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Ph.D. Thesis

Genetic studies on juvenile hormone signalling in insect metamorphosis

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ANNOTATION

This thesis consists of two published articles in both of which I am the first author, and unpublished results. My studies using RNA interference in the model beetle *Tribolium castaneum* (Coleoptera) and non-model insects contribute to defining the core of juvenile hormone signalling in insect metamorphosis. Results of studies in *Tribolium* presented in my first publication identify the JH-resistance gene *Methoprene-tolerant (Met)* as the first known transducer of the anti-metamorphic effect of juvenile hormone; my unpublished studies on a true bug *Pyrrhocoris apterus* (Hemiptera) demonstrate that role of *Met* in metamorphosis is shared by insects with hemimetabolous and holometabolous type of metamorphosis. The second publication demonstrates that Met exerts its function by regulating the *Broad-Complex (BR-C)* gene, and studies in *Tribolium* and the lacewing *Chrysopa perla* (Neuroptera) show that its central role of *BR-C* in holometabolan metamorphosis has changed during Holometabola evolution. My unpublished results show that the gene *Krüppel-homolog 1* is another *Met* target whose function in preventing precocious metamorphosis has been conserved between the holometabolous beetle and the hemimetabolous true bug *Rhodnius prolixus*.

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DECLARATION

I declare that I did all the work, presented in this thesis, by myself or in collaboration with the co-author of published articles, and using only the cited literature.

České Budějovice, October 23, 2008 Barbora Konopová

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CONTENT

RESEARCH OBJECTIVES

The objective of my Ph.D. study was to understand how insect metamorphosis is controlled by juvenile hormone (JH) at the genetic level. It has been known for decades that by its morphostatic function JH prevents insect larvae to metamorphose into adults, but the mechanism through which JH exerts this effect remained unresolved and the JH receptor unknown.

(1) By means of systemic RNA interference (RNAi) in the model beetle *Tribolium castaneum* I aimed to clarify the significance of the *Methoprene-tolerant (Met)* gene for JH signalling.

(2) After I had found out that *Met* was a key mediator of the anti-metamorphic JH function, I studied in *Tribolium* an epistatic interaction between *Met* and a JH-regulated gene that is necessary for metamorphosis, the *Broad-Complex (BR-C)*.

(3) To see whether the central role of *BR-C* in pupal morphogenesis has been conserved within holometabolans I studied the requirement for *BR-C* function in *Tribolium* and in a neuropteran lacewing, *Chrysopa perla*, both insects with a primitive type of holometaboly.

My ongoing research aims to investigate (4) what is the role of *Met* in insects with non-holometabolous development and (5) and what is the function of the JH-response gene *Krüppel-homolog 1* in insect metamorphosis and in *Met*-dependent JH signalling.

INTRODUCTION

Metamorphosis is a sudden and conspicuous morphological change that occurs at a specific time point during postembryonic development of species from many animal lineages. Prime attention has been given to metamorphosis in insects. Insects are ubiquitous and transformation of crawling juveniles to winged adults is a remarkable and notoriously known phenomenon. Moreover, insect metamorphosis appears as an ideal model situation for studies on how developmental extracellular signals co-ordinate complex morphogenesis at the tissue and genetic levels.

ORIGIN OF INSECT METAMORPHOSIS

Metamorphosis is a common feature of all recent winged insects (Pterygota). During metamorphosis some or many organs undergo marked and abrupt change of form or structure, the wing size increases dramatically and the wing articulation is formed (Kukalova-Peck 1991, Sehnal et al. 1996). With the exception of mayflies (Ephemeroptera) metamorphosis takes place at the transition from larval to adult stages.

Why do insects metamorphose? Clues from fossil record show that metamorphosis evolved as a necessary consequence of the emergence of wings (Kukalova-Peck 1978, 1983, 1991). Development of wings in recent larvae is retarded, wing pads are immobile, firmly fused with terga and lacking articulation. However, wings of primitive Paleozoic insects developed gradually, larval wings were articulated and older larvae probably could fly (Rasnitsyn 1981, Kukalova-Peck 1991) (Fig. 1). But laterally growing wing buds were vulnerable and useless during early instars, in which they could not provide effective flight, and instead prevented the larvae from moving into confined spaces and hiding from predators. Thus, selective pressure favoured individuals in which wing development was delayed until late instars. Finally, the suppression became so advanced that a sudden metamorphic change restoring wings into their functional condition became a necessity. Originally, metamorphosis occurred between larval instars and was followed by several instars of subimagoes (= flying juveniles) and imagoes; only recent pterygotes do not undergo postmetamorphic moulting. There is evidence from the fossil record that metamorphosis originated independently in several pterygote lineages: ephemeropterans, odonatoids, plecopteroids, orthopteroids, blattoids, hemipteroids and holometabolans (endopterygotes) (Kukalova-Peck 1978, 1983).

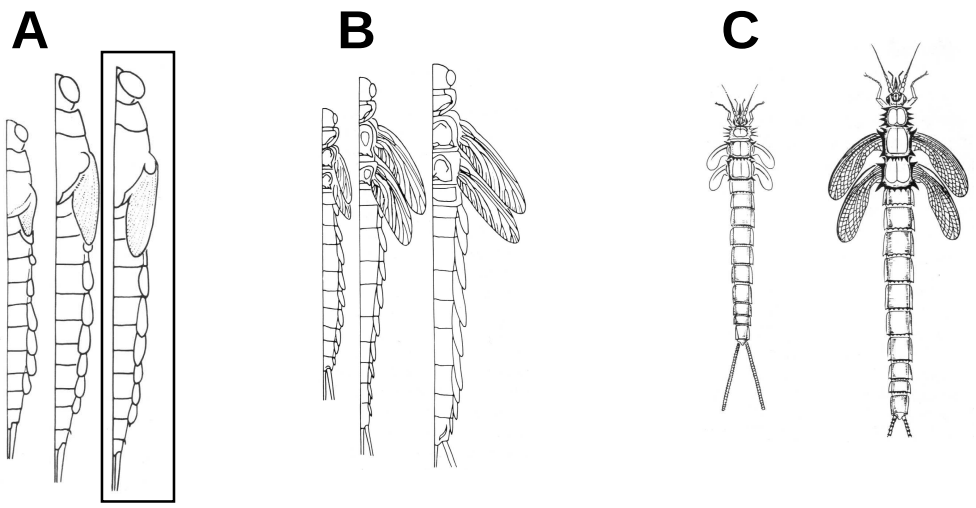

Figure 1: Larval wing development in metamorphic and ametamorphic Pterygota. (**A, B**) Comparison of larval development in metamorphic and ametamorphic insects on the example of recent and Permian mayflies. (**A**) Metamorphic mayfly larvae have immobile wing pads and a metamorphic instar (boxed). (**B**) Larvae of an extinct ametamorphic lineage have articulated, movable and gradually developing wing pads; there is no metamorphic instar. (**C**) A young and an older larva of ametamorphic Megasecoptera. Note the laterally outstretched wing pads. From Kukalova-Peck (1991).

TYPES OF METAMORPHOSIS

In modern insects, not only wings but also other organs, like the genitalia and cuticle, undergo metamorphosis. Within the over 250 million years since the emergence of the first metamorphosing insects, several types of postembryonic development and metamorphoses have evolved (reviewed in Sehnal et al. 1996, Stys and Sobotnik 1999, Hemming 2003); their main currently recognized categories are summarised in Table 1.

Mayflies (Ephemeroptera) are the only group of modern insects that retain two postmetamorphic (flying) instars, the non-reproductive subimago and the imago. In all other insects, metamorphosis is followed by a single adult stage. In hemimetaboly, manifested for example in true bugs (Hemiptera), cockroaches (Blattodea) and grasshoppers (Orthoptera), external wing rudiments (wing pads) begin to appear from a certain larval instar or as late as in the final larval instar. Functional wings, genitalia and other imaginal structures fully develop during metamorphosis at the end of the final larval instar.

The recently most widespread and best studied metamorphic strategy is holometaboly. Unlike hemimetabolans, holometabolous insects form a monophyletic unit, the Holometabola (= Endopterygota = Oligoneoptera) (Kristensen 1999). Larvae differ morphologically from

Table 1: Types of postembryonic development with regard to metamorphosis in recent insects.

developmental type	present in lineages	specific ontogenetic features	metamorphic change
ametaboly (no metamorphosis)	Archaeognatha, Zygentoma	gradual developemnt without metamorphosis, several imaginal instars	none
prometaboly	most Ephemeroptera	older larval instars with immobile wing pads, two postmetamorphic instars (final larva = subimago, imago)	penultimate larva with wing pads → flying final larva ("subimago")
hemimetaboly	some Ephemeroptera, Odonata, "Polyneoptera", most Paraneoptera	older larval instars with immobile wing pads	final larva with wing pads → flying imago
remetaboly[1]	Thysanoptera	wing pads first appear in the last two larval instars that are quiescent	*antepenultimate wingless larva → penultimate larva Q with wing pads* *penultimate larva Q with wing pads → final larva Q with wing pads* penultimate larva Q with wing pads → flying imago
allometaboly[1]	Aleyrodomorpha	none of the larvae provided with wing pads	final larva without external wings → flying imago
parametaboly[1]	male Coccomorpha	wing pads first appear in the last two larval instars that are quiescent	alike remetaboly
holometaboly	Holometabola = Endopterygota	larvae without external wings and rudiments of genitalia that first appear in the quiescent final juvenile (pupa), usually also ecological difference between larvae and adults	final larva without external wings → pupa (= Q juvenile with wing pads) pupa → flying imago

[1] commonly called neometaboly
Q marks a quiescent stage. Metamorphic changes in italics are less remarkable.
Based on Sehnal et al. 1996, Stys and Sobotnik 1999.

adults and lack external wing pads and genitalia, which first appear at the pupal stage (= a quiescent final juvenile instar). Wings and some other organs may develop already in larvae, but grow internally as so called imaginal discs that then externalize during larva-pupa metamorphosis. Imaginal discs have evolved independently in several lineages, for example several times only within beetles (Tower 1903, Svacha 1992). During holometabolous metamorphosis, larval tissues are reprogrammed and sequentially form pupal and adult structures whereas imaginal discs, if developed at all, give rise only to a few body parts, primarily the wings.

A remarkable deviation from this typical mode is seen in cyclorrhaphous Diptera that include the genetic model *Drosophila melanogaster* (Nijhout 1994). At metamorphosis, larval epidermal cells of the head, thorax and abdomen undergo programmed cell death, instead of being reprogrammed, and are replaced by epithelia that derive from large imaginal discs. Such imaginal discs form as early as in the embryo, then grow and become patterned during larval stages. Abdominal epidermis also degenerates after secreting a pupal cuticle and is replaced by adult abdominal epidermis that differentiates from histoblasts. Clusters of these blast cells are embedded in the larval epidermis and begin to proliferate rapidly at the onset of fly metamorphosis (Bodenstein 1994).

Metamorphosis occurring through striking morphogenetic changes and involving a quiescent penultimate stage, similar to the pupa of Holometabola, has evolved independently in several lineages of Paraneoptera (Sehnal et al. 1996). An interesting mode of this neometabolous development occurs in whiteflies (Aleyrodomorpha) (Weber 1934, cited in Hemming 2003). Wings are absent in all larval instars and larvae morphologically differ from adults. Metamorphosis to a fully winged imago involves considerable histolysis and histogenesis at the end of the quiescent final larval instar. The rate of metamorphosis thus exceeds that in Holometabola, with no intermediate pupal stage.

HORMONAL CONTROL OF METAMORPHOSIS

20-hydroxyecdysone and juvenile hormone regulate metamorphosis

Insect metamorphosis is regulated by antagonistic action of two hormones, the steroid 20-hydroxyecdysone (20E) and the sesquiterpenoid juvenile hormone (JH) (Nijhout 1994). While 20E induces metamorphosis, JH prevents its effect and ensures that metamorphosis takes place only at the right ontogenetic time.

Larval ecdysteroids are synthesized in the prothoracic glands (Gilbert 2004). In response to a signal from the brain, the prothoracic gland secretes ecdysone, a relatively inactive prohormone that is converted into the active 20E in the fat body and epidermal cells (Nijhout 1994). Apart from metamorphosis, 20E is required for periodic larval moulting (secretion of a new and ecdysis of the old cuticle) and thus 20E titer rises several times during larval development. It is due to the presence of JH, a product of the corpora allata glands, in young larvae that the pro-metamorphic function of 20E is blocked, and thus moulting to a next larval stage takes place. After reaching a critical body mass, JH level decreases and a surge of 20E induces the metamorphic changes.

The anti-metamorphic JH function was first demonstrated in the hemimetabolous true bug *Rhodnius prolixus* (Wigglesworth 1934, 1936). When larvae of the penultimate (thus non-metamorphic) instar were decapitated so that the corpora allata gland was removed, some showed partial development of adult characters such as wings, genitalia, or cuticle structures; later this effect was obtained also in younger instars (Wigglesworth 1985).

The essential role of JH in preventing precocious metamorphosis has been validated in a number of hemimetabolous and holometabolous insects. Experiments with ablation of corpora allata glands (allatectomy) (Wigglesworth 1954, 1985, Staal 1986) have recently been confirmed by genetic studies. Transgenic silkworms *Bombyx mori* overexpressing JH esterase, a JH degradative enzyme, metamorphosed to pupae and next to adults before reaching the final instar (Tan et al. 2005) just like silkworms that had been surgically allatectomized (Bounhiol 1938, Fukuda 1944). Precocious pupation was observed in the beetle *Tribolium castaneum* after RNA interference (RNAi) mediated silencing of the *juvenile hormone acid O-methyltransferase (JHAMT)* gene, encoding a key enzyme in JH biosynthesis (Minakuchi et al. 2008a). Interestingly, in some holometabolans the response to the absence of JH may be so strong that certain tissues directly metamorphose from the larval to the adult state (Williams 1961, Kiguchi and Riddiford 1978).

Ectopic JH and its analogs supplied to larvae block their metamorphosis and may instead cause moulting to supernumerary larval stages. Similarly in holometabolous pupae, which normally have low endogenous JH, ectopic hormone prevents metamorphosis to adults, and leads to moulting into a second pupal instar. Thus, JH function is to keep the current developmental state, the “status quo” (Williams 1959, 1961).

Hormonal regulation of metamorphosis of *Drosophila* and other cyclorrhaphous Diptera differs in that it mostly relies on ecdysteroids and is independent of JH. Ectopic JH cannot induce supernumerary larval instars and only in pupae it causes deposition of a second pupal cuticle on the histoblast-derived abdomen and deformities of the genitals (Srivastava and Gilbert 1968, Postlethwait 1974, Sehnal and Zdarek 1976, Zhou and Riddiford 2002). The latter phenotype was also observed upon misexpression of the JH-producing enzyme JHAMT (Niwa et al. 2008). However, unlike in *Tribolium* the presumed depletion of JH by RNAi silencing of *JHAMT* did not result in precocious metamorphosis in *Drosophila* (Niwa et al. 2008).

Genetic studies on hormonal signalling

There has been an ongoing effort to understand the hormonal signalling that directs insect development at the genetic level. An early model explaining how genes are activated by 20E derives from detailed studies on puffs of the *Drosophila* polytene chromosomes (Ashburner et al. 1974). In this model an ecdysteroid receptor directly activates a set of early puffs, which correspond to early-transcribed genes. The resulting early regulatory proteins then induce many late puffs, or genes, while repressing their own activity. With modifications, the Ashburner model is still accepted over 30 years after it had been formulated. Molecular characterization of the early puffs led to the identification of transcription factors, namely E74, E75 and Broad-Complex (BR-C) (reviewed in Thummel 1996, Kozlova and Thummel 2000). Many other players in the ecdysone cascade including the 20E receptor (EcR; Koelle et al. 1991, Yao et al. 1992, Thomas et al. 1993), other nuclear receptors, transcription factors, and some of the downstream genes have been described to date. Functional analyses of these proteins using *Drosophila* genetics have substantially improved our understanding of steroid hormone signalling. However, the mode of action of JH remains a mystery and the search for a JH receptor has not yet been concluded (Riddiford 2008).

Despite the evolutionarily derived *Drosophila* development this genetic model has provided vital clues by uncovering genes that function during metamorphosis and are regulated by JH, such as *BR-C*, *Met*, *usp* and *Kr-h1* (Berger and Dubrovsky 2005, Riddiford 2008). With current reverse-genetic methods and primarily with the advent of RNAi (Fire et al. 1998) it is now possible to extend genetic studies of JH action to other, suitable insect models.

The tenebrionid beetle *Tribolium castaneum* provides an excellent model for such a research. Apart from the ease to keep it, the beetle offers a wide spectrum of molecular genetic tools, including transgenesis (Berghammer et al. 1999, Pavlopoulos et al. 2004), efficient RNAi

(Bucher et al. 2002, Tomoyasu and Denell 2004, Tomoyasu et al. 2008) and the sequenced genome (Richards el al. 2008). Development of *Tribolium* and other tenebrionids is sensitive to JH, as ectopic JH induces extra larval and pupal instars (Connat et al. 1984, Bouhin et al. 1992, Konopova and Jindra 2007) while JH deficiency leads to precocious metamorphosis (Nakakita 1990, Quennedey and Quennedey 1999, Minakuchi et al. 2008a).

JUVENILE HORMONE RECEPTORS

Although receptor proteins for the pro-metamorphic and moulting hormone 20E were identified seventeen years ago (Koelle et al. 1991, Yao et al. 1992, Thomas et al. 1993), the JH receptor is unknown to date. Like ecdysone, JH is a small lipophilic molecule capable of penetrating cell membranes to the nucleus, where it presumably regulates transcription of specific genes. Two nuclear proteins, encoded by the *ultraspiracle (usp)* and *Methoprene-tolerant (Met)* genes, have been the most favoured JH receptor candidates of last years (Gilbert et al. 2000, Truman and Riddiford 2002, Berger and Dubrovsky 2005, Goodman and Granger 2005, Riddiford 2008). However, there is also evidence that JH signals by a non-genomic pathway via a plasma membrane receptor (Wheeler and Nijhout 2003). The main question is whether JH merely acts by modulating the ecdysteroid signalling molecules or whether it uses its own signal transduction pathway.

Ultraspiracle (Usp)

Usp is a nuclear hormone receptor, an insect homolog of the vertebrate retinoid X receptor (RXR) (Henrich et al. 1990, Oro et al. 1990, Shea et al. 1990). Like RXR, it functions as a heterodimeric partner of other nuclear receptors, including the receptor of 20E (EcR) (Yao et al. 1992, Thomas et al. 1993, Henrich et al. 1994, Sutherland et al. 1995, Hall and Thummel 1998). *usp* expression responds to both 20E and JH (Hiruma et al. 1999, Barchuk et al. 2004). These facts make Usp an attractive candidate through which JH might modulate 20E effects.

Drosophila Usp has a large hydrophobic pocket that could be occupied by a lipophilic ligand (Clayton et al. 2001, Billas et al. 2001). It has been proposed that the ligand might be JH, because JH can bind, even though with a low affinity, the *Drosophila* Usp protein and can cause its conformational change that in turn leads to a reporter gene activation (Jones and Sharp 1997, Jones et al. 2001, Xu et al. 2002, Maki et al. 2004, Fang et al. 2005). Later work has shown that Usp binds a JH precursor methyl farnesoate with a much higher affinity than that for JH itself (Jones et al. 2006), but whether methyl farnesoate can be a transcriptionally activating ligand is unclear. A recent finding that further complicates our understanding of

Usp function suggests that the Usp/RXR ortholog from the locust (*Locusta migratoria*) binds, at nanomolar rates, retinoic acid, which naturally occurs in the locust embryos (Nowickyj et al. 2008).

Other findings argue against the possibility that Usp is the JH receptor. (1) It has been shown that *usp* of mecopteroid insects, including dipterans, has undergone rapid evolution and thus differs markedly from *usp* of other insects (Bonneton et al. 2003, 2006) Crystallographic studies on the non-mecopteroid Usp/RXR ortholog from *Tribolium* have revealed that the ancestral type of this insect protein cannot be activated by either juvenile hormone or retinoic acid ligands in vitro or in vivo (Iwema et al. 2007, but see contradictory evidence for retinoic acid binding by locust Usp/RXR [Nowickyj et al. 2008]). (2) The non-mecopteroid locust Usp/RXR does not bind JH either alone or in combination with EcR (Hayward et al. 2003). (3) *Drosophila* Usp binds functional JH weakly, at non-physiological doses, and biological significance of higher-affinity ligands such as methyl farnesoate (Jones et al. 2006) is unclear. These ligands might be specific to the structurally derived type of *Drosophila* Usp. (4) Finally and perhaps most importantly, loss-of-function phenotypes observed for *usp* in *Drosophila* (Henrich et al. 1994, Hall and Thummel 1998) or in other insects (Martin et al. 2006, Barchuk et al. 2008, Tan and Palli 2008, and our unpublished data) do not support deficiency of JH signalling. Taken together, although Usp likely participates somehow in JH signalling, it probably does not function as the bona fide JH receptor.

Methoprene-tolerant (Met)

Role of *Met* in development

Met is a basic helix-loop-helix (bHLH)-PAS transcription factor (Ashok et al. 1998, Miura et al. 2005). *Met* mutations render *Drosophila* resistant to morphogenetic effects of JH and its mimics, e.g. methoprene (Wilson and Fabian 1986, Riddiford and Ashburner 1991, Wilson et al. 2003). Met binds JH and methoprene, but not structurally related compounds lacking JH activity, at nanomolar (physiological) concentrations (Miura et al. 2005). Met is present in embryos and larval and imaginal tissues (including larval and adult salivary glands, gut cells, imaginal discs, abdominal histoblasts in pupae and reproductive organs) that represent JH target sites (Pursley et al. 2000). *Drosophila Met* genetically interacts with a 20E- and JH-response gene *Broad-Complex (BR-C)* (Wilson et al. 2006a; and see below).

Drosophila Met has a paralog, the *germ-cell expressed (gce)* gene (Godlewski et al. 2006, Wang et al. 2006). *gce* mRNA occurs in early embryos and later in a subset of germ cells (Moore et al. 2000). In other insect genomes including basal dipteran mosquitoes, only single

Met/gce orthologs have been found (Wang et al. 2006). Based on sequence comparisons and intron positions *gce* was identified as the ancestral gene of the two *Drosophila* paralogs. Although neither *Drosophila gce* function nor sites of its expression during postembryonic stages are known, it is suggested to co-operate with *Met* based on direct interaction of the two protein products (Godlewski et al. 2006).

The existence of *gce* may explain the absence of notable developmental defects in *Met* mutants. Even flies with a *Met* null mutation develop normally to viable fertile adults (Wilson and Ashok 1998, Wilson et al. 2006b) Slight anomalies in these mutants include slower pupal development, increased pupal mortality and delayed onset of egg laying (Minkoff and Wilson 1992), lack of bristles between posterior ommatidia in compound eyes (Wilson et al. 2006a), lower interest in courtship and mating in males (Wilson et al. 2003), and reduced protein synthesis stimulated by JH in the male accessory glands (Shemshedini et al. 1990). Although functional redundancy between *gce* and *Met* could explain lack of more severe phenotypes that would reflect disrupted JH signalling (Wilson et al. 2006b), it is unclear how mere absence of Met causes resistance to JH toxicity. Overexpression of *Met* leads to death during larval stages and shifts methoprene-induced lethality from pharate adults to larvae; lethality is perhaps caused by changed Met/Gce ratio (Barry et al. 2008). In *Drosophila* S2 cells, Met is not required for JH-dependent suppression of antimicrobial peptides that become transcriptionally induced by an immune challenge in the presence of 20E (Flatt et al. 2008). Whether *gce* substitutes for *Met* in the innate immune response has not been tested.

Although the functional significance of each of the *Drosophila* paralogs for JH signalling has yet to be clarified, studies on their single ortholog in the beetle *Tribolium* have brought evidence that *Met* is critical for the JH-regulated entry into metamorphosis (Konopova and Jindra 2007), an affirmative proof that had been lacking in *Drosophila*. Finally, *Tribolium Met* is required for JH-regulated expression of the *BR-C* and *Krüppel-homolog 1 (Kr-h1)* genes. Whether Met acts as the JH receptor, its essential component or at another level of the JH signal transduction still needs to be determined, but as of now, Met can be considered the best JH receptor candidate.

How could Met transmit the JH signal?

Being a bHLH/PAS family member, Met is expected to function with a partner protein in gene regulation. This partner might most likely be a bHLH/PAS protein and/or a nuclear receptor.

A model bHLH/PAS transcription factor is the Aryl hydrocarbon (dioxin) receptor (AhR), the only vertebrate member of the family known to be bound and activated by small chemical ligands (reviewed in Furness et al. 2007). AhR resides in the cell cytosol bound to chaperones and upon ligand binding to the PAS-B domain, it translocates to the nucleus. Nuclear AhR releases chaperones and binds its partner, another bHLH/PAS family member, the Aryl hydrocarbon receptor nuclear translocator (ARNT), to upregulate expression of xenobiotic metabolising enzymes. Apart from mediating the xenobiotic response, AhR has a physiological function and can also be activated by endogenous ligands; the xenobiotic-detoxifying function could have been secondarily acquired (Barouki et al. 2007). The ligand-activated AhR/ARNT heterodimer was shown to bind the estrogen nuclear receptor and regulate its transcriptional activity (Ohtake et al. 2003). AhR also functions as a ubiquitin ligase that upon ligand binding selectively targets sex steroid receptors for protein degradation, which leads to suppressed response to sex hormones (Ohtake et al. 2007). ARNT serves as a universal partner for several bHLH/PAS proteins including AhR, the Hypoxia induced factor (HIF-1alpha, and others). The insect homologs of AhR and Arnt are called Spineless and Tango, respectively (Sonnenfeld et al. 1997, Emmons et al. 1999).

Immunoprecipitation assays showed that *Drosophila* Met had the capacity to form homodimers or heterodimers with Gce; these interactions were weakened in the presence of JH (Godlewski et al. 2006). Met did not interact with the promiscuous bHLH/PAS partner Tango (Godlewski et al. 2006). Yeast two-hybrid assays indicated that *Drosophila* Met formed complexes with each component of the ecdysone receptor dimer EcR-Usp (Li et al. 2007), thus suggesting a mechanism through which JH might modulate the ecdysteroid response (Dubrovsky 2005). How exactly could the presence of liganded or unliganded Met affect signalling by the ecdysone receptor complex is a matter of future studies.

Plasma membrane receptor

There is now evidence that JH might also act by a non-genomic pathway via a plasma membrane receptor and protein kinase C (Wheeler and Nijhout 2003, and references therein). The identity of the plasma membrane receptor is yet unknown. Probably both a genomic pathway regulating gene transcription and a faster operating non-genomic pathway affecting protein activity are integrated into execution of the JH effects, similarly as has been shown for ecdysone (Iga et al. 2007).

BROAD-COMPLEX (BR-C), A KEY JH-DEPENDENT REGULATOR OF METAMORPHOSIS

BR-C encodes several BTB-zinc finger transcription factors produced by alternative splicing from the single gene. Four *Drosophila* BR-C isoforms, Z1-Z4, differ in the carboxy-terminal DNA-binding domain containing C_2H_2 zinc-finger pairs (DiBello et al. 1991, Bayer et al. 1996). The common BTB is thought to enable BR-C homo- and heterodimerization and recruitment of corepressors (Bardwell and Treisman 1994, Perez-Torrado et al. 2006).

In *Drosophila BR-C* is a primary 20E-response gene whose expression sharply rises during larva to pupa metamorphosis. Loss of *BR-C* function in null mutants leads to death in the third (final) larval instar and inability to initiate metamorphosis (Kiss et al. 1988). Understanding of *BR-C* role in metamorphosis of diverse tissues comes primarily from studies on mutants of three fully complementing genetic loci: *br* (*broad*), *rbp* (*reduced bristle number on palpus*) and *2Bc* (Belyaeva et al. 1980, Kiss et al. 1988) that lack Z1, Z2 and Z3 isoforms, respectively (Bayer et al. 1997, Crossgrove et al. 1996, Emery et al. 1994). *BR-C* in *Drosophila* is thus required for secretion of glue proteins from larval salivary glands (Guai and Guild 1991, Karim et al. 1993, Crossgrove et al. 1996) and for the glands' subsequent degeneration by programmed cell death (Jiang et al. 2000). *BR-C* activity transforms the apolysed final larval cuticle into the sclerotized puparium (Hodgetts et al. 1995, Bayer et al. 1997). During pupal development, *BR-C* enables rebuilding the larval body into the adult one, being essential for elongation, eversion and fusion of imaginal discs (Kiss et al. 1988), for development of flight muscles (Restifo and White 1992) and specific bristles (Belyaeva et al. 1980), for compound eye morphogenesis and photoreceptor specification (Brennan et al. 2001), for remodelling of the central nervous system (Restifo and White 1991), the fat body (Emery et al. 1994, Bayer et al. 1997, Mugat et al. 2000) and the midgut (Restifo and White 1992), and also for degeneration of the prothoracic gland (Zhou et al. 2004).

Although *BR-C* transcripts were found in embryos as well as during larval stages (Fletcher and Thummel 1995, Zhou et al. 2004), development prior to metamorphosis proceeds normally without *BR-C* (Kiss et al. 1988). Embryonic and larval lethality was observed in double mutants for *BR-C* and *E74*, another 20E-response gene that alone also first becomes necessary at metamorphosis (Fletcher and Thummel 1995). This genetic interaction illustrates that diverse combinations of interacting proteins specify the fate of discrete organs during development.

Gain-of-function experiments in transgenic flies defined *BR-C* as a major specifier of pupal development and an essential mediator of the JH signal. After pupal ecdysis *BR-C* expression sharply drops and has to stay low or absent for proper pupa to adult metamorphosis to occur (Zhou and Riddiford 2002). Ectopic JH application that leads to the secretion of another pupal instead of adult cuticle on the abdomen re-induces *BR-C* expression in the entire pupal body. Mere *BR-C* misexpression in the pupa mimics this JH effect, thus bringing evidence that *BR-C* is a true executor of the JH signal at this stage. Conversely, early *BR-C* misexpression in second instar larvae caused premature expression of a pupal cuticle gene and suppression of a larval-specific cuticle gene, thus bringing evidence that *BR-C* in *Drosophila* functions as a "pupal specifier".

Further demonstration of BR-C role in *Drosophila* JH signalling is its genetic interaction with the JH binding protein Met (Wilson et al. 2006a). There is a shift in the lethal phase in *rbp Met* or *br Met* double mutants into earlier stages of metamorphosis, and absence of *Met* also reduces complementation between particular *BR-C* alleles. To explain why *BR-C* mutation and treatment with the JH mimic methoprene produce similar phenotypes, a hypothesis was proposed that during the period of low JH level at the beginning of metamorphosis BR-C binds the non-liganded Met and thus regulates expression of pro-metamorphic genes (Wilson et al. 2006a).

Functional studies in lepidopteran (*Bombyx mori*), coleopteran (*Tribolium castaneum*) and neuropteran (*Chrysopa perla*) species have demonstrated a conserved role of *BR-C* in the holometabolous metamorphosis. *BR-C* function was dispensable during larval growth, but its absence led to severe lethal defects during larva-pupa metamorphosis (Uhlirova et al. 2003, Konopova and Jindra 2008, Parthasarathy 2008a, Suzuki et al. 2008). For instance, *BR-C* absence perturbed pupal cuticle differentiation, wing extension and compound eye development like in *Drosophila*. On the contrary, loss of *BR-C* in *Tribolium* and *Chrysopa* slightly accelerated differentiation of adult characters in some tissues, while it still allowed transition to the pupal state in others (Konopova and Jindra 2008, Parthasarathy 2008a, Suzuki et al. 2008). The lethal period in *BR-C(RNAi)* animals was in accordance with low *BR-C* expression in larvae and its increase at the end of the final larval instar. It was found in the lepidopteran *Manduca sexta* that *BR-C* repression in larvae was caused by high hemolymph JH titer, and only JH decline in the middle of the final larval instar allowed pupal commitment and *BR-C* induction by ecdysone (Zhou et al. 1998). *BR-C* regulation by JH in

Tribolium pupae (Konopova and Jindra 2008) was similar to that in *Drosophila* (Zhou and Riddiford 2002).

In a JH-dependent manner, *BR-C* also regulates hemimetabolous development (Erezyilmaz et al. 2006). By contrast to holometabolans, *BR-C* in the hemimetabolous true bug (*Oncopeltus fasciatus*) was highly expressed in larvae but then, similar to the situation in the holometabolans, its expression ceased during adult differentiation. *BR-C* silencing by RNAi in *Oncopeltus* larvae prevented proper wing growth and changes in body color pattern between instars. Thus, if RNAi is similarly effective in *Oncopeltus* and holometabolans, then the hemimetabolous development is less dependent on *BR-C* function. Since the *BR-C* gene is found in several other non-holometabolans (Erezyilmaz et al. 2006, Konopova and Jindra, unpublished data) it will be of interest to examine the role of *BR-C* in other species' development a find out how *BR-C* function might have changed during evolution.

RESULTS

The following section is composed of two published articles and additional unpublished results.

Published articles:

Konopova B, Jindra M. 2007. Juvenile hormone resistance gene *Methoprene-tolerant* controls entry into metamorphosis in the beetle *Tribolium castaneum*. *Proc Natl Acad Sci USA* 104:10488-10493.

Konopova B, Jindra M. 2008. Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolan metamorphosis. *Development* 135:559-568.

Juvenile hormone resistance gene *Methoprene-tolerant* controls entry into metamorphosis in the beetle *Tribolium castaneum*.

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Besides being a spectacular developmental process, metamorphosis is key to insect success. Entry into metamorphosis is controlled by juvenile hormone (JH). In larvae, JH prevents pupal and adult morphogenesis, thus keeping the insect in its immature state. How JH signals to preclude metamorphosis is poorly understood, and a JH receptor remains unknown. One candidate for the JH receptor role is the Methoprene-tolerant (Met) Per-Arnt-Sim (PAS) domain protein [also called Resistance to JH, Rst (1)JH], whose loss confers tolerance to JH and its mimic methoprene in the fruit fly *Drosophila melanogaster*. However, *Met* deficiency does not affect the larval–pupal transition, possibly because this process does not require JH absence in *Drosophila*. By contrast, the red flour beetle *Tribolium castaneum* is sensitive to developmental regulation by JH, thus making an ideal system to examine the role of *Met* in the antimetamorphic JH action. Here we show that impaired function of the *Met* ortholog *TcMet* renders *Tribolium* resistant to the effects of ectopic JH and, in a striking contrast to *Drosophila*, causes early-stage beetle larvae to undergo precocious metamorphosis. This is evident as *TcMet*-deficient larvae pupate prematurely or develop specific heterochronic phenotypes such as pupal-like cuticular structures, appendages, and compound eyes. Our results demonstrate that *TcMet* functions in JH response and provide the critical evidence that the putative JH receptor Met mediates the antimetamorphic effect of JH.

Metamorfóza je pozoruhodný okamžik ve vývoji hmyzu, jehož spuštění je regulováno juvenilním hormonem (JH). U larev, JH brzdí morfogenezi v kuklu a dospělce, a udržuje tak jedince v juvenilním stadiu. Jen málo rozumíme tomu, jakým způsobem signál JH brání metamorfóze, receptor JH není znám. Jedním z kandidátů na JH receptor je Methoprene-tolerant (Met) [též zván Resistance to JH, Rst (1)JH], protein obsahující Per-Arnt-Sim (PAS) domény, jehož ztráta způsobuje u octomilky *Drosophila melanogaster* necitlivost k JH a jeho analogu methoprenu. Avšak absence genu *Met* nemá vliv na larválně-kuklovou přeměnu, pravděpodobně proto, že tento proces není u *Drosophila* tolik závislý na nepřítomnosti JH. Naopak vývoj brouka potemníčka *Tribolium castaneum* je citlivý k regulačním účinkům JH, což z tohoto druhu činí ideální model pro výzkum role *Met* v procesu, kterým JH brání metamorfóze. My zde ukazujeme, že ztráta funkce *Tribolium* orthologu genu *Met*, *TcMet*, způsobuje rezistenci vůči efektům ektopického JH a narozdíl od *Drosophila*, vede k předčasné metamorfóze mladých broučích larev. Larvy s narušenou funkcí *TcMet* se totiž kuklí předčasně anebo vykazují jiné známky vývojové heterochronie, jako přítomnost kuklově specifických kutikulárních útvarů, kuklově utvářených končetin a vývojem složených očí. Naše výsledky demonstrují, že *TcMet* funguje v odpovědi na JH a přinášíme významný důkaz, že domnělý receptor JH Met, zprostředkovává metamorfózu inhibující efekt JH.

BK’s contribution: 90%

Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolan metamorphosis.

Konopova B, Jindra M. 2008. Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolan metamorphosis. *Development* 135:559-568.

Metamorphosis of holometabolous insects, an elaborate change of form between larval, pupal and adult stages, offers an ideal system to study the regulation of morphogenetic processes by hormonal signals. Metamorphosis involves growth and differentiation, tissue remodeling and death, all of which are orchestrated by the morphogenesis-promoting ecdysteroids and the antagonistically acting juvenile hormone (JH), whose presence precludes the metamorphic changes. How target tissues interpret this combinatorial effect of the two hormonal cues is poorly understood, mainly because JH does not prevent larval-pupal transformation in the derived *Drosophila* model, and because the JH receptor is unknown. We have recently used the red flour beetle *Tribolium castaneum* to show that JH controls entry to metamorphosis via its putative receptor Methoprene-tolerant (Met). Here, we demonstrate that Met mediates JH effects on the expression of the ecdysteroid-response gene *Broad-Complex* (*BR-C*). Using RNAi and a classical mutant, we show that *Tribolium BR-C* is necessary for differentiation of pupal characters. Furthermore, heterochronic combinations of retarded and accelerated phenotypes caused by impaired *BR-C* function suggest that besides specifying the pupal fate, *BR-C* operates as a temporal coordinator of hormonally regulated morphogenetic events across epidermal tissues. Similar results were also obtained when using the lacewing *Chrysopa perla* (Neuroptera), a member of another holometabolous group with a primitive type of metamorphosis. The tissue coordination role of BR-C may therefore be a part of the Holometabola groundplan.

Metamorfóza holometabolního hmyzu je složitá vývojová změna mezi larvou, kuklou a dospělcem, a poskytuje ideální modelový systém pro studium hormonální regulace morfogeneze. Metamorfóza zahrnuje růst a diferenciaci, přetváření tkání a buněčnou smrt. Všechny tyto procesy jsou regulovány jednak ekdysteroidy, které podněcují morfogenezi a jednak antagonisticky působícím juvenilním hormonem (JH), jehož přítomnost brání metamorfickým změnám. Jen málo rozumíme tomu, jak cílové tkáně vyhodnocují společné působení těchto dvou hormonálních podnětů, a to především proto, že JH neovlivňuje larválně kuklovou proměnu u modelu *Drosophila,* a protože JH receptor není znám. My jsme na broukovi *Tribolium castaneum* nedávno ukázali, že JH řídí vstup do metamorfózy přes Methoprene-tolerant (Met), domnělý receptor JH. Zde demonstrujeme, že Met zprostředkovává JH efekt regulací exprese *Broad-Complex (BR-C)*, genu řízeného ecdysteroidy. Pomocí RNAi a klasického mutanta ukazujeme, že *BR-C* u T*ribolium* je nezbytný pro diferencici kuklových znaků. Heterochronní fenotypy vyvolané narušením funkce *BR-C* a projevující se zpožděním a urychlením vývoje naznačují, že kromě specifikace kuklového stavu, *BR-C* funguje jako časový koordinátor hormonálně řízených morfogenetických procesů v epidermálních tkáních. Podobné výsledky přinesly pokusy se zlatoočkou *Chrysopa perla* (Neuroptera), zástupcem další holometabolní skupiny s primitivním typem metamorfózy. Tkáňově koordinační role BR-C tak může být součástí základního plánu Holometabola.

BK’s contribution: 85%

ROLE OF THE *METHOPRENE-TOLERANT (MET)* GENE IN HEMIMETABOLOUS DEVELOPMENT (UNPUBLISHED RESULTS)

There is now strong evidence that *Met* is a key mediator of the JH signal and a regulator of metamorphosis in holometabolous insects (Konopova and Jindra 2007, 2008). However, it is unknown whether *Met* has a similar role in species with other types of development. To test Met function in a non-holometabolan insect, I chose the linden bug *Pyrrhocoris apterus* (Hemiptera). By touch-down PCR with degenerate primers followed by RACE reactions I isolated a fragment of a *Pyrrhocoris Met* ortholog, *PaMet*. A 953-bp *PaMet* sequence was used for RNAi studies.

Pyrrhocoris develops by five larval instars in which wings grow as black immobile wing pads (Fig. 2A). During metamorphosis, wings elongate, become articulated and change colour (Fig. 2A). Melanin pigment disappears from particular wing areas so that the underlying red epidermal pigment becomes visible (Socha 1993). When I injected early second instar larvae with *PaMet* dsRNA, all (n=11) moulted to normal third instar, but after another moult 8 (= 73%) showed precocious differentiation of adult characters (Fig. 2B). None of the control larvae (n=6) that had been injected with heterologous *egfp* dsRNA showed any developmental abnormalities.

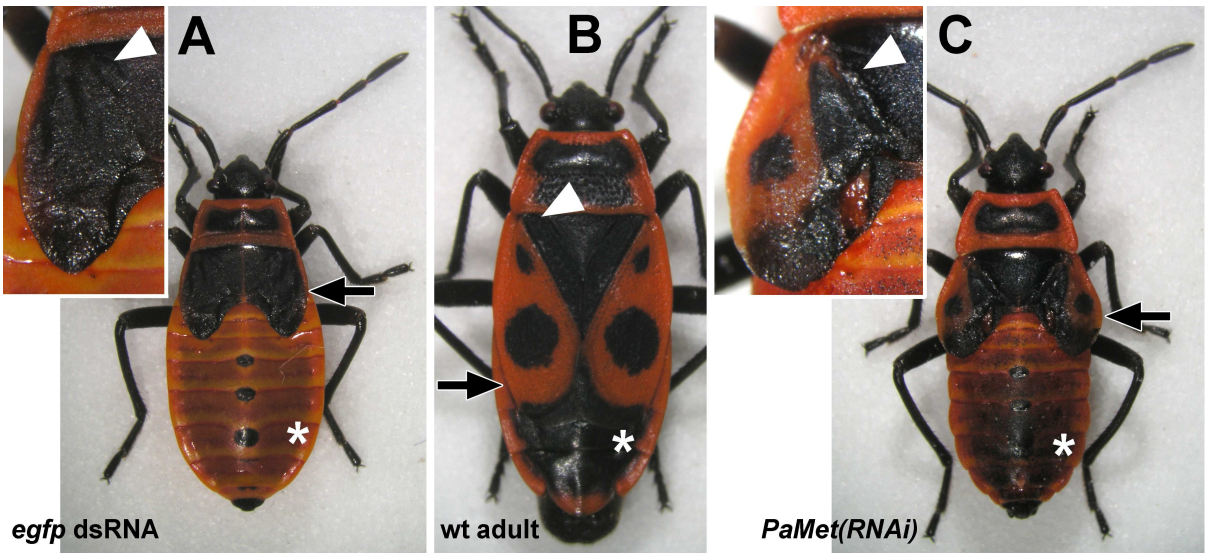

Figure 2: *PaMet* silencing in *Pyrrhocoris* induces precocious development of adult features. (**A**) Control larva that had been injected with *egfp* dsRNA, (**B**) wild-type adult (= a post-metamorphic stage), (**C**) a larva-adult intermediate that had been injected with *PaMet* dsRNA. *PaMet* silencing led to wing coloration similar to that of adults (compare arrow in C with A and B). While in control larvae wing pads were firmly attached to terga, wing pads of *PaMet(RNAi)* individuals were separated by a suture, suggesting that wing articulation enabling wing folding in adults is being formed (compare arrowheads in A-C). While abdomens of control larvae is orange, dark color (melanin) appears on abdomens of *PaMet(RNAi)* animals, thus mimicking the situation in adults (compare asterisks in A-C). Larvae had been injected in early second instar and photographed in the fourth instar. Anterior is to the top in all panels.

This experiment demonstrated that *Met* function in regulation of the entry into metamorphosis has been conserved between holometabolous and hemimetabolous insects. Next step in this project will be to test whether *PaMet* also mediates the JH signal.

ROLE OF THE *KRÜPPEL-HOMOLOG 1 (KR-H1)* GENE IN INSECT METAMORPHOSIS (UNPUBLISHED RESULTS)

Next to *BR-C*, *Kr-h1* is another, only less studied *Drosophila* zinc finger gene (Schuh et al. 1986) that mediates both ecdysone and JH response. In fly embryos *Kr-h1* expression is centered to neurons (Beck et al. 2004), but later it expands and becomes ubiquitous at the onset of metamorphosis (Beck et al. 2005). *Kr-h1* mutants show lethality already during embryogenesis and early larval development, but *Kr-h1* function becomes most critical during the prepupal stage (Pecasse et al. 2000). By mediating the ecdysone primary response, *Drosophila Kr-h1* regulates a battery of genes including *BR-C* (Beck et al. 2004, 2005). In *Drosophila* pupae, *Kr-h1* is epistatic to *BR-C* during the response to ectopic JH (Minakuchi et al. 2008b). *Kr-h1* gene responds to JH also in *Apis* and *Tribolium* (Hewes 2008, Minakuchi et al. 2008b, Parthasarathy et al. 2008a). In *Tribolium* larvae, *Kr-h1* mRNA expression decreased after *Met* silencing and increased after *BR-C* silencing (Parthasarathy et al. 2008a,b).

My ongoing research aims to clarify *Kr-h1* function in the holometabolous *Tribolium* and in the hemimetabolous true bug *Rhodnius prolixus*. Preliminary results are summarised below.

Tribolium Kr-h1 (TcKr-h1) expression declines at time points critical for metamorphosis

I have isolated a cDNA of the *Tribolium Kr-h1* gene *(TcKr-h1)* that predicts 82% amino acid identity with its *Drosophila* ortholog in the conserved zinc finger domain. Expression of the *Kr-h1* transcript peaked in late embryos; then moderate levels were seen throughout the non-metamorphic 5th larval instar (Fig. 3). In the metamorphic final (8th) larval instar, *TcKr-h1* mRNA expression was low at the beginning and declined to undetectable levels at the end of the feeding period (48 hours), at which time induction of pupal development (pupal commitment, Riddiford 1976) probably takes place. Moderate levels of expression appeared in prepupae (i.e. pharate pupae, the last two days of the *Tribolium* final larval instar when pupal characters differentiate). The *TcKr-h1* transcript was undetectable during the pupal stage when metamorphosis to adult takes place.

Thus, *TcKr-h1* expression profile reflects metamorphic changes and its mRNA levels decrease at time points when in holometabolans JH has to be low for metamorphosis to occur (Nijhout 1994). This suggests that *Kr-h1* regulates multiple JH-dependent steps during beetle development.

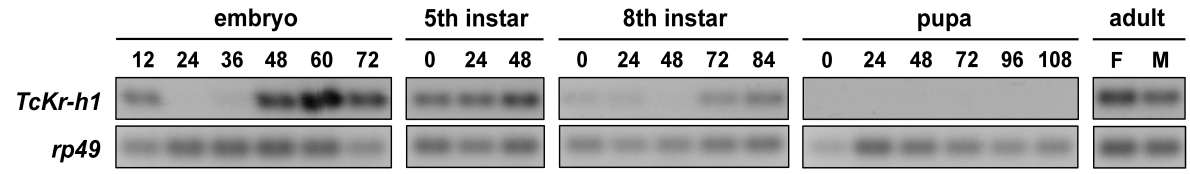

Figure 3: *TcKr-h1* expression in wild type *Tribolium*. Total RNA from the indicated stages was subjected to DNase treatment and RT-PCR with primers for *TcKr-h1* and control *rp49* genes. Numbers indicate hours since egg laying or ecdysis. F and M, 10-day old females and males, respectively. The prepupal stage begins at around 72 hours after the last larval ecdysis.

TcKr-h1 silencing in young *Tribolium* larvae induces precocious metamorphosis directly to imago

To see what is the role of *Kr-h1* in *Tribolium* metamorphosis, *TcKr-h1* was knocked down during the non-metamorphosing larval instar. Normally, *Tribolium* metamorphosis starts at the end of the final, seventh or eighth larval instar, when the larva enters the prepupal stage. Injection of *TcKr-h1* dsRNA at the beginning of the fifth larval instar led to formation of (eventually lethal) prepupae already at the end of the sixth instar (Fig. 4C) in all experimental animals (n=12). After I had removed the apolysed larval cuticle, seven of these animals resembled nearly perfect adults, while others looked like pupae or pupal-adult intermediates (compare Figs. 4D-F with A and B). No such heterochrony was seen in any individuals that had been injected with control *egfp* dsRNA.

These results suggest that the *TcKr-h1* function in the postembryonic beetle life is to block morphogenesis. First, *TcKr-h1* in young larvae keeps them at the larval stage, and decline of *TcKr-h1* triggers metamorphic changes. This is evidenced here by the fact that *TcKr-h1(RNAi)* larvae entered metamorphosis precociously. Second, *TcKr-h1* reappearance in prepupae normally interrupts the larva to adult metamorphosis that had just started and as a result the intermediary pupal stage appears. *TcKr-h1(RNAi)* prevented *Kr-h1* upregulation in the prepupal stage (Fig. 3), thus the larvae metamorphosed directly to adults, bypassing the pupa. Appearance of pupal features in some *TcKr-h1(RNAi)* animals was probably caused by a weaker RNAi effect. Pupal-adult intermediates (Fig. 4E) were likely caused by unequal

strength of RNAi among tissues. In summary, *TcKr-h1* is a vital regulator of holometabolous metamorphosis.

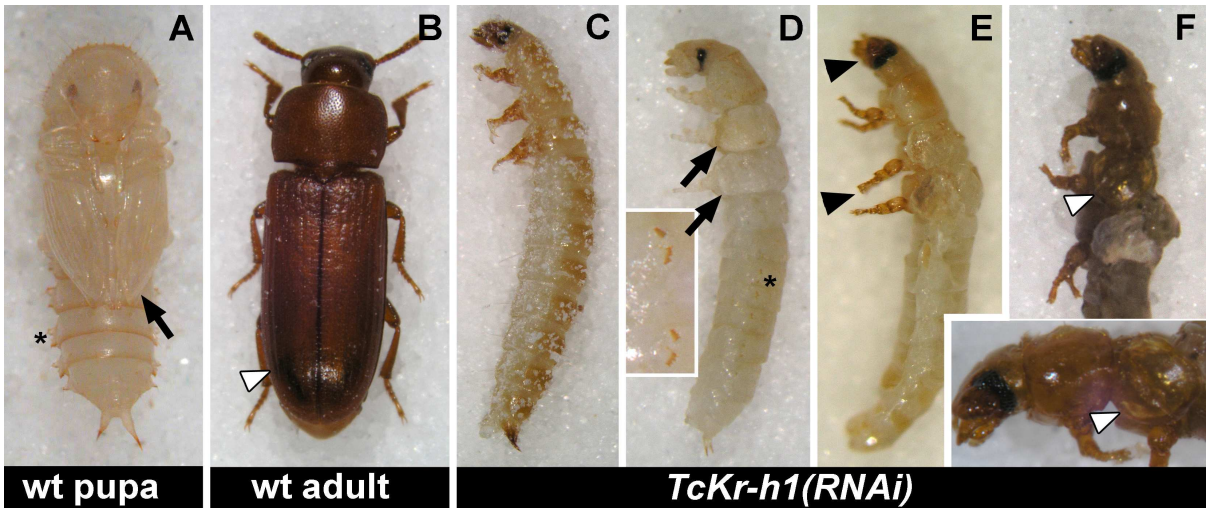

Figure 4: *TcKr-h1* silencing in *Tribolium* leads to precocious metamorphosis bypassing the pupal stage. (**A**) Wild-type pupa, (**B**) wild-type adult, (**C-F**) *TcKr-h1(RNAi)* phenotypes. *TcKr-h1* silencing in the fifth instar larvae leads to lethality in precocoius sixth instar prepupae (C). After manual removal of the larval cuticle the insects either resemble pupae with short wings (compare arrows in D and A) and pupal gin traps (asterisks in A and D and inset in D), or pupae with adult features (E), such as adult legs and anterior part of the head (black arrowheads), but most of them looked like adults (F) with short wings differentiated into elytrae (compare white arrowheads in F with B) and membraneous hind wings (not shown). Panels are not in scale. Anterior is to the top in all panels, to the left in the inset in F.

TcKr-h1 is a target of *TcMet*

Next, I wanted to see what is the relationship between *TcKr-h1* and *TcMet*. We have shown (Konopova and Jindra 2008) that in *Tribolium* pupae *TcMet* function is required for lethal effects of ectopic JH and for JH-dependent upregulation of the *TcBR-C* gene. I used cDNA samples from these same experiments and repeated the RT-PCR with primers for the *TcKr-h1* gene. Like *TcBR-C* (Fig. 5A, also see Konopova and Jindra 2008), expression of *TcKr-h*1 remains induced during all four days of pupal development once the JH mimic methoprene had been applied to freshly ecdysed pupae (Fig. 5A). *TcMet* silencing before methoprene treatment prevents this upregulation of *TcKr-h1* by methoprene (Fig. 5B) in the same way it prevents misexpression of *TcBR-C* (Fig. 5B, Konopova and Jindra 2008). These results showed that *TcKr-h1* is another gene regulated by *TcMet* in response to JH. Future experiments are necessary to clarify what is the relationship between *TcKr-h1* and *TcBR-C*.

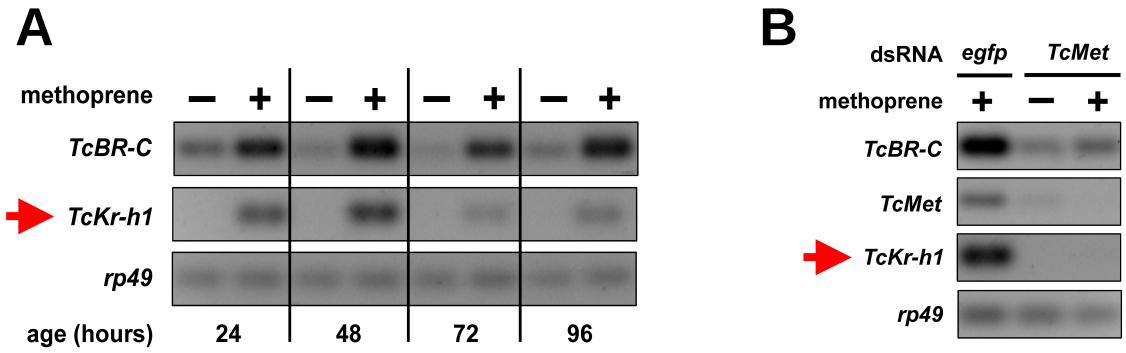

Figure 5: *TcMet* is required for *TcKr-h1* upregulation by the JH mimic methoprene. (**A**) *Tribolium* pupae aged up to 1 hour after ecdysis were briefly dipped into 0.3 mM methoprene or its solvent acetone (–) and tested (3-4 pupae per sample) for *TcKr-h1* and *TcBR-C* mRNA expression at the indicated times (adults normally emerge after 108-120 hours). *TcBR-C* expression was reported previously and is shown here to demonstrate similarity in the response. Although *TcKr-h1* expression in control pupae was undetectable, similar to that in intact pupae of corresponding age (see Fig. 3), it was markedly induced by methoprene. (**B**) Early prepupae were injected either with *egfp* or *TcMet* dsRNA, and within four hours after pupal ecdysis were treated with methoprene or acetone. Shown are examples of *TcKr-h1* mRNA expression in two individual pupae aged 48 hours. Methoprene could not induce *TcKr-h1* in *TcMet(RNAi)* pupae. Similar results were obtained with all examined pupae (at least eight for each treatment), aged either 48, 72 or 96 hours. Arrows highlight *TcKr-h1* expression. Expression of *rp49* serves for control.

Kr-h1 silencing in the hemimetabolous true bug *Rhodnius prolixus* leads to precocious wing metamorphosis

Finally, to see whether *Kr-h1* plays any essential role also in non-holometabolous development I examined loss-of-function phenotypes in the hemimetabolous true bug *Rhodnius prolixus*. A 940-bp fragment of *Rhodnius Kr-h1 (RpKr-h1)* cDNA that I isolated by RT-PCR was used as a template for dsRNA synthesis for RNAi experiments.

RpKr-h1 silencing in *Rhodnius* larvae leads to precocious wing metamorphosis (Fig. 6). Normally, *Rhodnius* develops by five larval instars. Wings grow externally as non-articulated lobes; articulation and venation of wings normally only develops during metamorphosis to the adult stage (Fig. 6B). In contrast, larvae injected with *RpKr-h1* dsRNA in the second instar possessed wings with marks of precocious articulation and venation already after the next moult (Fig. 6C). Nine of ten injected larvae had the strong phenotype shown in Fig. 6C; in one larva the effect was weaker. A few animals also displayed precocious development of the genitals (not shown). None of the larvae injected with the control *egfp* dsRNA (n=9) developed aberrantly, their wings remained firmly attached to terga and lacked venation (Fig. 6A).

These results demonstrate that regulation of metamorphosis by Kr-h1 is common to hemimetabolous and holometabolous insects. *Kr-h1* silencing in *Rhodnius* produced less pronounced phenotypes than it did in *Tribolium*; perhaps it was due to a weaker RNAi effect in *Rhodnius* or its a lower sensitivity to reduced *Kr-h1* function in tissues other than the wings. Future studies are needed to examine the role of *RpKr-h1* in detail, particularly changes of its expression during development and in response to hormonal cues.

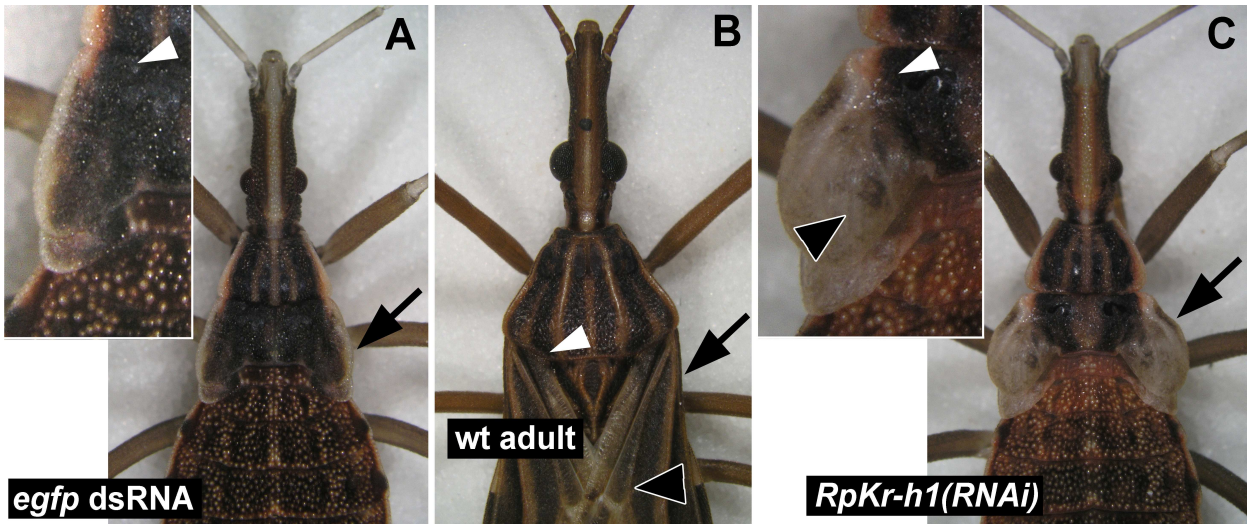

Figure 6: *RpKr-h1* silencing in *Rhodnius* larvae induces precocious wing metamorphosis. (**A**) Control larva injected with *egfp* dsRNA, (**B**) wild-type adult, (**C**) *RpKr-h1(RNAi)* larva. *RpKr-h1* silencing in second instar larvae led to premature maturation of wing pads during the next moult. Although these aberrant wing pads remained small (compare arrow in C with those in A and B; only part of adult wings covering the entire abdomen is shown) they developed wing venation like post-metamorphic wings of adults (compare black arrowheads in C and B). *RpKr-h1(RNAi)* wing pads showed hints of wing articulation that is normally lacking in larvae, but which later enables adult wing movement and folding (compare white arrowhead in C with A and B). No heterochrony was observed in control larvae (A). Anterior is to the top in all panels.

Preliminary project conclusions

In summary, the initial results of this project have characterized *Kr-h1* as a vital regulator of metamorphosis and a new molecular component of Met-mediated JH signalling. The finding that *Kr-h1* has a key role in metamorphosis of both holometabolous and hemimetabolous insects suggests that studies on *Kr-h1* function in insects of diverse developmental types will bring important information about the hormonal regulation of metamorphosis and its evolution.

CONCLUSIONS

***Met* is a core mediator of the anti-metamorphic JH function**

By using RNAi I showed that depletion of *Met* in larvae of the holometabolous beetle *Tribolium castaneum* as well as a hemimetabolous true bug *Pyrrhocoris apterus* was sufficient to induce their metamorphosis prematurely, i.e. before completing the normal number of larval instars. Such a phenotype was consistent with the anti-metamorphic JH effect because it phenocopied loss of the hormone itself. Loss of *Met* also protected *Tribolium* from lethal effects of ectopic JH, thus proving that *Met* was necessary for JH function (Konopova and Jindra 2007, and unpublished results). My results supported the long-debated role of *Met* as the missing JH receptor. *In vitro* and cell culture experiments with *Tribolium* Met aimed to clarify this role are underway.

Met* mediates JH response through downstream target genes, *BR-C* and *Kr-h1

Experiments in *Tribolium* pupae showed that *Met* function was required for ectopic induction of the *BR-C* and *Kr-h1* genes by added JH, thus demonstrating that *BR-C* and *Kr-h1* require Met to be induced by JH in vivo (Konopova and Jindra 2008, and unpublished results).

***BR-C* of holometabolans primarily serves as a co-ordinator of tissue morphogenesis at the larva-to-pupa metamorphosis rather than a strict pupal specifier**

In the complex *Drosophila* metamorphosis *BR-C* specifies the pupal stage. In my experiments, *BR-C* silencing in *Tribolium* and the neuropteran lacewing *Chrysopa perla*, both with primitive type of holometaboly, led to unexpected heterochronic phenotypes with larval, pupal and adult features all combined. I hypothesise that the ancestral role of *BR-C* in holometabolans was probably not to strictly specify the pupal stage but to coordinate precise developmental timing at the onset of metamorphosis (Konopova and Jindra 2008).

***Kr-h1* is an anti-metamorphic gene**

My unpublished results showed that *Kr-h1* silencing in *Tribolium* and in a true bug *Rhodnius prolixus* led to precocious metamorphosis, thus showing that *Kr-h1* functions as an essential anti-metamorphic factor whose role has been conserved between holometabolous and hemimetabolous insects.

In summary, my Ph.D. research has yielded two articles published in peer-reviewed journals and was presented at four international conferences. Unpublished data outlined in this thesis are being completed and prepared for publication in the near future.

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Konopova B, Jindra M. Key role of the Methoprene-tolerant gene in metamorphosis of the beetle Tribolium castaneum. 9th International Conference on Juvenile Hormones, York (UK), August 2007.

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Konopova B, Jindra M. Deciphering the genetics of insect metamorphosis. 2nd Meeting of the European Society for Evolutionary Developmental Biology, Ghent (Belgium), July-August 2008.