Factors associated to juvenile hormone signalling during embryonic and postembryonic development in the cockroach *Blattella germanica*

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A Xavier

"The biology of juvenile hormone is… one of the more elaborate results of the evolution of control and response mechanisms"

H. Frederik Nijhout

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Abstract

In the present thesis, we have studied the metamorphosis of the cockroach *Blattella germanica*, focusing on two main periods of development, the embryonic and the postembryonic. In the embryonic development, we have studied the role of the juvenile hormone (JH), analysing the genes Methoprene-Tolerant (*Met*), Taiman (*Tai*), Krüppel homolog 1 (*Kr-h1*), and JH acid methyltransferase (*JHAMT*). We have also studied the transcription factor E93, which has a key role in adult morphogenesis, but that has never been studied in the embryo. Results have shown that both, JH and *E93* play important roles during embryo development, especially in early stages. Regarding the postembryonic period, we studied the CREB binding protein (*CBP*) Nejire and the Transforming Growth Factor β (TGF-β) signalling pathway. Results have shown that both play relevant roles in the transition from the last nymphal stage to the adult.

Resumen

En la presente tesis hemos estudiado la metamorfosis de la cucaracha *Blattella germanica*, centrándonos en dos períodos principales de desarrollo, el embrionario y el postembrionario. En el desarrollo embrionario, hemos estudiado el papel de la hormona juvenil (JH), analizando los genes Methoprene-Tolerant (*Met*), Taiman (*Tai*), Krüppel homolog 1 (*Kr-h1*) y JH acid methyltransferase (*JHAMT*). También hemos estudiado el factor de transcripción E93, que tiene un papel clave en la morfogénesis adulta, pero que nunca se ha estudiado en el embrión. Los resultados han demostrado que tanto la JH como el factor *E93* desempeñan papeles importantes durante el desarrollo del embrión, especialmente en etapas tempranas. En cuanto al período postembrionario, estudiamos la proteína de unión a CREB (*CBP*) Nejire y la vía de señalización Transforming Growth Factor β (TGF-β). Los resultados han demostrado que ambos desempeñan papeles relevantes en la transición entre la última fase ninfal y el adulto.

Preface

During the last 20 years, at least, one of the main aims of the Xavier Bellés Laboratory has been the study of the mechanisms underlying the origin and evolution of metamorphosis in insects. Working in his group in the context of the present thesis, our objectives focused in adding new information on the regulation of process related to metamorphosis, using the German cockroach, *Blattella germanica*, as insect model. *B. germanica* shows a gradual metamorphosis, the so called hemimetabolan, which is the ancestral mode and from which derives the more modified complete metamorphosis (holometabolan). Therefore, a comprehensive knowledge of the hemimetabolan mode is strictly necessary to undertake the comparisons with the holometabolan (which has been more thoroughly studied, for example in the species *Drosophila melanogaster*) and then infer the mechanisms of the evolutionary transition from one to the other.

We have been working on two main periods of the cockroach development, the embryogenesis period and the transition from nymph to adult, in the postembryonic period. The transition from a nymph to an adult, by which adult morphogenesis proceeds and wings and genitalia are formed, is the transformation traditionally studied in metamorphosis studies. We have contributed to shed light to the mechanisms regulating adult morphogenesis that operate in the last instar nymph, by unveiling the role of a particular CREB-binding protein and the Transforming Growth Factor β (TGF-β) signalling pathway.

Embryogenesis has been little explored in the context of studies on metamorphosis. And this is a mistake, since in hemimetabolan insects the embryogenesis produces a nymph with the basic characteristics of the adult body structure, whereas embryogenesis in holometabolan species produces a larva, which is morphologically very divergent with respect to the adult. Indeed, the adult structure in holometabolans will only be achieved in postembryonic development, with the formation of the pupa and the subsequent adult. Therefore, the comparative study of embryogenesis in both metamorphosis models will undoubtedly produce essential information on the evolution from hemimetaboy to holometaboly. Therefore, we have studied the role of the juvenile hormone and the transcription factor *E93* in the embryo, since both factors play a key role in postembryonic morphogenesis.

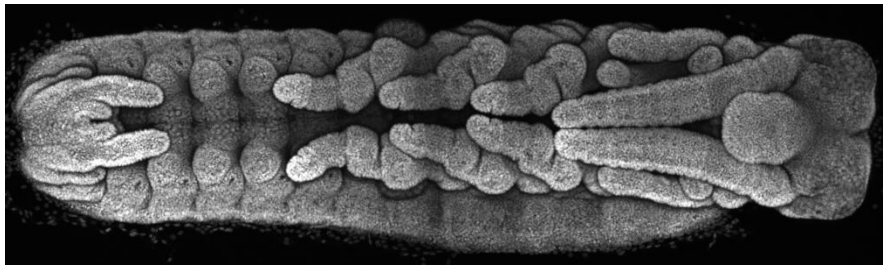
Abbreviations

20E	20-Hydroxyecdysone
bHLH-PAS:	Basic helix-loop-helix- Per-Arnt-Sim
Bi:	Bifid, also known as optomotor blind (omb).
BMP:	Bone morphogenetic protein.
BR-C:	Broad-Complex.
CBP:	CREB-binding protein.
CA:	Corpora allata.
cDNA:	Complementary DNA.
CREB:	cAMP response element-binding protein
DNA:	Deoxyribonucleic acid.
dsRNA:	Double-stranded RNA.
dsMock:	Double-stranded RNA used as control.
E:	Ecdysone.
E75:	Ecdysone-induced protein 75.
EcR:	Ecdyone receptor.
ED0:	Embryos on day 0.
ED6:	Embryo on day 6.
ED9:	Embryo on day 9.
ED13:	Embryo on day 13.
Eh:	Eclosion hormone.
HAT:	Histone acetyltransferase.
Hb:	Hunchback.
HR3A:	Nuclear hormone receptor 3, isoform A
JH:	Juvenile hormone.
JHAMT:	Juvenile hormone acid *O*-methyltransferase.

Kr:	Krüppel.
Kr-h1:	Krüppel homolog 1.
Ltl:	Larval translucida.
Mya:	Million years ago.
Mad:	Mothers against dpp.
Med:	Medea.
MF:	Methyl farnesoate.
MEKRE93:	Methoprene tolerant-Krüppel homolog 1-E93 pathway.
Met:	Methoprene-tolerant.
miRNAs:	microRNAs.
MTZ:	Maternal-to-zygotic transition.
NFE:	Non fertilized eggs.
Otd:	Orthodenticle.
PCNA:	Proliferating cell nuclear antigen.
RNAi:	RNA interference.
Sal:	Spalt
Sax:	Saxophone.
Smox:	Smad on X.
Sk:	Sulfakinin.
TALENs:	Targeted mutagenesis mediated by transcription activator-like effector nucleases.
TF:	Transcription factor.
TGF-β:	Transforming growth factor beta.
Tk:	Tachykinin.
Tkv:	Thick veins.
vg:	Vestigial.

Contents

1. INTRODUCTION



1. INTRODUCTION

The oldest know fossil insects are from the Early Devonian (~412 million years ago, Mya), although it is probable that insects originated in the Late Silurian with the earliest terrestrial ecosystems (Martin and Hempfling, 1976). Insects were among the first animals that colonized terrestrial ecosystems from the marine milieu. They shaped Earth's biota, exhibiting coevolved relationships with many groups, from flowering plants to humans. Insects were the first to invent flight and establish social societies. However, many aspects of insect evolution are still poorly understood (Trautwein et al., 2012), and one of them is metamorphosis.

Metamorphosis is one of the most widely used life-history strategy of animals. In insects, the ancestral forms developed in a direct way. Then, the innovation of metamorphosis fuelled their extraordinary radiation. In the most extreme case of metamorphosis the differences between the larva and the adult are dramatic, in terms of morphology and behaviour. This allows these stages to exploit different habitats and food sources, and acquired new adaptations. This explains, in part, the evolutionary success experienced by insects during evolution.

1.1. Different modes of metamorphosis in insects

There are three major types of metamorphosis. (1) The most primitive is ametaboly, or direct development, showed by

primitively wingless insect groups. In this mode, juvenile and adult stages essentially differ by the increasing size and the formation of reproductive structures in the adult. (2) Another mode is hemimetaboly, in which the juvenile stages already have the essential adult body structure, and the metamorphic transformation between juvenile stages or nymphs and the adult, primarily involves the formation of complete genitalia and functional wings. (3) The third mode is holometaboly, which is the most derived, characterized by the fact that juvenile stages or larvae are dramatically different from the adults. Thus, the transition from larvae to adult requires the formation of the pupa, which represents an intermediate stage (Yang, 2001) (Figure 1.1).

Much of insect diversity is represented by holometabolan insects, which comprise eleven insect orders (Yang, 2001) and include about three quarters of all described insects (Moczek, 2010). Holometabola include the insect orders Lepidoptera (moths and butterflies), Diptera (mosquitoes and flies), Hymenoptera (wasp, bees and ants), Coleoptera (beetles), Strepsiptera (twisted-wing parasites), Raphidioptera (snake flies), Megaloptera (alderflies, dobsonflies and fishflies), Neuroptera (lacewings, mantidflies and antlions), Mecoptera (scorpionflies), Siphonaptera (fleas) and Trichoptera (caddisflies).

The remaining one-quarter of insects on Earth are hemimetabolan, including the orders Odonata (dragonflies and damselflies), Ephemeroptera (mayflies), Orthoptera (grasshoppers and crickets), Mantodea (praying mantises), Blattodea (cockroaches), Isoptera (termites), Dermaptera (earwings),

Phasmatodea (stick insects), Phthiraptera (sucking lice), Plecoptera (stoneflies), Grylloblattodea (ice insects), Psocodea (bark and true lices), Thysanoptera (thrips) and Hemiptera (scale insects, aphids, cicadas, leafhoppers, true bugs and milkweed bug).

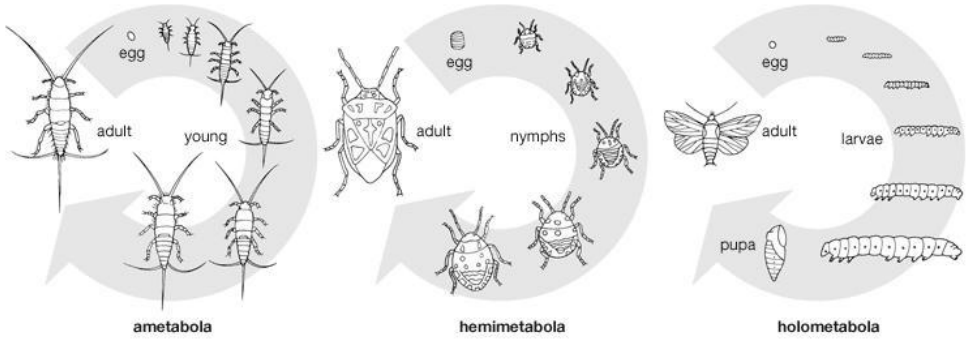


Figure 1.1: Types of insect development. Ametabolan, insects without metamorphosis, hemimetabolan or gradual metamorphosis and holometabolan or complete metamorphosis. Modified from Encyclopaedia Britannica, Inc. (2008).

The ametabolous insects are represented by the orders Protura (coneheads), Collembola (springtails), Diplura (two-pronged bristletails), Archaeognatha (jumping bristletails) and Zygentoma (silverfishes).

The earliest true insects included some of the ametabolous orders that are still alive today, the bristletails and silverfishes. Their juvenile stages look very much like the adult, except that they lack functional genitalia. Insects with incomplete metamorphosis (the hemimetabolan) are a polyphyletic assemblage that includes cockroaches, grasshoppers, dragonflies and true bugs. Their immature stages are usually called nymphs. Besides lacking

genitalia, the nymphs show wing buds more or less conspicuous, which will be finally transformed into articulated, functional wings during the moult to the adult stage (Kukalova-Peck, 1978). The oldest holometabolan insect fossil are from the Permian, and all present component holometabolan orders cluster together in a monophyletic group (Kristensen, 1975), the Holometabola. They have very different larval, pupal and adult stages and have achieved notably rapid life cycles.

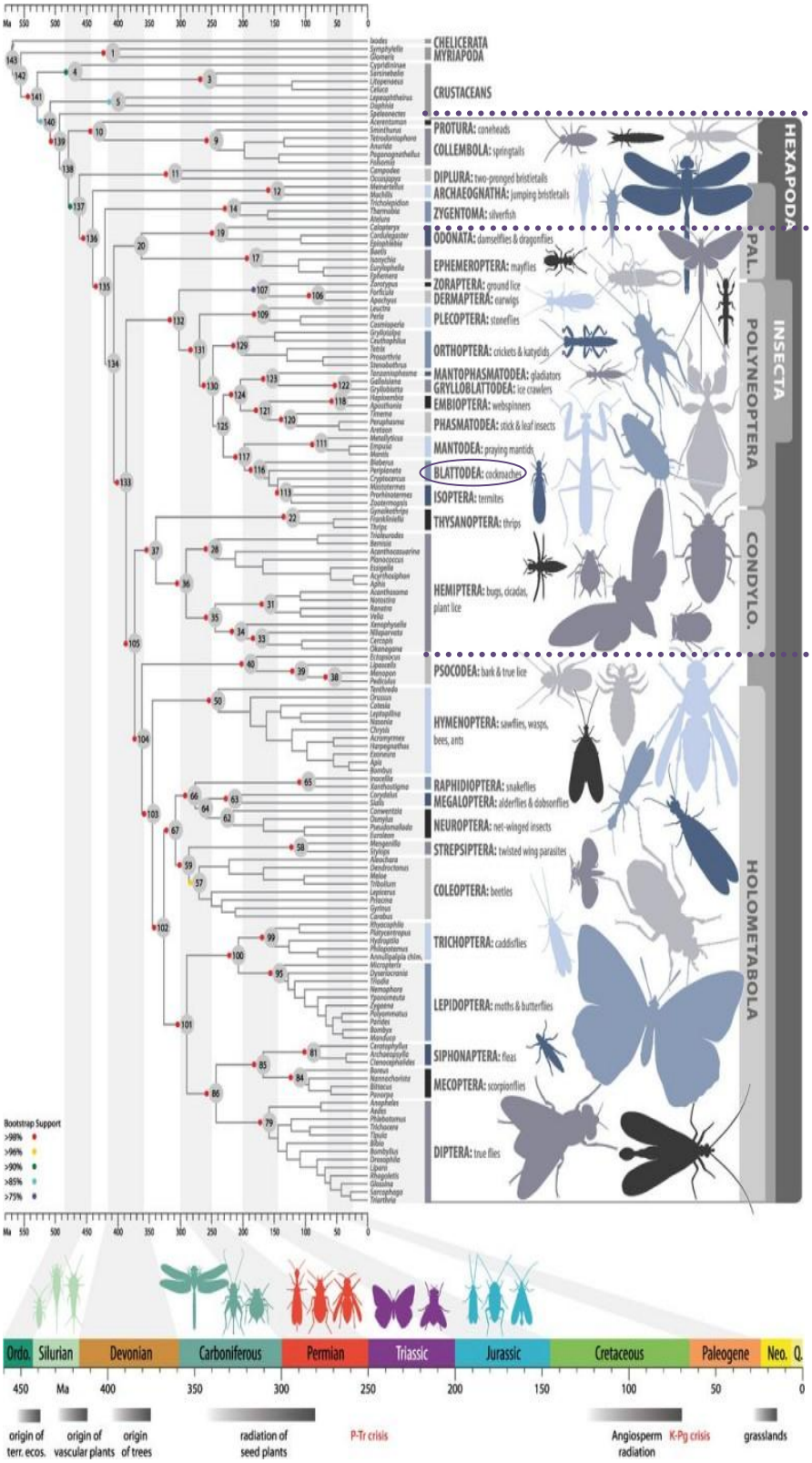
The Holometabola evolved from hemimetabolan ancestors during the Permian, 300 Mya (Labandeira and Phillips, 1996). Their hemimetabolan sister group would be the "Eumetabola", including Hemiptera and Thysanoptera (Figure 1.2).

1.2. Regulation of insect metamorphosis

Insect metamorphosis is essentially regulated by two hormones, 20-hydroxyecdysone (20E) and juvenile hormone (JH). 20E triggers the successive moults through the life cycle, whereas the main function of JH in morphogenesis is to repress metamorphosis (Nijhout, 1999), which has been qualified as an action for keeping the "status quo" in the juvenile form of the insect life cycle (Riddiford, 1996; Truman and Riddiford, 2007; Zhou and Riddiford, 2002). The 20E signalling pathway has been thoroughly studied, firstly in *Drosophila melanogaster* and then in other species, and the transcription factors involved in the transduction of the hormonal signal have been elucidated, most of them belonging to the nuclear receptor family (King-Jones and Thummel, 2005).

The JH signalling pathway has been elucidated in more recent years. The transcription factor Methoprene tolerant (*Met*) (Wilson and Fabian, 1986) has been recently shown to be the JH receptor in hemimetabolan and holometabolan insects (Jindra et al., 2015). JH bound to *Met* recruits the co-receptor Taiman, forming the complex that transduces the antimetamorphic signal of JH (Jindra et al., 2015). This complex activates the expression of the transcription factor Krüppel homolog 1 (*Kr-h1*), which is the master repressor of metamorphosis in hemimetabolan and holometabolan species (Konopova et al., 2011; Lozano et al., 2014; Lozano and Belles, 2011; Minakuchi et al., 2008).

The report describing the role of *E93* as trigger of adult metamorphosis in *Blattella germanica, Tribolium castaneum* and *D. melanogaster* (Ureña et al., 2014) indicates that this function of *E93* is conserved at least from cockroaches to flies. It was soon discovered that *Kr-h1* represses *E93* expression (Belles and Santos, 2014). Therefore, the basic pathway *Met* - *Kr-h1* - *E93* (known as "MEKRE93 pathway") was established as central to the status quo action of JH, transducing the hormonal signal that switches adult morphogenesis off and on. Importantly, this pathway is conserved across the Hexapoda, from cockroaches (basal hemimetabolan) to beetles (basal holometabolan) and flies (distal holometabolan), thus possibly predating the emergence of winged insects and the innovation of metamorphosis, more than 350 Mya (Martin and Hempfling, 1976).


CHELICERATA
MYRIAPODA
CRUSTACEANS
PROTURA: coneheads
COLLEMBOLA: springtails
DIPLURA: two-pronged bristletails
ARCHAEOGNATHA: jumping bristletails
ZYGENTOMA: silverfish
ODONATA: damselflies & dragonflies
EPHEMEROPTERA: mayflies
ZORAPTERA: ground lice
DERMAPTERA: earwigs
PLECOPTERA: stoneflies
ORTHOPTERA: crickets & katydids
MANTOPHASMATODEA: gladiators
GRYLLOBLATTODEA: ice crawlers
EMBIOPTERA: webspinners
PHASMATODEA: stick & leaf insects
MANTODEA: praying mantids
BLATTODEA: cockroaches
ISOPTERA: termites
THYSANOPTERA: thrips
HEMIPTERA: bugs, cicadas, plant lice
PSOCODEA: bark & true lice
HYMENOPTERA: sawflies, wasps, bees, ants
RAPHIDIOPTERA: snakeflies
MEGALOPTERA: alderflies & dobsonflies
NEUROPTERA: net-winged insects
STREPSIPTERA: twisted wing parasites
COLEOPTERA: beetles
TRICHOPTERA: caddisflies
LEPIDOPTERA: moths & butterflies
SIPHONAPTERA: fleas
MECOPTERA: scorpionflies
DIPTERA: true flies
HEXAPODA
PAL.
INSECTA
POLYNEOPTERA
CONDYLO.
HOLOMETABOLA
Ametabola
Hemimetabola
Holometabola
Bootstrap Support
>98%
>96%
>90%
>85%
>75%
Ordo.
Silurian
Devonian
Carboniferous
Permian
Triassic
Jurassic
Cretaceous
Paleogene
Neo.
Q.
450
Ma
400
350
300
250
200
150
100
50
0
origin of terr. ecos.
origin of vascular plants
origin of trees
radiation of seed plants
P-Tr crisis
Angiosperm radiation
K-Pg crisis
grasslands

Additionally, the MEKRE93 pathway, together with classical morphological and physiological criteria, appears to provide an important clue for establishing homologies between insect life-cycle stages.

1.3. *Blattella germanica* as a model insect

Fossil evidence indicates that cockroaches have been on Earth for over 300 million years (Grimaldi and Engel, 2005). They can be considered as one of the most successful hemimetabolan groups of insects, because their opportunistic ecology. Moreover, they have ancestral features, like the hemimetabolan mode of metamorphosis, which make them suitable as a baseline to study the evolution from hemimetaboly to holometaboly. Another interesting advantage of cockroaches as a model insect is that they are very sensitive to RNAi (Bellés, 2010).

The particular species *B. germanica*, the German cockroach, has been the model used in our experiments and observations. The size of adult is around 1.5-2 cm in length and show a general colouration light brown. Although they have fully developed

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Figure 1.2: Dated phylogenetic tree of insect relationships. All nodes up to orders are labelled with numbers (grey circles). Coloured circles indicate bootstrap support (left key). The time line at the bottom of the tree relates the geological origin of insect clades to major geological and biological events. CONDYLO, Condylognatha; PAL, Palaeoptera. Modified from Misof et al. (2014).

membranous metathoracic wings, they do not use to fly. Nymphs are similar in appearance to adults excepted that they are smaller and lack wings. The German cockroach is easily recognizable by its small size and by two dark parallel lines running longitudinally along the prothorax (Figure 1.3).

The German cockroach has three morphologically differentiated stages: egg, nymph and adult. Females produce a light brown, purse-shaped egg capsule called ootheca that is about 1 cm long and can contain around 30-40 eggs packaged in two rows, except at the both ends. The eggs within the ootheca are thus tightly packed together, laterally compressed. Each egg measures about 3 mm long, 1 mm wide and 0.3 mm thick (Tanaka, 1976). Adult females usually produce from four to eight oothecae during their lifetime. Oothecae are carried, adhered to the genital atrium, until hatching time. The period of transport, and embryogenesis, lasts around 18 days at 29ºC. Postembryonic development, comprising six nymphal stages, lasts between 22 to 24 days at the same temperature (Cornwell, 1968).

1.4. Postembryonic development and metamorphosis

B. germanica has become a significant model in insect development, especially with regards to endocrine regulation of metamorphosis. Firstly, the identification and pattern of production of the main hormones involved in metamorphosis, like JH (Maestro et al., 2010; Treiblmayr et al., 2006) have been established. Then, the transcription factors involved in 20E signalling have been thoroughly studied (Cruz et al., 2008, 2007, 2006; Maestro et al., 2005; Mané-Padrós et al., 2012, 2010, 2008). The peculiar transcription factor Broad-Complex (*BR-C*) has been also structurally and functionally studied in the embryo (Piulachs et al., 2010) and in postembryonic development (Huang et al., 2013).

Finally, the JH signalling pathway and the MEKRE93 pathway have been intensively studied in recent years (Belles and Santos, 2014; Lozano et al., 2014; Lozano and Belles, 2014, 2011; Ureña et al., 2014). With these antecedents, we have used *B. germanica* as model for our studies.

Figure 1.3: *Blattella germanica* adults. In the picture are shown the female and male, in ventral (left) and dorsal (right) view.

1.4.1. The tergal glands as a minimal model of metamorphosis

As commented above, in general, the hemimetabolan nymphs are morphologically very similar to the adult. The most remarkable differences between nymphs and adults are the wings and genitalia present in the later stage. However, adult male cockroaches have a very distinct structure called tergal glands, which produce substances attractive to the female and facilitates mating.

In our laboratory, a transcriptome analysis on male nymph tergites 7 and 8 (T7-8) (the epidermal tissue where the adult tergal gland will be formed) comparing with non-metamorphic (male nymph tergites 4 and 5) stages was carried out two years ago (Ylla and Belles, 2015) (Figure 1.4). The authors mapped all reads into a reference transcriptome in order to perform the analysis of differentially expressed genes between metamorphic and non-metamorphic stages, and a GO-enrichment test. A total of 5622 contigs appeared to be overrepresented in the transcriptome of metamorphosing specimens. Among these genes, there were six GO-terms with a p-value lower than 0.05 and among them GO: 0003676 ("nucleic acid binding") was especially interesting.

Among the differentially expressed factors, CREB binding protein (*CBP*) (=Nejire) and Medea, appeared especially interesting because, as reported in *D. melanogaster*, they interact with many other factors, thus constituting important hubs in regulatory networks involving different signalling pathways. Therefore, we

studied both factors in *B. germanica*, especially from a functional point of view, in the context of the present thesis.

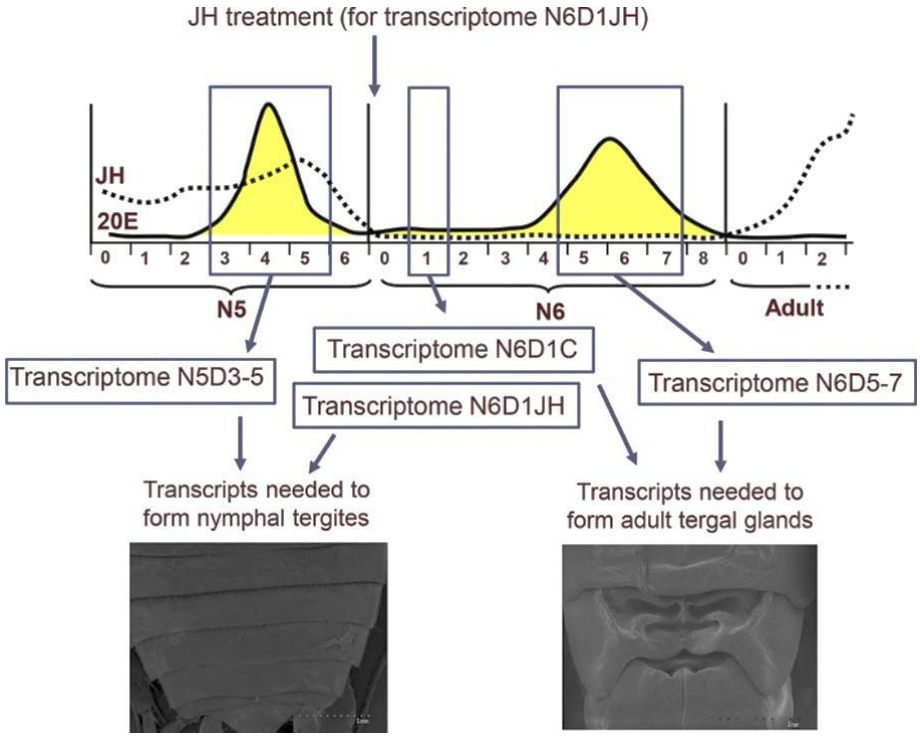


Figure 1.4: Hormonal context of the T7-T8 transcriptomes of *Blattella germanica*. The transcriptomes were prepared with RNA extracts of male tergites 7 and 8 fifth (N5) or sixth (N6) nymphal instar, on day 1 (D1) or during the production of the peak of 20-hydroxyecdysone (20E). N5D3-5 transcriptome correspond to high levels of juvenile hormone (JH), and the subsequent moult would result in new juvenile, flat tergites. N6D5-7 transcriptomes correspond to virtual absence of JH, and the subsequent moult result in adult tergites with the tergal gland formed. N6D1C corresponds to the stage where the adult program is being determined, when JH has just decreased. N6D1JH corresponds to the same stage, but using specimens that were treated with JH on N6D0, thus aborting the adult program (Ylla and Belles, 2015).

1.4.2. CREB binding protein (*CBP*)

CREB-binding protein (*CBP*) is an extremely promiscuous transcriptional coregulator, which, in addition to other properties, possesses histone acetyltransferase (HAT) activity (McManus and Hendzel, 2001). In insects, the functions of *CBP* have only been studied in *D. melanogaster*, which have a single homolog of *CBP*, known as *dCBP* or Nejire. As occurs in CBP/p300 knockout mice, flies mutant for *CBP* have highly pleiotropic phenotypes, indicating that it is required for multiple developmental processes (Akimaru et al., 1997a, 1997b). Both oogenesis and embryogenesis require *CBP* function, and RNAi depletion of *CBP* expression in *Drosophila* kc cells leads to cell death (Smolik and Jones, 2007).

The first roles of *CBP* reported in the embryogenesis of *D. melanogaster* were in relation to the regulation of the anterior/posterior polarity of embryonic segments through Hedgehog and Wingless signalling (Akimaru et al., 1997a; Goodman and Smolik, 2000). However, *CBP* is more fundamentally involved in dorsal–ventral patterning, especially through the regulation of the TGF-ß signalling pathway (Heldin and Moustakas, 2012; Waltzer and Bienz, 1999).

Available data on *CBP* roles in postembryonic development is scarce and focused on the HAT activity and the ecdysone signalling pathway in *D. melanogaster*. A study on prepupae of this species showed that *CBP* is essential for acetylating histone H3 at lysine 23 of early genes of the 20E signalling pathway, which is required for the proper activation of them (Bodai et al., 2012a). A

second study carried out in *Drosophila* S2 cells revealed that *CBP* is crucial for acetylating the histone H3 at lysine 27 of Sox14, which is a 20E-induced gene crucial for regulating dendrite development during metamorphosis (Kirilly et al., 2011b).

We focused in examining the roles of *CBP* in the post-embryonic development of *B. germanica*, especially in relation to metamorphosis and their endocrine regulation. Essentially, the approach followed was depleting *CBP* mRNA levels by RNAi in last nymphal stages, and examine the phenotype after the metamorphic moult.

1.4.3. Medea

Medea also emerged from the differential expression analyses of non-metamorphic and metamorphic tergal gland tissues of *B. germanica* (Ylla and Belles, 2015) . Medea is a Smad transcription factor common to the two branches of the Transforming growth factor-β (TGF-β) signalling pathway: the BMP branch and the TGF-β/Activin branch. *Mad* is the canonical Smad of the BMP branch, *Smox* is the canonical Smad of the TGF-β/Activin branch, whereas Medea participates in both branches. The TGF-β signalling pathway is fundamental for controlling development programs and cell behaviour in animals (Herpin et al., 2004).

The TGF-β ligand proteins bind to Ser/Thr kinase receptors and generally transduce the signal through the above Smad transcription factors (Massagué, 2008; Moustakas and Heldin, 2009). There are two Set/Thr kinase receptor types, type I and type

II, which typically form a tetramer receptor complex comprising two type I and two type II receptors. Type I transmit information from outside to inside the cell by binding extracellular ligands and phosphorylating intracellular transcription factors. Type II receptors are constitutively active kinases that phosphorylate and activate type I when forming a complex with them through ligand binding.

Receptor-activated (R-) Smad proteins are the most typical transcription factors phosphorylated by type I receptors. Upon receptor-induced phosphorylation, R-Smads typically form complex with a common-mediator (Co-) Smad, and the complex is translocated to the nucleus where, in cooperation with other transcription factors, contributes to regulating (co-activating or co-repressing) the expression of target genes (Heldin and Moustakas, 2012). These Smad complexes in turn induce the expression of the inhibitory (I-) Smads that negatively regulate the strength and duration of the signal (Itoh and ten Dijke, 2007).

In insects, the most thoroughly studied species has been *D. melanogaste*r, where the TGF-β/Activin branch operates through the type I receptor babo and signals through a single R-Smad (*Smox*) and a single Co-Smad (Medea), whereas the BMP branch has two type I receptors: thick veins (*tkv*) and saxophone (*sax*), although it also operates through a single R-Smad (*Mad*) and Medea. A single I-Smad (*Dad*, ortholog of Smad 6/7) acts in both branches of the TGF-β signalling pathway in this fly (Peterson and O'Connor, 2014).

The importance of the TGF-β signalling pathways in *D. melanogaster* has been comprehensively shown in embryogenesis

(Peterson and O'Connor, 2014). However, reports on postembryonic development are very few. In the BMP branch, the data refers to a handful of downstream targets that participate in wing formation, like spalt (*sal*), optomotor blind (*omb*), vestigial (*vg*) and larval translucida (*ltl*) (de Celis et al., 1996; Grimm and Pflugfelder, 1996; Kim et al., 1996; Szuperák et al., 2011). The TGF-β/Activin branch has been involved in wing disc patterning (Peterson et al., 2012; Peterson and O'Connor, 2014) and neuronal remodelling (Zheng et al., 2003), as well as the regulation of the metamorphic moult timing by influencing ecdysone production (Brummel et al., 1999; Gibbens et al., 2011).

In the context of our PhD thesis work, we aimed at examining the roles of the TGF-β signalling pathway in the postembryonic development of *B. germanica*, especially in relation to metamorphosis and their endocrine regulation. Using RNAi approaches, we studied the role of Medea, but also of *Mad* and *Smox*, in order to cover the two branches of the TGF-β pathway with the specific Smads of each one.

1.5. The interest of the embryonic development in the research of insect metamorphosis

Embryogenesis in hemimetabolan species gives rise to a creature with an essentially adult body structure. Conversely, in holometabolan species embryogenesis gives rise to an animal with a body structure considerably different from that of the adult, usually taking a vermiform shape. Therefore, the comparative study of

embryogenesis in hemimetabolan and holometabolan species should illuminate how was produced the transition from one metamorphosis mode to the other. The question would be how the embryogenesis of holometabolans diverged from that of hemimetabolans to give a kind of worm instead of a miniature adult.

1.5.1. Two basic modes of insect embryogenesis

Generally speaking, there are two basic mode of embryogenesis in insects. One is represented by long germ-band embryogenesis, by which all segments are established and defined at the blastoderm stage prior to gastrulation. A representative example is *D. melanogaster*, which has been thoroughly studied in this sense. This is a derived mode of embryogenesis presented by a number of species of Holometabola. Insects of more basally branching lineages follow a different mode of embryogenesis, by which only the most anterior segments are specified before gastrulation, while the more posterior segments are generated and patterned progressively from a posterior region called the growth zone. This is called short or intermediate germ-band embryogenesis, depending on the number of segments established at the blastoderm stage (Davis and Patel, 2002).

Given that short and intermediate germ-band modes are widely represented across the insect orders, whereas the long germ-band mode is restricted to holometabolan insects, it is likely that a mode of short or intermediate germ-band segmentation is ancestral (Davis and Patel, 2002) (Figure 1.5).

These developmental differences however, do not ultimately result in different morphogenesis. The insect body of the emerging nymph or larva consists of a head of six or seven segments, a thorax of three segments, and an abdomen of eight to eleven segments, a structure that is essentially invariant across species (Rogers and Kaufman, 1996; Schmidt-Ott et al., 1994).

1.5.2. Hormones and embryonic development

Although the occurrence of the two most important hormones in metamorphosis, 20E and JH, have been detected in embryos of different species, little is known about their functions in embryo development. In the case of 20E, correlation of the peaks of hormonal production and deposition of successive cuticular layers in the embryo indicates that its function is to promote embryo "moults", equivalently to those of the postembryonic stage. In apterygote species, like the silverfish *Thermobia domestica*, two embryonic cuticles are deposited during embryogenesis.

The embryo of the pterygote species (for example, *B. germanica*) deposit three embryonic cuticles. The embryo of *D. melanogaster*, and flies in general, exceptionally deposit two embryonic cuticles because it secondarily lost the prolarval cuticle (Konopová and Zrzavý, 2005). In the case of *B. germanica*, the three cuticle depositions coincide with respective bursts of 20E production (Piulachs et al., 2010).

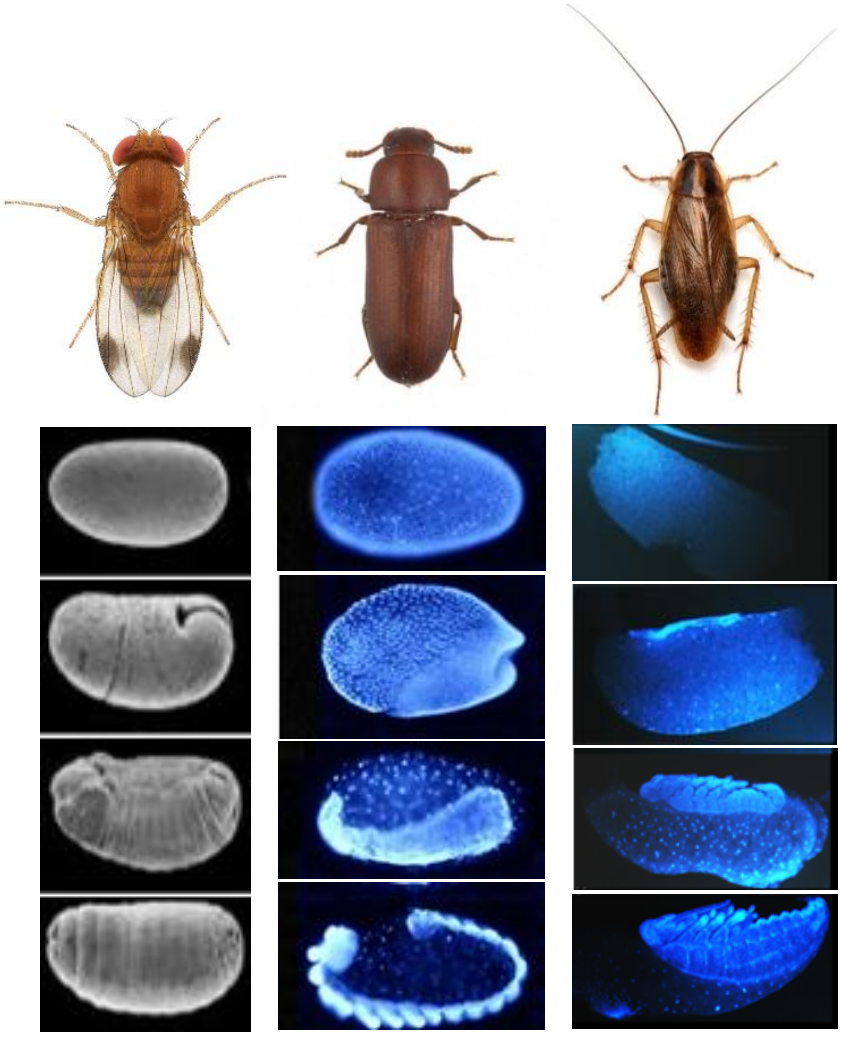


Figure 1.5: Early embryonic development in different insects. On the left *Drosophila melanogaster*, holometabolan species that shows long germ-band embryogenesis; in the middle, *Tribolium castaneum,* a short germ-band holometabolan species; on the right *Blattella germanica,* a hemimetabolan species showing short germ-band embryogenesis. Modified from Parkhurst lab and Diethard Tautz, University of Köln (1999).

Concerning JH, the situation is more enigmatic, as it has been suggested that embryonic JH could be a determining factor in hemimetabolan or holometabolan metamorphosis (Truman and Riddiford, 1999), whereas recent studies indicate that it only has rather accessory roles, if any, at least in holometabolan species (Daimon et al., 2015). In this context, it is worth remembering that the evolution of insect metamorphosis has been explained on the basis of a heterochrony: hemimetabolan species develop the basic adult body structure during embryogenesis, whereas holometabolan species delay the construction of the adult body structure until the pupal stage (Belles, 2011; Truman and Riddiford, 1999). Moreover, Truman and Riddiford proposed that embryonic JH might be an important factor in the evolutionary shift from hemimetaboly to holometaboly, after noticing that JH is produced earlier in holometabolan than in hemimetabolan embryogenesis (Truman and Riddiford, 2002, 1999). Thus, the conclusion inferred by these authors was that the premature production of JH might have precluded the formation of the adult body structure in holometabolan embryos. Nevertheless, eggs of the hemimetabolan species *Acheta domesticus* treated with JH during early embryogenesis resulted in a number of defects but did not impair formation of the adult body structure (Erezyilmaz et al., 2004).

To contribute to clarify the role of JH in insect embryogenesis, we used *B. germanica*, and the maternal RNAi technique to knock down key members of the JH signalling pathway. Robust information is available about *B. germanica* embryo development (Konopová and Zrzavý, 2005; Piulachs et al.,

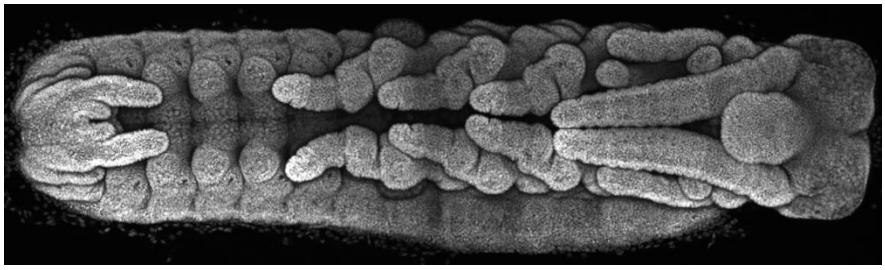
2010), including the classification of embryogenesis in 18 well defined stages (Tanaka, 1976), and about JH and 20E levels during embryogenesis (Belles and Maestro, 2005). In addition, maternal RNAi depletes embryo transcripts efficiently in this cockroach (Piulachs et al., 2010). As we will see in the Results section, we chose the following targets for interference, from downstream to upstream of the signalling axis (Jindra et al., 2015): *Kr-h1*, the main transducer of the JH signal during metamorphosis, *Met* (the JH receptor) and JH acid methyl transferase (*JHAMT*), the key enzyme that catalyses the last step of JH biosynthesis. The three genes had been structurally and functionally characterized in postembryonic stages of *B. germanica* in previous studies (Dominguez and Maestro, 2017; Lozano et al., 2014; Lozano and Belles, 2011).

1.5.3. Other factors involved in embryonic development

The transcription factor *E93* emerged as a crucial element in metamorphosis as trigger and specifier of adult morphogenesis (Belles and Santos, 2014; Ureña et al., 2014). However, nothing is known about its possible role in insect embryogenesis. Previous transcriptome analyses made in our laboratory indicated that *E93* appears as a significant maternal transcript in early embryos of *B. germanica*. Thus, the hypothesis emerging was that *E93* might have a key role in early embryogenesis, perhaps in the maternal to zygotic transition (MZT). We were also interested in ascertaining whether, in later embryo stages, when JH signalling becomes active,

the MEKRE93 pathway (Belles and Santos, 2014) operates in embryos as in postembryonic development.

2. OBJECTIVES

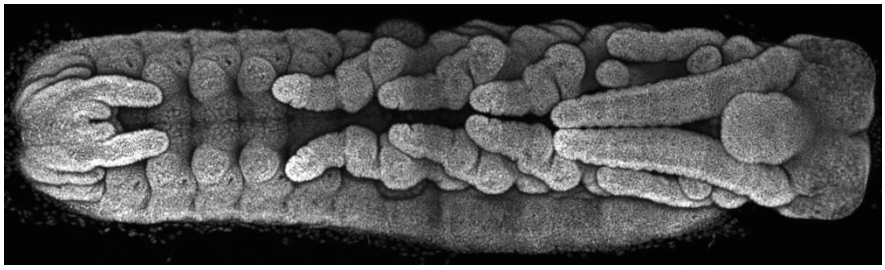


2. OBJECTIVES OF THE THESIS

The general objective of the present thesis was to contribute to the knowledge of the regulation of metamorphosis, using the German cockroach, *Blattella germanica*, as model. Part of the work focused in postembryonic development, in the transition from nymph to adult, and the specific objectives in this part were based on previous comparative transcriptomics data obtained in the laboratory. However, we realized that the study of embryogenesis is crucial to understand the evolution of metamorphosis. Therefore, we also studied the possible role in embryogenesis of hormones and transcription factors traditionally involved in postembryonic development. In these contexts, the specific objectives planned were the following.

1. To study the possible role of CREB binding protein (*CBP*) in the transition from nymph to adult.
2. To study the possible role of the Transforming growth factor-β (TGF-β) signalling pathway in the transition from nymph to adult.
3. To study the possible role of juvenile hormone signalling in the embryo, using as targets the transcription factors Krüppel homolog 1, Methoprene tolerant, and the enzyme Juvenile hormone acid *O*-methyl transferase.
4. To study the possible role of the transcription factor E93 in embryo development.

3. RESULTS



3.1. CREB-BINDING PROTEIN CONTRIBUTES TO THE REGULATION OF ENDOCRINE AND DEVELOPMENTAL PATHWAYS IN INSECT HEMIMETABOLAN PRE-METAMORPHOSIS

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3.2. SMADS AND INSECT HEMIMETABOLAN METAMORPHOSIS

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3.3. JUVENILE HORMONE SIGNALING IN SHORT GERM-BAND HEMIMETABOLAN EMBRYOS

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3.4. TRANSCRIPTION FACTOR E93 REGULATES MIR-309 AND ZELDA EXPRESSION IN EARLY EMBRYO DEVELOPMENT OF *BLATTELLA GERMANICA*

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3.4. TRANSCRIPTION FACTOR E93 REGULATES MIR-309 AND ZELDA EXPRESSION IN EARLY EMBRYO DEVELOPMENT OF *BLATTELLA GERMANICA*

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KEY WORDS: Juvenile hormone, Krüppel homolog 1, Methoprene tolerant, juvenile hormone acid *O*-methyltransferase, Broad complex, *Blattella*

ABSTRACT

Maternal RNAi of E93 in the short germ-band hemimetabolan species *Blattella germanica* effectively depleted the mRNA levels of this transcription factor in the embryo. In early embryo stages, depletion of E93 mRNA resulted in increased levels of Zelda and perturbed mRNA levels of early patterning genes and MIR-309 microRNAs. At phenotypical level, E93 depletion precluded the formation of the germ-band anlage in a group of embryos. In another group of embryos, development was interrupted in mid embryogenesis, involving defects related to dorsal closure and

formation of appendages. These phenotypes are possibly due to the low levels of juvenile hormone acid methyl transferase (JHAMT) and those of Broad-complex core (BR-C) resulting from E93-depleted conditions, as it has been previously shown that JHAMT and BR-C depletion in the embryo of *B. germanica* results in similar phenotypes. Hatchability was also reduced, which may have been due to premature upregulation of laccase 2, a promoter of cuticle tanning.

1. Introduction

E93 is a helix-turn-helix transcription factor containing a Pipsqueak motif, which was first reported to be involved in the regulation of programmed cell death in the prepupa of the fruit fly, *Drosophila melanogaster* (Baehrecke and Thummel, 1995; Lee et al., 2000). Subsequently, an E93 homolog called Mblk-1 was discovered in the large-type Kenyon cells of the mushroom bodies of brain of the honeybee, *Apis mellifera* (Takeuchi et al., 2001). This suggested that Mblk-1 (E93) could be involved in regulating the complex behaviours of the honeybee colony. A neat hint that E93 might be related to adult morphogenesis was afforded by Jafari et al.(2012) and Mou et al. (2012), who showed that it appeared to be a more general regulator of gene expression in *D. melanogaster* metamorphosis. More recently, Ureña et al. (2014) identified E93 when looking for genes controlling apoptosis in prothoracic glands and morphogenesis in wings of the cockroach species *Blattella germanica*, during metamorphosis. Ureña et al. (2014) showed that

E93 depletion by RNAi in preimaginal stages prevented the formation of the adult in the hemimetabolan *B. germanica* and in two holometabolan species, the red flour beetle *Tribolium castaneum* and *D. melanogaster*. In all three species, E93 is specifically expressed in the preimaginal stage, that is, in the last nymphal instar of *B. germanica* and in the prepupae and pupae of *T. castaneum* and *D. melanogaster* (Ureña et al., 2014). In *B. germanica*, E93 RNAi treatment carried out on penultimate (N5) or last (N6) instar male or female nymphs effectively depleted E93 mRNA levels in N6 and prevented metamorphosis to the adult stage. Instead, the specimens molted to a perfect supernumerary (N7) nymph (Belles and Santos, 2014; Ureña et al., 2014). Interestingly, Belles and Santos (2014) additionally showed that the transcription factor Krüppel homolog 1 (Kr-h1), which is the master transducer of the antimetamorphic signal of JH, represses the expression of E93. Thus, the essential pathway regulating metamorphosis would be constituted by JH interacting with their receptor, Methoprene tolerant (Met) that, after recruiting the co-receptor Taiman, would trigger the expression of Kr-h1 (the master repressor of adult morphogenesis), which in turn would repress the expression of E93 (the master promoter of adult morphogenesis). This regulatory axis, which has been called MEKRE93 pathway (Belles and Santos, 2014), regulates metamorphosis in hemimetabolan and holometabolan insects (Jindra et al., 2015).

We have recently studied the role of the JH signalling pathway in the embryo of *B. germanica* (Fernandez-Nicolas and Bellés, 2017) In particular, we studied the roles of JH acid methyl

transferase (JHAMT), which catalyses the last step of JH biosynthesis, Met, which is the JH receptor, and Kr-h1, the main transducer of the antimetamorphic signal of JH. We found that depletion of the expression of these three factors in the embryo triggered a number of defects in early, mid and late embryo development (Fernandez-Nicolas and Bellés, 2017). The purpose of the present work was to examine possible roles of E93 in the embryo of *B. germanica*, and to ascertain whether the MEKRE93 pathway operates in embryogenesis as it does in postembryonic metamorphosis. We followed the same approach of maternal RNAi used in previous studies (Fernandez-Nicolas and Bellés, 2017; Piulachs et al., 2010) and we found that E93 depletion resulted in defects in the formation of the germ band, which lead us to study a number of factors operating in early embryo development. Thus, we studied the expression of Proliferating Cell Nuclear Antigen (PCNA), a protein necessary for cell cycle progression and cellular proliferation (Kelman, 1997), which has been associated to nuclear division cycles in early insect embryogenesis (Yamaguchi et al., 1991). We additionally studied Zelda (*zld*) (also called Vielfaltig), a zinc-finger transcription factor and epigenetic effector that plays an essential role in the activation of transcription from the earliest zygotic genes, during the maternal-to-zygotic transition (MZT) (Liang et al., 2008), by binding specific sites in DNA and increasing chromatin accessibility (Schulz et al., 2015; Sun et al., 2015).

The MZT is a key step in early embryo development (Giraldez, 2010; Schier, 2007), during which most of maternal RNAs are degraded while zygotic genes are newly expressed

(Tadros and Lipshitz, 2009). Previously, Nien et al. (2011) reported that *zld* plays a key role during the MZT in *D. melanogaster*, collectively activating batteries of genes involved in early developmental processes, such as sex determination, cellularization and axis patterning (Nien et al., 2011). Interestingly, zld also activates the expression of the MIR-309 cluster of eight microRNAs (miRNAs) in *D. melanogaster* (Liang et al., 2008), which are involved in clearing many maternally loaded mRNAs (Bushati and Cohen, 2007). Given these antecedents, we also examined the expression of the MIR-309 miRNAs in *B. germanica*.

We also examined the expression of Hunchback (*hb*), Krüppel (*Kr*), Orthodenticle 1 (*otx1*), Caudal (*cad*), Even-skipped (*eve*), Nanos (*nos*), Odd-skipped (*odd*) and Piwi in E93-depleted embryos. These genes are involved in early embryo patterning in short (*T. castaneum*) and long germ-band insects (*D. melanogaster*, *Nasonia vitripennis*) (Lynch et al., 2012). Finally, as some of the E93-depleted embryos completed development but showed an intensely sclerotized cuticle, we examined the expression of laccase 2, a phenoloxidase involved in cuticle tanning (Arakane et al., 2005; Elias-Neto et al., 2010; Sugumaran and Barek, 2016).

2. Results

2.1. E93 has a peak of expression at the beginning of *B. germanica* embryogenesis

The expression pattern of E93 was studied by qRT-PCR during different days of embryogenesis. Expression levels oscillate between 0.5 and 12 mRNA copies per 1000 copies of Actin-5c mRNA (in ED16 and ED0 stages, respectively) (Figure 3.4.1A). The highest expression levels were observed in ED1. Then, the levels decrease and keep at relatively low values during all embryogenesis, including the period of JH production.

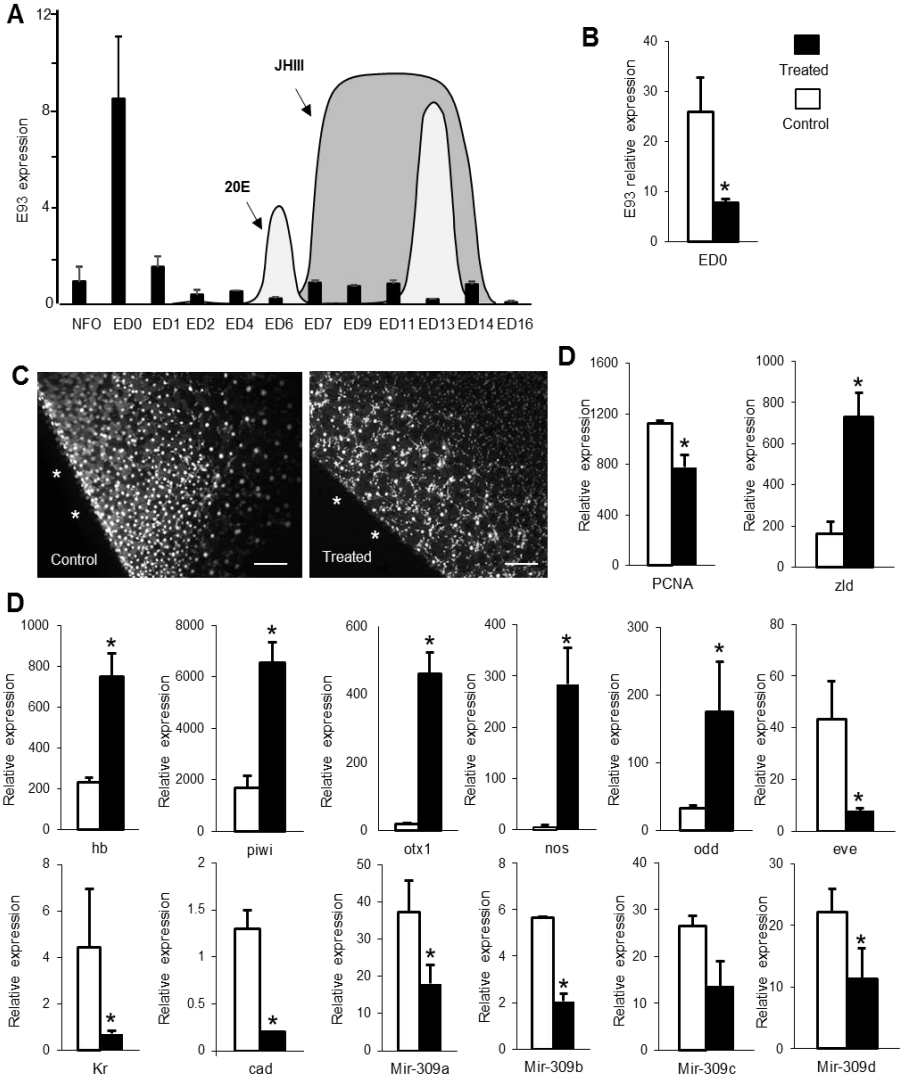
2.2. Effects of E93 depletion on ootheca fate

The maternal RNAi experiments consisted in injecting a single 3-µg dose of dsRNA targeting E93 (dsE93) into the abdomen of 5-days-old adult females. Controls received the same dose of unspecific dsRNA (dsMock). Control females (n=40) formed a normal ootheca on day 8 of adult life, which contained viable embryos that produced normal first instar nymphs 18 days later. Females treated with dsE93 (n= 30) also formed apparently normal oothecae on day 8, and 18 of them (50.0%) produced apparently well-formed first instar nymphs that eclosed normally on day 18, with no delay with respect to the controls. Nine out of the 30 oothecae formed (28.6%) were dropped between days 2 and 3 (12-17% embryogenesis), and six (21.4%) were transported beyond day 18 without hatching (they spontaneously dropped from the female between days 19 and 21). Measurement of E93 mRNA levels in 2-days-old embryos revealed that they were significantly lower (29.9%) in dsE93-treated specimens than in controls (Figure. 3.4.1B), which indicated that maternal RNAi was efficient.

2.3. Effects of E93 depletion on germ-band formation

The study of the embryos of the oothecae dropped between days 2 and 3 is interesting as this happened just after the occurrence of the highest levels of expression on day 1. In controls on ED2, we examined 350 embryos from 10 oothecae, and they were between Tanaka stages 1 and 2 (Tanaka, 1976), showing a high density of energids, which accumulated on the ventral side of the egg, where they cellularized and contributed to the formation of the germ-band anlage (Figure 3.4.1C). With regards to treated animals, we examined 235 embryos from 9 oothecae of dsE93-treated females that were spontaneously dropped on day 2. These embryos had interrupted development around the formation of the germ-band anlage, between stages 1 and 2, as defined by Tanaka (1976). Therefore, they were comparable in developmental terms with control embryos in ED2. The energids accumulated and cellularized in the germ-band anlage zone, although the accumulation was less dense and heterogeneous, forming discrete groups separated each other (Figure 3.4.1C), instead of showing the uniform distribution observed in controls. In ED2, PCNA expression in these E93-depleted embryos was lower than in the controls, whereas the expression levels of Zelda were dramatically higher (Figure 3.4.1D). We also examined a number of genes involved in the first patterning of the zygote, like *hb*, piwi, *otx1*, *nos* and *odd*, which were upregulated in E93-depleted embryos with respect to controls, as well as *eve*, *Kr* and *cad*, which were downregulated (Figure

3.4.1E). Moreover, the miRNAs of the MIR-309 family were downregulated with respect to controls (Figure 3.4.1F).



2.4. Effects of E93 depletion on mid-late embryogenesis and hatching

We studied the embryos from the oothecae that were transported beyond day 18 without hatching. Thus, on day 19 examined 278 embryos from 7 of these oothecae. A total of 71 out of the 278 embryos (25.53%) had interrupted development around the formation of the germ-band anlage, between Tanaka stages 1 and 2 (phenotype A, Figure 3.4.2A). Forty embryos (14.4%) were segmented and with appendages, but interrupted development between Tanaka stages 10 and 15, showing diverse malformations, such as imperfectly sealed dorsal closure, reduced abdomen or imperfect eyes (phenotype B, Figure 3.4.2B).

Figure 3.4.1. Expression of E93 during the embryogenesis of *Blattella germanica* and effects of maternal RNAi observed in 2-day-old embryos (ED2). (A) mRNA levels of E93 in non-fertilized eggs (NFE), and in embryos of different ages from day 0 (ED0) to day 16 (ED16); superimposed are the pattern of juvenile hormone III (JH III) and 20-hydroxyecdysone (20E). (B) Control and E93-depleted (treated) embryos showing the levels of E93 depletion. (C) Accumulation of energids in the ventral side of the embryo (white asterisks) in control and E93-depleted (treated) specimens; scale: 200 µm. (D) Expression of Proliferating cell nuclear antigen (*PCNA*) and Zelda (*zld*). (E) Expression of the genes Hunchback (*hb*), Piwi, Orthodenticle 1 (*otx1*), Nanos (*nos*), Odd-skipped (*odd*), expression of the pair-rules genes Even-skipped (*eve*), Krüppel (*Kr*), Caudal (*cad*) and MIR-309 mRNAs. Expression of measurement represents four biological replicates and results of qRT-PCR are expressed as copies of the examined transcript per 1000 copies of BgActin-5c mRNA. Data are represented as the mean ± SEM. The asterisk indicates statistically significant differences with respect to controls ($p < 0.05$), calculated on the basis of the REST (Pfaffl et al., 2002).

A total of 54 embryos (19.4%) were apparently well formed nymphs, but featured an intensely sclerotized cuticle (phenotype C, Figure 3.4.2C). Finally, 113 embryos (40.6%) were apparently well formed nymphs but did not hatch (phenotype D, Figure 3.4.2D).

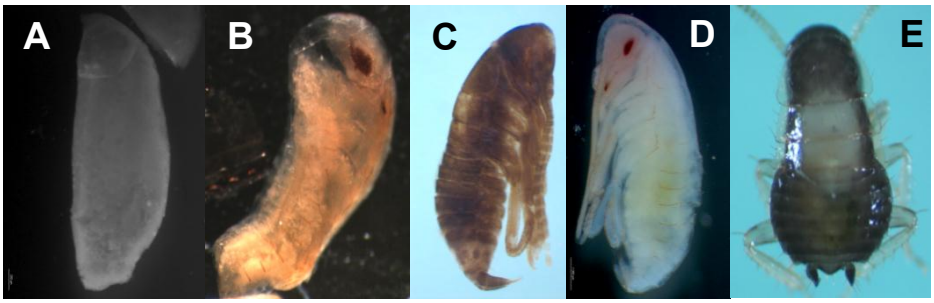


Figure 3.4.2. Embryo phenotypes resulting from maternal RNAi of E93 in *Blattella germanica*. (A) Phenotype A. (B) Phenotype B. (C) Phenotype C. (D) Phenotype D. (E) Normal freshly eclosed first instar nymph.

Phenotype D correspond to nymphs practically indistinguishable from control pre-hatched nymphs, showing, for example, the typical nymphal microsculpture and chaetotaxy, and sclerotized mandibles. Notably, if the chorion was artificially broken, then most phenotype D specimens behaved as naturally eclosed first instar nymphs, which apparently normal morphology and motility (Figure 3.4.2E). Phenotype A embryos resembled and appeared to correspond to those found in oothecae dropped between days 2 and 3, commented in the previous section. The phenotype B embryos were malformed, some of them with problems in eye development and others with problems in segmentation. The

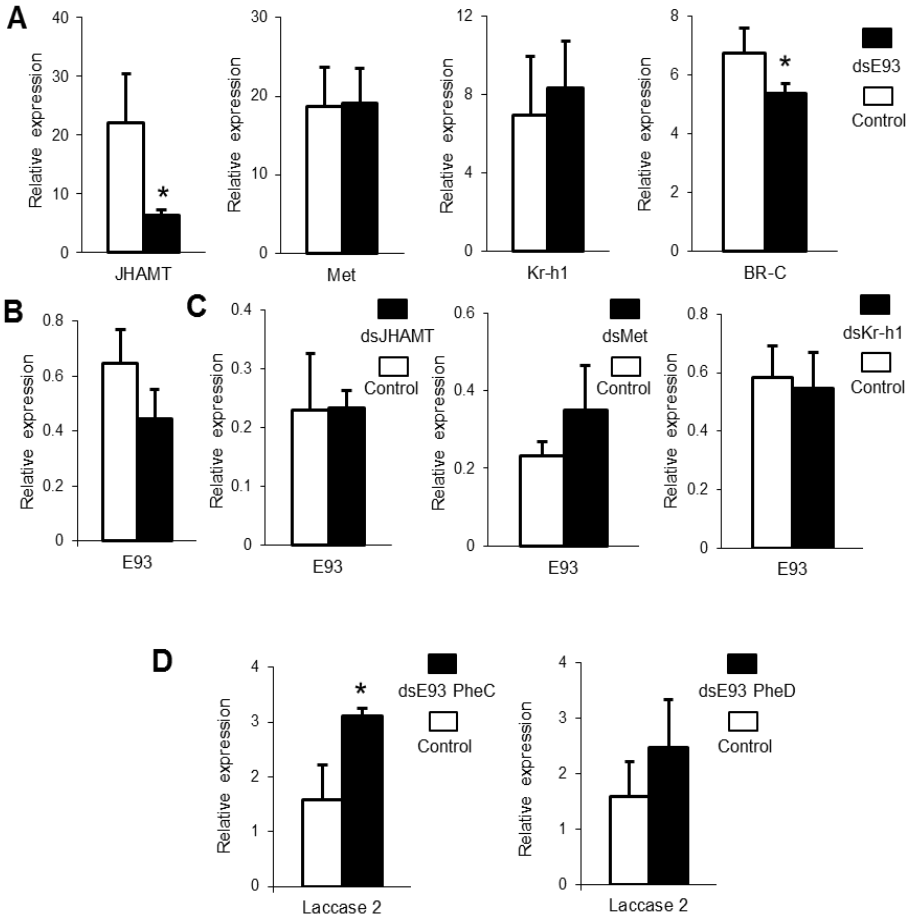
intensely sclerotized nymphs of phenotype C were suggestive of an effect on cuticle tanning.

Phenotypes B, C and D are reminiscent of those observed in embryos depleted for BR-C (Piulachs et al., 2010) and for factors involved in JH synthesis (JHAMT) and signalling (Met and Kr-h1) (Fernandez-Nicolas and Bellés, 2017). Therefore, we measured mRNA levels of BR-C, JHAMT, Met and Kr-h1 in ED6, at the onset of JH cycle of expression, when the second burst of ecdysteroids occur. Results showed that Met and Kr-h1 expression was unaffected, whereas that of JHAMT and BR-C was downregulated (Figure 3.4.3A). Transcript decrease in ED6 (Figure 3.4.3B) was 31.5%, thus at similar levels to that measured in ED2 (Figure 3.4.1B), although at this age differences between dsE93-treated specimens and controls were not statistically significant. We were also interested in studying whether depletion of JH signalling might affect E93 expression. Thus, in the samples of ED6 from JHAMT-, Met- and Kr-h1-depleted embryos previously obtained (Fernandez-Nicolas and Belles, 2017), we measured the mRNA levels of E93. Results indicated that none of these treatments affecting JH signalling significantly modified the expression of E93 (Figure 3.4.3C). In the experiments depleting JH factors (Fernandez-Nicolas and Bellés, 2017), we observed that the tanning factor laccase 2 was upregulated in hypersclerotized embryos (like in the present phenotype C) and in apparently normal embryos that did not hatch (like in the present phenotype D). Thus, we measured laccase 2 expression in embryos showing these two phenotypes.

Results (Figure 3.4 3D) showed that laccase 2 expression in these embryos was significantly higher than in controls.

3. Discussion

The significant amounts of E93 transcripts in ED0, as well as in NFE and ED1 suggest that they derive from a maternal origin and that they may have important functions in early development. This is consistent with the observation that a number of females treated



with dsRNA targeting E93 dropped the ootheca around 2 days after its formation, between Tanaka stages 1 and 2. The embryos of these oothecae showed less energids accumulating in the germ-band anlage, which is consistent with the observation that they expressed less *PCNA*, which is required for cell cycle progression and cell proliferation and has been associated with nuclear division cycles in early *D. melanogaster* embryogenesis (Yamaguchi et al., 1991). Thus, a role of E93 in very early embryogenesis would be to promote *PCNA* expression.

Figure 3.4.3. Effects of maternal RNAi of E93 in embryos of *Blattella germanica***.** (A) mRNA levels of broad complex core (*BR-C*), Juvenile hormone acid methyl transferase (*JHAMT*), Methoprene tolerant (*Met*) and Krüppel homolog 1 (*Kr-h1*). dsE93 (or dsMock, in controls) was administered on 5-day-old adult females, and measurements were carried out on embryo day six (ED6). (B) Control and E93-depleted (dsE93) embryos showing the levels of E93 depletion in ED6. (C) Effects on the expression of E93 in embryos with depleted *JHAMT*, *Met* of *Kr-h1* mRNA, measured on ED6. (D) Expression of laccase 2 in phenotype C (PheC) and phenotype D (PheD) embryos; PheC and PheD embryos were obtained from E93-depleted oothecae on day 19, which are compared with freshly emerged first instar nymphs from oothecae formed by control females (thus, measurements were carried out 19 days after ootheca formation in both cases). Each measurement represents three biological replicates and results of qRT-PCR are expressed as copies of the examined transcript per 1000 copies of BgActin-5c mRNA. Data are represented as the mean ± SEM. The asterisk indicates statistically significant differences with respect to controls ($p < 0.05$), calculated on the basis of the REST (Pfaffl et al., 2002).

Another important gene whose expression was altered by E93 depletion in these very early stages was *zld*, which plays an essential role in the activation of gene transcription in the earliest zygote (Schulz et al., 2015; Sun et al., 2015). Transcriptomic data from *B. germanica* obtained in our laboratory indicates that *zld* mRNA is present in maternally loaded transcripts, then the expression has a peak on ED1, decreasing to low levels on ED2, and keeping even lower levels afterwards, until the end of embryogenesis and in postembryonic stages. Therefore, the abnormally higher levels measured on ED2 with respect to controls suggests that *zld* was not downregulated from ED1 to ED2 in E93-depleted embryos. The abnormally high mRNA levels of genes expressed in the early zygote, like *hb*, piwi, *otx1*, *nos* and *odd*, can be due to the fact that *zld* expression was not downregulated, and the abnormally low mRNA levels of *eve*, *Kr* and *cad* might be derived from the perturbed epistatic relationships with the above factors. Of note, the above gap genes and these three pair-rule genes have crucial functions in early embryo patterning in short and long germ-band insect embryos (Lynch et al., 2012).

The four miRNAs of the MIR-309 family resulted downregulated in E93-depleted specimens, which suggests that E93 promotes the expression of MIR-309 miRNAs. In *D. melanogaster*, this stimulatory role on MIR-309 miRNAs expression is played by *zld* (Fu et al., 2014). Later, MIR-309 miRNAs play crucial roles as scavengers of maternally loaded transcripts in the MZT (Liang et al., 2008; Schulz et al., 2015; Sun et al., 2015). Our expression studies indicated that E93 has a peak of expression on ED1 (present

results), *zld* on ED1 (unpublished results) and MIR-309 miRNAs on ED2 (Ylla et al., 2017). These expression data and our mRNA measurements on ED2 in E93-depleted embryos led us to propose two hypotheses. First, that expression of E93 on ED1, possibly together with the peak of *zld* mRNA produced the same day, promoted the peak of expression of MIR-309 miRNAs of ED2. Second, that the peak of expression of MIR-309 miRNAs of ED2 would had repressed, in turn, the expression of *zld* (Figure 3.4 4A). Consistent with the first hypothesis is the downregulation of MIR-309 miRNAs in E93-depleted embryos. Consistent with the second hypothesis is the occurrence of a MIR-309 site in the 3'UTR region of *zld* (Figure 3.4.4B).

A percentage of 14.4% of the E93-depleted embryos examined in the oothecae transported beyond day 18 without hatching interrupted development between Tanaka stages 10 to 16. They exhibited unsealed dorsal closure, a reduction in the size of the abdomen, shorter appendages and imperfect eyes (what we called phenotype B). Most of the phenotype B embryos looked like some of the malformed embryos resulting from direct or indirect BR-C depletion (Fernandez-Nicolas and Bellés, 2017; Piulachs et al., 2010). This is not surprising as E93 depletion reduced JHAMT expression quite dramatically and reduced also that of BR-C, possibly in an indirect way (Figure 3.4.3A).

It is worth noting, however, that the molecular phenotype in early embryo stages of JHAMT-depleted embryos (Fernandez-Nicolas and Bellés, 2017) is different from that observed now in

E93 experiments, as in in the former, *PCNA* and *zld* expression was unaffected (Fernandez-Nicolas and Bellés, 2017).

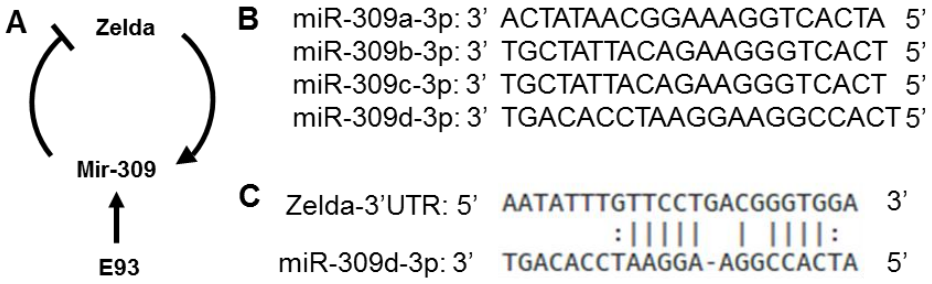


Figure 3.4.4. Hypothesis on the regulation of Zelda by MIR-309 miRNAs and E93 in *Blattella germanica*. (A). Schematic representation of the inferred interactions between E93, Zelda and Mir-309. (B) The four members of the MIR-309 family in *B. germanica*. (C) Predicted Mir-309 binding site in the 3'UTR region of Zelda mRNA aligned with Mir-309d.

This indicates that down-regulation of *PCNA* and up-regulation of *zld* (Figure 3.4.1D) are specific effects of E93 depletion, not derived from the down-regulation of *JHAMT*. Finally, the intensely sclerotized nymphs of phenotype C and the hatching problems of phenotype D embryos were associated to abnormally high levels of laccase 2 expression. This suggests that E93 depletion, while downregulating JHAMT expression, indirectly upregulated laccase 2 expression, consistent with our previous directly depleting JHAMT (Fernandez-Nicolas and Bellés, 2017). In this work, we proposed that JH signalling prevents the premature expression of laccase 2, and thus premature tanning in late embryo, which would impair hatching.

4. Materials and Methods

Insects and dissections

B. germanica specimens used in the experiments were from a colony reared in the dark at 30 ± 1ºC and 60-70% relative humidity. Freshly emerged females were maintained with males during the first gonadotrophic cycle; mating was confirmed at the end of experiments by assessing the occurrence of spermatozoids in the spermathecae. For dissections and tissue sampling, specimens were anesthetized with carbon dioxide. For RNA extractions we used non-fertilized eggs (NFE, see below), entire oothecae (to establish the expression patterns along embryogenesis and in oothecae from treated females dropped between days 2 and 3) or individual embryos dissected out from artificially opened oothecae.

RNA extraction and retrotranscription to cDNA

RNA extractions were performed with Gen Elute Mammalian Total RNA kit (Sigma- Aldrich, Madrid, Spain). An amount of 100 ng from each RNA extraction was treated with DNase (Promega, Madison, WI, USA) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers (Promega). RNA quantity and quality were estimated by spectrophotometric absorption 16 using a NanoDrop Spectrophotometer ND-1000® (452 NanoDrop Technologies, Wilmington, DE, USA).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) reactions were carried out in triplicate in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Madrid, Spain), using SYBR®Green Supermix (iTaq™ Universal Supermix; Applied Biosystems, Madrid, Spain). A control without a template was included in all batches. The primers used to measure the transcripts studied are indicated in Table S1. The efficiency of the primers was first validated by constructing a standard curve through four serial dilutions. mRNA levels were calculated relative to BgActin-5c expression, using the Bio-Rad iQ5 Standard Edition Optical System Software (version 2.0). Results are given as copies of mRNA per 1000 copies of BgActin-5c mRNA.

RNA interference

Detailed procedures for dsRNA preparation were as described previously (Ciudad et al., 2006). The dsRNA used to target E93 was that described by Belles and Santos (2014). A dsRNA from *Autographa californica* nucleopoydrovirus was used for control treatments (dsMock). The primers used to prepare the dsRNAs are detailed in Table S1. Maternal RNAi treatments were carried out essentially as previously reported (Fernandez-Nicolas and Bellés, 2017; Piulachs et al., 2010). A volume of 3 μL of dsRNA solution (3 μg/μL) was injected into the abdomen of 5-day-old adult females. Then the effects of the treatment were examined in the first oothecae formed by treated and control females 6, 9 and 13 days after oviposition.

Examination of embryos

Expression studies were carried out in non-fertilized eggs (NFE), and in embryos on days 0, 1, 2, 4, 6, 7, 9, 11, 13, 14 and 16 (ED0 to ED16). NFE are eggs obtained just before descending the oviduct to which the remains of follicular epithelium were removed. They should contain only maternal transcripts. ED0 to ED2 cover the MZT, while ED3, ED6 and ED13 coincide with respective pulses of 20-hydroxyecdysone (20E) (Piulachs et al., 2010); from ED6 to ED16 there is a cycle of JH production (Maestro et al., 2010). To examine the embryos microscopically, the oothecae were opened after 5 min in a water bath at 95ºC and the embryos were dechorionated and individualized. Then, they were fixed in 4% paraformaldehyde, permeabilized in PBS-0.2% tween (PBT) and incubated for 10 min in 1 µg/ml DAPI in PBT. They were then mounted in Mowiol (Calbiochem, Madison, WI, USA) and examined and photographed using epifluorescence with an AxioImager Z1 microscope (ApoTome System, Zeiss).

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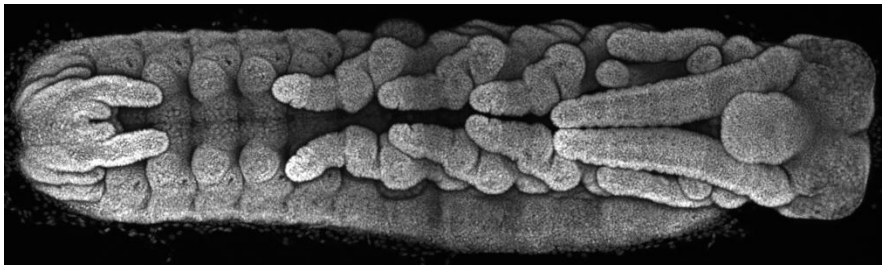
Table S1: **Primer sequences used for qRT-PCR measuremens and to prepare the dsRNA to target E93 and the dsMock.**

Gene name	Accession number	Primer sequence	Amplicon length (bp)
Actin 5c	AJ862721	F:5′-AGCTTCCTGATGGTCAGGTGA-3′ R:5′-ACCATGTACCCTGGAATTGCCGACA-3′	213
A. californica nucleopoydrovirus (for dsRNA)	K01149	F:5′-ATCCTTTCCTGGGACCCGGCA-3′ R:5′-ATGAAGGCTCGACGATCCTA-3′	307
Broad Complex Core (*BR-C*)	FN651774	F:5′-CGGGTCGAAGGGAAAGACA-3′ R:5′-CTTGGCGCCGAATGCTGCGAT-3′	76
Caudal (*cad*)	Submitted to GenBank	F:5′-GCAAAGTCATCAACCGTCCT-3′ R:5′-ATGCTGAGGGGGTGTTACTG-3′	127
Even-skipped (*eve*)	Submitted to GenBank	F:5′-AGGTGCGGGATTGTTAACTG-3′ R:5′-AGGGTTGGAAAAGCTTTGGT-3′	124
E93	HF536494	F:5′-TCCAATGTTTGATCCTGCAA-3′ R:5′-TTTGGGATGCAAAGAAATCC-3′	143
E93 (for dsRNA)	HF536494	F:5′-AAAGAGTTGTCGGGAGCAGA-3′ R:5′-CCACTGCTAGAAGCCACTCC-3′	500
Hunchback (*hb*)	LT717629	F:5′-TCTAAATTGCCCACCAGGTC-3′ R:5′-CCATGAGTTGGAGCCTGAAT-3′	120
Juvenil Hormone Acid Methyltransferase (*JHAMT*)	LT716988	F:5′-GACCTGGTGGTGAAGTCTTGG-3′ R:5′-TGACTCCATTTCGATTTTTTACTCTG-3′	91
Krüppel (*Kr*)	LT717630	F: 5′-CGTACACACACGGGAGAAAA-3′ R: 5′-ATTGTGACCGGCAATTTGTT-3′	84
Krüppel- homolog 1 (*Kr-h1*)	HE575250	F:5′-GCGAGTATTGCAGCAAATCA-3′ R:5′-GGGACGTTCTTTCGTATGGA-3′	77
Laccase 2	LT717634	F: 5′-TGGTGAATGTGGAACCAAGA-3′ R: 5′-TACGATGTTGGTCTCCCACA-3′	108
Methoprene-Tolerant (*Met*)	HG965209	F:5′-CTGTTGGGACATCAGCAGAA-3′ R:5′-GGCAGGTGATGGAGTGAAGT-3′	58
Mir-309a	MF574891	F:5′-TGGTAGTGACTTCCCAAGTGAT-3′ R:5′-CACTGGAAAGGCAATATCA-3′	59
Mir-309b	MF574892	F:5′-GATAGTGACTCCCCAAGTGATG-3′ R:5′-ACGATAATGTCTTCCCAGTGA-3′	59
Mir-309c	MF574893	F:5′-GATAGTGACTTCCCAGGTGAT-3′ R:5′-ACGATAATGTCTTCCCAGTG-3′	59
Mir-309d	MF574894	F:5′-AGTGGATTCTTTCTTGGGAATG-3′ R:5′-GTGGATTCCTTCCGGTGAT-3′	57
Nanos (*nos*)	Submitted to GenBank	F:5′-CGCATTTGACTGTAGTAACGC-3′ R:5′-CTCATCTCCGCTAGCATTGC-3′	464
Odd-skipped (*odd*)	Submitted to GenBank	F:5′-CATCCACTCGAAGGAGAAGC-3′ R:5′-CTCCTCCATGTGCAGGATCT-3′	100
Piwi	Submitted to GenBank	F:5′-GACCCAGGAATGAGGAATGA-3′ R:5′-TACCCATGATTCAGCTGGTG-3′	81
Proliferating cell nuclear antigen (*PCNA*)	LT717627	F: 5′-GAAGCTGGCACAGACAACAA-3′ R: 5′-GGCCTTGGTGAAAGAATTGA-3′	124
Zelda (*zld*)	LT717628	F:5′-TGTCCCAAACAGTTCAACCA-3′ R:5′-AAAGGGTTTCTCTCCCGTGT-3′	72

4. GENERAL DISCUSSION



4. GENERAL DISCUSSION

The present thesis has been planned and executed in the Evolution of Insect Metamorphosis Laboratory, headed by Xavier Belles, in the Institute of Evolutionary Biology (CSIC-UPF). Therefore, the objectives of the thesis fit and are in the context of the long term objectives of this Laboratory, which consists in reconstructing the regulatory basis of the evolutionary transition from gradual metamorphosis (hemimetaboly) to complete metamorphosis (holometaboly). As most of the data available on the subject relies in holometabolan models (especially the dipteran *Drosophila melanogaster*, as well as the lepidopteran *Bombyx mori* and, more recently, the coleopteran *Tribolium castaneum*), the approach in the Laboratory is to comprehensibly study the regulation of metamorphosis in a hemimetabolan model, the German cockroach *Blattella germanica*, and then compare the information generated with that available in holometabolan models. The idea is to increase the knowledge about how the divergence from hemimetaboly to holometaboly occurred.

In a previous project of the Laboratory, a series of transcriptomes from male tergites 7-8 in non-metamorphosing and metamorphosing stages of *B. germanica*, were constructed to study the morphogenesis of tergal glands, which are formed in these tergites (Ylla and Belles, 2015). The comparative analyses of the transcriptomes led to the identification of a series of genes that were candidates to play significant roles in the morphogenesis of the tergal glands, in particular, and in the metamorphosis, in general.

Three genes were considered especially interesting: *E93*, Nejire and Medea. The *E93* was functionally studied previously (Belles and Santos, 2014), whereas Nejire and Medea have been studied in the present thesis.

Nejire is a CREB-binding protein that acts as a transcriptional co-activator by interacting with a large number of transcription factors involved in development (Janknecht and Hunter, 1996; McManus and Hendzel, 2001). Medea is a key signal transducer common to the two branches of the transforming growth factor-β (TGF-β) signalling pathway, which is fundamental for controlling developmental programs and cell behaviour in animals (Herpin et al., 2004).

4.1. New factors operating in the regulation of adult morphogenesis

The figure 4.1 summarizes the main findings resulting from this part of the thesis, specifically related to the MEKRE93 pathway (Belles and Santos, 2014).

4.1.1. CREB-binding protein and postembryonic development

CREB-binding protein (*CBP*) is a promiscuous transcriptional co-regulator (Janknecht and Hunter, 1996; McManus and Hendzel, 2001). In insects, *CBP* functions had been studied only in *D. melanogaster,* where *CBP* is known as Nejire. Data obtained in this

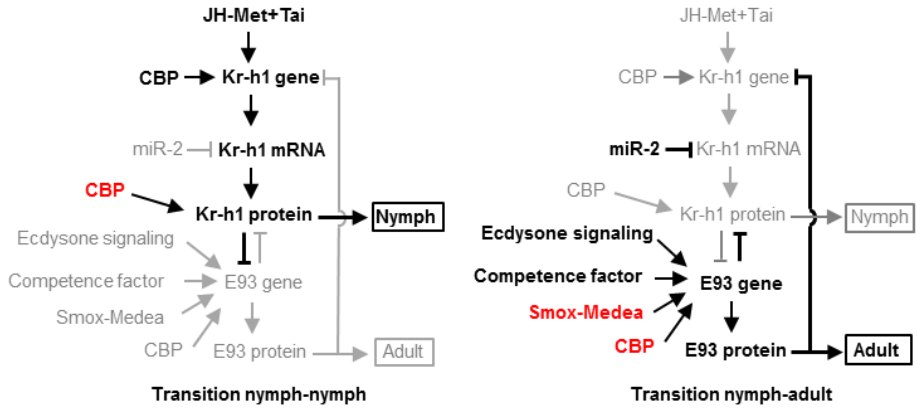


Figure 4.1: The TGF-β signalling pathways and *CBP* in *Blattella germanica* metamorphosis, in the context of the MEKRE93 pathway. Previous data had shown that in the transitions from nymph to nymph (left panel), juvenile hormone (JH) through *Kr-h1*, prevents metamorphosis; in the transition from the last instar nymph (N6) to adult (at the beginning of N6) (right panel), JH production decreases, transcription of *Kr-h1* begins declining, and the expression of E93, that had been repressed by *Kr-h1*, begins increasing; miR-2 contribute to remove *Kr-h1* transcripts at the beginning of N6. Our present results (highlighted in red) show that Medea-Mad would co-activate the transcription of *E93*, whereas *CBP* would co-activate *Kr-h1* and *E93* expression.

fly suggested that *CBP* functions are concentrated in the embryo, especially in anterior/posterior polarity, through Hedgehog and Wingless signalling, as well as in dorsal/ventral patterning, through TGF-β signalling (Akimaru et al., 1997a, 1997b; Goodman and Smolik, 2000; Holmqvist et al., 2012; Lilja et al., 2003; Waltzer and Bienz, 1999). In the postembryonic development of *D. melanogaster*, known data on *CBP* are limited to two contributions that describe the influence of *CBP* HAT activity on the ecdysone

signalling pathway (Bodai et al., 2012b; Kirilly et al., 2011a). Our studies using RNAi have shown that *CBP* contributes to the regulation of feeding and ecdysis during the pre-metamorphic nymphal instar of the cockroach *B. germanica*, and it participates in the regulation of the TGF- β, ecdysone and MEKRE93 pathways, in the latter case contributing to the activation of *Kr-h1* and *E93* expression. Further research into a higher diversity of models will probably reveal that the multiple post-embryonic roles of *CBP* observed in *B. germanica* are general in insects.

4.1.2. Medea and insect hemimetabolan metamorphosis

In contrast with *D. melanogaster*, practically nothing is known about the involvement of the TGF-β signalling pathway in the metamorphosis of hemimetabolan insects. To partially fill this gap and consistent with the data revealed by the transcriptomic analyses of tergal gland morphogenesis (Ylla and Belles, 2015), we studied the role of Medea and the other two Smads (*Smox* and *Mad*) in the metamorphosis of *B. germanica*. In *D. melanogaster*, *Mad* is the canonical R-Smad of the BMP branch of the TGF-β signalling pathway, *Smox* is the canonical R-Smad of the TGF-β/Activin branch and Medea participates in both branches. In insects, metamorphosis is regulated by the MEKRE93 pathway (Belles and Santos, 2014), which starts with juvenile hormone (JH), whose signal is transduced by Methoprene-tolerant (*Met*), which stimulates the expression of Krüppel homolog 1 (*Kr-h1*) that acts to repress *E93*, the metamorphosis trigger. The RNAi of *Mad*, *Smox* and

Medea in N6 of *B. germanica* revealed that the BMP branch of the TGF-β signalling pathway regulates adult ecdysis and wing extension, mainly through regulating the expression of bursicon, whereas the TGF-β/Activin branch contributes to increasing *E93* and decreasing *Kr-h1* at the beginning of N6, crucial for triggering adult morphogenesis, as well as to regulating the imaginal moult timing.

This can be considered as a seminal contribution to the involvement of the TGF-β signalling pathway in hemimetabolan metamorphosis. However, while preparing our results for publication, a paper reporting other relevant roles of the TGF-β signalling pathway in the cricket *Gryllus bimaculatus* (another hemimetabolan species) was published (Ishimaru et al., 2016). These authors reported that high expression of the TGF-β ligand myoglanin (which acts through the TGF-β/Activin branch) during the last nymphal instar induces metamorphosis by repressing the expression of the enzyme JH acid *O*-methyltransferase (*JHAMT*). *JHAMT* is the last and key enzyme of JH synthesis, thus high levels of myoglanin provoke a decline of JH and triggers metamorphosis. Conversely, *JHAMT* and JH production is up-regulated by the TGF-β ligands decapentaplegic and glass-bottom boat (which signal in the BMP branch) during nymphal stages. This keeps the status quo action of JH and prevents metamorphosis (Ishimaru et al., 2016). The authors hypothesize that these modulatory roles on JH production of the TGF-β signalling pathways could be extended to all insects, which still add another regulatory layer upstream the MEKRE93 pathway.

4.2. On the endocrine regulation of hemimetabolan embryogenesis

The evolution of metamorphosis has been explained on the basis of a heterochrony; hemimetabolan species develop the basic adult body structure during embryogenesis, whereas holometabolan species delay adult body structure construction until the pupal stage (Belles, 2011; Truman and Riddiford, 1999). In this context, an appealing hypothesis is that the premature production in the embryo of JH could be a determining factor in hemimetabolan or holometabolan metamorphosis (Truman and Riddiford, 1999). On the basis of these concepts, and given the possible implication of JH on the differences on the output of embryogenesis in both metamorphosis types, we were interested in studying in the embryo the role of the factors involved in regulating the postembryonic metamorphosis (essentially the MEKRE93 pathway).

The figure 4.2 summarizes the main findings resulting from this part of the thesis, specifically related to the regulation of Zelda in very early embryo development.

4.2.1. Juvenile hormone signalling in the embryo

Using maternal RNAi, we depleted the mRNA levels of *Kr-h1*, *Met* and *JHAMT* in embryos of *B. germanica*. In the three genes, mRNA depletion precluded the formation of the germ-band anlage in a group of embryos,

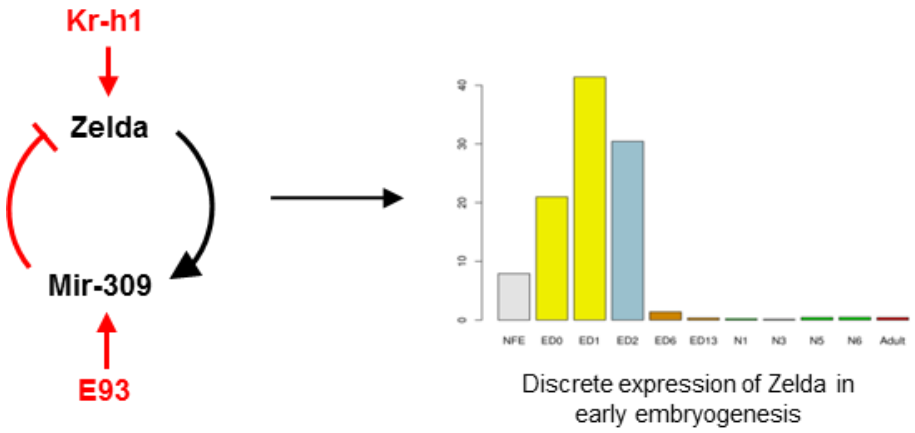


Figure 4.2: Hypothesis on the regulation of Zelda in *Blattella germanica*. The inferred interactions between *E93*, *Kr-h1*, Mir-309 and Zelda (left panel) modulate the discrete peak of expression of Zelda at the beginning of embryo development, between days 0 and 2 (right panel). Data from Zelda expression are from unpublished transcriptomic sources of our Laboratory. Highlighted in red are the interactions inferred from the results of the present thesis.

hatchability was reduced in others, whereas development was interrupted in mid embryogenesis in another group.

Concerning the formation of the germ-band anlage, our data suggest that *Kr-h1* and *JHAMT* play different roles in this process. *Kr-h1* would stimulate *PCNA* expression, a factor associated with nuclear division cycles in early embryogenesis (Yamaguchi et al., 1991). Moreover, *Kr-h1* would also stimulate the expression of Zelda in early embryogenesis, a factor that is crucial in the maternal to zygotic transition (MZT) (Liang et al., 2008; Schulz et al., 2015; Sun et al., 2015). On the other hand, *JHAMT* would appear to exert a negative regulation on the expression of Smads in the TGF-β

pathways, which are important for embryonic development (Deignan et al., 2016).

In the group of embryos that interrupted development in mid embryogenesis, we observed defects related to dorsal closure and appendages formation. We propose that these phenotypes are due to the low levels of Broad-complex (*BR-C*) produced under JH-depleted conditions, as previous work had shown that depletion of *BR-C* results in similar defects (Piulachs et al., 2010). Finally, the problems of hatchability observed may have been due to premature up-regulation of laccase 2, a promoter of cuticle tanning (Sugumaran and Barek, 2016).

The effects of *JHAMT*, *Met* and *Kr-h1* depletion in very early embryos are intriguing, as they may have nothing to do with JH, which starts to be detected around dorsal closure (Maestro et al., 2010). It is the first time that a role for maternal factors related to JH is reported, and they appear functionally important, especially the stimulatory role of *Kr-h1* on Zelda expression. This might be characteristic of hemimetabolan embryogenesis (or perhaps of short germ-band embryogenesis) as no effects on early embryogenesis were detected in the TALEN experiments carried out in the lepidopteran *B. mori* by Daimon et al. (2015) to knockout *Met* and *JHAMT* expression. However, in these experiments, homozygous mutant embryos were obtained from sibling-crosses of heterozygous adult moths. Therefore, given that heterozygous females produce both wild-type and mutated transcripts of *Met* and *JHAMT*, then both kinds of transcripts should had been maternally loaded, which led to conclude that the experiments of Daimon et al.

(2015) would not show whether maternal *Met* and *JHAMT* transcripts play a role in very early embryo development in *B. mori*.

Impaired hatchability in JH deficient embryos had been previously reported in hemimetabolan species, like the locust *Locusta migratoria* (Aboulafia-Baginsky et al., 1984) and in holometabolans, like *B. mori* (Daimon et al., 2015), which suggest roles of JH signalling in embryo hatchability that appear conserved in hemimetabolan and holometabolan insects. It remains to be assessed if premature tanning showed in *B. germanica* in our measurements of laccase 2 expression, might be also the cause of the impaired hatchability in *L. migratoria* and *B. mori*.

Especially interesting are the various malformations exhibited in mid-late development by *Kr-h1*, *Met* and *JHAMT* depleted embryos. These included unsealed dorsal closure, reduction in the size of the abdomen, shorter appendages and imperfected eyes. Since *Kr-h1*, *Met* and J*HAMT* depletion phenocopy the effects of *BR-C* depletion, as reported by Piulachs et al (2010), we propose that these phenotypes are due, at least in part, to the low levels of *BR-C* resulting from depleting JH signalling. It is worth remembering that, as observed in nymphs (Huang et al., 2013), *BR-C* expression is stimulated by JH in *B. germanica* embryos. In contrast, JH does not appear to affect the expression of *BR-C* in *B. mori* embryos (Daimon et al., 2015). The whole observations suggest that the stimulatory role of JH signalling on *BR-C* expression and the morphogenetic functions of JH in hemimetabolan embryos (possibly mediated by *BR-C*, at least in part), were lost in holometabolan embryos. If so, then this might

have been an important driver for the evolution towards holometabolan metamorphosis.

4.2.2. Transcription factor *E93* regulates miR-309 and Zelda expression in the embryo

In postembryonic development, *E93* promotes adult morphogenesis in hemimetabolan and holometabolan insects (Belles and Santos, 2014; Ureña et al., 2014). In young juvenile stages, E93 is repressed by JH through the signal transducer *Kr-h1*, within the axis known as MEKRE93 pathway (Belles and Santos, 2014). We were interested in studying whether this morphogenetic factor could play any role in embryo development, if it could have some relationships with JH signalling. mRNA measurements indicated that the greatest expression of *E93* occurs in very early embryogenesis, showing a peak on ED1. Maternal RNAi showed that in early embryogenesis *E93* promote *PCNA* expression, thus nuclear division cycles (Yamaguchi et al., 1991), and downregulates Zelda, a factor that activates the gene transcription in the zygote during the MZT (Sau and Sarma, 2012; Schulz et al., 2015; Sun et al., 2015). Our results also indicate that the expression of the four miRNAs of the MIR-309 family is stimulated by *E93*.

Interestingly, Zelda stimulates MIR-309 miRNAs expression in *D. melanogaster* and, in turn, these miRNAs contribute to eliminate maternal transcripts in the MZT (Sau and Sarma, 2012; Schulz et al., 2015; Sun et al., 2015). The whole data led us to propose that expression of E93 on ED1, possibly together with the

peak of Zelda produced the same day, promoted the peak of expression of MIR-309 miRNAs of ED2, and that this expression peak would repress, in turn, the expression of Zelda. In *D. melanogaster*, Zelda keeps moderate to high levels of expression practically during all embryogenesis (Ylla, Piulachs and Belles., unpublished), which suggests that it is constantly activating the zygote genome. Conversely, E93 limits the expression of Zelda in a narrow time window (from ED0 to ED2, in the MZT) in *B. germanica*. We propose that this might be an important difference between hemimetabolan and holometabolan embryogenesis.

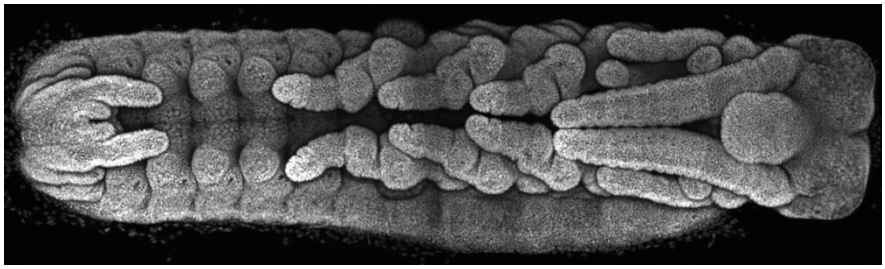
Our RNAi experiments also suggested that *E93* play morphogenetic roles in mid-embryogenesis, and inhibits premature cuticle tanning in late embryogenesis. These effects could be indirect, resulting from the fact that *E93* stimulates the expression of *JHAMT* and, consequently, that of *BR-C*, which promote the above process, as shown in our previous study on the roles of JH signalling in embryogenesis.

4.3. Final remarks

The study of the role of *CBP* and TGF-β signalling pathway in the regulation of adult morphogenesis in *Blattella germanica* has provided new insights into the regulation of insect metamorphosis in hemimetabolan insects. The contribution of *CBP* and TGF-β signalling appears particularly relevant in the regulation of the MEKRE93 pathway, which appears, thus, more complex than previously thought (Figure 4.1).

The study the regulation of embryogenesis in *B. germanica* has revealed that JH signalling pathway and *E93* play important roles, not only in mid and late embryo development, but also in very early embryo stages, around the maternal to zygotic transition, where the influence on the expression of Zelda (Figure. 4.2) appears to be relevant. This is completely new information, which contrasts with what has been described in holometabolan embryogenesis, where at least JH signalling appears to be dispensable. These differences could account for the evolutionary transition from hemimetaboly and holometaboly, and may be relevant to explain the evolution of insect metamorphosis, in general.

6. CONCLUSIONS



6. CONCLUSIONS

From the results obtained during the present work, the following conclusions can be inferred. All the conclusions refer to the species *Blattella germanica*. The first six concern the transition from last instar nymph to adult, and from the seven to the thirteen to embryo development.

1. In the last nymphal instar, *CBP* depletion results in decrease in feeding and in a delay in moulting. These observations suggest that *CBP* participates in the mechanisms controlling food intake and on those regulating the timing of the burst of ecdysone that triggers the imaginal moult.
2. In the last nymphal instar, *CBP* depletion impairs the decrease of *Kr-h1* expression and inhibits the increase of E93 expression. As *Kr-1* and *E93* are mutually repressed, we can conclude that *CBP* stimulates *Kr-h1* expression, inhibits that of *E93*, or both.
3. The expression of downstream targets of TGF- β pathway is lowered after *CBP* depletion in the last nymphal instar. This suggests that *CBP* directly or indirectly stimulates TGF- β signalling.
4. Depletion of the transcription factor *Mad* results in imperfect imaginal ecdysis and wing extension after the imaginal moult, and depletion of bursicon phenocopies these effects. We can conclude, thus, that the BMP branch of the TGF-β signalling pathway in involved in the regulation of

the imaginal ecdysis and wing extension, probably with the mediation of bursicon.

5. Depletion of Smox and Medea in the last nymphal instar impairs the decrease of Kr-h1 expression and inhibits the increase of *E93* expression. As *Kr-1* and *E93* are mutually repressed, we can conclude that the activin branch of the TGF- β signalling stimulates *Kr-h1* expression, inhibits that of *E93*, or both.
6. Depletion of *Smox* and Medea in the last nymphal instar results in a delay in moulting. These observations suggest that the activin branch of the TGF- β signalling participates in the mechanisms regulating the timing of the burst of ecdysone that triggers the imaginal moult.
7. Depletion of mRNA levels of *Kr-h1* in the embryo impairs the formation of the germ-band anlage. In parallel, *Kr-h1* depletion in these early stages results in downregulation of *PCNA* and Zelda. These results suggest that *Kr-h1* stimulates the expression of *PCNA* and Zelda, and that these two factors are essential for the formation of the germ-band and further embryo development.
8. Also in early embryogenesis, depletion of *JHAMT* results in an upregulation of the Smads of the TGF-β pathway, and in an impairment of the formation of the germ-band and further development. We can infer, thus, that J*HAMT* exerts a negative modulation on the TGF-β pathway, which would be important for correct embryonic development.

9. Depletion of *Kr-h1*, *Met* or *JHAMT* in the embryo also provokes developmental deficiencies in mid-late embryo stages. These deficiencies are similar to those previously described after depleting the transcription factor *BR-C* in the embryo. Given that *BR-C*, which is stimulated by JH, results downregulated in Kr-h1-, Met- or JHAMT-depleted embryos, we propose the deficiencies observed are due to the low levels of BR-C, and that this transcription factor is essential in mid-late embryo development.
10. Depletion of *Kr-h1*, *Met* or *JHAMT* in the embryo also affects hatchability. As the unhatched embryos (indeed, first instar nymphs) showed abnormally high levels of laccase 2 mRNA, we conclude that the above factors repress premature expression of laccase 2, thus preventing premature tanning, which would impair hatchability.
11. Depletion of mRNA levels of *E93* in the embryo impairs the formation of the germ-band anlage. In parallel, *E93* depletion in these early stages upregulates Zelda and downregulates MIR-309 miRNAs. As in *D. melanogaster*, Zelda stimulates MIR-309 miRNAs expression, which, in turn, eliminate maternal transcripts, we propose that in *B. germanica*, the expression of *E93* in day 1 of embryogenesis, possibly together with the peak of Zelda produced simultaneously, promotes the peak of expression of miR-309 miRNAs one day later, and that this expression peak repress, in turn, the expression of Zelda. This mechanism would be essential to regulate the maternal to

zygotic transition and the subsequent development of the embryo.

12. Depletion of *E93* in the embryo also provokes developmental deficiencies in mid-late embryo stages and affects hatchability. These deficiencies are similar to those previously described after depleting *JHAMT*. Given that depletion of *E93* downregulates *JHAMT* expression, we propose the deficiencies observed are due to the low levels of *JHAMT* (and consequent low levels of *BR-C* and high levels of laccase 2).
13. The importance of JH signalling in the hemimetabolan embryogenesis of *B. germanica*, together with the key roles of *E93* in very early embryo stages, contrasts with what has been described in holometabolan embryogenesis, where at least JH signalling appears to be dispensable and no roles for *E93* have been described. The peculiar features of *B. germanica* embryogenesis that we have reported could help to explain the evolutionary transition from hemimetaboly and holometaboly, and the evolution of insect metamorphosis, in general.

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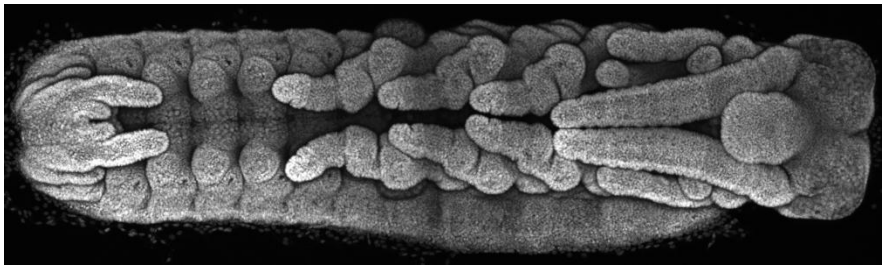

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