *et al.*, 2013; Jacobs *et al.*,2014a).

Insect development has classically been divided into three classes based on their segmentation pattern; short-germ, intermediate and long-germ-band development. These designations refer both to the initial size of the germ anlage and the differences in embryogenesis (Krause, 1939a, b; Sander, 1976, 1994). In many long-germ-band embryos (e.g. *D. melanogaster*, which is highly derived with respect to segmentation (Damen, 2007)), all

segments are specified almost simultaneously within the blastoderm before gastrulation (Davis and Patel, 2002). In short-germ-band embryos (e.g. *T. castaneum*) only head regions, including the procephalon as well as the gnathal segments (to form the mouth apparatus), are specified and formed at an early stage in the blastoderm. After gastrulation, the thoracic and abdominal segments are progressively generated from a posterior growth zone or segmentation addition zone (Davis and Patel, 2002).

The terms short- and long-germ-band development as described above are the extreme ends of a spectrum. Intermediate germ-band insects fall somewhere within this spectrum, depending on onset and rate of specification and differentiation of the segments (Davis and Patel, 2002). In particular, regulatory network modelling has shown that short, intermediate and long-germ-band development modes emerge from an evolving core network, displaying spatio-temporal variation in the expression of key regulatory genes (Fujimoto *et al.*, 2008; Salazar-Ciudad *et al.*, 2001; Ylla *et al.*, 2017). This results in shifts in the process of patterning and segmentation relative to gastrulation (Brown and Denell, 1996; Davis and Patel, 2002; Fujimoto *et al.*, 2008).

Phylogenetically, ancestral insects tend to display short-germ-band embryogenesis (Ylla *et al.*, 2017) and more derived insects appear long-germ (Élio *et al.*, 2014; Sander, 1976). Historically, Lepidopteran embryos have been classified as intermediate germ-band (Anderson, 1972). Although this is apt to an extent, Lepidoptera do highlight that germ-band mode of development is a continuous trait rather than a discrete one, as germ-band mode appears very variable in this order (Kraft and Jäckle, 1994; Masci and Monteiro, 2005; Nakao, 2010). Basal Lepidoptera, such as the Micropterigidae and Exoporia (both moth clades), display small embryonic anlagen and slow development, which are most often associated with short germ-band development (Davis and Patel, 2002; Kobayashi, 2003). The Ditrysia, which encompasses butterflies and the more derived moths like the popular model species *Bombyx mori* and

Manduca sexta (Mitter *et al.*, 2017), show more variation in development mode. *Bombyx mori* displays a classical intermediate germ-band development, while *M. sexta* and butterflies such as *Heliconius erato* and *Bicyclus anynana* have been described as being more at the long-germ end of the spectrum (Aymone *et al.*, 2014; Kobayashi, 2003; Kraft and Jäckle, 1994; Masci and Monteiro, 2005; Nakao, 2010). However, other studies suggest that *M. sexta* early development resembles that of *B. mori* and could thus be classified as being less at the long-germ end of the spectrum (Dorn *et al.*, 1987). These examples highlight the difficulty of assigning strict classification of the germ-band mode in Lepidoptera. The extreme ends of the spectrum (i.e. short- and long-germ) are in fact possibly even closer to each other than previously thought as shown by careful experimentation and modelling in the model species for long-germ development, *D. melanogaster* (Clark, 2017).

There is a need to develop more model species in the context of gauging the developmental modifications underpinning macro-evolutionary changes (e.g. in body plan), but also trait variability at the micro-evolutionary level, and thus in an ecological context and in combination with population genetics (Abouheif *et al.*, 2014; Jenner and Wills, 2007; Nunes *et al.*, 2013; Schmidt-Ott and Lynch, 2016). The Speckled Wood butterfly, *Pararge aegeria* is a prime candidate species for such an approach. The phylogeography and patterns of gene flow in this species have been characterised at great depth (Livraghi *et al.*, 2018b). It is currently of great ecological interest as it is expanding its range northwards, tracking climate change (Pateman *et al.*, 2016; Tison *et al.*, 2014), while other closely related species are not able to do the same (Palmer *et al.*, 2015; Weingartner *et al.*, 2006). Furthermore, many aspects of its life-history variation have been characterised in an ecological context; for example, life cycle regulation (Aalberg Haugen and Gotthard, 2015; Nylin *et al.*, 1995), (plasticity in) female reproduction (Gibbs *et al.*, 2009, 2010a, b, c) and development time (Sibly *et al.*, 1997). Concerning female reproduction, not only have many of the genes underpinning oogenesis *per se* been characterised in this species, but also the maternal regulation of early embryogenesis

(Carter *et al.*, 2013). Furthermore, using *P. aegeria*, it has been shown that butterflies display significantly divergent RNA localisation patterns of maternal transcripts to not only specify and regulate embryonic body axes (Carter *et al.*, 2015), but also the serosa (Ferguson *et al.*, 2014). The latter is not only unique in the sense that it is maternally regulated in the first place, but also in the sense that the genes involved are Ditrysian-specific paralogs of *Hox3* (Ferguson *et al.*, 2014). In terms of techniques, gene expression patterns are readily investigated through, for example, transcriptomics and *in-situ* hybridisations (Carter *et al.*, 2013; Carter *et al.*, 2015; Ferguson *et al.*, 2014), and we also have successfully developed CRISPR/Cas9 in this species (Livraghi *et al.*, 2018a; Mazo-Vargas *et al.*, 2017). As such, *P. aegeria* has been highlighted as an ideal eco-evo-devo model species (Schmidt-Ott and Lynch, 2016), but at present information regarding the development of its embryo and its extra-embryonic serosa is confined to the very early stages only (i.e. with maternal input, just after the maternal-to-zygotic transition and up to 48 hours), and to a large extent only allows us to address research questions in the context of (the evolution of) embryonic axes specification and differentiation. To also truly understand the impact of environmental perturbations on embryonic development we need a better insight into the whole of embryonic development in this species, including characterisation of the serosa.

2.2 Methods

2.2.1 Study species

The Speckled Wood butterfly (*P. aegeria)* is a temperate zone woodland species that has a widespread distribution throughout predominantly Europe and Northern Africa (Livraghi et al 2018, and references therein). Similar to many other species of Satyridae, *P. aegeria* feeds as caterpillars on a variety of grass species in the *Poaceae* family (Shreeve, 1986). The resources for oviposition are obtained during this stage (Karlsson, 1994). At the time of emergence females have few to no mature oocytes (Karlsson, 1987). They mate soon after eclosing and

are monoandrous, i.e. they usually only mate once (Wickman and Wiklund, 1983). When mating occurs on the day of eclosion oviposition usually starts 48h later on the third day of their adult life (Gibbs *et al.*, 2010c). *Pararge aegeria* oogenesis has been described in detail in Carter *et al.* (2013). An ovipositing female will lay eggs singly on suitable host plants, taking into account plant quality, location and temperature (Shreeve, 1986). The newly laid egg is cream coloured and spherical, slightly wider at the base (Fig. 2.1) where the egg is attached to the leaf. The size of the deposited eggs is roughly between 0.6-1.0 mm^2 but the size of the eggs is highly dependent on temperature (Gibbs *et al.*, 2010c), and dependent on resource allocation trade-offs as a result of maternal age and future reproduction (Karlsson, 1987), male harassment (Gibbs *et al.*, 2005), flight (Gibbs *et al.*, 2010b) and landscape of origin (Gibbs *et al.*, 2012). Fertilised eggs broadly maintain their size and shape throughout development. Unfertilised eggs become more yellow in appearance, flatten (i.e. collapse) and dry out. Developmental rate is dependent on egg size (and in particular the quality and amount of resources therein) and temperature (Gibbs *et al.*, 2010c). Development time is around 140 hours (approximately six days) when kept at incubation conditions of around 23 °C and 65% relative humidity, i.e. the conditions used in this study.

2.2.2 Egg collection

Eggs were collected from an outbred laboratory stock, established with Oxfordshire females (Carter *et al.*, 2013; Ferguson *et al.*, 2014). The eggs were dechorionated with 4% sodium hypochlorite (in 1x PBS). After dechorionation the eggs were fixed in a 1:1 mixture of heptane and 5% formaldehyde in 1XPBS in glass vials, for 30 minutes at room temperature followed by an overnight fixation at 4°C while slowly rotating (*cf.* Carter *et al.* (2015)). After fixation the eggs were washed in 1xPBS and gradually dehydrated in methanol (0%, 10%, 20%, 40%, 60%, 80%, and 100%) and stored in methanol at - 20°C. For imaging the eggs were rehydrated in 1x PBS (doing the dehydration series in reverse), after rehydration the eggs were dissected. The vitelline membrane was removed from each egg, and each egg was then either imaged for

serosa development or dissected further to remove the serosa (and when necessary the yolk) to image the embryo. This was done for the complete time series. Embryos and serosas were stained with 1:4000 4',6-Diamidine-2'-phenylindole, dihydrochloride (DAPI, ThermoFisher: 358⁄461nm) for 20 min. The embryos were imaged using a Zeiss Axio zoom v.16.

2.3 Results

Using significant transitions in external embryonic morphology as stage markers (after Broadie *et al.,* 1991) I have identified 22 distinct developmental stages in *P. aegeria* during embryogenesis.

Stage 1 is 0-5 hours after egg laying (AEL; 0-4% of Development Time, DT). In these first 5 hours AEL, the egg contains a modest amount of cytoplasm and a relatively large amount of yolk, is syncytial and fully enclosed by a shell formed from the vitelline envelope and the chorion (Chapman, 1998). Although the chorion is the thickest egg shell layer (e.g. 7-8 µm in *M. sexta*), respiration in insect eggs can take place through the permeable chorion, and/or through a series of tiny openings connecting the outer surface of the chorion with an inner respiratory plastron; the aeropyles (García-Barros and Martín, 1995; Hinton, 1981; Omelina *et al.*, 2013; Wigglesworth and Beament, 1950). These aeropyles allow for careful control of oxygen influx (i Monteys *et al.*, 2005; Woods *et al.*, 2005; Woods, 2010). However, not all Lepidoptera have them, there is variation reported between closely related species and even within a species (García-Barros and Martín, 1995). *Pararge aegeria* does not appear to have aeropyles (Fig.2.1), which has also been reported by García-Barros and Martín (1995). *Pararge aegeria* has been reported to have a variable number of micropyles (up to 4), which may be associated with female mating status (Iossa *et al.*, 2016), but we typically only detect two at either end of the egg (Fig. 2.1) in our UK stocks. Figure 2.2A shows a freshly laid egg with its chorion removed.

Although not visible externally, previous studies on *P. aegeria* have described mRNA localisation patterns at this stage of development (Carter *et al.*, 2015; Ferguson *et al.*, 2014), underpinning embryonic body plan formation. Indeed, many regions of a Ditrysian embryo are maternally specified from an early stage, driving differentiation of various embryological structures much later in development, including segments (Myohara ,1994; Carter *et al.,*2015 and references therein). Furthermore in *P. aegeria*, maternal localisation of three *Hox3* paralogs will direct the extra-embryonic serosa formation in stage 3; *ShxC* , *ShxD* and *zen* (Ferguson *et al.*, 2014).

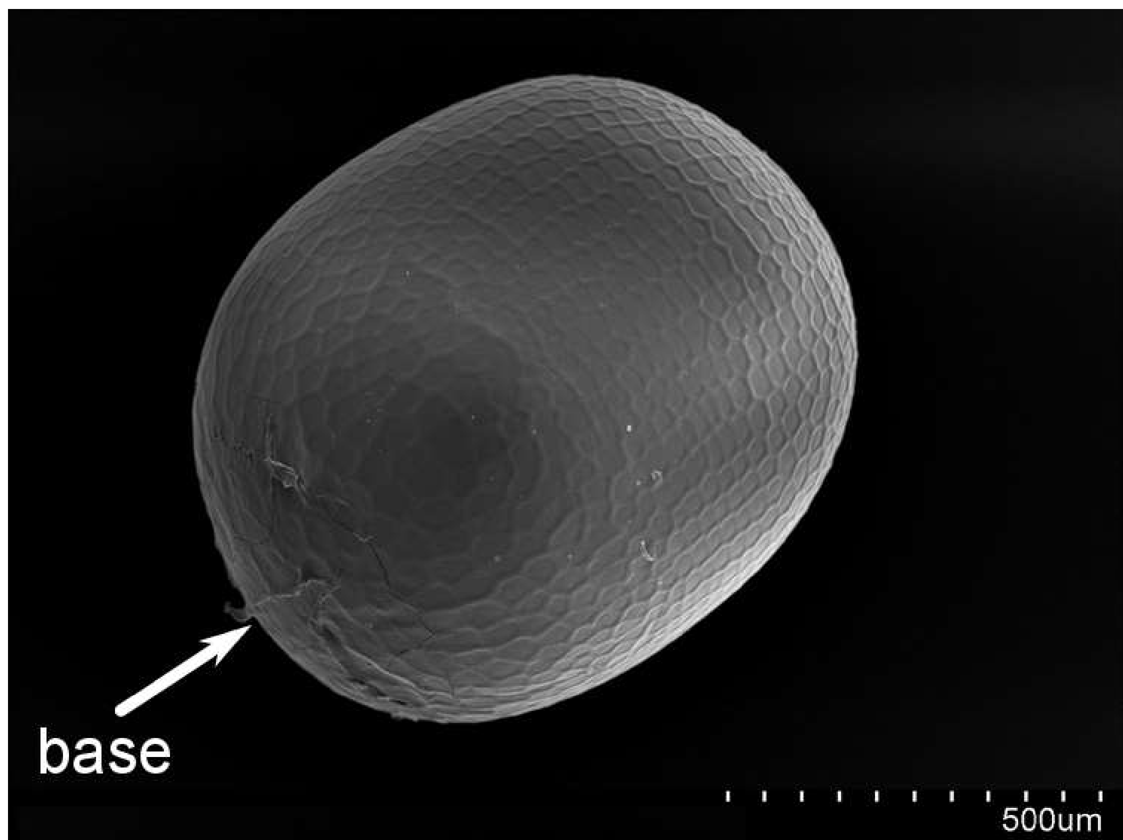

Figure 2.1: Scanning electron microscope image of a freshly laid *Pararge aegeria* egg. Indicated are the base of the egg and the opposite side where the micropyle(s) are located. The chorion displays a heliocoidal lamellar structure, lacking aeropyles.

Stage 2 is 6-7 hours AEL around 5% DT (Fig. 2.2B). There is only a relatively brief syncytial blastoderm stage, ending in this stage. Cellularisation takes place from the top of the egg to the base, asynchronously upon positioning of the nuclei. The cell density is initially higher at the anterior end, and cleavage does not so much proceed through invagination (furrows) but more through budding (Kobayashi, 2003; Myohara, 1994; Takesue *et al.*, 1980). For *P. aegeria*, and other butterflies, stages 1 and 2 with their easy access to the nuclei, are ideal for CRISPR/Cas9 injections (through the micropyle) (Livraghi *et al.*, 2018a).

Stages 3 and **4** are 8-12 hours AEL (6-8% DT)**.** Ferguson *et al.* (2014) have shown for *P. aegeria* that these stages are characterised by a transition from maternal effect gene transcripts to zygotic expression, as well as cellularised blastoderm formation (Fig. 2.2C-F). Following blastoderm formation two regions can be distinguished, which are considered to characterise the earliest stages of Ditrysian development (Ferguson *et al.*, 2014; Kobayashi, 2003; Miya, 2003) (Fig. 2.2D). At **stage 3** a presumptive extraembryonic region becomes polyploid taking on a large and flattened appearance while the presumptive embryonic region forms the germ anlage consisting of many small compact cells (Fig. 2.2C, D). The germ anlage thus forms initially as a wide domain wrapped around almost the entirety of the periphery leaving dorsal and polar regions to the extraembryonic fate (Ferguson *et al.*, 2014). Miya (2003) made a significant distinction between the terms germ anlage and germ-band, only once the germ anlage sinks slightly into the yolk is it referred to as germ-band (Nagy, 2006). At **stage 4** the extraembryonic region then undergoes significant expansion over the germ-band forming the protective serosal envelope (start of which can be seen in Fig. 2.2E, F). This serosal closure is comparable to that described in, for example Hymenoptera (Buchta *et al.*, 2013; Fleig and Sander, 1988). The formation of the serosa is characterised by the Hox3 paralogs (i.e. four *Shx* genes and *zen*) and stages 3 and 4 are characterised by a transition from maternal *Hox3* transcripts to zygotic (Ferguson *et al.*, 2014).

Stage 5 is around 14 hours AEL (10% DT; Fig. 2.2G, H). The serosa is fully formed, consisting of mononucleated cells, and surrounds the whole embryo and yolk. The germ-band is located directly underneath the serosa (Fig. 2.2H). The serosa will remain in place throughout development although its morphology changes towards the end of embryogenesis. During stage 5 the germ-band contracts. Other Ditrysia have been shown to form the primordial germ cells (PGC's) in this stage (Kobayashi, 2003; Nardi, 1993; Presser and Rutschky, 1957). Candidate genes involved in PGC's formation in *P. aegeria* have been discussed previously, and detailed analyses of the expression patterns of these genes would support the hypothesis that PGC's are formed around this stage (Carter *et al.*, 2015; Schmidt-Ott and Lynch, 2016). In other species it has been shown that PGCs differentiate from a cluster of cells situated along the midline of the germ-band in the course of germ-band formation. Before the germ anlage sinks into the yolk and becomes a cup-shaped germ-band, these PGC's invaginate into the yolk while dividing and then form a cell mass composed of around 30 cells at the dorsal side of the germ rudiment. The PGC's stop dividing in the next stage and they will become incorporated into the newly formed mesodermal cell layer (Kobayashi, 2003).

Stage 6 is around 16-17 hours (12% DT; Fig. 2.2I). The extending germ-band is cup-shaped; its concave side is located against the yolk. There is a differentiation of the protocephalon (i.e. head) region; the anterior end of the germ-band is characterised by auricular lobes, the presumptive head lobes. The caudal end of the embryo is wider than the anterior end of the embryo. At the end of this stage there are the first indications of the primitive groove. Germ-band extension takes place along the anterior-posterior axis. Mesodermal cell formation consists of the formation of a continuous sheet of cells towards the dorsal side of the embryo. I infer from observations on the butterfly *Pieris rapae*, that some dozens of cells fated to be mesodermal move from the dorsal of the embryo along its midline before the formation of the primitive groove (Eastham, 1927). The region along the midline of the embryo then becomes a 'middle plate' composed of tall and regular cells, the lateral parts

of this area are called ‘lateral plates’ (Eastham, 1927; Kobayashi, 2003). The middle plate soon sinks slightly and forms a shallow primitive groove as can be observed in Fig. 2.2I. The lateral plates extend toward the midline of the embryo pushing out the midline dorsally (Stage 7, Fig. 2.2J). The median edges of the lateral plates will fuse in the middle and thus the primitive groove will disappear (Stage 8, Fig. 2.2K) (Kobayashi, 2003).

Stage 7 is around 18 hours (14% DT; Fig. 2.2J) and is characterised by further germ-band extension, resulting in a more narrow and longer embryo, with a bilobed protocephalon (Broadie *et al.*, 1991). The primitive groove deepens and extends towards the posterior end of the embryo; the primitive groove can be observed on the ventral midline of the embryo (Fig. 2.2J). The auricular lobes on the anterior end of the embryo develop more shape, and slightly bend into the yolk. The embryonic posterior has not differentiated and is named the caudal pouch in this stage of development. Shortly after closing of the primitive groove the first external signs of patterning and segmentation outside of the head area can be observed.

Although not observed in this morphological study, as part of segmentation, the internal mesoderm will be segmented along with the external ectodermal segmentation (Kobayashi, 2003). From these segmentally arranged mesodermal regions a number of different tissues will originate during development; the muscles, blood cells, gonads, fat bodies, the suboesophagal body and the dorsal vessel or heart (Kobayashi, 2003).

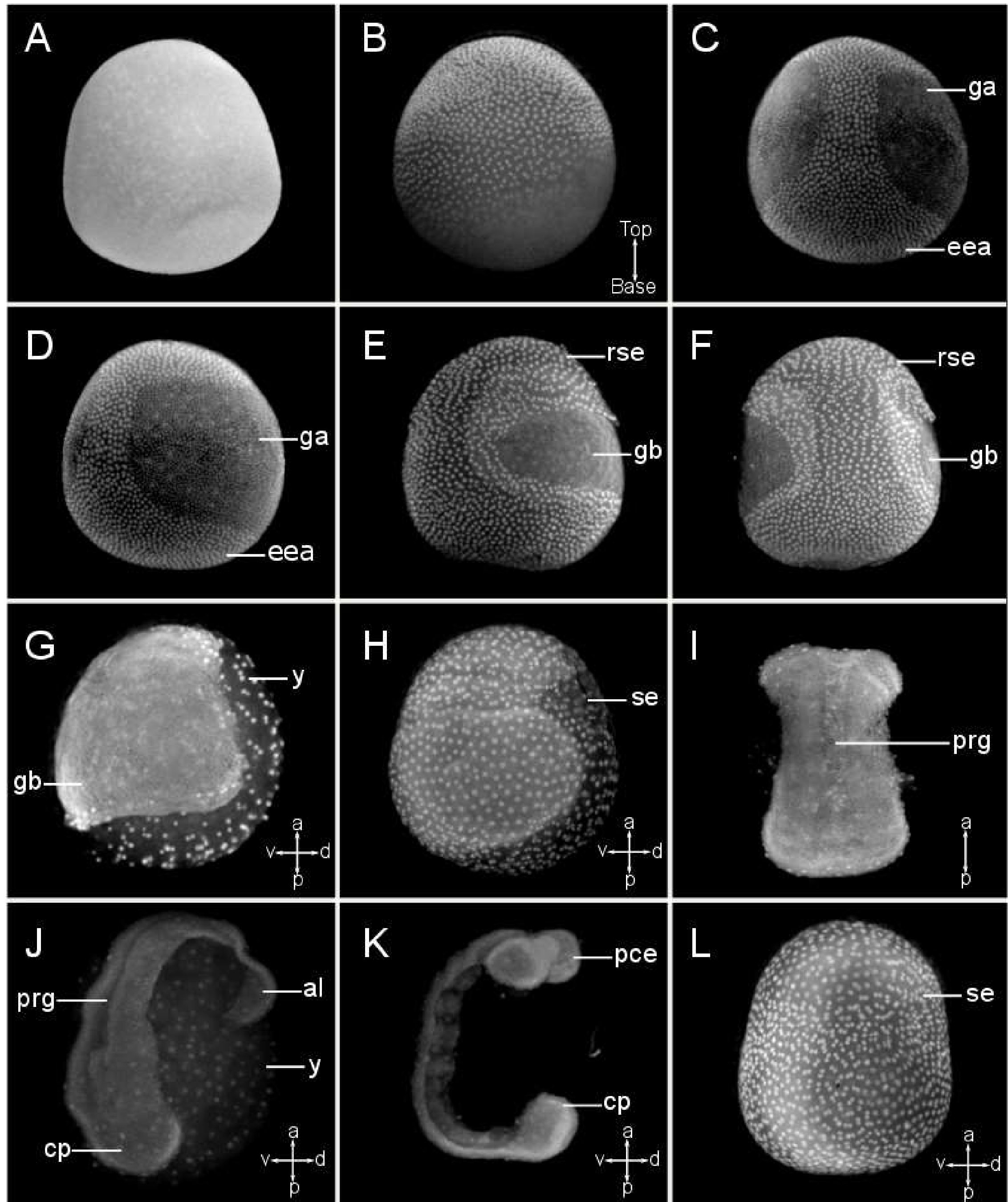

Figure 2.2: The first 8 stages of *Pararge aegeria* embryonic development (0% to 15% development time (DT)) stained with DAPI. Eggs have been dechorionated (see material and methods). A detailed description of the morphology at each stage can be found in the text. The embryo's orientation is indicated with the arrows in the figure with anterior (a) to posterior (p) and ventral (v) or dorsal (d), the top-base orientation indicated in **B** applies to all panels. The serosa has been removed from the specimens in panels **G, I, J** and **K.** Certain morphological features have also been indicated in the figure. (**A**) Stage 1- 0% DT freshly laid egg; syncytial; (**B**) Stage 2- 5% DT; (**C, D**) Stage 3- 6% DT; (**E,F**) Stage 4-8% DT Presumptive serosa folds over

the germ-band; (**G**) Stage 5- 10% DT; (**H**) Stage 5-6 11% DT The serosa envelops the embryo and yolk which can been under the serosa; (**I**) Stage 6- 12% DT , ventral view; (**J**) Stage 7- 14% DT; (**K**) Stage 8-15% DT; (**L**) Stage 8-15% DT. **Abbreviations**: al = auricular lobe, cp = caudal pouch, eea = extra-embryonic area, ga = germ anlage, gb = germ-band, pce = protocephalon, prg = primitive groove, rse= rudimentary serosa, se = serosa, y = yolk

Stage 8 is around 20 hours (15% DT; Fig. 2.2 K, L) and is characterised by germ-band extension. Protocorm formation (i.e. formation of the insect embryo posterior to the protocephalon) can be observed (Kobayashi, 2003) and the primitive groove closes. The head lobes are not differentiated yet. Segments are rapidly specified, anterior to posterior. The most anterior segments correspond to those that will differentiate into the gnathal appendages at later stages, followed by the thoracic ones. The more caudal abdominal segments are not immediately obvious at this stage, and the embryo has a relatively big caudal pouch. The first indications of the stomodaeal invagination or future foregut can be observed as a shallow invagination at the posterior end of the protocephalon. The embryo surrounds the yolk and lies mostly on top of it, but the head segments and the caudal end will fold into the yolk during this, and the following stages.

Though not directly observed in *P. aegeria*, the first neuroblasts appear in other Lepidoptera around a corresponding stage of development (Kobayashi, 2003), which is the start of the central nervous formation. These neuroblasts will eventually form the ventral nerve cord. The ventral nerve cord will form from ganglion in the mandibular to the 10th abdominal segment. Each of these ganglia is derived from dozens of neuroblasts formed at this and the following stages (Kobayashi, 2003; Presser and Rutschky, 1957).

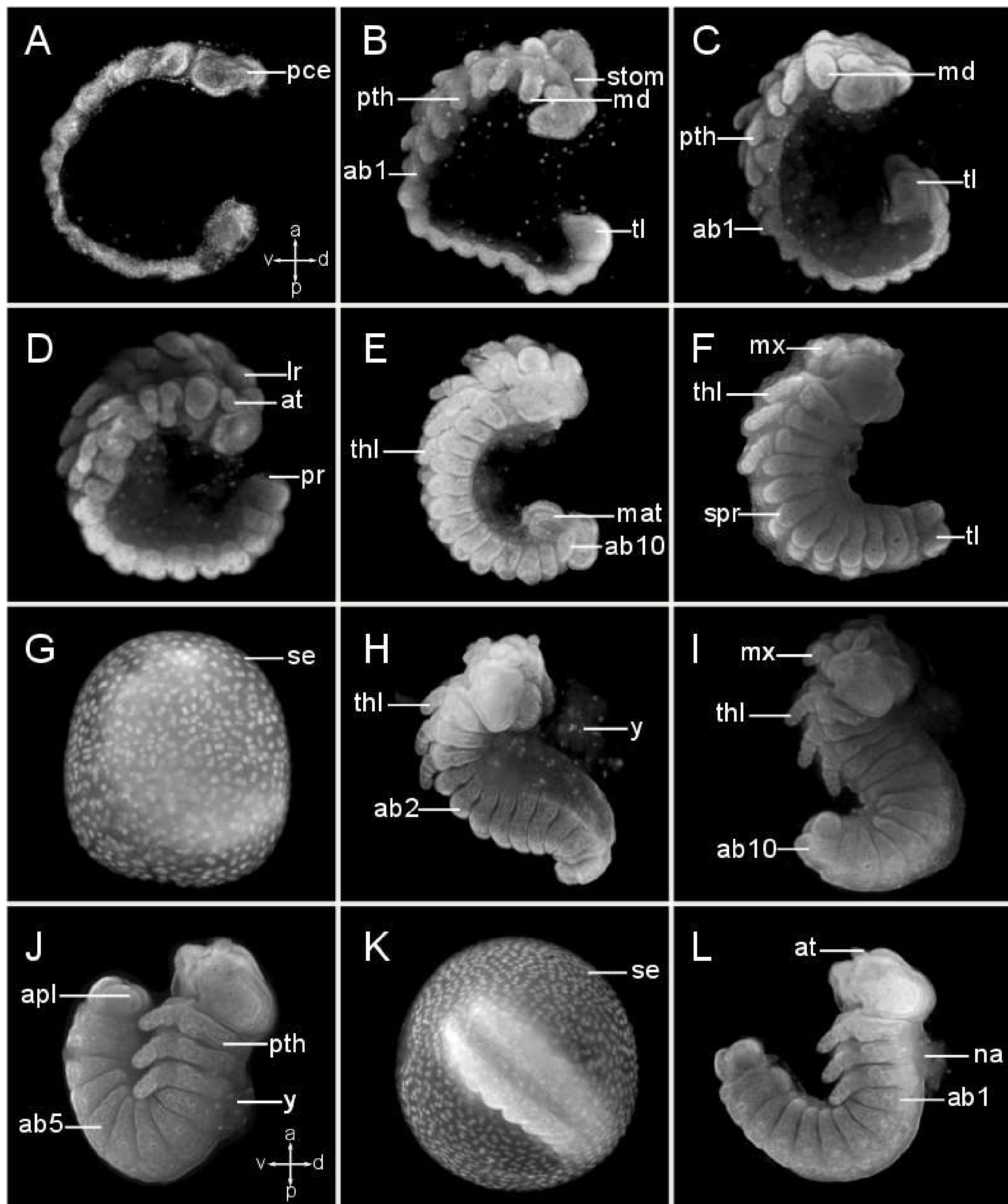

Figure 2.3: Stage 9 to stage 17 of *Pararge aegeria* embryonic development (17% to 60% development time (DT)) A detailed description of the morphology at each stage can be found in the text. All pictures are orientated as indicated in panel **A** and **J** (except **K)** with the anterior (a) to the top and posterior (p) to the bottom of the figure. The ventral (v) side is orientated left while the dorsal (d) side is on the right side of the figure. The serosa has been removed from the specimens in panels **A** to **F, H** to **I** and **L.** Certain morphological features have been indicted. (**A**) Stage 9-17% DT; (**B**) Stage 10-20% DT; (**C**) Stage 11- 25%DT; (**D**) Stage 12-30%DT; (**E**) Stage 13- 35%DT; (**F**) Stage 14-40% DT; (**G**) Stage 14-40% DT; (**H**) Stage 15-50% DT; (**I**) Stage

15-50% DT; **(J**) Stage 16-55% DT; (**K**) Stage 16-55% DT, the base of the egg, through the serosa the open dorsal side of the abdomen can be seen; (**L**) Stage 17-60% DT. **Abbreviations**: ab 1 to 10 = abdominal segment 1 to 10, apl= anal proleg, at = antennal rudiment, lr = labral lobe, mat = malpighian tubules, md = mandibular lobe, mx = maxillary lobe, na = navel, pce = protocephalon, pth = prothoracic segment, se = serosa, spr=spiracle, stom = stomodaeal invagination, thl = thoracic leg, tl = telson, y = yolk.

Stage 9 (24 hours, around 17% DT; Fig. 2.3A). This stage has been described in Carter *et al.* (2015) for *P. aegeria*. In this stage the specification of the segments gets finalised, as evidenced by means of *engrailed* (as a segment polarity gene) expression patterns. Intercalation between the specified segments takes place (*cf.* Benton *et al.* (2016)), resulting in significant germ-band (i.e. embryo) extension. Furthermore, segments start to differentiate, predominantly from the anterior to the posterior. On the ventrolateral side of the most anterior segments, six pairs of lobes appear indicating the start of the formation of the thoracic and gnathal appendages. The lobes on the first three (gnathal) segments are the largest at this time and constitute the developing mandibles, maxillae and labium. Around this time the formation of the protocephalic appendages, the labrum and antennae starts. The stomodeal invagination mentioned in stage 8 has become pronounced and further deepens, initiating the formation of the foregut. The development of the gut will span most of embryonic development (Aymone *et al.*, 2014; Presser and Rutschky, 1957).

Stage 10 (26 hours, around 20% DT; Fig. 2.3B, Fig. 2.4). Segments continue to differentiate, acquiring distinctive morphologies, with cell intercalation between segments finalising (*cf.* Benton *et al.* (2016)). The germ-band has reached it maximum length. The embryo now consists of three recognisable gnathal, three thoracic and 10 abdominal segments in addition to the head and a telson. Protocephalic appendages and the anterior segments display a distinct morphology, described below and indicated in Figure 2.4. The most anterior region of the embryo consists of the head which is differentiated into two head lobes on which the labral lobes are situated. An indentation at the centre of the head is the stomodaeal

invagination. The antennae will eventually form from two lobes at the ventral posterior end of the head region. The segments directly posterior to the head will differentiate into the gnathal segments. On the ventral-lateral side of each gnathal segment two lobes are distinguishable which will later integrate and form primarily the jaw. The first gnathal segment carries the mandibular lobes; the second segment will form the maxillary lobes whilst the third will develop labial lobes. Posterior to the gnathal segments, three thoracic segments with limb buds on the ventral –lateral side of the embryo can be observed; the prothoracic limb buds on the first segment, mesothoracic limb buds on the second segment, and metathoracic limb buds on the third thoracic segment.

Posterior to the thoracic segments are 10 abdominal segments. Although these are also differentiating, morphologically they appear very similar (Fig. 2.4). The telson is located at the most posterior end of the embryo and extends into the yolk (Presser and Rutschky, 1957; Wall, 1973). A shallow invagination or neural groove is clearly visible along the ventral midline and from *Heliconius erato* it is known that the formation of the proctodaeum or future hindgut begins at the posterior end of the telson (Aymone *et al.*, 2014). As the formation of the inner or mesodermal layer has been completed this stage is also characterised by mesodermal differentiation (Kobayashi, 2003; Presser and Rutschky, 1957).

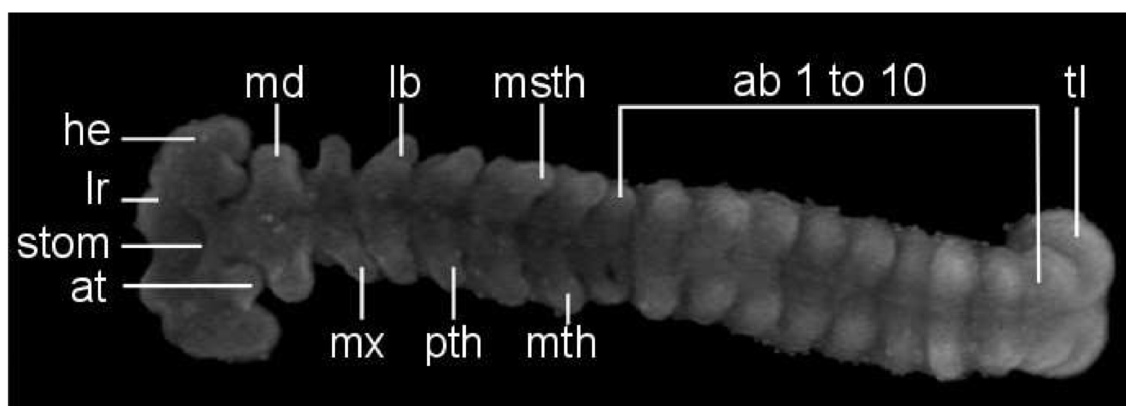

Figure 2.4: Flat mounted *Pararge aegeria* embryo around stage 10 or 20% of development stained with DAPI viewed from ventral side. Identity and location of the gnathal and thoracic appendages have been indicated. The neural groove is present along the ventral midline.

Abbreviations: ab 1 to 10 = abdominal segment 1 to 10, at = antennal rudiment, he = head lobe, lb = labial lobe, lr = labral lobe, md = mandibular lobe, msth = mesothoracic segment, mth = metathoracic segment, mx = maxillary lobe, pth = prothoracic segment, stom = stomodaeal invagination, tl = telson.

The maximally extended embryo now widens in **Stage 11** (32 hours around 25% DT; Fig. 2.3C). The protocephalic and gnathal appendages keep developing, while the abdominal segments still appear morphologically very similar. The head lobes become broader and move underneath the antennal lobes at the dorsal/ yolk side of the embryo. The labral lobes move towards each other, and they will meet in the centre in the following stage (i.e. Stage 12). The mandibular lobe expands, condenses, and shifts to a slightly more anterior position. The outgrowing anterior limb buds change position from the lateral side of the embryo thoracic segments to a more ventral medial position. A proctodaeal invagination appears in the telson; this ectodermal depression will eventually become the anal channel. Cells at the anterior and posterior ends of the C-shaped embryo migrate inward to form the foregut, hindgut and midgut. The foregut will arise from ectodermal cells of the stomodeum and the hindgut from the proctodaeum (Aymone *et al.*, 2014; Kobayashi, 2003). The median neuroblasts are known to arise in this stage along the inner neural groove, giving rise eventually to either the transverse connectives as was observed in *P. rapea* (Eastham, 1931) or the median nerve *Heliothis zea* (Presser and Rutschky, 1957).

The complete embryo, including the telson, which was previously located in the yolk, lies on top of the yolk in **Stage 12** (40 hours, around 30% DT; Fig. 2.3D). The thoracic limb buds extend distally and, morphologically, display a more pronounced segmental identity. I infer, on the basis of descriptions from other Lepidoptera (Presser and Rutschky, 1957), that the hypopharynx, maxillae and labium are now combined to form an integrated structure, which I see completed in *P. aegeria* in stage 13/14. The maxillary and labial buds become closely associated. Completing the medial shift initiated in Stage 11, the labral segments fuse. During

this stage abdominal segment differentiation becomes morphologically pronounced, with abdominal segment 9 and 10 being narrower than the other abdominal segments.

Based on the description of the morphological development in other Lepidoptera, as the intersegmental grooves deepen, some neuroblasts in segmental and paired clusters, differentiate into ganglions (Kobayashi, 2003). The neural groove then increases in depth as seen in the last two stages (stages 10 and 11, Fig. 2.3B and C), which separates each ventral ganglion in two distinct lateral cell mases in each segment (Presser and Rutschky, 1957). Paired ganglia in the three gnathal, three thoracic and 10 abdominal segments have been observed at this stage in other Lepidoptera (Kobayashi, 2003).

The labial appendages shift position to mid-ventral in **Stage 13** (50 hours, around 35% DT; Fig. 2.3E), and come to lie closely to the maxillary appendages (Presser and Rutschky, 1957). At the caudal-ventral end malpighian tubules arise at the dorsal side of the telson and proctodaeal, these also disappear from view again during this stage, becoming covered by a mesodermal sheath (Presser and Rutschky, 1957). The spiracles have by now a distinct appearance and are somewhat raised in the prothoracic segment. The 10th abdominal and telson come to fuse. The proctodaeal invagination cavity at the most caudal end of the embryo disappears. The thoracic legs become segmented. The labral lobes have properly fused, which means that based on morphological descriptions in the literature the neuropile begin to appear along the dorsal margins of the lateral cell masses in the ganglia (Kobayashi, 2003; Presser and Rutschky, 1957).

The embryo condenses, and the segments appear shorter and wider in **Stage 14** (60 hours, around 40% DT; Fig. 2.3F and G). The yolk remaining on the dorsum of the embryo is almost enclosed by the embryonic lateral walls extending dorsally (Kobayashi, 2003). This is the first step in eventual dorsal closure, which will be completed after katatrepsis (i.e. the embryo turning around in the egg) (Aymone *et al.*, 2014). The head becomes less flat, and

more rounded, and loses the clear midline through the middle of the head. The labial lobes lie next to each other in the middle of the head but are still to become separate lobes with the maxillary appendages forming closely associated lateral lobes on either side. The two antennae protruding from the head flank the labrum, which is located on the most anterior-centre of the head in the middle and anterior to the two mandibular lobes.

Prolegs begin to form on the 3rd, 4th, 5th and 6th abdominal segments. The ventrally located sphericals have become located more laterally through cell movements and condensation of the embryo. They are now clearly visible on all thoracic segments and the first eight abdominal segments. The position of the 10th abdominal segment is by now more dorsal, while the position of the 9th abdominal segment is now more immediately anterior to the telson. This segment is relatively small and lacks a spherical on each of its sides. There are two openings forming on the telson at the location where eventually the anal prolegs will form (Dorn *et al.*, 1987; Presser and Rutschky, 1957; Wall, 1973)

The telson is generally understood as a non-segmental posterior body region which lacks a neuromere, mesodermal sacs and appendages. However, the telson does develop anal prolegs, as it only does so after fusing with the 10th abdominal segment it could be that it assumes a segmental character after fusion. Although it has also been hypothesised that the telson has remnants from the vestigial 11th abdominal segment, facilitating the anal proleg formation (Kobayashi, 2003).

Although not observed, in other Lepidoptera the first cardioblasts appear as crescent shaped cells around this stage. These form from the somatic mesoderm of the prothoracic to 10th abdominal segment. As the body wall extends dorsally, the cardioblasts will move along in the growth direction (Kobayashi, 2003; Presser and Rutschky, 1957). Additionally, the three gnathal ganglia will together form the subesophageal ganglion shortly after neuropile

formation. The subesophageal ganglion enervates mouthparts, neck muscles and salivary glands (Presser and Rutschky, 1957).

The embryo makes a turn in **Stage 15** (72 hours, around 50% DT; Fig. 2.3H and I) ending up S-shaped in appearance. Dorsal closure will occur once the embryo is fully turned (Aymone *et al.*, 2014). During katatrepsis the now S-shaped embryo gradually moves from one side of the egg to the other side leading with the posterior end of the embryo. This movement leads into a J-shaped embryo (refer to Fig. 2.7G, H and I for the movement of the embryo through the egg). Before katatrepsis, the ventral side and appendages are located immediately below the egg surface and serosa (Fig. 2.3G and 2.7G). After katatrepsis that will be the still open dorsal side. The dorsal space, or space behind the dorsal side of the embryo, gets smaller during this stage with the yolk being absorbed by the embryo. All the main morphological structures of the body plan are clearly distinguishable by now. The serosa stays intact throughout this process (Fig. 2.6B).

Other Lepidoptera have been reported to unite the three protocerebral lobes into one during the revolution of the embryo which is also seen in *P. aegeria.* Not observed is the separation between the epidermis and neuromeres which occurs around this time as well (Kobayashi, 2003; Presser and Rutschky, 1957). Due to the morphogenetic movements of the head region in stage 14 in combination with katatrepsis the basic form of the proto-, deuto-, and tritocereberum is established during this stage (Kobayashi, 2003; Presser and Rutschky, 1957).

Dorsal closure, initiated in Stage 14 continues into **Stage 16** (78 hours, around 55% DT; Fig. 2.3J, K and 2.5), now largely closing the embryo dorsally. Only the dorsal part of both meso- and metathorax will not yet close and leave an opening by which the yolk mass enclosed by the embryo is connected with the yolk outside of the embryo. This cavity persists until Stage 18 after which it will close (Fig. 2.6A). The embryo looks like it is folded in two

around the second and third abdominal segment in a J-shape. It is difficult to observe ventral development during this stage. The serosa and amnion are not ruptured during katatrepsis nor do they seem to facilitate the process, unlike what is observed in other insects (Kobayashi, 2003; Panfilio, 2008). The serosa will surround the embryos throughout the whole process of dorsal closure, and afterwards (Fig. 2.3A and 2.6B). It does not seem to be at all involved in the process, unlike in other insects such as *T. castaneum* and *Oncopeltus fasciatus* (Panfilio, 2008; Panfilio *et al.*, 2013). A layer of yolk is present around the embryo, held between the amnion and the serosa (Chapman, 1998). The maxillary lobes have integrated into the head posterior to the labial lobes, which have fused to form a single median lobe. The antennae are now situated on top of the head in a more lateral position.

Reported for the butterfly *H. erato*, endodermal cells derived from the stomodeum and proctodaeum will enclose yolk in a tube, creating the midgut (Aymone *et al.*, 2014). Vitellophages in the yolk enclosed by the midgut will integrate into the midgut endoderm and form the midgut epithelium (Aymone *et al.*, 2014).

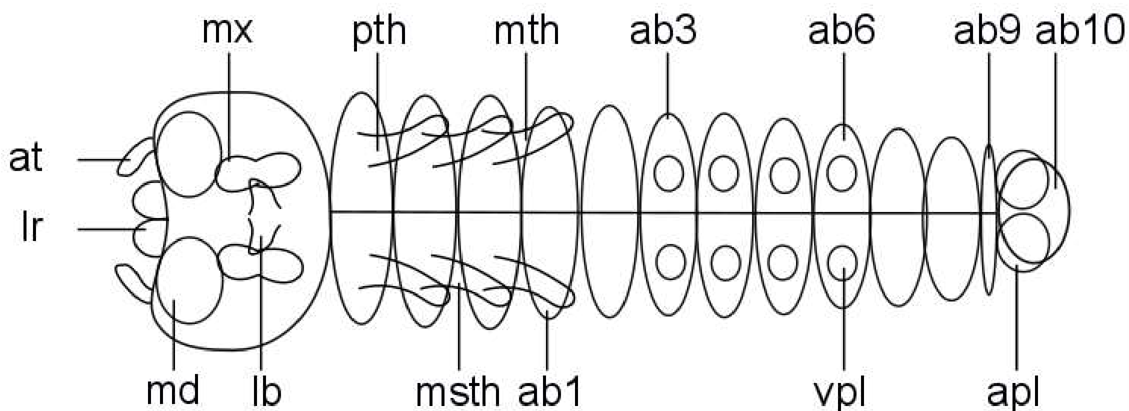

Figure 2.5: Ventral side of a stage 16 *Pararge aegeria* embryo. The identity and location of the gnathal, thoracic and abdominal appendages are indicated. **Abbreviations:** ab 1 to 10 = abdominal segment 1 to 10, apl = anal proleg, at = anttena, lb = labial lobe, lr = labrum, md = mandibular lobe, msth = mesothoracic leg, mth = metathoracic leg, mx = maxillary lobe, pth = prothoracic leg, vpl = ventral proleg.

In **Stage 17** (84 hours, around 60% DT; Fig. 2.3L) the embryo elongates and becomes less folded over but remains in a similar position in the egg as observed during Stage 16. The dorsal side closes except for the small cavity with protruding yolk just posterior of the head. The individual segment identity of the most posterior abdominal segments seems to be lost within this stage; the 7th, 8th and 9th abdominal segment cannot be clearly distinguished.

The antennae are getting thinner and become positioned lower down and more latero-centrally on the head. The mouthparts, the labium and maxilla become more pointed and extrude out of the head region. At this point the labium extrudes further than the maxilla. The mandibles are not visible from the lateral view in this stage. The labrum moves forward and is located between the antennae (Aymone *et al.*, 2014).

The cardioblasts have not been identified in this study but based on literature the cardioblast formed aroundStage 14, will have now moved dorsally during the process of dorsal closure, and the crescent-shaped cardioblasts meet and fuse beneath the dorso-midline of the embryo, forming a tubular heart. The fusion of cardioblasts occurs first on the posterior end of the abdomen and proceeds anteriorly finishing in the prothoracic segment (Kobayashi, 2003; Presser and Rutschky, 1957).

In both basal and derived Lepidoptera the gonads or reproductive structures reach their final position around this time of development. The position of the gonads, however, varies between species, in *B. mori* they are positioned in the 8th abdominal segment, in *H. zea* the fifth segment and in *Neomicropteryx nipponensis* the third abdominal segment (Kobayashi and Ando, 1983; Kobayashi, 2003; Miya, 1958; Presser and Rutschky, 1957). The location of *P. aegeria* gonads has not been determined.

As the embryo keeps growing, the embryo keeps taking up more space inside the egg, having to fold on itself by **Stage 18** (90 hours, around 65% DT; Fig. 2.6A and B). As the dorsal

side closes, so does the dorsal opening with extruding yolk cells. The 10th abdominal segment develops a pair of spikey lobes ventrally, precursors of the anal prolegs. The growing thoracic legs have a pointed appearance distally, as do the prolegs on abdominal segments 3 to 6. The labrum lobes become thinner and can be seen protruding from the mid-anterior of the head. The labium and maxilla are now of equal length, both are still protruding out on the most ventro-posterior part of the head.

In *H. erato,* the elongating hindgut becomes divided into the small intestine, large intestine and rectum (Aymone *et al.*, 2014). At this time the ganglia in the 8th, 9th and 10th abdominal segment fuse, resulting in a compound 8th abdominal ganglion (Eastham, 1931; Kobayashi, 2003; Presser and Rutschky, 1957). This takes place in Stage 18 in *P. rapae*, but before Stage 18 in some other Lepidoptera (Eastham, 1931; Kobayashi, 2003; Presser and Rutschky, 1957). The exact stage in *P. aegeria* could not be identified, but it is hypothesised to be Stage 18.

The yolk slowly depletes whilst the embryo grows and its head is now positioned on top of the curled tail in **Stage 19** (100 hours, around 75% DT; Fig. 2.6C). The two anal prolegs have invaginated. The mandibles can be distinguished from the overlying labrum. The other mouthparts cluster below the mandible. The development of the mouthparts is almost complete and takes on the larval morphology.

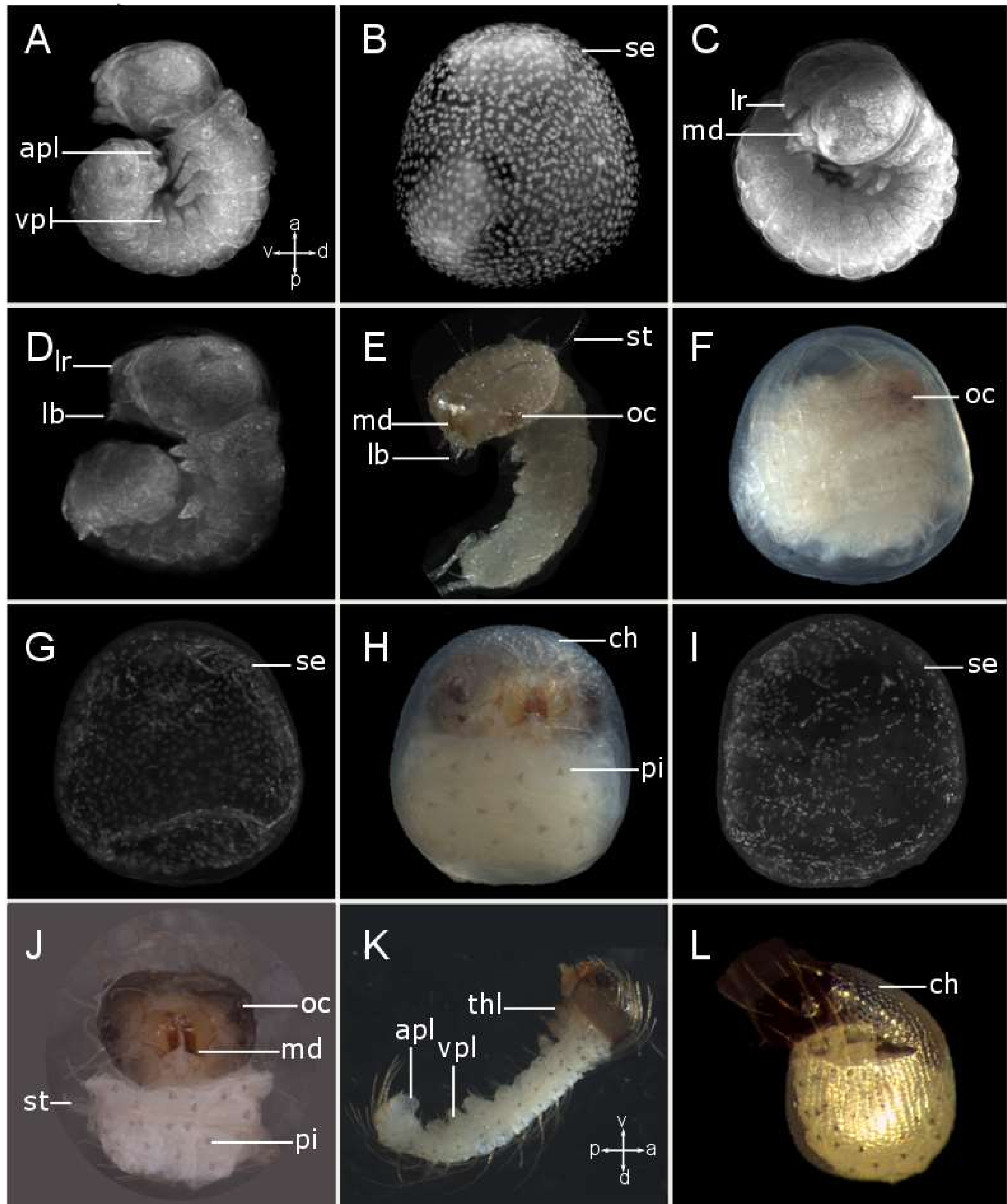

Figure 2.6: Stage 18 to hatching of *Pararge aegeria* embryonic development (65% to 100% development time (DT)) A detailed description of the morphology at each stage can be found in the text. Key morphological features discussed in the text are indicated, the orientation of the embryo is indicated where folding up of the embryo has not yet taken place with anterior (a) to posterior (p) and ventral (v) or dorsal (d). The serosa has been removed from the specimens in panels **A, C** to **E, J** and **K.** (**A**) Stage 18-65% DT; (**B**) Stage 18-65% DT ; (**C**) Stage 19 – 75% DT; (**D**) Stage 20-85% DT; (**E**) Stage 21-90% DT; (**F**) Stage 21-90% DT; (**G**) Stage 21-90% DT; Stage 22-95%; (**I**) Stage 22-95% DT; (**J-K**) Stage 22-95% DT; (**L**) Hatching-100% DT. **Abbreviations**: apl = anal proleg, ch = chorion, lb = labium, lr = labral lobe, md = mandibular

lobe, mx = maxillary lobe, oc = ocelli, pi = pinaculum, se = serosa, st = setae, thl = thoracic leg, vpl= ventral prolegs

The embryo is completely curled up in the egg, flattening somewhat by **Stage 20** (120 hours, around 85% DT; Fig. 2.6D) with little space above and below left for growth completion. Rows of setae display initial tanning, turning a very light shade of brown. Likewise, the mandibles are also a light brown as a result of cuticular sclerotization, which can be observed as light stripes on the anterior side of the head. There is still a very small amount of extraembryonic yolk left but this will disappear during this stage. The serosa is still present and appears morphologically-similar to that described in stage 5 when the serosa was first observed fully formed and functional (Fig. 2.2H and 2.6B).

The yolk has been completely consumed by **Stage 21** (126 hours, around 90% DT (Fig. 2.6E,F and G).The mandibles tan further and the serated edges get darker. The ocelli (eyes) are starting to tan on the lateral side of the head. The individual tanned occelli are hard to observe but seem to number two at each side at this stage. The observed darkening of various morphological structures is to a large extent due to melanin accumulation. Although the serosa is still in contact with the embryo its morphology has changed. Whilst it had a more or less regular distribution of cells and their nuclei since it was formed in stage 5; a number of the nuclei seem to have dissapeared around the base of the egg. It is changing its shape and becoming more closely attached to the embryo.

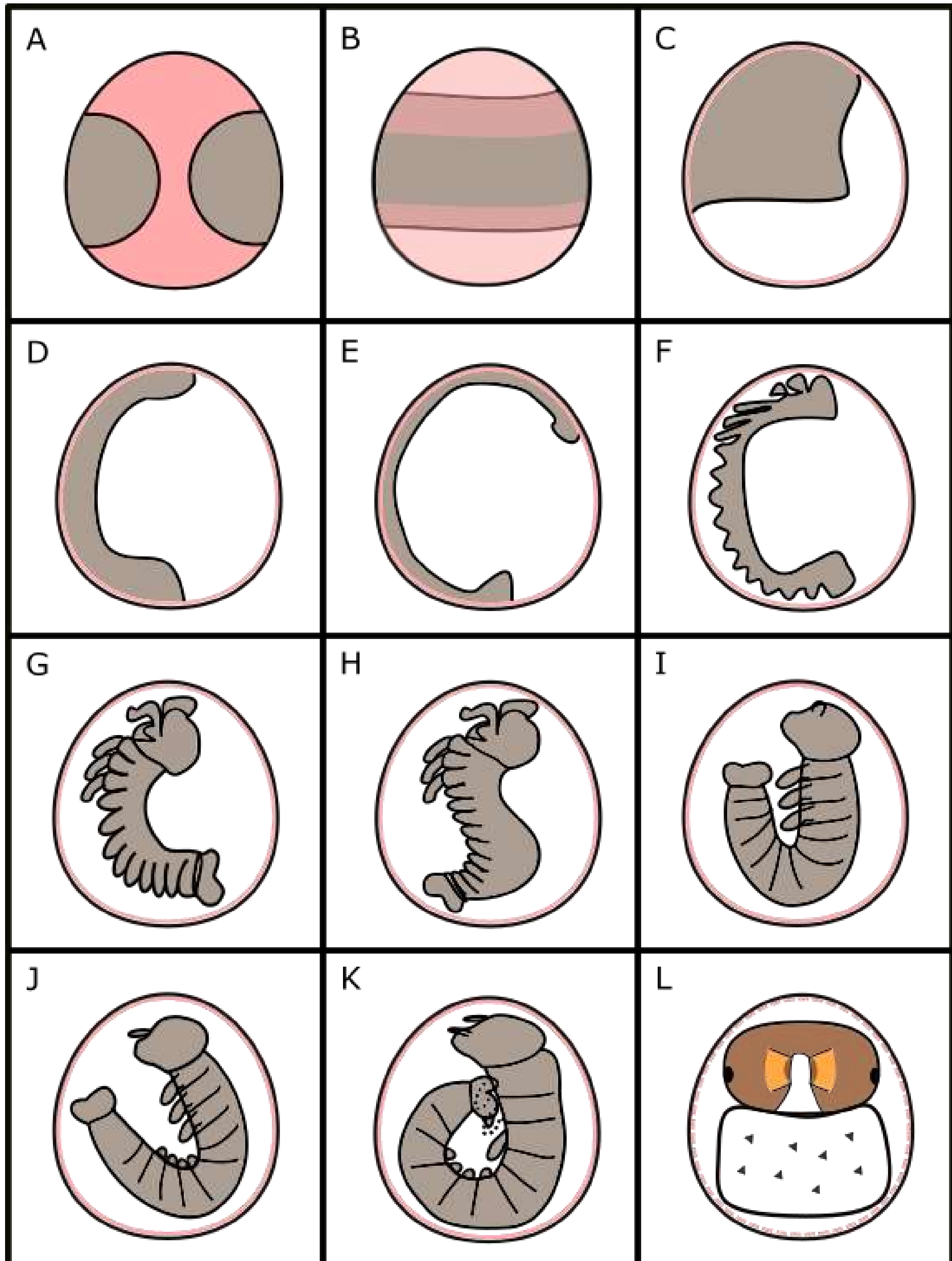

Figure 2.7: Summary for *Pararge aegeria* of key developmental stages of the embryo and serosa and the location of the embryo in the egg. The germ anlage/band is indicated in grey in panel A to K, the serosa is indicated in pink. (**A**) Stage 3: Differentiation of the germ anlage and presumptive serosa (**B**) Stage 4: The serosa folds over the germ-band; (**C**) Stage 5: Germ-band moves to the top of the yolk and both embryo and yolk are surrounded by the serosa; (**D**) Stage 6-7: Cup shaped embryo, the dorsal side of the embryo is located towards the yolk and

the ventral side towards the serosa and chorion; (**E**) Stage 8-9: Germ-band extension and completion of segmentation specification; (**F**) Stage 10+11: External differentiation of embryonic segments (**G**) Stage 12-14: The embryo condenses (**H**) Stage 15: Katatrepsis, the embryo turns around in the egg moving its caudal end towards the opposite end of the egg. The embryo appears S-shaped in this stage (**I**) Stage 16: The dorsal side of the embryo is located towards the serosa and chorion. Initiation of dorsal closure (**J**) Stage 17-18: Dorsal closure completed (**K**) Stage 19-21 Embryo grows and rolls itself up in the egg (**L**) Stage 22: The embryo is fully formed; it has a tanned head capsule and is ready to hatch from the egg. The serosa has lost its characteristic morphological appearance.

Stage 22 (138 hours, between 95-100%; Fig. 2.6H-K) is the final developmental stage before hatching. The embryo has completed its growth and fills the majority of the egg. The pair of head capsules are completely melanised, and the mandibles have a brown appearance. The rounded head is relatively large in comparison to rest of the body. The pinacula are now visible as dark triangular dots from which light brown setae protrude. The serosa completely changes its shape (as if it contracts) and ruptures. The mechanism underlying this is as yet unknown and I hypothesise that as the caterpillar is about to hatch it is both no longer actively maintained and needs to break to allow the caterpillar a way out of the egg. Shortly before hatching the blind end of the foregut and hindgut open and the continuity of the digestive tract is established (Aymone *et al.*, 2014).

When the larva is ready to hatch (Fig. 2.6L), it bites a circle of tiny holes out of the chorion, generating a "lid" (called the operculum) which is pushed upwards to allow the larva to make its exit through the now ruptured chorion (Sikes and Wigglesworth, 1931). The larva usually consumes the empty chorion as its first meal.

2.4 Discussion

Pararge aegeria displayed similar patterns of morphological (anterior to posterior) differentiation, involving the formation of the head, thoracic, abdominal segments and appendages as previously observed in other Lepidoptera, such as *B. mori, M. sexta, H. zea* and

H. erato phyliis (Aymone *et al.*, 2014; Broadie *et al.*, 1991; Dorn *et al.*, 1987; Dow *et al.*, 1988; Nagy *et al.*, 1994; Presser and Rutschky, 1957). Furthermore, the rapid (anterior to posterior) segment specification and embryo extension around stage 9 indicates that *P. aegeria* is an intermediate germ-band insect displaying significant long-germ characteristics (*cf.* Carter *et al.* (2015) – and supports the previous findings in two other butterfly species *H. erato phyllis* (Aymone *et al.*, 2014) and *B. anyana* (Masci and Monteiro, 2005). In intermediate and long germ-band lineages of Lepidoptera the germ anlage is covered by the serosa before the onset of proper embryonic gastrulation (Kobayashi, 2003). Long-germ species tend to have mononucleated serosal cells while short-germ species have multi-nucleated serosal cells (Fig. 2.2H) (Kobayashi and Ando, 1981; Wall, 1973). *Pararge aegeria*, just like other Ditrysians, such as *B. mori, M. sexta, B.anayna and H. erato phyllis* (Aymone *et al.*, 2014; Kraft and Jäckle, 1994; Masci and Monteiro, 2005) have mononucleated serosal cells (Fig 2.2H).

Insect embryos typically have 11 abdominal segments, excluding the telson (Aymone *et al.*, 2014). Holometabolous insects, such as the Lepidoptera, appear to partially or completely fail to develop this segment. Some basal moth embryos, such as those of the genera *Neomicropteryx* and *Endoclyta*, initially develop this segment, only for it to disappear during later developmental stages (Kobayashi and Ando, 1983; Tanaka, 1993). The reduction and/or absence of the 11th abdominal segment is considered to be apomorphic (i.e. derived) within Lepidoptera (Kobayashi and Ando, 1987), and also displayed by *P. aegeria,*

Pararge aegeria, somewhat unusually, appears to lack aeropyles. Although this was noted as well by García-Barros and Martín (1995), it is possible that there are population differences in the presence or absence of aeropyles in relation to prevalent humidity levels (García-Barros *pers. comm*.). Furthermore, although aeropyles appear to aid in careful control of gas exchange between the embryo and the outside world, they connect with a network of cavities inside the egg, not the embryo itself (i Monteys *et al.*, 2005). In the moth *M. sexta* limiting the permeability of the eggshell by means of a wax-layer and/or subchoral layers (and

which can include the serosa and its cuticle(s)) provides protection against excessive water efflux and oxygen influx (Woods *et al.*, 2005). Although, the genes involved in *P. aegeria* choriogenesis *per se* have been analysed previously (Carter *et al.*, 2013), the presence and development of the trabecauler, crystalline chorionic and wax layer, as identified in *M. sexta* (Woods *et al.*, 2005), still requires verification in *P. aegeria.* The serosa, identified here as being present throughout embryogenesis, is hypothesised to aid (directly, or indirectly via secreting cuticle(s)) in resisting water efflux (i.e. desiccation resistance) (Lamer and Dorn, 2001), but it remains to be investigated whether it could aid in controlling gas exchange. If it does, gas exchange would need to go directly through the serosa, as no openings for channels through them have been detected (Wigglesworth and Beament, 1950). Given that *P. aegeria* eggs are laid on leaves and require protection from desiccation by limiting chorion permeability, it is not clear what led to the loss of aeropyles, whilst a more basal species in the genus *Pararge*, *P. xiphia*, does have them (García-Barros and Martín, 1995; Iossa *et al.*, 2016). Given variability in aeropyle presence within some butterfly species (García-Barros *pers. comm*.), future studies should elucidate the ecological drivers behind aeropyle loss, in combination with within- and between-species variability in serosal functioning with respect to water and gas fluxes (Chapter 1). This is particularly relevant in the later stages of butterfly embryogenesis with its accelerated growth of the embryo, and when the serosa is still present (as opposed to other insects). It is also precisely these later developmental stages that we currently know little about.

Stages 3 and 4 are characterised by the formation of the serosa, covering the whole of the embryo. On the basis of other Ditrysia (Kobayashi, 2003; Presser and Rutschky, 1957), I infer that serosa formation will be followed by formation of the amnion in stage 5; another extra-embryonic tissue, although I could not observe that in this study. The edges of the germ disk are flexed ventrally during serosal closure and spread along the narrow space between the serosa and germ disk. The edges will meet and fuse with each other and form the amnion

(Fig. 2.8) (Kobayashi, 2003; Presser and Rutschky, 1957; Tanaka, 1968). The functionality of the butterfly amnion is still not clear. The amnion and the serosa play a role in morphogenetic movements and dorsal closure in other insects, such as *T. castaneum*, but not in butterflies (Hilbrant *et al.*, 2016; Panfilio, 2008). Not least because the serosa remains in place throughout embryonic development, and does not rupture during katatrepsis as observed in other insects (Hilbrant *et al.*, 2016).

The process of gastrulation in *P. aegeria* is not involved in the formation of the serosa, neither are the movements of gastrulation required for the germ anlage to be enveloped by the serosa, as is the case in short germ-band insects like *T. castaneum* (van der Zee *et al.*, 2005). The serosa envelops the *P. aegeria* germ anlage first (Stage 5) whilst gastrulation happens around stage 6 when the mesodermal cells liberate from the dorsal wall (Kobayashi, 2003). The formation of the gut is a process by means of invagination of the ectoderm on both the anterior and posterior end of the embryo and takes a large part of the embryonic development to complete (around stage 16-18) (Aymone *et al.*, 2014; Kobayashi, 2003).

In Lepidoptera, there are three types of embryonic membrane formation known; invagination, amnioserosa fold and fault types (Kobayashi, 2003). The invagination type is considered the most basal form and is found in *Neomicropteryx* and *Endoclyta.* In these genera, a relatively short germ-band invaginates into the yolk; extraembryonic ectoderm closes above it forming the serosa (Ando and Tanaka, 1980). The amnioserosa fold type is only found in *Eriocrania* whose embryonic membranes are formed by invagination of the anterior and posterior region of the germ anlage, which then form folds which extend to the ventral midline. These folds merge to form the amnion and serosa (Kobayashi and Ando, 1987). The final type of extra-embryonic membrane formation, and the type displayed by *P. aegeria* is the fault type. It is only known in the ditrysians and the montrysian *stigmella* (Aymone *et al.*, 2014). The fault mode of embryonic membrane formation is considered an apomorphic

characteristic of heteroneura (Kobayashi, 1996). With the fault mode, the germ anlage is cut-off from the extra-embryonic ectoderm and the serosa forms by cell-proliferation. This can be observed clearly in *P. aegeria* where the germ anlage appears to sink slightly into the yolk and the serosa then grows over the germ anlage (see Figures 2.7D and 2.8 for the schematic representation and Figure 2.2 E and F for an embryo mid process). In the fault type, the amnion forms independently from the serosa by the extension of the germ-band edges. The formation and location of the amnion has so far not been studied and observed in *P. aegeria* but is expected to follow this pattern. The amnion is of great interest to study as it might play an important role in dorsal closure as is the case in *D. melanogaster* (Panfilio *et al.*, 2013).

The serosa has been found to be able to protect embryos against desiccation, environmental toxins and even to mount an immune response in other insects (Berger-Twelbeck *et al.*, 2003; Jacobs *et al.*, 2013, 2014a; Jacobs and van der Zee, 2013; Lamer and Dorn, 2001). I suspect that it may play a similar role in *P. aegeria,* and after its formation around 14 hr after AEL or 10% DT I hypothesise that embryos may benefit from being better protected from environmental toxins such as pesticides. However, this remains to be tested. Transcripts of *ShxA* and *D* are no longer detectable from 15hr (Stage 6) onwards, but transcripts of *ShxC* and *zen* are still known to be present until 48h just before stage 13 (Ferguson *et al.*, 2014). Their role in serosa functionality (i.e. once formed) remains to be tested. However, the functionality of the serosa extends to playing a significant part in dorsal closure in insects, after which it disappears (Panfilio *et al.*, 2006, 2013; Panfilio, 2008; van der Zee *et al.*, 2005). In *P. aegeria* embryos, the serosa does not play this role as it stays around the embryo during dorsal closure and stays intact until just before hatching (Fig.2. 7 G and I). In addition, after its formation the serosa secretes a cuticle, the serosal cuticle which is located under the chorion and is also assumed to have a protective role for the embryo (Jacobs *et al.*, 2015). In *M. sexta* the serosal cuticle is secreted shortly after serosa formation starting around 12h AEL (or 10% DT) and is completed at around 22h AEL (or 19% DT) (Lamer and Dorn, 2001).

The serosal cuticle apolysis, separation of the cuticle from the serosa, starts immediately after cuticle completion. The detached serosa cuticle seems to persist until the end of embryogenesis (Lamer and Dorn, 2001). During this study I have not been able to determine the timing of formation of the serosal cuticle in *P. aegeria*. Nonetheless, based on Lamer and Dorn (2001) findings in *M. sexta*, I expect *P. aegeria* to have a serosa cuticle, and that the formation and structure of this cuticle will be similar to that observed in *M. sexta*. Given that the wax layer in the serosal cuticle is thought to contribute to defence against water loss (Chapman, 1998), It could be hypothesised that *P. aegeria* embryos may benefit from this protective function from around 14h AEL (10% of DT) until hatching. A transmission electron microscope (TEM) time series of the developing egg will give a good initial insight in the formation and breakdown of these cuticles.

To fully characterise embryonic development, especially extraembryonic membranes, the use of molecular markers could offer detailed insight as has been shown for the *Shx* genes and some other genes during early development (Ferguson *et al*., 2014; Carter *et al.,* 2015). This is of significance for tissues and processes that are otherwise hard to observe; for example, the formation of the amnion and the maternal-to-zygotic transition (Tadros and Lipshitz, 2009). To visualise the formation of the amnion an in-situ hybridisations for *iroquois* and certain genes in the *toll*-family, could give interesting new insights, both on the formation and possible breakdown of the amnion (Sharma et al., 2013; Benton *et al*., 2016). Special focus should be given to the butterfly homologues of *T. castaneum Toll-7* and *Toll-10* (Benton *et al*., 2016) Other genes of which *in situ* hybridisation could provide novel insights would be markers for the neural development such as *muscle segment homeobox (msh)* (Stollewerk, 2016)*, SOXB1* (Stollewerk, 2016)*, notch* and *achaete-scute* homologues (*ASH*) (Stollewerk, 2016) and germline marker genes like v*asa* (Raz, 2000) and the *NosO* (Nakao and Takasu, 2019). In addition to *in situ* hybridisation, the use of other molecular techniques such antibody staining for activated RNA-polymerase to pin down the time of the maternal to zygotic transition, and

phospho-histone H3 for timing and patterning of cell division could provide insights into early development (Tapia *et al.,* 2006).

Potential embryonic time points where the embryo may be most vulnerable to different environmental stressors are up to 10% DT or 14h AEL i.e. before the serosa is fully developed. During these sensitive time points, the developing embryo may be particularly vulnerable to environmental toxins and water loss, the latter being of particular importance if the eggs are exposed to desiccating conditions. These data provide important baseline information for the design of future studies examining the role of the serosa and serosal cuticle in protecting developing butterfly embryos from environmental stressors.

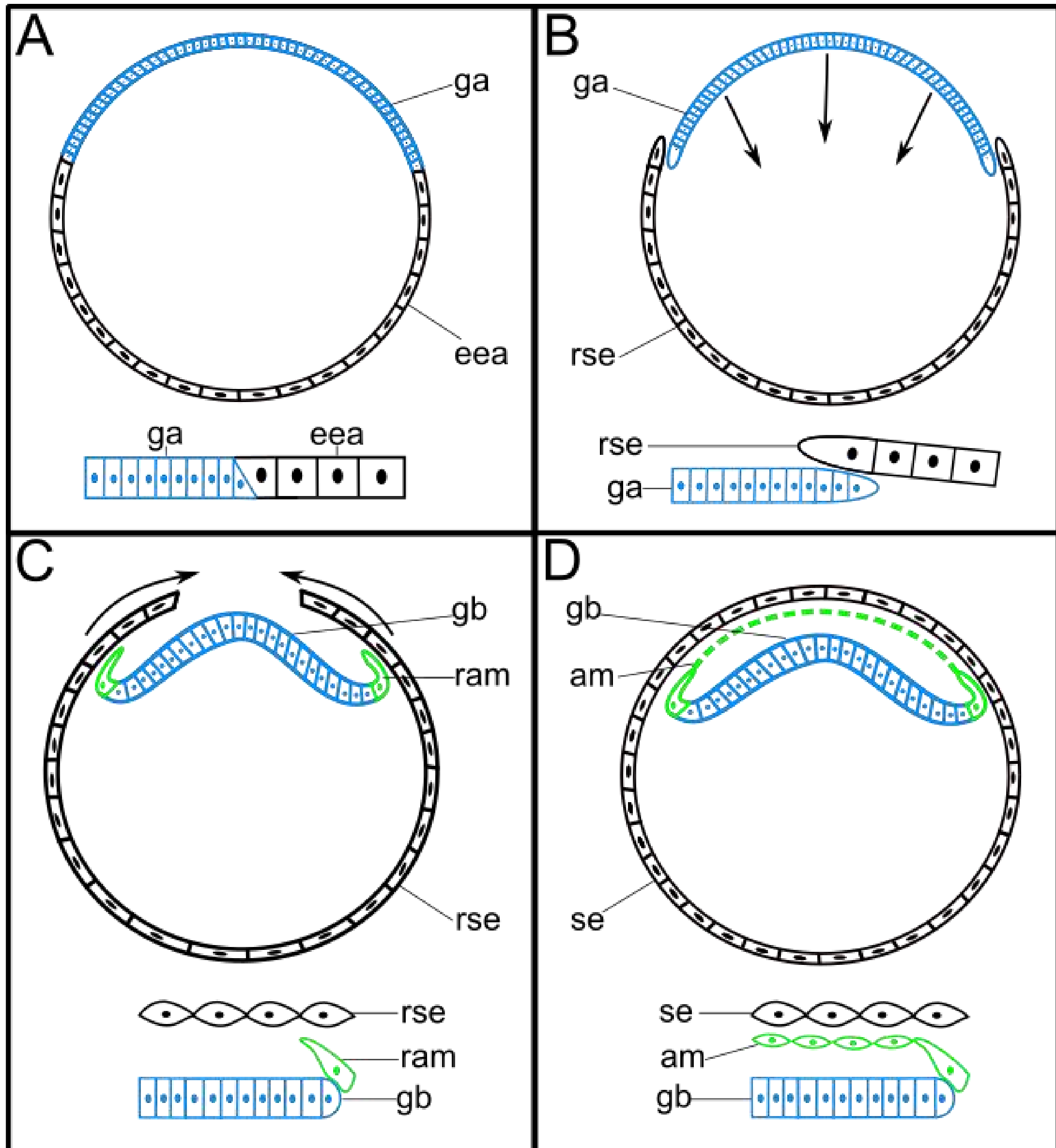

Figure 2.8: Diagram of the serosa and amnion formation in *Pararge aegeria*. (**A**) Differentiation of the germ disk and the extraembryonic area (Stage 2) (**B**) The germ disk is cut of along the margins of the extraembryonic area, and sinks slightly into the yolk. Thereafter the rudimentary serosa extends over the germ disk (Stage 3) (**C**) The amnion is formed by extension of the edges of the germ rudiment. (Stage 4) (**D**) The serosa has fully enclosed the embryo; the amnion is fully formed and lies closely under the serosa (Stage 5). **Abbreviations:** am= amnion, eea = extraembryonic area, ga= germ anlage, gb = germ-band, ram = rudimentary amnion, rse= rudimentary serosa, se= serosa (location of the amnion based on data from Panfilio, 2008 and Lamer and Dorn, 2001)

Chapter 3- The effects of insecticides on butterflies – a review

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The work presented in this Chapter has been done by me; Rebecca Neve made the phylogenetic trees in the supplementary information and Andrew Jones provided advice on neonicotinoid pesticides. Casper Breuker and Melanie Gibbs offered advice on the text and figures and C. J Breuker wrote the text accompanying the phylogenetic trees in the supplementary information.

3.1. Introduction

3.1.1 Non-target effects pesticides

There is no doubt that pesticides can be enormously beneficial in both agriculture and preventive medicine, for example to increase (the quality of) crop yields, to maintain healthy livestock and to prevent the spread of diseases (Aktar *et al.*, 2009; Benelli and Mehlhorn, 2016; Cooper and Dobson, 2007; Guedes *et al.*, 2016; Oerke, 2006). However, due care is needed for their use in an effective manner. Not only do we need to carefully establish the mode of action of pesticides, but also the effects of pesticides on both their intended targets and non-target species. It is clear that where innocent bystanders of pesticides find their natural habitat replaced or reduced by agricultural practices they are doubly affected (Potts *et al.*, 2016). One such group of insects are Lepidoptera, which may comprise good indicator species for the non-target impacts of pesticides. Our relationship with Lepidoptera is a complex one. On the one hand they are the focus of considerable conservation efforts, predominantly butterflies (Brereton *et al.*, 2011; Potts *et al.*, 2016), but on the other hand 70% of agricultural pests are insects in the order Lepidoptera, in particular many moth species and a few butterflies. Various studies on pest moth species have identified genes that could be targeted for pest control, either through pesticides, or genome editing techniques (Guan *et al.*, 2018). While there is a substantial body of literature on pesticide use and effects on moths (e.g. Shakeel *et al.,* 2017), a comprehensive overview for butterflies is lacking (Pisa *et al.*, 2015). Furthermore, although numerous studies have addressed the effects of land use *per se* on butterfly population dynamics and life-history strategies, very few have taken pesticide use into account (Hallmann *et al.*, 2017; Lebeau *et al.*, 2016; Malcolm, 2018). In this review I will therefore provide a comprehensive overview of what is known about the effects of pesticide use on butterflies, provide novel insights, highlight gaps in our knowledge, and propose future directions of study. Finally, it is hoped that although the focus will be on butterflies, extrapolation will be possible

to those benign moth species that have seen their numbers reduced, not least due to indiscriminate effects of pesticides (Fox, 2012).

Benefits of using pesticides in agriculture range from nutritional health and/or increased diversity of viable crops, to more derived secondary benefits such as a reduced migration by humans to cities and a better educated population (Aktar *et al.*, 2009; Cooper and Dobson, 2007). On the other hand, the increased use of pesticides can also result in harmful side-effects for wildlife (Bell *et al.*, 2001; Boutin *et al.*, 1999; Mineau, 2005). While such negative impacts of modern, intensive agriculture on biodiversity have been widely recognised, the contribution that agricultural pesticides make to this overall impact has largely been neglected (Gibbs *et al.*, 2009; Gilburn *et al.*, 2015). Insecticides are one of the biggest classes of pesticides used in the world (Aktar *et al.*, 2009), and this review reflects that insecticides are also the class of pesticides predominantly investigated in butterflies. Although insecticides are produced as a pest preventative method, the vast spectrum of their toxicity inadvertently leads to the suppression of non-target insects and organisms inhabiting the same niche or environment. Affected, non-target organisms might include pollinators, natural predators and parasites (Johansen, 1977).

The main focus of research on non-target pesticide effects has been the European honey bee (*Apis mellifera)* (Sanchez-Bayo and Goka, 2014). The honey bee is the most economically valuable pollinator of crop monocultures and their absence could cause a decrease in yield of up to 90% in some crops (Arena and Sgolastra, 2014; Southwick and Southwick, 1992; Winfree *et al.*, 2007). In recent years many (managed) bee colonies suddenly died over winter, through a phenomenon named Colony Collapse Disorder (CCD) (vanEngelsdorp *et al.*, 2009). The cause of CCD is unknown and is probably the result of a complex interaction between multiple factors. One of the factors implicated in CCD are pesticides, especially neonicotinoids (Lu *et al.*, 2014; Pisa *et al.*, 2015; Ratnieks and Carreck,

2010; van der Sluijs *et al.*, 2013). Neonicotinoids are the most widely used class of pesticides in the world. They are widely applied as seed dressing and work systemically throughout the plant. Neonicotinoids mimic the acetylcholine neurotransmitter and are highly neurotoxic to insects (Crossthwaite *et al.*, 2017; Goulson, 2013; van der Sluijs *et al.*, 2013). The indication of their role in CCD caused the European Union to ban three pesticides in the class of neonicotinoids in 2013, namely Clothianidin, Thiamethoxam and Imidacloprid (European-Commission, 2013, 2018a, b, c). The observation of CCD and the consequent neonicotinoid ban renewed and intensified the interest and research into the (non-target) effects of neonicotinoids in particular and pesticides in general (e.g. Pisa *et al.,* 2015; Wood and Goulson, 2017; Woodcock *et al.,* 2016, 2017)

Although honey bees are cheap, versatile, easy to manage and create their own economically valuable product they are not the most effective pollinator for a lot of crops (Klein *et al.*, 2007). Furthermore, honey bees are not the only non-target species affected. A recent review by Pisa *et al.* (2015) assessing the impact of pesticides on non-target species, identified a need for studies investigating the effect of pesticides on Lepidoptera, in particular butterflies (see also Wood and Goulson, 2017).

3.1.2 Butterflies as models for non-target effects of pesticides

Butterflies play an important role in ecosystems as plant pollinators (Feber *et al.*, 1997; Potts *et al.*, 2016) and as prey for other organisms (Strong *et al.*, 2000). Well-known to the general public, they are well monitored, recognised as indicators of environmental health (Whitworth *et al.*, 2018) and as such they have been used to measure impact of factors such as climate change (Schweiger *et al.*, 2012) and landscape fragmentation (Scriven *et al.*, 2017). Comparatively, their ecology and abundance is much better known than any other invertebrate taxa (New, 1997). This allows the possibility to investigate the impact of pesticides across a large ecological range (Fontaine *et al.*, 2016). Butterfly species diversity and

abundance has already been shown to be influenced by landscape complexity and type of farming (Rundlöf and Smith, 2006), quality of habitat (Pocewicz *et al.*, 2009) and habitat management (Marini *et al.*, 2009). Some butterfly species are agricultural pests, such as the cabbage white species (*Pieris* sp.), but nothing like the scale and species diversity observed for moths (Feber *et al.*, 1997). Understanding butterflies' sensitivity and responses to pesticide exposure more fully might help assess the overall risk of pesticide use (Pisa *et al.*, 2015). The availability of genomic data for an ever-increasing number of butterfly species allows one to investigate the observed sensitivity and responses at the underlying molecular level (Liu *et al.*, 2018; Shen *et al.*, 2016), but also how they may adapt to agricultural environments (Sikkink *et al.*, 2017). Research at the level of such integration in butterflies is far behind that of moths, and thus the detailed studies on pesticide development, usage and effects on pest moths can provide valuable starting points for such an approach (Troczka *et al.*, 2017).

The habitat of many butterfly species consists of hedgerows or the fragmented areas between arable lands (Krauss *et al.*, 2003; Warren *et al.*, 2001). Butterflies can therefore come into contact with pesticide treated plants and areas through foraging or translocation. Butterflies inhabiting hedgerows are susceptible to spray drift from insecticides (Çilgi and Jepson, 1995; Davis *et al.*, 1991a, b; Kjær *et al.*, 2014). Numbers of widespread butterflies on monitored farm land have declined by 58% between 2000 and 2009 (Brereton *et al.*, 2011), and a number of species are under threat. Some pesticides are applied in the form of a coating around seeds, this coating leaves a residue in the soil, and if water-soluble this residue can enter the ground water (Bonmatin *et al.*, 2015; Schaafsma *et al.*, 2015). Uptake from soil and soil water by non-target plants, particularly those in hedgerows and field margins is another potential route of (sub)lethal exposure in non-target species (Goulson, 2013). Butterflies that engage in mud-puddling behaviour can also be exposed to pesticide residues or run-off in soil water (Still *et al.*, 2015). Pesticides, such as neonicotinoids, that have systemic properties can translocate to pollen, nectar and guttation droplets, and become other potential routes of

exposure (van der Sluijs *et al.*, 2013). For example, via plant surfaces, as butterflies may collect honeydew/sap from trunks and leaves. However, little is known about the presence of pesticides in honey dew, but Corke (1999) suggested that 15 different species of honeydew/sap feeding UK butterfly species may have been negatively affected by exposure to particulate air pollution via this route. Therefore, there is the potential for these butterfly species to also be adversely affected by exposure to systemic pesticides, such as neonicotinoids, via honeydew/sap feeding. Adult feeding also has the potential to result in transovarial transport of pesticides from mothers to offspring, including bio-pesticides (Paula *et al.*, 2014). Insect growth regulators such as juvenile hormone analogs and chitin synthesis inhibitors are particularly amenable to transovarial transport (Campbell *et al.,* 2016; Chapter 4). However, much more work is required to explore the full range of potential routes by which butterflies may be exposed to pesticides in nature.

3.2. Data source and study selection

Here I provide a comprehensive review of research on the effects of pesticides on butterflies. The number of published studies on pesticide use and effects on butterflies is very small in comparison to that of moths, and I have set out to review every single study in this overview, making it therefore unique in its breadth. I have identified three main approaches to pesticide research on butterflies, each of which will be discussed in turn in this review. The first approach largely investigates the effects of pesticides on butterflies through the study of population trends. These studies use butterfly abundance and species richness data and compare these across places or times with different levels of pesticide usage. The second approach consists of field tests whereby researchers actively modify the use of pesticides in a (semi) natural environment. The third, and possibly the most used approach, is the examination of the direct effects of pesticides on all, or a selection of, stages in the butterfly lifecycle.

3.3 Effects of pesticide use on butterflies

3.3.1 Changes in butterfly abundance and species richness in response to pesticides

To our knowledge, eight studies have explicitly examined population trends to determine the non-target effects of pesticides on butterflies, usually as part of a population dynamics modelling approach (Brittain *et al.*, 2010; Feber *et al.*, 1997, 2007; Forister *et al.*, 2016; Gilburn *et al.*, 2015; Muratet and Fontaine, 2015; Pekin, 2013; Salvato, 2001). More often than not, studies merely infer the contribution of pesticide use on population trends (Malcolm, 2018). Six of these studies compared similar areas with different levels of pesticide usage and determined the differences in butterfly abundance and/or species richness between those areas (Brittain *et al.*, 2010; Feber *et al.*, 1997, 2007; Muratet and Fontaine, 2015; Pekin, 2013; Salvato, 2001). The approach taken by the two remaining studies, Gilburn *et al.* (2015) and Forister *et al.* (2016), differed from the other six. These two studies did not compare locations with different levels of pesticide use at the same point in time, but used time as a variable in their models and compared butterfly abundance before and after the introduction of neonicotinoids. These studies and the approaches used will be examined in more detail throughout this section.

Pekin (2013) used a large scale dataset, not focusing on absolute abundance of butterflies in the analyses, but rather on the number of butterfly species. This study found that variation in Turkish butterfly species composition was largely explained by the combination of agricultural chemical use, especially pesticides, with climate and land-cover variables. The significance of these variables varied per Turkish province, and thus location. Muratet and Fontaine (2015) used a large-scale dataset, collected by the public which considered pesticide use and butterfly abundance in their gardens. Pesticides, especially insecticides and herbicides were found to have a negative impact on butterfly abundance. This study examined an aspect

of pesticide use often overlooked; the non-industrial use of pesticides. Although these effects might be smaller, gardens can be very important refuges for butterflies (Fontaine *et al.*, 2016).

The other four studies compared sets of similar land types where the biggest difference across treatments was the amount of pesticide used. Feber *et al.* (1997, 2007) used paired sets of neighbouring organic and non-organic farms to compare butterfly abundance. Both of these studies found that irrespective of the type of crop present, non-pest butterfly species were more abundant on organic farms, especially in the uncropped field margins. Brittain *et al.* (2010) used a pair of intensively farmed basins in Italy versus a nature reserve and considered whether intensively farmed land with high pesticide use had lower species richness when compared with the nature reserve, which had negligible amounts of pesticide use. This study found that at the regional scale, butterfly species richness was lower in the intensely farmed basin with the high pesticide loads. Salvato (2001) surveyed ninetransects in South Florida and Lower Florida Keys for adult and larval densities of three species of butterflies; *Anaea troglodyta, Strymon acis bartrami* and *Hesperia meskei*. All pesticide treatment areas were compared against controls; areas where insecticide applications are restricted. In most cases, there was a lower butterfly density in the sprayed locations compared to the control sites. Larval density seems to be highest in unsprayed transects, and increased in transects that ceased insecticide application.

Finally, as mentioned previously, the studies of Gilburn *et al.* (2015) and Forister *et al.* (2016) differ from the other six studies in the approach they used to study the impact of pesticides on butterfly abundance. Gilburn *et al.* (2015) used UK-wide abundance data of 17 widespread resident butterfly species that routinely breed in any field or field margin habitats for their analysis. They modelled data from 1985 to 2012 and their model included a whole range of current and previous year weather measurements such as mean temperature and rainfall during the seasons, as well as the previous year's population index for each species and

previous year’s pesticide use. A strong negative correlation between butterfly population size and the amount of neonicotinoids used in previous years was observed. In 1998 neonicotinoid use in the UK exceeded 100,000 hectares for the first time. To examine the impact of this increase in neonicotinoid usage on butterfly abundance, Gilburn *et al.* (2015) split their data set up into two different time periods, one from 1985 to 1998 and one from 1998 to 2012. Remarkably, when the same model was applied to analyse variation in butterfly abundance across these two-time periods, the abundance of widespread butterflies showed a significant increase in the first -1985 to 1998- dataset, and a decrease in the second -1998 to 2012. These data suggest that increased usage of neonicotinoid pesticides may correlate with a decline in the abundance of 17 widespread UK butterfly species.

Forister *et al.* (2016) used a somewhat similar approach to the Gilburn *et al.* (2015) study but over a smaller geographical scale using longitudinal data from four North Californian locations experiencing butterfly declines since the late 1990’s. In each of the locations the presence of 67 butterfly species was monitored on a bi-weekly basis for 40 years. A negative relationship between neonicotinoid use and annual variation in butterfly species observations was readily detectable, while controlling for land use and other factors. Furthermore, smaller-bodied butterfly species and those with fewer generations per annum showed more severe declines in response to neonicotinoid exposure.

Even though these eight studies used a wide variety of different experimental and statistical approaches to examine the response of butterfly species over a range of spatial and temporal scales, a similar trend was reported by all; increased pesticide levels lead to reductions in butterfly abundance or species richness. The trends reported in these articles are in line with general expectations i.e. pesticide use can have detrimental non-target effects on butterflies. However, these studies do highlight some other important and interesting factors that require further consideration. One of these is consideration of how much non-industrial

use of pesticides might affect vulnerable species, especially in places like gardens which are increasingly being used in urbanised landscapes by many butterfly species as habitat patches that provide essential resources such as nectar sources and host plants for oviposition (Fontaine *et al.*, 2016). More detailed research into this area would be very valuable (Muratet and Fontaine, 2015), especially because butterfly abundance and species richness have been shown to be negatively correlated with pesticide use in gardens (Fontaine *et al.*, 2016).

Studies examining population trends to determine the non-target effects of pesticides on butterflies are very informative as the effects of pesticides are complex, and looking at the real-world effects can give vital insight into the actual scale of the effect. These studies also provide an opportunity to explore the impact of indirect effects, for example through complex interaction and by reducing the number of suitable host plants. Although factors, such as weather, interacting with pesticide use should be taken into account, this is not always done, through a lack of power in the dataset. Many butterfly species utilise host plants commonly considered to be weeds, which may be targeted by herbicides (Malcolm, 2018). Whilst crops may be genetically modified to develop herbicide resistance, other plants may be affected by herbicide spray drifts. This reduction in host plant availability or quality may also lead to reduction in butterfly abundance without having any direct toxicity effects on butterflies (Smart *et al.*, 2000). In Feber *et al.* (2007) this idea was explored by comparing differences in botanical compositions between the organic farms and conventional farms. Although no difference in grass and forb species between organic and conventional field boundaries was found, there may be differences in the abundances of particular nectar sources and host plants, which could impact butterfly population dynamics.

3.3.2 Field studies

Studies addressing the effects pesticides on butterflies, as well as genes involved, in a field context are based on butterflies that are considered pest species, including *Pieris brassicae*

(cabbage butterfly), *Pieris rapae* (small cabbage white butterfly), *Pieris napi* (green-veined white), *Virachola livia* (pomegranate butterfly), and *Papilio demoleus* (lemon butterfly) (Liu *et al.*, 2018). Such studies do not examine effects on non-target butterfly species. However, they do give a good insight into the actual field efficacy and thus the potential level of harmfulness to butterflies in general, particularly because the method of application, as well types of areas where some pesticides are applied suggest the potential for affecting non-target butterflies.

First, I will discuss studies focussing on *P. brassicae* as a target species. Davis *et al.* (1991b) compared the pesticide sensitivity of larvae from three butterfly species in the lab and established that *P. brassicae* as tested by Sinha *et al.* (1990) showed higher sensitivity to the following tested insecticides; Dimethoate, Phosalone, Fenitrothion and Diflubenzuron. This led them to conclude that *P. brassicae* might be a good indicator species for the effects of pesticides on butterflies in general (Davis *et al.*, 1991b). Subsequently both *P. brassicae* and *P. napi* larvae were exposed to the same spray drift at field-realistic concentrations, which again showed *P. brassicae* to be the more sensitive species to the pesticide Diflubenzuron, another insecticide. The molecular mechanisms or other reasons why *P. brassicae* seems to be more sensitive to pesticides than the other tested species were not addressed. Muthukumar *et al.* (2007) and Thakur and Deka (1997) combined, tested 19 different pesticides for their efficacy to kill or deter *P. brassicae* larvae. All of these 19 treatments had a significant effect, greatly reducing the number of larvae. Thakur and Deka (1997) mention six pesticides (Deltamethrin, Cypermethrin, Malathion, Fenitrothion, Endosulfan and Monocrotophos) with a field efficacy higher than 90%, and one, Fenvalerate, had a field efficacy of 100%. These numbers indicate that these pesticides are highly toxic to *P. brassicae* and potentially toxic to other butterfly species too. As these pesticides are applied by spray there is a high possibility of drift and thus contact with non-target butterflies.

Another frequently investigated pest species is the pomegranate butterfly (*Virachola livia*), in countries including Egypt, Cyprus and Jordan (Abd-Ella, 2015; Kahramanoglu and Usanmaz, 2013; Obeidat and Akkawi, 2002). *Virachola livia* lay their eggs on fruit, and after hatching the larvae bore into the fruit, causing crop damage. In contrast to the aforementioned *P. brassicae* studies, larval mortality levels were not measured. Instead, the reduction of fruit infestation and fruit damage after pesticide application was studied. Although a reduction in fruit damage was observed, the mechanism underlying this reduction is unknown, and it is unclear whether it is due to pesticides acting as an oviposition deterrent, or due to the pesticides directly killing eggs or larvae. A closer look into the mechanisms of crop protection could help to indicate the possible non-target toxicity effects on other butterflies and insects. These studies indicate that a wide range of pesticides may have high field toxicity to butterflies, suggesting that numerous, different pesticides are highly likely to have non-target effects.

In addition to chemical pesticides there are also bio-pesticides. Bio-pesticides are natural occurring substances that control pests (Copping and Menn, 2000). Fungi and the bacterium *Bacillus thuringiensis* (*Bt*) are commonly used as bio-pesticides but other kinds of bio-pesticides such as plant extracts are also used (Copping and Menn, 2000). Use of *Bt* as a bio-pesticide, including *Bt*-transgenic plants resistant to lepidopteran pests, appears effective against *P. brassicae* and *P. rapae* but less so for *P. demoleus* (Muthukumar *et al.*, 2007; Narayanamma and Savithri, 2003; Zafar *et al.*, 2002). However, this strategy is not without risks for non-target species through ingestion of GM *Bt* pollen (Manachini *et al.*, 2018) or through transmission of *Bt* toxins to offspring via eggs (Lang and Otto, 2015; Paula *et al.*, 2014). Treatment with fungi is again effective against *P. rapae* but not against *P. demoleus,* with fungi being even less effective against *P. demoleus* than *Bt* (Narayanamma and Savithri, 2003; Zafar *et al.*, 2002). The use of organisms that cause disease as bio-pesticides raise additional questions of possible negative non-target effects such as how long can they persist

in the environment? Can they be transmitted between individuals, and how far can these infections be carried (Duchet *et al.*, 2014; Tilquin *et al.*, 2008)? These types of questions are particularly relevant for *Bt* as this bio-pesticide is used extensively in aerial sprays for control of forest defoliators such as gypsy moth, *Lymantria dispar*, and western spruce budworm, *Choristoneura occidentalis*. Although the short half-life of *Bt* in the field is believed to minimise its impact on non-target Lepidoptera, some studies have demonstrated that it can be toxic to some non-target butterflies, such as *Papilio glaucus* for at least 30 days after the spray (Johnson *et al.*, 1995), and transgenerational effects have been reported (Paula *et al.*, 2014).

Non-target field studies can be divided into two categories; studies that look at the effects of pesticide spray drift (Davis *et al.*, 1991a; Davis *et al.*, 1991b; Davis *et al.*, 1993; Davis *et al.*, 1994; de Jong and van der Nagel, 1994; Zhong *et al.*, 2010) and studies that adjust the application of pesticides, mainly to leave the crop edges and hedgerows unsprayed (de Snoo *et al.*, 1998; Dover *et al.*, 1990; Rands and Sotherton, 1986). The latter category of studies examined how pesticides affect butterfly abundance in hedgerows, which are often considered as a safe-haven for butterflies, in particular when agricultural fields are turned into monocultures without suitable host plants. In their review, Dover and Sparks (2000) discuss the importance of hedgerows in detail; a total of 39 of the 61 UK resident or regular butterfly species have been recorded in hedgerows, making hedgerows an important biotope for conservation. Hedgerows and their grassy surroundings can provide larval host plants, shelter, flowering nectar sources and a corridor system for dispersal for adult butterflies (Fry and Robson, 1994; Longley and Sotherton, 1997). The severity of the impact of pesticides on each of the 39 hedgerow-associated species is likely to depend on the degree by which they utilise this important biotope. For example, some species can be totally supported by hedgerows, other species use them to breed, and some species only fly in from other core habitats to bask, feed or use them as transport corridors. As such it may be expected that species with a higher

association with hedgerows may be more greatly impacted by the non-target effects of pesticides. However, more studies would be required to confirm this (Dover and Sparks, 2000).

Rands and Sotherton (1986) compared a fully-sprayed plot of arable land with one that had the field edges left unsprayed with pesticides. The number of butterflies observed between May and August was significantly higher in the latter (868 vs. 297). Of the 17 species that were observed more than once, 13 were more abundant in the unsprayed plot. Similarly, Dover *et al.* (1990) monitored butterflies in each treatment across years 1995 to 1997 on 14 UK conservation headlands each of which fell into one of four types, short hedges, tall hedges, wood edges or railway embankments. The conservation headlands were selectively sprayed with some pesticides including an insecticide, although which insecticide was used and in what dose was not reported. The four types of headlands also had significantly fewer butterflies in the field areas with fully sprayed headlands. Furthermore, the pierids *Anthocharis cardamines, P. napi* and *P. rapae* all managed to lay eggs in the conservation headland on their host plants *Sinapis arvensis L.* and *Brassica napus*, be it in low densities. A similar study conducted in the Netherlands also reported fewer butterflies in sprayed margins than in unsprayed margins (de Snoo *et al.*, 1998). It did depend both on the crop type and the year examined (de Snoo *et al.*, 1998). It can be hypothesised that the favourable effects on butterfly abundance in the unsprayed margins were mainly due to the greater availability of flowering plants but could not be tested with the data from de Snoo *et al.* (1998). Such hedgerow studies also provide some insights not only into indirect effects of pesticides but also into potential interaction effects with other factors. An example includes the effects of herbicides and fertilisers on butterflies and their associated hostplants (Longley and Sotherton (1997)).

Spray drift is named as one of the main sources of non-target butterfly exposure to pesticides, as pesticides drift over from fields of arable land to areas with higher number of resources for butterflies such as hedgerows, wildflower patches or even nearby nature

reserves (Sinha *et al.*, 1990; Zhong *et al.*, 2010). Quite a few studies examine ground-level spraying effects on butterflies (Davis *et al.*, 1991a, b, 1993, 1994; de Jong and van der Nagel, 1994), while Zhong *et al.* (2010) addressed the impacts of aerial ultra-low volume spraying of Naled on the Miami blue butterfly in Florida. Naled is used to target mosquitoes and a small droplet of Naled created by the ultra-low volume spraying does not settle quickly and is capable of drifting extended distances both in and out of the target area. The Miami blue butterfly (*Cyclargus thomasi bethunebakeri*) is endemic to Florida and has been in serious decline. In addition to habitat loss, climate change and a handful of other factors, the use of the aerial application of Naled has been indicated as a possible contributory factor in their decline. Naled was found to negatively affect late instar Miami blue larvae at the concentration found in the target zone, but not at the concentrations found in the spray drift zones (Zhong *et al.*, 2010). However, whether the concentrations of Naled found in the spray drift zones affects other larval instars or life stages of these butterflies requires further work (Zhong *et al.*, 2010).

It was found that even at low wind levels pesticides could drift and cause high mortality to *P. brassicae* larvae up to 24 metres away from the spray site (studies reported in Table 3.1). For example, Davis *et al.* (1994) monitored two-day-old *P. brassicae* were placed on plants at different distances from a field sprayed with cypermethrin, recording a higher mortality of larvae for three days after spraying. They included an examination of how landscape features, especially hedgerows, could influence the spread of pesticides by spray drift, by acting as a barrier, and concluded that hedges may provide a sheltered area immediately behind the hedge, but as the distance from the hedge increases, larval mortality increases again minimising the shelter effect of the hedge. de Jong and van der Nagel (1994) also placed *P. brassicae* at different distances from a plot of land sprayed with diflubenzuron. In this study the LD-50 was established at only 0.16% of the sprayed dose, and the drift from the application was at a sufficiently high concentration to still cause larval mortality. As

expected, the closer the larvae were to the sprayed area the higher were the mortality levels. These studies indicate that pesticide spray drift has the potential to cause serious mortality in butterfly species over considerable distances from the sprayed area, and that landscape features, such as hedges, are ineffective barriers to spray drift.

3.3.3 Direct toxicity effects of pesticides on butterflies

Here, I was interested in determining how many different butterfly species have been used in direct toxicity tests, which pesticides have been tested on butterflies, in what dose and which butterfly life stages have been examined. For example, recent studies on *P. rapae* dissecting the sensitivity and response to pesticides at the molecular level (e.g. identification of relevant genes) do so in a life-stage specific way (Liu *et al.*, 2017, 2018).

In total, 24 species of butterflies were used in direct toxicity tests of pesticides (Table 3.1). It should be noted that these were all insecticides. Ten of these species were exposed to such pesticides in both the larval and adult stages. One species, *Papilio* spp was used only in the egg stage, while *P. brassicae* was used in egg and larval stage and another, *Pararge aegeria* was tested in the egg and adult stage. Three species, *Ascia monuste, Bicyclus anynana and Dryas julia*, were only tested in the adult stage and the remaining eight species were only tested in the larval stage. The number of studies published per species is highly variable, ranging from a single study for the majority of species studied, to 12 different studies on *P. brassicae.* As mentioned earlier in this review, *P. brassicae* has been demonstrated to be more sensitive to pesticides than some of the other species studied, and has therefore been suggested to be a good model species for examining the impact of pesticides on butterfly pest species (Davis *et al.*, 1991b). This may explain why the majority of studies examining effects of pesticides are on this species. In total, I found 32 studies that examined the direct effects of pesticide exposure on butterflies (Table 3.1). The majority of these studies performed direct toxicity tests on the larval stage (n= 26 studies), a few have considered the adult stage (n = 9

studies), but hardly any studies have examined the impact of pesticide usage in the egg stage (n = three studies) and none examined the pupal stages in butterflies (Table 3.1). Few studies have considered the sub-lethal effects of pesticides through the different stages of the life cycle to the adult stage or considered potential for transgenerational effects (i.e. the transfer of the effects of pesticides from parents to offspring and beyond). Although the larval stage is probably the most economically damaging phase of the butterfly life cycle, and thus the most suitable part of the life cycle to target for pest control, it would be valuable to examine how pesticides impact other life stages to provide further insights into the non-target and sub-lethal effects of pesticides on butterfly populations.

Table 3.1: A summary of the butterfly species, stages and pesticides used in direct pesticide exposure studies. First column contains the species tested, second column indicates which stages in the lifecycle were tested, and the third column the pesticides used. Definitions of terms in the table; *E* refers to egg stage, *L* refers to all possible instars of larval development, *A* refers to adult stage. The supplementary information of Braak *et al.* (2018) (https://doi.org/10.1016/j.envpol.2018.06.100) summarises the main findings of each paper in more detail, including the doses used.

Species	Stage	Pesticide	Reference(s)
Aglais urticae	L	p-p'-DDT, Dieldrin	Moriarty (1968)
Agraulis vanillae	L, A	Naled, Malathion	Eliazar and Emmel (1991), Salvato (2001)
Anartia jatrophae	L, A	Permethrin, Naled, Dichlorvos	Hoang *et al.* (2011), Hoang and Rand (2015)
Ascia monuste	A	Naled	Bargar (2012a); Bargar (2012b)
Bicyclus anynana	A	Pyriproxyfen	Steigenga *et al.* (2006)
Danaus plexippus	L, A	Clothianidin, Imidacloprid, Permethrin	Oberhauser *et al.* (2006), Krischik *et al.* (2015), Pecenka and Lundgren (2015)
Dryas julia	A	Naled	Bargar (2012a)
Eumaeus atala	L, A	Permethrin, Dichlorvos, Naled	Salvato (2001), Hoang *et al.* (2011), Hoang and Rand (2015)
Heliconius charitonia	L, A	Permethrin, Naled, Dichlorvos, Fenthion, Malathion	Eliazar and Emmel (1991), Salvato (2001), Hoang *et al.* (2011)
Icaricia icarioides blackmorei	L	Surfactant, Fluazifop-*p*-butyl, Sethoxydim	Russell and Schultz (2010)

Junonia coenia	L, A	Permethrin, Naled, Dichlorvos	Hoang *et al.* (2011), Bargar (2012a)
Neophasia menapia	L	SBP-138, Pyrethrins, Dewco-214, Methomyl, Chlorpyrifos, Tetrachlorvinphos, Sumithion, Phoxim, Zectran, Aminocarb, Malathion, Carbaryl, DDT, Trichlorfon	Lyon and Brown (1971)
Papilio cresphontes	L, A	Naled, Fenthion, Malathion, Resmethrin	Eliazar and Emmel (1991)
Papilio demoleus	L	β-Asarone, Diofenolan	Singh and Kumar (2011), Vattikonda *et al.* (2015)
Papilio spp	E	BHC, Dicrotophos, Chlorfenvinphos, Carbaryl, Diazinon, Dichlorovos, Dimethoate, Formothian, Malathion, Methamidophos, Parathion, Phosphamidon, Quinalphos, Tricholorofon	Siddappaji *et al.* (1977)
Pararge aegeria	E, A	Pyriproxyfen, 20E-analog	Chapter 4
Pieris brassicae	E, L	Paraoxon, Deltamethrin, Dimethoate, Pirimicarb, Phosalone, Endosulfan, Fenitrothion, Pirimiphos-methyl, Fenvalerate, Diflubenzuron, Cypermethrin, Permethrin, λ-cyhalothrin, Alphametrin, Bifenthrin, β-cyfluthrin, Fenpropathrin, Fenvalerate, DE / New silica, Spinosad, Diazinon, Diazoxon, Triazophos, Dimethoate, Dichlorvos, Quinolphos, Carbaryl, Pirimicarb	David (1959), Wahla *et al.* (1976), Tan (1981), Sinha *et al.* (1990), Davis *et al.* (1991a), Davis et al. (1993), de Jong and van der Nagel (1994), Çilgi and Jepson (1995), Bhat *et al.* (1997), Klokočar-Šmit *et al.* (2007), Dhingra *et al.* (2008), Mucha-Pelzer *et al.* (2010)
Pieris napi	L	Dimethoate, Phosalone, Fenitrothion, Diflubenzuron	Davis *et al.* (1991b)
Pieris rapae	L	Surfactant, Fluazifop-*p*-butyl, Sethoxydim, Deltamethrin, Pumpkin leaf acetone extract	Çilgi and Jepson (1995), Xu *et al.* (2008), Russell and Schultz (2010)
Polyommatus icarus	L	Fenitrothion, Clothianidin	Davis *et al.* (1991b), Basley and Goulson (2018)
Proteus urbanus	L, A	Naled, Malathion	Salvato (2001)
Pyrgus oileus	L, A	Naled	Salvato (2001)
Pyronia tithonus	L	Fenitrothion, Diflubenzuron	Davis *et al.* (1991b)
Vanessa cardui	L, A	Permethrin, Naled, Dichlorvos, Fenthion, Malathion, Resmethrin, Imidacloprid	Hoang *et al.* (2011)

In the studies detailed in Table 3.1 (and Appendix one in the supplementary data in www.dropbox.com/sh/26j5yp2jg8yt6rt/AADQ5oz-2Mrh07pYtG7s0GOza?dl=0), butterflies have been directly exposed to pesticides (i.e. insecticides) using three main methods; 1) direct physical exposure, bringing a droplet of pesticide of a specific concentration straight on to, often the thorax, of the larvae or adult butterfly, 2) using a similar method to 1 in which the egg, caterpillar or adult butterfly was sprayed with, or otherwise physically exposed, to a pesticide and 3) larvae are exposed to food plants treated with a pesticide. Additionally, in two studies the larvae were exposed via a plant grown on pesticide treated soil (Basley and Goulson, 2018; Krischik *et al.*, 2015).

A wide range of pesticides have been tested for their toxic effects on butterflies, and 19 of these studies report a LD-50 for that pesticide under their tested conditions (Appendix one in the supplementary data in www.dropbox.com/sh/26j5yp2jg8yt6rt/AADQ5oz-2Mrh07pYtG7s0GOza?dl=0). Although these values give a rough indication of the toxicity of each particular pesticide for butterflies, there are a number of factors that may affect the generality of these findings. First, the response to any given pesticide is likely to be very species-specific. The study by Hoang *et al.* (2011) provides a good example of why it is important to consider species-specific responses to pesticides. They exposed 5th instar larvae of four different butterfly species to the pesticide Naled. The range of LD-50 at 24 hours after exposure lies between 0.19 µg/g for *Anartia jatrophae* and 10.82 µg/g for *Vanessa cardui*, which means that a fifth instar *A. jatrophae* caterpillar is almost 57 times more sensitive to Naled than a fifth instar *V. cardui* caterpillar. This is a difference that cannot solely be explained by a difference in larval size as *V. cardui* 5th instar larvae (0.553±0.05 g) are only 1.3 times heavier than *A. jatrophae* 5th instar larvae (0.425±0.012 g).

Second, the response to a pesticide is highly dependent on the life stage of the butterfly examined; a first instar caterpillar might be more sensitive than the fourth instar

caterpillar of the same species (reviewed in Wood and Goulson, 2017). This effect is well demonstrated by the results of Eliazar and Emmel (1991), showing that different stages of the life cycle have different levels of sensitivity to pesticides and that these patterns are not predictable and depend on the pesticide examined. Fourth instar larvae of *Papilio cresphontes* have an LD-50 of 193.01 µg/g for Fenthion and an LD-50 of 62.463µg/g for Malathion whilst fifth instar larvae of the same species have LD-50s of 41.1 µg/g and 128.455 µg/g respectively. For both pesticides, the sensitivity of *P. cresphontes* depended on the instar of the larva but for Fenthion the sensitivity decreased, while for Malathion it increased with larval age. Additionally, Davis *et al.* (1993) shows that even a couple of days can have a big difference on the sensitivity of larvae to pesticides. Two-day old *P. brassicae* larvae have an LD-50 of 1.521 µg/g when Triazophos is topically applied, while four-day old larvae have an LD-50 of 3.283 µg/g. In the moth *Spodoptera frugiperda*, increased tolerance to the pesticides Methomyl, Diazinon and Permethrin with larval age was associated with increased midgut aldrin epoxidase and glutathione S-transferase activity (Yu *et al.*, 2015). However, more studies would be required to determine whether similar mechanisms are responsible for the age-specific variation in insecticide susceptibility observed in butterfly larvae. The mechanisms underlying these subtle changes in sensitivity and differences in trends between pesticides require further investigation. This could provide valuable insights into the modes of action of pesticides and determine when and how pesticides are most effective.

Lastly, the method of application could potentially have a large influence on the effect of pesticides. Dhingra *et al.* (2008) exposed third instar of *P. brassicae* to cypermethrin in two different ways; spraying the larvae with pesticide versus feeding the larvae with leaves dipped in the Cypermethrin. The larvae had an LD-50 of 9.0 µg/ml when fed with leaves dipped in Cypermethrin, versus an LD-50 of 11.6 µg/ml LD-50 when they were directly sprayed. Such differences in sensitivity could have major effects in the field.

In order to test what effects pesticides may have, field-realistic doses should be used as was done when testing the effects of the neonicotinoid Clothianidin on the development and survival of *Polyommatus icarus* (see Supplementary table 1 of Braak *et al.* (2018); Basley and Goulson (2018)). Reduced larval growth and elevated mortality levels were detected, but ideally the interaction between pesticide use and other factors (e.g. climatic variables and host plant quality) should be studied to get a more realistic indication of the potential effect of pesticides in the environment on multiple aspects of the butterfly development.

In conclusion, based on the values found in these studies alone it is difficult to estimate on the harmfulness of a specific pesticide to non-target butterflies, because the effects of the pesticide are likely to be influenced by the environmental context and the method of application used. To estimate the actual field harmfulness, I would need much more detailed knowledge about normal field doses the butterflies are exposed to, at what stages butterflies are most likely to be exposed, for how long or how often they will be exposed and what is the most likely exposure method that will be used. Additionally, looking only at lethal doses prevents the investigation of other negative sub-lethal effects of pesticides which could impact fitness-related traits and butterfly abundance at the population level. Sub-lethal effects of pesticides on beneficial arthropods have been found to include effects on neurophysiology, larval development, moulting, adult longevity, immunology, fecundity, sex ratio, mobility, navigation and orientation, feeding behaviour, oviposition behaviour and learning (Belzunces *et al.*, 2012; de França *et al.*, 2017; Desneux *et al.*, 2007). The compounding effect of these factors might have a negative impact on butterfly abundance even if the initial pesticide exposure is not lethal. Of the 32 studies detailed in Table 3.1 only 13 measured the sub-lethal impacts of pesticides on butterflies. 11 used larval traits (e.g. larval size, development time etc.), and four used adult traits (e.g. longevity, fecundity etc.) as a measure of sub-lethal effects. A very small number (n=4) measured behavioural traits, namely feeding adverse behaviour (Tan, 1981; Vattikonda *et al.*, 2015; Xu *et al.*, 2008) or egg laying choice (Oberhauser

et al., 2006). None of the studies to date have examined sub-lethal effects of pesticides on neurophysiology or immunology in butterflies. Consideration of whole-organism sub-lethal effects would be very valuable to provide more realistic estimates of the longer-term impact of pesticides on butterfly abundance. Synergistic effects may also play an important role in nature. Synergy occurs when the effect of a combination of stressors is higher than the sum of the effect of each stressor alone (van der Sluijs *et al.*, 2013). The impacts of immunity on moths are already known for three pesticide classes; botanical insecticides, inorganic insecticides and insect growth regulators (James and Xu (2012) provide an extensive review of mechanisms by which pesticides affect insect immunity). Synergy for pesticides and pathogen infection therefore has a high potential in butterflies and requires further investigation.

3.4 Defence mechanisms against pesticide exposure

As mentioned in the previous section, there is some evidence for differences in sensitivity to pesticides both within and across life stages. I will discuss the possible ways that butterflies may be able to defend themselves against exposure to pesticides across life stages.

There are numerous different classes of pesticides specifically designed to disrupt one or more different processes to cause insect mortality such as; the nervous system (e.g. organophosphates, carbonates, pyrethroids, avermectins, neonicotinoids), energy production (e.g. amidinohydrazone, pyrrole), cuticle production (insect growth regulators e.g. methoprene, Pyriproxyfen, fenoxycarb) and water balance (boric acid, silica aerogels, diatomaceous earth) (Sparks and Nauen, 2015). Some insecticides are very selectively toxic to Lepidopteran pests such as the bisacylhydrazine insect growth regulators Tebufenozide and RH-2485, both of which induce lethal larval moults via interaction with ecdysteroid receptor proteins (Dhadialla *et al.*, 1998). Other insect growth regulators such as aromatic non-terpenoidal insecticides like Pyriproxyfen (which mimic the action of juvenile hormone) are toxic to a broad spectrum of insects, including Lepidoptera, during their embryonic, last larval

or reproductive stages (Dhadialla *et al.*, 1998). The potential for non-target effects of these insecticides on butterflies is therefore very high, particularly because these types of modern insect growth regulators have been specifically designed to have a much greater metabolic and environmental stability so that they are better suited for use in agriculture (Dhadialla *et al.*, 1998). Currently, it is unknown why bisacylhydrazines have such a high lepidopteran pest specificity and aromatic non-terpenoidal insecticides do not, especially because most insects use ecdysteroid and/or juvenile hormone as moulting hormones (Dhadialla *et al.*, 1998). When first introduced for pest management it was widely believed that insects would not be able to develop resistance mechanisms to molecules that mimic their own hormones, but this has not proved to be the case (see Dhadialla *et al.* (1998) for an extensive review of the insecticidal, ecotoxiological and mode of action of bisacylhydrazines and non-terpenoidal insecticides). However, more work is required to explore the non-target impacts of insect growth regulators on butterflies and the capacity of butterflies to defend themselves against this class of insecticides.

Resistance to chemical insecticides can be caused by one or more of the following mechanisms; behavioural avoidance, reduced permeability (e.g. through the cuticle), increased metabolic detoxification or decreased sensitivity of the target (Heckel, 2009; Lilly *et al.*, 2016), with the latter two mechanisms being the most commonly encountered (Heckel, 2009).

If butterflies are able to recognise the presence of toxins visually, via olfaction or via contact, behaviours adopted by adult butterflies during oviposition or by larvae during feeding can aid in toxic plant avoidance (see e.g. Després *et al.* (2007)) for an extensive review of the evolutionary ecology of insect resistance to plant allelochemicals). For example, larvae of the butterfly *D. plexippus* feed on plants with secretory canals, and the larvae cut trenches to depressurise the canals and reduce toxic exudation at their feeding site (called canal trenching behaviour, Després *et al.* (2007)). Female butterflies are able to detect plant defensive compounds during oviposition, and the genes involved appear not only to evolve very rapidly,

but also duplicate readily with the resulting paralogs increasing the capacity of ovipositing females to detect a larger variety of (complex) plant compounds (Briscoe *et al.*, 2013; Engsontia *et al.*, 2014). It has been suggested that evolution in response to host plant defences may serve as a preadaptation to surviving exposure to modern synthetic insecticides (Després *et al.*, 2007; Heckel, 2009). In particular, there is potential for metabolic resistance to insecticides with a chemical structure similar to some of the plant-produced defensive chemicals, such as pyrethroids and neonicotinoids (Després *et al.*, 2007; Heckel, 2009). However, more work, and a greater integration of classical resistance studies with chemical ecology would be required to examine this further, but the long co-evolutionary history of insect-plant interactions in Lepidoptera would make them ideal models for such studies (Heckel, 2009).

Reduced permeability can occur via multiple routes including enhanced expression of metabolic resistance mechanisms in the integument, increased presence of binding proteins, lipids and/or sclerotisation that trap insecticides, a measurably thicker cuticle, or a combination of some or all of these mechanisms together (Lilly *et al.* (2016) and references therein). Only one study to date has demonstrated a role for reduced penetration in conferring resistance to a pesticide in Lepidoptera; changes in cuticular composition in response to DDT in the tobacco budworm (Vinson and Law, 1971). In other insects, reduced permeability has been implicated in insecticide-resistance to pyrethrin, organophosphates, carbonates and organochlorines, but ordinarily by itself reduced penetration does not provide a high level of resistance and typically is only found when other mechanisms are present (Lilly *et al.* (2016) and references therein). However, insect eggs are adaptively structured to provide a barrier that protects the embryo against penetration by environmental stressors, and are therefore considered the most difficult life stage to kill with pesticides (Campbell *et al.*, 2016). Campbell *et al.* (2016) have provided an extremely comprehensive review of the mechanisms by which insect embryos are protected against pesticides via both reduced penetration through egg

shell barriers, and by enzymatic resistance. Lepidopteran eggs have been shown to be susceptible to the following ovicidal insecticides; formamidine insecticides (tobacco budworm), paraoxon (*Pieris* butterflies), but not to essential oils (Mediterranean flour moth) (reviewed in Campbell *et al.* (2016)). Fumigation has been found to be effective against the Indian meal moth (*Plodia interpunctella*), a lepidopteran stored product pest (reviewed in Campbell *et al.* (2016)), and it is known that butterflies appear to have a high susceptibility to the transovarial transport of Pyriproxyfen (Steigenga *et al.*, 2006; Chapter 4). To date, no studies have examined the susceptibility of lepidopteran eggs to entomopathogenic fungi, or examined the potential for enzymatic resistance in lepidopteran embryos (Campbell *et al.*, 2016). Together, these data suggest that in Lepidoptera the chorion can form a very effective mechanical barrier against some, but not all pesticides. During early embryogenesis of pterygote insects, such as butterflies, another barrier forms which consists of an epithelial sheet of cells called the serosa that can actively express relevant genes to process environmental toxins (Berger-Twelbeck *et al.*, 2003; Orth *et al.*, 2003). As such, there is a huge potential for the serosa to play an active role in protecting butterfly embryos from pesticides, but at present, no studies have examined whether this is a mechanism of particular significance for butterflies.

Many studies of insects other than butterflies have demonstrated that alteration of the molecular targets of insecticides, most commonly by mutation, is associated with resistance (reviewed in Ffrench-Constant *et al.* (2016)). For example, a point mutation in the gene encoding the γ-aminobutyric acid (GABA) receptor RDL (resistant to dieldrin) gives rise to resistance to dieldrin and several other insecticides in a variety of species including the diamondback moth *P. xylostella* (Wang *et al.*, 2016). The presence of such mutations in butterflies may indicate exposure and adaption to certain insecticides. It is also emerging that species-specific isoforms of RDL generated by alternative splicing and RNA A-to-I editing may influence sensitivity to insecticides (reviewed in Taylor-Wells and Jones (2017)). It will be of

interest to investigate whether different butterfly species have such species-specific diversification in insecticide targets and whether this contributes to differential sensitivities to insecticides displayed in various species. Indeed, I found that many relevant genes in the context of pesticide targets, but also defence against pesticides, display divergence and expansion in butterflies with respect to other insects, including unique gene duplications (i.e. paralogs) and sequence divergence (Appendix 2.1 in the supplementary data in www.dropbox.com/sh/26j5yp2jg8yt6rt/AADQ5oz-2Mrh07pYtG7s0GOza?dl=0). I have demonstrated this for the *multidrug resistance* (*mdr*) genes (Appendix 2.1 in the supplementary data www.dropbox.com/sh/26j5yp2jg8yt6rt/AADQ5oz-2Mrh07pYtG7s0GOza?dl=0). Differential gene expression levels as well as sequence variation in *mdr* genes have been shown to be the cause of population differences in the response to toxic compounds, and the development of resistance in various insects (Begun and Whitley, 2000; Dermauw and Van Leeuwen, 2014), but these genes (including paralogs) have not been studied in Lepidoptera (Simmons *et al.*, 2013). Ryanodine receptors are targets for a class of insecticides known as diamides. These appear less divergent than the *mdr* genes (Appendix 2.2 in the supplementary data www.dropbox.com/sh/26j5yp2jg8yt6rt/AADQ5oz-2Mrh07pYtG7s0GOza?dl=0), illustrating divergence in evolutionary rate between gene families. Although well-studied in moths (including pesticide resistance; e.g. Bird (2016); Steinbach *et al.* (2015)), no data on these receptors and the effects of diamides exist for butterflies (Supplementary file in Braak *et al.* (2018)). Establishing natural variation in such genes (including the significance of the paralogs) and how it may underpin differences in pesticide sensitivity between butterfly populations is an exciting future research area.

3.5 Conclusions and future research

This review highlights the need for integrated studies examining the impact of pesticides on butterflies which combine data across multiple scales; from direct toxicity tests on individual

larvae in the laboratory to field studies that consider the potentiation of pesticides by ecologically relevant environmental biotic and abiotic stressors. Such integration would better inform population-level responses locally, regionally and nationally (e.g. see Fig. 3.1). There are several important areas which require further work in order to fully understand the impact of pesticides on butterflies in nature. Little is known about pesticide toxicity to butterflies, particularly in relation to differences in sensitivity across life stages and species, and further work is required to determine the potential routes by which butterflies may be exposed to pesticides in nature. One way to aid the determination of pesticide toxicity to butterflies is to do a meta-analysis on already available data. A meta-analysis approach was considered for the data collected in this chapter. However, many of the studies don't provided numerical information and the type of numerical data that is presented is very diverse. Thus, the range and depth of the current butterfly data is insufficient for a meta- analysis, however in combination with other lepidoptera data it might lead to interesting insights. In addition to lethal effects, sub-lethal pesticide effects could severely impact fitness, population recruitment and hence population size, but the larval effects also remain largely unexplored. Sub-lethal effects of pesticides can also result in strong selection. Transgenerational transfer of pesticides from mothers to offspring during oviposition adds an additional temporal effect, which may play an important role in the population dynamics of some species, and thus warrants further examination. For many pesticides, we have little information about the range of field doses likely to be encountered by butterflies, or the duration of exposure. We know that some pesticides, like neonicotinoids have half-lives in soil exceeding 1000 days (Bonmatin *et al.*, 2015; Yadav *et al.*, 2015), so there is a high potential for repeat exposure to some pesticides both within and across butterfly life stages. Yet, limited data are available on the sensitivity of butterflies to neonicotinoids within and across life stages (Wood and Goulson, 2017). Other questions that remain unanswered include; how do different land use types affect the impact of pesticides on non-target butterflies? How do pesticides other than insecticides affect

butterflies? Does time influence how butterflies react to pesticides? Can butterflies learn to avoid affected areas or even evolve resistance as seen in other species (Bass *et al.*, 2015; Konopka *et al.*, 2012; Sparks and Nauen, 2015; Tabashnik *et al.*, 2014; Wang *et al.*, 2013)? Is there the potential for the negative effects of pesticides to be missed if different populations of butterflies are well connected, and thus, when analysing data at the landscape level, is it worthwhile considering whether species repeatedly recolonise habitat patches or whether they are closed communities? As was demonstrated for the Diamondback moth, *Plutella xylostella* (Arena and Sgolastra, 2014; Hoang *et al.*, 2011; Steinbach *et al.*, 2015; Yao *et al.*, 2016), it is known that different species, and even populations of the same species, can respond differently to exposure to pesticides. These differences probably have a genetic underpinning, and exploring the underlying genetic mechanisms might help us to better understand species responses to pesticide exposure. Furthermore, we also need to consider the impact of non-industrial use of pesticides in gardens, parks and other recreation areas such as golf courses, which are increasingly important in agricultural and urbanised landscapes (Colding and Folke, 2009). Citizen-science will significantly aid in assessing the impact on butterflies of pesticide use in urban green spaces. In countries like the UK, where many people participate in yearly events like the big butterfly count adding a question about pesticide use in their gardens could provide relevant data (Dennis *et al.*, 2017a).

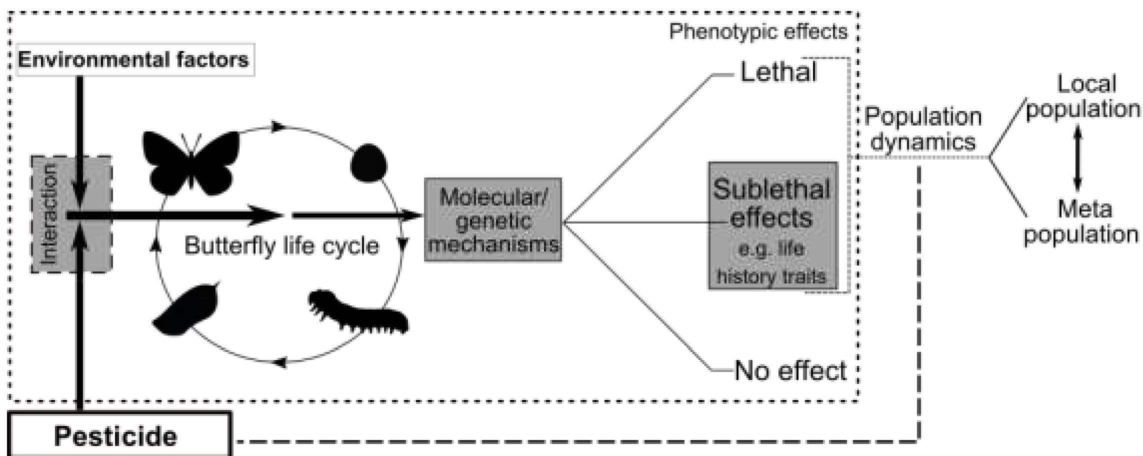

Figure 3.1: The complex effects of pesticides on butterflies: The effects of pesticides on butterflies are poorly understood; the dashed area outlined in the figure highlights where future research efforts are needed. Highlighted in grey are the three main areas where further research is required; 1) the effects of pesticides in interaction with biotic and abiotic environmental factors at different life stages, 2) the effects at the molecular level, particularly in non-target organisms, and determination of which genes are of importance in defence (and thus possibly resistance), and 3) how the effects of the pesticide manifest themselves at the phenotypic level (via lethal, sublethal, life history traits (e.g. reproduction) or even possibly from having no effect). Published meta-analyses have tried to infer from population dynamic trends what the pesticide effects were at the level of the individual (indicated by the broken line at the bottom of the figure joining pesticide and population dynamics).

Butterflies have a rich history of research in the field of evolutionary ecology, as well as their physiological responses to environmental variation. Recently these fields have become increasingly more integrated by investigating the underlying developmental genetic mechanisms involved in the response to a variety of environmental factors, in particular host plants (Schweizer *et al.*, 2017; Sikkink *et al.*, 2017; Yu *et al.*, 2016). Speckled Wood butterflies (*P. aegeria*), are an emerging developmental genetic model system to study growth, development (including embryogenesis) and the production of reproductive cells (Carter *et al.*, 2013; Carter *et al.*, 2015; Schmidt-Ott and Lynch, 2016). It is also a species whose habitat has expanded from forests to include agricultural fields and urbanised environments, providing an

opportunity to gauge the effects on pesticide exposure on local populations in a (meta) population network (Van Dyck and Holveck, 2016). Given that many pesticides affect development, growth and reproduction (e.g. hormone analogs such as Pyriproxyfen), as well as general metabolism, physiology and behaviour (e.g. neonicotinoids), it is timely to investigate the effects of pesticides on butterflies from the molecular level all the way to the population dynamic level using species such as *P. aegeria*. Research on relevant genes in moths, as well as other insect orders, in particular the Diptera (e.g. *Drosophila* and mosquitoes), provides us with a starting point to examine candidate mechanisms and genes (Feyereisen *et al.*, 2015). Having identified relevant genes involved in the pesticide response one can thus investigate which genes are likely to be under selection and involved in differential pesticide responses and resistance among populations within a species but also among species (Supplementary file in www.dropbox.com/sh/26j5yp2jg8yt6rt/AADQ5oz-2Mrh07pYtG7s0GOza?dl=0). Furthermore, different life-stages may differ in their sensitivity to pesticides to differential expression levels of the relevant genes. Finally, such detailed information will allow us to make more robust predictions of the fate of individual populations under a range of environmental conditions, and how they may affect life-history evolution.

Chapter 4- Direct and indirect effects of hormone analogs on *Pararge aegeria* eggs

The data on the effects of pyriproxyfen and 20E on *P. aegeria* females and the effects of maternal exposure pesticide exposure was collected by Tristan Rust and Casper J. Breuker as part of Tristan Rusts' undergraduate project. The data on the effects of direct exposure on eggs was collected by me and both datasets were analysed by me.

4.1 Introduction

Insecticides, the largest class of pesticides, are used widely to protect crops from damage by insect predation. However, insecticides also pose a great danger to non-target insects, as insecticides often target pathways and receptors that are highly conserved among insects (Casida, 2009). Non-target exposure to insecticides can be fatal but may also have a range of sub-lethal effects that are likely to affect life-history traits (e.g. survival and development, growth and reproductive outcome), which can make sub-lethal insecticide exposure a strong selective pressure. So far bees have been the main focus of the influence of pesticides on non-target organisms, but other insects are also at risk, such as butterflies (Pisa *et al.*, 2015). Although butterflies have been identified as non-target organisms and are a conservation concern we know surprisingly little about the influence of pesticides on butterflies (Braak *et al.*, 2018; Gilburn *et al.*, 2015). Butterflies are at risk of insecticide exposure at all life stages but most of the research so far has been focussed on the larval stages (Chapter 3). To fully understand the implications of non-target pesticide exposure we need to know more about the effects on all life stages and the possible transgenerational effects.

Of special interest and concern are a class of insecticides classified as endocrine receptors, which have significant effects on wildlife (Lyssimachou and Muilerman, 2016). Endocrine disruptors have been reasonably well studied in vertebrates (Matthiessen *et al.*, 2018), not least given the concern for the effects on human health, but insect endocrine disruptors are in many ways poorly understood in this context. Possibly the best known example is Pyriproxyfen (2-((1-(4-Phenoxyphenoxy)propan-2-yl)oxy)pyridine), which acts as a juvenile hormone (JH) analog (reviewed in Dhadialla *et al.*, 1998). Pyriproxyfen is a wildly used insecticide for the protection of crops, it has been added water supplies to reduce mosquito populations and it is commonly used as flea control for household pets (Hallman, 2015). Insect hormones, notably JH and 20-hydroxyecdysone (20E), are key regulators of growth and reproduction (Dhadialla *et al.*, 1998). Their concentrations and functionality are dependent on

many factors, including nutritional status and environmental factors such as temperature (reviewed in Carter *et al.*, 2013). Such hormone analogs in particular are thus ideal to investigate how (sub-lethal levels of) pesticides affect growth, development and reproduction in butterflies.

Maternally applied JH (analog) has been shown to act as an ovicide (Hicks and Gordon, 1992; Orth *et al.*, 2003; Ratna *et al.*, 1993), and in Lepidoptera maternal JH application has been shown to disrupt blastokinesis during embryogenesis (Panfilio, 2008; Riddiford and Williams, 1967). Likewise, ovicidal activity of maternally applied ecdysteroids such as 20E has also been demonstrated (Dorn and Buhlmann, 1982; Kadono-Okuda *et al.*, 1994), and changes in ecdysteroid levels can disrupt major morphological events during embryonic development such as; secretion of the serosal cuticle, formation of the embryonic cuticle, dorsal closure and gut morphogenesis (Bullière *et al.*, 1979; Gilbert, 2004; Lagueux *et al.*, 1979; Scalia *et al.*, 1987; Warren *et al.*, 1986). Less well studied are the whole organism and life history effects of JH and 20E (Dhadialla *et al.*, 1998), especially in Lepidoptera (Steigenga *et al.*, 2006), with studies to date mainly focusing on the effects of direct exposure of JH on adult longevity (Herman and Tatar, 2001; Steigenga *et al.*, 2006; Tatar and Yin, 2001) and reproductive output (Geister *et al.*, 2008b; Ramaswamy *et al.*, 1997; Steigenga *et al.*, 2006; Trumbo and Robinson, 2004; Webb *et al.*, 1999), and the role of JH and 20E on the growth and development of wing imaginal discs (Miner *et al.*, 2000; Nijhout *et al.*, 2007; Riddiford, 1993; Tobler and Nijhout, 2010; Truman *et al.*, 2006). Beyond examining the indirect effects of maternal application of JH and 20E on offspring development time and survival to adulthood, there have been few studies in insects examining the effects of these hormones on adult offspring traits (Dhadialla *et al.*, 1998). Consequently, little is known about how the maternal transfer of these types of pesticides to offspring may influence the ability of offspring to respond to environmental variation or maintain physiological homeostasis under stressful conditions, or how such maternal effects may influence adult traits in the offspring.

Lepidoptera have been categorised into four (physiological) groups based on the hormones used to initiate vitellogenesis and choriogenesis and thus the timing of egg production (Ramaswamy *et al.*, 1997). Nymphalids*,* like our model species the Speckled Wood butterfly, *P*ararge *aegeria* (L.) have been suggested to match the criteria for group 4 where JH is the only hormone necessary for; synthesis of vitellogenin in the fat body, inducing patency of ovarioles, uptake of vitellogenin and choriogenesis (Ramaswamy *et al.*, 1997). In support of this, a transcriptomic study by Carter *et al.* (2013) demonstrated that *P. aegeria* ovaries express genes to enable the immobilisation, regulate degradation or enable the transport of juvenile hormone (e.g. various juvenile hormone binding proteins). Interestingly, this study also demonstrated that *P. aegeria* females not only express genes involved in ovarian ecdysteroid signalling (e.g. *ecdysone receptor*, *EcR* and its partner *ultraspiracle*, *usp*), but also genes needed to produce 20E in the ovaries (*start1* and *dare*). This suggests that although JH may be the gonadotropic hormone in *P. aegeria*, 20E signalling also plays a significant role in vitellogenesis in this species (Carter *et al.*, 2013). Furthermore, transcripts of these genes as well as of *EcR* and *usp* are all transferred to the eggs, suggesting that mothers may significantly facilitate ecdysteroid signalling in their offspring. Additionally, transcripts of the JH signalling pathway, such as those of JH binding proteins are also transferred to the eggs (Carter *et al.*, 2013). There is thus clear evidence of potential for maternal regulation of both ecdysteroid and JH signalling in early embryos in *P. aegeria.* Taken together, these results suggest that maternal exposure to JH and 20E analogs have the potential to directly affect female reproduction as well as embryogenesis in non-target Nymphalid butterfly species like *P. aegeria*.

Pararge aegeria has been extending its range, presumably as a result of climate change and changes in land use (Brereton *et al.*, 2011; Hill *et al.*, 2001; Warren *et al.*, 2001). Although traditionally a woodland butterfly species, it is less restricted to the woodlands in regions with warmer winters and warmer and wetter summers, and populations of this species

have been found breeding in agricultural areas (Pateman *et al.*, 2016). Presence of populations in agricultural landscapes increases the likelihood of each of the life stages of this species being exposed to pesticides both directly and also indirectly via transfer of pesticides from the mother to her offspring during oogenesis.

I hypothesised that in egg-laying females exposure to hormone analogs may not only (adversely) affect their own reproductive output and longevity, but may also affect the growth and development of their offspring. Furthermore, I hypothesised that developing offspring indirectly exposed to hormone analogs will respond differently to environmental factors that affect their endocrine system, such as potentially stressful temperatures (i.e. cold or heat)(Neven, 2000). Using *P. aegeria*, I investigated the effects of the JH analog (Pyriproxyfen) and 20E on female reproductive output and longevity, as well as the effects on embryonic development time and egg hatching success. Furthermore, the transgenerational effects of maternal exposure to hormone analogs was investigated by examining the developmental effects of temperature extremes during embryonic development, offspring survival from egg hatching to adulthood, and the offspring traits in adulthood; forewing (FW) and hindwing size (HW) and shape (as indicators of growth).

4.2 Methods

4.2.1 The effect of hormone analog treatment on female longevity, reproductive output and egg hatching success.

On the day of eclosion 60 individually marked females from a large outbred laboratory stock of *P. aegeria* were weighed (AMD Instrument Ltd balance; accuracy: ± 0.1 mg). Each of the females was successfully mated (at 23 ± 2 ˚C, LD 16:8, 62 ± 5% RH), with the males removed from the cage the morning after mating. Twelve egg-laying cages were then set up (five females of the same age per cage), and each cage was randomly assigned to either one of three hormone treatment groups (i.e. 20 females per hormone treatment group): control (acetone), 20E (1 µg/µl in acetone) and Pyriproxyfen (6.67 µg/µl in acetone). 3 µl of the

relevant hormone solution was topically applied to the ventral-posterior cuticle of the female abdomen (Steigenga *et al.*, 2006). After hormone treatment the females were returned to their cages (in an incubator at 23 ± 2 ˚C, LD 16:8, 62 ± 5% RH) along with a fresh *Dactylis glomerata* plant for oviposition and an artificial flower containing 10% honey solution as a food source, which was provided freshly every day (*cf*. Gibbs *et al.*, 2005, 2010a, b, 2012). Cage position was randomised each day within the incubator. For each cage, eggs laid by the females in a cage were collected and both egg number and size were determined using a digital camera (Leica MZ6 Integrated Camera 80 High Definition) and customised egg size measurement plugins in Image J 1.38 (freely available at http://rsb.info.nih.gov/ij/) (Schneider *et al.*, 2012) (*cf.* Gibbs *et al.*, 2010b). Daily egg size was based on a maximum of 30 randomly chosen per cage and these eggs were separated into three batches of 10 (unless fewer than 30 eggs were laid on one day, then an equal number of eggs was divided three-ways). Following egg collection a new, fresh *D. glomerata* plant was placed into each cage. Each egg batch was kept in a labelled Eppendorf (sealed with a small piece of fine netting secured with an elastic band) in an incubator (23 ± 2 ˚C, LD 16:8, 62 ± 5% RH) for three days, with its position daily randomised. The duration of the experiment was determined by the lifespan of the females. For each female the longevity was recorded.

4.2.2 Interaction between maternal hormone treatment and temperature extremes during offspring development.

The negative effects on growth and development as a result of temperature extremes are known to be mediated to a large extent by changes in both JH and 20E signalling (Neven, 2000). It can thus be hypothesised that offspring from hormone analog treated mothers are likely to buffer their physiology less effectively against temperature extremes (Dingle and Winchell, 1997). To test this, three egg batches of 10 eggs were randomly assigned to either one of three temperature treatment groups on day three: control (23 ˚C, 62% RH), cold shock

(5 ˚C, 52% RH) or heat shock (35 ˚C, 62% RH) for two hours. These temperature treatments by themselves do not increase mortality.

Eggs were subsequently reared at 23 ± 2 ˚C (LD 16:8, 62 ± 5% RH) until hatching. The total number of days between being laid and hatching was used as a measure of embryonic development time. For eggs that failed to hatch we recorded whether they embryos died early (no larval growth visible) or late (larval growth visible - head capsule developed) during development.

4.2.3 Survival to adulthood

Survival during the larval stages in each of the treatment groups and controls was recorded. Due to space constraints we limited our investigation of the effects of maternally transferred hormones on offspring traits to a single temperature treatment group only; cold shock. Larvae were reared from each of the hormone treatment groups; Control (n = 64 larvae), JH (n = 39 larvae) and 20E (n = 60 larvae). Fewer larvae were reared from the JH treatment group because fewer larvae survived to hatch (see results section). On the day of hatching, larvae (4 per plant) were placed on potted host plants of the grass species, *Brachypodium sylvaticum*, had access to *ad libitum* food and were reared in under a direct development regime (23 ± 2˚C, LD 16:8, 62 ± 5% RH) until eclosion. The number of larvae that survived to eclose as an adult was determined, and at eclosion each adult was sexed. To avoid wing wear, butterflies were killed within 24 hours of emergence, after their wings had fully hardened and were stored at -20˚C.

4.2.4 Wing morphometrics

Both fore- and hindwings were carefully removed from the thorax, placed in between two glass slides and digital images were then taken of the ventral and dorsal wing surface with a LAS EZ Leica camera under carefully controlled light conditions. Eight landmarks were digitised on both FW and HW in ImageJ (Fig. 4.1)(freely available on http:// rsb.info.nih.gov/ij/) (after

Breuker *et al.*, 2007, 2010). These eight landmarks are homologous on both wings (Fig. 4.1) (Breuker *et al.*, 2010). The landmarks measured were those locations where a wing vein meets the edge of the wing, and as such, the landmarks provided an estimate of the overall outer wing shape and allow for calculation of the Centroid Size (i.e. measure of wing size) (Breuker *et al.*, 2007). Each wing was measured twice in order to be able to partition measurement error out for both size (i.e. Centroid Size) and shape by means of a Procrustes ANOVA (Klingenberg and McIntyre, 1998).

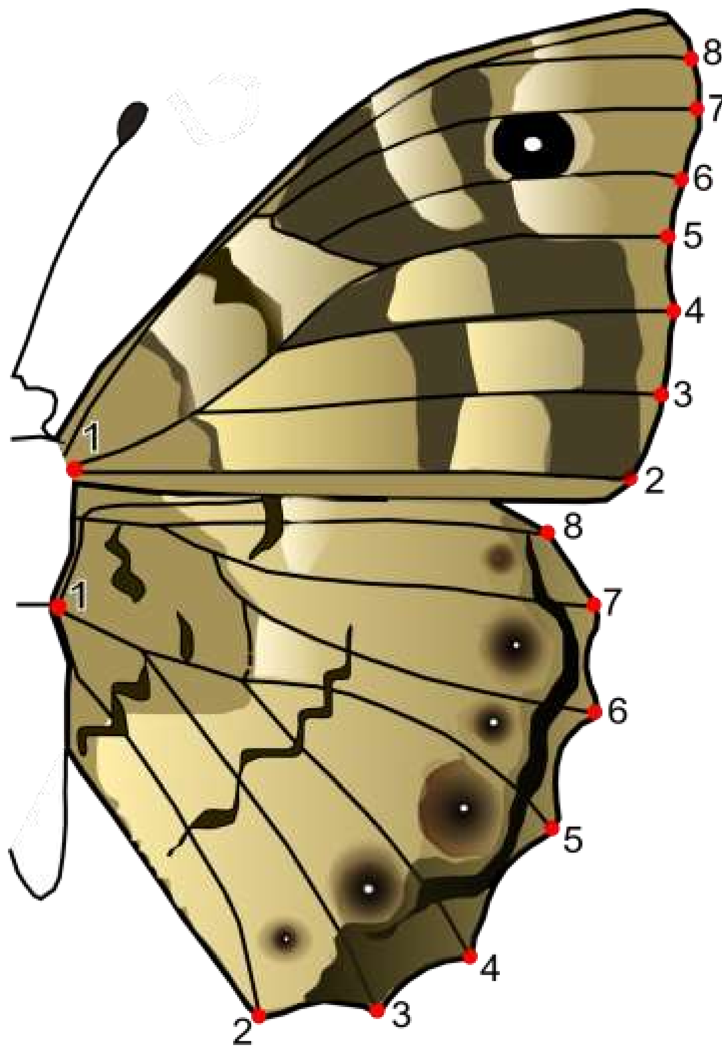

Figure 4.1: Ventral side of the *Pararge aegeria* wing surface highlighting landmarks used for the geometric morphometrics. The eight homologous landmarks used in the geometric morphometric analysis are shown on both the fore and hindwings (Figure adapted from Breuker *et al.,* 2007).

4.2.5 Direct exposure of eggs to JH analogs.

Eggs were collected every 20 minutes from an outbred laboratory stock of *P. aegeria* (27±2°C, LD 16:8, 62±5% RH) and placed in batches of up to 10 eggs in Petri dishes after which egg sizes were determined (see above). Eggs were subsequently individually placed in labelled Eppendorf's covered with gauze and placed in an incubator (27±2°C, LD 16:8, 62±5% RH) until their JH analog exposure at 6, 18, 42 or 66 hours after egg laying (AEL).

Eggs were randomly assigned to either a control treatment group (100% acetone) or one of four hormone treatment groups: Pyriproxyfen dissolved in acetone at a concentration of either 10 mg/l, 0.1 mg/l, 0.05 mg/l or 0.01 mg/l (Karatolos *et al.*, 2012). The eggs were placed in a permeable basket and submerged into one of the hormone treatments at one of four different time points: 6, 18, 42 or 66 hours AEL. After exposure eggs were transferred to individual wells of a 96 well plate. These plates were covered with netting and returned the incubator for further development of the eggs. Hatching success and rate was monitored during the day by checking the plates for hatched larvae at 20-minute intervals, while at night the hatching (and thus to assess hatching time) was monitored using time-lapse technology on an android phone and a freely available application Lapse It (http://www.lapseit.com).

4.2.6 Statistical analyses

Linear mixed effect (lme) models were fitted by means of restricted maximum likelihood (REML), which produces unbiased estimates of variance and covariance parameters. Model fit assessment and testing of the fixed effects was by means of the likelihood ratio test (LRT), which uses chi-square tests on the log-likelihood values to compare different models and produce fixed effect test statistics. Analyses were performed using R (3.4.0) (Core Team R, 2017) with packages 'lme4' (Bates *et al.*, 2015). Below I detail the full starting models for each trait examined in each experiment. Using a backward elimination procedure, only significant (i.e. $P < 0.05$) fixed terms and interaction effects were retained in the final model, and hence only significant terms and

interaction effects are presented in the results section. Residuals were examined for linearity and normality by inspecting normal probability plots and histograms of the residuals and by plotting the residuals versus the predicted values. For the analysis of female longevity a Kaplan-Meir survival curve was constructed using the R packages ' survival' and 'survminer' (Kassambara and Kosinski, 2018; Therneau, 2015; Therneau and Grambsch, 2000) .

4.2.6.1. The effect of hormone analog treatment on female longevity, reproductive output and egg hatching success

A model was constructed to assess the impact of hormone treatment (fixed effect) on female longevity, with female mass included as a covariate and female (nested within cage) being declared as a random factor. In addition to the model a log rank test was used to determine the effect of the hormone treatment on females survival rates. Reproductive output was assessed as mean number of eggs laid per day, with a hormone treatment x mean daily egg size interaction, female age and mean daily egg size included as continuous terms, and cage declared as a random factor. A model was constructed to assess the impact of hormone treatment (fixed effect) on egg hatching success, with female age and mean daily egg size included as continuous terms, and cage declared as a random factor.

4.2.6.2 Interaction between maternal hormone treatment and temperature extremes during offspring embryonic development

A model was constructed to assess the impact of maternal hormone treatment (fixed effect) on embryonic development time, with embryonic development temperature treatment (heat stress or cold stress) included as a fixed effect, female age and mean daily egg size included as continuous terms, and cage declared as a random factor.

4.2.6.3 The effect of maternal 20E hormone treatment on survival during offspring larval development

Chi-square analysis was performed to determine whether survival rates were significantly

different between the hormonal treatment groups compared to the controls.

4.2.6.4. Direct exposure of eggs to JH analogs

Models were constructed to investigate how each of the two offspring traits, embryonic development time and egg hatching success were affected by the dose of Pyriproxyfen and the time point at which Pyriproxyfen was applied.

4.2.6.5 Wing morphometric analyses

In order to test for differences in wing morphology of offspring of the hormone analog treated females versus controls, a Procrustes Analysis was performed for both FW and HW separately, and for both wing size and shape (Klingenberg and McIntyre, 1998). The test allows for testing among individual variability in shape (random effect), differences between the left and right of an individual, as well as portioning out measurement error (Klingenberg and McIntyre, 1998). As only a single individual from the Pyriproxyfen treatment reached adulthood the analyses consist of a shape and wing size comparison between the controls and the 20E treatment group. Sex and Treatment (i.e. controls versus 20E) were entered into the Procrustes ANOVA as fixed effects. Significances for the shape Procrustes ANOVA effects were determined by Pillai traces. In order to explicitly test for the effect of allometry on shape variation (e.g. when there is sexual dimorphism for both wing size and shape), as well as a possible interaction between Sex and Treatment (males and females have distinct hormonal control mechanisms (Bhardwaj *et al.*, 2018), a Principal Component Analysis (PCA) was conducted on the shape means of each individual as obtained from the Procrustes ANOVA (i.e. the so-called symmetrical shape component). A matrix of resulting PCs was used as the dependent variable in a MANCOVA, with log(Centroid Size) as covariate and Sex and Treatment as fixed main effects. Significances were determined by means of Pillai traces. All morphometrics tests conducted in MorphoJ 1.06d (Klingenberg, 2011), except for the MANCOVA (in R).

4.3 Results

4.3.1 The effect of hormone analog treatment on female longevity

There was a significant effect of hormone treatment on female longevity (χ^2 (2, N = 60) = 6.22, P = 0.04), with Pyriproxyfen treated females having shorter lifespans than control and 20E treated females (Fig. 4.2).

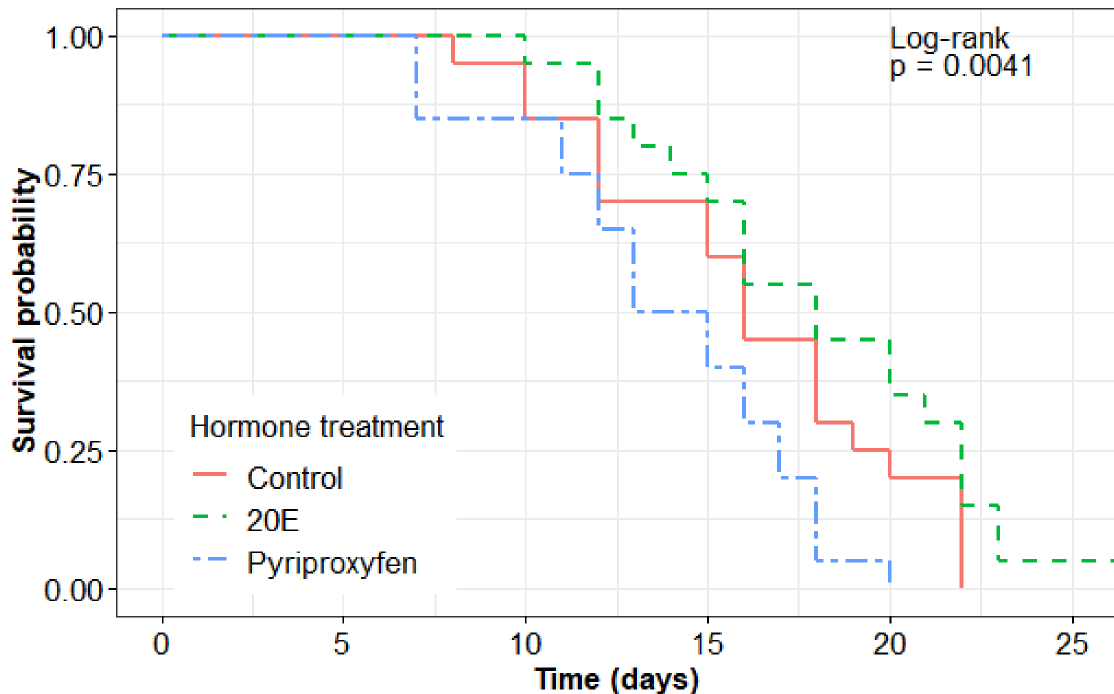

Figure 4.2: A Kaplan- Meir survival curve for the female butterflies under different hormone treatments. The probability of survival is displayed on the Y-axis and the longevity of the females in days on the X-axis. Control females are displayed in red, 20E in green and Pyriproxyfen treated females in blue. The Log-rank test show significant differences in survival between the different treatment groups (p= 0.0041).

4.3.2 The effect of hormone analog treatment on reproductive output

Variation in the mean number of eggs laid by each female per day was significantly explained by a hormone treatment by egg size interaction (χ^2 (2, N = 562) = 7.73, P = 0.02) and female age (χ^2 (1, N = 562) = 266.29, P < 0.0001). The interaction indicates that when females laid more eggs per day they also tended to lay larger sized eggs (Fig. 4.3). On average pyriproxyfen females lay larger eggs (0.704±0.05 mm^2) than control females (0.686±0.04 mm^2) and females

exposed to 20E (00.691±0.04 mm^2), although this is no longer the case when the average amount of eggs laid per female exceeds approximately 25 eggs per day (Fig. 4.3). There were also subtle differences in the egg laying pattern across hormone treatment groups. Hormone treated females showed a shift in the day of peak fecundity from day four in control females to day five in Pyriproxyfen treated females and day six in 20E treated females (Fig. 4.4). On their day of peak fecundity, Pyriproxyfen treated females laid fewer eggs than control and 20E treated females (Fig. 4.4). 20E treated females tended to lay eggs over a longer time period than control and Pyriproxyfen treated females (Fig 4.4). As females aged, fewer eggs were laid per day (Fig. 4.4).

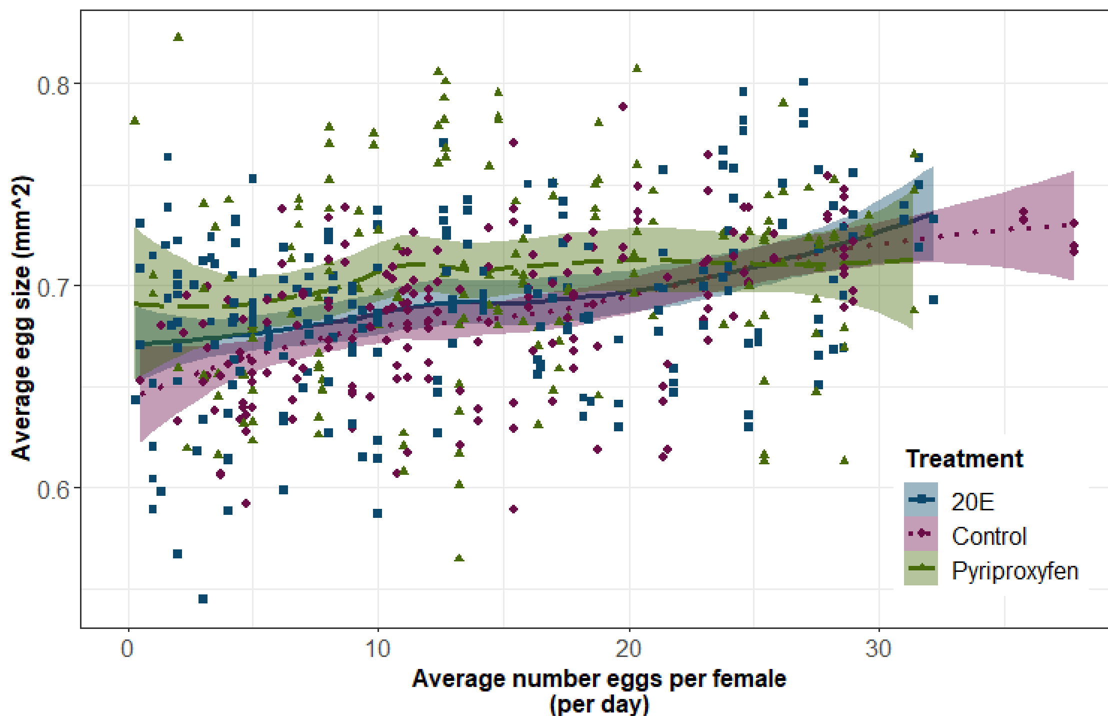

Figure 4.3: Female reproductive output following direct exposure to hormone treatment. Relationship between the mean number of eggs per female per day (X-axis) and the mean size of eggs laid per day (Y-axis). The lines represent the average relationship between egg size and number of eggs laid per female with the standard error surrounding the line. The blue squares and solid blue line indicate the number and size of the eggs laid by 20E exposed females, the red circles and dotted line indicate the control and the green triangles and dashed green line indicate eggs laid by pyriproxyfen treated females.

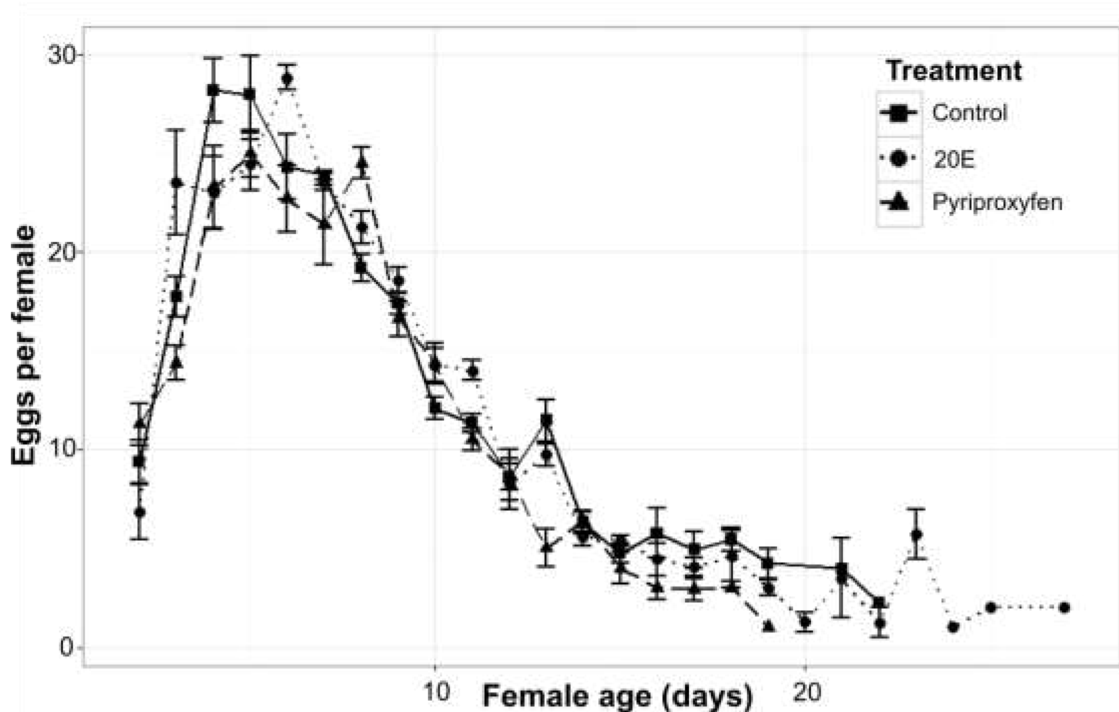

Figure 4.4: Female fecundity; average number of eggs laid per day as per female's age. Daily fecundity as the average number of eggs ± standard error per female is displayed on the Y-axis and the female age in days on the X-axis. The control group is indicated as a solid line with squares, 20E treatment as a dotted line with circles and Pyriproxyfen treatment as a dashed line with triangles.

4.3.3 The effect of hormone analog treatment on egg hatching success

Maternal hormone treatment, maternal age and egg size significantly explained variation in egg hatching success. Overall, eggs from hormone treated mothers had lower hatching success (χ^2 (2, N = 562) = 17.11, P = 0.0002), with eggs from Pyriproxyfen treated mothers having lower hatching success than eggs from control and 20E treated mothers (Fig. 4.5). Compared to eggs from control and 20E treated mothers, a higher proportion of offspring from Pyriproxyfen treated females died during the later stages of embryonic development (Table 4.1). There was a negative correlation between female age and egg hatching success; eggs laid by older females had lower hatching success (χ^2 (1, N = 562) = 25.98, P < 0.0001). There was a positive correlation between egg size and egg hatching success; larger sized eggs had higher hatching success (χ^2 (1, N = 562) = 5.43, P = 0.02).

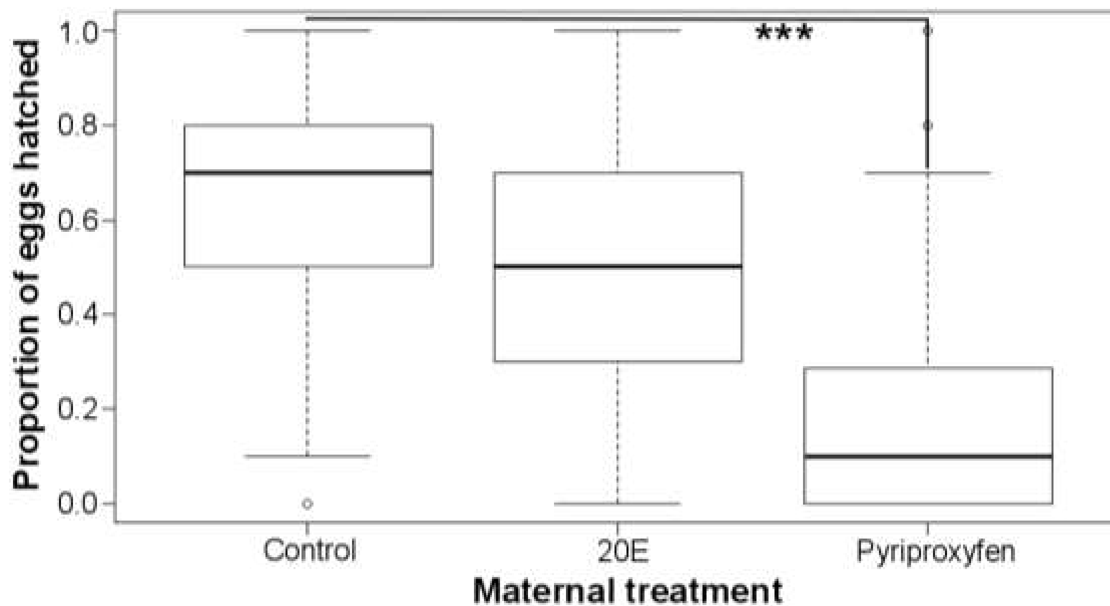

Figure 4.5: Egg hatching success across maternal hormone treatments. Proportion of eggs hatched indicated on the Y-axis and the maternal treatment on the X-axis. Significance codes: < 0.001 ***, < 0.01 **, < 0.05 *

Table 4.1: Maternal treatment and the influence on embryonic development Percentages of hatched eggs after treatment as well as the percentage of eggs that failed to hatch and in which stage of embryonic development they died.

Treatment	Died early in embryogenesis	Died late in embryogenesis (larval head capsule visible)	Egg hatched
Control	22.95%	13.69%	63.36%
Pyriproxyfen	31.3%	50.6%	18.1%
20E	25.1%	24.36%	50.48%

4.3.4 Interaction between maternal hormone treatment and temperature extremes during offspring embryonic development

Variation in embryonic development time was explained by a maternal hormone treatment and developmental temperature treatment interaction (χ^2 (1, N = 485) = 11.09, P = 0.03) and maternal age (χ^2 (1, N = 485) = 12.27, P = 0.0005). The embryonic development times of eggs from control (no hormone) mothers did not differ across temperature treatment groups (Fig. 4.6). Eggs from 20E treated mothers that received a heat treatment had longer embryonic

development times than eggs from control (no hormone) mothers (Fig. 4.6). Overall, irrespective of the type of temperature treatment received during development, eggs from Pyriproxyfen treated mothers had longer embryonic development times than eggs from control (no hormone) mothers (Fig. 4.6).

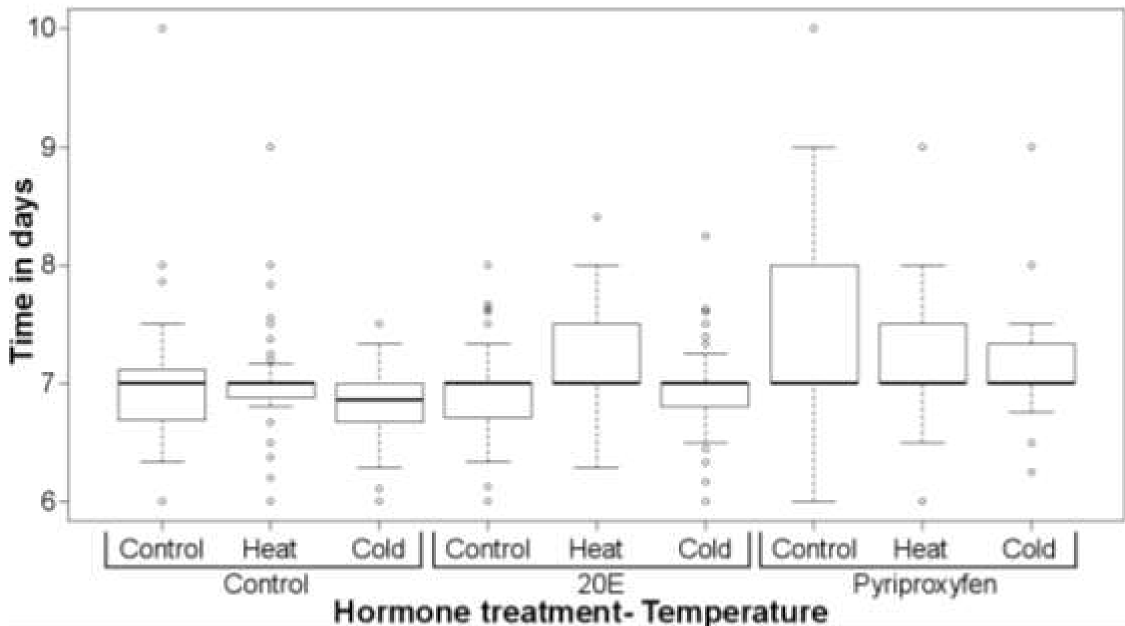

Figure 4.6: The effect of maternal hormone treatment and embryonic exposure to temperature stress on embryonic development time. Development time in days (Y-axis) for each of the treatment combinations. Temperature treatments; Control (23˚C), Heat (35˚C) and Cold (5˚C) and maternal hormone treatment; Control, 20E and Pyriproxyfen.

4.3.5 The effect of maternal hormone treatment on survival during offspring larval development

Maternal exposure to Pyriproxyfen had a significant impact on offspring survival from egg hatching to eclosion as an adult (χ^2 =38.18, $P < 0.0001$) with only 3% of larvae surviving to reach adulthood (1/39), compared to survival rates of 52% for offspring from control females (33/64) while the survival rates of 47% for offspring from 20E treated females (28/60) was not significantly different (χ^2 =0.6838, P =0.41). As a consequence, wing shape comparisons were only possible between offspring from control and 20E treated females.

4.3.6 Wing morphometrics

4.3.6.1 Wing size

Variation in fore- and hindwings was analysed separately by means of a Procrustes ANOVA (Table 4.2 and 4.3). Individuals differed significantly in wing size within each sex and treatment, and left and right wings were not always the same size, which is very common in butterflies (Breuker *et al.*, 2010). There was significant sexual size dimorphism for both wings, with females being larger than males (Log Centroid Size – FW: females 2.97 vs males 2.92; HW: females 2.99 vs males 2.94). However, there were no significant wing size differences between treatment groups.

Table 4.2: Analysis of FW Centroid Size variation by means of a Procrustes ANOVA with additional main fixed effects (Treatment and Sex). The error term consists of the measurement error (i.e. each wing was measured twice), and as such measurement error was partitioned out from size variation (Klingenberg and McIntyre, 1998). Significance codes: < 0.001 ***, < 0.01 **, < 0.05 *

Effect	SS	MS	df	F	P
Sex	39.68	39.7	1	11.05	0.0016 **
Treatment	0.0173	0.0174	1	0	0.9448
Individual	204.6	3.59	57	70.51	<.0001 ***
Side	0.0037	0.0037	1	0.07	0.7882
Ind * Side	3.00	0.0509	59	7.65	<.0001 ***
Error	0.799	0.0066	120		

Table 4.3: Analysis of HW Centroid Size variation by means of a Procrustes ANOVA with additional main fixed effects (Treatment and Sex). The error term consists of the measurement error (i.e. each wing was measured twice), and as such measurement error was partitioned out from size variation (Klingenberg and McIntyre, 1998). Significance codes: < 0.001 ***, < 0.01 **, < 0.05 *

Effect	SS	MS	df	F	P
Sex	40.7	40.695	1	8.91	0.0042 **
Treatment	0.0139	0.0139	1	0	0.9561
Individual	260.3	4.568	57	77.5	<.0001 ***
Side	0.250	0.2498	1	4.24	0.044 *
Ind * Side	3.48	0.0589	59	5.06	<.0001 ***
Error	1.39	0.0116	120		

4.3.6.2 Wing shape

Variation in fore- and hindwings was analysed separately by means of a Procrustes ANOVA (Table 4.4 and 4.5). As for wing size, individuals differed in wing shape within each sex and treatment, displayed significant asymmetries, and wing shape was sexually dimorphic (*cf.* Breuker *et al.,* 2007). For FW, not HW (significance disappears after applying Pillai trace); shape was significantly different between treatments (Fig. 4.7, Table 4. 4 and 4.5). Given that FW wing size did not differ, shape variation was not the result of allometric variation. This was confirmed by means of a MANCOVA (Table 4. 6 and 4.7; test results confirm that treatment only significantly affected FW shape, not HW shape). There was no significant interaction between sex and treatment.

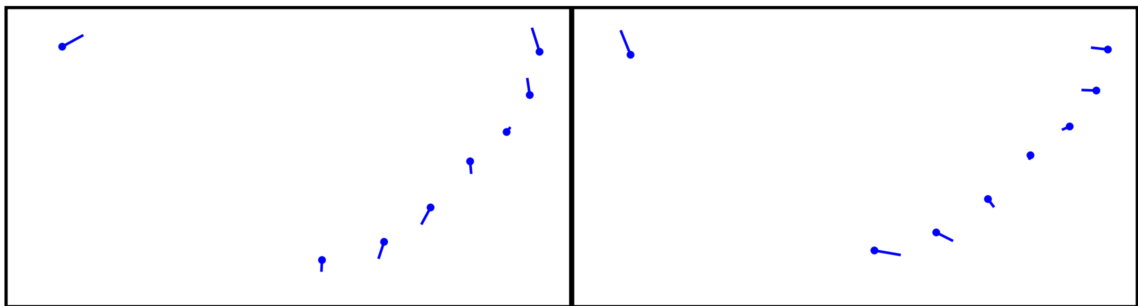

Figure 4.7: Shape differentiation associated with principal component 1 (PC1) for female (A) and male (B) forewings for the offspring of the two treatment groups control and 20E. The lollipops indicate the amount and direction of the variability.

Table 4.4: Analysis of FW shape variation by means of a Procrustes ANOVA with additional main fixed effects (Treatment and Sex). The error term consists of the measurement error (i.e. each wing was measured twice), and as such measurement error was partitioned out from size variation (Klingenberg and McIntyre, 1998). Significance codes: < 0.001 ***, < 0.01 **, < 0.05 *

Effect	SS	MS	Df	F	P	Pillai Tr.	P (Pillai)
Sex	0.129	0.0107	12	65.2	<.0001 ***	0.78	<.0001 ***
Treatment	0.00362	0.00030	12	1.83	0.0404 *	0.44	0.0036 **
Individual	0.112	0.00017	684	4.54	<.0001 ***	8.56	<.0001 ***
Side	0.00294	0.00025	12	6.73	<.0001 ***	0.49	0.0004 ***
Ind * Side	0.0257	3.63E-05	708	8.59	<.0001 ***	8.92	<.0001 ***
Error	0.00609	4.23E-06	1440				

Table 4.5: Analysis of HW shape variation by means of a Procrustes ANOVA with additional main fixed effects (Treatment and Sex). The error term consists of the measurement error (i.e. each wing was measured twice), and as such measurement error was partitioned out from size variation (Klingenberg and McIntyre, 1998). Significance codes: < 0.001 ***, < 0.01 **, < 0.05 *

Effect	SS	MS	Df	F	P	Pillai Tr.	P(Pillai)
Sex	0.064	0.0053	12	27.3	<.0001 ***	0.82	<.0001 ***
Treatment	0.0049	0.0004	12	2.11	0.0149 *	0.27	0.1875
Individual	0.134	0.00019	684	2.84	<.0001 ***	8.54	<.0001 ***
Side	0.0031	0.00026	12	3.84	<.0001 ***	0.39	0.011 *
Ind * Side	0.048	6.91E-05	708	4.97	<.0001 ***	8.64	<.0001 ***
Error	0.0200	1.39E-05	1440				

Table 4.6 MANCOVA results. Dependent variable was FW shape. Log (Centroid Size) was used as a measure of wing size. Significance codes: < 0.001 ***, < 0.01 **, < 0.05 *

	Df	Pillai tr.	approx F	num Df	den Df	P
Log(Centroid Size)	1	0.645	6.68	12	44	<.0001 ***
Sex	1	0.745	10.7	12	44	<.0001 ***
Treatment	1	0.450	3.00	12	44	0.00378 **
SEX x Treatment	1	0.119	0.496	12	44	0.9055
Residuals	55					

Table 4.7 MANCOVA results. Dependent variable was HW shape. Log (Centroid Size) was used as a measure of wing size. Significance codes: < 0.001 ***, < 0.01 **, < 0.05 *

	Df	Pillai tr	approx F	num Df	den Df	P
Log (Centroid Size)	1	0.58	5.14	12	44	<.0001 ***
Sex	1	0.79	14.51	12	44	<.0001 ***
Treatment	1	0.28	1.47	12	44	0.172
Sex x Treatment	1	0.27	1.38	12	44	0.2102
Residuals	55					

4.3.7 Direct exposure of eggs to JH analog

Following direct exposure to Pyriproxyfen, variation in egg hatching success was significantly explained by the time after egg laying at which the eggs were exposed (χ^2 (3, N=869) = 14.35, P =0.002), the dose of Pyriproxyfen received (χ^2 (4, N = 869) = 42.9, P < 0.001) and their interaction (X^2 (12, N = 869) = 46, P< 0.001) (Fig. 4.8). When eggs received the highest dose of

Pyriproxyfen (10 mg/L) there was a significant difference in egg hatching success across exposure time points (Fig. 4.6). Higher hatching success were observed at the 18, 42 and 66h AEL time points compared to the 6h AEL exposure time point (Fig. 4.8).

Embryonic development time is not affected by either the dose of Pyriproxyfen (X^2 (4, N = 653) = 1.13, P=0.89), the time of treatment s (X^2 (3, N = 653) = 2.82, P=0.42) nor their interaction (X^2 (12, N = 653) = 21.04, P=0.05).

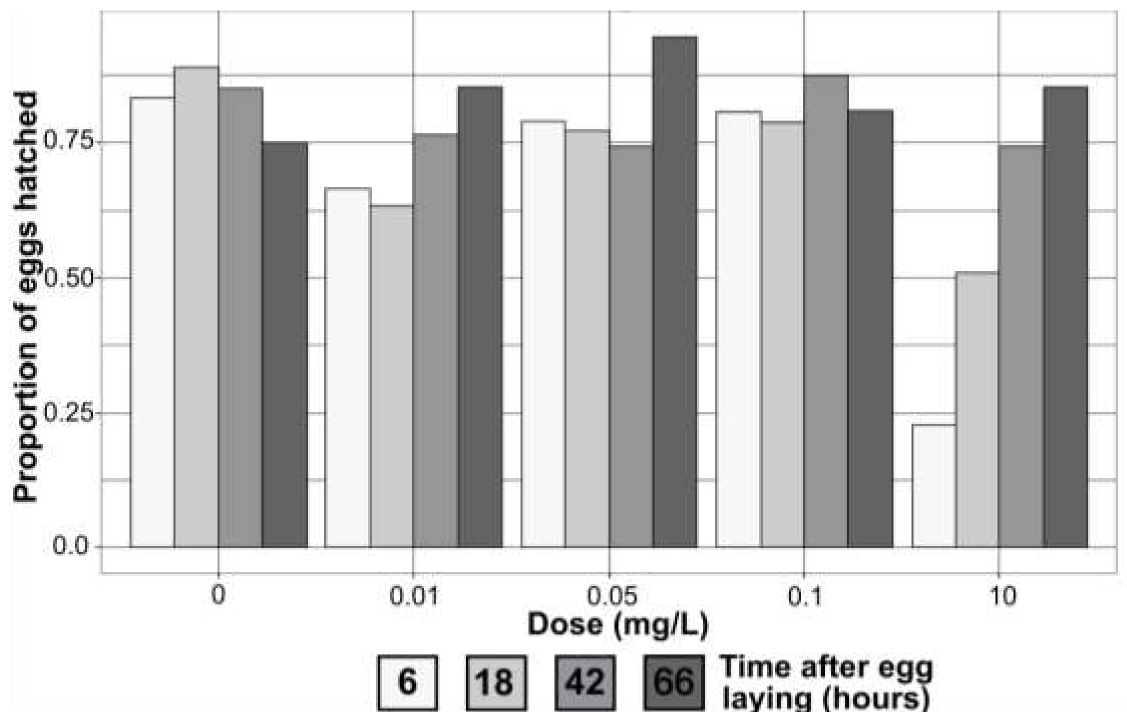

Figure 4.8: Direct exposure of eggs to Pyriproxyfen; the effect of dose and timing of exposure on egg hatching success. Proportion of eggs hatched versus the dose of Pyriproxyfen (X-axis). Colours indicate time after egg laying at which eggs were exposed to the treatment; the darker the colour the older the egg at time of exposure.

4.4 Discussion

4.4.1 The effect of hormone analog treatment on female longevity, reproductive output and egg hatching success

Comparable to other butterfly species (e.g. *Danaus plexippus*, (Herman and Tatar, 2001; Tatar and Yin, 2001), *Bicyclus anynana* (Steigenga *et al.*, 2006)) I found that exposure of adult *P. aegeria* females to Pyriproxyfen reduced their lifespan by 15% compared to control (not exposed) females. Exposure to Pyriproxyfen also resulted in changes in female reproductive output, with Pyriproxyfen treated females laying fewer and smaller sized eggs per day. A similar decrease in oviposition has been found in some Lepidopteran species (e.g. *Cydia pomonella* and *Spodoptera litura* (Hatakoshi, 1992; Webb *et al.*, 1999)), while in others (*B. anynana*, (Steigenga *et al.*, 2006)) it seems to have the opposite effect resulting in an increase in daily fecundity. Variation in response across studies may reflect species-specific differences (perhaps due to differences in the hormones used to initiate vitellogenesis and choriogenesis), differences in JH analog used, or differences in experimental design such as timing of exposure, dose used, and method of exposure. It has been suggested that in Nymphalids, like *P. aegeria*, JH is the only hormone necessary for synthesis of vitellogenin, inducing patency of ovarioles, uptake of vitellogenin and choriogenesis (Ramaswamy *et al.*, 1997). Our results add some support to this hypothesis, and suggest that direct exposure of adult *P. aegeria* females to the pesticide Pyriproxyfen negatively affects reproductive output and lifespan.

In addition to these direct effects of Pyriproxyfen on adult females, I also found a significant transgenerational effect of maternal exposure to Pyriproxyfen on offspring egg hatching success. Eggs laid by Pyriproxyfen treated mothers had a decreased hatching success. A large proportion of the eggs laid by Pyriproxyfen treated mothers died in the later stages of development; when development was around 95% complete and the embryos had black head capsules and brown mandibles (Chapter two). Singh and Kumar (2015) found a similar effect in

blowfly eggs (*Chrysomya megacephala*); following direct exposure to Pyriproxyfen, a proportion of the embryo's managed to reach advanced stages of development but did not hatch. Mondal and Parween (2000) discussed a range of potential reasons why an insect might be able to fully develop but fail to hatch including; differentiation failure, a failure of dorsal closure, muscle atrophy of the appendages or delayed toxic effects that kill the embryo. Further studies are required to determine whether any of these factors may play a role in the late-stage deaths of our *P. aegeria* embryos from Pyriproxyfen treated mothers.

Direct exposure to 20E had much more subtle effects on adult females resulting in slight (non-significant) increases in both longevity and the length of the egg laying period. As also observed for Pyriproxyfen treated females, exposure to 20E had a subtle effect on the timing of peak reproductive output, with 20E treated females reaching their peak reproductive output two days later than control females, and one day later than Pyriproxyfen treated females. I found no effect of maternal exposure to 20E on egg hatching success. It is possible that if I had used a different method of exposure, a different dose of 20E, or exposed females to 20E at a different time point during oviposition I may have observed stronger or even different responses by females. But further studies would be required to test these potential effects of 20E exposure in more detail.

Taken together our data suggest hormone-specific roles in the mediation of resource allocation trade-offs during oviposition in female *P. aegeria*, and strongly suggest that *P. aegeria* in agricultural landscapes have the potential to be negatively affected by non-target effects of hormone analog pesticides, particularly those containing JH analogs.

4.4.2 Interaction between maternal hormone treatment and temperature extremes during offspring embryonic development

This experiment was designed to test the hypothesis that developing offspring indirectly exposed to maternally-applied hormone analogs would respond differently to environmental

factors that affect their endocrine system such as potentially stressful (sub-lethal) cold or hot temperatures. In partial support of this hypothesis, I found that longer development times were observed in offspring from hormone treated mothers when embryos received a heat stress. Also, irrespective of the embryonic temperature treatment experienced, embryos from Pyriproxyfen treated mothers took significantly longer to develop. In nature, longer development times have the potential to indirectly impact offspring fitness, for example by increasing the offspring's exposure time to predators and parasitoids or other environmental threats (Chapter 1). This suggests that in nature, pesticides could have complex interaction effects with environmental factors such as temperature, which further negatively impact offspring fitness. In their natural environment, eggs will experience a daily cycle of natural temperature changes, in addition to unpredictable temperature changes due to variation in weather conditions. It would be interesting to investigate further how these natural environmental changes in temperature conditions may influence offspring sensitivity to pesticide exposure. Examining these synergistic effects would help to ascertain real world effects and establish a more comprehensive insight into the impact pesticides on non-target species like *P. aegeria*.

4.4.3 The effect of maternal hormone treatment on survival during offspring larval development

For offspring that survived to hatch from their egg, maternal exposure to Pyriproxyfen had a significant negative impact on *P. aegeria* larval survival to adulthood, with only 3% of offspring surviving to eclose as an adult. These negative transgenerational effects of maternal exposure on offspring survival have the potential to impact *P. aegeria* populations in agricultural landscapes via non-target effects of JH analog pesticide use. Maternal exposure to 20E did not affect the survival of their offspring post-hatching, but did affect their wing development. Compared to offspring from control (i.e. no hormone exposure) mothers, offspring from

mothers exposed to 20E had significantly different FW, but not HW, shapes. Previous studies on other butterfly species have demonstrated a direct effect of 20E application on wing shape (e.g. *Precis coenia*, Koch *et al.,* 2003 and *B. anynana*, Koch *et al.,* 1996). The effect of maternal exposure to excess 20E on her offspring wing shape is not a result of different wing growth (i.e. as a result of allometry), as there were no significant wing size differences between treatments. Wing size and shape are known to be able to respond independently to a variety of factors affecting wing development (e.g. Breuker *et al.,* 2006). As far as I am aware, this is the first study to show that maternal exposure to 20E can influence wing shape in their offspring. In nature, changes in wing shape have the potential to affect flight and hence behaviours that involve flight such as mate location, oviposition and dispersal. Additionally, flight in *P. aegeria* is sexually dimorphic with males using short often explosive types of flight to actively locate mates while females alternate long bouts of basking with oviposition and dispersive flight (Berwaerts *et al.*, 2006; Shreeve, 1986; Wickman and Wiklund, 1983). Furthermore, although the wing shape of both male and female offspring were affected (i.e. no significant interaction effect between sex and treatment), given their overall sexual wing dimorphism in morphology and flight patterns, there may well be sex-specific consequences for flight. The functional significance of these results remains to be tested.

4.4.4 Direct exposure of eggs to JH analogs

Pararge aegeria eggs were sensitive to direct exposure to Pyriproxyfen, and had reduced hatching success. However, this effect was only observable at high doses of Pyriproxyfen, and was most apparent in eggs that were exposed to Pyriproxyfen early in embryonic development (i.e. at 6h AEL). Previous studies on other species have recorded similar effects in *Heliothis virescens, S. littoralis, Tribolium confusum, Choristoneura fumiferana* and *Bemisia tabaci* where young eggs were extremely sensitive to exposure to juvenile hormone mimics and had significantly reduced hatching success, but older eggs hatched at, almost, normal rates (Ascher and Eliyahu, 1988; Benskin and Vinson, 1973; Hicks and Gordon, 1992; Ishaaya and Horowitz,

1992; Mkhize, 1993). The majority of winged insect, such as butterflies (Ferguson *et al.*, 2014), develop a protective sheet of cells surrounding the embryo during early embryogenesis (Jacobs *et al.*, 2013). This sheet of cells is called the serosa, which along with the chitin-rich membrane it produces (the serosal cuticle) have been found to protect insect embryos against desiccation, environmental toxins and even to mount an immune response (Berger-Twelbeck *et al.*, 2003; Chaudhari *et al.*, 2015; Jacobs *et al.*, 2013, 2014a, 2015; Jacobs and van der Zee, 2013; Lamer and Dorn, 2001; Vargas *et al.*, 2014). In *P. aegeria*, serosa formation occurs at 10-12h, or approximately 8-10% of the developmental time (Ferguson *et al.,* 2014; Chapter 2). Our finding that *P. aegeria* eggs exposed to Pyriproxyfen later than 6h AEL have higher hatching success, may be related to the presence of the serosa (and the serosal cuticle) which reduces the impact of the pesticide by providing either a physical or physiological defence. Especially as JH binding proteins have been observed in the serosa of both *Manduca sexta* and *Locusta migratoria* (Hartmann *et al.*, 1987; Orth *et al.*, 2003). Other possible reasons for this increased resistance with the age of the embryo could be associated with embryonic development. At the later developmental time points, the embryo has undergone most of its development and is more involved with growth, while the younger eggs have more developmental processes that could be disrupted by the application of JH mimics (Broadie *et al.,* 1991; Chapter 2). Which of these mechanisms may be responsible for the effects observed in *P. aegeria* requires further examination in future studies.

It is notable that the negative effects of direct exposure of eggs to Pyriproxyfen are less pronounced than those observed via transgenerational effects following maternal exposure to the pesticide. This could be due to several different reasons. For example, in our experiments mothers were exposed to relatively higher doses of Pyriproxyfen than the directly exposed eggs and this may have contributed to the larger indirect effects observed. Maternal exposure may also enable pesticides to be incorporated into the eggs as they are made, which could result in the developing embryo being exposed to relatively higher amounts. Another

reason could be due to JH having different functions at different developmental stages. It could for instance be that maternally applied Pyriproxyfen does not only negatively influence the embryo itself, but also other factors involved in the formation of the egg. For example, there are indications that it could negatively influence chorionation (Webb *et al.*, 1999). Furthermore, because there is maternal regulation of JH signalling in early embryos in *P. aegeria* (Carter *et al.*, 2015), it is possible that maternal exposure to juvenile hormone analogs may disrupt or influence this process.

4.4.5 Conclusion

Overall our data suggest that widespread use of insecticides that act as endocrine disruptors provide a real risk for non-target Lepidopteran species such as *P. aegeria* and potentially other nymphalid butterflies, both within and across generations. Maternal exposure to these types of insecticides has the potential to generate long-lasting, multi-generational effects. These effects not only negatively impact survival of offspring, but in surviving offspring, also generate sub-lethal effects that may impact offspring fitness in nature, possibly when acting in synergy with environmental factors such as temperature. Moreover, direct exposure of eggs to Pyriproxyfen also lowers hatching rates. This indicates that the influence of pesticides on eggs, often an under-investigated stage in the insect lifecycle, could be a very important piece in the puzzle necessary to accurately quantify how severely non-target insects, like butterflies, may be impacted by pesticide exposure.

Chapter 5- The role of the extra-embryonic serosa in mediating an immune response in *Bicyclus anynana*

5.1 Introduction

Insects are protected from infection in the first instance by physical barriers (e.g. cuticular layers across all life-stages, and a chorion in the egg stage) and in the second instance by both a local and systemic immune response (Ferrandon *et al.*, 2007; Moret and Moreau, 2012; Siva-Jothy *et al.*, 2005; Strand, 2008). In the absence of an equivalent of the vertebrate adaptive immune response, insects rely fully on innate immunity which is a rapid non-specific immune response requiring no prior exposure to the pathogen. Although priming of the immune system does facilitate a faster response, by which it shares the qualities of the adaptive response (Cooper and Eleftherianos, 2017; Kurtz, 2004). The systemic immune response is primarily exerted by the secretion of antimicrobial peptides (AMPs) into the hemolymph by the fat body (Ferrandon *et al.*, 2007). The epithelia and hemocytes play the main role in the local immune defences which comprises melanisation, phagocytosis and encapsulation (Lemaitre and Hoffmann, 2007; Nakhleh *et al.*, 2017). The fruit fly (*Drosophila melanogaster)* has been instrumental in elucidating much of the regulatory (molecular) mechanisms, including characterising relevant genes underpinning insect innate immunity (Eleftherianos and Castillo, 2012; Hoffmann and Reichhart, 2002; Mussabekova *et al.*, 2017). Apart from the fact that innate immune system can be primed, which may be of ecological relevance, it also shows life-cycle stage and age-specific characteristics, including immunosenescence in the adult stage (Eleftherianos and Castillo, 2012; Min and Tatar, 2018; Won *et al.*, 2018)

The innate immune system consists of two main widely conserved pathways, the Toll and Immune deficiency (IMD) pathway (Anderson, 2000; Kimbrell and Beutler, 2001; Lemaitre and Hoffmann, 2007). These immune pathways are activated when peptidoglycans released by the invading microbes are sensed by pattern-recognition receptors (PRRs), belonging to the peptidoglycan recognition protein (PGRPS) and Gram-negative binding protein (GNBP's) families (Lemaitre and Hoffmann, 2007). The lysine-type peptidoglycans of Gram-positive bacteria activate the Toll-pathway, whereas the meso-diaminopimelic acid type (DAP-type)

peptidoclogycans of Gram-negative bacteria activate the IMD-pathway (Ferrandon *et al.*, 2007; Myllymäki *et al.*, 2014; Valanne *et al.*, 2011). Fungal infections also activate the Toll pathway using GNBP3 (in *Drosophila*) which detects fungal β-(1,3) glucans while, in parallel to the PRRs, a fungal virulence factor, the PR1 protease, also triggers the Toll pathway (Gottar *et al.*, 2006; Lemaitre *et al.*, 1996; Xu *et al.*, 2012). The activation of the Toll pathway is mediated by the cleavage of the cytokine Spätzle, the ligand to the transmembrane receptor Toll (An *et al.*, 2010; Weber *et al.*, 2003). Activation of these immune signalling pathways leads to nuclear localisation of the NF-KappaB factors Dorsal, Dif or Relish that induce a number of effectors including AMPs, prophenoloxidases (proPOs which mediate melanisation) and dual oxidase (DUOX) (Lemaitre *et al.*, 1995b; Tanaka and Yamakawa, 2011; Yokoi *et al.*, 2012; Zhong *et al.*, 2012).

The activation of the Toll and IMD pathways in *Drosophila* is dependent on either Gram-positive or Gram-negative bacteria (Hultmark, 2003; Lemaitre *et al.*, 1995a, 1997; Lemaitre and Hoffmann, 2007). However, this separation between the two pathways is less strict in other insects such as *Tribolium castaneum* (Yokoi *et al.*, 2012). Core elements of the innate immunity, like the genes in the IMD and Toll signal transduction pathways are largely functionally conserved even between evolutionarily divergent (insect) species (Kimbrell and Beutler, 2001). However, species-specific family expansions (i.e. duplications) and sequence divergence in the recognition (PGRP) and effector (AMP) families are wide-spread (Christophides *et al.*, 2002; Evans *et al.*, 2006; Gerardo *et al.*, 2010; Little and Cobbe, 2005; Tian *et al.*, 2010; Waterhouse *et al.*, 2007). This is most likely required for effective recognition and elimination of fast evolving and species-specific pathogens (Mylonakis *et al.*, 2016; Vilcinskas, 2013). The increasing availability of tools such as RNA-seq has created a great possibility to assess the expression of genes upon an immune challenge and characterise new immune genes such as AMPs in non-model organisms (Bao *et al.*, 2013; Gunaratna and Jiang, 2013; Johnston and Rolff, 2013; Johnston *et al.*, 2014; Kim *et al.*, 2011; Viljakainen, 2015).

Lepidoptera have been an important model system for various aspects of (innate) immunity (Casanova-Torres and Goodrich-Blair, 2013; Kanost *et al.*, 2004). Until recently, research on moths has mostly focused on the cell biology of hemocytes and biochemical analysis of plasma proteins (Hultmark *et al.*, 1980; Jiang *et al.*, 2010; Lavine and Strand, 2002; Stephens, 1962). The larvae of moths have been instrumental in the discovery of a number of immune genes especially effector genes such as AMPs (Boman *et al.*, 1985; Carlsson *et al.*, 1991; Hultmark *et al.*, 1980; Hultmark *et al.*, 1983). The availability of genomic data for various moth species, for example genes *Bombyx mori, Plutella xylostella* and *Manduca sexta*, has allowed for inference of immune-related genes (Kanost *et al.*, 2016; Tanaka *et al.*, 2008; Xia *et al.*, 2015), and bioinformatics tools have been developed to aid comparison across all available insect genomes (Brucker *et al.*, 2012).The canonical set of immune genes is present within all these Lepidoptera but the copy numbers vary between species and are different from those in other species such as *D. melanogaster, Anopheles gambiae* and *Apis mellifera* (Kanost et al., 2016). RNA-seq analyses allow for elucidation of spatio-temporal expression patterns of immune-related genes. For example, Cao *et al.* (2015) scrutinised the expression of immune genes in 52 *M. sexta* RNA-seq datasets, representing different non-immune challenged adult and larval tissues. They found 187 genes encoding 198 members of the immune-related signal transduction pathways (Cao *et al.*, 2015). Gene-expression after an immune-challenge has been characterised in the larvae of a number of moth species (*Galleria mellonella* (Vogel et al., 2011), *Spodoptera exigua* (Bel *et al.*, 2013; Pascual *et al.*, 2012), *Antheraea mylitta* (Gandhe et al., 2006), *B. mori* (Wu and Yi, 2018), *M. sexta* (Gunaratna and Jiang, 2013) and *P. xylostella* (Lin et al., 2018)).

Immunity in butterflies has thus far largely been investigated in a life-history context. Within this context, expression of immune-related genes has been shown to be affected by, for example, age (Prasai and Karlsson, 2012), photoperiod (in relation to seasonal polyphenism) (Baudach *et al.*, 2018), flight (Fritzsche McKay *et al.*, 2016; Kvist *et al.*, 2015;

Woestmann *et al.*, 2017), food quality (Stoehr, 2007), sex specific differences (Lindsey and Altizer, 2009; Stoehr, 2007) and temperature (Karl *et al.*, 2011). There is a cost to mounting an immune response and there are often trade-offs with traits such as reproductive output and longevity (Freitak *et al.*, 2003; McKay *et al.*, 2016). Most data gathered to date has focussed on larval and adult stages, but the exact nature of the immune response is likely to be stage-dependent (Cao *et al.*, 2015; Eleftherianos and Castillo, 2012; Won *et al.*, 2018). In particular, embryogenesis is not only characterised by the development of morphological structures but also of the innate immune response. Thus, in the first instance maternal effects are important in the immune response of butterfly offspring (Gibbs *et al.*, 2010a), not least because the egg contains maternally deposited mRNA of immune defence genes (Carter et al., 2013). Female butterflies that have been forced to fly, deposit eggs with reduced immunocompetence (Gibbs *et al.*, 2010a). Similarly in butterflies, paternal food availability and quality has been shown to influence offspring immune competence, and maternal food deprivation has been shown to reduce larval immune response to parasitism (Saastamoinen *et al.*, 2013). *Danaus plexippus* males reared on certain milkweed can transfer protection against parasites to their offspring (Sternberg *et al.*, 2015). Few studies have investigated butterfly immunity through available genomic and transcriptomic data, and only recently has a study addressed the evolution of these genes in a micro-evolutionary context for the green veined butterfly (*Pieris napi*) (Keehnen *et al.*, 2018). Keehnen *et al.* (2018) showed differences between *P. napi* and model moth species both in the immune-related genes identified, and copy number variation (CNV) of these genes (Keehnen *et al.*, 2018). Many questions still remain unanswered, for example; what is the functional significance of observed CNV as well as sequence variation *per se*, what is the role of the environment in their expression and evolution, and how divergent are Lepidopteran clades? In particular, functional validation of immune genes is required, and the significance of the expression of these genes needs to be addressed across all life-stages. For example, at present we know very little about immune defence during the embryonic (i.e. egg)

stage, the conditions under which embryos may be able to mount an immune response, and what such a response would consist of.

Just like other life-cycle stages, insect eggs are exposed to pathogens (Kellner, 2002). These pathogens can be present at the oviposition location or even deposited on (or in) the egg by the mother at ovipostion (for a review see Kellner, 2002). Several entomopathogenic fungi have been found to penetrate eggs and have a pathogenic effect (Marta *et al.*, 2006) and entomopathogenic fungi are being developed as alternative bio-control agents to, for example, *S.litura* eggs (Anand and Tiwary, 2009). Wounding of the egg by failed predation or infestation also leaves the egg vulnerable to penetration of micro-organisms, and successful parasitism can introduce pathogenic microbes into the egg (Tanada and Kaya, 2012). For example, *Serratia* bacteria have been found inside the eggs of corn earworm and corn borers (*Ostrinia nubilalis*) (Bell, 1969; Lynch *et al.*, 1976) and can infect eggs in the laboratory (Sikorowski *et al.*, 2001). Eggs of the moth *M. sexta* are able to respond to parasitism by *Trichogramma evanescens* by upregulation of immune genes and a considerable percentage of the host eggs seems to be able to kill the parasitiod (Abdel-latief and Hilker, 2008). As such, mechanisms to protect embryos from invading pathogens should be beneficial, adaptive and thus selected for. Indeed, being able to circumvent such protection might lead to effective routes of pest control (Campbell *et al.*, 2016). Suprisingly, we have only a limited understanding of insect egg immune competance, especially in non-model organisms (Gorman *et al.*, 2004; Jacobs and van der Zee, 2013; Jacobs *et al.*, 2014a, b, 2017).

Insect embryos are partly protected from infection by physical barriers deposited around them by the mother during oogenesis, such as the chorion and underlying vitelline membrane (Hinton, 1981; Chapter 1; Chapter 2). Most pterygotes (winged insects) develop a protective sheet of cells surrounding the embryo during early embryogenesis (Ferguson *et al.*, 2014; Jacobs *et al.*, 2013; Panfilio, 2008). This serosa is the outermost cellular epithelium enclosing all other embryonic material. The serosa is the first layer that can actively adapt to a

changing and invading environment; all other layers offer a more passive protection. In butterflies, such as *Bicyclus anynana,* the serosa surrounds the embryo around 10% DT and stays in place until the embryo is ready to hatch (Holzem *et al.,* in prep; Chapter 2) while in most other insects the serosa disappears around half-way through development (Panfilio, 2008). The serosa has been implicated in providing the embryo with immunity (Jacobs and van der Zee, 2013; Jacobs *et al.*, 2014a). Compared with other insects, given that the butterfly serosa stays in place till hatching, the embryo could thus possibly rely on an extended serosa presence to aid in protection against immune challenges throughout embryonic development.

The serosa is present as an extra-embryonic membrane surrounding the whole embryo in most winged insects apart from the derived Dipteran clade the Schizophora, to which *Drosophila* belongs (Schmidt-Ott, 2000; Chapter 1). *Drosophilla* posses a highly derived serosa, called the amnioserosa, which doesn't surround the embryo. It is not until late stage 15 (after dorsal closure and embryonic culticle formation) that *Drosophila* eggs show significant upregulation of the antimicropial peptides in response to a immune challenge (Tan *et al.*, 2014). This upregulation is significantly different to that of an adult fly, in which upregulation of AMPs upon bacterial exposure is at least an order of magnitude larger (Tan *et al.*, 2014). Although the immune response is like to vary in intensity depended on life stage, *T. castaneum* eggs show an immune response comparable to that of an adult beetle when they are not even halfway through their development (Jacobs and van der Zee, 2013). This early embryonic upregulation of immune genes in *T. castaneum* was attributed to the serosa. Thus, I hypothesise that the presence of an amnioserosa, rather than a serosa, in combination with the very short development time might account for the relatively poor immune response in *Drosophila* eggs. Furthermore, Jacobs *et al.* (2014a) compared gene expression in wild-type and serosa-less *T. castaneum* eggs after an immune challenge, which showed that the serosa is essential for immune competence in the *T. castaneum* egg. Similarly, a decade earlier, several immune-related genes were detected in the extra-embryonic tissues of the tobacco hornworm

(*M. sexta*), although this response could not be specifically attributed to the serosa (Gorman *et al.*, 2004). However, not all insect eggs with a serosa appear to be able to always mount an immune response. For example, the eggs of the carrion beetle *Nicrophorus vespilloides* possess an extraembryonic serosa but lack endogenous production of antimicrobial peptides after an immune challenge, despite well-developed responses in larvae (Jacobs *et al.*, 2014b).

Given the results on other insects, I hypothesised that the eggs of the butterfly *B. anynana* are capable of mounting an immune response after the first 24 hours AEL and that the extra-embryonic serosa plays an essential role in this. Furthermore, I hypothesised that both wounding and mounting an immune response will have a fitness cost for the developing embryos. To test our hypotheses, I treated the *B. anynana* eggs in two different ways, alongside a control; sterile wounding (to assess the effect of wounding alone) or wounding with lyophilised bacteria (to test the effect of the immune response). I looked at the cost of an immune response and wounding in eggs at two different time-points; the first time-point being before serosa formation and the other after serosa formation (0-3 hours after egg laying (AEL) and 24 hours AEL respectively). Thus far, gene-expression in the serosa has been inferred through the altered gene expression patterns in an egg resulting from removal of the serosa by means of RNAi of a key gene regulating its development (i.e. *zen*) (Jacobs *et al.*, 2013, 2014a; Jacobs and van der Zee, 2013). However, absence of a serosa might affect the embryonic transcriptome, and thus the interpretation of the results. Therefore, in our experimental design, for all samples in each treatment group, the serosa was separated from the embryo, and both the embryo and the serosa were sequenced separately. This enabled a robust comparison between treatment groups and between serosa and embryo. Using *in-situ* hybridisation I confirmed the immune induced gene-expression in the serosa for a small set of differentially expressed genes.

5.2 Methods

5.2.1 Study species

The squinting bush brown (*B. anynana*) is a fruit feeding butterfly with a distribution ranging from Southern Africa to Ethiopia (Larsen, 1991). *Bicyclus anynana* exhibits striking phenotypic variation in the form of seasonal morphs which function as an adaptation to alternate wet-dry seasonal environments (Brakefield and Larsen, 1984; Brakefield *et al.*, 2009b). It has been used as model in a wide range of studies; *B. anynana* began as a model for the evolution of seasonal polyphenism (Beldade and Brakefield, 2002; Brakefield *et al.*, 1998) and eyespot evolutionary developmental biology (Beldade and Peralta, 2017; Breuker and Brakefield, 2003; Monteiro, 2015; Özsu *et al.*, 2017). *Bicyclus anynana* has since also been used in studies on for example; behaviour (Costanzo and Monteiro, 2007), life-history evolution (Fischer *et al.*, 2010; Geister *et al.*, 2008b), physiology (Zijlstra *et al.*, 2004) and (embryonic) development (Tong *et al.*, 2014). As a result a range of techniques and resources are available for *B. anynana* including *in-situ* hybridisations for embryos and larval and pupal wings (Brakefield *et al.*, 2009a; Ramos and Monteiro, 2007), transcriptomic data (Macias-Muñoz *et al.*, 2016), a high-quality draft genome (Nowell *et al.*, 2017) and other genomic resources (Beldade *et al.*, 2009), RNAi (Özsu *et al.*, 2017) and CRISPR/cas9 (Beldade and Peralta, 2017; Matsuoka and Monteiro, 2018; Prakash and Monteiro, 2018; Zhang *et al.*, 2017).

Two species of bacteria were used for this study. *Micrococcus luteus*, formerly known as *Micrococcus lysodeikticus*, a Gram-positive to Gram-variable, non-motile, coccus shaped actino bacterium (Kocur *et al.*, 2006). *Micrococcus luteus* is found in soil, dust, water and some foods (Kocur *et al.*, 2006). The other bacterium used is *Escherichia coli*, a Gram-negative rod-shaped bacterium from the family known as enterobacteriaceae (Tenaillon *et al.*, 2010). *Escherichia coli* is originally found in the lower intestines of endotherms (Tenaillon *et al.*, 2010). However, many strains are kept in culture as *E. coli* is widely used as a model organism and as a tool for

genetic manipulation and bioengineering (Barrick *et al.*, 2009; Zhang *et al.*, 2000). These bacterial species were chosen as they have been used previously to induce an immune response in *S. exigua* larvae (Pascual *et al.*, 2012) and adult *Melitaea cinxia* (Woestmann *et al.*, 2017)

5.2.2 Butterfly rearing and sample collection

Samples were collected from a large outbred *Bicyclus anynana* laboratory population kept at Oxford Brookes University, and established from a large outbred stock from Cambridge (over 350 egg-laying females). The experiment was performed over multiple consecutive generations, where all the eggs for RNA-sequencing experiment were collected from the first generation established in the lab, as were most of the eggs used for the *in situ* hybridisations, while the hatching experiments were performed on eggs from a later generation. The larvae were raised on *Brachypodium sylvaticum.* Adults were fed mashed banana, with *ad libitum* access to food. The butterflies were kept at (27±0.2°C and 70±3% RH) relative humidity (RH) at a 12h: 12h light to dark regime (Özsu *et al.*, 2017; Saastamoinen *et al.*, 2010). Eggs were collected in the afternoon (12.00 -18.00) within two hours after being laid on *B. sylvaticum* and placed in glass wells at 23±0.2°C and 70±3% RH until treatment.

5.2.3 Immune and wounding challenge

The embryos were exposed to their respective treatments (septic injury, sterile injury or control, i.e. not pricked) at 0-3 hours after egg laying (AEL) or 24 hours AEL. In preparation for their treatment, all eggs were lined up and secured in place by double sided tape (Ultratape™) on a microscope slide. After the eggs were secured on microscope slides, each slide was randomly assigned to one of the three treatment groups, standardising handling, conditions and sample sizes across treatments. The eggs in the sterile injury treatment groups were pricked through the cell and cuticular layers surrounding the embryo (Fig 1.1) with a tungsten needle with a 0.001 mm tip (FST™) (from now on named, Sterile injury). The eggs in the septic

injury treatment group (from now on named, Septic injury) were also pricked with the same type of tungsten needle, this time dipped in a 10 mg/ml mixture of lyophilised *M. luteus* and *E. coli* in 1X PBS before pricking each egg (concentration based on Pascual *et al.* (2012)). Pricking of the egg resulted in perforation of the serosa and surface pricking of the embryo. Lyophilised and heat-killed bacteria have previously been used to activate the immune system in moths (Gorman *et al.*, 2004; Pascual *et al.*, 2012; Wu and Yi, 2018). The control eggs were not pricked, but instead remained under the same temperature and humidity conditions (23±0.2°C and 70±3% RH) as the pricked eggs. All microscope slides were in a box covered with gauze. This approach was taken to ensure that differences in developmental time, hatching rate and gene expression were only the result of the different treatments and not the effect of handling, exposure to different temperature and humidity conditions, or the effects of being lined up on slides by means of tape (i.e. also taken into account when randomising slides).

The eggs used *in-situ* hybridisations were treated as described above at 24h AEL, after which they were placed back in the incubator to develop another 24h before fixation in preparation of *in-situ* hybridisation (5.2.8) or put in TRI-reagent for RNA-extraction (5.2.7) at 48h AEL. The eggs used for the RNA-sequence experiment were exposed to the above treatment at 24-28h AEL and placed back in the incubator to develop another 18h before fixation (5.2.5). The eggs were pricked at 24h after egg laying to ensure the presence of the serosa (Holzem *et al.,* in prep; Gorman *et al.,* 2004; Chapter 2). To aid comparison with previous studies on Lepidoptera, which used an experimental design to examine the immune response between 6h-24h after exposure to an immune stimulus (Gandhe *et al.*, 2006; Gorman *et al.*, 2004; Vogel *et al.*, 2011), in our study, all of the eggs were fixed 18h-24h after treatment

5.2.4 Hatching rates and development time

Eggs in each of the treatment groups not used for RNA-seq, or *in-situ* hybridisation, were monitored throughout embryogenesis, and hatching rates recorded. Eggs were checked multiple times per day (i.e. between 9 am and 6 pm) for hatching. Hatching rates (i.e. number hatched/total number of eggs in a treatment group) were thus determined. The time from the day that the egg was laid, until the day that the larva hatched from the egg was used as a measure of embryonic development time (in days).

5.2.5 RNA extraction and library preparation for transcriptomic analyses

Eggs in each of the treatment groups were either monitored for egg-hatching or sacrificed for transcriptomic analyses of embryos and their serosa's. In order to be able to separate an embryo from its serosa, eggs were fixed 18 hours after treatment at around 42h AEL (Fig. 5.1). Eggs were dechorionated in a 4% sodium hypochlorite in 1x PBS solution. After dechorionation eggs were fixed in a 1:1 mixture of heptane and 4% paraformaldehyde in 1XPBS in glass vials, for 50 minutes only at room temperature. After fixation eggs were gradually dehydrated in a methanol series and stored at -20°C in methanol for 2-4 weeks. For RNA extraction, eggs were gradually rehydrated in 1x MOPS and each egg was dissected separating an embryo from its serosa. For each treatment I thus had two types of samples; embryo and serosa. Each sample was furthermore collected in triplicate (90 to 100 eggs per replicate), resulting in 18 samples (Fig. 5.1). After dissection RNA was extracted using the Recover All™ Total Nucleic Acid isolation kit (Ambion™). The sample was homogenised in the digestion buffer provided in the kit, and the manufacturer's protocol was followed for all subsequent steps. The extracted total RNA concentrations were 200±68 ng/µl for the serosa samples and 768±246 ng/µl for embryonic samples, the RNA was stored at -80°C.

Total RNA was depleted of rRNA using the low input RiboMinus™ Eukaryote system v2 (Ambion™), following the manufacturer's protocol. The rRNA depleted RNA was stored at -

80°C in nuclease free water. The library preparation used the Ultra II Directional RNA Library Prep with Sample Purification Beads with NEBNext® Multiplex Oligos for Illumina® using Index Primers Set 1 and 2 following the protocol for rRNA depleted Formalin-Fixed Paraffin-Embedded (FFPE) RNA, as the samples were fixed in paraformaldehyde prior to RNA extraction.

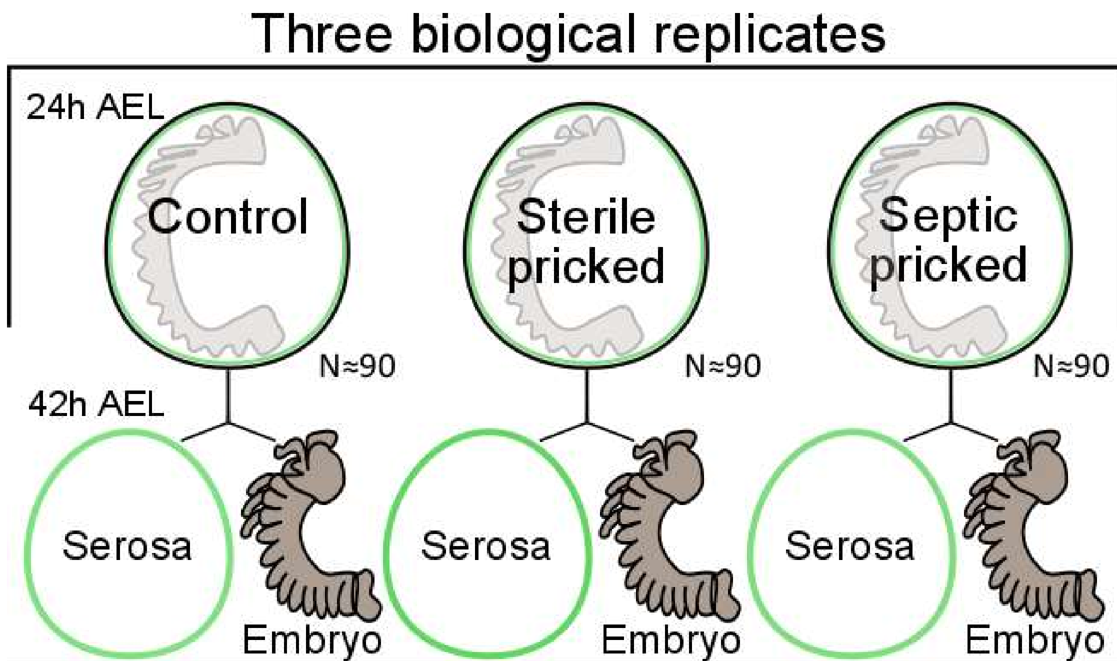

Figure 5.1: Experimental design for transcriptomic experiment. Eggs were collected between 0-3h old and kept at 23°C at 70% RH for 24h to ensure development and establishment of the serosa. At 24h after egg laying (AEL) eggs were either pricked with a sterile needle (sterile injury), pricked with a mix of lyophilized killed *Micrococcus luteus* and *Escherichia coli* in 1X PBS (septic injury) or remained untreated (control). Eggs were incubated for another 18h at 23°C after which, for each egg, an embryo was separated from its serosa. Each sample (consisting of material from 90 eggs) was collected in triplicate, thus RNA from 18 samples in total were sequenced.

5.2.6 Transcriptome analysis

5.2.6.1 Assembly and annotation

Transcriptomes were generated by Edinburgh Genomics using Illumina NovaSeq S1 50 PE. A total of 2 091 034 949 raw reads were used for a *de novo* assembly after running fastq. The

pipeline for transcriptome assembly used has been amended from the Illumina-based pipeline for butterflies from (Carter *et al.* (2016) and references therein) and is primarily based on the (latest) Trinity assembly software (v2.8.2) and downstream analyses in Trinity using integrated packages from Bioconductor (v3.8) (Gentleman *et al.*, 2004; Huber *et al.*, 2015), and the automatic annotation assembly pipeline Trinotate (v3.1.1) (including SignalP, tmHMM, and RNAMMER) (Grabherr *et al.*, 2011; Haas *et al.*, 2013; Krogh *et al.*, 2001; Lagesen *et al.*, 2007; Petersen *et al.*, 2011). As well as using the Swiss-Prot/UniProt and Pfam databases (Punta *et al.*, 2012) for standard annotation, the custom BLASTp and -x strategies (Altschul *et al.*, 1990) in the pipeline used in the present study also used custom databases largely compiled from LepBase (Challis *et al.*, 2016), in particular the annotated *B. anynana* (Nowell *et al.*, 2017) and *B. mori* genome (Shimomura *et al.*, 2009). BLASTp was performed on the longest predicted open reading frames of the transcripts. Assembled concatenated Trinity transcriptome (alignment rate 98.12%) consisted of 290 248 transcripts (273 828 Trinity genes), with an average contig length of 552bp (N50=622), 36.91% GC content. Summarised steps of the pipeline; raw reads for each of the samples were mapped back to the Trinity transcriptome to obtain an alignment-based read count estimation for each contig using Bowtie 2 (v.2.3.4.1) (Langmead *et al.*, 2009) and RSEM (Li and Dewey, 2011), after which expression matrices were generated for differential gene expression analyses. Read counts are in both fragments per kilobase transcript length per million fragments mapped (FPKM) and transcripts per million transcripts (TPM). For differential gene expression analyses Log_2 expression levels and fold changes (FC) were calculated and reported. In particular, significance is in the first instance here based on the False Discovery Rate (FDR) adjusted p-values (for multiple testing; based on Benjamini and Hochberg, 1995), with $log_2FC > 1$, and always reported alongside the FDR-values. Cut-off for significance was determined at $FDR<0.01$. Differential gene expression patterns were determined by means of voom/ limma (Bioconductor/R package) (Law *et al.*, 2014; Ritchie *et al.*, 2015) integrated in the Trinity environment, rather than EdgeR as in Carter *et al.*,

2016, given the design of the present study with biological replicates (Soneson and Delorenzi, 2013). A heat map of patterns of differentially expressed genes across treatments and replicates within treatments was generated using a stringent cut-off of FDR <0.001 and log_2FC >2. These cut-offs were chosen to illustrate the consistency in response to treatments across replicates and illustrate the most significant differences between treatment groups. Automatic annotations were manually verified for reported genes.

5.2.6.2 Detection of possible new antimicrobial peptides

To discover possible new AMPs-like proteins, eight protein sequences of differentially expressed genes (FDR<0.01) that could not be annotated but were predicted *B. anynana* proteins were run through the Antimicrobial Peptide Scanner Vr.2, a deep neural network designed to predict AMPs (Veltri *et al.*, 2018). Only the first 200 amino acids (AA) of each sequence were considered, the proteins with a prediction probability > 0.5 are considered to have AMP-like properties.

5.2.6.3 Identification of the canonical immune genes expression

A candidate gene approach was taken to assess the expression of the canonical immune genes, such as the genes in the IMD, Toll, MAPK-JNK-p38 and JAK-STAT pathways, as well as the pathogen-recognition genes, modulators (e.g. serpins and serine proteases) and effector genes (e.g. AMPs). Sequences of these canonical immune genes were compiled from the available literature in moths (Bel *et al.*, 2013; Cao *et al.*, 2015; Pascual *et al.*, 2012; Tanaka *et al.*, 2008), whereafter the orthologues of these genes were located in our transcriptome through homology-based searches using a local blast server (Camacho *et al.*, 2009).

5.2.7 Riboprobe generation

RNA from the eggs was obtained using a TRI-Reagent extraction followed by RNeasy purification as detailed in Ferguson *et al.* (2014). The riboprobes were generated following the protocol as described in Ferguson *et al.* (2014) and Carter *et al.* (2015). In short, cDNA was

generated using the QuantiTect® Reverse Transcription Kit (Qiagen) and stored at -20C. The products targeted were initially amplified by RT-PCR from RNA extracted from bacteria pricked eggs (Septic injury). In a second PCR, the gene-specific primers were adapted with a modified reverse or forward primer with a T7 5’ tail (5’-TAATACGACTCACTATAGGG+Fw/Rev-3’) resulting in an antisense or sense template. All riboprobes were synthesised from the second PCR product using DIG RNA labelling mix (Roche Applied Science, Penzberg, Germany). The resulting riboprobes were purified with an RNease Kit (Qiagen, Hilden, Germany) and stored at -20°C. All primers used for riboprobe generation can be found in Table 5.1.

With the *in-situ* hybridisations I verified the expression of four genes which were differentially expressed upon septic injury in our data set. The four genes are known to be important for different elements of the Lepidoptera innate immune response (Tanaka and Yamakawa, 2011). *Attacin-A* is one of several AMPs regulated by the IMD-pathway (Hultmark *et al.*, 1983). It works effectively against Gram-negative bacteria by inducing alterations in the structure and permeability of the outer membrane of the bacteria (Carlsson *et al.*, 1991; Hultmark *et al.*, 1983). *Cactus* is an essential part of the Toll signalling pathway which is activated upon Gram-positive bacteria, *cactus* degradation leads to nuclear localization of *dorsal* which activates AMP expression (Sun *et al.*, 2016; Valanne *et al.*, 2011). *Relish* is an essential part of the IMD-signalling pathway which enables the reaction to Gram-negative bacteria (Myllymäki *et al.*, 2014). After the IMD-pathway is activated *relish* is cleavaged by the IKK-complex where after the N-terminal of *relish* moves into the nucleus to start AMP expression (Myllymäki *et al.*, 2014). Finally, *hemolin* is a Lepidoptera-specific immune gene which functions as a pattern recognition receptor which recognises both Gram-positive and negative bacteria (Ladendorff and Kanost, 1990; Yu *et al.*, 2002; Yu and Kanost, 2002).

Table 5.1: *In-situ* hybridisation primers: forward and reverse primer sequence used for primary and secondary riboprobe template generation; annealing temperatures in degrees Celsius (Ta) and amplicon size in base pairs (bp).

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Ta (°C)	Amplicon
attacin-A	CCTTAGCTGGTAATGATCAG	TCGGCATATATGGTGAAATG	59.0	462
cactus	GACGGAGATACACAACTACAC	GTCATACTCATCCTCACTACTG	57.0	642
hemolin	GAGCACTCACCTGTAGAAG	AGTATCCTTGGTCACTCTTC	53.0	799
relish	ATTTGTCTGCCTGTGTACTC	CCCATTTCGTACAGGATAGC	59.0	631

5.2.8 Whole-mount *in-situ* hybridisation

In situ hybridisations were performed on eggs around 48h AEL, using an *in-situ* protocol that has been optimised for butterfly embryos, and their extra-embryonic tissues, as described in Ferguson *et al.* (2014) and Carter *et al.* (2015). For each *in-situ* hybridisation around 10 embryos and their extraembryonic membranes were used. For each probe the *in-situ* was successfully repeated at least twice (*cactus* and *relish)* and up to four times (*hemolin* and *attacin-A*). The protocol consists of the following (summarised) steps; eggs (i.e. embryos and their extra-embryonic tissues) were dechorionated in a 4% sodium hypochlorite in 1x PBS solution. After dechorionation eggs were fixed in a 1:1 mixture of heptane and 5.5% formaldehyde (in 1XPBS) in glass vials, for 30 minutes at room temperature followed by an overnight fixation at 4°C while slowly rotating. After fixation eggs were gradually dehydrated in methanol and stored at - 20°C (see Chapter 2). Before hybridisation eggs were rehydrated in PBS with tween-20, the left-over chorion and amnion were removed where after the eggs were digested for 13 minutes with proteinase-K. Embryos were post-fixed for 15 min in 5.5% formaldehyde where after the eggs were washed with 1X PBS and incubated in pre-hybridisation solution at 56°C. The hybridisation solution (50% Deionised formamide, 5x SSC, 0.02% Tween 20, 100 µg/ml denatured Yeast tRNA, 2 mg/ml Glycine) containing around 100 ng of riboprobe was applied and hybridisation proceeded overnight at 56°C with gentle rocking of samples. To increase the serosal staining the riboprobe was not denatured before applying it to the samples. Hybridised samples were washed and blocked (Roche Applied Science,

Penzberg, Germany) for 30 min before anti-DIG antibody incubation at room temperature for 3–4h. Eggs were washed thoroughly in 1x PBS including a final overnight wash at room temperature. Staining was developed in Alkaline Phosphatase buffer with NBT/BCIP. Embryos and their serosa's were counterstained with 1:4000 4',6 Diamidine-2'-phenylindole, dihydrochloride (DAPI, ThermoFisher: 358⁄461nm) for 20 min. Embryos and their serosa's were mounted in glycerol and imaged using a Zeiss Axio zoom v.16**.**

5.2.8 Statistical analyses

5.2.8.1 Hatching rate and development time

Linear mixed effect (lme) models were fitted by means of restricted maximum likelihood (REML) which produces unbiased estimates of variance and covariance parameters (Development time) or a generalized linear mixed effect model using binominal distribution (Hatching rate) (see also Chapter 4). Hatching rate was assessed by a glmer model using a binominal distribution as it was measured as a proportion hatched, while the development time was assessed using REML.

Development time was assessed using a model that included treatment (Septic, Sterile or Not) and time of treatment (3h AEL or 24h AEL) and their interaction as fixed factors, and the time that the egg was laid as random factor. Model fit assessment and testing of fixed effects was by means of the likelihood ratio test (LRT), which uses chi-square tests on the log-likelihood values to compare different models and produce fixed effect test statistics. Only significant interaction effects were included in final models and presented in the text. Analyses were performed using R (3.4.0) (Core Team R, 2017) with packages 'lme4' (Bates *et al.*, 2015). A Dunn test with Benjamini-Hochberg correction post-hoc test was performed to identify significant differences between the individual age*treatments, these results are reported in the figures (Benjamini and Hochberg, 1995; Dunn, 1964).

5.3 Results

5.3.1 The effects of an immune challenge and wounding on embryonic development

Eggs were treated at 3h AEL (i.e. before serosa formation) and at 24h AEL (i.e. when the serosa is fully established; see also Chapter 2). Treatment *per se* affected hatching rate (X^2 (2, N = 100) = 19.5, P<0.001), but not whether treatment occurred before or after serosa formation (i.e. a non-significance of time of treatment; X^2 (1, N = 100) = 2.37, P=0.12) (Fig. 5.2). Wounded eggs (i.e. pricked eggs) had a lower hatching rate than non-pricked eggs (Fig 5.2).

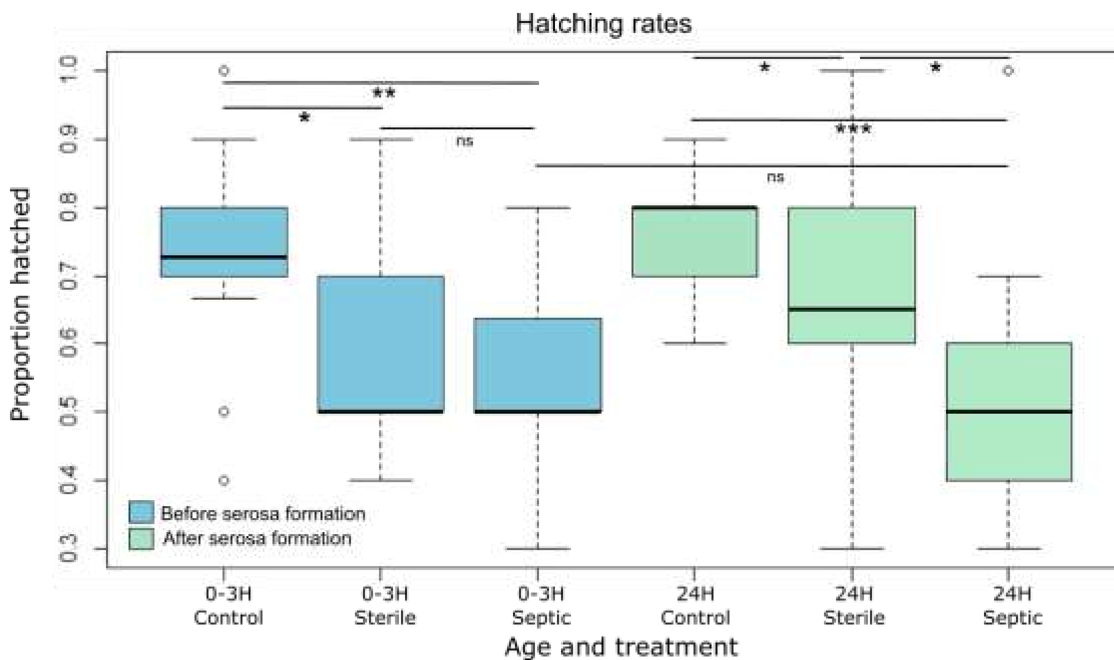

Figure 5.2: Hatching rates (as proportion hatched, 0-1) of eggs treated at 0-3h or 24h after egg laying (AEL). Eggs treated 3h AEL (i.e. before serosa formation) in blue versus eggs treated 24h AEL (i.e. after serosa formation) in green. P-values adjusted using from Benjamini-Hochberg post –hoc testing: ns=not significant, P< 0.001 ***, P < 0.01 **, P <0.05*

Embryonic development time of embryos that survived to hatch from the egg, was significantly dependent on time of treatment (X^2 (1, N = 613) = 14.62, P<0.001), treatment (X^2 (2, N = 613) = 42.7, P<0.001), and interaction (X^2 (5, N = 613) = 73.6, P<0.001). Overall, control eggs (5.3±0.03 days) developed significantly faster than pricked eggs (5.6±0.03 days) (Fig 5.3). The significant interaction effect was due to differences in response between sterile and septic injured

individual depending on the time point at which the treatment was administered; in the 0-3h AEL treated eggs sterile and septic injury eggs took a similar time to develop (Z=-1.42, P=0.09) while 24h AEL septic pricked eggs developed significantly faster than the sterile pricked eggs (Z=2.31, P=0.022).

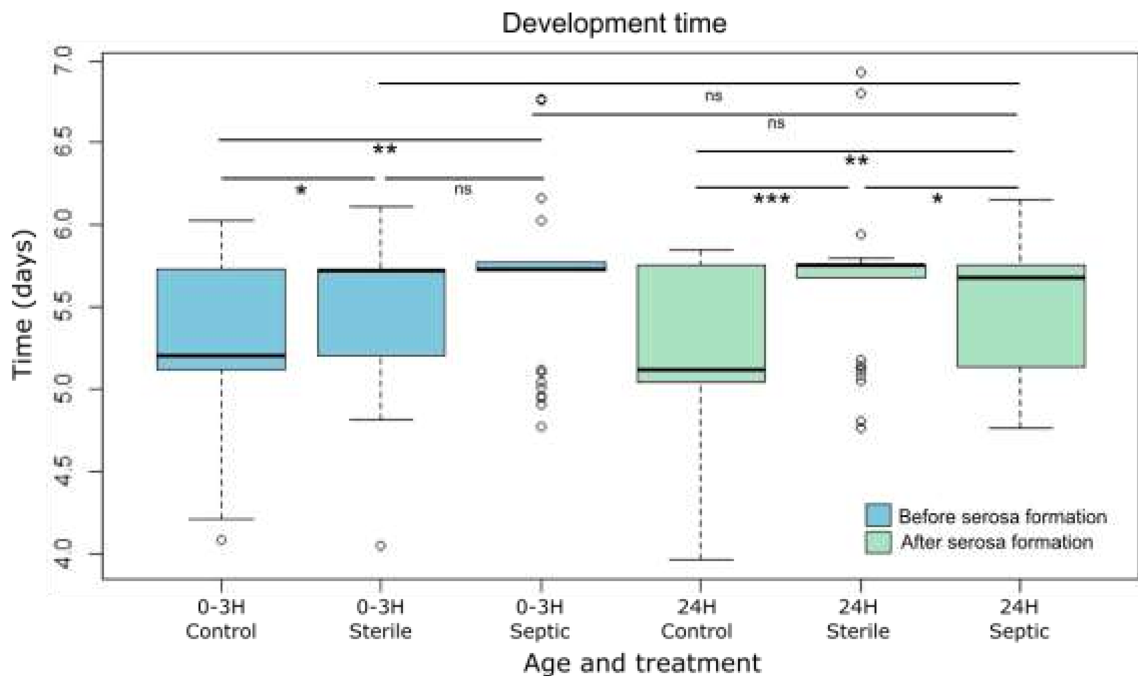

Figure 5.3: Development time of eggs treated at 0-3h or 24h after egg laying (AEL). Eggs treated 3h AEL (i.e. before serosa formation) in blue versus eggs treated 24h AEL (i.e. after serosa formation) in green. P-values adjusted using from Benjamini-Hochberg post –hoc testing: ns=not significant, P<0.001 ***, P < 0.01 **, P < 0.05 *

5.3.2 Transcriptomic Results

5.3.2.1 Biological replication of differential gene expression patterns

Gene expression in all of the nine serosa samples (see also Fig. 5.1 for experimental design), was significantly different from all nine embryonic samples. Furthermore, replicates for each the treatments in the serosal samples were similar in patterns of differentially expressed genes. This distinction was not evident for the embryonic samples (Fig. 5.4).

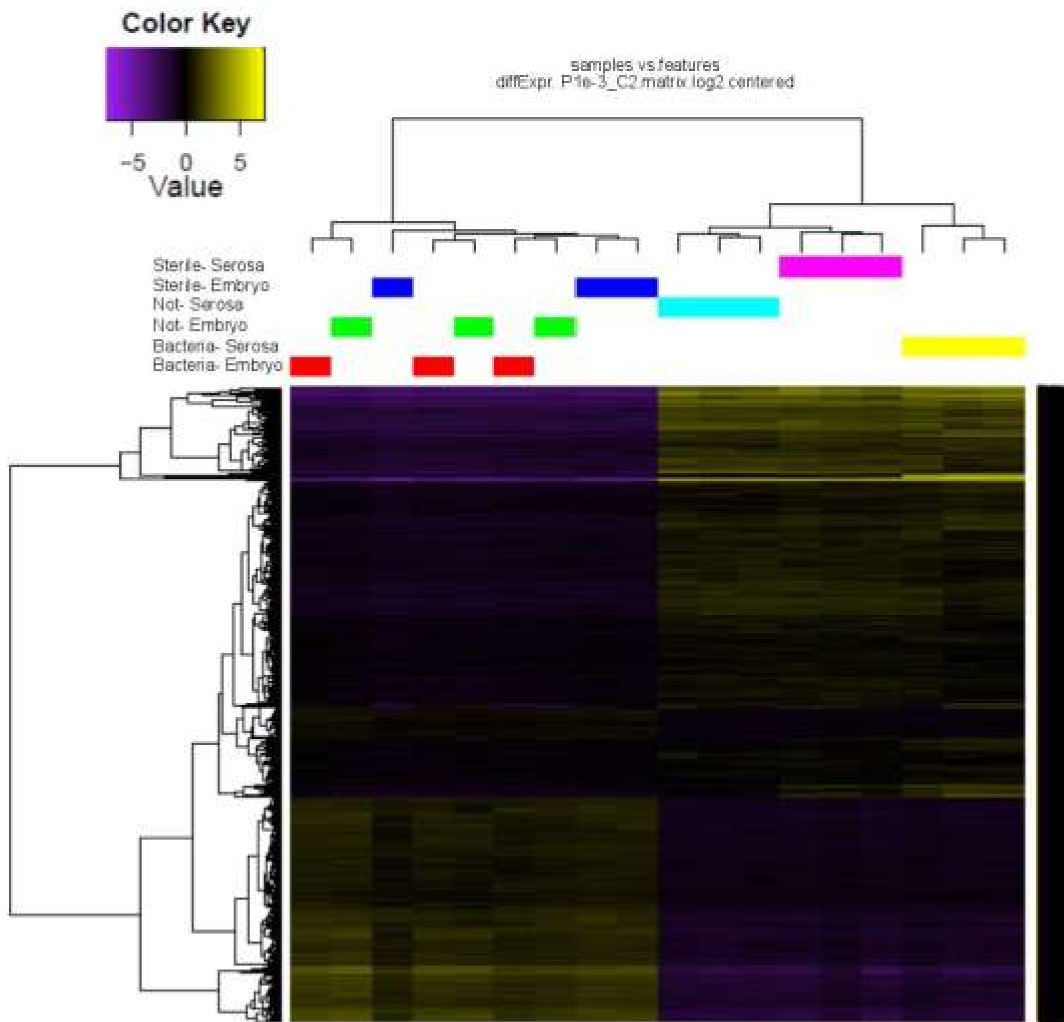

Figure 5.4: Heat map based on differential gene expression patterns across all samples. Only those genes are included with FDR <0.001 and log_2 fold change >2. It highlights how consistent differential gene expression patterns are between treatments across replicates.

5.3.2.2 Differential expression of genes upon treatment

No genes were differentially expressed as a result of the treatment in the embryonic samples, unlike for the serosal samples; 36 genes were differentially expressed between sterile pricked and control serosa, 56 between sterile and septic injury serosa and 237 between septic injury and control serosa's (Fig. 5.5) (Appendix three in the supplementary data www.dropbox.com/sh/26j5yp2jg8yt6rt/AADQ5oz-2Mrh07pYtG7s0GOza?dl=0). In order to fully analyse the immune and wounding response, 324 candidate immune-related genes were identified in the first instance in the concatenated transcriptome (Appendix three in the

supplementary data www.dropbox.com/sh/26j5yp2jg8yt6rt/AADQ5oz-2Mrh07pYtG7s0GOza?dl=0). The identity of these genes was based on relevant immunity studies on *B. mori, G. mellonella, S. exigua* and *M. sexta* (An *et al.*, 2010; Cao *et al.*, 2015; Kanost *et al.*, 2016; Pascual *et al.*, 2012; Tanaka *et al.*, 2008; Vogel *et al.*, 2011). Compared to the sterile injury treatment, 16 immune-related genes were differentially upregulated in the serosa upon septic injury. Compared to the control (i.e. not pricked) treatment, six genes were upregulated after sterile injury, and 32 were upregulated and one gene was down-regulated following septic injury (Table 5.2).

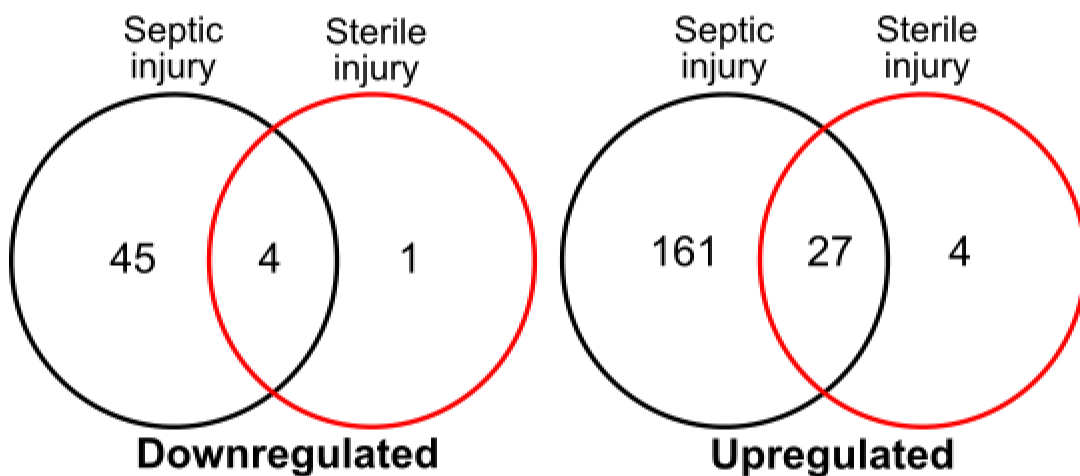

Figure 5.5: Differentially expressed genes in the serosa upon sterile and septic pricking compared to a control serosa (FDR<0.01 and log_2fold changes > 0.7).

5.3.2.3 Immune genes

Various genes of the IMD, Toll, MAPK-JNK-p38 and JAK-STAT pathways were found to be expressed by the serosa, and differentially so upon treatment. I hypothesise that the core elements of these pathways are conserved in *B. anynana* (Fig.5.6) based on the literature in moths and the expression data in our transcriptome (Cao *et al.*, 2015; Casanova-Torres and Goodrich-Blair, 2013; Hillyer, 2016; Tanaka and Yamakawa, 2011). Upon septic injury a number of the immune genes were upregulated in the serosa (FDR<0.01). The genes that were most highly upregulated upon septic injury are the effector genes such as the AMPs and lysozymes (Fig. 5.6). Other genes that were significantly upregulated are those coding for pathogen

recognition proteins such as *hemolin, serine protease* and *PGRP-like* genes. Finally, most genes in the Toll and IMD signalling pathways were not differentially expressed upon septic injury but there are exceptions, such as *spätzle, toll-7* and *cactus* in the Toll pathway. In the IMD pathway only *relish* and *IAP2* were upregulated in the serosa after septic injury.

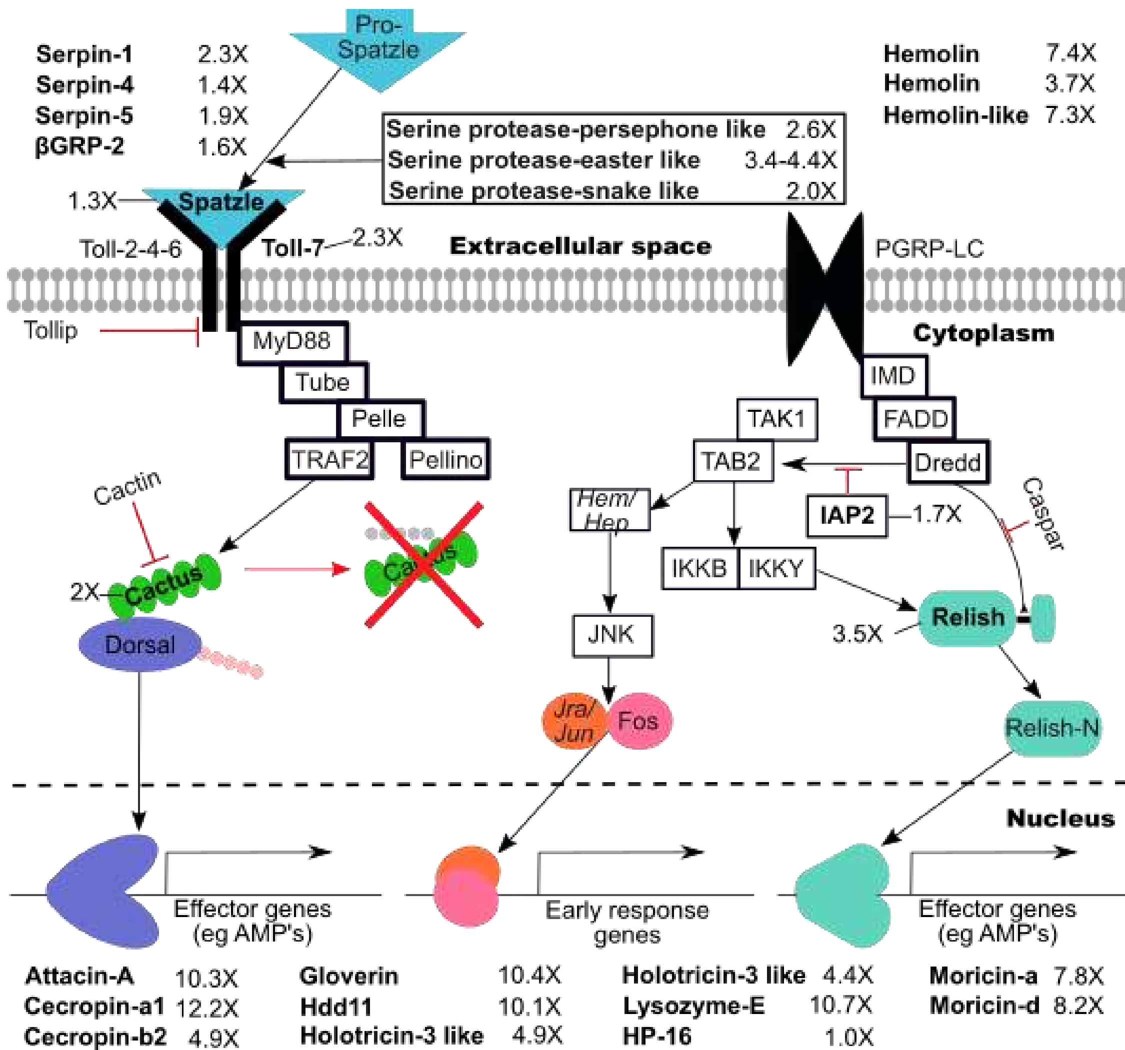

Figure 5.6: The immune signalling pathway in *B. anynana* as inferred from Tanaka and Yamakawa (2011), Casanova-Torres and Goodrich-Blair (2013), Cao *et al.* (2015), Jacobs *et al.* (2014a) and Hillyer (2016). Significantly induced genes in the serosa after septic injury (FDR<0.01) are indicated in bold. The log_2fold change upon septic injury is indicated with the number after the gene name.

Table 5.2: Differentially regulated immune genes (FDR<0.01 and $Log_2FC>1$) in the serosa comparing the three treatments, septic and sterile injury, and controls based on previously annotated Lepidoptera immune genes. Gene names in black upregulated, in red down-regulated

Description	Gene ID	Log_2FC	FDR	Log_2FC	FDR	Log_2FC	FDR
Effector		**Septic vs Control**		**Septic vs Sterile**		**Sterile vs Not**	
Attacin-A	TR170443|c0_g1	10.251	0.003	7.168	0.01		
Cathepsin O-like	TR63249|c0_g1	4.366	0.004	1.698	0.004		
Cecropin-a1	TR25098|c0_g2	12.187	0.007	6.5	0.006		
Cecropin-2	TR83865|c0_g2			6.214	0.004		
Cercropin-b2	TR191669|c0_g1	4.896	0	4.075	0.001		
Gloverin	TR37852|c0_g1	10.346	0.001				
Hdd11	TR31917|c0_g1	10.054	0.005	5.642	0.004		
Holotricin-3 like	TR145955|c0_g1	4.903	0	1.877	0.005	3.043	0.001
Holotricin-3 like	TR147073|c0_g1	4.368	0			2.777	0.002
HP16	TR78677|c0_g1	1.04	0.003				
Lysozyme-E	TR191719|c0_g1	10.687	0.007	6.233	0.004		
Moricin-a	TR64557|c0_g2	7.816	0.007	4.067	0.003		
Moricin-d	TR83971|c0_g1	8.232	0	2.48	0.001	5.754	0
Recognition							
βGRP-2a	TR54783|c0_g1	1.569	0.006				
Hemolin-like	TR153727|c0_g1	7.369	0.007				
Hemolin	TR169047|c0_g1	7.282	0.000	3.167	0.002	4.115	0.000
Hemolin	TR74733|c1_g1	3.744	0.000	2.751	0.003	0.991	0.003
Melanisation and wound healing							
Myosin heavy chain non-muscle-like	TR40944|c0_g1	3.056	0.007				
Serpin-1	TR63330|c0_g1	2.3	0.001				
Serpin-5	TR171430|c0_g1	1.852	0.009				
Serpin-4	TR168833|c1_g1	1.36	0.009				
Toll pathway							
Cactus	TR69126|c0_g1	2	0.004			1.180	0.008
ML/NCP2	TR100491|c0_g1	-1.176	0.005				
Serine protease easter-like	TR11436|c0_g2	4.429	0.006				
Serine protease easter-like	TR82515|c1_g1	3.356	0.005				
Serine protease persephone-like	TR34500|c0_g1	2.554	0.004				
Serine protease snake-like	TR67850|c1_g1	1.996	0.006				
Spätzle	TR61151|c0_g1	1.131	0.007				
Toll-7	TR40242|c0_g1	2.251	0.01				
IMD-pathway							
IAP2	TR51923|c0_g2	1.66	0.003	1.323	0.005		
Relish	TR84034|c0_g1	3.509	0	2.984	0.001		
Apoptosis							
IAP1	TR54217|c0_g1	1.113	0.008				
Other							
Immunoglobulin protein	TR15969|c0_g1	2.575	0.003	2.530	0.006		
Tryptase	TR124189|c0_g1	2.1	0.002	1.718	0.008		

5.3.2.4 Recognition and extra-cellular signal transduction

PGRP-LC, *PGRP-like 1* and *2* were expressed in the serosa, but not significantly upregulated after septic injury. *βGRP-2* was significantly upregulated after septic injury, *βGRP-1* and *3* were also expressed but not significantly upregulated. Different *serine protease-like* genes are upregulated after a septic injury, as are a number of *serpins*. But, not all of the *serpins* that were expressed in the serosa were differentially expressed across treatment groups. A number of *Tolls* have been identified in the serosa but only one *toll-7* was significantly upregulated after septic injury. Additionally, the Lepidoptera-specific *hemolin* was upregulated by both sterile and septic injury. I found three *hemolin (like)* transcripts in our transcriptome all of which were upregulated after septic injury, and of which two were upregulated through sterile injury.

5.3.2.5 Effector genes

Twenty-three AMPs, including *attacin-A, cecropins, defensins, gloverins, lebocins and moricins,* were expressed in our experimental samples. Of these 23 AMPs, 10 were significantly differentially expressed in the serosa after septic injury, and *moricin* and *Holotricin-3* were also upregulated by sterile injury (FDR<0.01). However, due to the presence of species specific AMPs and extreme sequence diversity of the genes, homology-based searches and our automatic annotation may have missed several AMPs. In the set of 237 differentially expressed genes (septic injury versus control) were three short proteins not currently annotated as AMPs but were predicted to be AMPs by the use of a deep neural network designed to predict AMPs (Veltri *et al.*, 2018) (Table 5.3). This is by no means a full and complete survey and other potential AMPs might have been missed through this approach, the potential AMPs will need to be functionally validated. Apart from the above mentioned antimicrobial peptides I also identified a number of other upregulated effector genes. There is upregulation of two *haemolymph proteinase* genes *lysozyme-E* and *HP16,* and the putative defence protein *Hdd-11* upon septic injury.

Table 5.3: Potential new Antimicrobial proteins: Log_2FC and FDR reported for the comparison of septic injury to controls in the serosa. The length of the protein in amino acid (AA), the transcript completion and the prediction probability with probabilities > 0.5 indicates a predicted AMP.

Sequence ID	Log_2FC	FDR	Open read frame (AA)	Transcript completion	Prediction probability
TR41770|c0_g1	2.611	0.0006	108	5' prime partial	0.7437
TR66743|c0_g1	1.795	0.008	148	5' prime partial	0.5114
TR47963|c0_g2	1.360	0.009	550	Internal	0.6993

5.3.2.6 Other immune responsive genes

Two hundred and thirty seven genes were differentially expressed in the serosa after septic injury (compared to controls), 188 genes were upregulated, of which 50 may be immune-related and only 34 of those were immune genes previously annotated in other Lepidoptera immune data sets (Appendix three in www.dropbox.com/sh/26j5yp2jg8yt6rt/AADQ5oz-2Mrh07pYtG7s0GOza?dl=0). A number of genes, especially enzymes, were also upregulated after sterile wounding and are described in the section for injury and wound healing. A number of additional cytochromes were upregulated after septic injury instead of injury *per se* (i.e. sterile injury). Other classes of genes that were upregulated in the serosa upon septic injury were genes related to cuticle formation, such as chitin related genes. A number of genes indicated in muscle formation like *tintins* and *myosin* and genes involved with hormones and hormone binding like *prothoracicotropic hormone-like* and *gonadotropin-releasing hormone receptor* were also upregulated after septic injury.

The 49 down-regulated genes in the serosa as a response to a septic injury include transporter proteins such as *organic cation transporter protein-like proton-coupled folate transporter-like* and *facilitated trehalose transporter Tret1-like.* Calcium channels like *Calcium-activated potassium channel slowpoke* and cytokinesis genes like *septin-1* are also down-regulated*.* As are development genes like *apterous-like* and *yellow-like* and genes involved in

the cell-wall like glycine-*rich cell wall structural protein-like.* Septic injury also resulted in downregulation of various *cytochrome P450* genes in the serosa.

5.3.3 Wound healing and melanisation

I found upregulation of a number of genes after sterile injury in the serosa. None of the canonical wound healing genes were upregulated after pricking but the sterile pricked serosas upregulated some reactionary immune genes as well as *cactus,* a member of the Toll pathway. Other genes that were upregulated after sterile injury are genes encoding cytochromes and enzymes such as esterases, dehydrogenases, proteases and transferases. Five genes were only differentially expressed upon sterile injury, four were upregulated and one was down-regulated (Fig. 5.6). Of these five genes, three were transcripts annotated as *Ejaculatory bulb specific protein (3) like (Ebp III)*. Two of the *Ebp III* transcripts were significantly upregulated and the other transcript is significantly down-regulated after sterile injury. The two other genes that were only upregulated after sterile injury were *cytochrome 5b-like* and *F-actin-capping protein subunit alpha.* After pricking, both the sterile and septic injured eggs showed melanisation at the prick site, this is present in both the eggs pricked at 3h AEL and 24h AEL. All pricked eggs seemed to show this melanisation. This melanisation spot was localised in the extra-embryonic membranes and yolk but the melanisation was never observed in the embryo (Fig. 5.7).

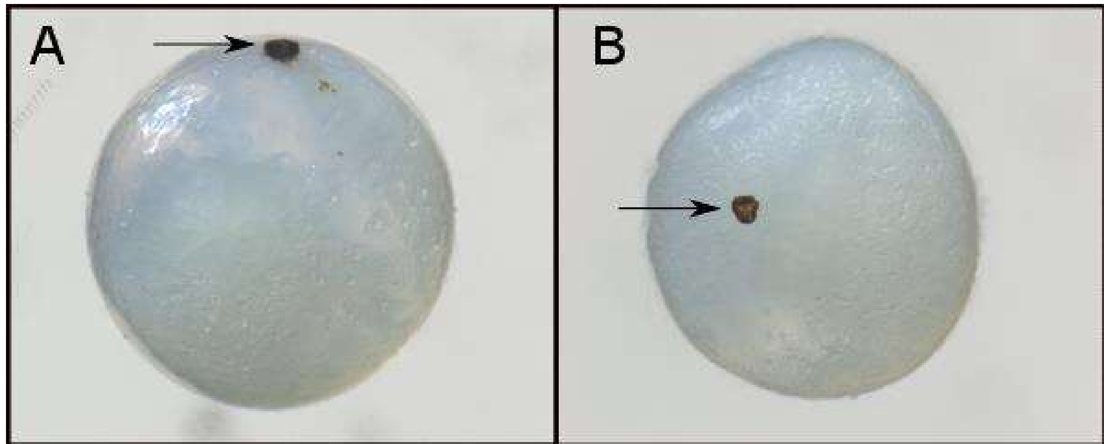

Figure 5.7: Wound healing and melanisation at the site of injury after both sterile and septic injury. The arrow indicates the site of injury and subsequent melanisation. **A** and **B** show melanisation in two different eggs from two different angles.

5.3.4 *In-situ* hybridisations

To visualise the expression of immune genes I used *in situ* hybridisation of four genes known to be involved in the Lepidopteran immune response and which showed significant upregulation after septic injury in our RNA-seq dataset. The four differentially expressed genes used were *hemolin* which encodes a recognition protein, *cactus* a part of the Toll-pathway, *relish* a part of the IMD-pathway and the AMP *attacin-A.* In control eggs, *hemolin* and *attacin-A* were not expressed, but I was able to detect *relish* expression in both the serosa and embryo (Fig. 5.10). In the septic injury embryos the expression of all of the genes was clearly associated with the serosa cells surrounding the embryo. For *hemolin* and *attacin-A* there was no expression in the embryo proper, but for *cactus* and *relish* embryonic expression can be readily observed (Fig. 5.8- 5.11). The *in-situ* probe will not penetrate the serosa easily unless the serosa has been ripped and (partly) detached from the yolk. The staining seen in the embryo in Figure 5.8A was non-specific staining which was observed in all embryos at this developmental stage. The expression patterns of each of the four genes were slightly different, as can been seen in the close up of the serosa in Figure 5.8 where expression of *attacin-A* overlaps with the DAPI stained serosal nuclei and surrounds these nuclei. The expression of *hemolin* was not located

in the nuclei but surrounds it, while *cactus* and *relish* expression seems to be solely localised to the serosal nuclei and do not surround the nuclei (Fig. 5.9-5.11)

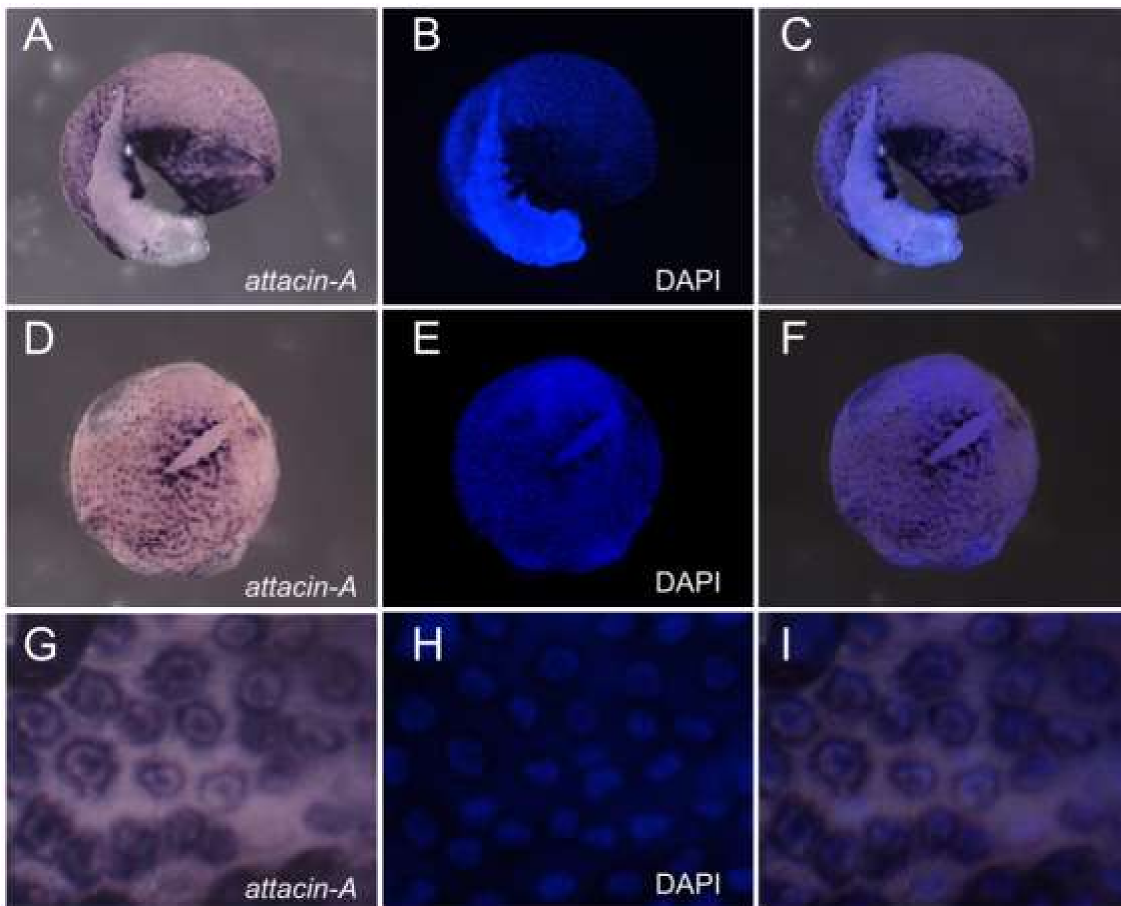

Figure 5.8: *In-situ* hybridisation showing expression of *attacin-A* upon septic injury. The first column shows the *in-situ* staining, the middle column is the embryo stained with DAPI and the last column is an overlay of the two previous images. Images **A-F** show whole embryo view where expression is localised in the overlaying serosa. The expression that can be observed in the legs of the embryo in A-C is non-specific staining. Panel **G-I** show a close up of the serosa, *attacin-A* expression is localised in and around the nuclei of the serosal cells.

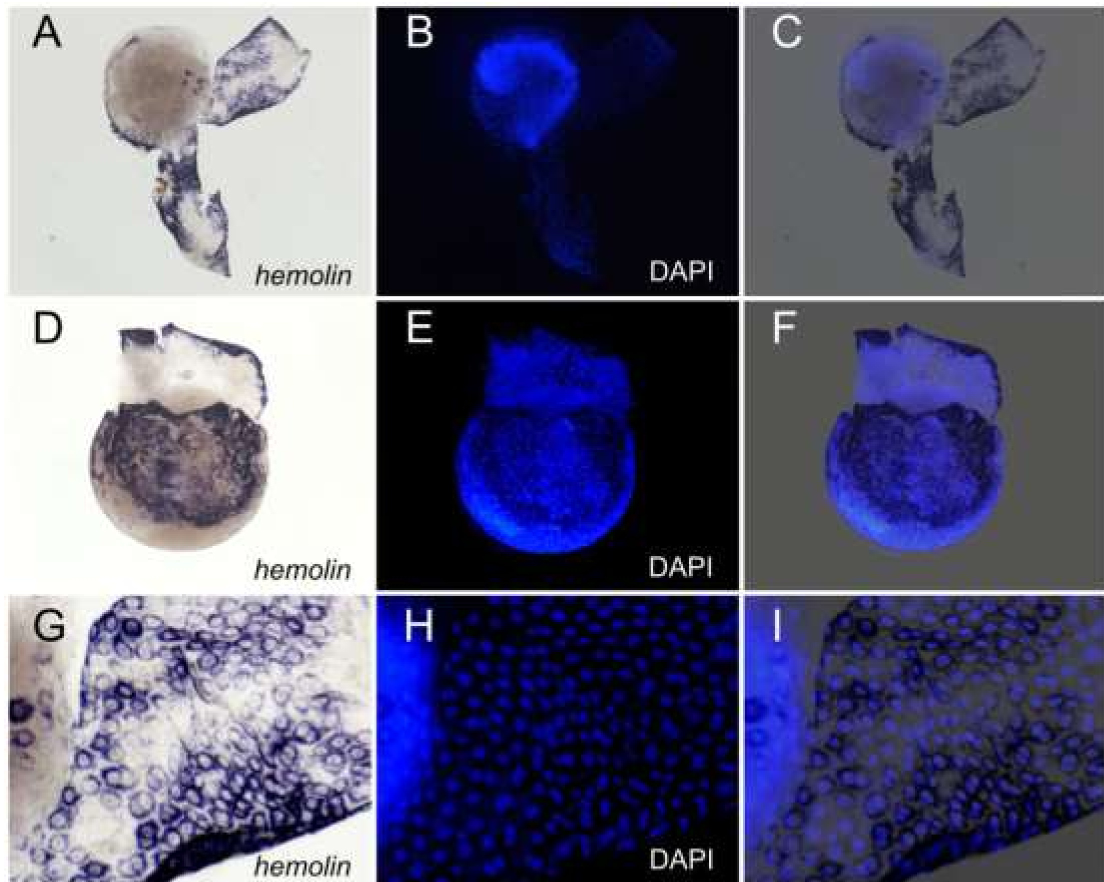

Figure 5.9: *In-situ* hybridisation showing expression of *hemolin* upon septic injury. The first column shows the *in-situ* staining, the middle column is the embryo stained with DAPI and the last column is an overlay of the two previous images. Images **A-F** show whole embryo view where expression is localised in the overlaying serosa. In panel **A-C** the embryo proper can be clearly distinguished below the serosa, the embryo shows no *hemolin* expression. In panel **G-I,** a higher magnification shows that the *hemolin* expression is localised around the nuclei of the serosal cells

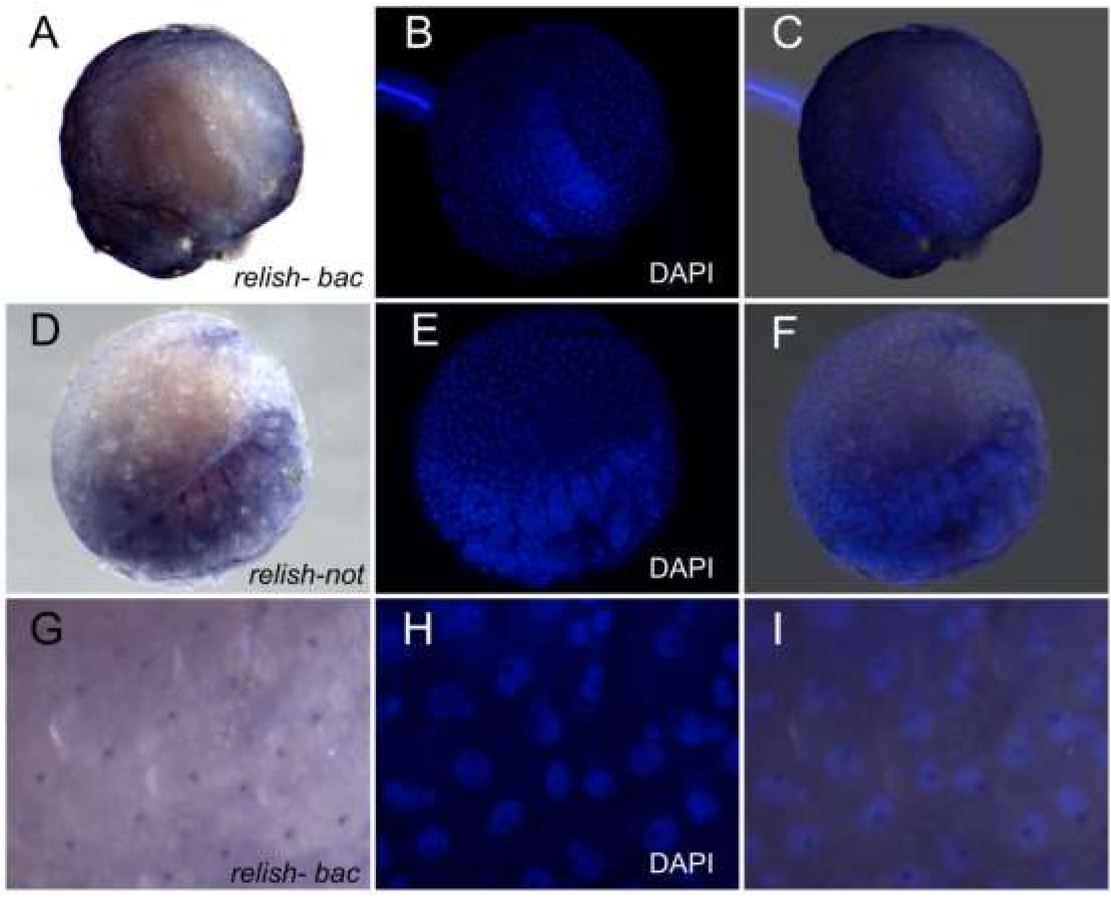

Figure 5.10: *In-situ* hybridisation showing expression of *relish*. The first column shows the *in-situ* staining, the middle column is the embryo stained with DAPI and the last column is an overlay of the two previous images. Images **A-F** show whole embryo view where expression is localised in the overlaying serosa and embryo. Panels **A-C** show expression in a septic pricked egg, while **D-F** shows expression in a control egg. In panel **D-F** the embryo proper can be clearly distinguished below the serosa, the embryo shows *relish* expression. In panel **G-I,** a higher magnification shows that the *relish* expression is localised in the nuclei of the serosal cells of a septic injury serosa, a similar pattern is seen in control serosas.

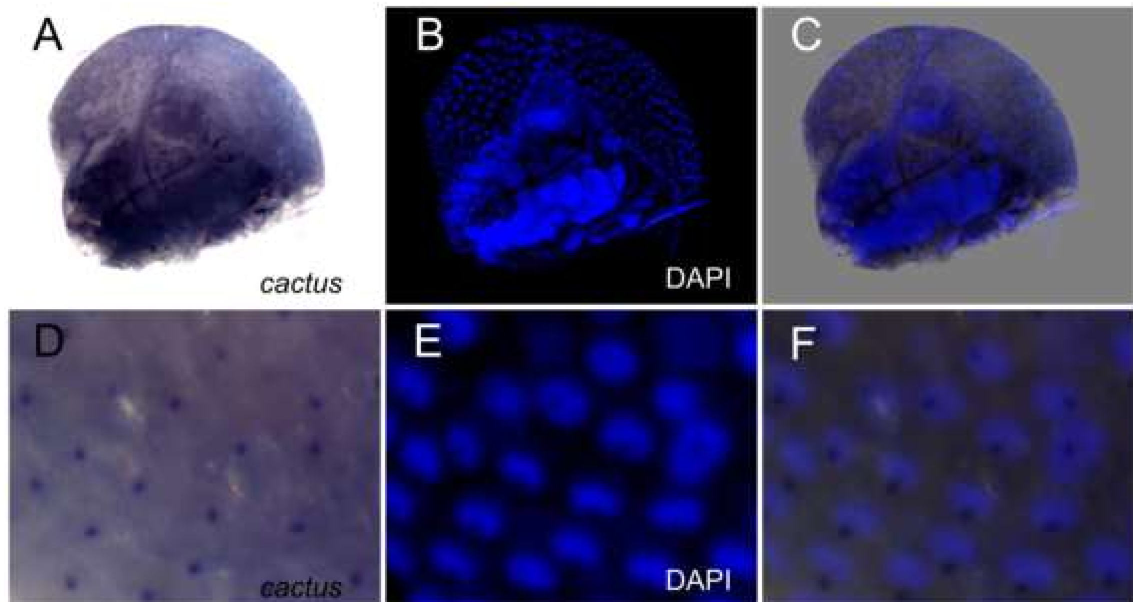

Figure 5.11: *In-situ* hybridisation showing expression of *cactus* up on septic injury. The first column shows the *in-situ* staining, the middle column is the embryo stained with DAPI and the last column is an overlay of the two previous images. Images **A-C** show whole embryo view where expression is localised in both the overlaying serosa and the embryo proper. In panel **G-I,** a higher magnification shows that the *cactus* expression is localised in the nuclei of the serosal cells.

5.4 Discussion

In this chapter I investigated whether eggs of the *B. anynana* butterfly were able to mount an immune response and the role of the extra-embryonic serosa therein. I sequenced the whole transcriptome of the separated serosa and embryo of control, sterile or septic injured eggs, which enabled us to directly compare gene expression in the serosa and embryo. This data showed that the serosa is the main and only source of the immune competence within the developing butterfly egg. Using *in-situ* hybridisation I confirmed the immune induced gene-expression in the serosa.

5.4.1 Embryonic survival and development rate

Wounding, regardless of when eggs were pricked, significantly reduced hatching rate, but less so when a serosa was present (Fig. 5.2). This could be because the serosa aids in wound

healing or because older embryos were less sensitive to wounding (Jacobs *et al.*, 2014a). When the eggs with a serosa were pricked with lyophilised bacteria, the hatching rate was lower than those of sterile pricked eggs (Fig. 5.2). This reduction in hatching suggests that mounting an immune response might come with a cost, as the lyophilised bacteria themselves were not a threat (i.e. being dead). However, this immune response might serve to prime the immune system and thus be beneficial when the eggs were exposed to live pathogens. Jacobs *et al.* (2014a) showed that bacterial growth in eggs with a serosa is reduced compared to those without a serosa suggesting that the immune response is effective and might increase hatching upon pathogen exposure. Therefore, to really understand the fitness costs or benefits of mounting an immune response in the egg further investigation is needed, such as comparing the hatching rates of eggs exposed to bacteria with and without a serosa.

Injury delayed development, both before and after serosa formation (Fig. 5.3). This delay could have been mediated by resource allocation trade-offs (Lazzaro and Little, 2009; Siva-Jothy *et al.*, 2005). Surprisingly, developmental rate of eggs exposed to lyophilised bacteria after serosa-formation was faster than when they were sterile wounded. This response needs further investigation, but one hypothesis might be that the embryo is forced to speed up their development as egg resources are running out faster, and it would reduce pathogen exposure time.

In *P. aegeria,* whether a mother has been forced to fly as well as her age affect affects hatching success (in combination with egg size) and embryonic developmental time after their offspring were exposed to viral pathogens (Gibbs *et al.*, 2010a). What has not been considered in this chapter is the fact that mothers may immune prime their eggs by including mRNA of immune-related genes into the eggs, which could defend the eggs before serosa formation (Carter *et al.,* 2013; Chapter 6). Furthermore, I only considered embryonic development, but mounting an immune response might also affect other (post-hatching) life history-traits

(Diamond and Kingsolver, 2011; Lazzaro and Little, 2009; Siva-Jothy *et al.*, 2005; Zuk and Stoehr, 2002).

5.4.2 Differential gene expression

The gene expression data show significant differences in the repertoire of genes expressed in serosa and embryo. Furthermore, the serosal replicates showed a distinctive gene expression pattern based on treatment but the embryonic samples did not. This indicates that gene expression of the embryo was not influenced by treatment, and was not involved in mounting an immune response at this time in development. This is comparable to *T. castaneum* (Jacobs *et al.* (2014a)), where the serosa is also solely responsible for the eggs immune response. This was shown using eggs with and without a serosa, although one should note that the absence of the serosa may affect embryonic gene expression patterns. Using our approach I eliminated the confounding effects of a serosa-less development and possible serosa to embryo signalling.

5.4.3 Immune gene expression

All canonical genes in the Toll, IMD, Mapk-Jnk-P38 and JAK-STAT pathways were found expressed in *B. anynana,* which suggest that these pathways are generally highly conserved and possibly functionally similar to the well characterised pathways in *Drosophila*, *T. castaneum*, and other Lepidoptera (Kleino and Silverman, 2014; Myllymäki *et al.*, 2014; Tauszig *et al.*, 2000; Valanne *et al.*, 2011; Yokoi *et al.*, 2012). Most of these genes have been found to be expressed in our serosal transcriptome (Appendix three in the supplementary data www.dropbox.com/sh/26j5yp2jg8yt6rt/AADQ5oz-2Mrh07pYtG7s0GOza?dl=0), which supports that the serosa has the capability for a full and complete immune response. However, the copy number of these canonical immune genes seems highly variable across Lepidoptera species (Kanost *et al.*, 2016; Keehnen *et al.*, 2018). Further investigation into these genes and their copy number in the *B. anynana* genome would yield valuable insights into the evolvability of

immune genes, including regulatory evolution in relation to co-option in wing patterning (Özsu and Monteiro, 2017).

Genes in the Toll pathway are involved in immunity to Gram positive bacteria and fungi (Valanne *et al.*, 2011). The Toll pathway is not directly activated by binding to any microbial pathogen-associated molecular patterns (PAMPS) but instead it utilises the cytokine-like ligand Spätzle (Valanne *et al.*, 2011). Spätzle is synthesised as a pro-protein which is processed by a cascade of serine proteinases to an active form which then binds to the ectodomain of Toll to trigger signal transduction and production AMPs (Ferrandon *et al.*, 2007). After septic injury multiple *serine proteases* are upregulated in the serosa, as is *spätzle*. Toll receptors are evolutionarily conserved and are transmembrane proteins with extracellular Leu-rich repeats and a cytoplasmic toll/interleukin 2 (Cao *et al.*, 2015). I have identified *toll-2, 4, 6, 7* and *tollip* (based on orthology to *Drosophila toll* genes) to be expressed in the serosa. Structural similarity to immune reactive *toll 1* of *Drosophila* and transcript location *toll 2* and *toll 5* have been suggested as immune responsive toll candidates in *M.sexta* (Cao *et al.*, 2015). In our dataset, *toll-7* is differentially expressed in the serosa after septic injury, suggesting a role for *B. anynana toll-7* in an innate immune response. In *P. napi toll7-3*, one of the copies of *toll 7* identified in this species, is observed to be under positive selection as is *toll 6* (Keehnen *et al.*, 2018). As these *toll* genes are under positive selection, it might indicate they are of significance in the innate immune response in *P. napi* (Keehnen et al., 2018). In *D. melanogaster toll-7* has a virus autophage role (Buchon *et al.*, 2014).

The IMD-pathway, considered to be mainly activated by Gram-negative bacteria, regulates the transcription of immune-effector genes that overlap with those controlled by the Toll pathway (Kleino and Silverman, 2014). The IMD-pathway is activated by the recognition of bacterial peptidoglycan (PGNs) by so-called PGN-recognition proteins. In *Drosophila* PGRP-LE and PGRP-LC can activate the IMD-pathway (Myllymäki *et al.*, 2014). In moths there is no

PGRP-LE ortholog (Zhang *et al.*, 2015), and it is hypothesised that PGRP-LCa and PGRP-LCb instead recognise PGNs and activate the IMD-pathway. I found very low transcript levels *of* a PGRP-LE-like gene, while PGRP-LC was expressed both in the serosa and embryo but not upregulated after septic injury. Other genes upregulated in this pathway were *inhibitor of apoptosis 2 (IAP2)* and *relish* which is cleaved to *relish-N* which moves into the nucleus to activate the transcription of the immune effector genes (Myllymäki *et al.*, 2014).

The MAPK-JNK-P38 pathways are responsive to growth factors, cytokines and stress signals and regulate cell-proliferation, differentiation, inflammation and death (Arthur and Ley, 2013). The JNK-pathway is essential for wound healing in *Drosophila* (Rämet *et al.*, 2002). Interestingly, genes in these pathways were only upregulated when the serosa was exposed to septic wounding. The transcripts of MAPK were upregulated in the septic injury serosa compared to control and sterile pricked serosa. This suggests that in a *B. anynana* egg this pathway might be more important for the immune reactionary function than in wound-healing.

Another upregulated gene was *hemolin*, a Lepidoptera-specific immune gene which functions as a pattern recognition receptor similar is to C-type lectins, b-1,3-glucan-binding proteins and peptidoglycan-binding proteins (Kanost *et al.*, 2004; Yu *et al.*, 2002; Yu and Kanost, 2002). In adult Lepidopteran insects it may be synthesised in the fat body in response to both Gram-negative and positive bacteria (Ladendorff and Kanost, 1990) and has been associated with the cellular immune response and to be key in haemocyte functions (Eleftherianos *et al.*, 2007). Here I have shown that the serosa can also upregulate *hemolin*, both after sterile and septic wounding, suggesting *hemolin* might also have a function in wound healing. As I found three transcripts of *hemolin* and *hemolin-like* proteins the evolution and possibly alternative splicing of this gene requires more attention (Chapter 6). As two of the transcripts were upregulated by both sterile and septic injury and one only by septic injury, the

transcripts may have functionally diverged. To explore the responses of these different pathways in more detail, I would need to expose the eggs to either Gram-positive bacteria, Gram-negative bacteria or a fungus but not a mix.

All these pathways lead to the expression of reactionary proteins, mainly AMPs. AMPs are short polycationic peptides constituting a crucial part of the innate immune response. The classical action of an AMP is causing cell membrane damage (Ageitos *et al.*, 2017). AMPs show extreme sequence divergence, are fast evolving and species-specific rapid changes in AMP gene copy number are found in several insect gene families including Lepidoptera (Waterhouse *et al.*, 2007; Yang *et al.*, 2011), making homology-based AMP identification challenging. I identified 23 AMPs in our data set, 10 of which were significantly upregulated in the serosa upon septic injury. I also identified three significantly upregulated previously unannotated AMPs, however their function and structure will have to be further investigated and confirmed, especially as relevant contigs were partial. In addition to AMPs I identified other upregulated effector genes upon septic injury; e.g. *haemolymph proteinase* genes, *lysozyme-E*, *HP16* and the putative defence protein *Hdd-11*.

5.4.4 Wound healing

I detected no differential gene expression of the canonical genes involved in wound healing and wounding response such as the MAPK-JNK pathway (Lee and Miura, 2014). However, both sterile and septic pricked eggs showed melanisation and wound healing at the injury site around the time of RNA-extraction (Fig. 5.6). This is contrary to what has been found by Gorman *et al.* (2004), where only a fraction of the septic pricked eggs and none of the sterile pricked eggs displayed melanisation at the injury site. The reason there was no differential expression of the canonical melanisation and wound healing genes might be the result of the time between wounding and RNA-extraction. A wound healing response needs to be fast and efficient and consequently this reaction might have happened and abated in the 18 hours

between wounding and RNA-extraction (Galko and Krasnow, 2004; Lee and Miura, 2014). The serosa did show upregulation of other genes after sterile injury, such as a number of reactionary immune-genes and *cactus* (Toll pathway). The upregulation of the Toll pathway by sterile injury has been found before (Chen and Nuñez, 2010) and in *Drosophila* the Toll pathway is required in epidermal wound repair (Carvalho *et al.*, 2014). Other genes that were upregulated after wounding were enzyme-like proteins, such as cytochromes, esterases, dehydrogenases, proteases and transferases. Glucose dehydrogenase [FAD, quinone]-like has been associated with cuticle formation in *D. melanogaster*, which suggests that it might play a role in restoring the serosal cuticle after wounding (Cox-Foster *et al.*, 1990). Cytochromes are heme containing enzymes known for their oxidizing capabilities; cytochromes P450 specifically are known to be involved in detoxification and pesticide resistance (Li *et al.*, 2006; Liu *et al.*, 2018). This detoxifying function is also known for esterases which are a group of hydrolysing enzymes (Li *et al.*, 2006). The function of these enzymes and other enzymes after wounding in the serosa requires further investigation. Three transcripts that were annotated as *ejaculatory bulb-specific protein (3)-like (Ebp III)* were only differentially expressed in the serosa after sterile injury but not after septic injury and are both up and down-regulated. Ebp III is an OS-D-like protein, also known as chemosensory (CSP) or sensory appendage proteins (SAP). Some of OS-D genes like pherokine-2 and 3 are related to virus response (Sabatier *et al.*, 2003). While other are, like the names suggests, related to the olfactory system or pheromone binding proteins (Pelosi *et al.*, 2006). The function of these proteins in the serosa and their reaction to wounding needs to be looked into more, especially as septic wounding did not upregulate these genes. The same is the case for the other two genes that were only expressed upon sterile injury, *cytochrome 5b-like* and *F-actin-capping protein subunit alpha*.

5.4.5 *In-situ* hybridisations show immune gene expression localised to the serosa

Both *hemolin* and the AMP *attacin-A* were clearly expressed by the serosa but only after septic injury, as shown by *in-situ* hybridisation (*cf. T. castaneum* for *attacin* (Jacobs *et al.*, 2014a)). Both *cactus* and *relish* were found expressed in the serosa as well as the embryo. The Toll pathway plays an important role during development thus the embryonic expression of pathway genes is expected, and is probably not immune related (Belvin *et al.*, 1995; Benton *et al.*, 2016). Additionally, you would expect expression of genes in these pathways at low levels at all times as the pathways need to be in place to sense unexpected microbial invasion. This was shown by the expression of *relish* in the serosa of the control eggs.

5.4.6 Conclusion

Butterfly eggs can mount a full-range innate immune response involving AMPs and melanisation upon wounding. A reduction in hatching upon septic injury compared to sterile injury after serosa formation showed that an immune response might come with a fitness cost. The egg-stage immune response at our chosen time point in development depended entirely on the extra-embryonic serosa.

Chapter 6- Discussion

6.1 Butterflies as an eco-evo-devo model system

The extinction rate of insect biodiversity, including the abundance of individual species (Hallmann *et al.*, 2017) appears to be increasing alarmingly (Biesmeijer *et al.*, 2006; De Vos *et al.*, 2014). This decline has potentially severe ramifications, both ecologically and economically (Hallmann *et al.*, 2014; Potts *et al.*, 2016; Vanbergen *et al.*, 2014; Yang and Gratton, 2014). The underlying causes are a continued source of debate and research. Significant causative factors appear to be anthropogenic disturbance in general, and land-use change (including urbanisation), climate change and the (large-scale) introduction of toxins into the environment (e.g. breakdown products, industrial waste-material but also the use of pesticides) in particular (Ewald *et al.*, 2015; Merckx *et al.*, 2018; Potts *et al.*, 2010; Scherber *et al.*, 2013; Winfree *et al.*, 2009). Furthermore, how such factors interact with each other is often complex, with region- and species/population-specific responses (Potts *et al.*, 2010; Winfree *et al.*, 2009). Particular taxonomic groups have been singled out as "environmental health" indicators, given that even small harmful and stressful levels of such factors can relatively easy be detected by monitoring their population dynamics and species richness (Whitworth *et al.,* 2018; Dennis *et al.,* 2017b; New, 1997; Chapter 3). One such taxon sensitive to environmental change is the order of Lepidoptera, which consists of moths and butterflies. Although moths are increasingly popular for assessing environmental health and change (Fox, 2012; Fuentes-Montemayor *et al.*, 2011), butterflies are possibly better investigated in this respect having been the subject of a long-term UK monitoring scheme as well as extensive ecological research programmes (Brereton *et al.*, 2017; Dennis and Whiteley, 1992; New, 1997; Thomas, 2005). Butterfly abundance and species diversity have been in decline as a result of aforementioned factors (Bonebrake *et al.*, 2016; Chen *et al.*, 2011; Gallou *et al.*, 2017; Gilburn *et al.*, 2015; Malcolm, 2018; Stenoien *et al.*, 2018; Wilson *et al.*, 2007).

Currently, most studies on the impact of environmental change on butterflies are largely confined to population dynamics, species trends, and more theoretical studies on the adaptive capabilities in general of butterfly populations (Gilburn *et al.*, 2015; Hanski and Thomas, 1994; McDermott Long *et al.*, 2016; Oliver *et al.*, 2015). However, to assess the full impact of environmental change, both biotic and abiotic factors, natural as well as anthropogenic we need to go beyond studying demography and population dynamics alone. We should incorporate a detailed analysis of the traits that respond to and appear to be involved in adaption to environmental variation (and stress) (Campbell *et al.*, 2017). The study of these traits should include investigating their development, functioning, and the significance of both regulation (including epigenetics) and sequence variability of relevant genes therein (Campbell *et al.*, 2017). Shifts in environmental conditions can directly affect gene expression patterns and thus development (i.e. developmental plasticity with the environment as causative agent of phenotypic variation) and this would likely precede any demographic or evolutionary changes (with the environment as selective agent) (Campbell *et al.*, 2017; Crozier and Hutchings, 2014; Gilbert and Epel, 2009).

Environmental change affects all life-stages, though in different ways. In the embryonic stage, development is likely to be predominantly affected, whilst in the larval stage the effects could be developmental, physiological and metabolic. In the adult stage the effects will be mainly physiological and metabolic, and can ultimately impact life-history strategies (see Fig. 1.1). In a life-cycle such as this, the parental environment and the resulting reproductive investment decisions a parent makes can also contribute to the environment experienced by the offspring, affecting their development; both in the embryonic and also larval stages (see e.g. Woestmann and Saastamoinen (2016) for an in-depth review). Environmental stress can thus become incorporated into the life-cycle through transgenerational effects (Gluckman, 2004; Vaiserman *et al.*, 2017). For butterflies, examples include differential resource investment in eggs based on the suitability of the environment such as host plant quality

(Braby, 1994; Jaumann and Snell-Rood, 2017; Wiklund, 1984), and when forced to fly long distances (Gibbs *et al.*, 2010a). Furthermore, females are able to direct early development of their offspring by incorporating, mRNA and proteins of specific (maternal effect) genes into the egg (Carter *et al.*, 2013, 2015; Ferguson *et al.*, 2014; Gibbs and Breuker, in prep), the exact contents varying in response to environmental factors (Gibbs and Breuker, in prep). Transgenerational effects such as these are largely instructive; the development of the offspring is parentally directed. On top of that, mothers can ensure that their offspring are protected from potentially non-adaptive changes to their development. The egg stage is subjected to a range of biotic and abiotic factors, some of which may be harmful (Fig. 1.1). To protect the embryo from undue direct environmental influences, a number of protective layers, both maternal and embryonic in origin, surround the embryo (Panfilio, 2008; Chapter 1; Chapter 2). Some of these layers such as the chorion might offer passive protection while others such as the extra-embryonic serosa offer a more active protection against environmental perturbations (Jacobs *et al.*, 2013, 2014a; Jacobs and van der Zee, 2013). Although the serosa is deployed by a number of insect orders for protection, what makes the serosa in butterflies unique is that it is maternally specified (Ferguson *et al.*, 2014), and remains in place throughout the duration of embryonic development (Chapter 2). This makes the extra-embryonic serosa an interesting trait to investigate in relation to the effects of environmental stress upon eggs.

Despite there being a number of studies on the effects of environmental stress on the larval and adult stage in the butterflies (e.g. in terms of growth and life-history variation) (Fischer *et al.*, 2010; Franke *et al.*, 2014), not much is known about how the environment affects embryonic development, either directly or through transgenerational effects. In my thesis I have used two model species to investigate direct environmental effects on early butterfly development (Chapters 4 and 5), as well as transgenerational effects (Chapter 4); the

emerging eco-evo-devo model *Pararge aegeria* (Schmidt-Ott and Lynch, 2016) and the established model species *Bicyclus anynana* (Brakefield *et al.*, 2009b).

In terms of responses to environmental change, *P. aegeria* is a very suitable model species, as it displays a relatively rapid (although population-specific) rate of recovery after drought episodes (Oliver *et al.*, 2015 and references therein). It has northwards range-expansion, tracking climate change (Hill *et al.*, 1999; Pateman *et al.*, 2016; Tison *et al.*, 2014), while other species may not be able to do the same, such as the closest relative of *P. aegeria* in the UK; the Wall Brown (*Lasiommata megera*) (Palmer *et al.*, 2015; Van Dyck *et al.*, 2014; Weingartner *et al.*, 2006). The phylogeography and patterns of gene flow in this species have been characterised at great depth (Habel *et al.*, 2013; Livraghi *et al.*, 2018b; Tison *et al.*, 2014; Weingartner *et al.*, 2006). *Bicyclus anynana* is a well-established model organism for the research on seasonal polyphenism (i.e. developmental plasticity) and evolution thereof in wing morphology and patterning, size, behaviour and reproductive strategies (Brakefield and Larsen, 1984; Brakefield *et al.*, 2009b; Fischer *et al.*, 2010; Geister *et al.*, 2008a; Geister *et al.*, 2008b). Additionally, *B. anynana* is a grass-feeding Satyrid, as is *P. aegeria*, which lends them to cross-species comparisons (Peña *et al.*, 2006; Wahlberg *et al.*, 2009). The genome of both of these species has been sequenced, the one of *B. anynana* to a great depth (Ferguson *et al.*, 2014; Nowell *et al.*, 2017), and genomic resources for both are available on LepBase (Challis *et al.*, 2016). In order to study gene expression patterns (e.g. in response to environmental stress, or generally in embryonic and wing development) a variety of techniques has been optimised for these species. These include transcriptomics and custom bioinformatic pipelines (Chapter 5 and Carter *et al.*, 2016), *In situ* hybridisation and most recently CRISPR/Cas9 (Beldade and Peralta, 2017; Carter *et al.*, 2013, 2015; Ferguson *et al.*, 2014; Livraghi *et al.*, 2018b; Mazo-Vargas *et al.*, 2017; Zhang *et al.*, 2017). Furthermore, in-depth morphological staging of embryogenesis (i.e. both embryo and its serosa) of both species is necessary to facilitate phenotyping the effects of the environment and the role of specific genes therein, during

(early) embryogenesis. In Chapter 2, Idescribed the 22 embryonic developmental stages in full for external morphology for *P. aegeri*a. In line with findings from other butterfly species, these data confirmed that *P. aegeria* is an intermediate germ-band insect displaying significant long-germ characteristics such as *B. anynana* (Holzem *et al.,* in prep). *Pararge aegeria* was also found to display a fault-type embryonic membrane formation and a serosa with mononucleated cells.

In Chapter two, I hypothesised that embryonic time points before 14h AEL (i.e. before 10% DT) may be more vulnerable to environmental stressors, because serosa formation does not take place until 10-12h AEL, followed by cuticle deposition, and therefore embryos are not afforded protection from this tissue prior to this times point. Support for the hypothesis that embryos before 14h AEL may be more vulnerable to environmental stressors was found in Chapter 3; embryos exposed to the endocrine disruptor Pyriproxyfen at 6h AEL had lower survival to hatching than embryos that were exposed later in development (Ascher and Eliyahu, 1988; Chapter 4). However, in Chapter 5 I found that the presence of the serosa did not necessarily improve survival to hatching in embryos that had been wounded and/or exposed to bacteria during development. It is only after serosa formation, that septic injured embryos developed faster than sterile injured embryos, suggesting age-specific differences in response to wounding and/or exposure to bacteria in this species (Chapter 5). This is an intriguing result which warrants further investigation in future studies. Although the serosa remains in place and appears consistent in its morphology until just before hatching, it has been suggested that it displays functional differences across development, and thus may respond differently to different environmental stressors depending on the developmental stage (Lamer and Dorn, 2001).

Holzem *et al.,* (in prep) characterised an embryonic time-series for *B. anynana. Bicyclus anynana* development is faster than that of *P. aegeria*, but early embryogenesis of

both species is very similar, in particular with regards to segment specification, differentiation and axis elongation, which may have been conserved across Ditrysia (Aymone *et al.*, 2014; Eastham, 1927; Eastham, 1931; Kobayashi, 2003). Although the initial area covered by the presumptive serosa in early embryogenesis is bigger in *P. aegeria* than in *B. anynana,* eventually, in both species, the serosa comes to extend across the whole the egg and thus cover the embryo.

6.2 Environmental stress

When it comes to studying the effects of environmental stressors, a large number of studies concentrate on temperature stress (Fischer *et al.*, 2003a; Fischer *et al.*, 2003b; Geister *et al.*, 2008b). However, recent research has highlighted that butterflies increasingly come into contact with various other stressors such as pesticides (in particular insecticides). Other environmental stressors, like temperature extremes, may potentiate the effects of pesticide exposure. As reviewed in Chapter 3, surprisingly little research has been carried out to investigate these effects in butterflies. In particular, we do not know the field- realistic doses of pesticides nor do we know how or when in the life cycle (the egg, larva or adult) butterflies are exposed. Ultimately, we do not know how pesticides directly affect butterfly development, physiology, metabolism, and indirectly population dynamics and demography. A firm understanding of the direct effects is necessary, as we need to understand the mechanisms by which butterflies can protect themselves (and thus the genes involved in this protection). Furthermore, we need to investigate how other environmental stressors potentiate the effects of pesticides in butterflies. When effects are sub-lethal, life-history traits may be affected (Dhadialla *et al.*, 1998; Herman and Tatar, 2001; Steigenga *et al.*, 2006), and via transgenerational effects development may also be affected in subsequent generations. This was shown in Chapter 4 for *P. aegeria* using two endocrine disruptors mimicking juvenile hormone (JH) and 20-hydroxyecdysone (20E). Importantly, I found that maternal exposure to 20E generated long-lasting transgenerational effects on forewing shape (but not size) in adult

offspring. Changes in wing shape have the potential to impact offspring fitness via its effects on flight-dependent life history traits such as mate location, territorial defence and oviposition behaviour (Chapter 4).

Comparable research in *B. anynana* has shown that the severity of the transgenerational effects for the JH-analog Pyriproxyfen was dependent on maternal age (Steigenga *et al.*, 2006). When wet-season females were exposed just before or during oviposition they had increased daily fecundity and egg-laying rate, but reduced longevity (Steigenga *et al.*, 2006). By contrast, daily fecundity of *P. aegeria* was lower upon Pyriproxyfen exposure, although longevity was also reduced in this species. Differences in response across species may be due to timing differences and in general a heterochrony in egg maturation, which needs further investigation (Chapter 4). In both species, exposure to Pyriproxyfen resulted in females laying smaller sized eggs, suggesting a potential for transgenerational maternal effects to affect subsequent offspring development (Steigenga *et al.*, 2006). These transgenerational effects were further investigated for *P. aegeria* in Chapter 4. Eggs from females exposed to the same JH-analog, Pyriproxyfen, had poor survival, and larvae hatching from such eggs had a very low probability of reaching the adult stage (3%). The prospects for egg survival and subsequent development were better when mothers were exposed to 20E, but as mentioned previously, subtle effects on the offspring development were detected in the form of wing shape variability when the offspring reach the adult stage (Chapter 4). These results demonstrate that transgenerational effects are not confined to early development but can be exerted across each stage of the life-cycle of the subsequent generation. In this case the endocrine disruptors managed to escape the protection mechanisms of the embryo, bypassing them by affecting oogenesis (i.e. how the eggs were made and which components were included). Therefore, I also tested the eggs themselves, by exposing these directly to Pyriproxyfen, which is much more readily absorbed through tissues than 20E (Chapter 4).

The direct effects of Pyriproxyfen on *P. aegeria* eggs were much less severe than the indirect (i.e. maternal) effects, and high doses were needed to exert an effect (Chapter 4). This might in part be because of the protective function of the serosa especially, as Orth *et al.* (2003) showed that the serosa expressed JH- binding proteins which might be able to bind the Pyriproxyfen before they reach the embryo. This defensive function of the serosa against pesticides needs further investigation, including the significance and efficiency of the serosa and the JH-binding proteins, in protecting the embryo against maternally incorporated excess JH (analogs). For example, if the serosa and the JH-binding proteins can protect the embryo against such transgenerational effects, then the endocrine disruptors must have affected the quality of the eggs per se explaining the poor survival of such embryos, and thus that serosal protection comes too late. Furthermore, life-history effects, other than embryonic survival and development time, were not examined. Potentially, by examining variation in post-hatching life history traits I may have uncovered further effects of direct exposure to pesticides, but this hypothesis remains to be tested.

In Chapter 5, I examined immune response during embryonic development of *B. anynana* and investigated whether the extra-embryonic serosa played a role in immune and wounding response. I confirmed that in butterflies, just like in *Tribolium castaneum,* the serosa plays an important role in the protection of the embryo against both wounding and pathogens (Jacobs *et al.*, 2013; Jacobs *et al.*, 2014a). The serosa in butterflies is divergent from that of other insects as it stays in place until hatching (Chapter 2) instead of disappearing during the time of dorsal closure as observed for example in *T. castaneum* (Panfilio *et al.*, 2013; van der Zee *et al.*, 2005). So far, the (immune) function of the serosa in *P. aegeria* has only been assessed at one embryonic time point. Further investigation of the function of the serosa at other, especially later time stages, of development is needed. This is of special interest as innate immune response has been shown to have life-cycle stage and age-specific

characteristics, including immunosenescence (Eleftherianos and Castillo, 2012; Min and Tatar, 2018).

Key pathways underlying the immune response in insects, such as the Toll, IMD, Mapk-Jnk-P38 and JAK-STAT pathways (Kleino and Silverman, 2014; Myllymäki *et al.*, 2014; Tauszig *et al.*, 2000; Valanne *et al.*, 2011; Yokoi *et al.*, 2012), are conserved in *B. anynana* and were expressed in the serosa upon septic injury (Chapter 5). Septic injury resulted in upregulation of *hemolin.* The gene *hemolin* (formerly named P4), a gene from the immunoglobin superfamily, is of particular interest when studying the innate immune response in Lepidoptera as it appears to be unique to this insect order. Furthermore, its expression pattern in response to bacterial infections and viral infections has been studied in detail in moths (Casanova-Torres and Goodrich-Blair, 2013; Eleftherianos *et al.*, 2007; Ladendorff and Kanost, 1990; Terenius, 2008; Yu *et al.*, 2002; Yu and Kanost, 2002), as well as in butterflies (Baudach *et al.*, 2018). *Hemolin* is highly derived, and appears to diverge between Lepidopteran clades. The significance of this variation across species in combatting viral and bacterial challenges requires more detailed attention, not least of all because it appears unique to Lepidoptera. A recent study on the butterfly *Pieris napi* (Keehnen et al., 2018) investigated the micro-evolution of innate response genes and showed that such genes show increased genetic diversity, and at times display population-specific dynamics; but unfortunately they did not investigate *hemolin* in this context. Studies on larvae of the moth *M. sexta* have shown that careful *hemolin* regulation is important in a response to one of the bacteria I have used in Chapter 5, *Micrococcus luteus* (Ladendorff and Kanost, 1991). Not only does *hemolin* display sequence variability between species but its expression pattern, both baseline and after a bacterial challenge, may also differ between species and within a species between life cycle stages and even seasonal forms. In the map butterfly *Araschnia levana*, *hemolin* is only upregulated within the first three hours after a bacterial infection in short-day larvae, but not long-day larvae (Baudach *et al.*, 2018). Similar to *A. levana* larvae, *attacin* and *gloverin* were

upregulated many hours after the bacterial challenge (12-24 hours for the larvae, and 18 hours in the serosa). Unlike *A. levana* larvae, where *hemolin* levels have returned to normal 3 hours after a bacterial challenge, I found that *hemolin* levels were still very high in the serosa some 18 hours after septic injury (Chapter 5), indicating a possibly sustained role for *hemolin*. Furthermore, many (innate) immune genes, and possibly *hemolin*, appear to be expressed during oogenesis in butterflies, as was shown for *P. aegeria* (Carter *et al.*, 2013). Their transcripts were found to be incorporated into an egg (Carter *et al.*, 2013), possibly to equip the embryo before there is a serosa in place. Rather interestingly, the identity of maternally expressed immune genes shows only some overlap with those found differentially expressed in the serosa in Chapter 5. The significance of this requires further study. There is of course a possibility that proteins of maternally expressed immune defence genes may also be incorporated into eggs, but this has thus far not been verified (Carter *et al.*, 2013). Maternal transcripts disappear around the time the serosa develops (Ferguson *et al.* (2014); Chapter 2), and thus any maternal protection against bacteria and/or viruses gets supplanted by the serosa, possibly using a somewhat different repertoire of genes.

Insect innate immune pathways are not only involved in immune defence, and appear curiously pleiotropic. An iconic example in this respect is the Toll pathway, which plays a key role in insect embryonic dorsal-ventral patterning (Lynch and Roth, 2011) and is also involved in anterior posterior axis elongation (Benton *et al.*, 2016). In butterflies the immune and wound healing pathways have been hypothesised to be co-opted in wing-patterning (Özsu and Monteiro, 2017). The expression of these genes during (embryonic) development in response to an immune challenge might therefore have additional (fitness) effects as well as affecting life-history traits (Ardia *et al.*, 2012; Freitak *et al.*, 2003).

A significant challenge for future studies on the (genetic) mechanism underpinning responses to environmental change in butterflies will be to study exactly how stressors

potentiate each other, thus constituting complex selection pressures, and which specific genes get up- and downregulated as a result. Particular attention should be paid to disentangling the contributions made by selection in different life-stages on 1) gene expression patterns (i.e. regulatory regions such as enhancers) *per se*, not least because many of the genes involved are highly pleiotropic, 2) sequence variability (i.e. specific polymorphisms), and 3) lepidopteran-specific paralogs (see also Chapter 3 for a discussion on genes involved in pesticide resistance). In order to fully appreciate evolvability of butterflies when confronted with environmental changes, these are topics that need to be urgently addressed.

6.3 Future directions

The data in this thesis have made a significant contribution to elucidate how embryos respond to environmental stress, and some of the genes involved, but ultimately raised many more questions than it answered. Importantly, the full extent of serosal functioning during butterfly development needs more in-depth investigation, especially when challenged by the environment. The serosa, both its formation and role during development is divergent in butterflies compared to other insect species (Ferguson *et al.*, 2014; Panfilio, 2008). The full extent of the function of the *Shx* genes in the serosa formation and functioning remains unknown. In particular how they interact with each other, and whether they could contribute to serosal diversity between species given their rapid evolution (Ferguson *et al.*, 2014; Livraghi, 2017). Their function should be looked into through gene knock-out experiments, using CRISPR/Cas9 designed to investigate the role of each of the four *Shx* genes individually, and in combination, across a developmental time-series of both the embryo and serosa (Livraghi, 2017). Additionally, if CRISPR/cas9 could result in developing serosa-less butterfly embryo the function of the serosa in protection against various environmental stressors could be further investigated (*cf. T. castaneum,* Jacobs *et al.*, 2013, 2014a; Jacobs and van der Zee, 2013). The preference for CRISPR/Cas9 over RNAi in butterflies is related to the limited efficacy of RNAi in butterflies, although tried exhaustively RNAi has never worked in *P. aegeria.*

At present we still lack data on other structures that could protect the embryo; the extra-embryonic amnion and the cuticle(s) secreted by the serosa (see also Chapter 2). We lack data of the developmental periods butterfly serosal cuticles may be formed and broken down, although some data exists for *M. sexta* (Lamer and Dorn, 2001). In *M. sexta* the cuticle formation starts immediately after serosa formation, after it is fully formed the serosa cuticle detaches from the serosa and stays intact until the end of embryogenesis (Lamer and Dorn, 2001). A serosal cuticle is of particular interest as differences in structure and developmental timing of this cuticle could be involved in the varying levels of drought resistance observed in butterfly eggs (Farnesi *et al.*, 2015; Jacobs *et al.*, 2015; Klockmann and Fischer, 2017; Rezende *et al.*, 2008; Vargas *et al.*, 2014).

It has been shown that genes are differentially expressed in the serosa following a direct immune challenge (Jacobs *et al.*, 2014a; Chapter 5), but it is not known whether the serosa would also respond in this way to other stressors, and what the effect of a combination of stressors would be. Given the results of Chapters 3 and 4, a logical class of stressors to test (including and in combination with bacterial infections) would be insecticides, in particular the endocrine disruptors (Orth *et al.*, 2003).

Given the maternal contribution to serosa formation, as well as the maternal contribution in general in directing the development of their offspring (this thesis and; Gibbs *et al.*, 2010a; Gibbs *et al.,* 2010c; Woestmann and Saastamoinen 2016), a challenge is to take the stressors a mother experiences into account as well. We know that maternal egg provisioning varies depending on the prevailing environmental conditions at oviposition (Gibbs *et al.*, 2010a), and that female *P. aegeria* deposits maternal RNAs (i.e. maternal effect genes) in the egg (Carter *et al.*, 2013). What at present is insufficiently known is the identity of the relevant maternal effect genes related to any given environmental stressor, and how these transcripts

change gene expression after the transition from maternal effect gene transcription to zygotic expression.

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