School of Doctoral Studies in Biological Sciences

University of South Bohemia in České Budějovice

Faculty of Science

Juvenile hormone signaling in insect development and reproduction

Ph.D. Thesis

Mgr. Vlastimil Smýkal

Supervisor: Prof. RNDr. Marek Jindra, CSc.

Biology Centre of the Academy of Sciences of the Czech Republic,

Institute of Entomology, České Budějovice

České Budějovice 2014

This thesis should be cited as:
Smýkal, V., 2014, Juvenile hormone signaling in insect development and reproduction. Ph.D. Thesis. University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 52 pp.

Annotation

This thesis comprises three published papers and one manuscript, all focused on the role of juvenile hormone (JH), the JH receptor Methoprene-tolerant (Met) and its target gene *Krüppel-homolog 1* (*Kr-h1*) in insect development and reproduction. The JH-Met-Kr-h1 pathway is critical for metamorphic transition in hemimetabolan *Pyrrhocoris apterus* (Hemiptera) and holometabolan *Bombyx mori* (Lepidoptera) but seems to be dispensable during early larval postembryonic development. The results also show that Met and its heterodimeric partner Taiman (Tai) but not Kr-h1 are critical for ovarian development and vitellogenesis in *Pyrrhocoris* females. *In vitro*, *in vivo* and cell-based techniques in *Drosophila melanogaster* have demonstrated that Met and its paralog Gce are a *bona fide* receptor for JH. Only Gce capable of binding JH rescued *Drosophila* deficient for Met and Gce proteins, and the capacity of Gce to bind JH was necessary for JH-dependent transcriptional activation by Gce and Tai.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice, 29. 9. 2014 .....

Vlastimil Smýkal

This thesis originated from a partnership of Faculty of Science, University of South Bohemia, and Institute of Entomology, Biology Centre of the ASCR, supporting doctoral studies in the Molecular and Cellular Biology and Genetics study program.

Financial support

This work was supported by grants IAA500960906 from the Academy of Sciences, 6007665801 from the Ministry of Education of the Czech Republic and 204/09/H058 from the Czech Science Agency to Marek Jindra, grant 14-32654J from the Czech Science Agency and LH14029 (Kontakt II) from the Ministry of Education, Youth and Sports to David Doležel and Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No.25252059) to Tetsuro Shinoda.

Acknowledgements

I would like to thank my supervisor, prof. RNDr. Marek Jindra, CSc., for his guidance, financial support during my Ph.D. studies, help with writing the thesis and generally for introducing me into molecular genetics and science. I would like to thank Mgr. David Doležel, Ph.D. for financial support and for sharing information on gene sequences, Bc. Hana Vaněčková for help with insect rearing, and Dr. Martina Hajdušková, Ph.D. (http://www.biographix.cz/) for preparing figures used in my publications. My special thanks go to Dr. Tetsuro Shinoda (NIAS, Japan) for giving me the opportunity to work on my project in his lab and also to Dr. Takaaki Daimon (NIAS, Japan) for teaching me how to work with *Bombyx* and for his help with experiments and also for sharing unpublished information on *Bombyx*. I would also like to thank Dr. Masako Asahina Jindrová for help, and members of all above-mentioned labs for friendly working atmosphere. Finally, I want to thank my family and my wife for their overall support during my studies.

List of published papers and author's contribution

The thesis is based on the following papers (listed chronologically):

* equal contribution

I. Konopova*, B., **Smykal*, V.**, Jindra, M. (2011). Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. *PLoS ONE* 6 (12)**:** e28728. (IF = 4.092)
Vlastimil Smýkal participated in embryonic and larval staging, injecting of dsRNA into Pyrrhocoris larvae, their hormonal treatment and scoring of phenotypes. He also dissected larvae, collected tissue samples and analyzed gene expression using qRT-PCR. He also participated in data analysis and interpretation of results.

II. **Smykal*, V.**, Bajgar*, A., Provaznik, J., Fexova, S., Buricova, M., Takaki, K., Hodkova, M., Jindra, M., Dolezel, D. (2014). Juvenile hormone signaling during reproduction and development of the linden bug, *Pyrrhocoris apterus. Insect Biochem Mol Biol.* 45: 69-76. (IF = 3.234)
Vlastimil Smýkal performed most of the experiments on Pyrrhocoris larvae, including RNAi, hormonal treatments, dissecting and scoring of phenotypes. He also participated in dissecting adults, collecting tissue samples and analyzing gene expression using qRT-PCR. He also participated in data analysis and statistical evaluation of the results and writing of the manuscript.

III. **Smykal, V.**, Daimon, T., Kayukawa, T., Takaki, K., Shinoda, T., Jindra, M. (2014). Importance of juvenile hormone signaling arises with competence of insect larvae to metamorphose. *Dev Biol.* 390(2): 221-30. (IF = 3.868)
Vlastimil Smýkal generated vast majority of the data. He staged Pyrrhocoris and Bombyx larvae, injected Pyrrhocoris larvae with dsRNA and treated Bombyx larvae with methoprene, scored for the phenotypes, dissected larvae, processed the samples and analyzed gene expression using qRT-PCR. He also analyzed and statistically evaluated all the results and participated in writing of the manuscript.

IV. Jindra, M., **Smykal, V.**, Charles, J.P., Uhlirova, M., Hill, R.J. A Homolog of the Vertebrate Dioxin Receptor Is a Receptor for Arthropod Juvenile Hormone. (submitted)
Vlastimil Smýkal contributed by designing reporter plasmids for luciferase reporter assay in Drosophila S2 cells and performing these assays. He tested the transcriptional activation by the Drosophila wild-type Met and Gce and Tai, and mutated Gce proteins, dose-dependent response to JH, the precursor methyl farnesoate, and JH mimics. He also analyzed gene expression using qRT-PCR and participated in data analysis.

CONTENT

LIST OF ABBREVIATIONS

20E – 20-hydroxyecdysone

AhR – Aryl hydrocarbon receptor

bHLH – basic helix-loop-helix

BR-C – Broad complex

CA – Corpus allatum

Clc – Clock

Cyc – Cycle

E – Ecdysone

EcR – Ecdysone receptor

ET – Early trypsin

FA – farnesoic acid

FC – follicle cells

Ftz-F1 – Fushi tarazu transcription factor 1

FPP – farnesyl diphosphate

FISC – βFtz-F1-interacting steroid receptor coactivator

Gce – Germ cell-expressed

HCS – head capsule slippage

Hsp83/Hsp90 – Heat-shock protein83/Heat-shock protein 90

HRE – hormone response element

JH – juvenile hormone

JHAMT – JH acid O-methyltransferase

JHB3 – JH bisepoxide

JHBP – JH binding protein

JHEpo – JH epoxidase

JHEH – JH epoxide hydrolase

JHE – JH esterase

JHM – JH mimic

JHRE – JH response element

JHRR – JH response region

JHSB3 – JH skipped-bisepoxide

Kr-h1 –Krüppel-homolog 1

MEKRE93 – Met-Kr-h1-E93

Met – Methoprene-tolerant

MF – methyl farnesoate

NC – nurse cells

NES – nuclear export signal

NLS – nuclear localization signal

NR – nuclear receptor

PAC – PAS-associated C-terminal

PAS – Per-ARNT-Sim

PG – Prothoracic gland

PTTH – prothoracicotropic hormone

RNAi – RNA interference

RXR – Retinoid X Receptor

SRC – Steroid receptor coactivator

Tai – Taiman

TALEN – Transcription activator-like effector nuclease

Usp – Ultraspiracle

Vg – Vitellogenin

XRE – xenobiotic response element

Juvenile hormone signaling in insect development and reproduction

RESEARCH OBJECTIVES

Since the pioneering work of Wigglesworth in 1930's, juvenile hormone (JH) has been known as a critical regulator of insect development that controls the entry to metamorphosis. Molecular mechanism of JH function has been enigmatic for many years. In 1986, a screen for *Drosophila* mutants resistant to the JH analog methoprene uncovered Methoprene-tolerant (Met), later identified as a bHLH-PAS transcription factor, as a possible transducer of the JH signal. *Drosophila Met* null mutant larvae did not enter metamorphosis prematurely and adults were viable with only minor developmental defects and reduced fecundity. *Met* orthologs were found in all insects, and knockdown experiments in the basal holometabolan beetle *Tribolium* established Met as a JH-dependent regulator of metamorphosis that controls development through expression of target genes. The transcription factor Krüppel-homolog 1 was identified as a primary Met target in the antimetamorphic JH signaling.

The **first** goal of our studies was to demonstrate whether the JH-Met-Kr-h1 signaling pathway plays a conserved role in insects with hemimetabolan mode of development. We achieved this by RNAi-mediated silencing of *Met* and *Kr-h1* in juveniles of the linden bug, *Pyrrhocoris apterus*.

The **second** aim was to determine whether larval identity requires the JH-Met-Kr-h1 signaling from the very start of larval development, as proposed by Wigglesworth, or only during late instars. We approached this problem by using RNAi in *P. apterus* and by exploiting a recently characterized "JH-free" *mod* mutant of the silkworm, *Bombyx mori*.

Third, we addressed the question whether Met and its putative heterodimeric partner Taiman (Tai), also a bHLH-PAS protein, transmit the stimulatory effect of JH on oogenesis in *Pyrrhocoris*. Since JH is the main regulator of vitellogenesis in hemimetabolan insects, we tested whether the JH-Met-Tai complex executes a diapause-to-reproduction switch in the adult female bugs. We also examined possible role of Tai in juvenile *Pyrrhocoris*.

Finally, we aimed to establish Met/Gce as the *bona fide* JH receptor using the *Drosophila* model. We employed hormone binding and luciferase reporter assays with Gce mutated in its ligand-binding pocket to ultimately prove that the capacity of Gce to physically bind JH is critical for Gce to activate transcription in response to JH, and *in vivo* for *Drosophila* development. Since bHLH-PAS proteins mostly transactivate as heterodimers, we tested the requirement of Tai for Met and Gce transcriptional activation. We also tested whether JH mimics and the JH precursor methyl farnesoate are true JH agonists.

INTRODUCTION

Hormonal control of insect development

Growth of arthropods is fully dependent on physical properties of their relatively inextensible exoskeleton. Insect development from the embryo to the adult stage consists of several molts, when the new cuticle is synthesized and deposited and the old cuticle is shed (ecdysed). In hemimetabolan insects, such as in true bugs (Hemiptera), grasshoppers and crickets (Orthoptera), and cockroaches (Blattodea), larvae (=nymphs) develop through several larval instars and metamorphose into adults (Figure 1). In holometabolan insects, such as in beetles (Coleoptera), butterflies (Lepidoptera) or flies (Diptera), advanced larval specialization resulted in addition of an intermediate pupal stage following the final larval instar (Figure 1). In both modes of development, metamorphosis gives rise to winged and fertile adults.

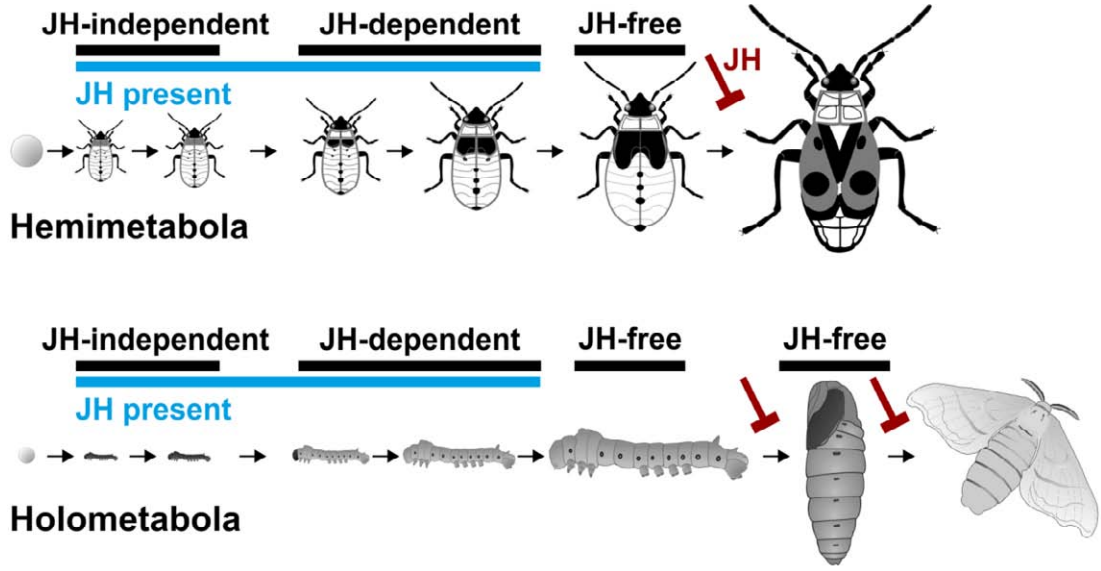

Figure 1. Schematic of JH-independent and JH-dependent phases of larval development in Hemi- and Holometabola.
Although JH is present during most of the larval development (blue bars), early larval instars develop independently of JH titer and only the presence of JH in late larval stage is required to maintain an insect at a larval stage (black bars). Cessation of JH synthesis in the ultimate larval instar allows entry to metamorphosis (rightmost black bars). Ectopic JH (purple line) can prevent pupal or adult development and results in reiteration of the larval or pupal instars, respectively. Adapted from (Smykal *et al.*, 2014b).

Juvenile hormone (JH) is a signaling molecule with fundamental roles in postembryonic development, reproduction, caste determination, behavior, stress

response, diapause and various polyphenisms in insects (Nijhout, 1994). During development, insects undergo a number of molts and ecdyses to reach the adult stage. An orchestrated interplay of JH, ecdysone (E) and other factors (Weaver and Audsley, 2009; Hiruma and Kaneko, 2013; Nijhout *et al.*, 2014) integrate nutritional status with developmental timing to proceed through the insect life cycle. In the following text I will outline some aspects of the function of JH and E including molecular function of their receptors, Methoprene-tolerant (Met) and EcR-USP, respectively.

Ecdysone signaling

A steroid hormone ecdysone (Figure 2A) is the main regulatory molecule of insect molting. During larval instars, the main source of E is the prothoracic gland (PG). Secretion of synthesized E is regulated by the prothoracicotropic hormone (PTTH) (Marchal *et al.*, 2010) and other neuropeptides in correspondence to nutritional status, mediated by insulin signaling (Yamanaka *et al.*, 2013). E is released into the hemolymph and converted to the biologically active form 20-hydroxyecdysone (20E; Figure 2B) in peripheral tissues (Petryk *et al.*, 2003).

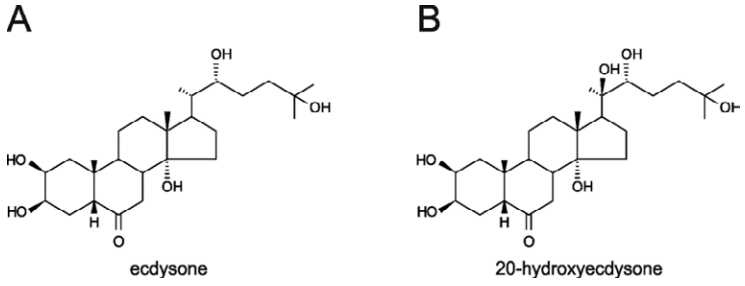

Figure 2. Ecdysone (A) and 20-hydroxyecdysone (B).

20E binds to the heterodimeric nuclear receptor consisting of the EcR and USP proteins (Oro *et al.*, 1990; Koelle *et al.*, 1991; Yao *et al.*, 1993; Hall and Thummel, 1998). EcR and USP are members of the nuclear receptor (NR) superfamily, where the EcR is the ligand-sensing partner and the Retinoid X Receptor (RXR) homolog USP is the heterodimeric partner. They both share the typical NR protein structure including N-terminal and C-terminal trans-activation domains, a DNA binding domain containing two zinc-fingers, and a ligand-binding domain (Mangelsdorf *et al.*, 1995). In general, upon 20E binding and a

conformational change, the EcR/USP heterodimer bound to hormone response elements (HRE) in gene promoters (Cherbas *et al.*, 1991) initiates expression of early ecdysone-inducible transcription factors such as E74, E75, E93, Broad-Complex (BR-C), etc. that regulate expression of late ecdysone-inducible target genes involved in molting and metamorphosis (Ashburner *et al.*, 1974; Thummel, 1996; Dubrovsky, 2005; Yamanaka *et al.*, 2013).

The EcR/USP heterodimer constitutes the core ecdysone receptor cooperating with a number of cofactors to regulate gene expression in time and tissue specific manner (Henrich, 2012; Yamanaka *et al.*, 2013). Some of these cofactors are involved in epigenetic DNA modifications and some of them, such as p160/SRC/FISC/Taiman (Tai), p300/CBP, SMRTER, or DART1 are known ligand-dependent EcR/USP coactivators or repressors. (Tsai *et al.*, 1999; Bai *et al.*, 2000; Kimura *et al.*, 2008; Kirilly *et al.*, 2011)

Juvenile hormone signaling

JH synthesis and degradation

Juvenile hormone is a sesquiterpenoid molecule synthesized in an endocrine gland, the corpus allatum (CA), located just posterior to the brain. Early steps of JH biosynthesis are facilitated by enzymes of the mevalonate pathway, common to Ecdysozoa and vertebrates (Sin *et al.*, 2014; Figure 3 and 4A). The synthesis is based on reductive polymerisation of Acetyl-CoA and gives rise to a number of isoprenoid compounds, such as mevalonate, and produce farnesyl diphosphate (FPP) (Bellés *et al.*, 2005) that is the precursor compound in the sterol and JH branches. In vertebrates, FPP is a precursor for cholesterol synthesis in the sterol branch (Liscum, 2002; Figure 3) However, sterol branch is missing in arthropods (Clark and Bloch, 1959).

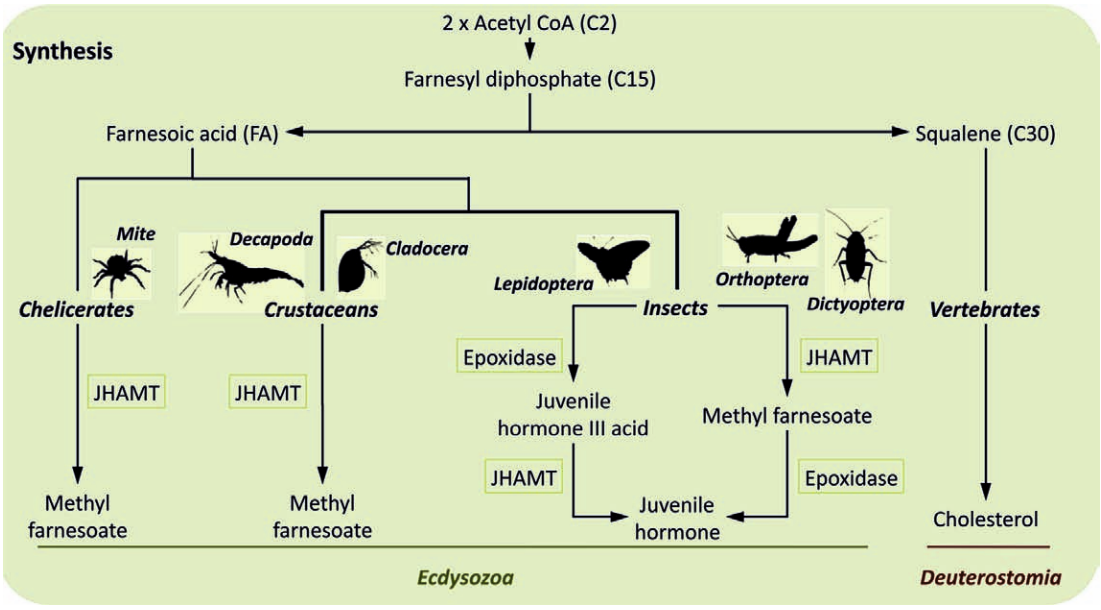

Figure 3. Juvenile hormone biosynthesis pathway overview.
Mevalonate pathway produces farnesyl diphosphate (FPP) from Acetyl-CoA. FPP enters the steroid branch to synthesize cholesterol in vertebrates. In arthropods, FPP enters the JH synthesis branch that produces farnesoic acid (FA), methyl farnesoate (MF) and JH. Chelicerates and crustaceans use MF as the main JH. In insects, epoxized JH is synthesized either by epoxidation and methylation in Lepidoptera or by methylation followed by epoxidation in Orthoptera, Dictyoptera, etc. The crustacean water flea *Daphnia pulex*, shrimp *Neocaridina denticulata*, and chelicerate spider mite *Tetranychus urticae* contain putative JHAMT proteins in their genomes, but may not produce JH. Adapted from (Sin *et al.*, 2014).

Instead, FPP is a precursor for synthesis of farnesoic acid (FA), methyl farnesoate (MF) and JH in the JH branch (Bellés *et al.*, 2005; Goodman and Cusson, 2012; Hiruma and Kaneko, 2013; Sin *et al.*, 2014; Figure 4A). Arthropod groups differ in the final products of the pathway. MF is considered the main sesquiterpenoid in chelicerates and crustaceans (Tobe and Bendena, 1999). Insects use epoxidized forms of farnesoids. The most common JH III can be synthesized either via epoxidation of FA by Juvenile hormone epoxidase (JHEpo) and subsequent methylation by Juvenile hormone acid O-methyltransferase (JHAMT) in Lepidoptera, or via methylation of FA to MF by JHAMT and epoxidation of MF to JH III by JHEpo in most other insects (Defelipe *et al.*, 2011; Figure 3). JH III is the main epoxidized JH in most insect orders, but additional forms are also present: JH 0, JH I, 4-methyl JH I and JH II in the Lepidoptera, JH bisepoxide (JHB3) in Diptera and JH skipped-bisepoxide in the Heteroptera (JHSB3) (Kotaki *et al.*, 2009; Goodman and Cusson, 2012; Figure 4B).

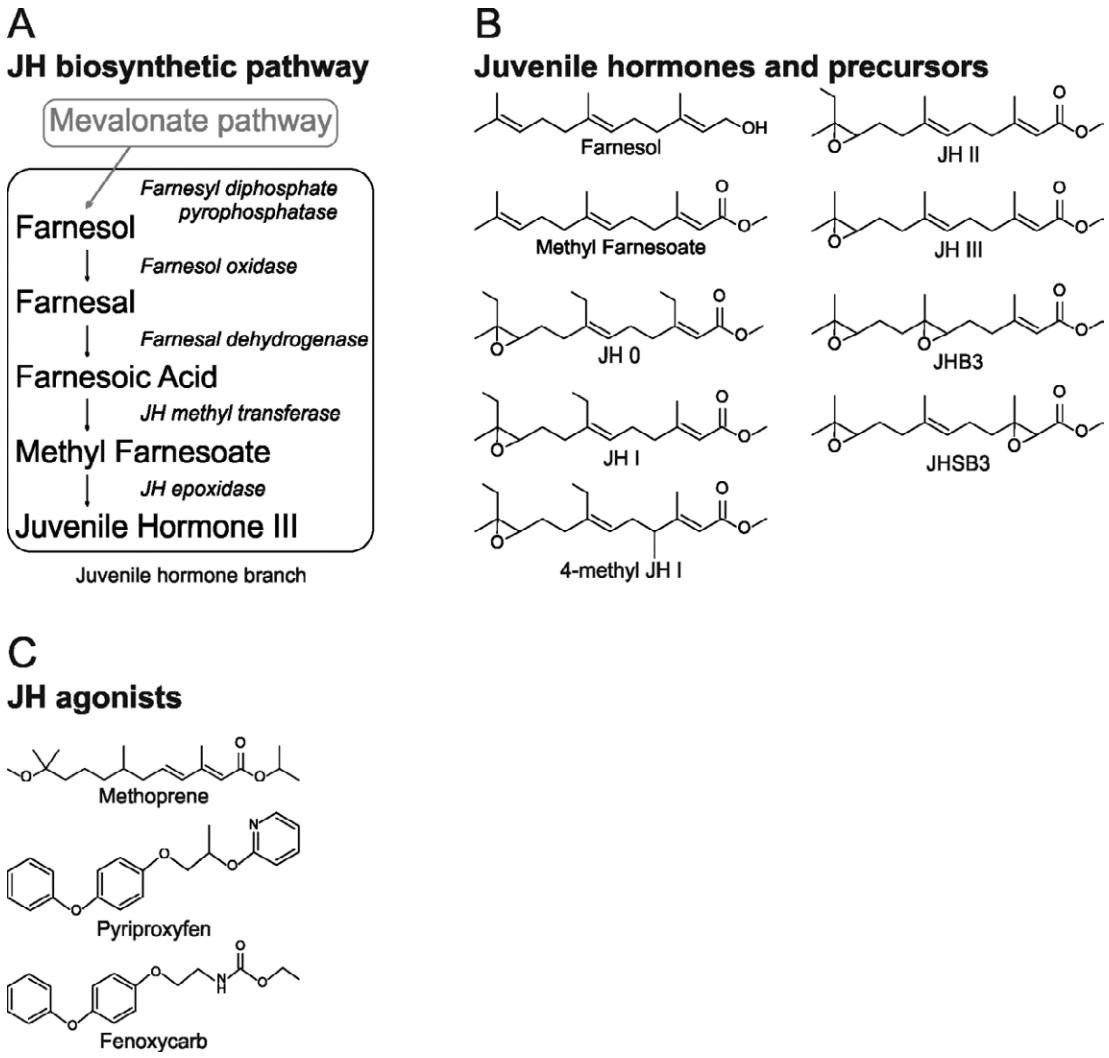

Figure 4. Schematic of JH biosynthesis.
(A) JH branch of JH III biosynthesis in the corpus allatum insect endocrine gland. (B) Examples of JH precursors, farnesol and methyl farnesoate, and individual JH species found in various insects. (C) Synthetic JH mimics. Abbreviations: JH, juvenile hormone; JHB3, JH bisepoxide; JHSB3, JH skipped bisepoxide. Adapted from (Bellés *et al.*, 2005; Goodman and Cusson, 2012, in Gilbert ed. 2012).

Upon synthesis and secretion from the CA, JH transport in the haemolymph is performed by JH binding proteins (JHBPs) (Goodman and Chang, 1985).

Interestingly, methyl farnesoate (MF), a precursor of JH synthesis, can be secreted from CA in some insects such as *Drosophila melanogaster* (Jones *et al.*, 2013a). MF is a putative ligand of dipteran and lepidopteran (=Mecopterida) USP (Iwema *et al.*, 2007; Jones *et al.*, 2010, 2013a, 2013b) and might play a significant role in development (Jones *et al.*, 2013a). More interestingly, MF and its precursor farnesoic acid (FA) are suggested as important crustacean regulators of reproduction (Nagaraju, 2011), a role mostly pertaining to epoxized JH in insects.

Precise regulation of development requires precise and rapid control of JH titer in the hemolymph (Figure 5). Various inputs from the growing insect body are integrated in the insect brain and CA (Weaver and Audsley, 2009; Hiruma and Kaneko, 2013) to regulate JH synthesis. Enzymes performing final steps of JH synthesis, such as JHAMT and JHEpo are imminent targets of the regulation. Experimental removal of JHAMT in *Tribolium* (Minakuchi *et al.*, 2008) or loss of the JH epoxidase CYP15C1 in the silkworm *Bombyx* "*mod"* mutants (Daimon *et al.*, 2012) lead to precocious entry to metamorphosis. When a synthetic JH mimic (JHM) such a methoprene (Figure 4C) — a molecule eliciting the same antimetamorphic effect as JH — was applied to *mod* mutant larvae, normal development was restored and larvae metamorphosed after the normal number of five larval instars (Daimon *et al.*, 2012).

After JH synthesis ceases, JH clearance is accomplished by JH-degrading enzymes, Juvenile hormone esterases (JHEs; Tsubota *et al.*, 2010b) and Juvenile hormone epoxide hydrolases (JHEHs; Tsubota *et al.*, 2010a). Experimental constitutive JHE overexpression in *Bombyx* lowered JH levels and drove larvae to precocious metamorphosis (Tan *et al.*, 2005), thus mimicking the lack of JH.

Antimetamorphic effect of JH

Insects grow during immature stages to attain the critical weight sufficient for successful metamorphosis (Mirth *et al.*, 2005; Mirth and Riddiford, 2007; Nijhout *et al.*, 2014). Number of larval instars varies among and within insect species (Esperk *et al.*, 2007) but in vast majority of them three larval instars are the minimum. However, members of several specialized insect groups can develop through one or two larval instars. Parasitoid wasps (Jarjees and Merritt, 2002) and some Leptodirini cave beetles (Cieslak *et al.*, 2014) can go through a single larval instar and some histerid (Achiano and Giliomee, 2005) and Leptodirini species develop through two larval instars (Cieslak *et al.*, 2014). The larvae of the latter group hatch from a large (macrolecital) egg, where the copious yolk provides sufficient nutrients to complete larval development without feeding (Cieslak *et al.*, 2014).

After the larvae attain appropriate size, a surge of ecdysone initiates molt, and molting behavior leading to ecdysis (Figure 5). The identity of the newly ecdysed individuals is set beforehand by the presence or absence of JH: when high titer of JH

occurs, larvae molt into another larval instar. When naturally absent during the ultimate larval instar, or experimentally removed during the penultimate instar, larvae become committed towards metamorphosis (Wigglesworth, 1934; Bounhiol, 1938; Fukuda, 1944; Slama, 1965; Tan *et al.*, 2005; Minakuchi *et al.*, 2008; Figure 5). Ectopic applications of JH or JHM to the final larvae or pupae cause reiteration of the stage, larval or pupal, respectively (Jindra *et al.*, 2013).

In contrast, early larval development seems to be unaffected by JH absence. *Bombyx* "*mod*" mutant, one of several "moltinism" mutants of *Bombyx* (Tazima 1978; Banno *et al.*, 2005; Daimon *et al.* 2012), lacks epoxized JH throughout the entire life cycle but still metamorphoses no earlier than after three larval instars (Daimon *et al.* 2012; Figure 2). Pupation after the minimum of three larval instars was also obtained in *Bombyx* allatectomized larvae (Bounhiol, 1938; Fukuda, 1944; K. Hiruma, personal communication) and in transgenic larvae constitutively overexpressing JHE (Tan *et al.*, 2005). Takaaki Daimon developed *Met1* knockout larvae using the TALEN approach (T. Daimon, personal communication). Most of the *Met1*$^{-/-}$ larvae showed developmental arrest at molting from L2 to L3. L3 larvae that succeed in shedding the old cuticle looked like perfect larvae, but died without feeding. Detail examination of the survivors showed that L3 larvae had very small patches of pupal cuticle on the dorsal side. Taken together, although some tissues of L2 larvae become competent to metamorphose, only larvae going through three larval instars can complete metamorphosis and form pupae (T. Daimon, personal communication).

Precocious entrance to metamorphosis in highly derived *Drosophila* has been induced by compromising ecdysone signaling (Bialecki *et al.*, 2002; Mirth *et al.*, 2005) but not by genetic depletion of JH (Liu *et al.*, 2009; Riddiford *et al.*, 2010; Abdou *et al.*, 2011). This again suggests that the first three *Drosophila* instars are larval by default, independently of JH.

In the hemimetabolan true bug, *Pyrrhocoris apterus,* some larvae of the *rl(17)* (*reddish lobes 17*) mutant strain were reported to lack the CA (Socha, 1987), and these larvae developed adult characters at the fourth instar, instead after the normal five instars (Socha and Hodkova, 1989). Consistently, *Pyrrhocoris* larvae with experimentally silenced JH-signaling genes (discussed below) initiated metamorphosis no earlier than during the third instar (Smykal *et al.*, 2014b). Similar

results were observed in allatectomized L1 and L2 larvae of the stick insect, *Carausius morosus* (Pflugfelder, 1937, 1952).

Along with the above evidence, our recent work has confirmed that although early nymphal/larval instars are independent of JH, larvae gradually attain the competence to metamorphose. Active JH signaling becomes progressively more important for protecting larvae from precocious metamorphosis while allowing them sufficient time spent on feeding and growth (Suzuki *et al.*, 2013; Smykal *et al.*, 2014b).

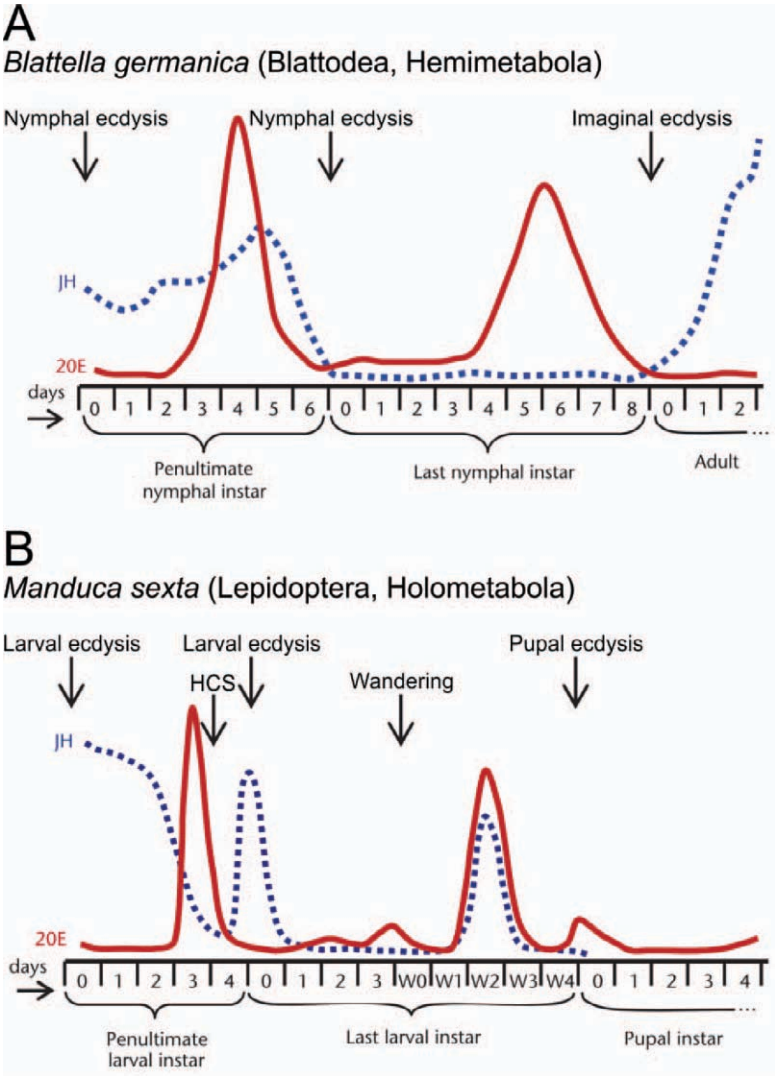

Figure 5. JH and 20E titers during late larval/nymphal development and metamorphosis in hemimetabolan and holometabolan insects.
Diagram of hormone profiles in a hemimetabolan cockroach *Blattella germanica* (upper part) and holometabolan tobacco hornworm *Manduca sexta* (lower part). A surge of 20E in the absence of JH during the last hemimetabolan nymphal instar causes metamorphic molt (upper part). In holometabolan insects, simultaneous surge of 20E and JH in last larval instar permits larval/pupal and blocks larval/adult molt (lower part). HCS – head capsule slippage. Adapted from (Bellés, 2011).

JH receptor Methoprene-tolerant

Discovery of the *Methoprene-tolerant* gene in *Drosophila*

First indication that the *Methoprene-tolerant* (*Met*) gene might play a role in transmission of antimetamorphic effect of JH came from an unbiased genome-wide screen for methoprene-resistant mutants in *Drosophila* (Wilson and Fabian, 1986; Figure 6). The absence of Met function prevented lethal morphogenetic defects caused by methoprene during the prepupal and pupal stages, permitting survival of *Met* mutants (Wilson and Fabian, 1986; Wilson and Ashok, 1998). In 1998, Wilson and Ashok demonstrated that homozygous Met^{27} mutants completely lacked the *Met* transcript (Wilson and Ashok, 1998). Yet, surprisingly, Met^{27} homozygotes were viable with only minor defects in the adult compound eye and reduced oviposition in females. Supplementing mutant Met^{3} or Met^{27} flies with genomic or DNA clones containing the full *Met* ORF restored sensitivity to methoprene (Ashok *et al.*, 1998; Baumann *et al.*, 2010; Abdou *et al.*, 2011).

One would expect more deleterious effects for defective JH signaling than those observed in the viable *Met* mutant flies. A possible explanation for the relatively weak phenotype could be the occurrence of a paralogous gene in the *Drosophila* genome. Such a gene was initially described as *germ cell-expressed* (*gce)* by Moore *et al.* in 2000 (Figure 6). Similarly to Met^{27} null mutants, *gce* homozygous null mutants, $gce^{2.5k}$, were viable. Only double Met^{27} $gce^{2.5k}$ mutants died during prepupal stage (Baumann *et al.*, 2010; Abdou *et al.*, 2011) and the phenotype was similar to JH-deficient flies with genetically ablated CA (Liu *et al.*, 2009; Riddiford *et al.*, 2010). Rescue experiments in Met^{27} $gce^{2.5k}$ background showed that overexpression of either Met or Gce alone restored normal development (Baumann *et al.*, 2010; Abdou *et al.*, 2011), indicating a certain level of functional redundancy between Met and Gce. However, the redundancy is limited, as loss of Met, but not of Gce, caused premature differentiation of the photoreceptors in the lobula of the optic lobe and subsequent emergence of blind adults (Riddiford *et al.*, 2010).

Met/Gce is a conserved JH receptor in insects

The expected outcome of disrupted JH signaling during larval development is a precocious entry to metamorphosis. Although *Drosophila* Met^{27} $gce^{2.5k}$ double mutant fails to complete development, mutant animals enter the metamorphosis after the normal number of three larval instars. Therefore, less derived insect species had to be employed to elucidate the role of Met in the antimetamorphic JH action. Orthologs of *Met* (in fact of *gce*, which is ancestral of the two *Drosophila* genes) have been found in all examined insect species including ametabolous groups (Konopova *et al.*, 2011), in crustaceans (Miyakawa *et al.*, 2013), and even in ticks (http://www.genome.jp/dbget-bin/www_bget?isc:IscW_ISCW008805). Besides *Drosophila*, duplicated *Met*/*gce* paralogous genes occur in lepidopterans such *Bombyx* (*Met1* and *Met2*; Kayukawa *et al.*, 2012), the monarch butterfly (*Danaus plexippus*) and other species. Like *gce* in *Drosophila*, *Met2* appears to be the more ancestral gene in *Bombyx*. However, the gene duplication must have occurred independently at least in the Lepidoptera and in the *Drosophila* lineages as lower flies and mosquitoes only possess one *Met*/*gce*-like gene.

Met was shown to be the key transmitter of the JH antimetamorphic action in several holo- and hemimetabolan insect orders. The beetle *Tribolium castaneum* (Konopova and Jindra, 2007) and the silkworm *Bombyx mori* (T. Daimon, personal communication) enter pupation precociously after RNAi (*Tribolium*) or TALEN-mediated (*Bombyx*) knock-out of *Met1* in early larval stages. Precocious adult development was observed upon *Met* RNAi in nymphs of the hemimetabolan true bug, *Pyrrhocoris apterus* (Konopova *et al.*, 2011) and the German cockroach, *Blattella germanica* (Lozano and Bellés, 2014). Moreover, treatment of final-instar larvae/nymphs or pupae with JH or JHM induced supernumerary larval/nymphal or pupal instar(s), an effect that was abolished by previous *Met* knockdown (Konopova and Jindra, 2007; Parthasarathy *et al.*, 2008b; Konopova *et al.*, 2011).

Met and Gce are members of the bHLH-PAS domain family

Sequence analysis of Met and Gce revealed that both proteins belong to the family of basic helix-loop-helix (bHLH) Per-ARNT-Sim (PAS) protein family (Ashok *et al.*, 1998; Moore *et al.*, 2000; Figure 6).

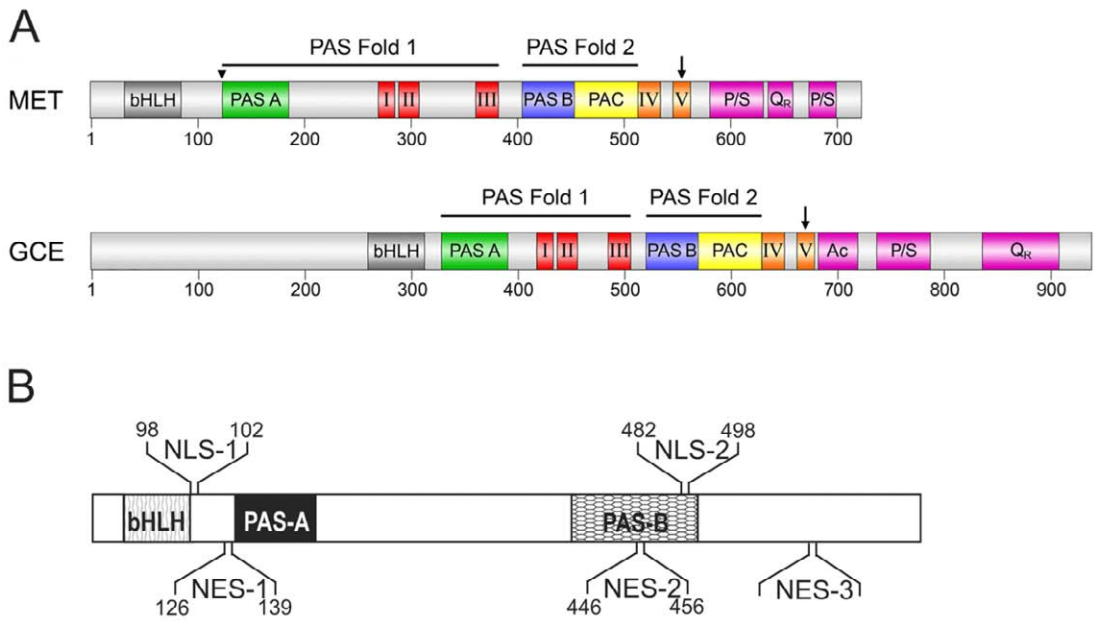

Figure 6. Schematic illustration of Met and Gce protein structures.
(A) Shown are the bHLH domain, the PAS-A, PAS-B/PAC domains, conserved sequence blocks between Met and Gce (I-V), and the acidic (Ac), proline/serine (P/S), and glutamine (QR) regions. Arrowhead indicates the location of an LxxLL sequence in Met; vertical arrow indicates LIxxL NR box in Met and Gce. Numbers at the bottom of each diagram designate residue position. (B) Schematic representation of NLS and NES signals residing within the Met protein. Adapted from (Greb-Markiewicz *et al.*, 2011; Bernardo and Dubrovsky, 2012).

bHLH-PAS transcription factors are conserved throughout the tree of life, from bacteria to eukaryotes. In *Drosophila* they control many aspects of fly life, ranging from development (e.g. Met, Gce, Tango, Spineless, Taiman), sensing and responding to environmental signals and cell homeostasis (Similar) to circadian rhythms (Period, Clock, Cycle). Members of the bHLH-PAS family usually act as heterodimers and share common molecular organization (Kewley *et al.*, 2004; Figure 6). They contain the N-terminal basic helix-loop-helix domain (bHLH), where the basic regions of two heterodimerizing partners constitute the DNA-binding function, while the HLH motif mediates dimerization. The following PAS-A and PAS-B/PAC (PAS-associated C-terminal) domains are involved in dimerization and, depending on a protein, contribute to DNA binding and protein-protein interactions with heat-shock proteins and transcription factors (Kewley *et al.*, 2004; Furness *et al.*, 2007; Soshilov and Denison, 2008; He *et al.*, 2014). In some cases such as the vertebrate Aryl hydrocarbon receptor (AhR) (Soshilov and Denison, 2008; Pandini *et al.*, 2009; Xing *et al.*, 2012) or Met/Gce (Charles *et al.*, 2011), the PAS-B domain mediates binding to activating low-molecular-weight ligands. The poorly conserved

C-terminal part of bHLH-PAS proteins contains the acidic (Ac), proline/serine (P/S), and glutamine (QR) regions responsible for transactivation or transrepression (Figure 6A). Both Met and Gce *Drosophila* proteins possess a nuclear receptor (NR) box motif LIxxL localized behind the PAS-B/PAC domain. LIxxL motif mediates interaction with the FTZ-F1 nuclear receptor and is conserved among Met homologs from some other species (Bernardo and Dubrovsky, 2012). Additionally, *Drosophila* Met contains an LxxLL NR motif, typically found in some other PAS domain proteins, such as in *Drosophila* Taiman, and responsible for protein-protein interactions (Plevin *et al.*, 2005; Bernardo and Dubrovsky, 2012; Figure 6A). Both Met and Gce also possess Nuclear Localization Signals (NLS) and Nuclear Export Signals (NES) (Greb-Markiewicz *et al.*, 2011; Figure 6B). *Drosophila* Met has NLS-1 adjacent to the bHLH domain, NLS-2 and NES-2 in the PAS-B domain, NES-1 in PAS-A, and an unidentified NES-3 in the C-terminal part of the protein (Greb-Markiewicz *et al.*, 2011; Figure 6B). *Drosophila* Met specific NLS-1 is a monopartite Simian Virus 40 T large antigen type of NLS (Kalderon *et al.*, 1984) not present either in Gce or Met homologs from other species. NLS-1 is dominant over all localization signals in JH-independent manner. Met with mutated NLS-1 (and functional NLS-2) translocates to the nucleus only in the presence of JH. Both PAS-B located NLS-2 and NES-2 are highly conserved among insect Met homologs. Although NES-1 sequence similarity among insect Met proteins is low, a similar hydrophobic α-helix is found in many Met homologs (Greb-Markiewicz *et al.*, 2011).

Met/Gce subcellular localization

Met has been reported to be strictly nuclear in S2 cells (Miura *et al.*, 2005) and in various tissues *in vivo* (Pursley *et al.*, 2000). Similarly, Gce has been found in nuclei of the larval fat body cells regardless of whether or not it can bind JH (Jindra *et al.*, submitted). However, He *et al.* (2014) demonstrated JH-inducible Met nuclear translocation (He *et al.*, 2014) in *Drosophila* larval fat body cells and in the *Kc* cell line. The translocation required Heat shock protein 83 (Hsp83), a homolog of vertebrate Hsp90, and thus resembled “modus operandi” of the vertebrate ligand-activated AhR (Kazlauskas *et al.*, 2001; Soshilov and Denison, 2011).

Met/Gce binds JH in nanomolar concentrations

In 2005 Miura *et al.* demonstrated that *in vitro* translated *Drosophila* Met binds JH III (K_d = 5.3 nM), an affinity within the physiological JH concentration (Jones *et al.*, 2013a, 2013b). A somewhat lower affinity for JH III was found for *Drosophila* Gce (K_d = 19.3 nM; Jindra *et al.*, submitted). Met of the mosquito *Aedes aegypti* bound JH III with a K_d = 4.4 nM (Li *et al.*, 2014), and Met from the beetle *Tribolium castaneum* showed a K_d = 2.9 nM (Charles *et al.*, 2011). Based on deletion and mutational analyses, the C-terminal PAS-B domain of Met has been determined as required and sufficient for the hormone binding (Charles *et al.*, 2011). Interestingly, even the PAS-B domain of Met from the ametabolous wingless firebrat *Thermobia domestica* (Zygentoma) bound JH III (Charles *et al.*, 2011).

Although the crystal structure of Met is still missing, homologous modeling of *Tribolium* and *Aedes* Met PAS-B domains based on the crystal structure of the Hypoxia inducible factor 2α (HIF2α) PAS-B domain revealed a hydrophobic cavity accessible for a small lipophilic molecule. The model emphasized some amino acid residues that have indeed proven to be critical for JH binding by *Tribolium* (Charles *et al.*, 2011) and *Aedes* (Li *et al.*, 2014) Met proteins (Figure 7). Docking experiments suggested that the putative ligand-biding pocket can accommodate JH III as well as methoprene or a chemically unrelated JH mimic pyriproxyfen (Charles *et al.*, 2011; Figure 4C). Indeed, both JHMs competed with JH III for binding to the PAS-B domain of *Tribolium* Met (with pyriproxyfen exceeding the affinity of JH III), showing that both methoprene and pyriproxyfen were true JH agonists (Charles *et al.*, 2011).

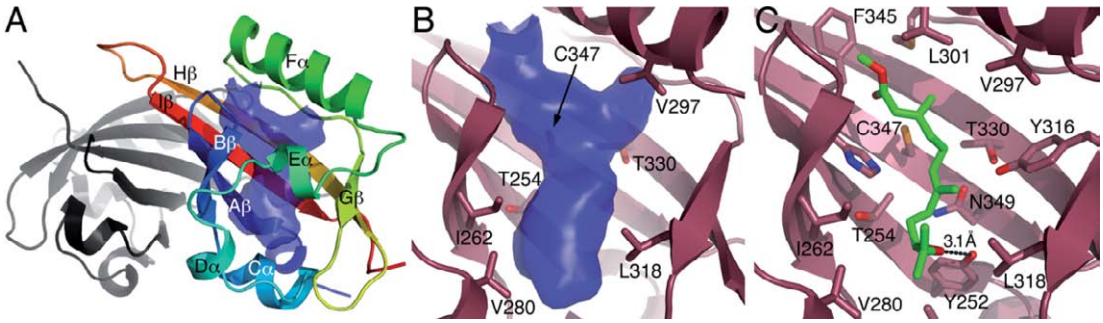

Figure 7. Model of the ligand-binding cavity of the *Tribolium* Met PAS-B domain.
(A) Overall structure of Met PAS-B (blue, N terminus; red, C terminus) with the cavity. Position of a hypothetical heterodimeric partner is shown in gray. (B) A closer view of the pocket. (C) Docking model of a Met–JH III complex. A hydrogen bond (dotted line) is predicted between the hydroxyl group of Tyr-252 and the epoxide moiety of JH III. Orientation is the same in all models. Adapted from (Charles *et al.*, 2011).

Recently, Jindra *et al.* (submitted) showed that the routinely used JH mimics, methoprene and pyriproxyfen, as well as the JH precursor MF, can effectively compete with JH III for binding to *in vitro* translated Gce with K_i values of 45.8 nM for methoprene, 5.7 nM for pyriproxyfen and 87.9 nM for MF. Gce affinity for MF lies within the natural MF concentration in larval hemolymph (Jones *et al.*, 2013a). Thus, Gce might be, together with USP, an *in vivo* sensor of MF action.

Met binding partners and interacting proteins

Met-Met and Met-Gce

bHLH-PAS proteins can form either homo- or heterodimers. *Drosophila* Met forms a homodimer or a Met-Gce heterodimer that both dissociate in the presence of JH or JHM (Godlewski *et al.*, 2006). In agreement with the *Drosophila* proteins, homodimerization of *Tribolium* Met is also JH-sensitive, and the dissociation is blocked if homodimer is formed by Met proteins incapable of JH binding (Charles *et al.*, 2011).

Taiman/SRC/FISC

In *Aedes aegypti* Met forms a JH-dependent complex with the βFtz-F1-interacting steroid receptor coactivator (FISC) (Li *et al.*, 2011), also a member of the bHLH-PAS protein family (Zhu *et al.*, 2006). In other species, orthologs of FISC have been named either Steroid receptor coactivator (SRC) based on homology to the mammalian SRC-1/NCoA/p160 proteins, or Taiman (Tai) based on *Drosophila* (FlyBase) nomenclature. *Tribolium* Tai interacted with Met in a JH binding-dependent manner (Charles *et al.*, 2011) and was together with Met required for expression of several JH-inducible genes (Zhang *et al.*, 2011). Both *Drosophila* Met and Gce require Tai to stimulate transcription in *Drosophila* L57 or S2 cells (Li *et al.*, 2011; Jindra *et al.*, submitted). Met-Tai heterodimer formation was also described in *Bombyx* (Kayukawa *et al.*, 2012) where only one of *Bombyx* Met paralogs (Met2) formed Met-Tai complex in JH-dependent manner.

Interestingly, in crustaceans *Daphnia pulex* and *D. magna* orthologs of Met and Taiman/SRC stimulated gene transcription in two-hybrid luciferase assay in ligand-dependent manner (Miyakawa *et al.*, 2013). MF, the main JH of crustaceans (Figure

4B), stimulated Met-Tai/SRC interaction and MF was >10-fold more potent inducer than JH III. A threonine to valine single amino-acid substitution in *D. pulex* (T292V) and *D. magna* (T296V) Met proteins corresponding to Val-297 of *Tribolium* increased Met sensitivity to JH III, and surprisingly also to MF (Miyakawa *et al.*, 2013).

These results suggested a similar "modus operandi" for the crustacean and insect MF/JH signaling, acting through a Met-Tai complex, raising the question what was the original role of MF/JH signaling in arthropods. Since the ametabolous firebrat possesses Met that binds JH III (Charles *et al.*, 2011), reproduction seems to be the primary function of MF/JH-Met in insects. Whether MF/Met signaling has a role in crustacean reproduction or metamorphosis is to be investigated.

Cycle

Interestingly, in newly emerged *Aedes* mosquitoes Met dimerizes with Cycle (Cyc), a bHLH-PAS factor regulating the circadian clock. The Met-Cyc heterodimer mediates target gene (*Hairy* and *Kr-h1*) expression in a manner dependent on both JH and the daily photoperiod (Shin *et al.*, 2012).

Moreover, Bajgar *et al.* (2013) demonstrated that *Met*, *cyc*, and another circadian gene *Clock* (*Clk*) are all required in the gut of *Pyrrhocoris apterus* for organ-autonomous switch between a diapause and a reproductive program. All three proteins participate on expression of the *Par domain protein 1* (*Pdp1*) during the reproductive phase, while Pdp1 in turn represses *cryptochrome 2* (*cry2*), a gene that specifies diapause.

Ftz-F1

Dubrovsky *et al.* (2011) proposed that the nuclear receptor Ftz-F1 functions as a competence factor of Gce for the up-regulation by JH of the ecdysone-inducible *E75A* gene in *Drosophila* S2 cells. Although Tai/FISC can interact with Ftz-F1 in mosquitoes (Zhu *et al.*, 2006), whether the *Drosophila* Gce-Ftz-F1 complex involves Tai is not known.

Chd64 and FK506

Drosophila Met can also interact with a calponin-like protein Chd64 and a peptidyl-prolyl cis-trans isomerase FK506-binding protein (FKBP39) in *Drosophila*

L57 cells (Li *et al.*, 2007). The complex binds to specific DNA JHREs and induces gene expression. Both Chd64 and FKBP39 are nuclear proteins that form complexes with EcR-USP and thus might be among factors mediating a crosstalk between ecdysone and JH signaling (Li *et al.*, 2007). However, the significance of Chd64 and FKBP39 for hormonal signaling *in vivo* is unclear.

Hsp83/Hsp90

The cotton bollworm *Helicoverpa armigera* (Lepidoptera) Met1 was immunoprecipitated with an anti-*Helicoverpa*-Hsp90 antibody in epidermis-derived HaEpi cells in the presence of JH or methoprene (Liu *et al.*, 2013). In vertebrates, Hsp90 and other cofactors are stably associated with AhR (Kewley *et al.*, 2004) in the cytoplasm, masking the nuclear localization signal (NLS) in the PAS-B domain of AhR. The NLS becomes exposed upon ligand binding, and Hsp90 facilitates translocation of the AhR/Hsp90 complex to the nucleus (Kewley *et al.*, 2004). There the AhR/Hsp90 complex disassociates, AhR heterodimerizes with AhR nuclear translocator (ARNT), the complex binds xenobiotic response elements (XRE) and regulates gene transcription (Kazlauskas *et al.*, 2001; Kewley *et al.*, 2004; Soshilov and Denison, 2011).

Drosophila Met/Gce has been reported to interact with Heat shock protein Hsp83, a homolog of the vertebrate Hsp90 (He *et al.*, 2014). Hsp83 binds Met through its PAS-B and bHLH domains and may be important for Met to bind JH (He *et al.*, 2014). JH-Met/Gce-Hsp83 translocates to the nucleus, binds a JH response region (JHRR) within the *Kr-h1* promoter, and regulates *Kr-h1* transcription in *Drosophila Kc* cells. He *et al.* (2014) also speculate that a nuclear pore protein Nup358 might be part of the Met-importing complex.

Transcriptional factors downstream of JH-Met signaling

Met executes the humoral signal of JH through expression of target genes. Probably the best characterized JH-Met responding gene encodes a transcription factor, Krüppel-homolog 1 (Kr-h1). Precisely regulated Kr-h1 subsequently contributes to regulation of the ecdysone-inducible E93 and BR-C transcription factors (Figure 8), and thus represents a key mechanism in control of insect metamorphosis (Jindra *et al.*, 2013; Ureña *et al.*, 2014; Bellés and Santos, 2014).

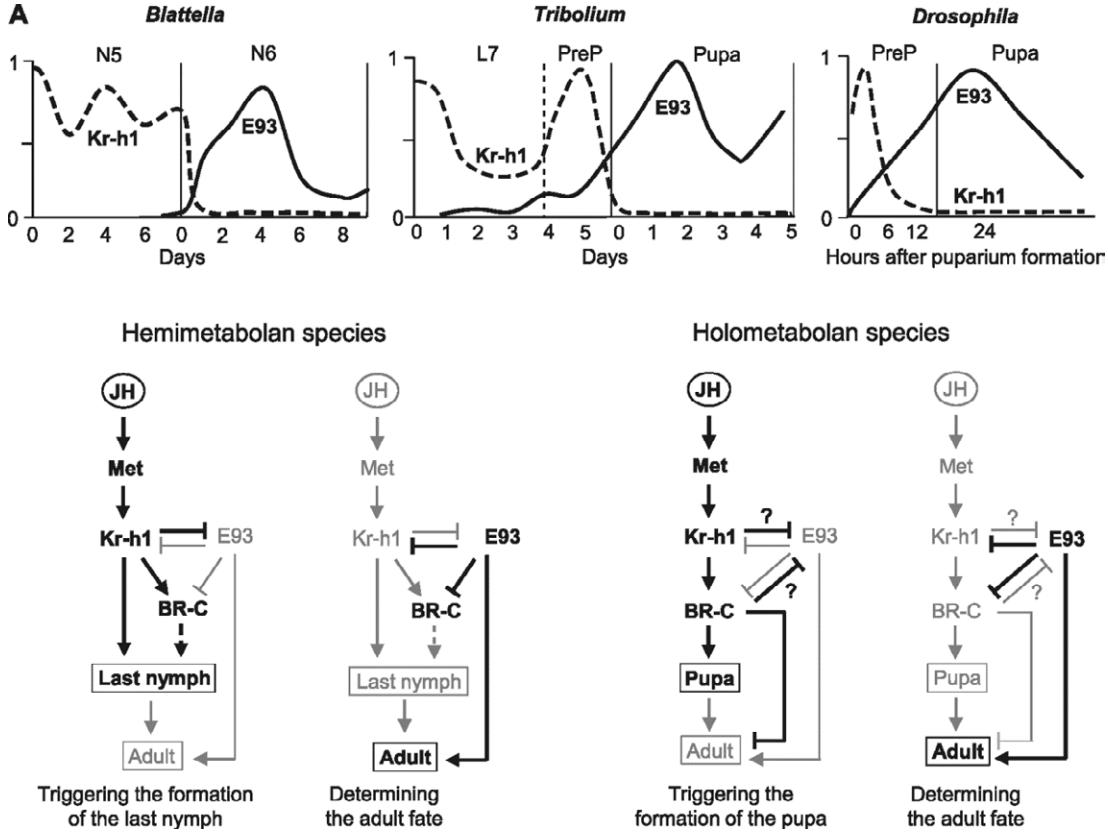

Figure 8. (A) Expression profiles of *Kr-h1* and *E93* mRNAs during the pre-adult stages in *Blattella germanica*, *Tribolium castaneum* and *Drosophila melanogaster*. Larval and nymphal instars are abbreviated as L and N, respectively, prepupa is indicated as PreP. *Kr-h1* expression is high until ultimate nymphal or prepupal instar. When JH titer drops (not shown) fall of *Kr-h1* expression permits *E93* induction and adult transition.
(B) Model of the JH signaling pathway representing important players triggering last nymph formation at the time of the 20E peak in late penultimate nymphal instar (left) and determining the adult fate at the beginning of the last nymphal instar (right) in a hemimetabolan insect. BR-C stands as a side branch of E93 repression because BR-C does not determine the nymphal or adult states in hemimetabolan species. (C) Equivalent model for triggering pupal formation at the time of the last 20E peak in the prepupal stage (left) and determining adult fate at the beginning of the pupal instar (right) in a holometabolan insect. Black and grey lines represent interactions occurring or not occurring, respectively, in a given pathway. Adapted from (Bellés and Santos, 2014).

Krüppel-homolog 1

Kr-h1 is a C2H2-type zinc-finger transcription factor identified as a P-element-induced mutation disrupting *Drosophila* prepupal metamorphosis (Pecasse *et al.*, 2000). Although *Kr-h1* expression is partly regulated by 20E (Pecasse *et al.*, 2000; Kayukawa *et al.*, 2014) and at its basal level partly dependent on as yet unknown factors (Kayukawa *et al.*, 2014; Smykal *et al.*, 2014b), the major inducer of *Kr-h1* expression is JH. Thus, *Kr-h1* mRNA follows closely the JH titer in the hemolymph, and the shutdown of JH synthesis concomitant with a drop in *Kr-h1* expression in the last larval/nymphal instar and during the pupal stage (Jindra *et al.*, 2013; Bellés and Santos, 2014) permits entry to metamorphosis (Figure 8A). Endogenous JH or ectopic JHM induce *Kr-h1* expression *in vivo* (Minakuchi *et al.*, 2008, 2009, 2011; Konopova *et al.*, 2011; Lozano and Bellés, 2011; Kayukawa *et al.*, 2012, 2014; Smykal *et al.*, 2014a), and this induction has been shown to require Met/Gce (Minakuchi *et al.*, 2009; Abdou *et al.*, 2011; Charles *et al.*, 2011; Konopova *et al.*, 2011; Li *et al.*, 2011; Smykal *et al.*, 2014a; He *et al.*, 2014; Figure 8B). Knockdown of *Kr-h1* in penultimate-instar larvae or nymphs leads to precocious metamorphosis, resembling phenotypes upon *Met* knockdown (Minakuchi *et al.*, 2009; Konopova *et al.*, 2011; Lozano and Bellés, 2011; Smykal *et al.*, 2014b). Interestingly, while ectopic JH or JHM induce supernumerary larval instars through up-regulation of *Kr-h1* (Minakuchi *et al.*, 2009; Konopova *et al.*, 2011; Lozano and Bellés, 2011), direct overexpression of Kr-h1 in *Bombyx* larvae blocked formation of the pupa but was insufficient to elicit supernumerary larval molting (Kayukawa *et al.*, 2014). Moreover, experiments on the *E93* gene (discussed below) in *Blattella* nymphs confirmed that abnormal *Kr-h1* expression during the ultimate JH-free nymphal instar is not a sufficient condition for reiteration of larval development (Ureña *et al.*, 2014).

E93

Recently, the ecdysone-inducible transcription factor E93 has been proposed as a master regulator of adult metamorphosis in both hemi- and holometabolan insects (Ureña *et al.*, 2014; Bellés and Santos, 2014; Figure 8). E93, originally named Eip93F, is a helix-turn-helix protein with a Pip-squeak (Psq) DNA-binding motif. E93 is involved in *Drosophila* development (Mou *et al.*, 2012), autophagy and

programmed cell death (Lee *et al.*, 2000; Thummel 2001). E93 is expressed at negligible levels during young instars and becomes up-regulated in metamorphic stages – the last nymphal instar of the hemimetabolan *Blattella* and the prepupal and pupal stages of the holometabolans *Tribolium* and *Drosophila* (Ureña *et al.*, 2014). *E93* RNAi in penultimate (N5) or early ultimate (N6) instar of *Blattella* drove nymphs to additional nymphal-nymphal molts, leading to giant (up to N10) nymphs but not adults (Ureña *et al.*, 2014). At the same time, *E93* RNAi animals showed ectopic expression of *Kr-h1* and *BR-C*. Similarly, *Tribolium* pupae lacking *E93* function ectopically expressed *Kr-h1* and *BR-C* and formed second pupae (Ureña *et al.*, 2014). Finally, when deprived of *E93* by transgenic RNAi, *Drosophila* pupae failed to down-regulate *BR-C* and other pupal-specific genes, whereas induction of adult-specific genes was abrogated and formation of adult structures blocked (Ureña *et al.*, 2014).

Thus, while Kr-h1 is a JH-dependent repressor of metamorphosis, E93 is the missing adult-specifying factor. Bellés and Santos (2014) have named the extended JH signaling pathway "MEKRE93" (for Met-Kr-h1-E93) to emphasize its common conserved players. They have clearly demonstrated that *Kr-h1* is epistatic to *E93*, thus linking the JH-Met-mediated antimetamorphic signaling to E93-dependent adult determination (Figure 8). The current model essentially states that drop of JH and subsequently of *Kr-h1* level at the final pre-adult stage de-represses *E93*, which in turn further inhibits expression of *Kr-h1* and *BR-C* via a negative regulatory loop, thus enforcing the adult commitment (Ureña *et al.*, 2014; Bellés and Santos, 2014).

Broad-Complex (BR-C)

Broad-Complex (also known as Broad) is one of the key factors regulating metamorphosis. BR-C is a C2H2-type zinc-finger transcription factor with a broad/tramtrack/bric-à-brac (BTB)/Pox virus and Zinc finger (POZ) protein interaction motif in the N-terminal region (DiBello *et al.*, 1991; Bayer *et al.*, 1996). *BR-C* is an early ecdysone response gene but its expression is partly regulated by the JH-Met-Kr-h1 signaling pathway, particularly in holometabolan insects (Jindra *et al.*, 2013; Figure 8B). In hemimetabolan insects, *BR-C* is required for embryonic development and for the growth of the nymphal wing pads (Erezyilmaz *et al.*, 2006, 2010; Piulachs *et al.*, 2010; Konopova *et al.*, 2011). In the Holometabola, *BR-C* has

been recruited to specify the pupa. *Drosophila broad* (*npr1*) null mutants develop normally as larvae but then die before pupariation (Kiss *et al.*, 1988). In lepidopterans, 20E induces *BR-C* in the absence of JH and thereby commits final instar larvae toward pupation while simultaneously blocking precocious appearance of adult hallmarks (Zhou and Riddiford, 2002; Uhlirova *et al.*, 2003). In *Bombyx*, JH prevents *BR-C* induction during penultimate-to-final larval instar molt, but surprisingly JH seems to be dispensable for suppressing *BR-C* during early larval instars, thus again suggesting that the early larval status may be independent of JH (Smykal *et al.*, 2014b). In more basal holometabolan species such as *Tribolium* or the lacewing *Chrysopa perla* (Neuroptera) *BR-C* is expressed at low levels throughout larval development and peaks at the prepupal stage to (a) inform the pupal development, and (b) to prevent premature emergence of adult features (Konopova *et al.*, 2008; Parthasaraty *et al.*, 2008a; Minakuchi *et al.*, 2009).

Transcriptional regulation of target genes by Met

In response to JH, a complex comprising Met and its partner binds JH-response elements (JHREs) in upstream regions of target genes. Several studies employed high-throughput methods to search for JHREs and JH-regulated genes (Li *et al.*, 2007; Zhang *et al.*, 2011; Zou *et al.*, 2013; Li *et al.*, 2014). They have found many differently expressed genes. However, most of our knowledge about how JH-Met target genes are regulated comes from *in vitro* and cell culture assays using upstream sequences of *Kr-h1* genes from several species and from the promoter of the *Aedes aegypti early trypsin* (*ET*) gene.

JH-response elements of *Kr-h1*

Reporter-based assays using the promoter of the *Kr-h1* gene in a *Bombyx* cell line revealed that JH-dependent induction of *Kr-h1* transcription requires the Met and Tai/SRC proteins (Kayukawa *et al.*, 2012). The *Kr-h1* JHRE contained an E-box sequence CACGTG, conserved upstream of *Kr-h1* genes in several other species. In the presence of the Met and Tai proteins, the JHRE–luciferase reporter was rapidly induced upon JH administration (Kayukawa *et al.*, 2012). A similar mechanism controlling *Kr-h1* expression was described also in *Tribolium* (Kayukawa *et al.*,

2013) and *Aedes* (Cui *et al.*, 2014; Li *et al.*, 2014) cell lines. JH-Met-Tai complex of *Locusta migratoria* is also required for transcriptional induction of a reporter containing the CACGTG E-box sequence from *Locusta Kr-h1* promoter in S2 cells (Song *et al.*, 2014). A conserved consensus sequence GCACGTG has also been identified in the honeybee *Apis mellifera Kr-h1* promoter. Surprisingly, *Drosophila Kr-h1* promoter contains two distinct E-box sequences, a single C box CACGCG and two additional B boxes CATGTG. Although B boxes contribute to the full JH-Met/Gce dependent transcription induction from the reporter in *Drosophila* Kc cells, C box seems to represent the main JHRE in the *Drosophila Kr-h1* promoter (He *et al.*, 2014).

Besides the JH-Met-Tai complex, *Aedes Kr-h1* and *Hairy* gene expression is at least partially regulated by JH through a complex of Met and Cyc in a circadian manner in newly emerged adult mosquito females (Shin *et al.*, 2012). The JH-Met-Cyc complex binds a JHRE within the *Kr-h1* upstream regulatory region that harbors a CACGCG sequence. Taken together, CACGC/TG appears to be a common *cis*-regulatory element of *Kr-h1* in insects.

JH-response element of the *Aedes aegypti early trypsin* gene

The *Aedes early trypsin* (*ET*) is a female-specific protease involved in initial digestion of blood proteins in the midgut (Noriega *et al.*, 1996). A JHRE with the CACGCG E-box, similar to the one described in *Aedes Kr-h1* promoter but diverse in flanking sequences, was identified in the *ET* gene promoter. A reporter containing the *ET* JHRE is activated by *Aedes* JH-Met-Tai (Li *et al.*, 2011; Li *et al.*, 2014) and by *Drosophila* JH-Met/Gce-Tai complexes in *Drosophila* L57 (Li *et al.*, 2011) and in S2 cells (Jindra *et al.*, submitted). RNAi-mediated knockdown of either *tai* or *gce* but not of *Met* prevented JH from inducing the luciferase expression under *ET* JHRE, suggesting prevalence of JH-Gce-Tai complex in S2 cells. Interestingly, Gce-Tai can activate the *ET* JHRE reporter after application of methoprene, pyriproxyfen or methyl farnesoate (Jindra *et al.*, submitted). Transcriptional activation of the Gce-Tai complex by MF is interesting in an evolutionary perspective, since MF may be the JH of some non-insect arthropods, and it has been shown to stimulate formation of the Met-Tai/SRC complex in the crustacean *Daphnia* (Miyakawa *et al.*, 2013).

Juvenile hormone in female reproduction

Insect oogenesis and vitellogenesis

Highly effective reproduction is one of the main reasons for the great insects' success. Ovarioles are the egg producing units of female ovaries. An ovariole has two main functional parts – the germanium and the vitellarium. The germarium harbors and releases germ cells to the vitellarium, where the oocytes are enclosed with the somatic follicle cells (FC) to form the follicles that grow to become the eggs (Dobens and Raftery, 2000; Trauner and Büning, 2007). We recognize two main types of ovaries (Büning, 1994) based on the structure of the ovarioles: (1) panoistic, in which every germ cell becomes an oocyte that is not equipped with nutritive or nurse cells (NC) (Figure 9A) and (2) meroistic in which the oocyte is connected with one or more NC through stable cytoplasmic bridges or nutritive cords (Figure 9B,C).

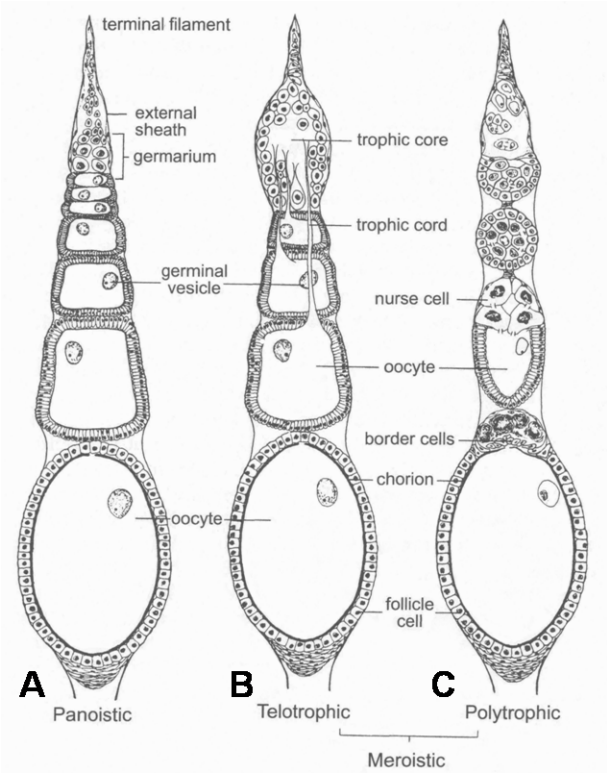

Figure 9. The basic types of ovarioles. (A) Panoistic, (B) telotrophic meroistic and (C) polytrophic meroistic ovarioles. Germinal vesicle is the nucleus of the oocyte (2n) before meiosis. (Adapted from Schwalm, 1988).

Meroistic ovarioles have two subtypes based on the position of NC – polytrophic and telotrophic. In telotrophic ovarioles, found e.g. in *Tribolium* or *Pyrrhocoris*, the oocyte is enclosed with FC but remains connected to NC located in the germarium

that is called tropharium (Figure 9B). In polytrophic ovarioles, such as in *Drosophila*, the oocyte and NC are all enclosed by FC within each individual follicle (Büning, 1994; Figure 9C). NC are non-reproductive sister cells of the oocyte that are formed by asymmetric divisions of the oogonia (de Cuevas *et al.*, 1997; Huynh and St. Johnston, 2004) and the main role of these polyploid cells is to supply the oocyte with RNAs, proteins, mitochondria, centrioles, ribosomes and other materials (Berleth *et al.*, 1988; Spradling 1993; Mahajan-Miklos and Cooley, 1994; Theurkauf and Hazelrigg, 1998; Nicolas *et al.*, 2009).

Newly established follicles undergo previtellogenic and vitellogenic phases of growth. During the previtellogenic phase, oocytes grow either on their own or with the support from the NC. The FC mitotically divide to cover the growing oocyte with the single cell layer. The FC communicate with each other and with the oocyte to form the anterio-posterior and dorso-ventral axes of the future embryo (Roth *et al.*, 1995; González-Reyes *et al.*, 1995; Deng and Ruohola-Baker, 2000). At the onset of vitellogenesis, the FC switch from proliferation to endoreplication and become polyploid (Klusza and Deng, 2011). After polyploidization, the FC change their shape to become columnar and separate from each other to create large intercellular spaces or channels in the follicular epithelium in the process called patency (Telfer, 1965; Pascual *et al.*, 1992; Wyatt and Davey, 1996). Intercellular channels enable contact of the hemolymph with the plasma membrane of the oocyte (oolema) and the receptor-mediated endocytosis of vitellogenins (Vg) (Sappington and Raikhel, 1998; Schonbaum *et al.*, 2000) and other yolk proteins (Sappington, 2002) from the hemolymph. Vitellogenins are storage glycolipoproteins synthesized mainly in the fat body of adult females or, to a lesser extent, also in the FC of the ovaries (Rousset and Bitsch, 1989; Raikhel *et al.*, 2005). When vitellogenesis is completed, the somatic FC deposit the vitelline membrane and the chorion (Cernilogar *et al.*, 2001).

JH in female reproduction

Female reproduction is controlled by the gonadotropic effects of ecdysone, JH, and various neurohormones (Raikhel *et al.*, 2005; Wielendaele *et al.*, 2013). Regulation of reproduction by the two non-peptide hormones has evolved along with their effects on molting and metamorphosis, and it differs considerably among insect orders and species. In most species JH has an important role in preparing the

reproductive tissues (fat body and ovaries) for vitellogenesis and later in inducing the FC patency to facilitate yolk uptake (Raikhel *et al.*, 2005). In adult insects, the ovaries are the main source of ecdysteroids, which further stimulate reproduction by inducing *Vg* expression in the fat body of species such as mosquitoes (Hagedorn *et al.*, 1975; Adams *et al.*, 1985). JH is the main regulator of female reproduction and the *Vg* gene expression in a majority of hemimetabolan insects (Wigglesworth, 1936; Socha *et al.*, 1991; Wyatt and Davey, 1996; Comas *et al.*, 1999) but not in all of them (Bradley *et al.*, 1995; Hoffmann and Sorge, 1996). Vitellogenesis in holometabolan insects can be regulated by JH (de Kort *et al.*, 1997; Parthasarathy *et al.*, 2010), by JH and E (Sun *et al.*, 2003; Riddiford, 2013) or can be JH and E independent (Wyatt and Davey, 1996; Handler, 1997).

JH signaling pathway in reproduction

Advances in molecular methods enabled partial unveiling of the mechanism of JH action on regulation of oogenesis. In 1986 Wilson and Fabian showed that *Drosophila Met* mutants exhibited delayed onset of vitellogenesis and oviposition. The finding was later supported by the presence of Met protein in the nuclei of the FC of the vitellogenic follicles (Pursley *et al.*, 2000). Flies mutant for the *Met* paralog *gce* also showed reduced fecundity (Abdou *et al.*, 2011). *Met* RNAi in *Tribolium* caused more than 80% reduction of *Vg* mRNA levels, although *Kr-h1* RNAi affected *Vg* expression only mildly (Parthasarathy *et al.*, 2010). Additionally, Sheng *et al.* (2011) demonstrated that JH and Met regulate *Vg* expression in *Tribolium* indirectly through the insulin-like peptide pathway.

Vitellogenesis in adult *Aedes aegypti* females is regulated mainly by ecdysteroids but females require a JH-dependent posteclosion (PE) development that prepares their reproductive tissues and the fat body for ecdysteroid mediated processes (Raikhel *et al.*, 2005). During the JH-regulated PE period JH titer rises and peaks at 48-50 hours PE and declines slowly thereafter (Shapiro *et al.*, 1986). Zou *et al.* (2013) used the microarray time-course analysis to assign genes to three major gene clusters according to their response to JH in the female fat body during the first 72 hours PE: early posteclosion (EPE), mid posteclosion (MPE) and late posteclosion (LPE) clusters. EPE genes are maximally expressed at a low JH titer, MPE at an intermediate JH level and LPE genes require a high JH titer for their

maximal expression. Though a large percentage of EPE, MPE, and LPE genes appear not to be controlled by Met, *Met* RNAi downregulated genes that were maximally expressed during LPE and up-regulated those that were expressed during EPE. Thus, Met can activate or repress gene expression of diverse gene sets both at low and high JH titer and prepare the mosquito female for the blood meal and reproduction (Zou *et al.*, 2013). Moreover, *Met* RNAi in *Aedes* caused retardation of ovarian follicle growth (Zou *et al.*, 2013) and either *Met* or *tai* (*FISC*) knockdown substantially reduced egg production (Li *et al.*, 2011).

In *Pyrrhocoris*, knockdown of *Met* or *tai*, but not of *Kr-h1* blocked ovarian development and *Vg* mRNA expression in the bug fat body (Smykal *et al.*, 2014a) and thus phenocopied the absence of JH upon allatectomy or during reproductive diapause (Smykal *et al.*, 2014a). More recently, Song *et al.*, 2014 demonstrated that *Met* RNAi in adult *Locusta migratoria* females caused substantial reduction of *Vg* expression in the fat body as well as arrested ovarian development. Unlike in *Pyrrhocoris* (Smykal *et al.*, 2014a), *Kr-h1* knockdown had the same reducing effect on locust female reproduction, suggesting that the canonical JH-Met-Kr-h1 pathway may regulate both development and reproduction in some species (Song *et al.*, 2014). *Met* RNAi in the viviparous cockroach *Diploptera punctata* blocked Vg synthesis in the fat body and the vitellogenic growth of the oocytes (Marchal *et al.*, 2014). The arrested follicles showed no signs of patency and their FC displayed abnormal morphology of the rough endoplasmic reticulum and failed in the vitelline membrane and chorion deposition. None of the phenotypes could be rescued by application of the JHM methoprene (Marchal *et al.*, 2014). However, Met cell-autonomous function in the establishment of the FC patency stays unclear since the systemic nature of *Met* RNAi might suppress other signaling pathways including JH biosynthesis in the CA (Marchal *et al.*, 2014).

Discussed studies from several hemi- and holometabolan species suggest that Met not only mediates the effects of JH on in diverse types of metamorphosis, but also on reproduction of insects with diverse organization of the ovaries. Since JH stimulates vitellogenesis and egg production even in ametabolous insects such as in firebrats (Rohdendorf and Watson, 1969; Bitsch *et al.*, 1985), regulation of reproduction might be the original role of JH/Met signaling in insects.

RESULTS

Research article I

Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects

Barbora Konopova, Vlastimil Smykal and Marek Jindra

OPEN ACCESS Freely available online

PLoS one

Common and Distinct Roles of Juvenile Hormone Signaling Genes in Metamorphosis of Holometabolous and Hemimetabolous Insects

Barbora Konopova[1☯], Vlastimil Smykal[2☯], Marek Jindra[1]*

1 Biology Center, Academy of Sciences of the Czech Republic, Ceske Budejovice, Czech Republic, **2** Department of Molecular Biology, University of South Bohemia, Ceske Budejovice, Czech Republic

Abstract

Insect larvae metamorphose to winged and reproductive adults either directly (hemimetaboly) or through an intermediary pupal stage (holometaboly). In either case juvenile hormone (JH) prevents metamorphosis until a larva has attained an appropriate phase of development. In holometabolous insects, JH acts through its putative receptor Methoprene-tolerant (Met) to regulate *Krüppel-homolog 1* (*Kr-h1*) and *Broad-Complex* (*BR-C*) genes. While Met and Kr-h1 prevent precocious metamorphosis in pre-final larval instars, BR-C specifies the pupal stage. How JH signaling operates in hemimetabolous insects is poorly understood. Here, we compare the function of *Met*, *Kr-h1* and *BR-C* genes in the two types of insects. Using systemic RNAi in the hemimetabolous true bug, *Pyrrhocoris apterus*, we show that Met conveys the JH signal to prevent premature metamorphosis by maintaining high expression of *Kr-h1*. Knockdown of either *Met* or *Kr-h1* (but not of *BR-C*) in penultimate-instar *Pyrrhocoris* larvae causes precocious development of adult color pattern, wings and genitalia. A natural fall of *Kr-h1* expression in the last larval instar normally permits adult development, and treatment with an exogenous JH mimic methoprene at this time requires both *Met* and *Kr-h1* to block the adult program and induce an extra larval instar. Met and Kr-h1 therefore serve as JH-dependent repressors of deleterious precocious metamorphic changes in both hemimetabolous and holometabolous juveniles, whereas BR-C has been recruited for a new role in specifying the holometabolous pupa. These results show that despite considerable evolutionary distance, insects with diverse developmental strategies employ a common-core JH signaling pathway to commit to adult morphogenesis.

Citation: Konopova B, Smykal V, Jindra M (2011) Common and Distinct Roles of Juvenile Hormone Signaling Genes in Metamorphosis of Holometabolous and Hemimetabolous Insects. PLoS ONE 6(12): e28728. doi:10.1371/journal.pone.0028728

Editor: Amit Singh, University of Dayton, United States of America

Received October 11, 2011; **Accepted** November 14, 2011; **Published** December 8, 2011

Funding: This work was supported by grants 500960906 from the Academy of Sciences and 6007665801 from the Ministry of Education of the Czech Republic, and by projects 204/07/1032 and 204/09/H058 from the Czech Science Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: jindra@entu.cas.cz

☯ These authors contributed equally to this work.

Introduction

Winged insects have evolved diverse modes of metamorphosis [1]. Hemimetabolous insects such as grasshoppers, true bugs or cockroaches develop from larvae (also called nymphs) that resemble adults, possess externally growing wing pads, and metamorphose during the final molt by acquiring perfect wings and genitalia. In contrast, larvae of holometabolous insects including flies, butterflies or beetles can differ dramatically from the adults. They undergo a two-stage "complete" metamorphosis (holometaboly), first forming an intermediate called the pupa before changing into a winged adult. Holometaboly has evolved from hemimetaboly and, judging by the number of known species, has become the most successful developmental strategy on land [2].

In both hemimetabolous and holometabolous insects, the developmental switch between juvenile and adult forms depends on juvenile hormone (JH), a sesquiterpenoid produced by the corpora allata gland [3]. The presence of JH in pre-final larval instars ensures that the next molt, promoted by ecdysteroids, produces another, only a larger larva [4,5]. At an appropriate stage, a natural drop of JH secretion permits metamorphosis. Experimental removal of JH at earlier times activates the metamorphic program prematurely, whereas supply of ectopic JH to final-instar larvae or pupae causes repetition of larval or pupal instars, respectively [6–8].

Although the anti-metamorphic effect of JH was discovered in the hemimetabolous true bug, *Rhodnius prolixus*, [9,10], our knowledge on the molecular mode of JH action almost exclusively derives from studies in holometabolans. JH signals through its putative intracellular receptor, the bHLH-PAS protein Methoprene-tolerant (Met), originally identified in the fruit fly *Drosophila melanogaster* [11,12]. In the red flour beetle, *Tribolium castaneum*, loss of Met triggers pupation of larvae during pre-final instars [13] – a classic precocious metamorphosis phenotype caused by deficiency of JH itself [14]. In response to JH, Met regulates expression of transcription factor genes *Krüppel-homolog 1* (*Kr-h1*) and *Broad-Complex* (*BR-C*) [15–18], and loss of *Kr-h1* also elicits precocious metamorphosis of beetle larvae [17]. *BR-C* is dispensable in holometabolous larvae until the onset of metamorphosis, when it specifies pupal features [16,19–23]. Upon pupation both *BR-C* and *Kr-h1* are naturally down-regulated by the absence of JH to allow

adult development [16–18,20,22]. Of the three JH-signaling genes, *BR-C* has been functionally studied in hemimetabolous insects, where, unlike in holometabolans, it is required for development of the embryonic germ band [24,25] and for anisometric growth of the larval wing pads [26].

To provide a direct comparison of JH signaling in holometaboly and hemimetaboly, we have examined the function of *Met*, *Kr-h1* and *BR-C* in the hemimetabolous firebug, *Pyrrhocoris apterus* (true bugs, Hemiptera). We show that despite the diverse developmental strategies and the vast evolutionary distance between them, transduction of the anti-metamorphic JH signal relies on the common-core elements, Met and Kr-h1. In both insect types, Kr-h1 acts as a strictly JH- and Met-dependent repressor of metamorphosis. In contrast, the function of BR-C has changed from promoting progressive development of hemimetabolous larvae to a new role in specifying the holometabolous pupa.

Results

JH Signaling Genes Are Conserved in Insects with Diverse Types of Development

As the first step towards functional comparison between holometaboly and hemimetaboly, we have isolated cDNAs encoding the putative JH receptor Met (JN416984) and its target genes *Kr-h1* (JN416987) and *BR-C* (JN416990), from *Pyrrhocoris apterus*, and *Met* (JN416985) and *Kr-h1* (JN416988) cDNAs from another true bug, *Rhodnius prolixus*. Alignments amongst the orthologs reveal conservation of the main functional domains (Fig. S1), namely the basic helix-loop-helix (bHLH) region and two Per-Arnt-Sim (PAS) domains in Met, eight zinc-finger motifs in Kr-h1, and a Broad-Tramtrac-Bric-a-brac (BTB) domain followed by one of the alternative zinc-finger isoforms (Z2) in BR-C. Therefore, the three JH signaling genes are common to insect orders developing through holometaboly and hemimetaboly. In fact, their conservation predates the origin of metamorphosis, as we have found *Met* (JN416986), *Kr-h1* (JN416989) and *BR-C* (JN416991) orthologs in the firebrat, *Thermobia domestica*, a representative of the primitive lineage of non-metamorphosing wingless insects (Fig. S1).

Developmental Regulation of the JH-Response Genes in *Pyrrhocoris*

The firebug invariantly undergoes four larval molts demarcating five larval instars (L1–L5), followed by a metamorphic molt, which produces an adult possessing external genitals and wings with a specific color pattern. Maintenance of the larval state in *Pyrrhocoris* requires JH, as has been demonstrated by removal of the JH-producing corpora allata gland (allatectomy) from penultimate (L4) larvae [27]. Conversely, metamorphosis is permitted by a natural decline in JH titer during the last larval instar (L5), as supplying L5 larvae with JH mimics induces an extra larval stage (reference [28] and this work).

We examined how expression of *Met*, *Kr-h1* and *BR-C* during *Pyrrhocoris* development correlates with this regulation by JH. From embryogenesis to adulthood, *Met* mRNA persisted without major fluctuations through all larval stages (Fig. 1). Such a constitutive presence agrees with the presumed JH receptor role of Met and with the ability of L5 larvae that naturally lack JH to respond to exogenous JH mimics by forming an extra larval instar. Temporal profiles of *Kr-h1* and *BR-C* were more dynamic. Both transcripts reached their highest levels during the second half of embryogenesis, then *BR-C* oscillated throughout larval development, whereas *Kr-h1* was not detected in last-instar larvae, L5 (Fig. 1A). A more detailed profile revealed that upon ecdysis to L5, *BR-C* mRNA

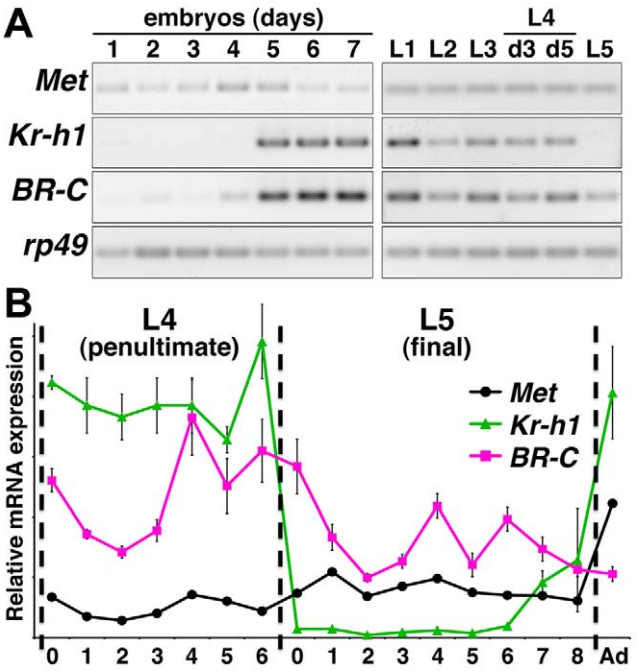

Figure 1. Developmental expression of *Pyrrhocoris* JH signaling genes. A) Semi-quantitative RT-PCR on total RNA from embryos (26 temperature cycles) or larvae (28 cycles) of indicated stages. d3, d5, mid- and late-L4 instar larvae, respectively. **B**) Relative mRNA levels on successive days of larval development were assessed with qRT-PCR and normalized to *rp49* mRNA. Dashed lines mark ecdyses to L4, L5 and adult stages. Values are mean ± SD from three measurements on RNA isolated from individual animals.
doi:10.1371/journal.pone.0028728.g001

decreased but remained expressed to adulthood, while *Kr-h1* expression plummeted from its L4 level by more than 50-fold and was virtually undetectable for the first six days of L5 until the next rise, likely induced by a new surge of JH in pharate adults (Fig. 1B). Allatectomy of adult *Pyrrhocoris* females drastically reduced *Kr-h1* mRNA (494-fold, $n = 3$), confirming that *Kr-h1* transcription absolutely requires the natural source of JH. The expression pattern suggested that Kr-h1 prevents metamorphosis until the final larval instar, when the Kr-h1-free (and JH-free) period may be necessary to initiate metamorphosis.

Kr-h1 Is a Met-Dependent Repressor of Precocious Adult Development in *Pyrrhocoris* Larvae

To test whether Met and Kr-h1 are indeed required to maintain the juvenile character of hemimetabolous larvae, we utilized systemic RNAi in *Pyrrhocoris*. Injection of *Met* dsRNA into early-L4 larvae caused an 86% knockdown of *Met* mRNA (Fig. 2B), followed by precocious appearance of adult attributes after the ensuing molt, which would have otherwise produced an L5 larva (Fig. 2C and Table 1). Despite their smaller body size compared to normal adults, *Met(RNAi)* males had external genitals (Fig. 2C). Instead of the solid black and fully attached L5 wing pads, these adultoids developed small but movable, articulated wings, liberated from the scutellum and the tergites. The black melanin pigment disappeared from specific regions of these wings to leave black spots and edges on the red background, a pattern only seen in adults (Fig. 2C). On the thorax, the notum expanded posteriorly and also displayed an adult-specific color pattern. Like in adults, abdominal cuticle tanned with melanin. We did not determine whether particular regions of the cuticle, produced by individual

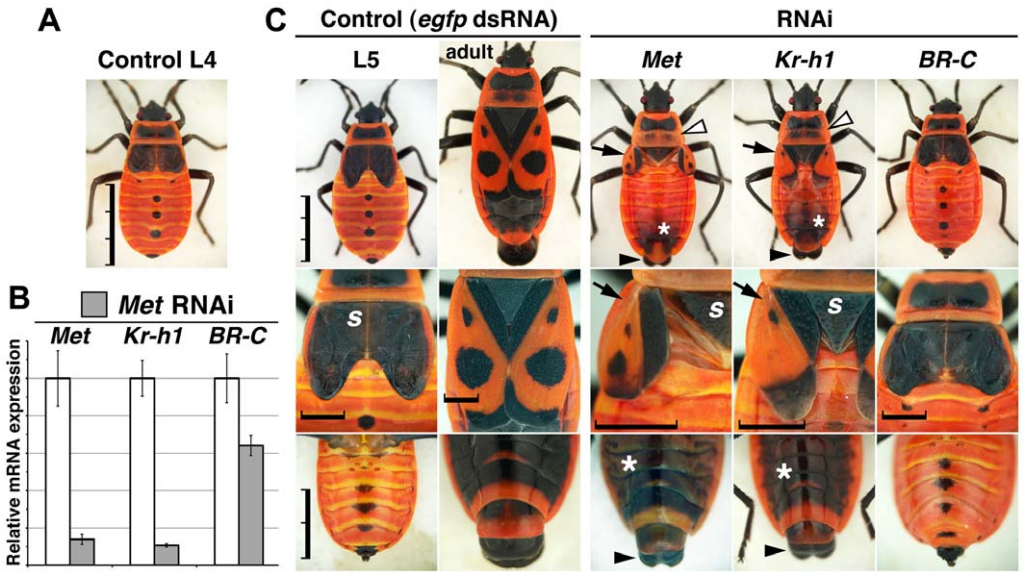

Figure 2. Loss of *Met* or *Kr-h1* causes precocious metamorphosis in *Pyrrhocoris* larvae. A) Larvae newly ecdysed to the L4 instar were injected with control (*egfp*) or *Met* dsRNA. **B**) Efficacy of *Met* mRNA depletion and its effect on expression of *Kr-h1* and *BR-C* expression were determined by qRT-PCR three days after injection of *Met* dsRNA (gray columns) relative to *egfp* dsRNA controls (open columns) arbitrarily set to 100%. Values are mean ± SD from $n = 5$ animals. **C**) *Met*, *Kr-h1* and *BR-C* RNAi phenotypes after ecdysis to the L5 stage as compared to control L5 larvae and adults (left two columns). Animals in the top row are to the same scale; the middle and bottom rows show details of wings and of the ventral abdomen, respectively. Precocious adult attributes upon *Met* and *Kr-h1* RNAi include color-patterned articulated wings (arrows) separated from the scutellum (*s*), extended notum (open arrowheads) with two posterior black spots, external male genitals (solid arrowheads), and dark abdominal cuticle (asterisks). Compared to control L5, *BR-C(RNAi)* larvae display retarded wing growth but no precocious adult development. For quantitative data see Table 1. (Scale bars: A and C, top row, 3 mm; C, middle row, 1 mm; C, bottom row, 2 mm).
doi:10.1371/journal.pone.0028728.g002

epidermal cells, were purely adult or whether they had a mixed larval-adult identity, so this intriguing possibility remains [29]. Unable to molt again, all L5 adultoids were terminally arrested.

Kr-h1 mRNA levels were reduced by 90% in L4 larvae subjected to *Met* RNAi (Fig. 2B), demonstrating that *Kr-h1* in *Pyrrhocoris* was a Met-dependent target gene as in *Tribolium* [15,17]. This reduction paralleled the natural fall of *Kr-h1* expression upon ecdysis to L5 (Fig. 1B), suggesting that the normal function of Met during the L4 stage is to respond to endogenous JH by maintaining high expression of *Kr-h1*, which in turn prevents adult development. Consistent with this prediction, larvae injected with *Kr-h1* dsRNA at the L4 stage formed L5 adultoids similar to *Met(RNAi)* animals (Fig. 2C and Table 1). Occasionally their wings grew larger than upon *Met* RNAi, and some individuals only showed part of the adult color pattern (Fig. 2C). Clearly, the loss-of-function phenotypes of both genes were in good concert and demonstrated precocious metamorphosis of *Pyrrhocoris* larvae.

Table 1. Loss of *Met* or *Kr-h1* triggers precocious adult development in *Pyrrhocoris* larvae.

dsRNA	n	L4 Death[a]	Abnormal L5 phenotypes		Normal adults
			Adult hallmarks[b]	Short wings	
Met	29	3	26	0	0
Kr-h1	31	3	28	0	0
BR-C	34	15	0	19	0
control	12	1	0	0	11

[a]Lethality without a specific external phenotype.
[b]Precocious metamorphosis.
doi:10.1371/journal.pone.0028728.t001

To verify the function of *Kr-h1* in an independent system, we performed RNAi in the blood-sucking bug *Rhodnius*, the very model in which juvenile hormone had been postulated nearly eight decades ago [9,10]. Upon molting to the L4 penultimate instar, all *Rhodnius* larvae ($n = 20$) injected with *Kr-h1* dsRNA at the previous L3 instar showed accelerated growth of wings and genitals (Fig. 3), both typical hallmarks of adult morphogenesis.

In contrast to *Met* and *Kr-h1*, injection of *BR-C* dsRNA into *Pyrrhocoris* L4 larvae produced no signs of premature adult development (Fig. 2C and Table 1). *BR-C* RNAi administered early during the L4 instar reduced *BR-C* mRNA levels to 17.7±2.0% ($n = 5$ animals) and caused either lethality at the end of the L4 instar or compromised growth of the wing pads in animals that successfully molted to the L5 instar (Table 1). A similar wing defect was observed after ecdysis to the L4 instar in 100% of animals ($n = 22$) that had been given *BR-C* dsRNA as L3 larvae. The retarded wing growth was previously shown for *BR-C* RNAi in the milkweed bug, *Oncopeltus fasciatus* [26]. When compared to *Kr-h1*, *BR-C* mRNA expression was neither as strongly dependent on Met at the L4 instar (Fig. 2B) nor did it completely cease upon ecdysis to L5 (Fig. 1B). Therefore, Kr-h1 but not BR-C functions as a JH- and Met-dependent repressor of hemimetabolous metamorphosis.

Met and Kr-h1 Mediate the Anti-Metamorphic Effect of Methoprene

When treated with JH mimics, final instar *Pyrrhocoris* larvae fail to metamorphose to adults and instead repeat the larval program in the succeeding supernumerary "L6" instar [28]. We achieved the same effect by topical treatment of early-L5 larvae with the JH

Figure 3. Precocious development of adult features after *Kr-h1* RNAi in the blood sucking bug, *Rhodnius prolixus*. When subjected to *Kr-h1* dsRNA injection as L3 larvae, the animals displayed abnormal growth and venation of wing lobes after molting to the L4 stage (right) as compared to control L4 larvae. We also noticed premature development of external genitals in *Kr-h1(RNAi)* animals (arrowhead).
doi:10.1371/journal.pone.0028728.g003

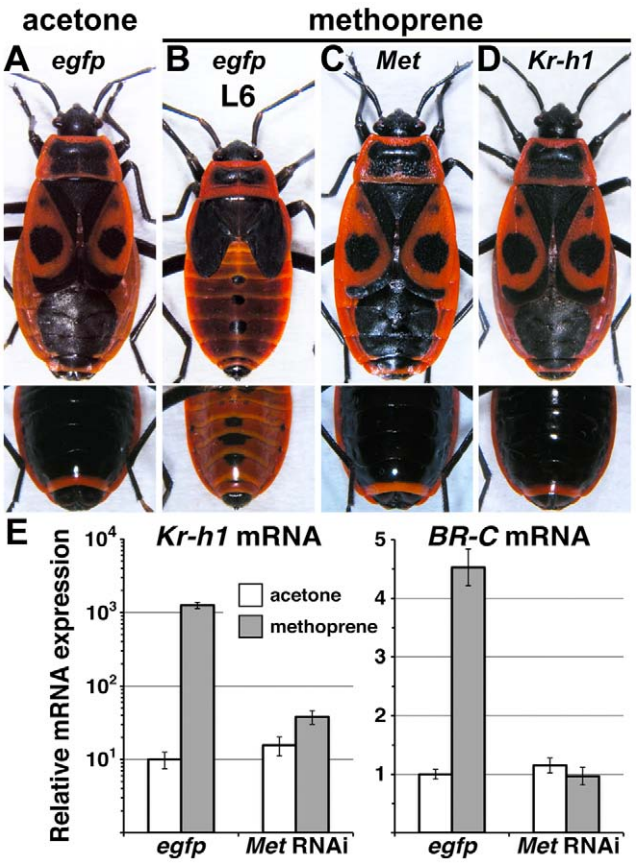

Figure 4. *Met* and *Kr-h1* mediate the anti-metamorphic effect of exogenous JH mimic. A–D) Animals received either control (*egfp*), *Met* or *Kr-h1* dsRNA as late-L4 larvae, followed by mock (acetone) or JH mimic (methoprene) treatment early at the L5 instar. Bottom row shows ventral view of the abdomens. Acetone-treated larvae produced normal adults (A). Methoprene induced a supernumerary larval instar (L6) whose wing pads remained black and attached to the tergites, while the abdominal cuticle tanned only partly (B). Knockdown of *Met* (C) or *Kr-h1* (D) prior to methoprene treatment restored normal adult development. **E**) Ectopic Met-dependent induction of *Kr-h1* and *BR-C* by methoprene at the L5 instar. Relative mRNA levels of *Kr-h1* and *BR-C* in the abdominal epidermis of animals injected with control (*egfp*) or *Met* dsRNA and subjected to hormonal treatment as described above were assessed on day 4 of the L5 instar. Values are mean ± SD from $n = 5$ animals. Note that the much higher *Kr-h1* induction (left) is on the logarithmic scale.
doi:10.1371/journal.pone.0028728.g004

mimic methoprene (Fig. 4A,B) in 100% ($n = 18$) of animals. In this background we then tested whether *Met* and *Kr-h1* mediated the response to the exogenous JH activity.

When *Met* was silenced by dsRNA injection at the end of the L4 instar, the following application of methoprene no longer perturbed adult development in 96% ($n = 23$) of treated animals, and these rescued adults were externally indistinguishable from controls receiving no methoprene (Fig. 4A,C). The "status-quo" effect of methoprene was likewise prevented by *Kr-h1* RNAi (Fig. 4D) in 12 out of 13 animals. Therefore, both *Met* and its target *Kr-h1* were necessary for the capacity of methoprene to block metamorphosis and induce the L6 larva. In addition, neither *Met* nor *Kr-h1* appeared to be required for the normal L5-to-adult transition, since dsRNA injection to late-L4 larvae, as opposed to RNAi applied early during the L4 stage, did not interfere with metamorphosis. This result corresponded with the natural absence of *Kr-h1* expression during most of the final larval instar (Fig. 1B).

The above data indicated that the Met-dependent reiteration of the larval program, induced by methoprene, occurred via ectopic transcriptional re-activation of *Kr-h1* during the L5 instar. Indeed, levels of *Kr-h1* mRNA in the epidermis (or in the whole body, data not shown) of mid-L5 larvae, which had been treated with methoprene as L4, exceeded levels normally observed at the L5 instar by 125-fold (Fig. 4E). This induction was the opposite to the natural drop of *Kr-h1* expression upon L4 to L5 ecdysis (Fig. 1B), and it was abolished by *Met* RNAi (Fig. 4E). By contrast, *BR-C* mRNA was only induced 4.5-fold with methoprene, again in a Met-dependent manner (Fig. 4E). The striking difference in the fold mRNA induction between *Kr-h1* and *BR-C* did not result from difference in the mRNA levels induced by methoprene but from the extremely low expression of *Kr-h1* in mid-L5 larvae (Fig. 1).

Discussion

The likely JH receptor Met and its targets *Kr-h1* and *BR-C* play key roles during holometabolous insect metamorphosis. Our present data afford a direct comparison with how the three genes function in the ancestral hemimetabolous development (Fig. 5). We provide three lines of evidence that the JH-free and Kr-h1-free period in the final larval instar (L5) of the *Pyrrhocoris* bug is critical for adult transition: (*i*) administration of methoprene to L5 larvae induces Met-dependent *Kr-h1* expression and an extra larval stage,

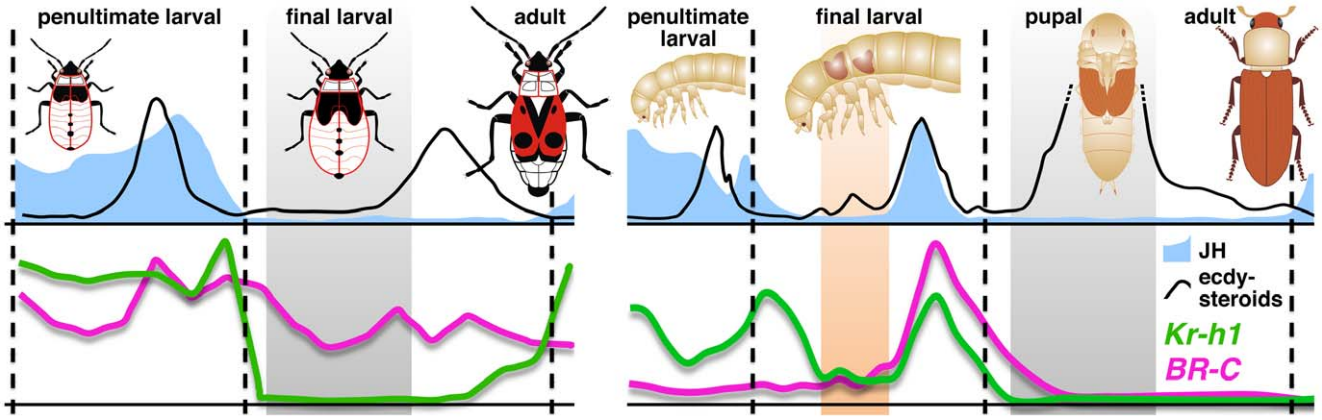

Figure 5. Regulation of hemimetaboly and holometaboly. *Pyrrhocoris* (left) and *Tribolium* (right) cartoons signify the main innovations – postponement of wing development and the resting pupal stage in holometabolans. The absence of JH-dependent *Kr-h1* expression in pupae and final instar hemimetabolous larvae (gray shaded areas) is prerequisite to adult development in both types of metamorphosis, supporting the view that these final juvenile stages of both insects types may be homologous [1,35]. The orange shaded area marks a period of low *Kr-h1* activity in the absence of JH, which is necessary to permit partial metamorphosis during the pupal molt, specified by the newly acquired function of *BR-C* in holometabolans. Gene expression profiles for *Pyrrhocoris* and *Tribolium* are from Figure 1B and from [17], respectively. JH and ecdysteroid titers are from *Blattella germanica* [34] (left) and *Manduca sexta* [5] (right).
doi:10.1371/journal.pone.0028728.g005

(*ii*) this phenotype is averted if *Met* or *Kr-h1* are silenced prior to methoprene treatment, and (*iii*) premature suppression of *Kr-h1*, either direct or through depletion of Met, triggers precocious adult development. Therefore, the JH/Met-dependent *Kr-h1* activity ensures the larval program, and only when JH disappears from the blood in the last larval instar, transcription of *Kr-h1* ceases for six days to create an opportunity for adult transition (Figs. 1B and 5). Interestingly, the same-length time window for adult commitment was previously defined as a methoprene-sensitive period during the final larval instar of the *Rhodnius* bug [30]. *Kr-h1* now provides a molecular determinant of that window.

Similar regulation applies to holometaboly. In pre-terminal instars of *Tribolium* larvae, *Met* and *Kr-h1* respond to JH by blocking precocious metamorphosis [13,17]. By mid-final larval instar, expression of *Kr-h1* declines to reappear at the pupal molt, this time together with *BR-C* mRNA (Fig. 5). While the transient down-regulation of *Kr-h1* permits partial metamorphosis (pupation), its co-expression with *BR-C* likely ensures that development does not go too far. This is suggested by appearance of not only pupal but also of adult features in *Tribolium* larvae subjected to *Kr-h1* or *BR-C* RNAi [16,17,22,23]. After the pupal program has been installed, a JH-free period ensures that both *Kr-h1* and *BR-C*, acting downstream of *Kr-h1* in this context [17,18], are shut down in order for adult morphogenesis to take place (Fig. 5). Giving exogenous JH or its mimics to pupae will re-activate both genes and block adult development [16–18,20].

While the function of *Kr-h1* as a JH-induced repressor of adult morphogenesis is clearly a common trait of holometaboly and hemimetaboly, the role of *BR-C* is not. Studies on the hemimetabolan *Oncopeltus* bug have revealed *BR-C* requirement during embryogenesis and for the anisometric growth of larval wing pads but no *BR-C* expression or function connected with metamorphosis [24,26]. Conversely, *BR-C* is essential for pupal development but not at earlier stages in representatives of four holometabolan insect orders [16,19–22]. The delay of *BR-C* activity until the pupal stage in Holometabola has been ascribed to an early surge of JH during embryogenesis, which is thought to preserve the seemingly undeveloped ("embryonic") nature of holometabolous larvae [24,31–33]. However, while *BR-C* is necessary for hemimetabolous embryogenesis [24,25], its function has not been causally linked with JH in insect embryos.

Unlike in *Oncopeltus*, *BR-C* expression continues, albeit at a lower rate, throughout the last larval instar of *Pyrrhocoris* or another hemimetabolan, the cockroach *Blattella germanica*, in the absence of JH [34] (Fig. 5). Consistent with this pattern, our data show that compared to *Kr-h1*, expression of *BR-C* much less depends on JH and that in contrast to *Kr-h1* or *Met*, removal of *BR-C* cannot accelerate metamorphosis in bug larvae. The changed need for *BR-C* function from hemimetabolous embryos and larvae to holometabolous metamorphosis suggests that during the evolution of holometaboly, *BR-C* has been recruited for the new function in specifying the pupal state.

Kr-h1 is intimately regulated by JH and Met to safeguard juveniles of both hemimetabolous and holometabolous insects against precocious and hence fatal metamorphic changes. Therefore, regardless of the disparate life histories, insects undergoing both types of metamorphosis use a common signaling pathway to commit to adult development. The parallel timing of the critical down-regulation of *Kr-h1* in the final-instar *Pyrrhocoris* larva and in the holometabolous pupa (Fig. 5) supports the view that these stages represent ontogenetically homologous units [1,35], rather than hypotheses building on the assumption that the pupa has originated via compression of all hemimetabolous larval instars into one [24,32,33].

Materials and Methods

Insects

Pyrrhocoris apterus (short-winged form) was maintained at 25°C and a photoperiod of 18 h light to 6 h dark, on dry linden seeds and was supplemented with water. Eggs were collected daily and larvae of particular instars were identified based on the size of the body and wing pads; staging within instars relied on measuring time after ecdysis.

cDNA Cloning

Partial sequences for *Pyrrhocoris Met*, *Kr-h1* and *BR-C* genes were isolated by using touch-down nested RT-PCR with degenerate

primers (Table S1), mapping to conserved domains. cDNA ends of selected genes were amplified with the GeneRacer Kit (Invitrogen, Carlsbad, CA).

mRNA Expression Analysis

Total RNA was isolated from *Pyrrhocoris* embryos, whole larvae or abdominal epidermis with the TRIzol reagent (Invitrogen, Carlsbad, CA). After TURBO DNase (Ambion, Austin, TX) treatment, 2 μg of RNA were used for cDNA synthesis with Superscript II reverse transcriptase (Invitrogen). Relative transcript levels were measured by quantitative RT-PCR using the iQ SYBR Green Supermix kit and the C1000 Thermal Cycler (both from Bio-Rad Laboratories, Hercules, CA). All data were normalized to the relative levels of ribosomal protein (Rp49) mRNA as described [36]. Primer sequences used for qRT-PCR are listed in Table S2.

RNAi and Methoprene Treatments

dsRNAs comprising 952 bp (*Met*), 844 bp (*Kr-h1*) and 1026 bp (*BR-C*) of the *Pyrrhocoris* cDNA sequences and control dsRNAs encoding the EGFP and MalE proteins (720 bp and 901 bp, respectively) were synthesized by using the T3 and T7 MEGAscript kit (Ambion, Austin, TX). Approximately 2–5 μg of dsRNA (depending on the size of larvae) were injected into the abdomen of CO_2-anesthesized bugs. For JH mimic treatment, late-L4 stage *Pyrrhocoris* larvae were injected with dsRNA and within 2–3 hours after ecdysis to the L5 instar, a 4-μl drop of acetone-diluted 0.3 mM methoprene (VUOS, Pardubice, Czech Republic) or acetone alone (control) was applied on their dorsal side.

Supporting Information

Figure S1 Conservation of JH signaling genes. Alignments of Met (*A*), Kr-h1 (*B*) and BR-C (*C*) protein sequences from insects representing diverse developmental strategies. Holometaboly: the fruit fly *Drosophila melanogaster*, mosquitoes *Aedes aegypti* and *Culex quinquefasciatus* (Diptera); the silk moth *Bombyx mori* (Lepidoptera); the flour beetle *Tribolium castaneum* (Coleoptera); the honey bee *Apis melifera* (Hymenoptera); the lacewing *Chrysopa perla* (Neuroptera). Hemimetaboly: true bugs *Pyrrhocoris apterus, Rhodnius prolixus* and *Oncopeltus fasciatus* (Hemiptera); the cockroach *Blattella germanica* (Blattodea); the louse *Pediculus humanus corporis* (Phthiraptera). The thrips, *Frankliniella occidentalis* (Thysanoptera), represents neometaboly, an aberrant type of hemimetaboly with multiple resting stages. The firebrat, *Thermobia domestica* (Zygentoma), represents a basal lineage of wingless insects without metamorphosis (ametaboly). *Dm*_Met, *Dm*_Gce, and *Bm*_Met1, *Bm*_Met2 are products of paralogous *Met* genes that have duplicated independently in the *Drosophila* and *Bombyx* lineages, respectively. Database accession numbers for the aligned Met proteins (*A*) are NP_511126.2 (*Drosophila melanogaster* Met), NP_511160.1 (*Drosophila melanogaster* Gce), XP_001660262.1 (*Aedes aegypti*), BAJ05085.1 (*Bombyx mori* Met1), BAJ05086.1 (*Bombyx mori* Met2), ABR25244.1 (*Tribolium castaneum*), XP_395005.3 (*Apis melifera*), JN416984 (*Pyrrhocoris apterus*), JN416985 (*Rhodnius prolixus*), XP_002430841.1 (*Pediculus humanus corporis*), and JN416986 (*Thermobia domestica*). Accession numbers for the Kr-h1 proteins (*B*) are CAA06544.2 (*Drosophila melanogaster*), XP_001863529.1 (*Culex quinquefasciatus*), NP_001129235.1 (*Tribolium castaneum*), NP_001011566.1 (*Apis melifera*), BAJ41258.1 (*Frankliniella occidentalis*), JN416987 (*Pyrrhocoris apterus*), JN416988 (*Rhodnius prolixus*), XP_002428656.1 (*Pediculus humanus corporis*), and JN416989 (*Thermobia domestica*). Accession numbers for the BR-C proteins (*C*) are CAA38476.1 (*Drosophila melanogaster*), AAS80327.1 (*Aedes aegypti*), BAD23979.1 (*Bombyx mori*), joined ABW91135.1 and ABW91137.1 (*Tribolium castaneum*), ABW91140.1 (*Chrysopa perla*), BAJ41241.1 (*Frankliniella occidentalis*), JN416990 (*Pyrrhocoris apterus*), ABA02191.1 (*Oncopeltus fasciatus*), CBJ05858.1 (*Blattella germanica*), and joined GQ983556.1 and JN416991 (*Thermobia domestica*).
(PDF)

Table S1 Degenerate primers for isolation of *Met*, *Kr-h1* and *BR-C* cDNAs from *Pyrrhocoris apterus*, *Rhodnius prolixus* and *Thermobia domestica*.
(PDF)

Table S2 Primers for RT-PCR expression analysis of *Pyrrhocoris apterus Met*, *Kr-h1* and *BR-C* mRNAs.
(PDF)

Acknowledgments

We thank David Dolezel for sharing *Pyrrhocoris* culture and information on gene sequences, Jan Erhart for *Rhodnius*, Magdalena Hodkova for allatectomy, Martina Hajduskova for insect drawings, and Aida Svestkova for technical help.

Author Contributions

Conceived and designed the experiments: BK MJ. Performed the experiments: VS BK MJ. Analyzed the data: VS BK MJ. Contributed reagents/materials/analysis tools: VS BK MJ. Wrote the paper: MJ.

References

1. Schnal F, Svacha P, Zrzavy J (1996) Evolution of insect metamorphosis. In: Gilbert LI, Tata JR, Atkinson BG, eds. Metamorphosis. Postembryonic reprogramming of gene expression in amphibian and insect cells. San Diego: Academic Press. pp 3–58.
2. Kristensen N (1999) Phylogeny of endopterygote insects, the most successful lineage of living organisms. Eur J Entomol 96: 237–253.
3. Gilbert LI, Granger NA, Roe RM (2000) The juvenile hormones: historical facts and speculations on future research directions. Insect Biochem Mol Biol 30: 617–644.
4. Nijhout H (1994) Insect hormones. Princeton: Princeton University Press. 267 p.
5. Riddiford L (1994) Cellular and molecular actions of juvenile hormone. I. General considerations and premetamorphic actions. Adv Insect Physiol 24: 213–274.
6. Fukuda S (1944) The hormonal mechanism of larval molting and metamorphosis in the silkworm. J Fac Sci Tokyo Univ sec IV 6: 477–532.
7. Wigglesworth V (1954) The physiology of insect metamorphosis. Cambridge: Cambridge University Press. 152 p.
8. Williams C (1961) The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the *Cecropia* silkworm. Biol Bull 116: 323–338.
9. Wigglesworth V (1934) The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II. Factors controlling moulting and ''metamorphosis.'' Quart J Micr Sci 77: 191–222.
10. Wigglesworth V (1936) The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). Quart J Micr Sci 79: 91–121.
11. Wilson TG, Fabian J (1986) A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. Dev Biol 118: 190–201.
12. Ashok M, Turner C, Wilson TG (1998) Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. Proc Natl Acad Sci U S A 95: 2761–2766.
13. Konopova B, Jindra M (2007) Juvenile hormone resistance gene Methoprene-tolerant controls entry into metamorphosis in the beetle *Tribolium castaneum*. Proc Natl Acad Sci U S A 104: 10488–10493. doi:10.1073/pnas.0703719104.
14. Minakuchi C, Namiki T, Yoshiyama M, Shinoda T (2008) RNAi-mediated knockdown of juvenile hormone acid O-methyltransferase gene causes precocious metamorphosis in the red flour beetle *Tribolium castaneum*. FEBS J 275: 2919–2931. doi:10.1111/j.1742-4658.2008.06428.x.
15. Parthasarathy R, Tan A, Palli SR (2008) bHLH-PAS family transcription factor methoprene-tolerant plays a key role in JH action in preventing the premature development of adult structures during larval–pupal metamorphosis. Mech Dev 125: 601–616. doi:10.1016/j.mod.2008.03.004.

16. Konopova B, Jindra M (2008) Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolan metamorphosis. Development 135: 559–568. doi:10.1242/dev.016097.
17. Minakuchi C, Namiki T, Shinoda T (2009) Krüppel homolog 1, an early juvenile hormone-response gene downstream of Methoprene-tolerant, mediates its anti-metamorphic action in the red flour beetle *Tribolium castaneum*. Dev Biol 325: 341–350. doi:10.1016/j.ydbio.2008.10.016.
18. Minakuchi C, Zhou X, Riddiford LM (2008) Krüppel homolog 1 (Kr-h1) mediates juvenile hormone action during metamorphosis of *Drosophila melanogaster*. Mech Dev 125: 91–105. doi:10.1016/j.mod.2007.10.002.
19. Kiss I, Beaton AH, Tardiff J, Fristrom D, Fristrom JW (1988) Interactions and developmental effects of mutations in the Broad-Complex of *Drosophila melanogaster*. Genetics 118: 247–259.
20. Zhou X, Riddiford LM (2002) Broad specifies pupal development and mediates the "status quo" action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. Development 129: 2259–2269.
21. Uhlirova M, Foy BD, Beaty BJ, Olson KE, Riddiford LM, et al. (2003) Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of Broad-Complex in insect metamorphosis. Proc Natl Acad Sci U S A 100: 15607–15612. doi:10.1073/pnas.2136837100.
22. Suzuki Y, Truman JW, Riddiford LM (2008) The role of Broad in the development of *Tribolium castaneum*: implications for the evolution of the holometabolous insect pupa. Development 135: 569–577. doi:10.1242/dev.015263.
23. Parthasarathy R, Tan A, Bai H, Palli SR (2008) Transcription factor broad suppresses precocious development of adult structures during larval–pupal metamorphosis in the red flour beetle, *Tribolium castaneum*. Mech Dev 125: 299–313. doi:10.1016/j.mod.2007.11.001.
24. Erezyilmaz DF, Rynerson MR, Truman JW, Riddiford LM (2010) The role of the pupal determinant broad during embryonic development of a direct-developing insect. Dev. Genes Evol 219: 535–544. doi:10.1007/s00427-009-0315-7.
25. Piulachs M-D, Pagone V, Bellés X (2010) Key roles of the Broad-Complex gene in insect embryogenesis. Insect Biochem Mol Biol 40: 468–475. doi:10.1016/j.ibmb.2010.04.006.
26. Erezyilmaz DF, Riddiford LM, Truman JW (2006) The pupal specifier broad directs progressive morphogenesis in a direct-developing insect. Proc Natl Acad Sci U S A 103: 6925–6930. doi:10.1073/pnas.0509983103.
27. Sláma K (1965) Effect of hormones on growth and respiratory metabolism in the larvae of *Pyrrhocoris apterus* L. (Hemiptera). J Insect Physiol 11: 113–122.
28. Sláma K, Williams CM (1965) Juvenile hormone activity for the bug *Pyrrhocoris apterus*. Proc Natl Acad Sci U S A 54: 411–414.
29. Willis J, Rezaur R, Sehnal F (1982) Juvenoids cause some insects to form composite cuticles. J Embryol Exp Morph 71: 25–40.
30. Nijhout H (1983) Definition of a juvenile hormone-sensitive period in *Rhodnius prolixus*. J Insect Physiol 29: 669–677.
31. Novak V (1966) Insect hormones. London: Methuen & Co. Ltd. 478 p.
32. Truman JW, Riddiford LM (1999) The origins of insect metamorphosis. Nature 401: 447–452. doi:10.1038/46737.
33. Truman JW, Riddiford LM (2002) Endocrine insights into the evolution of metamorphosis in insects. Annu Rev Entomol 47: 467–500. doi:10.1146/annurev.ento.47.091201.145230.
34. Belles X (2011) Origin and evolution of insect metamorphosis. Chichester: John Wiley & Sons, Ltd. doi:10.1002/9780470015902.a0022854.
35. Hinton H (1963) The origin and function of the pupal stage. Proc R Ent Soc Lond 38: 77–85.
36. Dolezel D, Zdechovanova L, Sauman I, Hodkova M (2008) Endocrine-dependent expression of circadian clock genes in insects. Cell Mol Life Sci 65: 964–969. doi:10.1007/s00018-008-7506-7.

Supplemental material

Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects

Barbora Konopova, Vlastimil Smykal and Marek Jindra

A
Met
bHLH
PAS-A
PAS-B
B
Kr-h1
Zn1
Zn2
Zn3
Zn4
Zn5
Zn6
Zn7
Zn8
C
BR-C
BTB
Zn Z2

Table S1. Degenerate primers for isolation of *Met*, *Kr-h1* and *BR-C* cDNAs from *Pyrrhocoris apterus*, *Rhodnius prolixus* and *Thermobia domestica*.

Target sequence	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
Met	GARATGMGIAAYHKIGCNGARAA	ACIARIGTRTTIAYRCANAYRAA
Met, nested	AARCARMGIMGISAIAARYTNAA	GCIAYIATIACCCANCKNACRTC
Kr-h1	CAYTAYMGIACICAYACNGGNGA	TTIARYTGYTTISWRCAIGTRAANCC
Kr-h1, nested	GTICAYMGIMGIATHCAYACNAARG	CAIAYRTAIGGYTTYTCICCNGTRTG
BR-C_Z2	TTYTGYYTIMGNTGGAAYAAYTAYC	TTRTGRTAIGTRTAIATRTGNGTCAT
BR-C_Z2, nested	TTYGARAAYYTIMGIGAYGAYGARG	CKISWRCARTAIACICKYTCRCA

Table S2. Primers for RT-PCR expression analysis of *Pyrrhocoris apterus Met*, *Kr-h1* and *BR-C* mRNAs.

Target sequence	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
Met	TTCTGATGATGGTGAAAAGATG	TATCGCCCCTGACTACTTGG
Kr-h1	GAACGTCTTGTTACACACACC	CCCTACCAGTGTAACTTTTGC
BR-C	TCTCCAAGATGTCATGTTTGAAG	AACCTCGACCGTCTGCCAAAC
*rp49**	CCGATATGTAAAACTGAGGAGAAAC	GGAGCATGTGCCTGGTCTTTT

*Levels of the ribosomal protein *rp49* mRNA were used for normalization of all expression data.

RESULTS

Research article II

Juvenile hormone signaling during reproduction and development of the linden bug, *Pyrrhocoris apterus*

Vlastimil Smykal, Adam Bajgar, Jan Provaznik, Silvie Fexova, Marcela Buricova, Keiko Takaki, Magdalena Hodkova, Marek Jindra and David Dolezel

Insect Biochemistry and Molecular Biology 45 (2014) 69–76

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Juvenile hormone signaling during reproduction and development of the linden bug, *Pyrrhocoris apterus*

Vlastimil Smykal [a,b,1], Adam Bajgar [a,b,1], Jan Provaznik [a,b], Silvie Fexova [a,b], Marcela Buricova [a,b], Keiko Takaki [a], Magdalena Hodkova [a], Marek Jindra [a,c,*], David Dolezel [a,b,*]

[a] Biology Center, Academy of Sciences of the Czech Republic, 37005 Ceske Budejovice, Czech Republic
[b] Department of Molecular Biology, Faculty of Sciences, University of South Bohemia, 37005 Ceske Budejovice, Czech Republic
[c] Animal, Food and Health Sciences Division, Commonwealth Scientific and Industrial Research Organization, North Ryde, NSW 2113, Australia

ARTICLE INFO

Article history:
Received 31 October 2013
Received in revised form
9 December 2013
Accepted 11 December 2013

Keywords:
Methoprene-tolerant
bHLH-PAS
Diapause
Oogenesis
Vitellogenesis
Metamorphosis

ABSTRACT

Juvenile hormone (JH), a sesquiterpenoid produced by the insect corpus allatum gland (CA), prevents metamorphosis in larvae and stimulates vitellogenesis in adult females. Whether the same JH signaling pathway regulates both processes is presently unknown. Here, we employ the robust JH response during reproduction and development of the linden bug, *Pyrrhocoris apterus*, to compare the function of key JH-signaling genes encoding the JH receptor, Methoprene-tolerant (Met), its binding partner Taiman (Tai), and a JH-inducible protein, Krüppel-homolog 1 (Kr-h1). RNA interference (RNAi) with Met or Tai, but not Kr-h1, blocked ovarian development and suppressed vitellogenin gene expression in the fat body of females raised under reproduction-inducing conditions. Loss of Met and Tai matched the effects of CA ablation or the natural absence of JH during reproductive diapause. Stimulation of vitellogenesis by treatment of diapausing females with a JH mimic methoprene also required both Met and Tai in the fat body, whereas *Kr-h1* RNAi had no effect. Therefore, the Met-Tai complex likely functions as a JH receptor during vitellogenesis. In contrast to Met and Kr-h1 that are both required for JH to prevent precocious metamorphosis in *P. apterus* larvae, removal of Tai disrupted larval ecdysis without causing premature adult development. Our results show that while Met operates during metamorphosis in larvae and reproduction in adult females, its partner Tai is only required for the latter. The diverse functions of JH thus likely rely on a common receptor whose actions are modulated by distinct components.

1. Introduction

The sesquiterpenoid juvenile hormone (JH) controls multiple events in insect's life, from development to reproduction, seasonal diapause, and various polyphenisms (Nijhout, 1994). How JH exerts all of its functions is unclear as our knowledge of the mode of JH action remains limited. JH was discovered and named for its capacity to maintain the juvenile state of insect larvae until they have attained an appropriate stage (Wigglesworth, 1934). Only during the final larval instar, a temporal drop in JH secretion from the corpora allata (CA) glands permits metamorphosis to the adult form (Hiruma and Kaneko, 2013; Jindra et al., 2013). In adult insects, JH reappears to fulfill its other major function during reproduction, particularly in oogenesis (Raikhel et al., 2005). Since JH occurs even in wingless insects that do not undergo metamorphosis, stimulating reproduction is thought to be the evolutionarily older of the JH roles (Sehnal et al., 1996).

The molecular basis of JH signaling has been partially unveiled owing to studies in several insect models [see (Jindra et al., 2013) for a review]. A candidate JH receptor gene, *Methoprene-tolerant* (*Met*), was originally uncovered through resistance to the JH mimic methoprene in *Drosophila melanogaster* mutants (Wilson and Fabian, 1986). Met was identified as a novel member of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family of transcription factors (Ashok et al., 1998). RNA interference (RNAi)-mediated knockdown of a *Met* ortholog in the flour beetle, *Tribolium castaneum*, revealed that Met was required for JH to prevent precocious metamorphosis (Konopova and Jindra, 2007). The anti-metamorphic JH pathway was soon extended to the zinc-finger

* Corresponding authors. Biology Center, Academy of Sciences of the Czech Republic, 37005 Ceske Budejovice, Czech Republic.
E-mail addresses: jindra@entu.cas.cz (M. Jindra), david.dolezel@entu.cas.cz (D. Dolezel).
[1] These authors contributed equally to this work.

0965-1748/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibmb.2013.12.003

transcription factor Krüppel-homolog 1 (Kr-h1) that acts immediately downstream of JH and Met to block metamorphosis in holometabolous (Minakuchi et al., 2009, 2008) as well as in hemimetabolous (Konopova et al., 2011; Lozano and Bellés, 2011) insects. Met was shown to bind JH with a nanomolar affinity (Charles et al., 2011; Miura et al., 2005), and its ligand-binding domain was defined through specific amino acid mutations (Charles et al., 2011). In response to JH, Met forms complexes with at least two other bHLH-PAS family proteins: Taiman (Tai), also called FISC or steroid receptor coactivator (SRC) (Charles et al., 2011; Li et al., 2011; Zhang et al., 2011), and the circadian clock protein Cycle (Cyc) (Shin et al., 2012).

Besides regulating metamorphosis, the JH receptor Met has been implicated in oogenesis. *Drosophila Met* mutants showed delayed onset of vitellogenesis and oviposition (Wilson and Fabian, 1986), and a milder reduction of fecundity was also found in flies lacking *germ cell-expressed* (*gce*), a gene paralogous to *Met* (Abdou et al., 2011). RNAi knockdown of *Met* in females of *Tribolium* nearly stalled egg production and prevented expression of a vitellogenin gene (Parthasarathy et al., 2010). JH and Met were shown to regulate *Tribolium* vitellogenesis indirectly, via activating insulin signaling (Sheng et al., 2011). In the *Aedes aegypti* mosquito, where JH is required for female reproductive maturation prior to blood meal that then stimulates vitellogenesis (Raikhel et al., 2005), *Met* RNAi retarded ovarian development (Zou et al., 2013) and lowered egg production (Li et al., 2011).

Tai is currently the best-known protein partner of Met, but its function in JH-regulated processes is much less evident than it is for Met itself. A role for Tai/SRC in metamorphosis has been postulated in recent models (Hiruma and Kaneko, 2013; Riddiford, 2012) but not yet experimentally demonstrated. A role for Tai in oogenesis is suggested by reduced number of eggs laid by *A. aegypti* females deficient for the Tai ortholog FISC (Li et al., 2011). One intriguing possibility is that Met might engage in interactions with multiple protein partners in order to exert the distinct functions of JH.

We have chosen to address the roles of Met and Tai in reproduction and in the maintenance of the larval state using the linden bug, *Pyrrhocoris apterus* (Hemiptera). Besides efficient systemic RNAi, this species offers a robust model of JH-dependent reproduction that can be manipulated by the photoperiod. Under short day length, the bug females have inactive CA and remain in a state of reproductive diapause, whereas extended photoperiod promotes JH synthesis and oogenesis. The animals can be repeatedly switched between diapause and reproduction using the photoperiod alone, without changing temperature or access to food, and oogenesis can be triggered in short-day diapausing females upon JH mimic treatment. In this experimental setup, we have shown recently that diapause to reproduction reprogramming of gene expression in the gut requires JH signaling from the CA that is mediated by Met and two circadian clock genes, *cyc* and *Clock* (*Clk*) but not Tai (Bajgar et al., 2013a, 2013b).

Our present data indicate that in *P. apterus*, JH-dependent development and reproduction both rely on the same JH receptor, Met. However, other components of the JH signaling pathway may have specific functions, as Tai is required for JH to stimulate vitellogenesis but not to maintain the juvenile program in penultimate-instar larvae.

2. Methods and materials

2.1. Insect rearing

P. apterus bugs (short-winged form) were maintained at 25 °C on dry linden seeds and were supplemented with water. The cultures were kept at either of two photoperiod regimes: long-day (LD; 18 h light, 6 h dark) that permits reproduction, or short-day (SD; 12 h light, 12 h dark) that induces adult reproductive diapause. Adult females of specific age after adult ecdysis or newly ecdysed penultimate (fourth instar) larvae were selected for experiments.

2.2. cDNA cloning

Cloning of *P. apterus* cDNAs encoding Met and Kr-h1 (Konopova et al., 2011), Tai, Cyc, and Clk (Bajgar et al., 2013b) was described in the cited references. Sequences encoding Vitellogenin (Vg1 and Vg2) and Hexamerin proteins were retrieved by using BLAST from *P. apterus* cDNA subjected to 454 pyrosequencing (GATC Biotech), PCR-amplified, and verified by Sanger sequencing. Sequences are available from the GenBank under the following accession numbers (*Vg1*, KF583751; *Vg2*, KF583752; *Hex*, KF583750).

2.3. RNA interference (RNAi), methoprene treatment, and organ culture

Double-stranded RNA (dsRNA) was prepared using the T3 and T7 RNA polymerases with the MEGA script kit (Ambion) from plasmids containing the appropriate gene fragments and injected into *P. apterus* adult females or larvae as described previously (Bajgar et al., 2013b; Konopova et al., 2011). Adult females received 4 μl of dsRNA at a concentration of 4 μg/μl in Ringer's solution; fourth-instar larvae were injected with 1 μl containing 3 μg of dsRNA. Control animals were injected with heterologous dsRNAs derived from bacterial *β-galactosidase* (adults) and *malE* (larvae) genes, *egfp* (larvae) or with the Ringer's solution alone.

For JH mimic treatments, diapausing SD adult females were anesthetized under CO_2 and treated on the dorsal side with 5 μl of 0.3 mM methoprene (VUOS Pardubice, Czech Republic) dissolved in acetone; controls were treated with acetone only. When RNAi and JH mimic treatments were to be combined, methoprene application followed dsRNA injection by four days. Animals were then sacrificed and subjected to mRNA expression analysis four days after methoprene administration. For experiments on isolated fat body, the tissues were dissected from adult females four days after dsRNA injection. Part of fat body with the Ringer's solution (approximately 50 μl) from a single female was placed in a small culture dish with 100 μl of Grace's medium and exposed to 10 μM methoprene or solvent (acetone) alone for 24 h before mRNA expression was analyzed.

2.4. mRNA quantification

Analyzed tissues (fat body, epidermis) were dissected in RNAse-free Ringer's solution. Total RNA was isolated with the Trizol reagent (Invitrogen). After Turbo DNAse (Ambion) treatment, 1 μg of total RNA was used for cDNA synthesis using the SuperScript III reverse transcriptase (Invitrogen). Relative transcript levels were measured by quantitative PCR using the iQ SYBR Green Supermix kit and the C1000 Thermal Cycler (both Bio-Rad). All measured transcripts were normalized to relative levels of the ribosomal protein (*rp49*) mRNA as described previously (Dolezel et al., 2007). Sequences of primers used for quantification of the specific mRNAs are listed in Table 1.

2.5. Ablation of the corpus allatum

Females selected from the colony within 24 h after adult ecdysis were deprived of linden seeds. One day later, females were anesthetized by submergence in water for 15 min and the corpus allatum was removed through the neck membrane incision under

Table 1
Primers used for quantification of the specific mRNAs.

Gene	Forward primer (5′–3′)	Reverse primers (5′–3′)
Vg1	CACAGCTCACGTGTAATTCC	CAGAGAAAGGGATACTTGACGA
Vg2	GGAAAGACCTGCTTCTCCATT	GGGAGACTGATTGATTGCTTG
hex	TGGGCTGGGAGGAAGGTCGC	CCTCCGCAGGCCATGTCTGG
tai	ACACGGGAAGCAGCATAAAC	AGAGGCGAGAACGCATAAGA
Clk	GTCAACCAGCTCCAGAAGTGTA	CTAGGGTTAAAGAGGGCATCGG
cyc	ACAGATTGCCGAGGAAGTGATT	GGGTCGGTAAGGAAATGTCACT

Ringer's insect saline. Immediately after operation, females were supplied with linden seeds.

2.6. Statistical analysis

Differences between mRNA expression levels were tested for statistical significance using the Statistica 10 software (StatSoft). For comparisons of multiple samples with a single control group, we used One-Way Anova with Dunnett's post hoc test or Student's *t*-test. Student's *t*-test was used for all pairwise comparisons.

3. Results

3.1. Met and Tai are required for oogenesis in P. apterus

Adult *P. apterus* females are reproductive under long photoperiod, but shortening the day length leads to inactivation of their CA and to a reproductive arrest or diapause (Hodkova, 1976). Reproduction can be restored in females experiencing short days by treatment with the JH mimic methoprene (Bajgar et al., 2013b), indicating that JH acts downstream of the photoperiodic sensory input to promote oogenesis. To confirm the requirement of JH for oogenesis directly, we removed the source of JH by performing allatectomy on pre-vitellogenic females, aged 24–48 h after adult ecdysis (AAE), that were reared under the long-day (LD) regime. As expected, allatectomy prevented ovarian growth and oogenesis, resulting in small ovaries, similar to those observed during diapause under short day (SD) length (Fig. 1A–C).

To determine whether endogenous JH stimulates oogenesis through the JH receptor Met and its partner Tai, we examined ovaries of LD females that had been injected during the pre-vitellogenic phase (24 h AAE) with double-stranded RNA (dsRNA) targeting either protein. Although individual ovarioles in *Met* RNAi and *tai* RNAi LD females five days after dsRNA injection appeared thicker than those in JH-deficient, either diapausing (SD) or allatectomized (CAX) females, they likewise developed no vitellogenic oocytes (Fig. 1B–E). Knockdown of the JH-response gene *Kr-h1* did not prevent oogenesis (Fig. 1F).

Table 2
Requirement of *Met* and *tai* for methoprene-mediated induction of ovarian development in diapausing SD females.

dsRNA	Treatment[a]	Vitellogenic ovaries	*n*
Control	Acetone	0	15
Control	Methoprene	15	15
Met	Acetone	0	15
Met	Methoprene	0	15
tai	Acetone	1	15
tai	Methoprene	1	15
cyc	Acetone	0	15
cyc	Methoprene	14	15
Clk	Acetone	0	15
Clk	Methoprene	13	15
Kr-h1	Acetone	0	15
Kr-h1	Methoprene	15	15

n, total number of females dissected.
[a] Females kept under the short-day photoperiod were treated topically four days after injection of the indicated dsRNAs, and their ovaries were examined four days later.

To further determine which genes are required for JH to induce oogenesis, we used reproductively arrested SD females. As expected, administration of methoprene stimulated oogenesis in control SD females, but not in those that had been deprived of either Met or Tai prior to the methoprene treatment (Table 2). In contrast, most of the diapausing females that had been subjected to RNAi against two other bHLH-PAS proteins (Clk and Cyc) or Kr-h1 responded to methoprene normally, by developing vitellogenic oocytes (Table 2). Together, the above data show that both Met and Tai are essential for oogenesis in the presence of endogenous JH under LD conditions that naturally permit reproduction, as well as for methoprene-induced oogenesis under short photoperiod.

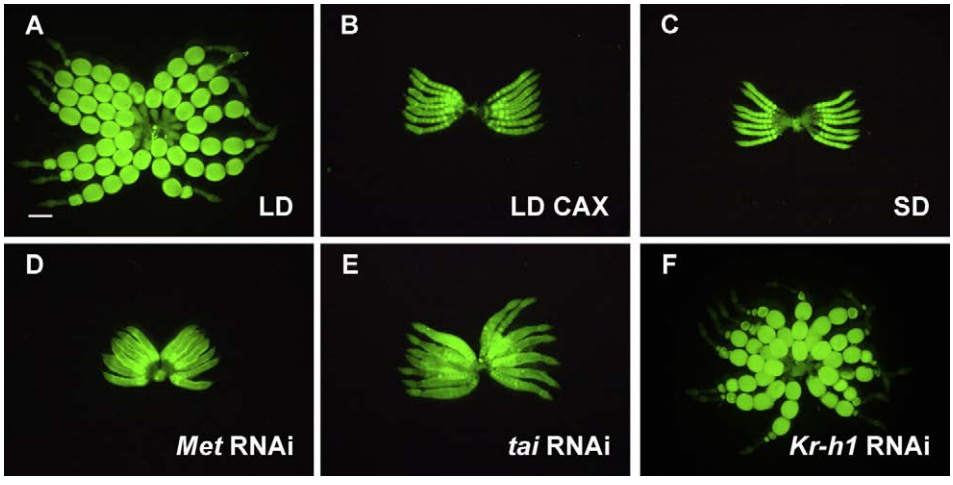

Fig. 1. Oogenesis in *P. apterus* requires JH and the JH receptor Met with its partner Tai. Ovaries were dissected from adult females subjected to indicated light regimes or treatments, stained with Bodipy (Invitrogen), and photographed using epifluorescence microscopy. Vitellogenic oocytes (3–4 per ovariole) occurred in control females under long-day (LD) conditions (A, $n = 10$). No vitellogenic oocytes developed in LD females deprived of JH by allatectomy (LD CAX, $n = 6$) (B) or in females kept under diapause-inducing short-day (SD) conditions (C, $n = 12$). Vitellogenesis was prevented in LD females subjected during the pre-vitellogenic phase to RNAi-mediated silencing of *Met* (D, $n = 14$) and *tai* (E, $n = 12$) but not of *Kr-h1* (F, $n = 13$). Scale bar, 1 mm.

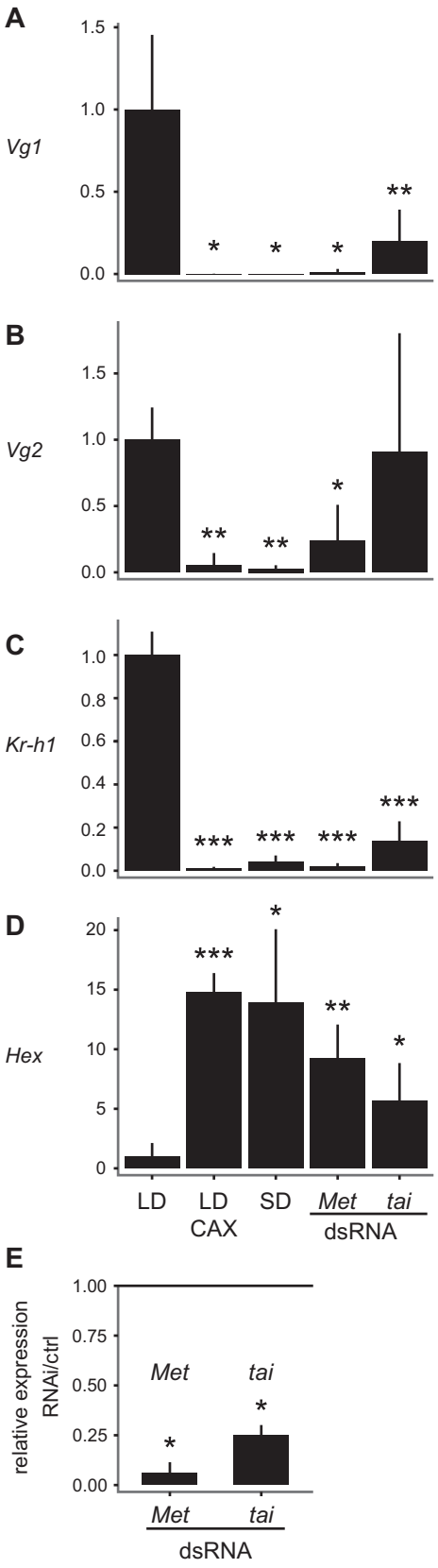

Fig. 2. JH, Met and Tai are required for vitellogenin expression in the fat body of reproductive females. mRNA levels of *Vg1* (A), *Vg2* (B), *Kr-h1* (C) and *Hex* (D) genes were determined in the fat body of reproductive (LD) females, diapausing (SD) females, and LD females subjected either to allatectomy (LD CAX) or RNAi silencing of *Met* or *tai*. The efficiency of the RNAi knockdown relative to *Met* and *tai* mRNA levels in LD females injected with control dsRNA is shown in (E). In all panels, average expression levels in control LD females are arbitrarily set to 1. Values are mean + s.d. based on three (LD, LD CAX, SD, and *Met* RNAi) or six (*tai* RNAi) independent biological replicates. All data were normalized using expression of the *rp49* mRNA. Asterisks indicate statistical significance of differences against the control values as determined by Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3.2. Met and Tai mediate effects of endogenous JH on vitellogenin and hexamerin genes

It has been established previously (Socha et al., 1991) that yolk proteins disappear from the fat body (the main site of vitellogenin synthesis), the hemolymph, and the ovaries upon allatectomy of *P. apterus* females, whereas subsequent application of JH mimics reinstates yolk production. Consistently, we found that relative to reproductive LD females, expression of vitellogenin genes in the fat body was either eliminated (*Vg1*) or reduced to 3–5% of remaining mRNA (*Vg2*) both in CAX LD females and in diapausing SD females (Fig. 2A and B). As expected, a similar reduction occurred for the JH-inducible *Kr-h1* gene (Fig. 2C). Conversely, mRNA of a JH-suppressible storage protein, Hexamerin (Hex) (Sula et al., 1995), accumulated in the fat bodies of either CAX or diapausing females to about 14-fold the level occurring in LD controls (Fig. 2D).

Our present data show that both proteins involved in JH reception, Met and Tai, are necessary for vitellogenic follicles to develop (Fig. 1D and E and Table 2). We therefore examined the effect of *Met* and *tai* knockdown on vitellogenin gene expression in the fat bodies of LD females that had been injected with dsRNA during the pre-vitellogenic phase (24 h AAE). *Met* RNAi was highly efficient, as judged by 6% of residual *Met* mRNA on average (Fig. 2E), and it reduced *Vg1* and *Vg2* transcripts to about 1% and 23%, respectively, of the control levels (Fig. 2A and B). Somewhat less effective *tai* RNAi (25% of residual *tai* mRNA) (Fig. 2E) resulted in a significant loss of expression only in the case of *Vg1* (Fig. 2A and B). The lower sensitivity of *Vg2* to perturbed function of both JH signaling genes correlated with the small but detectable amount of *Vg2* but not of *Vg1* mRNA, either in CAX or in diapausing SD females (Fig. 2A and B). Like the absence of endogenous JH itself (CAX and SD females), deficiency in either Met or Tai function led to a marked reduction of *Kr-h1* (Fig. 2C) and activation of *Hex* (Fig. 2D).

No decrease in *Vg* mRNAs or increase in *Hex* mRNA was observed when LD females were subjected to *Kr-h1* RNAi (data not shown). However, we note here that we were unable to consistently suppress *Kr-h1* mRNA below 50% of its normal level in LD females. This might be due to naturally high JH titer in these females, which keeps *Kr-h1* transcription at levels 25-fold and 110-fold exceeding those in SD and CAX females, respectively (Fig. 2C). We will therefore address the potential role of *Kr-h1* in vitellogenesis in a different setting (see Section 3.3.).

3.3. Methoprene induces Vg1 and Vg2 expression through Met and Tai in the fat body

Administration of methoprene elicited ovarian development in diapausing SD females (Table 2). In the fat bodies of these females, methoprene massively induced mRNAs of both *Vg1* and *Vg2* while suppressing *Hex* transcription (Fig. 3A). In this background, we tested which transcription factors were required for the response to methoprene. Adult SD females were injected with dsRNA, treated with methoprene or solvent four days later, and gene expression was analyzed in their fat bodies after another four days. Depletion of either Met or Tai prevented induction of both *Vg1* and *Vg2* genes by methoprene (Fig. 3A). Interestingly, the capacity of methoprene to suppress *Hex* expression was completely blocked by *Met* RNAi,

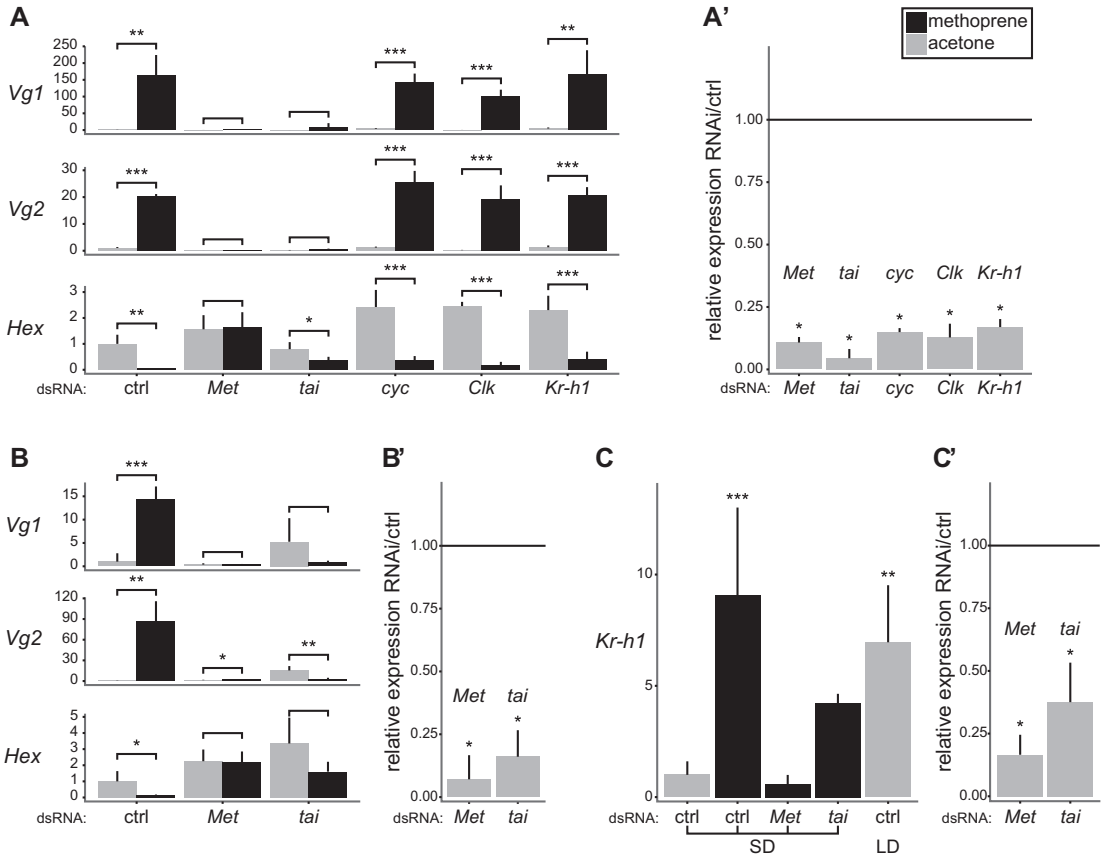

Fig. 3. Met and Tai mediate methoprene effects on gene expression autonomously in the fat body. (A) Diapausing females under short-day conditions (SD) were injected with dsRNAs targeting the indicated genes, and treated with methoprene (black columns) or acetone only (gray columns) four days later. After another four days, levels of *Vg1*, *Vg2*, and *Hex* mRNAs in the fat body was compared to levels occurring in the fat body of LD females injected with control dsRNA (arbitrarily set to 1 in all panels). (B) Gene expression analysis in isolated fat bodies. The experimental setup was as described for (A) except that four days after dsRNA injection, fat body was dissected from the treated females and exposed to 10 μM methoprene (or acetone) in vitro for 24 h. (C) Low *Kr-h1* expression in the fat body of diapausing SD females was induced to or above levels observed in LD females treated with acetone and control dsRNA; the full induction required Met and partially also Tai. Knockdown efficiencies in each set of experiments are shown in (A′, B′, and C′) as mRNA levels of the RNAi-targeted transcripts relative to control expression levels (arbitrarily set to 1). In all panels, values are mean + s.d. based on four (A, B), or five (C, except LD, where $n = 3$) independent biological replicates. All data were normalized using expression of *rp49* mRNA. Asterisks indicate statistical significance of differences against the control values as determined by Dunnett's post hoc test (C) and Student's *t*-test (all other panels): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

but only partially limited by the removal of *tai* relative to *Hex* mRNA levels in solvent-treated *tai* RNAi specimens (Fig. 3A). In contrast to *Met* and *tai*, silencing of *cyc*, *Clk* and *Kr-h1* affected neither the ability of methoprene to induce the yolk genes nor to suppress *Hex* mRNA (Fig. 3A), even though mRNA depletion for each of the five targeted genes was comparably effective (Fig. 3A').

We next tested whether the action of Met and Tai in the methoprene response was autonomous to the fat body. To this end, diapausing SD females were injected with dsRNA as above, but four days later their fat bodies were dissected and cultured with or without methoprene. As was the case with intact animals, loss of either *Met* or *tai* function precluded methoprene-induced expression of both *Vg* genes in the cultured fat body (Fig. 3B). Again, only *Met* RNAi but not *tai* RNAi fully prevented decrease of *Hex* expression (Fig. 3B).

Finally we examined whether Met and Tai are essential for *Kr-h1* expression in the adult fat body. As expected, *Kr-h1* mRNA was low in diapausing SD females, and administration of methoprene to these females induced *Kr-h1* expression to levels comparable to reproductive LD females (Fig. 3C). *Met* RNAi delivered prior to the methoprene treatment completely blocked *Kr-h1* induction, whereas *tai* RNAi reduced it only partially (Fig. 3C). We cannot rule out that the apparently lower dependence of *Kr-h1* expression on Tai was due to a somewhat weaker efficiency of *tai* mRNA knockdown compared to *Met* RNAi (Fig. 3C′).

The above results show that Met and Tai mediate methoprene-dependent transcriptional activation of the two yolk genes, whereas induction of *Kr-h1* and suppression of *Hex* mainly requires the presence of Met. Both Met and Tai function organ-autonomously in the fat body.

3.4. *Tai is required for larval ecdysis but not to prevent precocious metamorphosis*

In juvenile *P. apterus*, the JH → Met → Kr-h1 pathway prevents metamorphosis until JH naturally declines and *Kr-h1* expression falls in the fifth (L5), final larval instar. RNAi silencing of either *Met* or *Kr-h1* early during the penultimate (L4) instar thus causes precocious adult development instead of the normal final larval molt in 100% of ecdysed animals (Konopova et al., 2011). To test whether Tai, as a known partner of Met, was part of the anti-metamorphic JH/Met signaling, we examined *tai* RNAi phenotype. Upon injection of early-L4 larvae with *tai* dsRNA, we observed developmental arrest and lethality at the next ecdysis in all injected larvae ($n = 16$). These pharate L5 animals underwent apolysis but were unable to shed the old cuticle. However, even after manual peeling of the old cuticle, we saw none of the signs of accelerated development of adult characters, such as coloration patterns or premature growth of the wing pads (Fig. 4A) and external genitalia (not shown) that

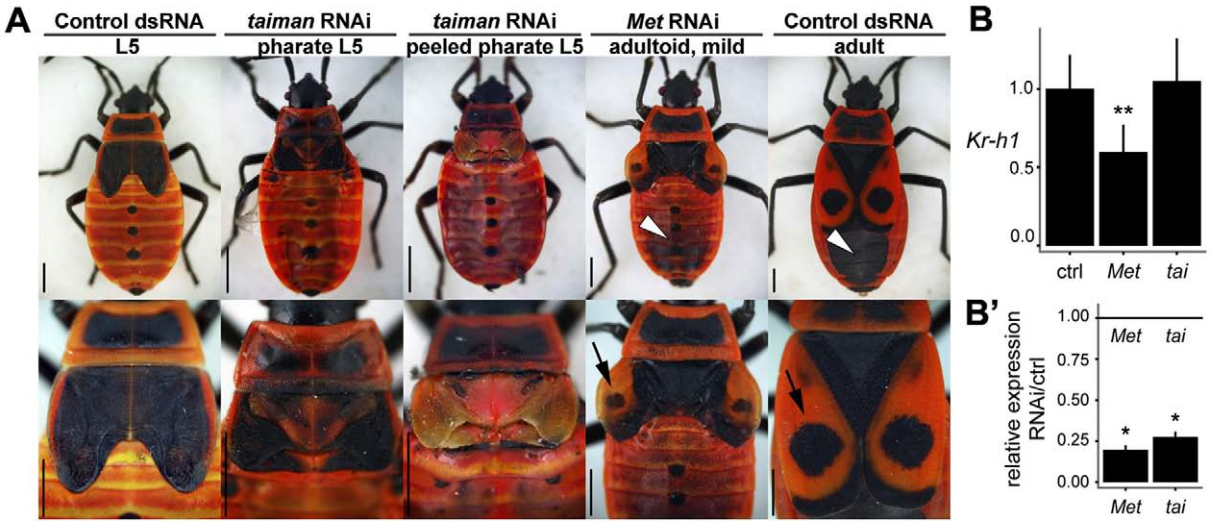

Fig. 4. Loss of *tai* does not induce precocious metamorphosis. (A) Animals were injected with control, *tai*, and *Met* dsRNAs early during the L4 instar. Control larvae ecdysed to normal L5 instar. *Met* RNAi ecdysed to adultoids showing outgrowth and adult-specific color patterning of the wing pads (arrows) and dark pigmentation (arrowhead) of the abdomen (compare to control adult, right). A mild version of the *Met* RNAi effect is shown; for fully expressed phenotype, see (Konopova et al., 2011). Larvae injected with *tai* dsRNA underwent apolysis but died as pharate L5 without being able to ecdyse. They showed no signs of precocious adult development even when the old cuticle was peeled off. Scale bar, 1 mm. (B) Levels of *Kr-h1* mRNA were reduced upon *Met* but not *tai* RNAi (left panel). The right panel shows knockdown efficiency for *Met* and *tai* RNAi as assessed in the epidermis of L4 larvae three days after dsRNA injection. Average expression levels in control larvae are arbitrarily set to 1. Values are mean + s.d. based on 8 (control) or 7 (*Met* RNAi, *tai* RNAi) independent biological replicates. All data were normalized using *rp49* mRNA expression. Asterisks indicate statistical significance of differences against the control values as determined by Dunnett's post hoc test (*Kr-h1* expression) and Student's *t*-test (knockdown efficiencies): *, $P < 0.05$; **, $P < 0.01$.

are obvious for loss of either *Met* or *Kr-h1* [Fig. 4A and (Konopova et al., 2011)]. Moreover, in contrast with reduced *Kr-h1* expression upon *Met* RNAi (Konopova et al., 2011), the level of *Kr-h1* mRNA in the epidermis of L4 larvae before the lethal phase was unaffected by *tai* knockdown (Fig. 4B). The absence of the expected precocious metamorphosis phenotype and the sustained *Kr-h1* expression in *tai* RNAi larvae suggested that Tai may not be necessary for JH and Met to maintain the larval program.

4. Discussion

An intriguing question concerning JH action is how does the single hormone control several distinct developmental and physiological processes (Wheeler and Nijhout, 2003)? We have used the *P. apterus* bug to see if the two main functions of JH, those in reproduction and metamorphosis, may rely on different or identical molecular pathways. Our previous study (Konopova et al., 2011) has demonstrated that the JH receptor Met and its immediate target gene *Kr-h1* are both required for the anti-metamorphic effect of JH in *P. apterus* larvae. Here, we provide three lines of evidence that Met also is essential for JH action in *P. apterus* oogenesis. First, knockdown of *Met* in pre-vitellogenic females prevented both vitellogenin synthesis and ovarian development, thus matching the effects of JH absence, either natural during reproductive diapause or after allatectomy. Second, Met was required for the JH mimic methoprene to stimulate ovarian development in diapausing females. Third, *Met* RNAi prevented methoprene from inducing *Vg* gene expression in the fat body. Therefore, Met acts as a JH-activated regulator that ensures both the juvenile status in larvae and oogenesis in adult females (Fig. 5).

The role of Met in promoting oogenesis is not unprecedented and it may apply to various and evolutionarily distant insect orders. In the Diptera, *Drosophila Met* and *gce* mutants (Abdou et al., 2011; Wilson and Fabian, 1986) as well as *A. aegypti* mosquitoes subjected to *Met* RNAi (Li et al., 2011; Zou et al., 2013) suffer from retarded ovarian development and low fecundity. Oogenesis was blocked by *Met* knockdown in *Tribolium* (Coleoptera) (Parthasarathy et al., 2010), where Met was shown to stimulate vitellogenin gene expression by acting upstream of insulin signaling (Sheng et al., 2011). Specifically, expression of the insulin-like peptide 2 (ILP2) in the brain and fat body required JH and Met, and depletion of either the hormone or its receptor could be compensated by addition of bovine insulin (Sheng et al., 2011). *Tribolium* thus seems to differ from other insects including *Drosophila* (Tu et al., 2005), the cockroach *Blattella germanica* (Maestro et al., 2009), the *A. aegypti* (Pérez-Hedo et al., 2013) and *Culex pipiens* (Sim and Denlinger, 2008) mosquitoes, where the studies have placed JH biosynthesis downstream of the nutrient-sensing insulin/TOR pathways. Although our in-vitro experiments with isolated *P. apterus* fat bodies show that methoprene induces vitellogenin expression through Met acting tissue-autonomously, we cannot currently exclude involvement of an insulin-like signal downstream of Met in the fat body.

Met belongs to the bHLH-PAS family of proteins that form heterodimeric transcription factors (Kewley et al., 2004). Without JH, Met occurs as a homodimer (Godlewski et al., 2006). In the presence of JH, Met has thus far been shown to interact with two other bHLH-PAS proteins, Tai/FISC/SRC (Charles et al., 2011; Li et al., 2011; Zhang et al., 2011) and Cycle (Shin et al., 2012). While Tai itself does not engage in binding JH, the hormone-binding capacity of Met is necessary for the Met-Tai complex to assemble (Charles et al., 2011). In the gut of *A. aegypti* adult females, both Met and Tai (FISC) are required for normal expression of the JH-induced *Kr-h1* and *early trypsin* genes (Li et al., 2011). Indeed, Met and Tai/FISC/SRC comprise a JH-activated transcription factor complex in several insect species (Kayukawa et al., 2012; Li et al., 2011; Zhang et al., 2011). Although the function of Tai as a partner or a coactivator for the JH receptor Met has been established through the above cited studies, direct evidence for the involvement of Tai in either of the JH-regulated processes, vitellogenesis and metamorphosis, has been missing.

Our present data clearly support a role for Tai in *P. apterus* vitellogenesis. Like Met, Tai was required for oogenesis under reproductive (LD) conditions (Fig. 1D and E) and for methoprene to trigger ovarian development in previously diapausing SD females

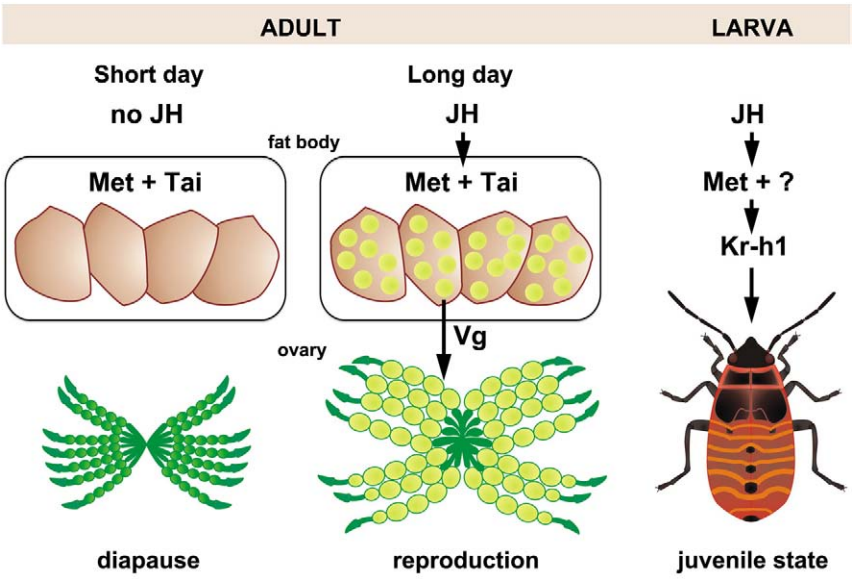

Fig. 5. A model for JH signaling in reproduction and development of *P. apterus*. Both vitellogenesis (this work) and metamorphosis (Konopova et al., 2011) rely on the same JH receptor, Met. However, Tai only appears to be required for vitellogenesis but not for maintenance of the larval status. Conversely, while Kr-h1 is an essential repressor of adult development (Konopova et al., 2011), our data do not support its role in vitellogenesis. We propose that JH may achieve its different functions through Met interacting with alternative partner proteins and regulating diverse target genes.

(Table 2). Both Met and Tai mediated the strong induction of vitellogenin genes by methoprene in the fat body (Fig. 3). These matching effects of Met and Tai suggest that the Met-Tai complex acts as a JH receptor during oogenesis (Fig. 5). By contrast, knocking down either *Kr-h1* or the circadian genes *Clk* and *cyc* did not affect vitellogenesis.

Not all Met effects necessarily depend on Tai. We have shown recently that while Met, Clk and Cyc are all required for JH to reprogram gene expression from a diapause mode to a reproductive mode in the gut of adult *P. apterus* females, Tai is not (Bajgar et al., 2013b). Here, we have tested whether Tai is involved in the control of *P. apterus* metamorphosis. When either *Met* or *Kr-h1* are silenced by RNAi early during the penultimate (L4) larval instar, expression of *Kr-h1* prematurely declines and adultoids, rather than final-instar L5 larvae, result from the next molt (Konopova et al., 2011). In the same test, *tai* RNAi did not reduce *Kr-h1* mRNA levels in the larval epidermis, and inflicted no signs of precocious metamorphosis (Fig. 4), suggesting that Tai may not be part of the JH → Met → Kr-h1 anti-metamorphic pathway. From these data we conclude that in contrast to vitellogenesis, JH and Met ensure the larval status independently of Tai (Fig. 5). Whether Met controls the entry to metamorphosis through an interaction with another protein partner remains to be determined.

Nonetheless, Tai does play an essential role during development, as all *tai* RNAi animals died at apolysis as pharate L5 larvae, unable to shed their old cuticle (Fig. 4A). Because Tai serves as a coactivator to nuclear hormone receptors including the ecdysone receptor (EcR) (Bai et al., 2000) and Ftz-F1 (Zhu et al., 2006), it is conceivable that disrupted ecdysis in *tai* RNAi larvae reflects cooperation of Tai with the ecdysone pathway rather than with JH signaling. Indeed, knockdown of *EcR* during the L4 instar of *P. apterus* (K.T., V.S., and M.J., unpublished observations) caused a pre-ecdysis arrest closely resembling the *tai* RNAi phenotype.

Reappearance of *Kr-h1* mRNA after metamorphosis implies an adult function for this gene. The adult expression of *Kr-h1* in the fat body depends on JH as revealed by its severe reduction in CAX LD females or in SD females during diapause. Met, and to a large extent also Tai, were required for *Kr-h1* expression in the fat body of reproductive LD females (Fig. 2C). It was therefore surprising that in contrast to *Met* and *tai*, *Kr-h1* RNAi did not interfere with ovarian development or vitellogenin gene expression in LD females. This result agreed with a previous report that *Vg* transcription was only suppressed to a minor degree by *Kr-h1* RNAi in *Tribolium* females (Parthasarathy et al., 2010). However, like in the beetles, *Kr-h1* mRNA could only be depleted by about 50% in our *P. apterus* females raised under the LD condition. The inefficient knockdown might be due to a high JH titer in reproductive females. When we applied *Kr-h1* dsRNA to diapausing SD females before challenging them with methoprene, *Kr-h1* RNAi did not reduce the level of *Vg1* and *Vg2* induction by methoprene, whereas depletion of *Met* or *tai* abolished it completely (Fig. 3). Thus, although we cannot strictly exclude a role for Kr-h1 in oogenesis, we obtained no evidence that the JH-Met-Tai complex achieves its pro-vitellogenic effect through Kr-h1.

Together, work in the *P. apterus* model suggests that the same receptor, Met, mediates the effects of JH on metamorphosis (Konopova et al., 2011), on diapause-to-reproduction reprogramming of the gut (Bajgar et al., 2013b), and on vitellogenesis in the fat body (this study) (Fig. 5). However, as exemplified by the selective action of Tai in oogenesis, not all of the few currently known components of the JH signaling pathway uniformly engage in all JH functions. Future discoveries of new players that modulate JH-Met signaling should explain how JH exerts its multiple developmental and physiological roles.

Acknowledgments

We thank Martina Hajduskova (www.biographix.cz) for Fig. 5. This work was supported by Grant Agency of the Czech Republic project P502/10/1612 to M.H. and Academy of Sciences grant 500960906 to M.J. We acknowledge the use of research equipment funded from the European Union program FP7/2007–2013, grant No. 316304. D.D. and M.B. were supported from INsecTIME (FP7-PEOPLE-2012-ITN, grant No. 316790), and M.J. received support from a Marie Curie Fellowship Award 276569 from the European Union.

References

Abdou, M.A., He, Q., Wen, D., Zyaan, O., Wang, J., Xu, J., Baumann, A.A., Joseph, J., Wilson, T.G., Li, S., Wang, J., 2011. *Drosophila* Met and Gce are partially redundant in transducing juvenile hormone action. Insect Biochem. Mol. Biol. 41, 938–945.

Ashok, M., Turner, C., Wilson, T.G., 1998. Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. Proc. Natl. Acad. Sci. U S A 95, 2761–2766.

Bai, J., Uehara, Y., Montell, D.J., 2000. Regulation of invasive cell behavior by taiman, a *Drosophila* protein related to AIB1, a steroid receptor coactivator amplified in breast cancer. Cell 103, 1047–1058.

Bajgar, A., Dolezel, D., Hodkova, M., 2013a. Endocrine regulation of non-circadian behavior of circadian genes in insect gut. J. Insect Physiol. 59, 881–886.

Bajgar, A., Jindra, M., Dolezel, D., 2013b. Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. Proc. Natl. Acad. Sci. U S A 110, 4416–4421.

Charles, J.-P., Iwema, T., Epa, V.C., Takaki, K., Rynes, J., Jindra, M., 2011. Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. Proc. Natl. Acad. Sci. U S A 108, 21128–21133.

Dolezel, D., Sauman, I., Kostal, V., Hodkova, M., 2007. Photoperiodic and food signals control expression pattern of the clock gene, period, in the linden bug, *Pyrrhocoris apterus*. J. Biol. Rhythms 22, 335–342.

Godlewski, J., Wang, S., Wilson, T.G., 2006. Interaction of bHLH-PAS proteins involved in juvenile hormone reception in *Drosophila*. Biochem. Biophys. Res. Commun. 342, 1305–1311.

Hiruma, K., Kaneko, Y., 2013. Hormonal regulation of insect metamorphosis with special reference to juvenile hormone biosynthesis. Curr. Top. Dev. Biol. 103, 73–100.

Hodkova, M., 1976. Nervous inhibition of corpora allata by photoperiod in *Pyrrhocoris apterus*. Nature 263, 521–523.

Jindra, M., Palli, S.R., Riddiford, L.M., 2013. The juvenile hormone signaling pathway in insect development. Annu. Rev. Entomol. 58, 181–204.

Kayukawa, T., Minakuchi, C., Namiki, T., Togawa, T., Yoshiyama, M., Kamimura, M., Mita, K., Imanishi, S., Kiuchi, M., Ishikawa, Y., 2012. Transcriptional regulation of juvenile hormone-mediated induction of Krüppel homolog 1, a repressor of insect metamorphosis. Proc. Natl. Acad. Sci. U S A 109, 11729–11734.

Kewley, R.J., Whitelaw, M.L., Chapman-Smith, A., 2004. The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. Int. J. Biochem. Cell Biol. 36, 189–204.

Konopova, B., Jindra, M., 2007. Juvenile hormone resistance gene methoprene-tolerant controls entry into metamorphosis in the beetle *Tribolium castaneum*. Proc. Natl. Acad. Sci. U S A 104, 10488–10493.

Konopova, B., Smykal, V., Jindra, M., 2011. Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. PLoS One 6, e28728.

Li, M., Mead, E.A., Zhu, J., 2011. Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression. Proc. Natl. Acad. Sci. U S A 108, 638–643.

Lozano, J., Bellés, X., 2011. Conserved repressive function of Krüppel homolog 1 on insect metamorphosis in hemimetabolous and holometabolous species. Sci. Rep. 1, 163.

Maestro, J.L., Cobo, J., Bellés, X., 2009. Target of rapamycin (TOR) mediates the transduction of nutritional signals into juvenile hormone production. J. Biol. Chem. 284, 5506–5513.

Minakuchi, C., Namiki, T., Shinoda, T., 2009. Krüppel homolog 1, an early juvenile hormone-response gene downstream of Methoprene-tolerant, mediates its anti-metamorphic action in the red flour beetle *Tribolium castaneum*. Dev. Biol. 325, 341–350.

Minakuchi, C., Zhou, X., Riddiford, L.M., 2008. Krüppel homolog 1 (Kr-h1) mediates juvenile hormone action during metamorphosis of *Drosophila melanogaster*. Mech. Dev. 125, 91–105.

Miura, K., Oda, M., Makita, S., Chinzei, Y., 2005. Characterization of the *Drosophila* methoprene -tolerant gene product. FEBS J. 272, 1169–1178.

Nijhout, H., 1994. Insect Hormones. Princeton University Press, Princeton.

Parthasarathy, R., Sun, Z., Bai, H., Palli, S.R., 2010. Juvenile hormone regulation of vitellogenin synthesis in the red flour beetle, *Tribolium castaneum*. Insect Biochem. Mol. Biol. 40, 405–414.

Pérez-Hedo, M., Rivera-Perez, C., Noriega, F.G., 2013. The insulin/TOR signal transduction pathway is involved in the nutritional regulation of juvenile hormone synthesis in *Aedes aegypti*. Insect Biochem. Mol. Biol. 43, 495–500.

Raikhel, A.S., Brown, M.R., Bellés, X., 2005. Hormonal control of reproductive processes. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), Comprehensive Insect Science. Elsevier/Pergamon, Amsterdam, pp. 433–491.

Riddiford, L.M., 2012. How does juvenile hormone control insect metamorphosis and reproduction? Gen. Comp. Endocrinol. 179, 477–484.

Sehnal, F., Svacha, P., Zrzavy, J., 1996. Evolution of insect metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), Metamorphosis. Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells. Academic Press, San Diego, pp. 3–58.

Sheng, Z., Xu, J., Bai, H., Zhu, F., Palli, S.R., 2011. Juvenile hormone regulates vitellogenin gene expression through insulin-like peptide signaling pathway in the red flour beetle, *Tribolium castaneum*. J. Biol. Chem. 286, 41924–41936.

Shin, S.W., Zou, Z., Saha, T.T., Raikhel, A.S., 2012. bHLH-PAS heterodimer of methoprene-tolerant and cycle mediates circadian expression of juvenile hormone-induced mosquito genes. Proc. Natl. Acad. Sci. U S A 109, 16576–16581.

Sim, C., Denlinger, D.L., 2008. Insulin signaling and FOXO regulate the overwintering diapause of the mosquito *Culex pipiens*. Proc. Natl. Acad. Sci. U S A 105, 6777–6781.

Socha, R., Sula, J., Kodrik, D., Gelbic, I., 1991. Hormonal control of vitellogenin synthesis in *Pyrrhocoris apterus* (L.) (Heteroptera). J. Insect Physiol. 37, 805–816.

Sula, J., Kodrik, D., Socha, R., 1995. Hexameric haemolymph protein related to adult diapause in the red firebug, *Pyrrhocoris apterus* (L.) (Heteroptera). J. Insect Physiol. 41, 793–800.

Tu, M.-P., Yin, C.-M., Tatar, M., 2005. Mutations in insulin signaling pathway alter juvenile hormone synthesis in *Drosophila melanogaster*. Gen. Comp. Endocrinol. 142, 347–356.

Wheeler, D.E., Nijhout, H.F., 2003. A perspective for understanding the modes of juvenile hormone action as a lipid signaling system. Bioessays 25, 994–1001.

Wigglesworth, V., 1934. The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II. Factors controlling moulting and "metamorphosis." Q. J. Microsc. Sci. 77, 191–222.

Wilson, T.G., Fabian, J., 1986. A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. Dev. Biol. 118, 190–201.

Zhang, Z., Xu, J., Sheng, Z., Sui, Y., Palli, S.R., 2011. Steroid receptor co-activator is required for juvenile hormone signal transduction through a bHLH-PAS transcription factor, methoprene tolerant. J. Biol. Chem. 286, 8437–8447.

Zhu, J., Chen, L., Sun, G., Raikhel, A.S., 2006. The competence factor beta Ftz-F1 potentiates ecdysone receptor activity via recruiting a p160/SRC coactivator. Mol. Cell. Biol. 26, 9402–9412.

Zou, Z., Saha, T.T., Roy, S., Shin, S.W., Backman, T.W.H., Girke, T., White, K.P., Raikhel, A.S., 2013. Juvenile hormone and its receptor, methoprene-tolerant, control the dynamics of mosquito gene expression. Proc. Natl. Acad. Sci. U S A 110, E2173–E2181.

RESULTS

Research article III

Importance of juvenile hormone signaling arises with competence of insect larvae to metamorphose

Vlastimil Smykal, Takaaki Daimon, Takumi Kayukawa, Keiko Takaki, Tetsuro Shinoda and Marek Jindra

Developmental Biology 390 (2014) 221–230

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Importance of juvenile hormone signaling arises with competence of insect larvae to metamorphose

Vlastimil Smykal [a,b], Takaaki Daimon [c], Takumi Kayukawa [c], Keiko Takaki [a], Tetsuro Shinoda [c], Marek Jindra [a,d,*]

[a] Biology Center, Academy of Sciences of the Czech Republic, 37005 Ceske Budejovice, Czech Republic
[b] Department of Molecular Biology, Faculty of Sciences, University of South Bohemia, 37005 Ceske Budejovice, Czech Republic
[c] National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8634, Japan
[d] Animal, Food and Health Sciences Division, Commonwealth Scientific and Industrial Research Organization, North Ryde, NSW 2113, Australia

ARTICLE INFO

Article history:
Received 22 January 2014
Received in revised form
14 March 2014
Accepted 17 March 2014
Available online 22 March 2014

Keywords:
Insect metamorphosis
Hormonal signaling
Juvenile hormone
Heterochronic development
Methoprene-tolerant
Krüppel homolog 1

ABSTRACT

Juvenile hormone (JH) postpones metamorphosis of insect larvae until they have attained an appropriate stage and size. Then, during the final larval instar, a drop in JH secretion permits a metamorphic molt that transforms larvae to adults either directly (hemimetaboly) or via a pupal stage (holometaboly). In both scenarios, JH precludes metamorphosis by activating the *Kr-h1* gene through a JH receptor, Methoprene-tolerant (Met). Removal of Met, Kr-h1, or JH itself triggers deleterious precocious metamorphosis. Although JH is thought to maintain the juvenile status throughout larval life, various methods of depleting JH failed to induce metamorphosis in early-instar larvae. To determine when does JH signaling become important for the prevention of precocious metamorphosis, we chose the hemimetabolous bug, *Pyrrhocoris apterus*, and the holometabolous silkworm, *Bombyx mori*. Both species undergo a fixed number of five larval instars. *Pyrrhocoris* larvae subjected to RNAi-mediated knockdown of *Met* or *Kr-h1* underwent precocious adult development when treated during the fourth (penultimate) instar, but younger larvae proved increasingly resistant to loss of either gene. The earliest instar developing minor signs of precocious metamorphosis was the third. Therefore, the JH-response genes may not be required to maintain the larval program during the first two larval instars. Next, we examined *Bombyx mod* mutants that cannot synthesize authentic, epoxidized forms of JH. Although *mod* larvae expressed *Kr-h1* mRNA at severely reduced levels since hatching, they only entered metamorphosis by pupating after four, rarely three instars. Based on findings in *Pyrrhocoris* and *Bombyx*, we propose that insect postembryonic development is initially independent of JH. Only later, when larvae gain competence to enter metamorphosis, JH signaling becomes necessary to prevent precocious metamorphosis and to optimize growth.

Introduction

Insect larvae grow through a number of successive instars, each terminated by a molt and ecdysis that replace the old larval cuticle with a new one. The molts are promoted by surges of ecdysteroids (Yamanaka et al., 2013). Secretion of the sesquiterpenoid juvenile hormone (JH) signals that a molt will produce another larva, thus enabling further growth (Hiruma and Kaneko, 2013; Nijhout et al., 2014). Only when larvae have attained an appropriate stage or size, the temporal absence of JH permits a metamorphic molt that transforms them into adults, either directly (hemimetaboly) or via an intermediate pupal stage (holometaboly). In either case, it is during the final larval instar that the animals commit to metamorphosis (Nijhout, 1994; Nijhout and Williams, 1974; Riddiford, 1994). The anti-metamorphic ("status quo") action of JH has been documented in a broad variety of insects by two types of experiments: First, untimely administration of JH early during the final larval or pupal stages blocks metamorphosis, causing a molt that repeats the previous juvenile stage. Second, premature removal of JH during pre-final larval instars leads to a precocious metamorphic molt, manifested by heterochronic development of adult characters such as wings and external genitals or, in holometabolans, formation of miniature pupae.

The effect of JH deprivation, achieved through decapitation or allatectomy (extirpation of the JH-producing corpora allata glands),

* Corresponding author at: Biology Center, Academy of Sciences of the Czech Republic, Branisovska 31, 37005 Ceske Budejovice, Czech Republic.
Tel.: +420 387775232.
E-mail address: jindra@entu.cas.cz (M. Jindra).

http://dx.doi.org/10.1016/j.ydbio.2014.03.006
0012-1606/

was initially demonstrated for the hemimetabolous blood-sucking bug, *Rhodnius prolixus* (Wigglesworth, 1936, 1934) and the holometabolous silkworm, *Bombyx mori* (Bounhiol, 1938; Fukuda, 1944). Wigglesworth observed that when decapitated at the right time after feeding, even first-instar *Rhodnius* larvae could undergo a single molt to "precocious adults" with small genitals, partially grown wing pads, and an adult-specific cuticle pattern (Wigglesworth, 1934). He therefore argued that at least some insects were competent to metamorphose already as early larvae, and that any gradual development observed during the larval life occurred due to delayed or reduced JH secretion (Wigglesworth, 1954). Consequently, the notion that JH is required to maintain the larval character of all pre-metamorphic molts has been a paradigm of insect endocrinology to this day. However, the earliest time at which precocious *Bombyx* pupae could be obtained was after three, instead of the normal five larval instars (Bounhiol, 1938; Fukuda, 1944), even when allatectomy was performed on early second-instar larvae (K. Hiruma, personal communication). This record has not been broken even with modern techniques.

Indeed, various genetic methods of depleting JH have failed to induce precocious metamorphosis during early larval instars, suggesting that contrary to the paradigm, the juvenile character may not depend on JH until a later phase of larval development. Transgenic *Bombyx* silkworms expressing a JH-degrading esterase (JHE) throughout development pupated no earlier than after three larval instars (Tan et al., 2005). The recently characterized *dimolting* (*mod*) *Bombyx* mutants that lack a JH biosynthetic enzyme, JH epoxidase CYP15C1, metamorphosed to miniature pupae and adults only after completing three or four larval instars (Daimon et al., 2012). In *Drosophila melanogaster* that normally undergoes three larval instars, genetic ablation of the corpora allata cells did not reduce the instar number (Abdou et al., 2011; Liu et al., 2009; Riddiford et al., 2010) even though second-instar *Drosophila* larvae can be caused to pupate by other genetic manipulations (e.g., (Bialecki et al., 2002; Mirth et al., 2005; Zhou et al., 2004).

Interestingly, although instar numbers vary among and within insect species (Esperk et al., 2007), three larval instars are the lowest consensus. Exceptions include some parasitoid wasps with a single larval instar (Jarjees and Merritt, 2002) and histerid beetles that go through two larval instars (Achiano and Giliomee, 2005). Thus, it appears that in vast majority, juvenile insects must experience a minimum of three instars (or two larval molts) before gaining competence to metamorphose. The role of JH in the acquisition of this competence is unclear.

Recent progress in understanding of JH reception and molecular action (Jindra et al., 2013) now enables studies of the function of JH signaling genes throughout development. JH controls metamorphosis by activating a gene *Krüppel-homolog 1* (*Kr-h1*) that prevents precocious pupal and/or adult development in holometabolous (Kayukawa et al., 2014; Minakuchi et al., 2009, 2008) and hemimetabolous (Konopova et al., 2011; Lozano and Bellés, 2011) insects. The JH-dependent activation of *Kr-h1* requires the JH receptor, Methoprene-tolerant (Met) (Charles et al., 2011; Kayukawa et al., 2012; Konopova et al., 2011; Minakuchi et al., 2009; Parthasarathy et al., 2008). RNAi-mediated knockdown of *Met* or *Kr-h1* function triggers precocious pupation in larvae of the flour beetle, *Tribolium castaneum* (Konopova and Jindra, 2007; Minakuchi et al., 2009), and accelerates adult development in larvae of the linden bug, *Pyrrhocoris apterus* (Konopova et al., 2011).

In this study, we examine the role of the JH→Met→Kr-h1 anti-metamorphic pathway in preserving the juvenile status throughout larval development. We employ two complementary insect models with distinct development, the hemimetabolous *Pyrrhocoris* bugs and the holometabolous *Bombyx* silkworms. Systemic RNAi in *Pyrrhocoris* has previously revealed that silencing of *Met* and *Kr-h1* during the penultimate (fourth) larval instar induces precocious adult development (Konopova et al., 2011). Here, we find that *Pyrrhocoris* larvae are incapable of responding to disruption of JH signaling by developing adult-like features until in the third instar. Using the JH-deficient *Bombyx mod* mutants (Daimon et al., 2012), we show that while expression of the anti-metamorphic *Kr-h1* gene is drastically reduced in the absence of JH since hatching, this reduction is not sufficient for metamorphosis to take place. We conclude that insect larvae are initially incompetent to undergo metamorphosis, and do not rely on JH for maintenance of the juvenile status until late instars.

Materials and methods

Insect rearing and staging

Pyrrhocoris apterus bugs (short-winged form) were maintained at 25 °C under long-day photoperiod (18 h light/6 h dark) on dry linden seeds, and were supplemented with water. Eggs and hatchlings were collected daily. The five larval instars (hereafter referred to as L1 through L5; Fig. 1A) were distinguished based on body size, shape, and the degree of development of the wing pads. Staging within instars relied on measuring time after ecdysis.

The *Bombyx mori dimolting* (*mod*) mutants (Daimon et al., 2012) were maintained as a homozygous stock (*w-1*; *mod*) in the *white egg 1* (*w-1*) genetic background. The *w-1*; *pnd-1* (*pigmented and non-diapausing 1*) strain (hereafter referred to as "control") served for reference. Larvae of both strains were reared at 25 °C under 12 h light/12 h dark regime on mulberry leaves, and were synchronized just after ecdysing to each instar (day 0) and collected for mRNA expression analysis on the daily basis. During molts, animals were staged using the slippage of the old head capsule (HCS stage) as a morphological marker.

RNAi in Pyrrhocoris larvae

Double-stranded RNA (dsRNA) was synthesized using the T3 and T7 RNA polymerases with the MEGAscript kit (Ambion) from plasmids containing the appropriate gene fragments as described previously (Konopova et al., 2011). Using a glass capillary needle mounted in a micromanipulator, dsRNA was injected ventro-laterally into the abdomen of CO_2-anesthesized *Pyrrhocoris* larvae. Approximately 0.2 μg, 0.5 μg, 1 μg and 3 μg of dsRNA were delivered into larvae of L1, L2, L3 and L4 instars, respectively. Control animals were injected with equal amounts of heterologous dsRNA, derived from the *egfp* gene.

mRNA Quantification

In *Pyrrhocoris*, total RNA was extracted either from whole larvae (instars L1 and L2) or from their abdominal epidermis (instars L3-L5) using either TRIzol (Invitrogen) or the RNeasy Plus mini kit (Qiagen). After TURBO DNase treatment (Ambion), 150 ng of total RNA was used for cDNA synthesis using the SuperScript II reverse transcriptase (Invitrogen) and oligo$(dT)_{18}$ primers. Relative transcript levels were measured by quantitative PCR (qPCR) using the iQ SYBR Green Supermix kit and the C1000 Thermal Cycler (both Bio-Rad) and were normalized to mRNA levels of the *Pyrrhocoris* ribosomal protein (Rp49) as described previously (Konopova et al., 2011). Primers used for qPCR are listed in Table 1.

In *Bombyx*, either whole larvae were homogenized (instars L1 and L2), or the dorsal epidermis of the anterior four (L2), two (L3) or one (L4, L5) abdominal segments was dissected. RNA was then extracted using the RNeasy Plus mini kit (Qiagen), and 50 ng of total RNA was taken per cDNA synthesis reaction using the PrimeScript RT reagent kit (Takara Bio) with mixed oligo

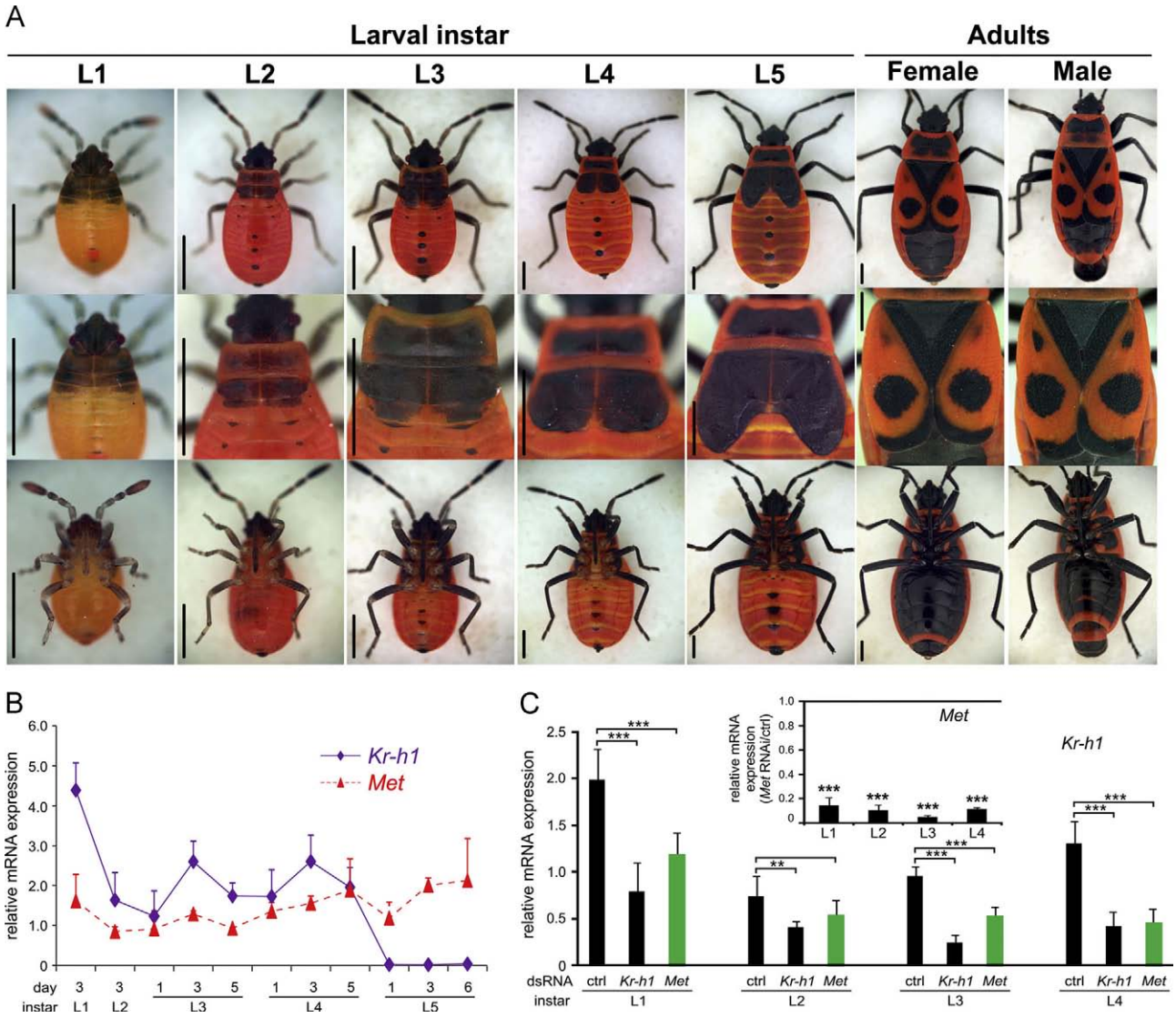

Fig. 1. Developmental profile and RNAi-mediated knockdown of *Met* and *Kr-h1* mRNA expression during larval instars of *Pyrrhocoris apterus*. (A) Postembryonic development of *Pyrrhocoris*. The bugs invariably undergo five larval instars (L1–L5) before molting to adults. Note the progressive increase in body size (scale bars = 1 mm) and growth of the wing pads. Only adults possess articulated wings with a specific color pattern, external genitalia (visible particularly in males), and tanned abdominal cuticle. (B) Expression of *Met* and *Kr-h1* mRNAs on the indicated days of the larval instars. Downregulation of *Kr-h1* during the final (L5) instar results from a natural absence of JH that permits the metamorphic molt (Konopova et al., 2011). Values are mean + s.d. based on three independent biological replicates. (C) *Met* and *Kr-h1* RNAi. Newly ecdysed (L2–L4) or hatched (L1) *Pyrrhocoris* larvae were injected with the indicated dsRNAs and mRNA expression was determined three days later. Levels of *Kr-h1* mRNA were significantly depleted by RNAi targeting *Kr-h1* itself in each larval instar compared to treatment with control dsRNA. Full expression of *Kr-h1* required Met (green columns; decrease not significant for L2). *Met* knockdown was equally efficient during all pre-ultimate instars (inset; horizontal line indicates control levels of *Met* mRNA, arbitrarily set to 1). Values are mean + s.d. based on 4–7 independent biological replicates. Asterisks indicate statistical significance of differences against the control values as determined by Dunnett's test for *Kr-h1* or by Student's *t*-test for *Met* expression. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 1
Primers used for quantification of the specific mRNAs.

mRNA	Forward primer (5′→3′)	Reverse primer (5′→3′)
PaKr-h1	CCCTACCAGTGTAACTTTTGC	GAACGTCTTGTTACACACACC
PaMet	TTCTGATGATGGTGAAAAGATG	TATCGCCCCTGACTACTTGG
PaRp49	CCGATATGTAAAACTGAGGAGAAAC	GGAGCATGTGCCTGGTCTTTT
BmKr-h1α	CACAACCTACGCCAACATTAGAAACG	ACTGATGAACTCGCTCCTCGTCAC
BmKr-h1β	GAAACAATTTCGTTCTTCAGGTGACG	TCGTGCGTGTGCTGTAAGCG
BmBR-C	CGCAACACTTCTGTCTCCGATGG	TTGAGGCTTTTCCCGTCGCA
BmRp49	CAGGCGGTTCAAGGGTCAATAC	TGCTGGGCTCTTTCCACGA

Pa, *Pyrrhocoris apterus*; *Bm*, *Bombyx mori*.

$(dT)_{18}$ and random hexamer primers. qPCR reactions were performed with gene-specific primers (Table 1) as described previously, and data were normalized against *Bombyx rp*49 expression levels (Kayukawa et al., 2012).

Methoprene treatment of Bombyx larvae

Two microliters of 1 μg/μl solution of methoprene (SDS Biotech) in acetone were applied topically to the dorsal abdomen

of *Bombyx mod* larvae; control larvae received acetone only. After 24 h, the whole larvae were collected or abdominal epidermis was dissected for RNA isolation and mRNA quantification as described above.

Statistical analysis

Differences between mRNA expression levels were tested for statistical significance using the Statistika10 software (StatSoft). For comparison of multiple experimental groups to a single control group, we used the One-Way Anova test with Dunnett's post hoc comparison analysis. Student's *t*-test was used in all other comparisons.

Results

Sensitivity to perturbed JH signaling increases with instar number in Pyrrhocoris larvae

The *Pyrrhocoris* bugs undergo five larval instars (hereafter referred to as L1-L5) before metamorphosing to winged, reproductive adults. Each of the larval instars is distinguished by body size and shape, and by the degree of development of the wing pads (Fig. 1A). Expression of the JH receptor gene *Met* is stable throughout the larval life, whereas levels of *Kr-h1* mRNA are highest in early L1 larvae following a peak during late embryogenesis (Konopova et al., 2011), intermediate during L2–L4, and undetectable for most of the final (L5) instar (Fig. 1B). Premature suppression of *Kr-h1* during the penultimate (L4) instar, whether direct or through *Met* RNAi, induces precocious metamorphosis to adultoids instead of the normal molt to L5 larvae. This heterochronic phenotype is observable after the ecdysis as appearance of external genitals (particularly in males), wings, elongated pronotum, and adult-specific color patterning on the wings and the abdominal cuticle ((Konopova et al., 2011) and Fig. 2).

If JH is required to maintain the juvenile character throughout the entire larval life, one would expect to induce precocious adult development by interfering with JH signaling also during the earlier L1–L3 instars. To test this assumption, we applied *Met* and *Kr-h1* RNAi to L4 and to the progressively younger larval instars of *Pyrrhocoris*. In all instars, the dsRNAs were injected to larvae within two hours after ecdysis (or hatching in the case of L1) to insure that JH signaling was compromised before the animals committed to the larval type of molt. The effect of RNAi on *Met* and *Kr-h1* mRNA levels was determined in the epidermis of L4 and L3 larvae, or in the whole body of L2 and L1 larvae, three days after the dsRNA injection in each instar (Fig. 1C). These experiments showed that the full extent of *Kr-h1* expression during all of the pre-ultimate instars required *Met* function (Fig. 1C). Administration of *Met* RNAi in L4 larvae depleted *Met* mRNA to 12% of its control level (Fig. 1C, inset), whereas *Kr-h1* expression only decreased to about one-third upon silencing of either *Kr-h1* itself or *Met* (Fig. 1C). Yet 100% of all *Met* and *Kr-h1* RNAi animals that succeeded to ecdyse from the L4 instar produced adultoids incapable of further molting (Fig. 2B and C and Table 2). Thus, penultimate-instar larvae were fully sensitive to experimental suppression of JH signaling.

When L3 larvae received *Met* dsRNA, the hallmarks of precocious metamorphosis also appeared, but only after the animals had completed two molts. Thus, of 87 injected L3 animals, all except one developed to externally normal L4 larvae of which 72 were able to molt again, and 52 of these survivors formed adultoids instead of L5 larvae (Fig. 2C and Table 2). The delayed onset of precocious metamorphosis was not due to inefficient *Met* knockdown, as *Met* mRNA was depleted to 5% of its control level by mid-L3 instar (Fig. 1C, inset). Interestingly, although less effective than *Met* RNAi in removing its own transcript (Fig. 1C), *Kr-h1* dsRNA applied during the L3 instar produced mild versions of the precocious adult features already after one molt in 67% individuals (Fig. 3A and Table 2). These animals were unable to molt again and displayed outgrowth and partial adult-specific coloration of the wing pads, appearance of external male genitals, and tanning of the abdominal cuticle (Fig. 3A). Their wing phenotype was much less pronounced compared to L5 adultoids, likely because of the smaller initial size of the L3 wing pads.

The above results showed that, compared to the L4 penultimate instar, L3 larvae were less sensitive to perturbation of JH signaling. In addition, removal of the JH receptor Met was slow-acting and less effective in provoking premature metamorphosis than was interference with its target gene, *Kr-h1*.

The trend of decreasing sensitivity of the larval program to loss of JH signaling genes continued towards the youngest instars. Knockdown of *Met* in L2 larvae, although equally effective as in the older instars (Fig. 1C, inset), did not accelerate metamorphic development at L3 and only induced visible cuticle tanning and slightly enlarged genitals in few L4 individuals, i.e., again after two molts (Fig. 3A and Table 2). Injections of *Kr-h1* dsRNA at the L2 instar yielded L3 larvae, of which many died without detectable signs of metamorphosis, and only a minority arrested as L3 with isolated spots of dark abdominal cuticle and extremely subtle outgrowth of the wing pads and genitalia (Fig. 3B and Table 2). The same phenotypes (not shown) became more pronounced in several animals arresting in the L4 instar, and six out of eight *Kr-h1* RNAi larvae reaching the final L5 instar formed adults (Table 2).

Injections of L1 hatchlings with dsRNA allowed nearly all larvae to ecdyse into the L2 instar (Table 2). For reasons unclear to us, most animals then died either during the second instar or upon molting to L3, including those injected with control heterologous dsRNA. Nonetheless, of the resulting 117 *Met* RNAi and 95 *Kr-h1* RNAi L2 larvae, none showed any precocious metamorphic development at that stage (Table 2). Of 46 *Kr-h1* RNAi individuals that ecdysed to the L3 instar, 10 arrested with local tanning on the abdominal cuticle, a phenotype similar to that of animals subjected to *Kr-h1* RNAi at L2 (Fig. 3B).

Taken together, the experiments in *Pyrrhocoris* reveal that the capacity of *Met* and *Kr-h1* RNAi to elicit a terminal, precocious metamorphosis phenotype gradually increases from nil at L1 to 100% penetrance when applied at the L4 instar. The earliest stages capable of developing even the slightest signs of precocious adult characters are L3 (*Kr-h1* RNAi) and L4 (*Met* RNAi). We cannot discern whether the early larval instars do not respond to the knockdown by initiating metamorphosis because their larval state does not require the JH→Met→Kr-h1 pathway or because of the residual *Kr-h1* expression that may or may not depend on JH and Met.

Expression of Kr-h1 is strongly reduced in JH-deficient Bombyx larvae

To shed light on the requirement of JH for the anti-metamorphic signaling during early larval instars, we turned to the *Bombyx mod* mutants, in which JH biosynthesis is blocked due to the lack of JH epoxidase (Daimon et al., 2012). *Bombyx* normally undergoes five larval instars before pupation. The earliest the *mod* mutants can pupate is after L3 (dimolters). In our present study, only 2% of homozygous *mod* larvae were dimolters while 98% pupated as trimolters after L4, i.e., one instar earlier than normal. Fortunately, the lack of *mod* function did not alter the timing of larval molts (Daimon et al., 2012), enabling us to directly compare developmental gene expression profiles between *mod* larvae deficient in epoxidized JH and equally aged controls.

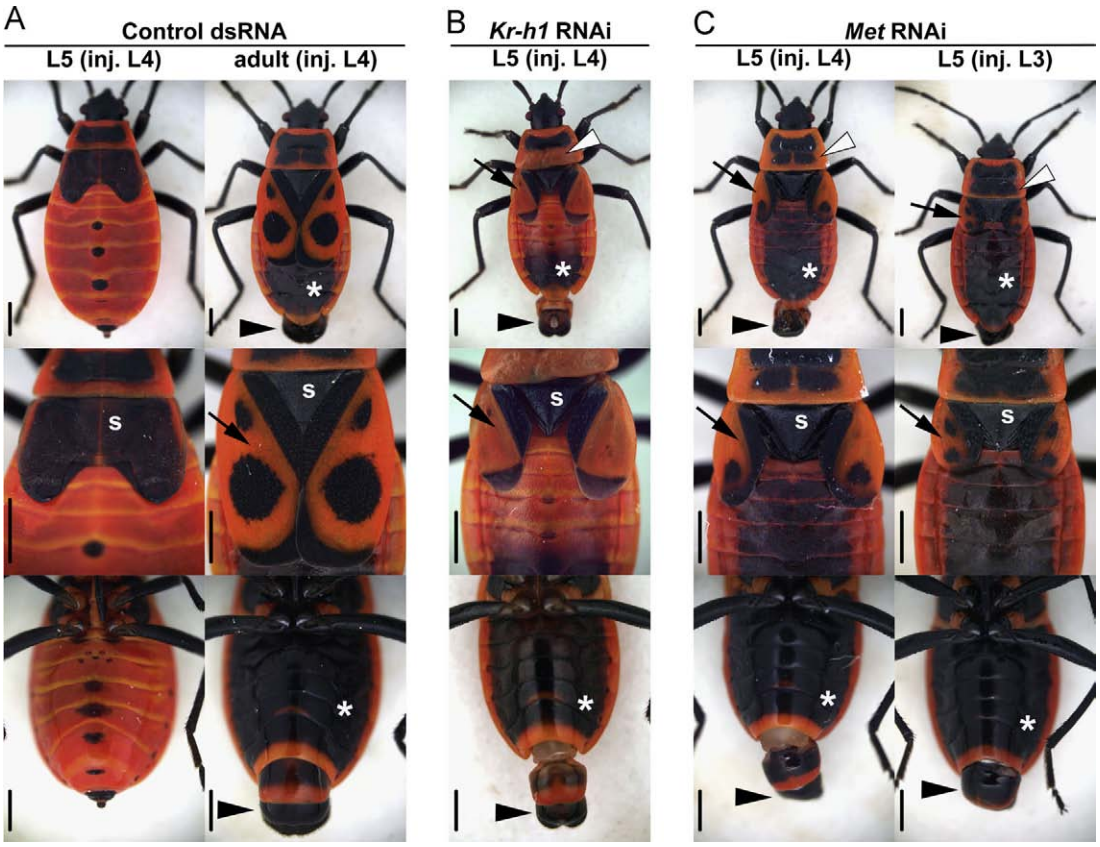

Fig. 2. Loss of *Met* or *Kr-h1* leads to precocious metamorphosis in late instars of *Pyrrhocoris*. (A) Normal final-instar (L5) larva and adult male after injection of control dsRNA during the penultimate (L4) instar. (B, C) Larvae injected with *Kr-h1* (B) or *Met* (C, left column) dsRNA at early L4 invariably ecdysed to adultoids incapable of further molting (see Table 2). Note the precocious development of color-patterned, articulated wings (arrows) separated from the scutellum (s), extended notum (white arrowheads), dark abdominal cuticle (asterisks), and external male genitals (black arrowheads), often with protruding genital and posterior abdominal segments. *Met* RNAi delivered during the L3 instar induced the same adult characters, but only after two completed molts (C, right column). Scale bars, 1 mm.

Table 2
Capacity of *Met* and *Kr-h1* RNAi to induce precocious metamorphosis in the successive larval instars of *Pyrrhocoris*.

dsRNA	Instar injected	*n*	Death[a] at L1	Death[a] at L2	Death[a] at L3	Adult hallmarks at L3	Death[a] at L4	Adult hallmarks at L4	Death[a] at L5	Adult hallmarks at L5	Normal adults
control	L1	54	1	30	4	0	2	0	5	0	12
	L2	31	n.a.	19	0	0	1	0	1	0	10
	L3	24	n.a.	n.a.	3	n.a.	2	0	2	0	17
	L4	27	n.a.	n.a.	n.a.	n.a.	2	n.a.	7	0	18
Kr-h1	L1	97	2	49	24	10	7	0	3	0	2
	L2	50	n.a.	9	16	11	0	6	2	0	6
	L3	21	n.a.	n.a.	0	n.a.	4	14	2	0	1
	L4	46	n.a.	n.a.	n.a.	n.a.	5	n.a.	0	41	0
Met	L1	119	2	58	20	0	19	0	10	0	10
	L2	64	n.a.	20	14	0	12	4	5	0	9
	L3	87	n.a.	n.a.	1	n.a.	14	0	6	52	14
	L4	68	n.a.	n.a.	n.a.	n.a.	6	n.a.	0	62	0

Gray fields denote incidence of precocious metamorphosis with at least one of the adult characters present.
n.a., not applicable.

[a] Lethality without a specific external phenotype.

Expression of the *Kr-h1* gene depends on endogenous JH in the epidermis of penultimate (L4) instar larvae (Kayukawa et al., 2012). We therefore compared *Kr-h1* mRNA profiles between control larvae and *mod* trimolters, in which L3 is the penultimate instar. Expression of two alternatively spliced *Kr-h1* transcripts, α and β, was markedly reduced throughout the first three instars of *mod* larvae (Fig. 4A and B). During eight days of the L1 and L2 instars, both *Kr-h1* mRNAs in the whole body reached on average only 8.3% of the levels in control larvae of corresponding age ($n=3$ for each time point). A similar reduction occurred in the epidermis of L3 *mod* larvae (Fig. 4A and B). The *Kr-h1* mRNA profile in L2 and L3 control epidermis was dynamic, with marked upregulation as the animals approached the larval molts (head capsule slippage stage, HCS) and ecdyses (Fig. 4A and B). A minor but reproducible increase in the expression of both *Kr-h1* isoforms, culminating at the HCS stage of L2 and L3 instars, was also apparent in *mod* mutants (Fig. 4A and B), suggesting that part of this expression may not depend on epoxidized forms of JH.

In agreement with data for the L4 and L5 instars (Kayukawa et al., 2012, 2014), *Kr-h1*β mRNA was about 10-fold less abundant

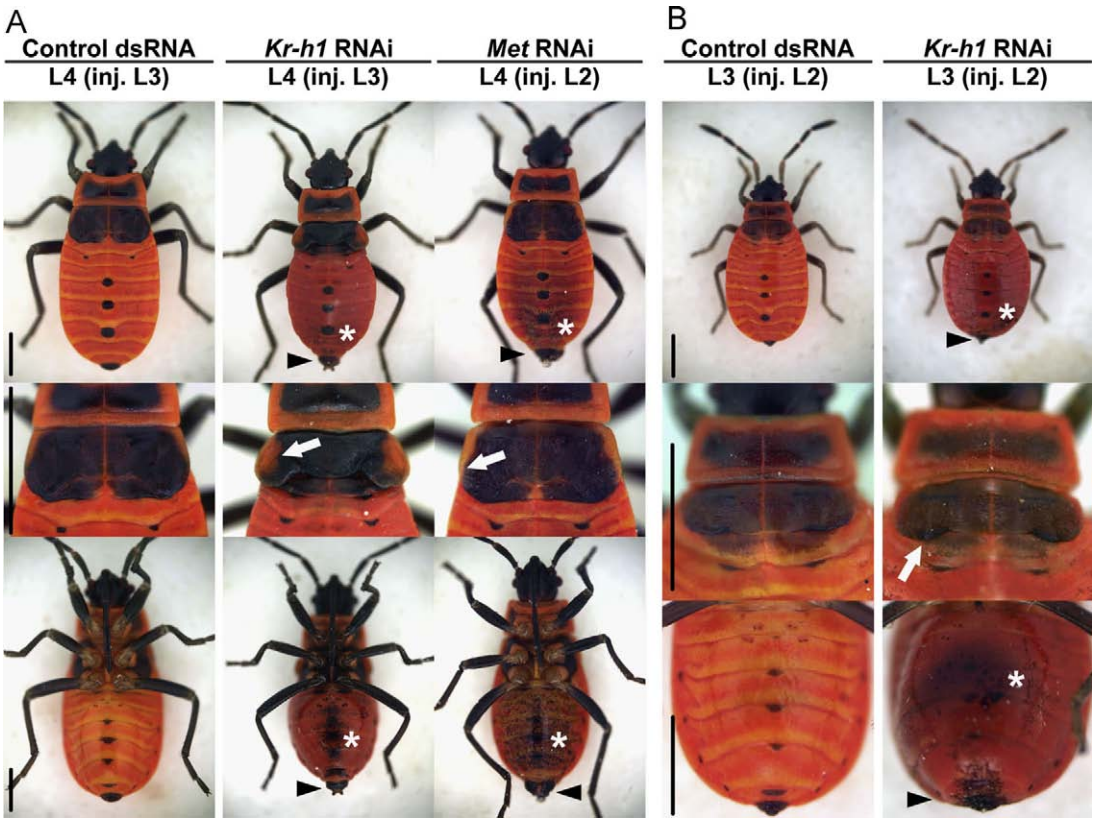

Fig. 3. Third larval instar of *Pyrrhocoris* is the earliest stage to show signs of precocious metamorphosis. (A) Larvae subjected to *Kr-h1* RNAi at early L3 showed mild adult characters upon ecdysis to the L4 instar, including partially outgrown and color-patterned wing pads (arrow), tanned cuticle (asterisks) and rudimentary external genitalia (arrowhead). They did not molt further. A similar, yet milder phenotype resulted from *Met* dsRNA injection at the early L2 instar (right). (B) Larvae after *Kr-h1* knockdown in the L2 instar showed minor tanning of the abdominal cuticle (asterisk) and barely detectable outgrowth of the wing pads (arrow), and no change in external genitalia (arrowhead) upon ecdysis to L3. Scale bars, 1 mm.

than *Kr-h1α* during L1-L3 but the temporal patterns of both isoforms were similar. Therefore, we hereafter followed only the predominant *Kr-h1α* transcript. Interestingly, even from its already low level in *mod* larvae, *Kr-h1α* mRNA decreased further after the animals had ecdysed to the L4 instar (Fig. 4C). This resembled very faithfully the natural fall of *Kr-h1* expression following ecdysis to the final instar in control larvae (Fig. 4C and (Kayukawa et al., 2012, 2014)). Indeed, L4 is the final larval instar for *mod* trimolters. After five days of feeding, *Kr-h1α* mRNA increased as *mod* animals began to spin their cocoons (Fig. 4C). The increase again resembled, albeit on a smaller scale, the rise of *Kr-h1* expression in spinning L5 control larvae (Fig. 4C and (Kayukawa et al., 2012)). Thus, although transcription of *Kr-h1* was severely diminished in *mod* mutant larvae, its residual level could still be downregulated when the trimolters committed to pupation at the L4 instar, and then upregulated just prior to metamorphosis.

Finally, we tested whether the diminished *Kr-h1* expression in *mod* larvae could be induced with a JH mimic, and therefore whether the mutants retained functional JH response. When treated with methoprene early during L3 or L4, *mod* larvae pupate and form adults normally after five instars (Daimon et al., 2012), suggesting that the pathway downstream of JH is unaffected by the *mod* mutation. Indeed, we found that mRNAs for both *Kr-h1* isoforms were elevated, to various degrees, when assessed one day after methoprene administration to newly ecdysed *mod* larvae of L1–L4 instars (Fig. 5). In agreement with the preserved response to methoprene, the *mod* mutants showed normal expression of *Met1* (data not shown), one of the two JH receptor genes present in *Bombyx* (Kayukawa et al., 2012). These results confirm that *mod* mutant larvae are capable of activating the anti-metamorphic *Kr-h1* gene when supplied with the exogenous hormone.

Regulation of the pupal-specifying BR-C gene in JH-deficient Bombyx larvae

BR-C is required for pupal development of holometabolous insects (Konopova and Jindra, 2008; Suzuki et al., 2008; Uhlirova et al., 2003; Zhou and Riddiford, 2002), and its upregulation in the final larval instar marks pupal commitment of the epidermis (Muramatsu et al., 2008; Zhou et al., 1998). Prior to the pupal commitment, 20E-dependent induction of *BR-C* is prevented by JH, at least beginning from the mid-penultimate larval instar of *Manduca* (Zhou et al., 1998) or from the L4 to L5 molt in *Bombyx* (Muramatsu et al., 2008). Whether JH suppresses *BR-C* during the previous larval instars has not been clarified. In this context, it was of interest to examine the temporal pattern of *BR-C* expression in *mod* trimolters.

We discovered that the developmental profile of *BR-C* mRNA during the first two instars was remarkably similar in control and *mod* larvae (Fig. 6). Upon ecdysis to L3 and throughout this instar (penultimate in *mod* trimolters), *BR-C* expression in the epidermis of the mutants substantially exceeded that in control larvae (Fig. 6). Surprisingly, *BR-C* mRNA then dropped as the *mod* trimolters ecdysed to their last, L4 instar, and remained low for four days, closely following the profile in the normal L5 final instar of control larvae (Fig. 6). Before the onset of spinning (late L5 in controls and late L4 in *mod* trimolters), there was a strong upregulation of *BR-C* associated with the pupal commitment of the epidermis in both control and *mod* animals (Fig. 6 and (Muramatsu et al., 2008)).

These data unexpectedly suggest that in the JH-deficient *mod* mutants, *BR-C* does not become prematurely upregulated until in the third instar. Therefore, epoxidized JH is not necessary for keeping *BR-C* expression at low levels prior to L3. However,

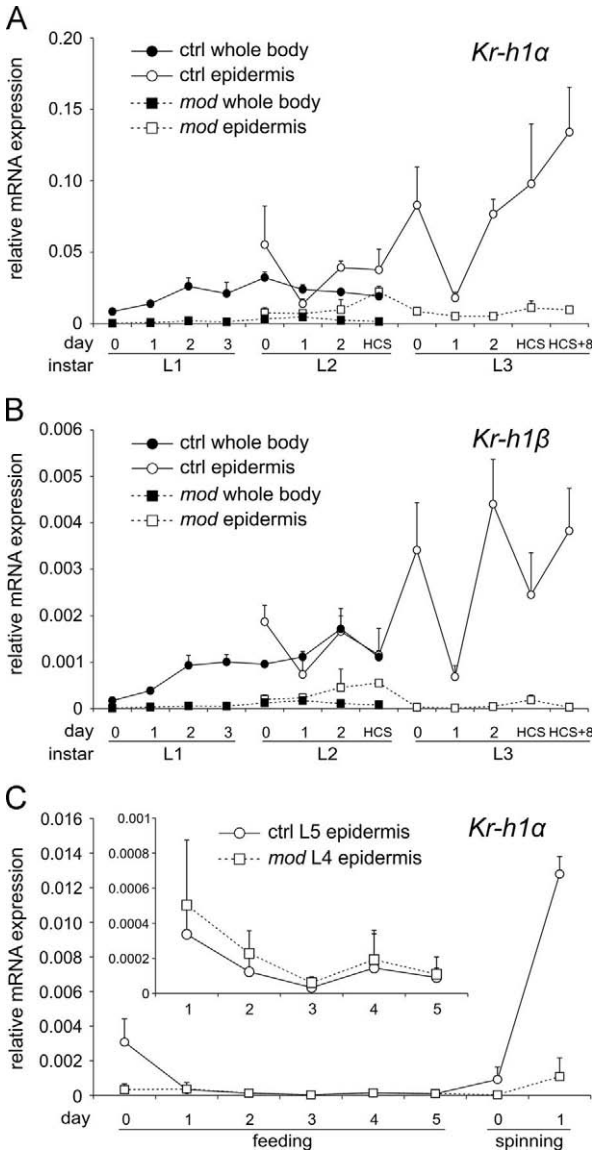

Fig. 4. Expression of *Kr-h1* is greatly reduced throughout the first three larval instars of JH-deficient *Bombyx mod* mutants. Expression profiles of *Kr-h1α* (A) and *Kr-h1β* (B) mRNA isoforms were determined in whole body (L1 and L2, solid symbols) or in the epidermis (L2 and L3, open symbols) of control (solid lines) and *mod* (broken lines) larvae. Both *Kr-h1* isoforms followed the same pattern but *Kr-h1α* was the prevalent transcript. (C) *Kr-h1α* mRNA declined to extremely low levels during the final instar of both control (L5) and *mod* (L4) larvae, reaching its minimum on day 3 (inset). The expression then increased at the onset of spinning. HCS (head capsule slippage) marks the onset of ecdysis. HCS+8, HSC stage plus 8 h. Values are mean+s.d. based on three (A and B) or 3–6 (C) independent biological replicates.

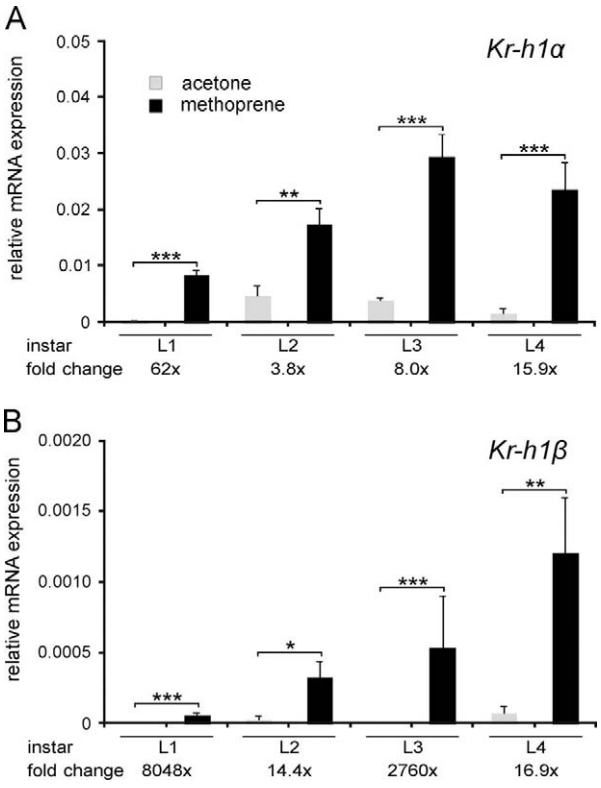

Fig. 5. *mod* mutant larvae retain functional JH response. Newly ecdysed *mod* larvae were topically treated with 2 μg/μl of methoprene or solvent (acetone) alone, and levels of *Kr-h1α* (A) and *Kr-h1β* (B) mRNAs were determined after 24 h either in whole larvae (L1) or abdominal epidermis (L2–L4). The high fold induction of *Kr-h1β* in L1 and L3 instars (B) was due to extremely low *Kr-h1β* expression in control animals at those times. Values are mean+s.d. based on 3–4 independent biological replicates. Data for statistical analysis were log transformed to improve normality. Asterisks indicate statistical significance of differences against the control values as determined by Student's *t*-test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

relative to the major rise during the pupal commitment at L5 in normal animals or at L4 in *mod* trimolters (Fig. 6), this increase of *BR-C* mRNA over control L3 levels is probably too low to divert the epidermis towards the pupal program.

Discussion

The necessity of JH for preserving the larval character of insects from the start of their postembryonic life is a tenet of insect endocrinology that has been challenged by several lines of experimentation. Particularly, genetic depletion of JH in *Bombyx* (Daimon et al., 2012; Tan et al., 2005) and in *Drosophila* (Abdou et al., 2011; Liu et al., 2009; Riddiford et al., 2010) argues that the absence of JH alone is insufficient to provoke metamorphosis prior to the third larval instar.

Here, we addressed the issue by examining the function and expression of genes that are currently known to mediate the anti-metamorphic effect of JH. Using the facility of systemic RNAi in the *Pyrrhocoris* bugs, we confirmed that removal of the JH receptor Met during the penultimate (L4) larval instar caused a precocious metamorphic molt in all treated animals (Konopova et al., 2011). A similar albeit weaker effect of *Met* RNAi administered during the L3 instar only occurred after two molts, of which the first produced apparently normal L4 larvae. Similarly delayed and yet weaker was the effect of *Met* RNAi delivered at L2, and silencing of *Met* at the L1 instar could not induce precocious development of adult features at all. As RNAi does not eliminate its target completely, we cannot exclude residual *Met* function. However, this possibility seems unlikely, considering that injections of *Met* dsRNA proved equally effective in depleting *Met* mRNA at all instars, yet the first two instars were insensitive to the knockdown, L3 was marginally sensitive, and only L4 larvae fully depended on Met for the prevention of precocious metamorphosis.

Silencing of the JH/Met target gene *Kr-h1* essentially confirmed the results obtained with *Met* RNAi except that the effect of *Kr-h1* RNAi was stronger and more immediate, requiring only a single molt for mild but clear adult characters to develop after *Kr-h1* dsRNA had been injected to L3 larvae. The faster effect of *Kr-h1* relative to *Met* RNAi may reflect the direct knockdown of *Kr-h1* as opposed to the indirect loss of *Kr-h1* expression upon depletion of its activator Met. In summary, the earliest stage of *Pyrrhocoris* capable of attaining a terminal phenotype with any

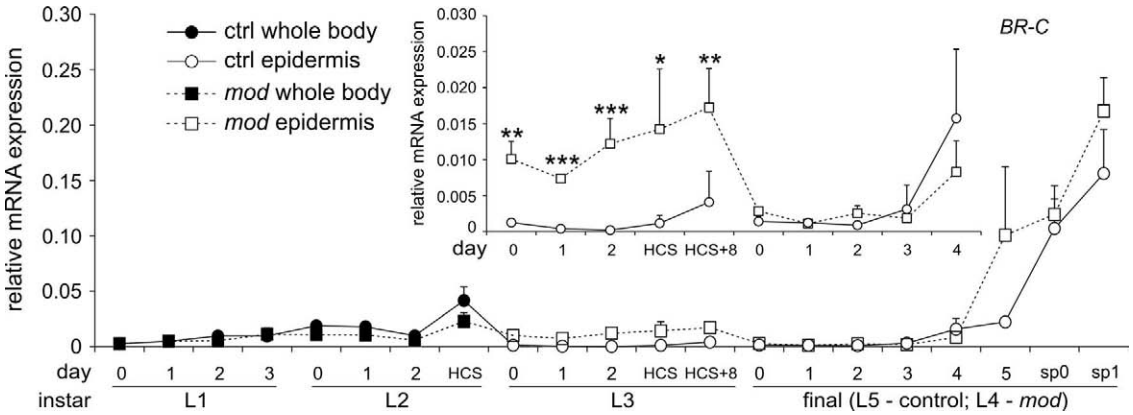

Fig. 6. The pupal-specifying gene *BR-C* is not repressed by JH during the first two larval instars of *Bombyx*. Expression of *BR-C* mRNA (without discriminating isoforms) was determined in whole body (L1 and L2, solid symbols) and in the epidermis (L3 and the final instar, open symbols); note that data for the L4 instar of control larvae are omitted (interrupted solid line). The temporal profile was very similar between control and *mod* larvae during L1, L2, and the final instar (i.e., L5 in controls and L4 in *mod* mutants). Only during the L3 instar, *BR-C* expression in the epidermis of *mod* larvae significantly exceeded control levels (inset). Values are mean + s.d. based on 3–6 independent biological replicates. Asterisks indicate statistical significance of differences against the control values as determined by Student's *t*-test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

signs of precocious adult characters was L3, following *Kr-h1* dsRNA injection during the L2 instar.

The above gene knockdown data match previously described effects of JH deficiency in *Pyrrhocoris*. The fully penetrant precocious metamorphosis phenotype achieved by *Met* or *Kr-h1* RNAi in L4 larvae corresponds with allatectomy, which likewise caused L4 larvae to molt to adultoids (Sláma, 1965). Allatectomy was not performed on earlier larval instars. However, a *Pyrrhocoris* mutant strain *rl(17)* that sporadically yielded individuals without detectable corpora allata (CA) had been described (Socha, 1987; Socha and Hodkova, 1989). Those CA-deficient larvae displayed the same precocious outgrowth of male genitalia and the adult-specific pigmentation on the wing pads and abdominal cuticle at the L4 instar (Socha and Hodkova, 1989) that we observed at that stage upon dsRNA injection of L3 (*Kr-h1*) or L2 (*Met*) larvae (Fig. 3A). Interestingly, the *rl(17)* mutants did not begin to metamorphose earlier than at L4 although they probably lacked the CA from the outset of development, leading the authors to suggest that JH was not required for the early larval status (Socha and Hodkova, 1989).

The situation in *Pyrrhocoris* therefore differs from results obtained with *Rhodnius*, where L1 larvae decapitated at a proper time after their first blood meal developed into "precocious adults" (Wigglesworth, 1934). However, decapitation is not exactly comparable with pure allatectomy, as the animals deprived of the brain obviously lacked many factors in addition to JH. Some of those experiments also involved fusion of the decapitated young larvae to final-instar larvae. While this was necessary to supply an early-decapitated larva with the molting hormone, ecdysone, the exchange of blood between the two individuals inevitably meant transfer of other signals and nutrients. Thus, although of seminal importance, the original experiments on *Rhodnius* may not accurately reflect the anti-metamorphic function of JH in most insects.

Indeed, early reports on other hemimetabolous insects concluded that withdrawal of JH alone was not sufficient to induce metamorphosis at any stage of larval life. The stick insect, *Carausius morosus*, has seven larval instars. Allatectomy of L3 larvae produced precocious fertile adults only after two molts that were considerably delayed, and appearance of partial adult characters also required two molts when the CA were removed from L1 or L2 larvae (Pflugfelder, 1952, 1937). Juveniles of the *Leucophaea maderae* cockroach molted directly to adultoids when deprived of the CA as penultimate (L7) instar larvae, whereas two molts were necessary for the adult features to develop after allatectomy during the previous, L5 or L6, instars (Scharrer, 1946). This delay corresponds with the postponed effect of Met depletion in young *Pyrrhocoris* larvae (Table 2) and with the delayed pupation of the JH-deficient larvae of *Bombyx* (Daimon et al., 2012; Tan et al., 2005). The result in *Leucophaea* had in fact been interpreted as a lack of competence of the early juveniles to metamorphose (Scharrer, 1946), although arguments were also raised that residual JH, persisting after allatectomy, might have blocked metamorphosis (Wigglesworth, 1954).

Our present data support the lack-of-competence view. They show that *Pyrrhocoris* larvae deficient in the JH signaling genes, *Kr-h1* and *Met*, do not commence precocious metamorphosis prior to their third or fourth instar, respectively. Results obtained with the JH-deficient *mod* mutants of *Bombyx* lead to the same conclusion. The *mod* larvae do not begin to pupate until after three or, much more frequently, four instars despite *Kr-h1* expression is severely reduced in L1 through L3 animals. Yet, a functional response to JH is in place as *Kr-h1* mRNA can be induced with the exogenous JH mimic during the early instars. Importantly, this hormonal treatment restores the normal course of five-instar development (Daimon et al., 2012).

Interestingly, the residual *Kr-h1* mRNA level in L1-L3 *mod* trimolter larvae was not nil, suggesting that a minor component of the *Kr-h1* gene activity is independent of authentic, epoxidized JH. Ecdysone may be one possibility, as 20-hydroxyecdysone has been shown to stimulate *Kr-h1* transcription in *Drosophila* (Pecasse et al., 2000) and to potentiate *Kr-h1* induction by JH in *Bombyx* (Kayukawa et al., 2014). Alternatively, *Kr-h1* could respond to a non-epoxidized JH precursor such as methyl farnesoate that might accumulate in the *mod* mutants, deficient in the next enzymatic step of JH biosynthesis (Daimon et al., 2012). Indeed, methyl farnesoate induced *Kr-h1* expression in cultured *Bombyx* cells, albeit at a concentration 1000-fold higher than the 0.1 nM dose that was required for similar activation by the authentic hormone, JH I (Kayukawa et al., 2012). Whether the residual *Kr-h1* expression suffices to prevent metamorphosis cannot be ruled out until *Kr-h1*-null mutant insects become available. However, we consider this scenario unlikely for two reasons. First, a similar degree of incomplete depletion of *Kr-h1* mRNA in *Pyrrhocoris* was sufficient to trigger precocious metamorphosis in 100% of L4 larvae, but both the penetrance and the expressivity of this phenotype decreased progressively in younger instars. Second, in agreement with the JH deficiency phenotype (Daimon et al., 2012; Tan et al., 2005), our preliminary TALEN-mediated knockout of the *Bombyx Kr-h1* gene led to precocious development of pupal cuticle only when the animals have reached the third instar (T.D. and T.S., unpublished data).

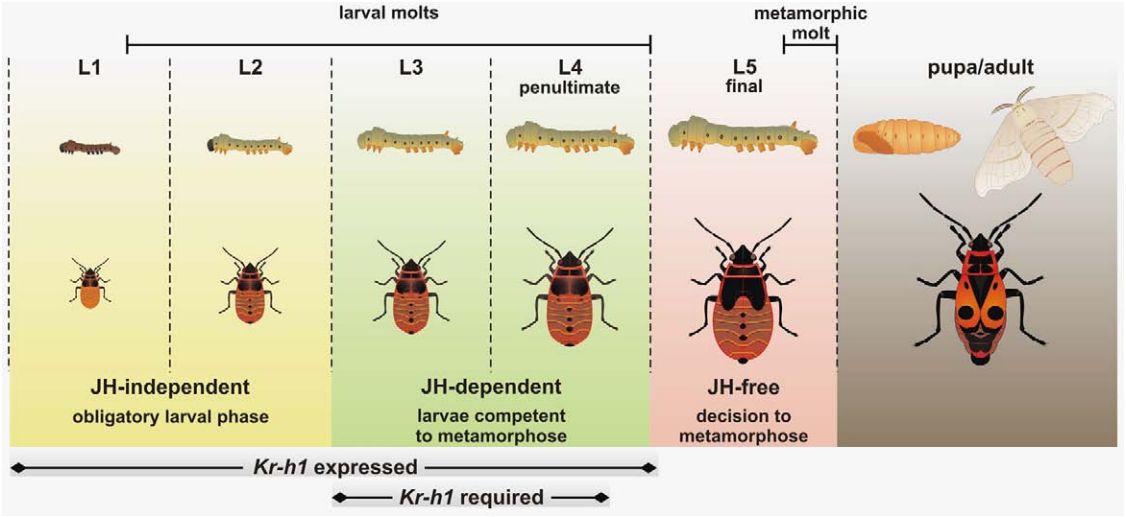

Fig. 7. Schematic of JH-independent and JH-dependent phases of larval development. Note that the diagram only considers roles of JH and Kr-h1 in pre-metamorphic (larval) development but not in the pupa-adult transition or during the adult life.

Expression of the *BR-C* gene that marks and specifies the onset of pupal development provides yet another indication that early *Bombyx* larvae do not rely on JH signaling for prevention of metamorphosis. Despite the absence of JH, *BR-C* mRNA levels in *mod* larvae do not surpass levels in control larvae until at the third instar, and this increase in *BR-C* expression is likely too mild to trigger the pupal program. *BR-C* is then equally suppressed in both *mod* mutants and controls until day four of the respective final instars (Fig. 6). Clearly, the absence of JH alone is insufficient for full extent of precocious expression of the pupal-specifying gene.

Conclusions

In summary, we propose that the successive larval instars are not iterations of the same program, but that the early and late instars represent qualitatively different phases of insect development (Fig. 7). During early postembryonic life, juveniles are incompetent to enter metamorphosis and their larval character is maintained independently of JH. Only when the animals have gradually acquired the competence by the second and third instars, the JH→Met→Kr-h1 pathway becomes increasingly important for protecting them against precocious metamorphosis until the final larval instar (Fig. 7).

During the latter, JH-dependent phase of larval development, the main role of JH is to permit the optimal growth. Indeed, the effect of JH on the attainment of critical body weight for metamorphosis has been clearly established in the tobacco hornworm, *Manduca sexta* (Nijhout and Williams, 1974). A recent study (Suzuki et al., 2013) has determined that JH is in fact necessary for the ability of penultimate and last-instar *Manduca* larvae to prolong their feeding time in response to starvation, such that the critical body weight is achieved. When deprived of JH, starved larvae fail in this compensatory feeding and pupate after a fixed period of time irrespective of their size (Suzuki et al., 2013).

If the absence of JH alone does not render insect larvae competent for metamorphosis, then what is the "competence signal"? *Drosophila* larvae invariably develop in three instars, and neither genetic allatectomy (Abdou et al., 2011; Liu et al., 2009; Riddiford et al., 2010) nor deficiency in JH receptor genes, *Met* and *gce* (Abdou et al., 2011), could reduce this number. Nonetheless, precocious pupation (or at least pupariation) of L2 *Drosophila* larvae has been accomplished in a number of ways, all of which compromised ecdysone production in the prothoracic gland (Bialecki et al., 2002; Gibbens et al., 2011; Mirth et al., 2005; Sliter and Gilbert, 1992; Venkatesh and Hasan, 1997; Zhou et al., 2004). In all cases, this led to a protracted feeding period, thus potentially allowing the animals to reach the weight critical for metamorphosis still within the second instar. Although our current data do not address the nature of the competence to metamorphose, we suspect that body size and/or time spent feeding is likely the key factor.

Acknowledgments

We thank Martina Hajduskova (www.biographix.cz) for Fig. 7, Kiyoshi Hiruma for sharing unpublished information on *Bombyx* allatectomy, and Jean-Philippe Charles for translation of Bounhiol's thesis (1938) from French. This work was supported by the Czech Academy of Sciences Grant 500960906 and by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 25252059). M.J. was supported by a Marie Curie Fellowship Award 276569 from the European Union.

References

Abdou, M.A., He, Q., Wen, D., Zyaan, O., Wang, J., Xu, J., Baumann, A.A., Joseph, J., Wilson, T.G., Li, S., Wang, J., 2011. *Drosophila* Met and Gce are partially redundant in transducing juvenile hormone action. Insect Biochem. Mol. Biol. 41, 938–945.

Achiano, K.A., Giliomee, J.H., 2005. Biology of the house fly predator *Carcinops pumilio* (Erichson) (Coleoptera: Histeridae). Biocontrol 50, 899–910.

Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W.A., Thummel, C.S., 2002. Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. Dev. Cell 3, 209–220.

Bounhiol, J., 1938. Recherches experimentales sur le determinisme de la metamorphose chez les Lepidopteres. Bull. Biol. Fr. Belg 24, 1–199.

Charles, J.-P., Iwema, T., Epa, V.C., Takaki, K., Rynes, J., Jindra, M., 2011. Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. Proc. Natl. Acad. Sci. USA 108, 21128–21133.

Daimon, T., Kozaki, T., Niwa, R., Kobayashi, I., Furuta, K., Namiki, T., Uchino, K., Banno, Y., Katsuma, S., Tamura, T., Mita, K., Sezutsu, H., Nakayama, M., Itoyama, K., Shimada, T., Shinoda, T., 2012. Precocious metamorphosis in the juvenile hormone-deficient mutant of the silkworm *Bombyx mori*. PLoS Genet. 8, e1002486.

Esperk, T., Tammaru, T., Nylin, S., 2007. Intraspecific variability in number of larval instars in insects. J. Econ. Entomol. 100, 627–645.

Fukuda, S., 1944. The hormonal mechanism of larval molting and metamorphosis in the silkworm. J. Fac. Sci. Tokyo Univ. Sec. IV 6, 477–532.

Gibbens, Y.Y., Warren, J.T., Gilbert, L.I., O'Connor, M.B., 2011. Neuroendocrine regulation of *Drosophila* metamorphosis requires TGFβ/Activin signaling. Development 138, 2693–2703.

Hiruma, K., Kaneko, Y., 2013. Hormonal regulation of insect metamorphosis with special reference to juvenile hormone biosynthesis. Curr. Top. Dev. Biol. 103, 73–100.
Jarjees, E.A., Merritt, D.J., 2002. Development of *Trichogramma australicum* Girault (Hymenoptera: Trichogrammatidae) in *Helicoverpa* (Lepidoptera: Noctuidae) host eggs. Austr. J. Entomol. 41, 310–315.
Jindra, M., Palli, S.R., Riddiford, L.M., 2013. The juvenile hormone signaling pathway in insect development. Annu. Rev. Entomol. 58, 181–204.
Kayukawa, T., Minakuchi, C., Namiki, T., Togawa, T., Yoshiyama, M., Kamimura, M., Mita, K., Imanishi, S., Kiuchi, M., Ishikawa, Y., Shinoda, T., 2012. Transcriptional regulation of juvenile hormone-mediated induction of Krüppel homolog 1, a repressor of insect metamorphosis. Proc. Natl. Acad. Sci. USA 109, 11729–11734.
Kayukawa, T., Murata, M., Kobayashi, I., Muramatsu, D., Okada, C., Uchino, K., Sezutsu, H., Kiuchi, M., Tamura, T., Hiruma, K., Ishikawa, Y., Shinoda, T., 2014. Hormonal regulation and developmental role of Krüppel-homolog 1, a repressor of metamorphosis, in the silkworm *Bombyx mori*. Dev. Biol. 388, 48–56.
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.
Konopova, B., Smykal, V., Jindra, M., 2011. Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. PLoS ONE 6, e28728.
Liu, Y., Sheng, Z., Liu, H., Wen, D., He, Q., Wang, S., Shao, W., Jiang, R.J., An, S., Sun, Y., Bendena, W.G., Wang, J., Gilbert, L.I., Wilson, T.G., Song, Q., Li, S., 2009. Juvenile hormone counteracts the bHLH-PAS transcription factors MET and GCE to prevent caspase-dependent programmed cell death in *Drosophila*. Development 136, 2015–2025.
Lozano, J., Bellés, X., 2011. Conserved repressive function of Krüppel homolog 1 on insect metamorphosis in hemimetabolous and holometabolous species. Sci. Rep. 1, 163.
Minakuchi, C., Namiki, T., Shinoda, T., 2009. Krüppel homolog 1, an early juvenile hormone-response gene downstream of Methoprene-tolerant, mediates its anti-metamorphic action in the red flour beetle *Tribolium castaneum*. Dev. Biol. 325, 341–350.
Minakuchi, C., Zhou, X., Riddiford, L.M., 2008. Krüppel homolog 1 (Kr-h1) mediates juvenile hormone action during metamorphosis of *Drosophila melanogaster*. Mech. Dev. 125, 91–105.
Mirth, C., Truman, J.W., Riddiford, L.M., 2005. The role of the prothoracic gland in determining critical weight for metamorphosis in *Drosophila melanogaster*. Curr. Biol. 15, 1796–1807.
Muramatsu, D., Kinjoh, T., Shinoda, T., Hiruma, K., 2008. The role of 20-hydroxyecdysone and juvenile hormone in pupal commitment of the epidermis of the silkworm, *Bombyx mori*. Mech. Dev. 125, 411–420.
Nijhout, H., 1994. Insect hormones. Princeton University Press, Princeton
Nijhout, H.F., Riddiford, L.M., Mirth, C., Shingleton, A.W., Suzuki, Y., Callier, V., 2014. The developmental control of size in insects. WIREs Dev. Biol. 3, 113–134.
Nijhout, H.F., Williams, C.M., 1974. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): growth of the last-instar larva and the decision to pupate. J. Exp. Biol. 61, 481–491.
Parthasarathy, R., Tan, A., Palli, S.R., 2008. bHLH-PAS family transcription factor methoprene-tolerant plays a key role in JH action in preventing the premature development of adult structures during larval–pupal metamorphosis. Mech. Dev. 125, 601–616.
Pecasse, F., Beck, Y., Ruiz, C., Richards, G., 2000. Krüppel-homolog, a stage-specific modulator of the prepupal ecdysone response, is essential for *Drosophila* metamorphosis. Dev. Biol. 221, 53–67.
Pflugfelder, O., 1937. Bau, Entwicklung und Funktion der Corpora allata und cardiaca von *Dixippus morosus* Br. Z. wiss. Zool. A 149, 477–512.
Pflugfelder, O., 1952. Entwicklungsphysiologie der Insekten. Leipzig.
Riddiford, L., 1994. Cellular and molecular actions of juvenile hormone. I. General considerations and premetamorphic actions. Adv. Insect Physiol. 24, 213–274.
Riddiford, L.M., Truman, J.W., Mirth, C.K., Shen, Y.C., 2010. A role for juvenile hormone in the prepupal development of *Drosophila melanogaster*. Development 137, 1117–1126.
Scharrer, B., 1946. The role of the corpora allata in the development of *Leucophaea maderae* (Orthoptera). Endocrinology 38, 35–45.
Sláma, K., 1965. Effect of hormones on growth and respiratory metabolism in the larvae of *Pyrrhocoris apterus* L. (Hemiptera). J. Insect Physiol. 11, 113–122.
Sliter, T.J., Gilbert, L.I., 1992. Developmental arrest and ecdysteroid deficiency resulting from mutations at the dre4 locus of *Drosophila*. Genetics 130, 555–568.
Socha, R., 1987. Occurrence of precocious adults in the rl(17) strain of *Pyrrhocoris apterus* (Heteroptera, Pyrrhocoridae). Acta Entomol. Bohemoslov. 84, 10–14.
Socha, R., Hodkova, M., 1989. The absence of corpus allatum in prothetelic adultiforms in a strain of *Pyrrhocoris apterus* (Heteroptera, Pyrrhocoridae). Acta Entomol. Bohemoslov. 86, 167–171.
Suzuki, Y., Koyama, T., Hiruma, K., Riddiford, L.M., Truman, J.W., 2013. A molt timer is involved in the metamorphic molt in *Manduca sexta* larvae. Proc. Natl. Acad. Sci. USA 110, 12518–12525.
Suzuki, Y., Truman, J.W., Riddiford, L.M., 2008. The role of Broad in the development of *Tribolium castaneum*: implications for the evolution of the holometabolous insect pupa. Development 135, 569–577.
Tan, A., Tanaka, H., Tamura, T., Shiotsuki, T., 2005. Precocious metamorphosis in transgenic silkworms overexpressing juvenile hormone esterase. Proc. Natl. Acad. Sci. USA 102, 11751–11756.
Uhlirova, M., Foy, B.D., Beaty, B.J., Olson, K.E., Riddiford, L.M., Jindra, M., 2003. Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of Broad-Complex in insect metamorphosis. Proc. Natl. Acad. Sci. USA 100, 15607–15612.
Venkatesh, K., Hasan, G., 1997. Disruption of the IP3 receptor gene of *Drosophila* affects larval metamorphosis and ecdysone release. Curr. Biol. 7, 500–509.
Wigglesworth, V., 1934. The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II. Factors controlling moulting and "metamorphosis. Quart. J. Micr. Sci. 77, 191–222.
Wigglesworth, V., 1936. The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). Quart. J. Micr. Sci. 79, 91–121.
Wigglesworth, V., 1954. The physiology of insect metamorphosis. Cambridge University Press, Cambridge.
Yamanaka, N., Rewitz, K.F., O'Connor, M.B., 2013. Ecdysone control of developmental transitions: lessons from *Drosophila* research. Annu. Rev. Entomol. 58, 497–516.
Zhou, B., Hiruma, K., Shinoda, T., Riddiford, L.M., 1998. Juvenile hormone prevents ecdysteroid-induced expression of broad complex RNAs in the epidermis of the tobacco hornworm *Manduca sexta*. Dev. Biol. 203, 233–244.
Zhou, X., Zhou, B., Truman, J.W., Riddiford, L.M., 2004. Overexpression of broad: a new insight into its role in the *Drosophila* prothoracic gland cells. J. Exp. Biol. 207, 1151–1161.
Zhou, X., Riddiford, L.M., 2002. Broad specifies pupal development and mediates the "status quo" action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. Development 129, 2259–2269.

RESULTS

Research article IV

A Homolog of the Vertebrate Dioxin Receptor Is a Receptor for Arthropod Juvenile Hormone

Marek Jindra, Vlastimil Smykal, Jean-Philipe Charles, Mirka Uhlirova and Ron J. Hill

CONCLUSIONS

i) JH receptor Methoprene-tolerant and its target gene *Kr-h1* are universal mediators of anti-metamorphic JH function in insect development

Knockdown of either *Met* or *Kr-h1* in *Pyrrhocoris* larvae of the penultimate larval instar elicited precocious metamorphosis and mimicked results from JH-depleted larvae. The JH-Met-Kr-h1 pathway seems to be critical for proper metamorphic transition in all insect orders with JH-dependent development.

ii) JH-Met-Kr-h1 signaling is dispensable during early larval development of hemi- and holometabolan insects

Knockdown of *Met* and *Kr-h1* in *Pyrrhocoris* hatchlings and early larval instars showed signs of precocious metamorphosis no earlier than in third larval instar of normal five-instar development. Similarly, JH-deficient *mod* mutant larvae of the silkworm *Bombyx mori* entered precocious metamorphosis after three or four larval instars instead of normal five. We have also shown that both suppression of *BR-C* and a minor portion of *Kr-h1* expression are JH-independent in early *Bombyx* larvae. Thus, the postembryonic development in both models is initially independent of JH and later, when larvae gain competence to enter metamorphosis, JH signaling becomes necessary to prevent precocious metamorphosis and to optimize growth.

iii) Met and its heterodimeric partner Tai but not Kr-h1 are critical for reproduction of *Pyrrhocoris*

Silencing of *Met* and *tai* blocked JH-dependent induction of vitellogenins in the fat body, retarded ovarian development and switched adult *Pyrrhocoris* females from reproduction to diapause. *Kr-h1* RNAi neither interfered with ovarian development or vitellogenin gene expression in reproductive females, nor did prevent methoprene from inducing vitellogenesis in diapausing females. Therefore, the Met-Tai complex acts as a JH receptor during oogenesis.

iv) The effect of JH is mediated by Gce-Tai rather than Met-Tai in a luciferase-based reporter assay

We tested whether Met, Gce, and their partner Tai mediated JH-dependent transcriptional activation in *Drosophila* S2 cells. The luciferase reporter was activated by adding JH III, its precursor methyl farnesoate, and JH mimics in a dose-dependent manner and was enhanced after overexpression of Tai. RNAi-mediated knockdown of either *tai* or *gce* but not of *Met* prevented JH III from inducing the transcription. Introducing amino acid mutations that prevented ligand-binding to the Gce protein blocked transcriptional activation, demonstrating that the ability of Gce to bind the hormone is indeed required for its function.

v) Gce restores sensitivity to JH mimicking insecticides in *Met^{27} gce$^{2.5k}$* mutant *Drosophila*

We demonstrated that ubiquitous expression of Gce^{WT} in *Drosophila Met^{27} gce$^{2.5k}$* double-null mutants rescued a major proportion of *Met^{27} gce$^{2.5k}$/Y* hemizygous males. In contrast, expression of the mutated Gce^{T272Y}, Gce^{V315F}, or Gce^{C366M} proteins allowed not a single *Met^{27} gce$^{2.5k}$/Y* adult to emerge. Thus, only Gce capable of binding JH could substitute for the absent *Met* and *gce* function. Similarly, only Gce^{WT} could restore JH mimic sensitivity of formerly viable but methoprene-resistant *Met^{27}* single mutants. These results constitute the first genetic evidence establishing a member of the bHLH-PAS family as a receptor for a *bona fide* hormone.

REFERENCES

Abdou, M.A., He, Q., Wen, D., Zyaan, O., Wang, J., Xu, J., Baumann, A.A., Joseph, J., Wilson, T.G., Li, S. and Wang, J. 2011. *Drosophila* Met and Gce are partially redundant in transducing juvenile hormone action. *Insect Biochem. Mol. Biol.* 41(12): 938-45.

Achiano, K.A. and Giliomee, J.H. 2005. Biology of the house fly predator *Carcinops pumilio* (Erichson) (Coleoptera:Histeridae). *BioControl* 50: 899-910.

Adams, T.S., Hagedorn, H.H. and Wheelock, G.D. 1985. Haemolymph ecdysteroid in the house fly, *Musca domestica*, during oogenesis and its relationship with vitellogenin levels. *J. Insect Physiol.* 31: 91-97.

Ashburner, M., Chihara, C., Meltzer, P. and Richards, G. 1974. Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harb. Symp. Quant. Biol.* 38: 655-62.

Ashok, M., Turner, C. and Wilson, T.G. 1998. Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proc. Natl. Acad. Sci. USA* 95(6): 2761-6.

Bai, J., Uehara, Y. and Montell, D.J. 2000. Regulation of invasive cell behavior by taiman, a *Drosophila* protein related to AIB1, a steroid receptor coactivator amplified in breast cancer. *Cell* 103(7): 1047-58.

Bajgar, A., Jindra, M. and Dolezel, D. 2013. Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. *Proc. Natl. Acad. Sci. USA* 110(11): 4416-21.

Banno, Y., Fujii, H., Kawaguchi, Y., Yamamoto, K., Nishikawa, K., Nishisaka, A., Tamura, K. and Eguchi, S. 2005. *A Guide to the Silkworm Mutants 2005: Gene Name and Gene Symbol.* Silkworm Genetics Division, Institute of Genetic Resources, Kyushu University, Fukuoka, Japan.

Baumann, A., Barry, J., Wang, S., Fujiwara, Y. and Wilson, T.G. 2010. Paralogous genes involved in juvenile hormone action in *Drosophila melanogaster*. *Genetics* 185(4): 1327-36.

Bayer, C.A., Holley, B. and Fristrom, J.W. 1996. A switch in *broad-complex* zinc-finger isoform expression is regulated posttranscriptionally during the metamorphosis of *Drosophila* imaginal discs. *Dev. Biol.* 177(1): 1-14.

Bellés, X., Martín, D. and Piulachs, M.D. 2005. The mevalonate pathway and the synthesis of juvenile hormone in insects. *Annu. Rev. Entomol*. 50: 181-99.

Belles, X. 2011. Origin and Evolution of Insect Metamorphosis. In: Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd: Chichester.

Belles, X. and Santos, C.G. 2014. The MEKRE93 (Methoprene tolerant-Krüppel homolog 1-E93) pathway in the regulation of insect metamorphosis, and the homology of the pupal stage. *Insect Biochem. Mol. Biol.* 52: 60-8.

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. and Nüsslein-Volhard, C. 1988. The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J.* 7(6): 1749-56.

Bernardo, T.J. and Dubrovsky, E.B. 2012. The *Drosophila* juvenile hormone receptor candidates Methoprene-tolerant (Met) and germ cell-expressed (gce) utilize a conserved LIXXL motif to bind the FTZ-F1 nuclear receptor. *J. Biol. Chem.* 287: 7821-33.

Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W.A. and Thummel, C.S. 2002. Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. *Dev. Cell* 3(2): 209-20.

Bitsch, C., Baehr, J. C. and Bitsch J. 1985. Juvenile hormones in *Thermobia domestica* females: Identification and quantification during biological cycles and after precocene application. *Experientia* 41(3): 409-10.

Bounhiol, J. 1938. Recherches experimentales sur le determinisme de la metamorphose chez les Lepidopteres. *Bull. Biol. Fr. Belg* 24, 1-199.

Bradley, J.T., Masetti, M., Cecchettini, A. and Giorgi, F. 1995. Vitellogenesis in the allatectomized stick insect *Carausius morosus* (Br.) (Phasmatodea: Lonchodinae). *Comp. Biochem. Physiol. B* 110: 255-66.

Büning, J. 1994. The insect ovary: ultrastructure, previtellogenic growth and evolution. Chapman and Hall, London, UK.

Cernilogar, F. M., Fabbri, F., Andrenacci, D., Taddei, C. and Gargiulo, G. 2001. *Drosophila* vitelline membrane cross-linking requires the *fs(1)Nasrat*, *fs(1)polehole* and chorion genes activities. *Dev. Genes Evol.* 211: 573-80.

Charles, J.P., Iwema, T., Epa, V.C., Takaki, K., Rynes, J. and Jindra, M. 2011. Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. *Proc. Natl. Acad. Sci. USA* 108(52): 21128-33.

Cherbas, L., Lee, K. and Cherbas, P. 1991. Identification of ecdysone response elements by analysis of the *Drosophila Eip28/29* gene. *Genes Dev.* 5(1): 120-31.

Cieslak, A., Fresneda, J. and Ribera, I. 2014. Life-history specialization was not an evolutionary dead-end in Pyrenean cave beetles. *Proc. R. Soc. B* 281(1781): 20132978.

Clark, A.J. and Bloch, K. 1959. The absence of sterol synthesis in insects. *J. Biol. Chem.* 234: 2578-82.

Comas, D., Piulachs, M.D. and Bellés, X. 1999. Fast induction of vitellogenin gene expression by juvenile hormone III in the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae). *Insect Biochem. Mol. Biol.* 29(9): 821-7.

Cui, Y., Sui, Y., Xu, J., Zhu, F. and Palli, S.R. 2014. Juvenile hormone regulates *Aedes aegypti* Krüppel homolog 1 through a conserved E box motif. *Insect Biochem. Mol. Biol.* 52: 23-32.

Daimon, T., Kozaki, T., Niwa, R., Kobayashi, I., Furuta, K., Namiki, T., Uchino, K., Banno, Y., Katsuma, S., Tamura, T., Mita, K., Sezutsu, H., Nakayama, M., Itoyama, K., Shimada, T. and Shinoda, T. 2012. Precocious metamorphosis in the juvenile hormone-deficient mutant of the silkworm, *Bombyx mori*. *PLoS Genet.* 8(3): e1002486.

de Cuevas, M., Lilly, M.A. and Spradling, A.C. 1997. Germline cyst formation in *Drosophila*. *Annu. Rev. Genet.* 31: 405-28.

Defelipe, L.A., Dolghih, E., Roitberg, A.E., Nouzova, M., Mayoral, J.G., Noriega, F.G. and Turjanski, A.G. 2011. Juvenile hormone synthesis: "esterify then epoxidize" or "epoxidize then esterify"? Insights from the structural characterization of juvenile hormone acid methyltransferase. *Insect Biochem. Mol. Biol.* 41(4): 228-35.

de Kort, C.A.D., Koopmanschap, A.B. and Vermunt, A.M.W. 1997. Influence of pyriproxyfen on the expression of hemolymph protein genes in the Colorado potato beetle, *Leptinotarsa decemlineata*. *J. Insect Physiol.* 43: 363-71.

Deng, W.M. and Ruohola-Baker, H. 2000. Laminin A is required for follicle cell-oocyte signaling that leads to establishment of the anterior-posterior axis in *Drosophila*. *Curr. Biol*. 10(11): 683-6.

DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W. and Guild, G.M. 1991. The *Drosophila Broad-Complex* encodes a family of related proteins containing zinc fingers. *Genetics* 129(2): 385-97.

Dobens, L.L. and Raftery, L.A. 2000. Integration of Epithelial Patterning and Morphogenesis in *Drosophila* Ovarian Follicle Cells. *Dev. Dyn.* 218(1): 80-93.

Dubrovsky, E.B. 2005. Hormonal cross talk in insect development. *Trends Endocrinol Metab.* 16(1): 6-11.

Dubrovsky, E.B., Dubrovskaya, V.A., Bernardo, T., Otte, V., DiFilippo, R. and Bryan, H. 2011. The *Drosophila* FTZ-F1 nuclear receptor mediates juvenile hormone activation of E75A gene expression through an intracellular pathway. *J. Biol. Chem.* 286(38): 33689-700.

Erezyilmaz, D.F., Riddiford, L.M. and Truman, J.W. 2006. The pupal specifier *broad* directs progressive morphogenesis in a direct-developing insect. *Proc. Natl. Acad. Sci. USA* 103: 6925-30.

Erezyilmaz, D.F., Rynerson, M.R., Truman, J.W. and Riddiford, L.M. 2010. The role of the pupal determinant *broad* during embryonic development of a direct developing insect. *Dev. Genes Evol.* 219: 535-44.

Esperk, T., Tammaru, T. and Nylin, S. 2007. Intraspecific variability in number of larval instars in insects. *J. Econ. Entomol.* 100: 627-45.

Fukuda, S. 1944. The hormonal mechanism of larval molting and metamorphosis in the silkworm. *J. Fac. Sci. Tokyo Univ.* Sect. IV 6: 477-532.

Furness, S.G., Lees, M.J. and Whitelaw, M.L. 2007. The dioxin (aryl hydrocarbon) receptor as a model for adaptive responses of bHLH/PAS transcription factors. *FEBS Lett.* 581(19): 3616-25.

Godlewski, J., Wang, S. and Wilson, T.G. 2006. Interaction of bHLH-PAS proteins involved in juvenile hormone reception in *Drosophila*. *Biochem. Biophys. Res. Commun.* 342(4): 1305-11.

González-Reyes, A., Elliott, H. and St Johnston, D. 1995. Polarization of both major body axes in *Drosophila* by *gurken-torpedo* signalling. *Nature* 375(6533): 654-8.

Goodman, W.G. and Chang, E.S. 1985. Juvenile hormone cellular and hemolymph binding proteins. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, ed G.A. Kerkut and L.I. Gilbert, Vol. 7, pp. 491-510. Pergamon Press, New York.

Goodman, W.G. and Cusson, M. 2012. The Juvenile Hormones, in Insect Endocrinology, ed. Gilbert LI (Elsevier, Amsterdam), pp. 310-365.

Greb-Markiewicz, B., Orłowski, M., Dobrucki, J. and Ożyhar, A. 2011. Sequences that direct subcellular traffic of the *Drosophila* methoprene-tolerant protein (MET) are located predominantly in the PAS domains. *Mol. Cell Endocrinol.* 345(1-2): 16-26.

Hagedorn, H.H., O'Connor, J.D., Fuchs, M.S., Sage, B., Schlaeger, D.A. and Bohm, M.K. 1975. The ovary as a source of alpha-ecdysone in an adult mosquito. *Proc. Natl. Acad. Sci. USA* 72(8): 3255-9.

Hall, B.L. and Thummel, C.S. 1998. The RXR homolog ultraspiracle is an essential component of the *Drosophila* ecdysone receptor. *Development* 125(23): 4709-17.

Handler, A.M. 1997. Developmental regulation of yolk protein gene expression in *Anastrepha suspensa*. *Arch. Insect Biochem. Physiol.* 36(1): 25-35.

He, Q., Wen, D., Jia, Q., Cui, C., Wang, J., Palli, S.R. and Li, S. 2014. Heat Shock Protein 83 (Hsp83) Facilitates Methoprene-tolerant (Met) Nuclear Import to

ModulateJuvenile Hormone Signaling. *J. Biol. Chem.* doi: 10.1074/jbc.M114.582825 [Epub ahead of print]

Henrich, V.C. 2012. The Ecdysteroid Receptor, in Insect Endocrinology, ed Gilbert LI (Elsevier, Amsterdam), pp. 177-206.

Hiruma, K. and Kaneko, Y. 2013. Hormonal regulation of insect metamorphosis with special reference to juvenile hormone biosynthesis. *Curr. Top. Dev. Biol.* 103: 73-100.

Hoffmann, K.H. and Sorge, D. 1996. Vitellogenin levels in allatectomized female crickets, *Gryllus bimaculatus* de Geer (Ensifera, Gryllidae). *Arch. Insect Biochem. Physiol.* 32: 549-58.

Huynh, J.R. and St Johnston, D. 2004. The Origin of Asymmetry: Early Polarisation of the *Drosophila* Germline Cyst and Oocyte. *Curr. Biol.* 14(11): 438-49.

Iwema, T., Billas, I.M., Beck, Y., Bonneton, F., Nierengarten, H., Chaumot, A., Richards, G., Laudet, V. and Moras, D. 2007. Structural and functional characterization of a novel type of ligand-independent RXR-USP receptor. *EMBO J.* 26(16): 3770-82.

Jafari, S., Alkhori, L., Schleiffer, A., Brochtrup, A., Hummel, T. and Alenius, M. 2012. Combinatorial activation and repression by seven transcription factors specify *Drosophila* odorant receptor expression. *PLoS Biol.* 10(3): e1001280.

Jarjees, E.A. and Merritt, D.J. 2002. Development of *Trichogramma australicum* Girault (Hymenoptera: Trichogrammatidae) in *Helicoverpa* (Lepidoptera: Noctuidae) host eggs. *Austr. J. Entomol.* 41(4): 310-15.

Jindra, M., Palli, S.R. and Riddiford, L.M. 2013. The juvenile hormone signaling pathway in insect development. *Annu. Rev. Entomol.* 58: 181-204.

Jones, D., Jones, G., Teal, P., Hammac, C., Messmer, L., Osborne, K., Belgacem, Y.H. and Martin, J.R. 2010. Suppressed production of methyl farnesoid hormones yields developmental defects and lethality in *Drosophila* larvae. *Gen. Comp. Endocrinol.* 165(2): 244-54.

Jones, G., Teal, P., Henrich, V.C., Krzywonos, A., Sapa, A., Wozniak, M., Smolka, J. and Jones, D. 2013a. Ligand binding pocket function of *Drosophila* USP is necessary for metamorphosis. *Gen. Comp. Endocrinol.* 182: 73-82.

Jones, D., Jones, G. and Teal, P.E. 2013b. Sesquiterpene action and morphogenetic signaling through the ortholog of retinoid X receptor, in higher Diptera. *Gen. Comp. Endocrinol.* 194: 326-35.

Kalderon, D., Roberts, B.L., Richardson, W.D. and Smith, A.E. 1984. A short amino acid sequence able to specify nuclear location. *Cell* 39(3): 499-509.

Kayukawa, T., Minakuchi, C., Namiki, T., Togawa, T., Yoshiyama, M., Kamimura, M., Mita, K., Imanishi, S., Kiuchi, M., Ishikawa, Y. and Shinoda, T. 2012. Transcriptional regulation of juvenile hormone-mediated induction of Krüppel homolog 1, a repressor of insect metamorphosis. *Proc. Natl. Acad. Sci. USA* 109(29): 11729-34.

Kayukawa, T., Tateishi, K. and Shinoda, T. 2013. Establishment of a versatile cell line for juvenile hormone signaling analysis in *Tribolium castaneum*. *Sci. Rep.* 3: 1570.

Kayukawa, T., Murata, M., Kobayashi, I., Muramatsu, D., Okada, C., Uchino, K., Sezutsu, H., Kiuchi, M., Tamura, T., Hiruma, K., Ishikawa, Y. and Shinoda, T. 2014. Hormonal regulation and developmental role of Krüppel homolog 1, a repressor of metamorphosis, in the silkworm *Bombyx mori*. *Dev. Biol.* 388(1): 48-56.

Kazlauskas, A., Sundstrom, S., Poellinger, L. and Pongratz, I. 2001. The hsp90 chaperone complex regulates intracellular localization of the dioxin receptor. *Mol. Cell Biol.* 21(7): 2594-607.

Kewley, R.J., Whitelaw, M.L. and Chapman-Smith, A. 2004. The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *Int. J. Biochem. Cell Biol.* 36(2): 189-204.

Kimura, S., Sawatsubashi, S., Ito, S., Kouzmenko, A., Suzuki, E., Zhao, Y., Yamagata, K., Tanabe, M., Ueda, T., Fujiyama, S., Murata, T., Matsukawa, H., Takeyama, K., Yaegashi, N. and Kato, S. 2008. *Drosophila* arginine methyltransferase 1 (DART1) is an ecdysone receptor co-repressor. *Biochem. Biophys. Res. Commun.* 371(4): 889-93.

Kirilly, D., Wong, J.J., Lim, E.K., Wang, Y., Zhang, H., Wang, C., Liao, Q., Wang, H., Liou, Y.C., Wang, H. and Yu, F. 2011. Intrinsic epigenetic factors cooperate with the steroid hormone ecdysone to govern dendrite pruning in *Drosophila*. *Neuron* 72(1): 86-100.

Kiss, I., Beaton, A.H., Tardiff, J., Fristrom, D. and Fristrom, J.W. 1988. Interactions and developmental effects of mutations in the *Broad-Complex* of *Drosophila melanogaster*. *Genetics* 118(2): 247-59.

Klusza, S. and Deng, W.M. 2011. At the crossroads of differentiation and proliferation: precise control of cell-cycle changes by multiple signaling pathways in *Drosophila* follicle cells. *Bioessays* 33(2): 124-34.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P. and Hogness, D.S. 1991. The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* 67: 59-77.

Konopova, B. and Jindra, M. 2007. Juvenile hormone resistance gene *Methoprene-tolerant* controls entry into metamorphosis in the beetle *Tribolium castaneum*. *Proc. Natl. Acad. Sci. USA* 104(25): 10488-93.

Konopova, B. and Jindra, M. 2008. Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolan metamorphosis. *Development* 135: 559-68.

Konopova, B., Smykal, V. and Jindra, M. 2011. Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. *PLoS One* 6(12): e28728.

Kotaki, T., Shinada, T., Kaihara, K., Ohfune, Y. and Numata, H. 2009. Structure determination of a new juvenile hormone from a heteropteran insect. *Org. Lett.* 11(22): 5234-7.

Lee, C.Y., Wendel, D.P, Reid, P., Lam, G., Thummel, C.S. and Baehrecke, E.H. 2000. *E93* directs steroid-triggered programmed cell death in *Drosophila*. *Mol. Cell.* 6(2): 433-43.

Li, M., Mead, E.A. and Zhu, J. 2011. Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression. *Proc. Natl. Acad. Sci. USA* 108(2): 638-43.

Li, M., Liu, P., Wiley, J.D., Ojani, R., Bevan, D.R., Li, J. and Zhu, J. 2014. A steroid receptor coactivator acts as the DNA-binding partner of the methoprene-tolerant protein in regulating juvenile hormone response genes. *Mol. Cell Endocrinol.* 394(1-2): 47-58.

Li, Y., Zhang, Z., Robinson, G.E. and Palli, S.R. 2007. Identification and characterization of a juvenile hormone response element and its binding proteins. *J. Biol. Chem.* 282(52): 37605-17.

Liscum, L. 2002. Cholesterol biosynthesis. Chapter 15. In "Biochemistry of Lipids, Lipoproteins, and Membranes: D.E. Vance and J. Vance, Eds. Elsevier Science. 4th Edition. pp. 409-431.

Liu, Y., Sheng, Z., Liu, H., Wen, W., He, Q., Wang, S., Shao, W., Jiang, R.-J., An, S., Sun, Y., Bendena, W.G., Wang, J., Gilbert, L.I., Wilson, T.G., Song, Q. and Li, S. 2009. Juvenile hormone counteracts the bHLH-PAS transcriptional factor MET and GCE to prevent caspase-dependent programmed cell death in *Drosophila*. *Development* 136(12): 2015-25.

Liu, W., Zhang, F.X., Cai, M.J., Zhao, W.L., Li, X.R., Wang, J.X. and Zhao, X.F. 2013. The hormone-dependent function of Hsp90 in the crosstalk between 20-hydroxyecdysone and juvenile hormone signaling pathways in insects is determined by differential phosphorylation and protein interactions. *Biochim. Biophys. Acta.* 1830(11): 5184-92.

Lozano, J. and Bellés, X. 2011. Conserved repressive function of Krüppel homolog 1 on insect metamorphosis in hemimetabolous and holometabolous species. *Sci. Rep.* 1: 163.

Lozano, J. and Bellés, X. 2014. Role of Methoprene-Tolerant (Met) in Adult Morphogenesis and in Adult Ecdysis of *Blattella germanica*. *PLoS One* 9(7): e103614.

Mahajan-Miklos, S. and Cooley, L. 1994. Intercellular cytoplasm transport during *Drosophila* oogenesis. *Dev. Biol.* 165(2): 336-51.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R.M. 1995. The nuclear receptor superfamily: the second decade. *Cell* 83: 835-39.

Marchal, E., Vandersmissen, H.P., Badisco, L., Van de Velde, S., Verlinden, H., Iga, M., Van Wielendaele, P., Huybrechts, R.,Simonet, G., Smagghe, G. and Vanden Broeck, J. 2010. Control of ecdysteroidogenesis in prothoracic glands of insects: a review. *Peptides* 31(3): 506-19.

Marchal, E., Hult, E.F., Huang, J., Pang, Z., Stay, B. and Tobe, S.S. 2014. Methoprene-Tolerant (Met) Knockdown in the Adult Female Cockroach, *Diploptera punctata* Completely Inhibits Ovarian Development. *PLoS One* 9(9): e106737.

Minakuchi, C., Namiki, T., Yoshiyama, M. and Shinoda, T. 2008. RNAi-mediated knockdown of *juvenile hormone acid O-methyltransferase* gene causes precocious metamorphosis in the red flour beetle *Tribolium castaneum*. *FEBS J.* 275: 2919-31.

Minakuchi, C., Namiki, T. and Shinoda, T. 2009. *Krüppel homolog 1*, an early juvenile hormone-response gene downstream of Methoprene-tolerant, mediates its anti-metamorphic action in the red flour beetle *Tribolium castaneum*. *Dev. Biol.* 325(2): 341-50.

Minakuchi, C., Tanaka, M., Miura, K. and Tanaka, T. 2011. Developmental profile and hormonal regulation of the transcription factors *broad* and *Krüppel homolog 1* in hemimetabolous thrips. *Insect Biochem. Mol. Biol.* 41(2): 125-34.

Mirth, C., Truman, J.W. and Riddiford, L.M. 2005. The role of the prothoracic gland in determining critical weight for metamorphosis in *Drosophila melanogaster*. *Curr. Biol.* 15(20): 1796-807.

Mirth, C.K. and Riddiford, L.M. 2007. Size assessment and growth control: how adult size is determined in insects. *Bioessays* 29(4): 344-55.

Miura, K., Oda, M., Makita, S. and Chinzei, Y. 2005. Characterization of the *Drosophila Methoprene-tolerant* gene product. Juvenile hormone binding and ligand-dependent gene regulation. *FEBS J.* 272(5): 1169-78.

Miyakawa, H., Toyota, K., Hirakawa, I., Ogino, Y., Miyagawa, S., Oda, S., Tatarazako, N., Miura, T., Colbourne, J.K. and Iguchi, T. 2013. A mutation in the receptor Methoprene-tolerant alters juvenile hormone response in insects and crustaceans. *Nat. Commun.* 4: 1856.

Moore, A.W., Barbel, S., Jan, L.Y. and Jan, Y.N. 2000. A genomewide survey of basic helix-loop-helix factors in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 97(19): 10436-41.

Mou, X., Duncan, D.M., Baehrecke, E.H. and Duncan, I. 2012. Control of target gene specificity during metamorphosis by the steroid response gene *E93*. *Proc. Natl. Acad. Sci. USA* 109(8): 2949-54.

Nagaraju, G.P. 2011. Reproductive regulators in decapod crustaceans: an overview. *J. Exp. Biol.* 214: 3-16.

Nicolas, E., Chenouard, N., Olivo-Marin, J.C. and Guichet, A. 2009. A dual role for actin and microtubule cytoskeleton in the transport of Golgi units from the nurse cells to the oocyte across ring canals. *Mol. Biol. Cell* 20(1): 556-68.

Nijhout, H.F. 1994. Insect hormones. Princeton University Press, Princeton.

Nijhout, H.F., Riddiford, L.M., Mirth, C., Shingleton, A.W., Suzuki, Y. and Callier, V. 2014. The developmental control of size in insects. *Wiley Interdiscip. Rev. Dev. Biol.* 3(1): 113-34.

Noriega, F.G., Wang, X.Y., Pennington, J.E., Barillas-Mury, C.V. and Wells, M.A. 1996. Early trypsin, a female-specific midgut protease in *Aedes aegypti*: isolation, aminoterminal sequence determination, and cloning and sequencing of the gene. *Insect Biochem. Mol. Biol.* 26(2): 119-26.

Oro, A.E., McKeown, M. and Evans, R.M. 1990. Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. *Nature* 347: 298-301.

Pandini, A., Soshilov, A.A., Song, Y., Zhao, J., Bonati, L. and Denison, M.S. 2009. Detection of the TCDD binding-fingerprint within the Ah receptor ligand binding domain by structurally driven mutagenesis and functional analysis. *Biochemistry* 48(25): 5972-83.

Parthasarathy, R., Tan, A., Bai, H. and Palli, S.R. 2008a. Transcription factor broad suppresses precocious development of adult structures during larval-pupal metamorphosis in the red flour beetle, *Tribolium castaneum*. *Mech. Dev.* 125(3-4): 299-313.

Parthasarathy, R., Tan, A. and Palli, S.R. 2008b. bHLH-PAS family transcription factor methoprene-tolerant plays a key role in JH action in preventing the premature development of adult structures during larval-pupal metamorphosis. *Mech. Dev.* 125(7): 601-16.

Parthasarathy, R., Sun, Z., Bai, H. and Palli, S.R. 2010. Juvenile hormone regulation of vitellogenin synthesis in the red flour beetle, *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* 40(5): 405-14.

Pascual, N., Cerdá, X., Benito, B., Tomás, J., Piulachs, M.D. and Bellés, X. 1992. Ovarian ecdysteroid levels and basal oöcyte development during maturation in the cockroach *Blattella germanica* (L.). *J. Insect Physiol.* 38(5): 339-48.

Pecasse, F., Beck, Y., Ruiz, C. and Richards, G. 2000. *Krüppel-homolog*, a stage-specific modulator of the prepupal ecdysone response, is essential for *Drosophila* metamorphosis. *Dev. Biol.* 221(1): 53-67.

Petryk, A., Warren, J.T., Marques, G., Jarcho, M.P., Gilbert, L.I., Kahler, J., Parvy, J.P., Li, Y., Dauphin-Villemant, C. and O'Connor, M.B. 2003. Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl. Acad. Sci. USA* 100(24): 13773–78.

Pflugfelder, O. 1937. Bau, Entwicklung und Funktion der Corpora allata und cardiaca von *Dixippus morosus* Br. *Z. wiss. Zool.* 149: 477-512.

Pflugfelder, O. 1952. Entwicklungsphysiologie der Insekten. Leipzig.

Piulachs, M.D., Pagone, V. and Bellés, X. 2010. Key roles of the *Broad-Complex* gene in insect embryogenesis. *Insect Biochem. Mol. Biol.* 40(6): 468-75.

Plevin, M.J., Mills, M.M. and Ikura, M. 2005. The LxxLL motif: a multifunctional binding sequence in transcriptional regulation. *Trends Biochem. Sci.* 30(2): 66-9.

Pursley, S., Ashok, M. and Wilson, T.G. 2000. Intracellular localization and tissue specificity of the *Methoprene-tolerant* (*Met*) gene product in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 30(8-9): 839-45.

Raikhel, A.S., Brown, M. and Belles, X. 2005. Endocrine Control of Reproductive Processes. In: Comprehensive Molecular Insect Science (L Gilbert, S Gill and K. Iatrou eds.) Vol. 3, Endocrinology, pp. 433-491. Elsevier Press.

Rohdendorf, E.B. and Watson J.A.L. 1969. The control of reproductive cycles in the female firebrat, *Lepismodes inquilinus*. *J. Insect Physiol.* 15(11): 2085-101.

Riddiford, L.M., Truman, J.W., Mirth, C.K. and Shen, Y.C. 2010. A role for juvenile hormone in the prepupal development of *Drosophila melanogaster*. *Development* 137(7): 1117-26.

Riddiford, L.M. 2013. Microarrays reveal discrete phases in juvenile hormone regulation of mosquito reproduction. *Proc. Natl. Acad. Sci. USA* 110(24): 9623-4.

Roth, S., Neuman-Silberberg, F.S., Barcelo, G. and Schüpbach, T. 1995. *cornichon* and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in *Drosophila*. *Cell* 81(6): 967-78.

Rousset, A. and Bitsch, C. 1989. Synthesis and maturation of the major yolk protein by the ovaries of the firebrat *Thermobia domestica* (Insecta, Thysanura). *Comp. Biochem. Physiol. B* 94(3): 599-06.

Sappington, T.W. and Raikhel, A.S. 1998. Molecular characteristics of insect vitellogenins and vitellogenin receptors. *Insect Biochem. Mol. Biol.* 28(5-6): 277-300.

Sappington, T.W. 2002. The major yolk proteins of higher *Diptera* are homologs of a class of minor yolk proteins in lepidoptera. *J. Mol. Evol.* 55(4): 470-75.

Schonbaum, C.P., Perrino, J.J. and Mahowald, A.P. 2000. Regulation of the vitellogenin receptor during *Drosophila melanogaster* oogenesis. *Mol. Biol. Cell* 11(2): 511-521.

Schwalm, F.E. 1988. Insect morphogenesis., In: Monograph in developmental biology, Vol. 20, Ed. by Sauer, H.W., Karger, Basel pp. 1-356.

Shapiro, A.B., Wheelock, G.D., Hagedorn, H.H., Baker, F.C. Tsai, L.W. and Schooley, D.A. 1986. Juvenile hormone and juvenile hormone esterase in adult females of the mosquito *Aedes aegypti*. *J. Insect Physiol.* 32(10): 867-77.

Sheng, Z., Xu, J., Bai, H., Zhu, F. and Palli, S.R. 2011. Juvenile hormone regulates vitellogenin gene expression through insulin-like peptide signaling pathway in the red flour beetle, *Tribolium castaneum*. *J. Biol. Chem.* 286(49): 41924-36.

Shin, S.W., Zou, Z., Saha, T.T. and Raikhel, A.S. 2012. bHLH-PAS heterodimer of methoprene-tolerant and cycle mediates circadian expression of juvenile hormone-induced mosquito genes. *Proc. Natl. Acad. Sci. USA* 109(41): 16576-81.

Sin, Y.W., Kenny, N.J., Qu, Z., Chan, K.W., Chan, K.W., Cheong, S.P., Leung, R.W., Chan, T.F., Bendena, W.G., Chu, K.H., Tobe, S.S. and Hui, J.H. 2014. Identification of putative ecdysteroid and juvenile hormone pathway genes in the shrimp *Neocaridina denticulata*. *Gen. Comp. Endocrinol.*
doi: 10.1016/j.ygcen.2014.07.018.

Slama, K. 1965. Effect of hormones on growth and respiratory metabolism in the larvae of *Pyrrhocoris apterus* L. (Hemiptera). *J. Insect Physiol.* 11: 113-12.

Smykal, V., Bajgar, A., Provaznik, J., Fexova, S., Buricova, M., Takaki, K., Hodkova, M., Jindra, M. and Dolezel, D. 2014a. Juvenile hormone signaling during reproduction and development of the linden bug, *Pyrrhocoris apterus*. *Insect Biochem. Mol. Biol.* 45: 69-76.

Smykal, V., Daimon, T., Kayukawa, T., Takaki, K., Shinoda, T. and Jindra, M. 2014b. Importance of juvenile hormone signaling arises with competence of insect larvae to metamorphose. *Dev. Biol.* 390(2): 221-30.

Socha, R. 1987. Occurrence of precocious adults in the *rl(17)* strain of *Pyrrhocoris apterus* (Heteroptera, Pyrrhocoridae). *Acta Entomol. Bohemoslov.* 84: 10-14.

Socha, R. and Hodkova, M. 1989. The absence of corpus allatum in prothetelic adultiforms in a strain of *Pyrrhocoris apterus* (Heteroptera, Pyrrhocoridae). *Acta Entomol. Bohemoslov.* 86: 167-71.

Socha, R., Sula, J., Kodrik, D. and Gelbic, I. 1991. Hormonal control of vitellogenin synthesis in *Pyrrhocoris apterus* (L.) (Heteroptera). *J. Insect Physiol.* 37(11): 805-16.

Song, J., Wu, Z., Wang, Z., Deng, S. and Zhou, S. 2014. Krüppel-homolog 1 mediates juvenile hormone action to promote vitellogenesis and oocyte maturation in the migratory locust. *Insect Biochem. Mol. Biol.* 52: 94-101.

Soshilov, A. and Denison, M.S. 2008. Role of the Per/Arnt/Sim domains in ligand-dependent transformation of the aryl hydrocarbon receptor. *J. Biol. Chem.* 283(47): 32995-3005.

Soshilov, A. and Denison, M. S. 2011. Ligand displaces heat shock protein 90 from overlapping binding sites within the aryl hydrocarbon receptor ligand-binding domain. *J. Biol. Chem.* 286(40): 35275-82.

Spradling, A.C. 1993. Developmental genetics of oogenesis. In: Bate M, Martinez Arias A, editors.The Development of *Drosophila* melanogaster. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 1-70.

Sun, X., Song, Q. and Barrett, B. 2003. Effect of ecdysone agonists on vitellogenesis and the expression of EcR and USP in codling moth (*Cydia pomonella*). *Arch. Insect Biochem. Physiol.* 52(3): 115-129.

Suzuki, Y., Koyama, T., Hiruma, K., Riddiford, L.M. and Truman, J.W. 2013. A molt timer is involved in the metamorphic molt in *Manduca sexta* larvae. *Proc. Natl. Acad. Sci. USA* 110(31): 12518-25.

Tan, A., Tanaka, H., Tamura, T. and Shiotsuki, T. 2005. Precocious metamorphosis in transgenic silkworms overexpressing juvenile hormone esterase. *Proc. Natl. Acad. Sci. USA* 102(33): 11751-6.

Tazima, Y. 1978. The silkworm: an important tool. Tokyo: Kodansha.

Telfer, W. 1965. The mechanism and control of yolk formation. *Annu. Rev. Entomol.* 10: 161-84.

Theurkauf, W.E. and Hazelrigg, T.I. 1998. In vivo analyses of cytoplasmic transport and cytoskeletal organization during *Drosophila* oogenesis: characterization of a multi-step anterior localization pathway. *Development* 125(18): 3655-66.

Thummel, C.S. 1996. Flies on steroids--*Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet.* 12(8): 306-10.

Thummel, C.S. 2001. Steroid-triggered death by autophagy. *Bioessays* 23(8): 677-82.

Tobe, S.S. and Bendena, W.G. 1999. The regulation of juvenile hormone production in arthropods. Functional and evolutionary perspectives. *Ann. NY Acad. Sci.* 897: 300-10.

Trauner, J. and Büning, J. 2007. Germ-cell cluster formation in the telotrophic meroistic ovary of *Tribolium castaneum* (Coleoptera, Polyphaga, Tenebrionidae) and its implication on insect phylogeny. *Dev. Genes Evol.* 217(1): 13-17.

Tsai, C.C., Kao, H.Y., Yao, T.P., McKeown, M. and Evans, R.M. 1999. SMRTER, a *Drosophila* nuclear receptor coregulator, reveals that EcR-mediated repression is critical for development. *Mol. Cell* 4(2): 175-86.

Tsubota, T., Nakakura, T. and Shiotsuki, T. 2010a. Molecular characterization and enzymatic analysis of juvenile hormone epoxide hydrolase genes in the red flour beetle. *Insect Mol. Biol.* 19(3): 399-408.

Tsubota, T., Minakuchi, C., Nakakura, T., Shinoda, T. and Shiotsuki, T. 2010b. Molecular characterization of a gene encoding juvenile hormone esterase in the red flour beetle, *Tribolium castaneum*. *Insect Mol. Biol.* 19(4): 527-35.

Uhlirova, M., Foy, B.D., Beaty, B.J., Olson, K.E., Riddiford, L.M. and Jindra, M. 2003. Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of Broad-Complex in insect metamorphosis. *Proc. Natl. Acad. Sci. USA* 100(26): 15607-12.

Ureña, E., Manjón, C., Franch-Marro, X. and Martín, D. 2014. Transcription factor E93 specifies adult metamorphosis in hemimetabolous and holometabolous insects. *Proc. Natl. Acad. Sci. USA* 111(19): 7024-9.

Van Wielendaele, P., Badisco, L. and Vanden Broeck, J. 2013. Neuropeptidergic regulation of reproduction in insects. *Gen. Comp. Endocrinol.* 188: 23-34.

Weaver, R.J. and Audsley, N. 2009. Neuropeptide regulators of juvenile hormone synthesis: structures, functions, distribution, and unanswered questions. *Ann. NY Acad. Sci.* 1163: 316-29.

Wigglesworth, V. 1934. The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II. Factors controlling moulting and "metamorphosis." *Quart. J. Microscop. Sci.* 77: 191-222.

Wigglesworth, V. B. 1936. The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus*. *Quart. J. Microscop. Sci.* 79: 91-120.

Wilson, T.G. and Fabian, J. 1986. A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. *Dev. Biol.* 118(1): 190-201.

Wyatt, G.R. and Davey, K.G. 1996. Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormones in adult insects. *Adv. Insect Physiol.* 26: 1-155.

Xing, Y., Nukaya, M., Satyshur, K.A., Jiang, L., Stanevich, V., Korkmaz, E.N., Burdette, L., Kennedy, G.D., Cui, Q. and Bradfield, C.A. 2012. Identification of the Ah-receptor structural determinants for ligand preferences. *Toxicol. Sci.* 129(1): 86-97.

Yamanaka, N., Rewitz, K.F. and O'Connor, M.B. 2013. Ecdysone control of developmental transitions: lessons from *Drosophila* research. *Annu. Rev. Entomol.* 58: 497-516.

Yao, T.P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., McKeown, M., Cherbas, P. and Evans, R.M. 1993. Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature* 366(6454): 476-9.

Zhang, Z., Xu, J., Sheng, Z., Sui, Y. and Palli, S.R. 2011. Steroid receptor co-activator is required for juvenile hormone signal transduction through a bHLH-PAS transcription factor, methoprene tolerant. *J. Biol. Chem.* 286(10): 8437-47.

Zhou, X. and Riddiford, L.M. 2002. Broad specifies pupal development and mediates the 'status quo' action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. *Development* 129(9): 2259-69.

Zhu, J., Chen, L., Sun, G. and Raikhel A.S. 2006. The competence factor beta Ftz-F1 potentiates ecdysone receptor activity via recruiting a p160/SRC coactivator. *Mol. Cell Biol.* 26(24): 9402-12.

Zou, Z., Saha, T.T., Roy, S., Shin, S.W., Backman, T.W., Girke, T., White, K.P. and Raikhel, A.S. 2013. Juvenile hormone and its receptor, methoprene-tolerant, control the dynamics of mosquito gene expression. *Proc. Natl. Acad. Sci. USA* 110(24): 2173-81.