University of South Bohemia in České Budějovice

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Novel Agonists of the Juvenile Hormone Signaling

Master thesis

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ANNOTATION

Juvenile hormone (JH) plays a key role in insect development. In larval stage, JH prevents the insect to metamorphose. While JH is unique to arthropods and absent from vertebrates, it represents potential tool for insect control. Combination of cell-based assay and high-throughput screening enabled to find candidate JH agonists (JHags) that activate JH signaling via juvenile hormone receptor (JHR). This thesis aims to elucidate the effect of these JHags on three evolutionary distinct insect species *in vivo* and possible selectivity of the JHags for individual insect species *in vitro*. In addition, we provide evidence that JHR from hemimetabolous species *Blattella germanica* and *Prorhinotermes simplex* binds JH *in vitro*. This thesis provides useful findings and tools for development of new JHags that could be selective for particular insect taxa.

DECLARATION

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1   INTRODUCTION

1.1   General introduction

This thesis is part of a greater project which aims to discover new potential agonists of juvenile hormone. The project stems from automated cell-based high-throughput screening (HTS) of a chemical library consisting of ~90,000 compounds. The goals of this thesis are to develop suitable methods for evaluating the identified compounds *in vivo*, elucidate the species-selectivity of tested compounds, and prepare DNA constructs to broaden the spectrum of insect species in which the compounds will be tested.

1.2   Juvenile hormone and its signaling

Juvenile hormone (JH) is one of the two main lipophilic players regulating insect metamorphosis. Together with ecdysone, whose active form is 20-hydroecdysone (20E), JH regulates the main event in insect development. While 20E causes insect larvae to molt and metamorphose, the presence of JH modifies the effect of 20E by maintaining the larval status (Jindra 2019; Jindra et al. 2015a; Konopova et al. 2011; Riddiford 1994; Wigglesworth 1958). Despite both being lipophilic, their chemical structures differ substantially as 20E is a steroid and JH has sesquiterpenoid nature (Fig. 1). Hormones with this unique sesquiterpenoid structure are essential for arthropods and they are not known to play any role in vertebrates. This makes JH a promising tool for the control of insects.

JH is produced by the corpora allata, a pair of endocrine glands located near the insect brain (Jindra 2019; Riddiford 2020). In the haemolymph, juvenile hormone binding proteins transport the JH to the target tissues, however the mechanism of transfer through the cell membrane is not fully elucidated (Dupas et al. 2020; Suzuki et al. 2011).

An important step in the research of JH signaling was the discovery of the JH receptor (Charles et al. 2011; Konopova and Jindra 2007). Using RNA interference (RNAi) in the red flour beetle *Tribolium castaneum*, it was demonstrated that the absence of gene *Methoprene-tolerant* (*Met*), originally identified in 1986 (Wilson and Fabian 1986), has an effect similar to the absence of JH – precocious metamorphosis. Consequently, it was shown that in the fruit fly *Drosophila melanogaster*, a paralogous gene *germ cell-expressed* (*gce*) is functionally redundant with *Met* (Abdou et al. 2011; Atchley et al. 1997; Wang et al. 2007). Thus, expression of either *Met* or *gce*

is sufficient for *D. melanogaster* to develop normally (Abdou et al. 2011; Jindra et al. 2015b). Although the orthologs in other insect species are named *Met*, the *gce* appears to be the ancestral gene that duplicated in derived flies (Baumann et al. 2010b). Except for lepidopterans, which possess two paralogs of *Met*, (Kayukawa and Shinoda 2015) only a single ortholog of *Met/gce* is found within most insect species.

The protein structure of Met implies its molecular behaviour. Being a member of the basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) family of transcription factors, Met possesses one bHLH and two PAS (PAS-A and PAS-B) domains (Fig. 2A) (Ashok et al. 1998; Godlewski et al. 2006; Charles et al. 2011; Li et al. 2011). bHLH-PAS proteins are known to dimerize to form either homodimers or heterodimers with other bHLH-PAS proteins (Partch and Gardner 2010). In absence of JH, Met forms Met-Met dimers or Gce-Met dimers (in *D. melanogaster*) (Godlewski et al. 2006). This dimerization is diminished by the binding of JH or its mimics to the PAS-B domain (Charles et al. 2011; Godlewski et al. 2006). It was shown that Met/Gce contains nuclear localization signals and with the aid of Hsp90 (insect homolog Hsp83) and nucleoporin Nup358, Met/Gce is transported to the cell nucleus in JH dependent manner (Greb-Markiewicz et al. 2015; Greb-Markiewicz et al. 2011; He et al. 2017; He et al. 2014; Liu et al. 2013).

In the cell nucleus, Met/Gce forms a heterodimer with another bHLH-PAS protein Taiman (Tai), creating a JH receptor (JHR) complex (Kayukawa et al. 2012; Li et al. 2014; Li et al. 2011; Lozano et al. 2014; Miyakawa et al. 2013). Tai is also called βFtz-F1-interacting steroid receptor coactivator (FISC) or steroid receptor coactivator (SRC). For clarity, I will use Tai as the prevailing term in literature. Tai contains a single bHLH and two PAS domains. In the presence of JH, bHLH domains of both Tai and Met/Gce compose a bipartite DNA-binding domain which associates with specific JH response elements (JHREs) (Charles et al. 2011; He et al. 2014; Jindra et al. 2015b; Li et al. 2011; Zhang et al. 2011). The JHREs have been localised upstream of JH-inducible genes *Krüppel-homolog 1* (*Kr-h1*) and *early trypsin* (He et al. 2014; Kayukawa et al. 2012; Li et al. 2014; Li et al. 2011; Minakuchi et al. 2009; Minakuchi et al. 2008; Noriega et al. 1997; Zhang et al. 2011; Zhu et al. 2010). The JHREs contain conserved sequence important for activation of transcription of JH-target genes. In case of *Kr-h1* of various insect species including *D. melanogaster*, *T. castaneum*, and *A. mellifera*, it was shown that the conserved sequence corresponds to E-box palindrome CACGTG (Kayukawa et al. 2013; Kayukawa et al. 2012). The

JHRE1 of early trypsin gene from *A. aegypti* contains conserved E-box-like imperfect palindrome CACGCG (Li et al. 2014; Li et al. 2011).

In JH signaling, Kr-h1 was shown to play a key role in preventing metamorphosis. Induction of *Kr-h1* by treatment with JH mimics pyriproxyfen and methoprene disrupted adult development of *D. melanogaster* and *T. castaneum*, respectively (Minakuchi et al. 2009; Minakuchi et al. 2008). Subsequently, Kr-h1 was shown to be an effector in preventing metamorphosis in hemimetabolous *Pyrrhocoris apterus* and *Blattella germanica* (Konopova et al. 2011; Lozano and Belles 2011). RNAi of *Kr-h1* in last-instar larvae caused precocious metamorphosis, phenotype identical to that provoked by RNAi of *Met* in *P. apterus* and *T. castaneum* (Konopova et al. 2011; Konopova and Jindra 2007; Parthasarathy et al. 2008). These data suggest that the absence of Kr-h1 is a prerequisite for metamorphosis which was corroborated by studies in silkworm *Bombyx mori* (Kayukawa et al. 2017; Kayukawa et al. 2014; Kayukawa et al. 2012). Therefore, in hemimetabolous last-instar larva and holometabolous pupal stage, the level of *Kr-h1* plummets almost to zero and metamorphosis can proceed, however, the level of *Met* does not change dramatically (Belles 2020; Belles and Santos 2014; Jindra 2019; Konopova et al. 2011; Lozano and Belles 2011; Parthasarathy et al. 2008; Riddiford 2020; Truman and Riddiford 2019).

Kr-h1 acts as a repressor of 20E target gene *E93*. The protein structure of Kr-h1 involves a DNA-binding domain with eight zinc fingers (Pecasse et al. 2000). Employing electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP), the Kr-h1 binding site was identified in the upstream region of *B. mori E93* (*BmE93*), immediately next to the ecdysone response element. Therefore, one could think that binding of BmKr-h1 to its binding site represses the transcription of *BmE93*. However, it was proven that it is the C-terminal conserved domain of BmKr-h1 that inhibits *BmE93* transcription, not the DNA binding alone (Kayukawa et al. 2017). E93 is a helix-turn-helix transcription factor containing the Pipsqueak motif (Baehrecke and Thummel 1995). Called adult-specifier, *E93* plays a key role in the transition to the adult stage in both hemimetabolous and holometabolous species (Ureña et al. 2014). Reciprocally, *E93* represses *Kr-h1* in order for metamorphosis to proceed normally (Belles 2020; Belles and Santos 2014; Jindra 2019; Jindra et al. 2015a).

1.3 Structural variability of JHR ligands

1.3.1 Juvenile hormone native homologs

The JH receptor proteins Met/Gce can bind a broad repertoire of structurally distinct compounds. As mentioned above, Met/Gce is composed of bHLH motif near the N-terminus and two PAS domains towards the C-terminus, where the PAS-B domain is responsible for ligand binding (Ashok et al. 1998; Charles et al. 2011). Despite JH is the natural ligand of Met/Gce, different structural homologs of JH have been found in distinct insect taxa. The main crustacean JH, methyl farnesoate (MF) is a precursor in the synthesis of JH and is composed of farnesol backbone but lacks epoxide moiety (Cusson et al. 1991). Whereas JH 0, JH I, and JH II (Fig. 1) are found only within Lepidoptera, JH III prevails in most insect species (Cusson et al. 1991; Jindra et al. 2015a; Riddiford 2020). JH III bisepoxide (Fig. 1) was identified in *D. melanogaster*, together with MF and JH III (Richard et al. 1989; Wen et al. 2015). Recently, *in vitro* binding competitive assays based on the tritiated ligand ($[10-^3H(N)]$-JH III) showed that Gce is able to bind MF with moderate affinity, however relative to JH III, the affinity is about 8-fold lower (Bittova et al. 2019; Jindra et al. 2015b). The original JH discovered by Wigglesworth in *Rhodnius prolixus* (Wigglesworth 1958) was not identified until 2020 when it was shown that it is JH III skipped bisepoxide (Villalobos-Sambucaro et al. 2020). JH III skipped bisepoxide is prevalent within the Hemiptera order (Matsumoto et al. 2021). The biological significance of structural variability of the native JH homologues in individual insect taxa is unclear.
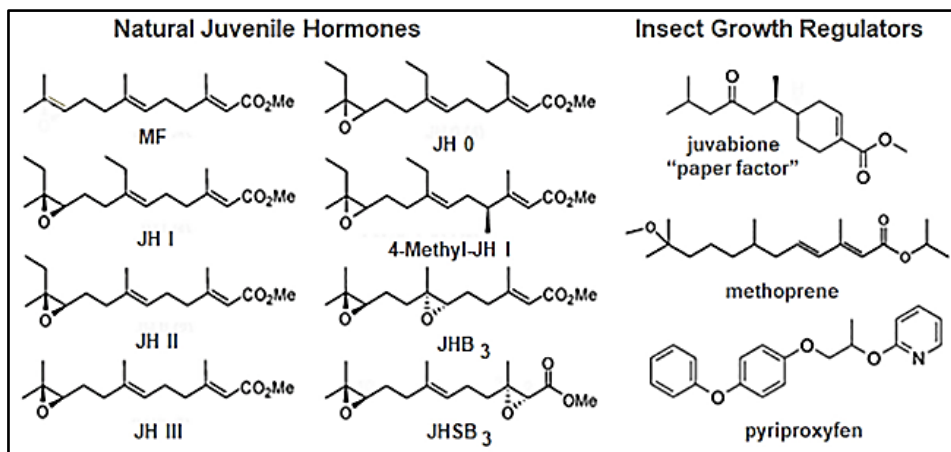

Figure 1: Structures of natural JHs and JH analogues called insect growth regulators (IGRs). MF, methyl farnesoate; JHB$_3$, JH bisepoxide; JHSB$_3$, JH skipped bisepoxide. Adapted from (Riddiford 2020).

1.3.2 Insect growth regulators – established ligands of JHR

Remarkable serendipity led to the discovery of JH-mimicking compounds structurally different from JH. When moving to Boston, Dr. Sláma noticed that *P. apterus* reared in North America did not undergo metamorphosis but molted into supernumerary 6th larval instar or adultoid that combined larval and adult traits. The source of the effect was found to originate from paper manufactured from American balsam fir. Since the chemical nature was unknown it was called "paper factor" (Sláma and Williams 1965). Later, the paper factor was chemically identified and called juvabione (Fig. 1) (Bowers et al. 1966). Juvabione is unique in its selectivity for the Pyrrhocoridae family leaving more evolutionarily distant species unaffected (Sláma 1971). Besides natural JHs and juvabione, there is a number of potent JH mimics, known as insect growth regulators (IGR) (Fig. 1).

These biologically active compounds predate the discovery of the JHR gene *Methoprene-tolerant* which was named after the widely used insecticide methoprene, introduced in 1973 by Zoecon. Another IGR is pyriproxyfen that was introduced in 1984 by Sumitomo. Unlike methoprene, pyridine-based pyriproxyfen has a structure unrelated to the JHs (Fig. 1). Another IGR structurally unrelated to JH is a carbamate derivative fenoxycarb (not shown) introduced in 1981 by Maag (Ramaseshadri et al. 2012). Although the topical treatment by these IGRs produced phenotypes seen after treatments by JHs (Grenier and Grenier 1993; Minakuchi et al. 2009; Minakuchi et al. 2008; Ramaseshadri et al. 2012; Riddiford and Ashburner 1991; Wilson and Fabian 1986), the evidence that they function as JH ligands was missing. Recent *in vitro* binding studies showed that methoprene, pyriproxyfen, and fenoxycarb are true agonist ligands of Met/Gce of *T. castaneum* and *D. melanogaster* (Bittova et al. 2019; Charles et al. 2011; Jindra et al. 2015b; Miura et al. 2005). Pyriproxyfen and fenoxycarb even surpassed the affinity of natural JH III to the Gce protein (Bittova et al. 2019). Juvabione shows that JH mimics can be selective for individual insect taxa. To design such compounds, it is essential to know the properties ligand-binding site of JHR.

Even though the precise crystallographic structure of the whole Met/Gce or its PAS-B domain remains to be solved, *in silico* models could be developed based on homology to the nuclear magnetic resonance (NMR) and crystal structures of hypoxia-inducible factor-2α PAS-B domain (Erbel et al. 2003; Scheuermann et al. 2009). Using ligand-docking to such models, a number of residues have been shown to interact with bound ligand. In *T. castaneum*, mutation

T254Y, V280F, V297F, T330Y, and C347M (highlighted in Fig. 2B) substantially diminished the ligand binding by Met (Charles et al. 2011). Corresponding residues were mutated in Met of the yellow fever mosquito *Aedes aegypti* with equivalent results, suggesting the vital role of these amino acids in ligand binding (Li et al. 2014). The importance of these residues was corroborated in a bioassay with *D. melanogaster*. When fed with methoprene, Met^{27} null mutant flies are abnormally insensitive to deleterious methoprene effect, however, expression of transgenic gce^{+} is sufficient to reinstate the methoprene sensitivity (Baumann et al. 2010a). Indeed, the methoprene sensitivity was restored by expressing transgenic gce^{+}, but not by its mutated variants Gce^{T272Y}, Gce^{V315F}, and Gce^{C366M} (Jindra et al. 2015b). These results are consistent with findings described previously for *T. castaneum* and *A. aegypti* Met (Charles et al. 2011; Li et al. 2014).
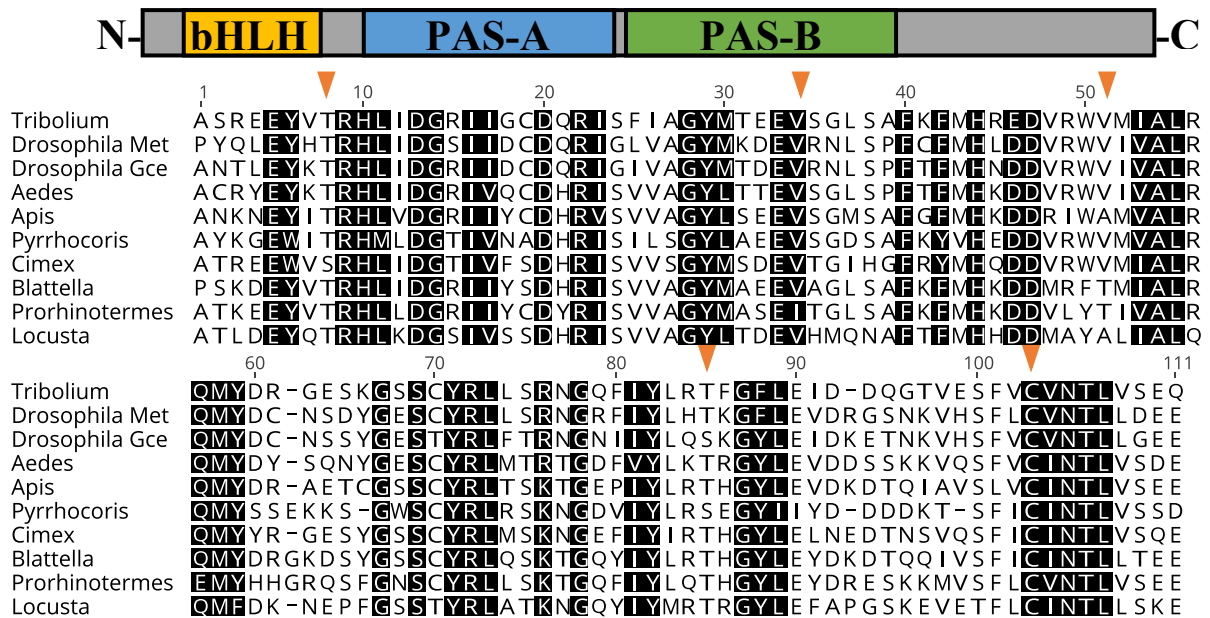
Figure 2: Protein domains of Met and alignment of its PAS-B domain across insect species. Top: Conserved domains of TcMet and their positions, adapted from (Charles et al. 2011). Bottom: Alignment of PAS-B domains in Met/Gce of different insect species. *T. castaneum* sequence (aa 247-355) was used as a query. Accession numbers of reference sequences are in Tab. 2. Only positions with 100% similarity (BLOSUM62, threshold = 2) are highlighted. Orange arrowheads mark residues vital for JH binding, in TcMet: T254, V280, V297, T330, and C347 (Charles et al. 2011; Jindra et al. 2015b; Li et al. 2014).

1.4 Methods for screening and verifying new JH agonists

Recent progress in the understanding of JH signaling enabled methods suitable to search for new agonist ligands of Met/Gce. In connection with automated platforms, the systems can be highly effective in screening through a large number of candidate compounds. In the following paragraphs, I will present two cell-based assays used in our project to discover and validate new putative JH agonists. The third paragraph will describe an *in vitro* assay which examines the binding of the putative JH agonists by Met/Gce proteins.

1.4.1 JHRE-luc reporter assay

The JHRE-luc reporter assay uses a DNA construct composed of enhancer (JHRE), promoter, and a reporter gene, luciferase (Fig. 3). This assay measures the ability of a tested compound to trigger the transcription of the reporter gene via JH signaling. The resulting signal is produced by the luciferase enzyme, whose activity is easy to quantify. When using heterologous cell line, such as human HEK293 cells, in addition to reporter plasmid, the components of JHR (*Met/Gce* and Tai) must be co-transfected (Kayukawa et al. 2012; Kayukawa and Shinoda 2015; Yokoi et al. 2020). Our approach exploits insect cells that endogenously express Gce/Met and Tai and therefore only the reporter construct is transfected (Bittova et al. 2019; Kayukawa et al. 2021; Kayukawa et al. 2012; Li et al. 2011).

For the JHRE-luc reporter assay used in the HTS that generated candidate JH agonists tested in this study, the *D. melanogaster* Kc167 embryonic cell line was chosen. The reporter DNA construct containing eight copies of JHRE and basal promoter (both from *A. aegypti early trypsin* gene) was inserted into the pGL4.17 vector (Figure 12) upstream of the firefly (*Photinus pyralis*) *luc2* gene. The pGL4.17 encodes resistance to neomycin which was used in the selection of stable line of Kc167 cells containing the reporter plasmid. For negative control, mutJHRE-luc reporter deviating by each nucleotide in the E-box-like sequence (Fig. 3) was cloned and selected identically as JHRE-luc reporter. The mutJHRE-luc reporter was shown inefficient in triggering the transcription in a JH-dependent manner (Jindra et al. 2015b).
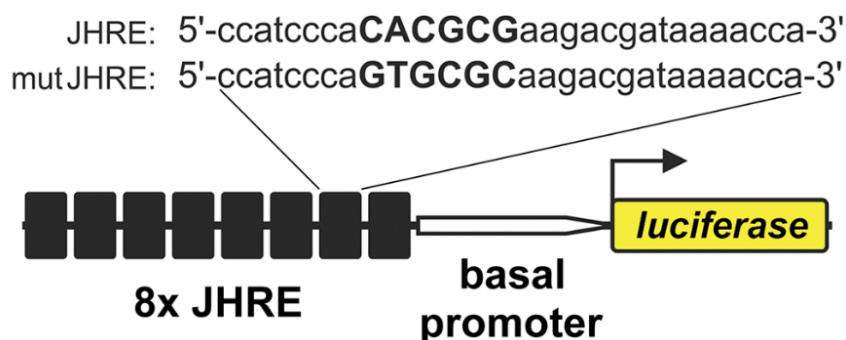
Figure 3: Scheme of JHRE-luc reporter and its mutated form. Eight copies of JHRE containing key E-box-like imperfect palindrome from *early-trypsin* gene of *A. aegypti* together with the basal promoter and firefly (*Photinus pyralis*) *luc2* gene creates reporter DNA construct. Mutated E-box-like sequence (mutJHRE) was generated by mutating each nucleotide. Adapted from (Jindra et al. 2015b).

1.4.2 Two-hybrid assay

Recently, the two-hybrid assay was developed to measure ligand-binding dependent interaction of Met/Gce and Tai proteins in human cell lines such as HEK293 (Bittova et al. 2019; Miyakawa and Iguchi 2017). Based on CheckMate Mammalian Two-Hybrid system (Promega), the assay employs fusion proteins VP16-Met/Gce in pACT plasmid (Promega) and GAL4-Tai in pBIND plasmid (Promega). The ligand binding triggers the heterodimerization of Met/Gce with Tai while activating transcription of the luciferase gene in reporter plasmid through GAL4 and VP16 by binding to UAS and TATA-box sequences, respectively. Therefore, the signal intensity corresponds to the ability of tested JH agonists to induce the interaction of fusion proteins VP16-Met/Gce and GAL4-Tai. Additionally, the pBIND plasmid encodes the second luciferase from *Renilla reniformis*. This luciferase is expressed independently of the JH agonist and is used for normalization to account for differences in the amount of live cells and other variables in the assay. However, the *Renilla* luciferase interferes with Nano-Glo Luciferase Assay System (Promega) used in HTS. Thus, to select stable cell lines for HTS, we used the pcDNA3.1 plasmid (Fig. 15), instead of pBIND. The two-hybrid assay can be used not only for unbiased screening for JH agonists of any insect species but also for validation of JH agonist activity.

1.4.3 [³H]JH III binding assay

In order to validate candidate compounds as true JHR agonist ligands, the JH-binding assay with tritium-labelled JH III represents a key experiment and was used in numerous studies

(Bittova et al. 2019; Charles et al. 2011; Jindra et al. 2015b; Miura et al. 2005). It is based on incubation of $[^3H]$JH III with *in vitro* translated Met/Gce and absorption of free $[^3H]$JH III by dextran-coated charcoal. Subsequently, the radioactivity of $[^3H]$JH III bound to the Met/Gce protein is measured by scintillation counter. However, this approach requires the tritium-labelled ligand. This requirement can be bypassed by a competition assay, in which the constant amount of $[^3H]$JH III compete with the tested compound for Met/Gce binding (Bittova et al. 2019; Charles et al. 2011). Provided that the tested compound is a true agonist, one can expect that increasing concentration of the tested compound will decrease the radioactive signal produced by $[^3H]$JH III as the two ligands compete for Met/Gce binding. Therefore, this method can be used either as proof of JH-binding ability or for validation of potential new JHR ligands.

1.4.4 The pipeline of HTS searching for novel JHR agonist

The JHRE-luc reporter assay has been adapted for HTS, which provides excellent precision and enables to screen library as large as almost 90,000 compounds with drug-like properties. This part was conducted in the collaborating group led by Dr. David Sedlák using CZ-OPENSCREEN facility in Prague. The JH agonists tested in this thesis were selected via an approach described in following paragraphs (Fig. 4).

The selection of tested agonists was based on *D. melanogaster* JHRE-luc reporter assay described above. The whole library consisting of almost 90 thousand compounds was screened in duplicates for JH-activity with 10 µM concentration using 1536-well plates. This first round of HTS produced 842 primary hits (Fig. 4). These compounds were subjected to two rounds of validation in which the reactions were measured as triplicates in each round. Since the mutJHRE should not produce a JH-dependent signal, the compounds were tested with mutJHRE for false-positive results from the first round. Also, the dose-response of agonists was measured in order to evaluate the potential of the agonists by determining the half-maximal effective concentration (EC_{50}). To see potential toxicity of the tested compounds, cell viability was measured based on free ATP released by dead cells to the medium. These two confirmational rounds produced 141 confirmed hits. In the next round, fresh stocks of compounds were used as a prevention from

degradation side effects. The dose-response was tested in concentration range from 0.2 nM - 50 μM using both JHRE-luc reporter and mutJHRE-luc reporter. Possible activators of the firefly luciferase used in the JHRE-luc reporter were filtered out by counter screening with JHRE-luc reporter using NanoLuc (Nano-Glo, Promega), whose substrate and properties differ from the firefly luciferase (Fig. 4)

As next, the primary unbiased HTS will be repeated while employing two-hybrid assay with Met and Tai proteins from different insect species. This step promises to find novel JH agonists that would exhibit species selectivity as the existing JH agonists lack such selectivity.

Following the HTS, the compounds were examined in our laboratory by various methods. Cell-based assays were used to verify JH activity, species-selectivity, and Met/Gce binding. *In vivo* experiments using distinct insect species were conducted to validate the effect of tested compounds on live animals and the JH-activity was corroborated by measuring JH-target gene *Kr-h1* induction. Although the HTS eventually yielded 132 validated JH agonists, only 15 were used in follow-up methods for evaluating their activity (Fig. 5). For clarity, the 15 selected JH agonists will be referred to as JHag(1-15).
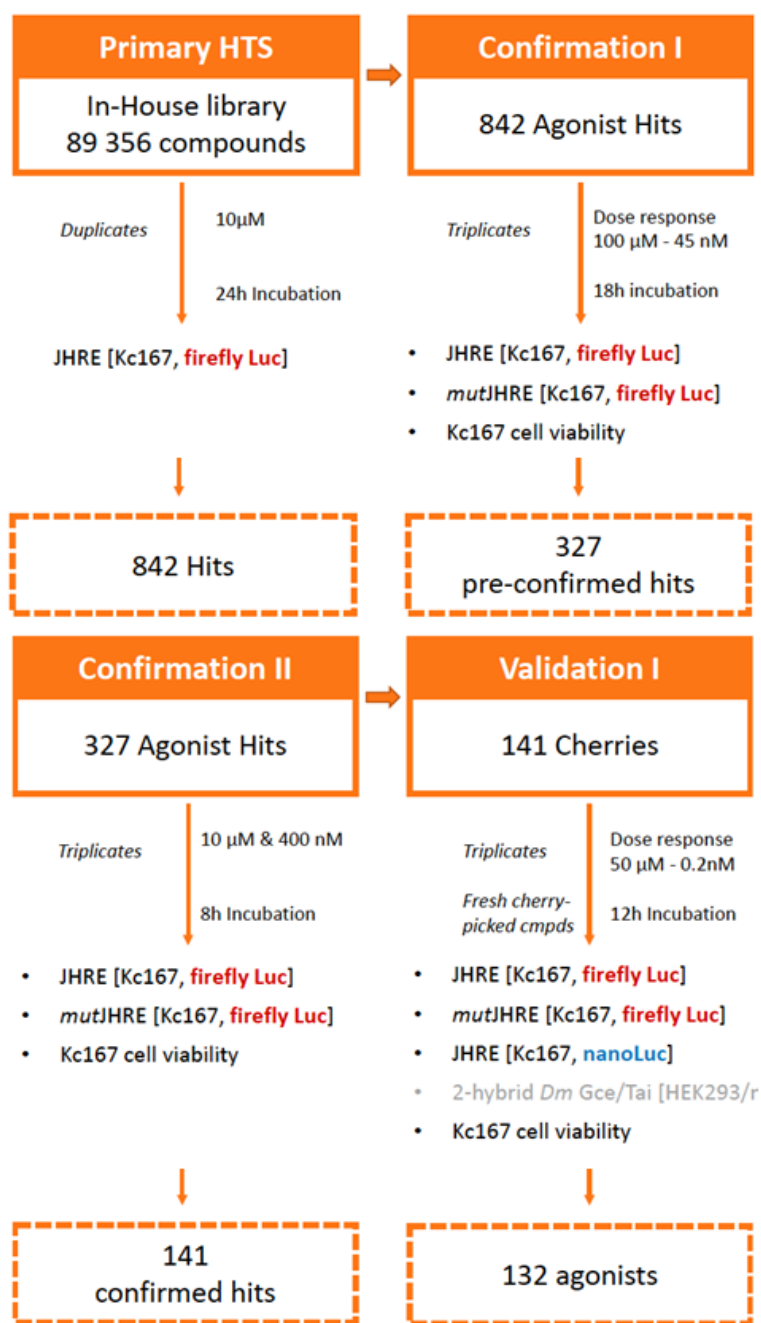
Figure 4: Pipeline of HTS showing individual steps in searching forn novel JH agonists. The figure was provided by Dr. David Sedlák.
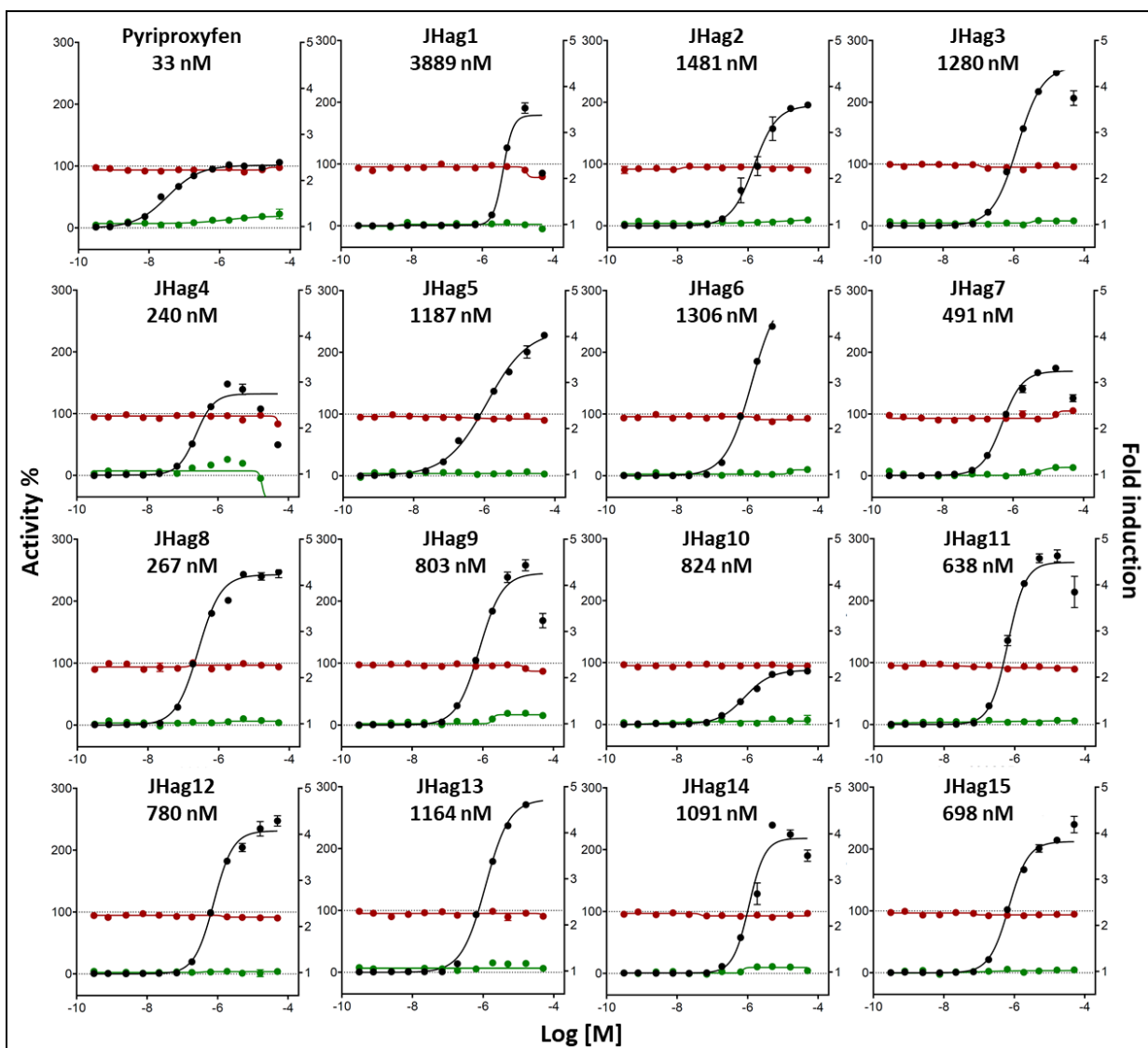
Figure 5: **High throughput screening employing JHRE-luc reporter assay discovered potential JH agonists.** The EC$_{50}$ values are indicated below the agonist names. Black data points represent % of JHRE-luc activity induced by tested agonists relative to maximal activity achieved with the reference compound and established JHR agonist pyriproxyfen (top row, left). The activity of mutJHRE (green) is plotted as fold-change relative to solvent-only values. The red data points represent cell viability. The data were provided by Dr. David Sedlák.

The aims of the thesis:

- To test the selected JH agonists for JH effects *in vivo* using different insect species.

- To determine whether the compounds disrupt insect development via JH signaling.

- To confirm selectivity of the compounds for different insect species.

- To prepare reagents for testing new potential JH agonists in additional insect systems.

2 METHODS

2.1 *T. castaneum* - keeping and treatments

Vermilion white strain of *Tribolium castaneum*, was reared in constant darkness at the temperature of 32°C. Beetles were fed with whole wheat flour with 5% of yeast. Stock populations were kept in plastic boxes, whereas approximately 50 individuals were separated into 60-mm Petri dishes for breeding. In order to produce a synchronised population, adults were moved into a new Petri dish daily. For JH agonist treatment, freshly molted pupae were collected every 12 hours. To complete the pupal cuticle development, pupae were kept in an incubator for another 12 hours. Afterwards, they were washed with water to remove excess flour, dried on a paper tissue, and on glass cover slip, topically treated with 0.6 µL of JH agonist, S-methoprene (VUOS Pardubice, Czech Republic), or control DMSO (Honeywell) in acetone (Sigma-Aldrich, Merck) using IM-9B microinjector (Narishige) as a dispenser. Phenotypes were evaluated after approximately 5 days. For RT-qPCR, 3 pupae from each sample were frozen in liquid nitrogen 6 hours post treatment and stored at -80°C.

2.2 *P. apterus* - keeping and treatments

Short-winged form of *Pyrrhocoris apterus,* a strain isolated in Oldřichovec (Czech Republic), was reared in long-day conditions – 16 hours of light, 8 hours of darkness. At a constant temperature of 25°C, bugs were kept in jars and were provided with dry linden seeds, water, and filter paper for cover. Individuals in larval instar 5 (L5) were collected for treatment on a daily basis. Collected larvae were anaesthetized by CO_2 and topically treated with 3 µL of JH agonist, S-methoprene, or control DMSO in acetone. After 10 days, animals were visual inspected and their phenotypes were documented and photographed.

2.3 *D. melanogaster* – keeping and treatments

Strains of *Drosophila melanogaster* were reared at a constant temperature of 26°C with a natural dark/light period. In glass vials, on diet composed of 1% agar, 6.25% cornmeal, 6.25% molasses, and 3.5% yeast, capped with cotton plugs. The *white* (w^{1118}) strain was used as a "wild-type" control stock for compound testing. To increase susceptibility to JH agonists, ubiquitous expression of transgenic Gce was induced using the Gal4-UAS system (Jindra et al. 2015b). Homozygous *arm-Gal4* females were mated with *UAS-gce* males for 24 hours and then were

transferred to another vial. Subsequently, their progeny was used for the treatments. To test JH agonists, 10 mM JH agonists or S-methoprene in DMSO were diluted in ethanol (VWR) to appropriate concentration and 150 µL of JH agonists, S-methoprene, or DMSO was applied onto food with 3rd instar larvae. For RT-qPCR, 3 white puparia were treated with 0.2 µL of 0.2 mM JH agonists or control and 24 hours post treatment were frozen in liquid nitrogen and stored at -80°C.

2.4 RNA isolation

Collected samples for RNA isolation were frozen in liquid nitrogen and stored at -80°C in 1.5 mL Eppendorf tubes. After homogenization with a plastic pestle in 1 mL of TRIzol reagent (Ambion), brief vortexing and incubation at room temperature (RT) for 5 min, 200 µL of chloroform (Sigma-Aldrich, Merck) was added and the samples were hand-shaken for 20 s. Then followed incubation at RT for 3 min and centrifugation at 12,000g for 15 min at 4°C. 500 µL of the aqueous phase was transferred into a fresh 1.5 mL tube and 450 µL of isopropyl alcohol (Lachner) was added. Incubated at RT for 10 min, samples were centrifuged at 12,000g for 10 min at 4°C and almost all supernatant was aspired. The remaining supernatant was removed after a brief spin in a table-top centrifuge. Immediately, 1 mL of chilled (-20°C) 75% ethanol was added and the samples were centrifuged at 7500g for 5 min at 4°C. Then the supernatant was removed identically as in the previous step and the RNA pellet was resuspended in 40 µL of nuclease-free water (Ambion). The concentration and purity of RNA were measured using NanoDrop 2000 spectrometer (ThermoFisher).

2.5 cDNA synthesis

For the synthesis of first-strand cDNA, the Superscript III system (ThermoFisher) was employed. In order to anneal oligo(dT)$_{18}$ primers, 12 µL of mixture including 2 µg of total RNA, 1 µL of 10 mM dNTPs, 1 µL oligo(dT)$_{18}$ primers, and nuclease-free water was incubated at 65°C for 5 min, chilled on ice. Premix comprising 2 µL of nuclease-free water, 4 µL of 5x First-strand buffer, 1 µL of 0.1M dithiothreitol (DTT), 1 µL of RNase OUT, and 1 µL of SuperScript III was prepared and added to the mixture containing DNA and primers. Gently mixed by pipetting, the whole 20 µL reaction was incubated at 50°C for 50 min. Eventually, the reaction was heat-inactivated at 70°C for 15 min.

2.6 RT-qPCR

To confirm the effect of JH agonist the relative expression of JH response gene *Krüppel-holomog* 1 mRNA was analysed. For this purpose, the total RNA was isolated as mentioned above, however, it was necessary to carry out DNase treatment to remove DNA from the samples. Therefore, the premix for DNase treatment comprising 14 µL of nuclease-free water, 6 µL of 10x Turbo DNase buffer, and 1 µL of Turbo DNase (all from Ambion) was added and the samples were mixed by pipetting up and down. After 30-min incubation at 37°C, 60 µL of phenol (Sigma-Aldrich, Merck):chloroform:isoamyl alcohol (Sigma-Aldrich, Merck) (25:25:1) was added. Samples were shaken by hand for 20 sec and centrifuged at 12,000g for 10 min at 4°C and 50 µL of the aqueous phase was transferred into a fresh 1.5 mL tube. Then the samples were extracted with another 50 µL of chloroform:isoamyl alcohol (50:1) and 40 µL of the aqueous phase was transferred into a new tube. To precipitate RNA, 4 µL of 3M Na-acetate (pH 5.2) was added and the samples were washed in 110 µL of chilled (-20°C) absolute ethanol overnight at -20°C. Finally, the samples were centrifuged at 12,000g for 30 min at 4°C, ethanol was removed as mentioned above and the RNA pellet was resuspended in 20 µL of nuclease-free water. After the cDNA was synthesized, it was diluted 20-fold. The 14 µL RT-qPCR reaction was mixed from 7 µL 2x qPCR SYBR Master Mix (Top-Bio), 3 µL of 20-fold diluted cDNA, 0.35 µL + 0.35 µL of 10µM gene-specific primers (Table 1) and 3.3 µL of PCR ultra H_2O (Top-Bio). Samples were measured either in technical triplicates (*T. castaneum/D. melanogaster*) or in two biological replicates and two technical replicates (*P. apterus*). RT-qPCR was conducted using 96-well plates covered with Microseal 'B' seal and 96-well Optical Pad in CFX Connect Real-Time System (all Bio-Rad). Obtained Quantification Cycle (Cq) values for *Krüppel-homolog 1* were standardized relative to Cq values of Rp49.

RT-qPCR used the following protocol:

95°C for 3 min

95°C for 30 s

58°C for 30 s (*T. castaneum/D. melanogaster*), 59°C for 30 s (*P. apterus*)   ⎤
                                                                                  ⎬ 40 cycles
72°C for 30 s   ⎦

Fluorescence detection

Melt curve analysis 65-95°C, increment 0.5°C/step

Table 1: Primers used for RT-qPCR.

Target mRNA	Forward primer 5'-3'	Reverse primer 5'-3'
TcKr-h1	GTTTGCTCCAAGGGGTTCACG	GGTTGTAGCCGAAGGATTTGCC
TcRp49	TTATGGCAAACTCAAACGCAAC	GGTAGCATGTGCTTCGTTTTG
PaKr-h1	CCCTACCAGTGTAACTTTTGC	GAACGTCTTGTTACACACACC
PaRp49	CCGATATGTAAAACTGAGGAGAAAC	GGAGCATGTGCCTGGTCTTTT
DmKr-h1α	CCGAATACGACATAACAGCC	CGATTTCCGTGAATATGTTCT
DmRp49	GAAGAAGCGCACCAAGCACT	CACGTTGTGCACCAGGAACT

2.7 Design of expression vectors

2.7.1 Two-hybrid assay

Constructs from *D. melanogaster* and *T. castaneum* were described previously in (Bittova et al. 2019; Miyakawa and Iguchi 2017). The sequences of *A. aegypti* (*Met, Tai*) *P. apterus* (*Met,Tai*), *Cimex lectularius* (*Met*), *Prorhinotermes simplex* (*Met,Tai*), and *A. mellifera* (*Met, Tai*) were commercially synthetized (GenScript) according to reference and length in (Tab. 2). BamHI and KpnI restriction sites were added to the 5' and 3' end, respectively. The restriction digest, separation of fragments on gel, purification, ligation and bacteria transformation followed the identical approach as described below.

In order to complete plasmid pairs or add other species, DNA templates for *Met* and *Tai* from *L. migratoria* were kindly provided by Dr. Shutang Zhou, live individuals of *C. lectularius* were kindly provided by Dr. Onrej Balvin, and live adults of *B. germanica* were taken from insectarium of Institute of Entomology, Biology Centre CAS. Isolation of total RNA and subsequent cDNA synthesis were done as described above. Appropriate DNA fragments were amplified in 150μL Phusion PCR comprising of 99 µL of nuclease-free water, 30 µL of 5x Phusion HF buffer (*LmTai*, *ClTai*, *BgTai*), or 5x Phusion CG buffer (*BgMet*, *LmMet*), 3 µL of 10 mM dNTPs, 7.5+7.5 µL of gene-specific primers (Tab. 3), 3 µL of template cDNA/DNA, and 2 µL of Phusion HF Polymerase (ThermoFischer). PCR run after following protocol:

98°C for 3 min

98°C for 30 s
55°C for 30 s ⎱ 40 cycles
72°C for 180 s

72°C for 10 min

After purification by QIAquick PCR & Gel Cleanup Kit (Qiagen), the PCR products were digested in reaction comprising of 27.5 µL of purified PCR products diluted in nuclease-free water, 3 µL of 10x Cutsmart buffer (NEB), 1+1 µL of respective HF restriction enzyme (NEB) (Tab. 3). Also, 3 µg of respective plasmid DNA was digested. The restriction digest reaction was incubated at 37°C for 150 min, then 1 µL of Shrimp alkaline phosphatase (NEB) was added to the vector for 30 min. Then the DNA was separated on 0.8% agarose gel in 1xTAE buffer, and desired bands were cut out and purified using QIAquick Gel Extraction Kit (Qiagen). Afterwards, ligation reaction was set up: 8 µL of insert DNA, 2 µL of vector DNA, 1.2 µL of 10x T4 ligase buffer, and 1 µL of T4 DNA ligase enzyme (both ThermoFisher). Incubated overnight, 6 µL of ligase reaction was added to 200 µL of competent cells and incubated on ice for 30 min. Heat shock at 42°C for 45 sec was used for transformation and 750 µL of LB medium was added to the cells. After 1 hour at 37°C of vigorous shaking, the cells were plated on LB-carbenicillin plates and incubated overnight. Plasmid isolation from bacteria colonies was done using GenElute Plasmid Miniprep Kit (Sigma-Aldrich, Merck).

Table 2: Summary of DNA constructs used in the two-hybrid assay. The gene length is counted from the N-terminus of the protein. Accession number indicates the template in NCBI.

Species	Gene	Accession number/source	Origin	Length (aa)	Plasmid
D. melanogaster	Gce	NP_001188593.1	Myiakawa and Iguchi 2017; Bittova et al 2019	769	pACT
	Tai	NP_001245949.1		740	pBIND
T. castaneum	Met	NP_001092812		452	pACT
	Tai	XM_008195407.2		418	pBIND
A. aegypti	Met	AY955097.1	Synthetic	596	pACT
	Tai	DQ469817.3	Synthetic	609	pBIND
P. apterus	Met	AEW22976.1 (and V. Smykal)	Synthetic	683	pACT
	Tai	AGI17570.1	Synthetic	413	pBIND
C. lectularius	Met	XM_024229424.1	Synthetic	503	pACT
	Tai	XM_014394537.2	Native	527	pBIND
P. simplex	Met iso1	Ondrej Luksan	Synthetic	517	pACT
	Tai	Ondrej Luksan	Synthetic	409	pBIND
A. mellifera	Met	XP_006568391.2	Synthetic	480	pACT
	Tai	XP_016772488.2	Synthetic	473	pBIND
B. germanica	Met	PSN54894.1, HG965209.1	Native	952	pACT
	Tai	HG965205.1	Native	524	pcDNA3.1
L. migratoria	Met	AHA42531.1	S. Zhou	989	pACT
	Tai	ANG56298.1	S. Zhou	529	pcDNA3.1

Table 3: Primers used for cloning. Primers contain sequence not exactly complementary to the template, which creates a restriction site.

Primer name	Position	Sequence 5'-3'	Restriction site
Bam-ClTai	Forward	AGTGGATCCGCGAGTGGTCAGTGCCTGATTG	BamHI
ClTai-Kpn1	Reverse	AAGGTACCTTAGGTAGGCGTGAGGGATGCTCG	KpnI
Sbf-BgTai	Forward	ATTCCTGCAGGGATGAGTGCAGCAGTCTCTGGA	SbfI
BgTai-Kpn	Reverse	GTAGGTACCAGGGGTAAGGCTCTGGCGAG	KpnI
Mlu-BgMet	Forward	ATAGACGCGTCACCAGCCATGTTGGATTGG	MluI
BgMet-Kpn2	Reverse	TATGGTACCTTACACGCCAACATCTTGGTTCATCC	KpnI
Bam-LmTai	Forward	AGAGGATCCCCATGTCAACAGTCATCGCTGA	BamHI
LmTai-Kpn	Reverse	TGGGGTACCCACAGGTGTCAAGCTCTGGCTC	KpnI
Mlu-LmMet	Forward	ATAGACGCGTTTATGATTAGCTGGGAGCACTA	MluI
LmMet-Kpn	Reverse	TATGGTACCGAGTTGTAGGTGTTTGCGTG	KpnI

2.7.2 JH-binding assay

To prepare appropriate expression vectors for the JH-binding assay, *PsMet* and *BgMet* were cloned into pK-Myc-C2 vector that provides Myc tag on N-terminus (Valenta et al. 2003). To that end, 2 µg of *BgMet* and *PsMet* in pACT (Tab. 2) and pK-Myc-C2, was digested in 30 µL reaction using SalI HF (*BgMet*) or BglII (*PsMet*) and KpnI HF (both) restriction enzymes and 10x CutSmart buffer (all NEB). After 4.5 hours of incubation at 37°C, 1 µL of Shrimp alkaline phosphatase (NEB) was added to the vector reaction and samples were incubated at 37°C for another 50 min. Afterwards, the fragment separation on the gel, purification, and ligation was done as in 2.7.1. After bacteria transformation, the cells were plated on LB-kanamycin plates and the colonies of bacteria were used for plasmid isolation using Plasmid Midi kit (Qiagen). Subsequently, the DNA was purified by phenol:chloroform:isoamyl alcohol extraction as described in 2.6. Nuclease-free water was used for resuspension of DNA pellet and the concentration was set to 500 ng/µL.

2.8   Mutagenesis of BgMet and PsMet

To create appropriate control for [^{3}H]JH III binding assay, *BgMet* and *PsMet* in pK-Myc-C2 were mutated in the critically important homologous residues T324Y and T316Y, respectively. The importance of the threonine for the ability of Met to bind JH was discovered previously (Charles et al. 2011; Jindra et al. 2015b; Li et al. 2014). To mutate the genes, specific primers

with an altered codon for threonine to tyrosine mutation were designed (Table 4). The Quikchange site-directed mutagenesis employed 25 µL PCR comprising of 16 µL of nuclease-free water, 5 µL of 5x Phusion buffer (ThermoFisher), 1 µL 10mM dNTPs, 1+1 µL of primers, 1 µL of DNA template (50 ng/µL), and Phusion HF DNA polymerase (ThermoFischer). The reverse primer was added after the 7th cycle of following protocol:

98°C for 3 min

98°C for 30 s

55°C for 30 s (PsMetT316Y), 50°C for 30 s (BgMetT324Y) ⎱ 18 cycles

72°C for 420 s

72°C for 10 min

      To digest methylated DNA, which contained the non-mutated version of genes, 1 µL of DpnI enzyme (NEB) was added and the reaction was incubated at 37°C for 1 hour. Afterwards, 10 µL of the reaction was added to 200 µL of competent cells and incubated on ice for 30 min. Heat shock at 42°C for 45 sec was used for transformation and 750 µL of LB medium was added to the cells. After 1 hour at 37°C of vigorous shaking, the cells were plated on LB-kanamycin plates and incubated overnight. For isolation of plasmids from bacteria, the GenElute Plasmid Miniprep kit (Sigma-Aldrich, Merck) was used. The resulting genes were verified by Sanger sequencing (SeqMe).

Table 4: Primers used for mutagenesis

Primer name	Primer sequence 5'-3'
QC_BgMet_T324Y_F	CCAAGCAAAGATGAGTATGTGTATCGCCACCTCATCGATG
QC_BgMet_T324Y_R	CATCGATGAGGTGGCGATACACATACTCATCTTTGCTTGG
QC_PsMet_T316Y_F	CCACAAAGGAAGAGTACGTGTACAGACACCTGCTGGACGG
QC_PsMet_T316Y_R	CCGTCCAGCAGGTGTCTGTACACGTACTCTTCCTTTGTGG

2.9 JH-binding assay

      The JH-binding and competition assays were performed using the dextran-coated charcoal method as previously described (Charles et al. 2011; Jindra et al. 2015b). For JH-binding assays, proteins in pK-Myc-C2 plasmid were produced *in vitro* using the rabbit reticulocyte lysate TnT Quick T7 Coupled transcription/translation system (Promega). For one 50-µL reaction, 500 ng

($BgMet/BgMet^{T324Y}$) or 1000 ng ($PsMet/PsMet^{T316Y}$) of template plasmid was used. After 90 min at 30°C, the reaction was divided into 15-µL aliquots, making three replicates. In all assays, the the rabbit reticulocyte lysate without DNA input was used as (mock) control.

Synthetized and kindly provided by Dr. Aleš Marek (IOCB, Prague), the 77.82 µM stock of [^{3}H]JH III (25.7 Ci/mmol) in toluene was diluted 100-fold in hexane. In polyethylene glycol (PEG)-coated vial, ~1.56 pmol (~89000 dpm) of [^{3}H]JH III diluted in hexane was evaporated under a gentle stream of nitrogen and dissolved in 85 µL of buffer C [20 mM Tris (pH 7.8), 5 mM magnesium acetate, 1 mM EDTA, 1 mM DTT]. The 15-µL protein aliquot was mixed with 85 µL aliquot of [^{3}H]JH III, mixed gently and incubated for 60 min at RT. Subsequently, 20 µL of dextran-coated charcoal suspension [10 mM Tris HCl (pH 7.5), 1.5 mM EDTA (pH 8), 1% dextran from *L. mesenteroides* (Sigma-Aldrich, Merck), 5% Norit A activated charcoal (Acros)] was added, gently vortexed and incubated for 2 min at RT. After 3 min of centrifugation at 12000g, the 100 µL of supernatant was added to 7 mL of scintillation liquid (PerkinElmer) for quantification.

In the competition assay, the 85 µL aliquot of [^{3}H]JH III in buffer C contained a constant amount of [^{3}H]JH III plus increasing input (0.003-3000 pmol) of the competitor JH III (Sigma-Aldrich). The radio-labelled and cold JH III were premixed before adding the protein input.

3   RESULTS

3.1   JHags selectively induce interaction of JHR subunits of different species *in vitro*

In order to validate the ability of selected JHags, discovered in the HTS, to activate JH signaling, we employed two-hybrid assay. In this assay, binding of a JH agonist to the Met/Gce protein induces interaction with the partner protein Tai. In this assay we were able to confirm that all of the 15 selected JHags induced the interaction between the *D. melanogaster* Gce-Tai JHR subunits to at least 30% relative to the level achieved with a reference compound, the synthetic JHR agonist S-methoprene in (Tab. 5). These data correspond well with the capacity of the individual JHags to trigger transcription in JHRE-luc reporter assay in the original HTS (Fig. 5).

To explore potential effects of the JHags on different species, we employed orthologues of Met-Tai from diverse insect species in two-hybrid assay. In order to cover a broad spectrum of insects we chose species representing distant orders, namely, hemimetabolous Hemiptera (*P. apterus*, *C. lectularius*) and Blattodea (*P. simplex*), and holometabolous Hymenoptera (*A. mellifera*), Coleoptera (*T. castaneum*), and Diptera (*D. melanogaster, A. aegypti*). The JHag's ability to induce Met-Tai interaction in two-hybrid assay was normalized to established JH agonists fenoxycarb (AmMet-Tai) or S-methoprene (all other species).

JHag8 was shown to be the most versatile compound as it activated interaction of 6 out of 7 examined Met/Gce-Tai pairs by ≥30% relative to the respective reference (Tab. 5). Conversely, JHag2, -3, -6, and -10 effectively activated (≥30%) interaction of only the DmGce-Tai pair, and thus may be considered selective for *D. melanogaster*. JHag2 was most active of the compounds selective for the *Drosophila* pair. JHag1, -7, -12, -13, and -15 were shown to effectively induce (≥30%) the interaction of Met/Gce-Tai pair of at least one but up to three insect species. This suggests they are potentially selective agonists for a narrower spectrum of species. These data from two-hybrid assay indicate that specific JHags obtained from the HTS could exhibit species selectivity.

JHag8, -9, and -11 were shown to stimulate (>30%) the Met/Gce-Tai interaction in 5 out of 7 examined protein pairs. Since those JHags induced the Met-Tai interaction of some species more than reference compound (S-methoprene/fenoxycarb), they might represent functional alternative to established JH agonists (Tab. 5).

Table 5: Relative ability of selected JH agonists to induce Gce/Met-Tai interaction from different species. To compare the individual experimental measurements, the baseline of solvent-only value (1) was subtracted from value of relative luciferase activity induced by 2 μM JHag. Subsequently, the excess value was standardized as % activity of relevant control (1 μM fenoxycarb for *A. mellifera*, 2 μM S-methoprene for the rest). For clarity, the color shade indicates the value of luciferase activity. The expression plasmids used for this experiment are listed in Tab. 2.

JH agonist	*A. aegypti* Met-Tai	*D. melanogaster* Gce-Tai	*T. castaneum* Met-Tai	*P. apterus* Met-Tai	*C. lectularius* Met-Tai	*P. simplex* Met-Tai	*A. mellifera* Met-Tai
Reference	100%	100%	100%	100%	100%	100%	100%
JHag1	117%	122%	1%	5%	-1%	33%	-4%
JHag2	0%	94%	4%	6%	9%	15%	-4%
JHag3	0%	64%	-2%	-6%	-3%	1%	-7%
JHag4	131%	119%	72%	27%	12%	137%	14%
JHag5	109%	76%	115%	2%	4%	50%	-5%
JHag6	11%	105%	17%	-2%	21%	6%	-6%
JHag7	2%	82%	37%	0%	-1%	39%	-3%
JHag8	128%	30%	150%	42%	1%	117%	102%
JHag9	62%	67%	38%	89%	4%	212%	-3%
JHag10	2%	89%	-1%	3%	-1%	6%	-7%
JHag11	18%	109%	83%	59%	2%	152%	35%
JHag12	84%	117%	44%	0%	0%	16%	-6%
JHag13	39%	80%	7%	8%	18%	9%	-3%
JHag14	2%	100%	58%	91%	9%	93%	5%
JHag15	77%	110%	48%	9%	1%	7%	-6%

3.2 Selected JH agonists disrupt insect development

3.2.1 In *D. melanogaster*, selected JHags mimics the effect of JH

To examine the effect on live animals, the three concentrations of JHags were incorporated into the food of w[1118] *D. melanogaster* 3rd instar larvae. The increased rate of pupal lethality was expected as a result of the JH agonist action described previously (Jindra et al. 2015b; Wilson and Fabian 1986). Approximately 5 days after pupariation, the dead pupae were counted. Whereas the dietary 1mM S-methoprene caused 100% lethality, only JHag2, JHag3, and JHag4 were able to exceed the average 10% at the lowest 1 mM concentration and 20% at the highest 5 mM

concentration (Fig. 6A). The most effective compound showed to be JHag4 (61%), albeit the 0.5 mM S-methoprene was more effective than a 10-fold higher concentration of JHag4.

Although some JHags showed to effectively mimic the effect of S-methoprene, to prove the specificity of the effect, the JHags were incorporated into the food of 3rd instar larvae overexpressing *gce*. Such larvae were previously shown to be more sensitive to the effect of JH agonists (Jindra et al. 2015b). As expected, 0.1 mM S-methoprene was sufficient to induce 100% pupal lethality (Fig. 6B). A notable increase in lethality rate was observed in the case of dietary JHag3 and JHag4 as they killed a substantial proportion of *arm>gce* pupae in comparison to the rest of the tested JHags.

In order to test the molecular effect of JHags on *D. melanogaster*, the white puparia were treated with 0.2 mM JHag, S-methoprene, or DMSO and the induction of JH-target gene *Kr-h1* was measured. Since in pupal stage the *Kr-h1α* mRNA level is almost undetectable, the treatment with JH analogue should induce a marked increase of *Kr-h1α* transcription (Minakuchi et al. 2008). Results consistent with the effect on pupal lethality were observed, as the pupae treated with S-methoprene, JHag3, and JHag4 showed higher levels of *Kr-h1α* mRNA than those treated with control solvent DMSO (Fig. 6C). Even though the induction of *Kr-h1α* caused by JHag3 and JHag4 was <3% relative to S-methoprene, it exceeded the effect of the other tested JHags.

In conclusion, however, JHag3 and JHag4 are the only active compounds, their activity was corroborated by results from both pupal lethality data and *Kr-h1α* induction data.
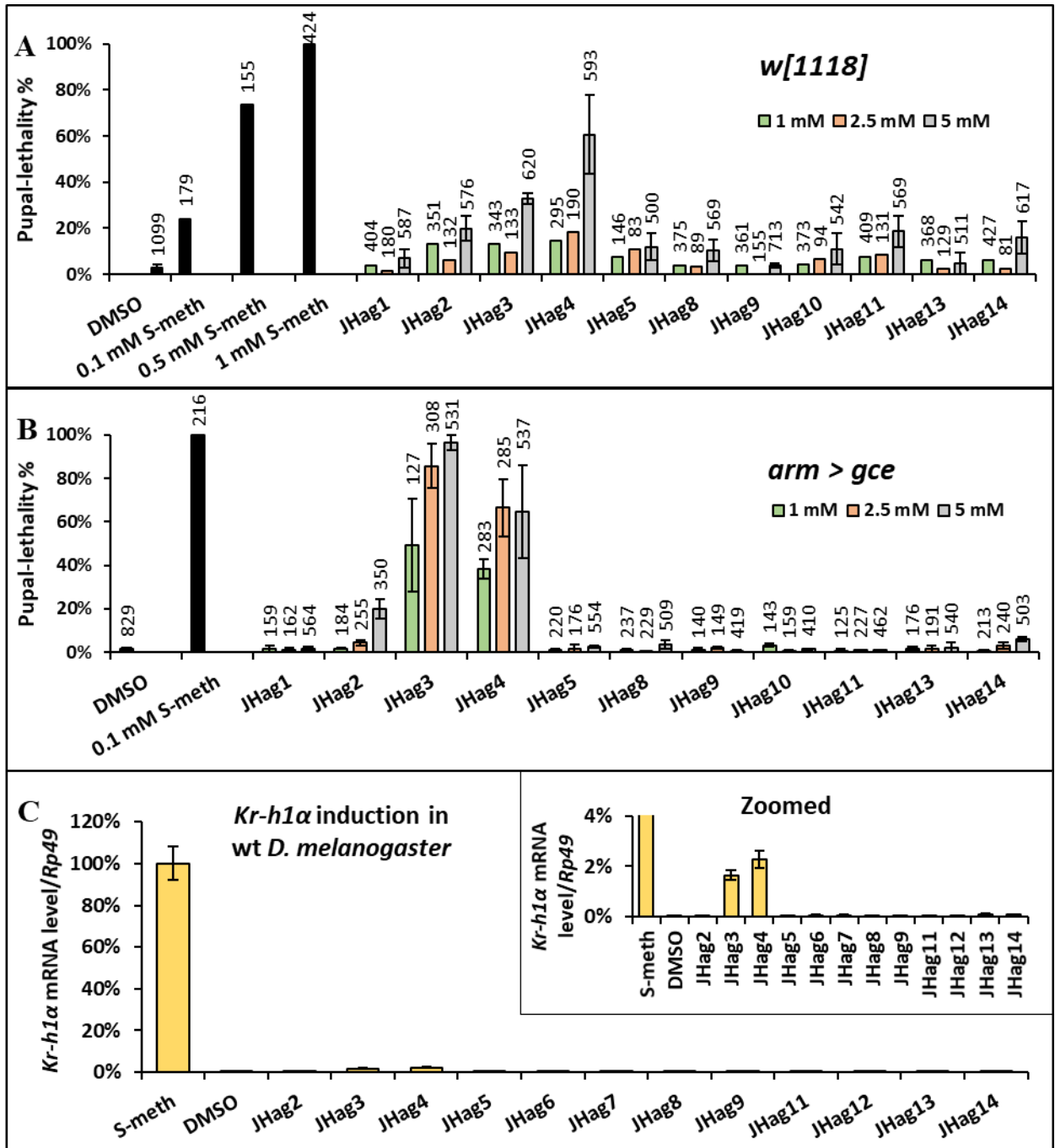
Figure 6: The effect of treatment with JH agonists on pupal lethality and Kr-h1 induction in *D. melanogaster.* (A, B) Pupal lethality after treatment of *w[1118]* (A), or *arm>gce* (B) 3rd instar larvae by JH agonists, S-methoprene, or DMSO in ethanol. Values are averages ±SD of 1-4 independent experiments, n of flies is indicated above columns. (C) Induction of Kr-h1 mRNA level by treatment with JH agonist, S-methoprene, or DMSO. Values are normalized mean RT-qPCR data from three treated white puparia, which were measured as technical triplicates. Plotted values are normalized to control S-methoprene, which was arbitrarily set as 1; error bars represent SD.

3.2.2 Selected JHags affect the development of *T. castaneum* via JH signaling

Exploiting the greater sensitivity of red flour beetle *Tribolium castaneum* to JH agonists, the selected JHags were topically applied on pupae of *T. castaneum*. When exposed to compounds with JH activity in the pupal stage, the *T. castaneum* produces the second larval cuticle, or die as pharate-adult (Konopova and Jindra 2007). The emergence of the second pupa was observed after treatment with S-methoprene, JHag8, or JHag11 in 100% pupae (Fig. 7A,B). While 91% of pupae treated with JHag12 produced pharate-adult phenotype, JHag4 and JHag5 showed weaker potency, as they provoked only 18% and 9% pharate-adults, respectively (Fig. 7A,B).

Analogously to *D. melanogaster*, the expression of *Kr-h1* in *T. castaneum* pupae is almost undetectable. The effect of JH agonist should induce the *Kr-h1* as previously shown with established JH agonists (Minakuchi et al. 2009). To confirm the JH activity of selected compounds, mRNA levels of *Kr-h1* were measured in pupae treated with JHags, S-methoprene, or DMSO. The induction of *Kr-h1* mRNA level was observed in pupae treated with S-methoprene, JHag4, JHag5, JHag8, JHag11, and JHag12 (Fig. 7C). The most potent compounds from HTS were JHag8 and JHag11, which were able to cause $37\pm5\%$ and $21\pm5\%$ induction of *Kr-h1*, respectively, relative to S-methoprene. Notably, although the induction of *Kr-h1* by JHag12 was similarly low as in the case of JHag4 and JHag5, the efficiency of producing lethal phenotype deviates greatly (Fig. 7). To sum up, JHag8 and JHag11 proved to be effective in mimicking the JH effect in *T. castaneum*. Such results were corroborated by measurements of *Kr-h1* induction. Despite the lower induction of *Kr-h1*, JHag12 exhibited solid penetrance in producing the lethal pharate-adult phenotype.
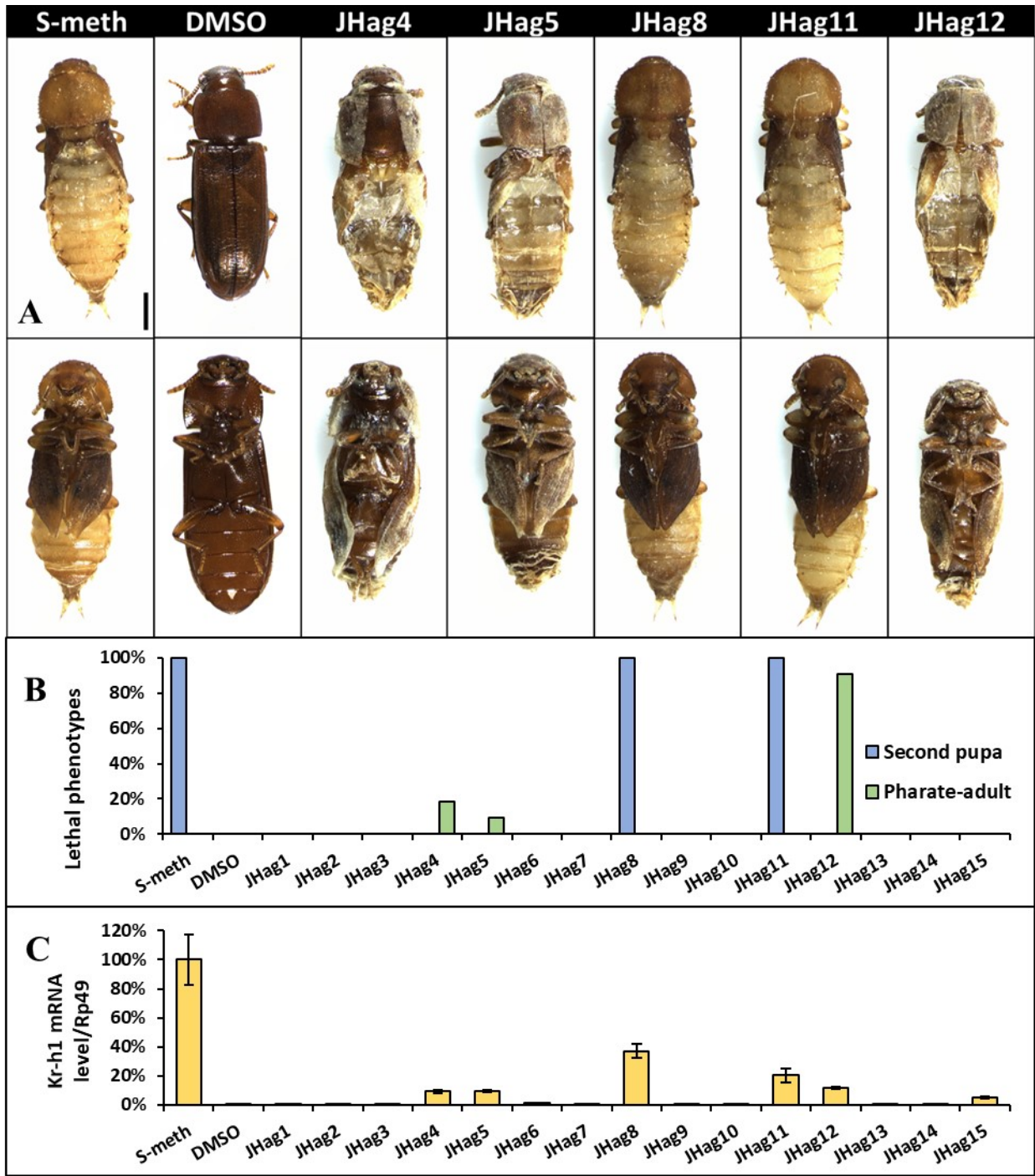
Figure 7: **JH agonists selected in HTS block metamorphosis** in *T. castaneum* **pupae via JH-signaling.**
(A) *T. castaneum* pupae treated with 0.6 µL of 0.2mM JHag4, JHag5, JHag8, JHag11, JHag12, or control S-methoprene died either as second pupa (S-methoprene, JHag8, JHag11), or as a pharate-adult (JHag4, JHag5, JHag12). Scale bar: 0.5 mm. (B) Rate of lethal second-pupa or pharate-adult phenotypes after treatment with JH agonists, S-methoprene, or DMSO (n=11). (C) Induction of *Kr-h1* mRNA by JH agonists relative to induction by S-methoprene. Values are normalized mean RT-qPCR data from three

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treated pupae measured as technical triplicates. Plotted values are normalized to control S-methoprene, which was arbitrarily set as 1; error bars represent SD.

3.2.3 Selected JHags provoke iteration of last larval instar in hemimetabolous *P. apterus*

Since it was shown that JHags mimic the JH effect in holometabolan insects, we aimed to elucidate the effect of JHags on hemimetabolous linden bug *P. apterus*. The treatments of last instar larvae (L5) with analogues of JH led to the formation of supernumerary 6th larval instar (L6) or adultoids that are more reminiscent to adults (Babu and Sláma 1972; Konopova et al. 2011; Sláma and Williams 1965). L6 instar exhibits typical larval traits, such as black wing pads without any color pattern, or untanned abdominal cuticle. Adultoids display a combination of larval and adult traits, having mostly tanned abdominal cuticle and wing pads with color pattern to some extent (Sláma and Williams 1965). As expected, the L6 phenotype was induced by treatment with S-methoprene, JHag8, JHag9, and JHag14 but not by solvent DMSO (Fig. 8A). Although active, JHag11 was able to only produce the intermediate adultoid phenotype, suggesting weaker JH activity. These data indicate that JHag8, -9, and -14, are effective JH agonists inducing supernumerary larval instar in hemimetabolous *P. apterus*.
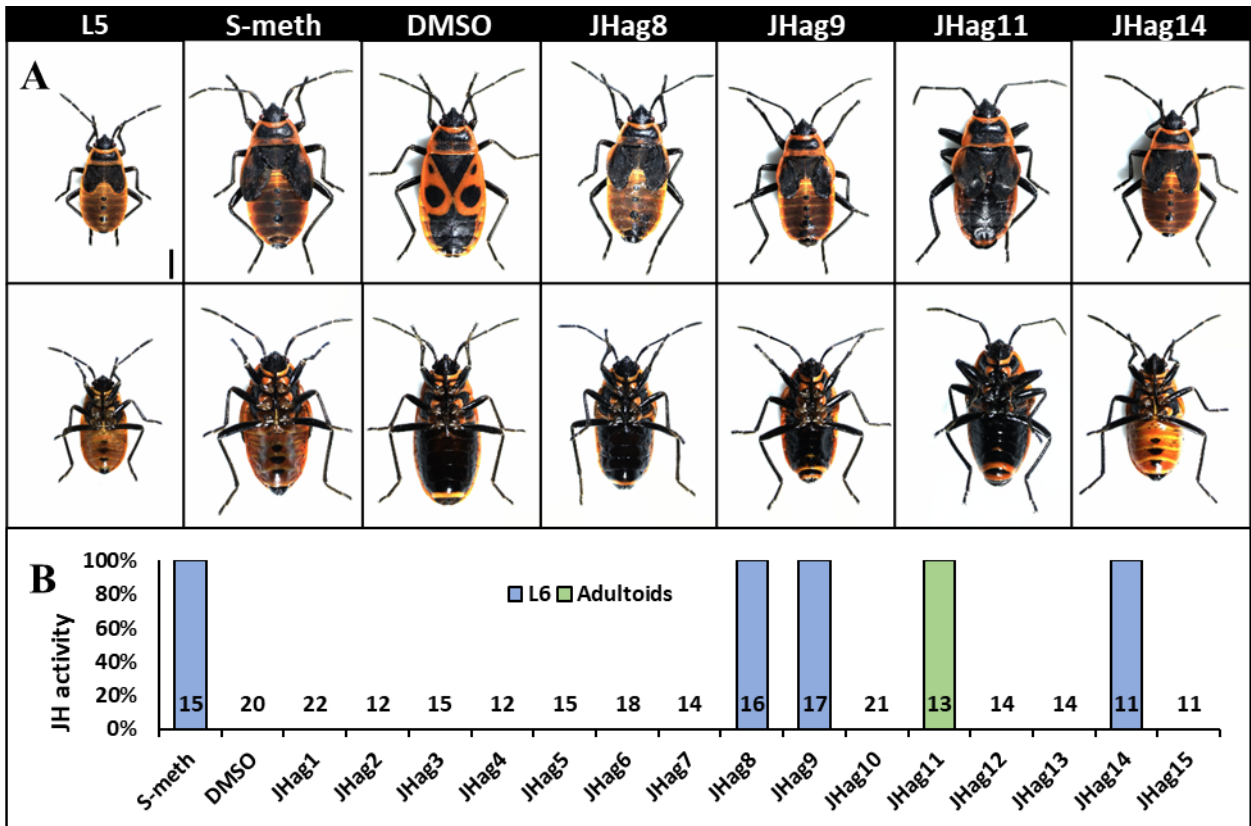
Figure 8: JH agonists selected via HTS induce supernumerary 6th larval instar (L6) or adultoids in *P. apterus* L5 larvae. (A) Last instar larvae treated with 3µL of 0.2mM JHags or S-methoprene ecdysed to L6 instar with typical hallmarks such as black wing pads or untanned abdominal cuticle (S-meth, JHag8, JHag9, JHag14). Treatment with JHag11 produced the adultoid phenotype, whose cuticle exhibits traits more reminiscent of the adult. Scale bar: 2 mm. (B) Rate of induced L6s or adultoids relative to the treated individuals, which did not die as L5 (n is indicated above columns).

3.3 Versatile JHag8 binds TcMet and DmGce *in vitro*

To prove that JHag8 is a true JHR agonist, the ability to bind JHR was tested in the JH-binding competition assay. JHag8 exerted the function as JHR agonist in the two-hybrid assay in *D. melanogaster* and both in two-hybrid assay and *in vivo* in *T. castaneum* and *P. apterus*. While the JH-binding assay does not work with the PaMet protein *in vitro*, we obtained data with TcMet and DmGce. The constant amount of [^{3}H]JH III was incubated with the increasing amount of JHag8 in order to reach the equilibrium between the competing ligands bound to the JHR. The results show that JHag8 competed against [^{3}H]JH III for TcMet and also for DmGce (Fig. 9). These results show that JHag8 is the true agonist ligand of JHR.
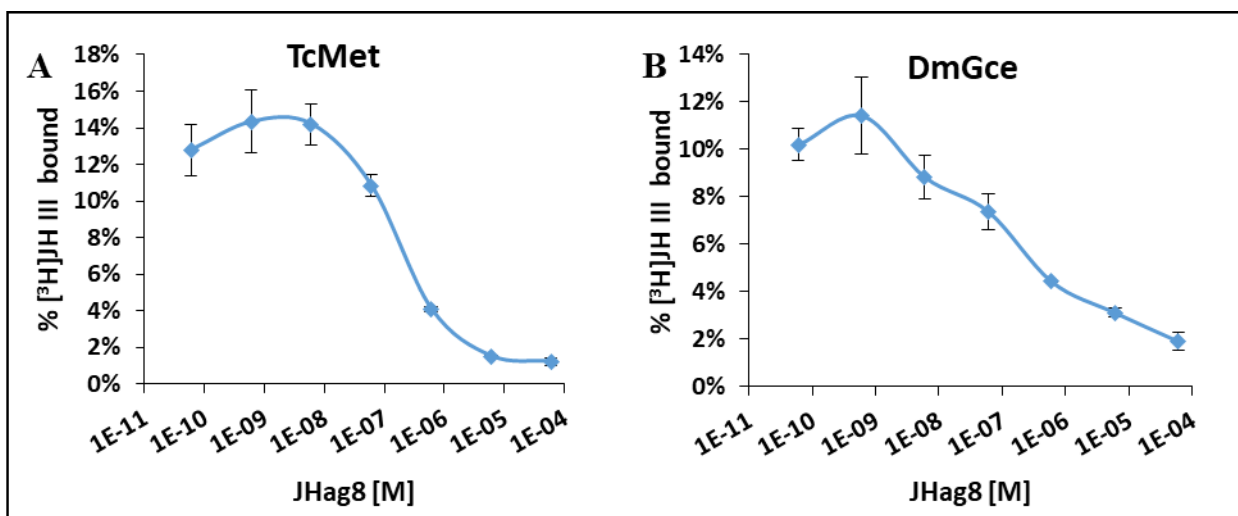
Figure 9: JHag8 shows to bind TcMet and DmGce. JHag8 binds TcMet (A) or DmGce (B) competitively against [³H]JH III, each data point represent the mean from three replicates ± SD.

3.4 Met-Tai from *B. germanica* and *L. migratoria* provide new systems for two-hybrid assay

In order to broaden the spectrum of insect species for HTS using two-hybrid assay, Met and Tai sequences from hemimetabolous species *Blattella germanica* (Blattodea) and *Locusta migratoria* (Orthoptera) were cloned into appropriate plasmids (Tab. 2). As described in the introduction, the pcDNA3.1 plasmid was used since it does not encode *Renilla* luciferase that interferes with Nano-Glo Luciferase Assay System (Promega). The response of JH agonist-induced interaction of Met-Tai was confirmed by the two-hybrid assay in HEK293 cells. In the presence of 1 μM fenoxycarb, Met-Tai from *L. migratoria* and *B. germanica* showed 18-fold and 104-fold activation of NanoLuc relative to DMSO (Fig. 10). Thus, both pairs provide new systems for searching for novel compounds with JH activity in the two-hybrid assay.
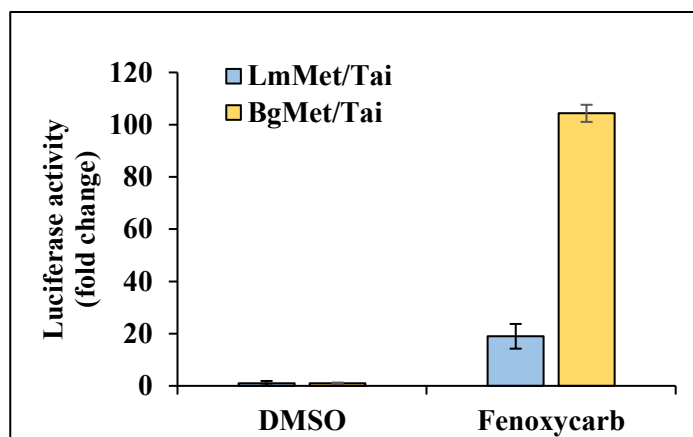
Figure 10: Fenoxycarb induces Met-Tai interaction of *B. germanica* and *L. migratoria*. 1 µM fenoxycarb induces Met-Tai interaction relative to DMSO in both species. Values are mean ± SD from three replicates.

3.5 JH III is a competitive ligand of PsMet and BgMet

In order to elucidate whether Met proteins from *P. simplex* and *B. germanica* specifically bind JH III, PsMet and BgMet were cloned into pK-Myc-C2 mammalian expression vector, translated *in vitro,* and used in JH-binding assay. The results from the assay show the dose-response of competitive binding of JH III by PsMet and BgMet against [^{3}H]JH III (Fig. 11A,B).

To prove that the JH III is bound by the PAS-B domain of the PsMet and BgMet, we mutated conserved threonine to a bulkier tyrosine residue. This mutation was shown to prevent ligand binding in different insect species (Charles et al. 2011; Jindra et al. 2015b; Li et al. 2014). The JH III binding by PsMet^{T316Y} and by BgMet^{T324Y} was reduced by 91% and 85%, respectively (Fig. 11C). These data suggest that the conserved threonine plays important role in JH III binding by the BgMet and PsMet proteins.
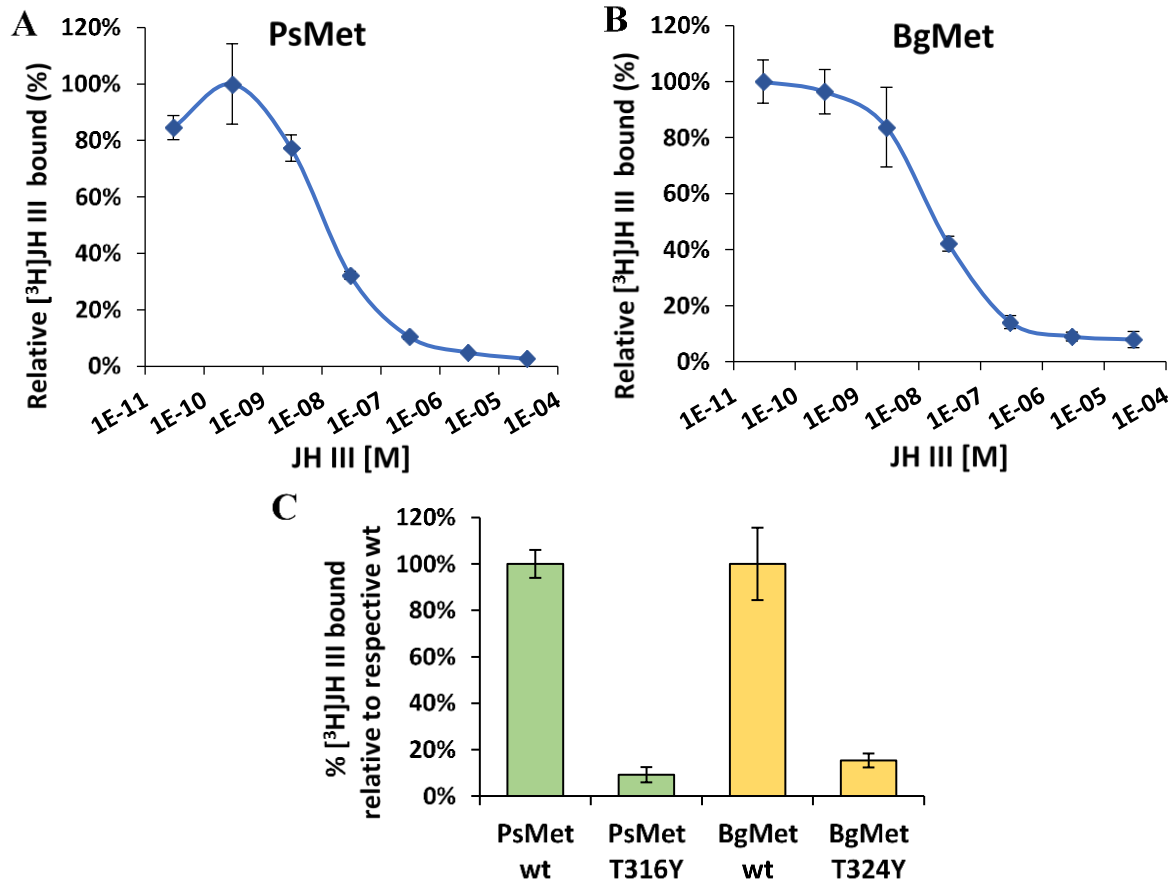
Figure 11: JH III is a competitive ligand of PsMet and BgMet, mutation in the PAS-B domain substantially diminishes binding ability. *P. simplex* Met (A) and *B. germanica* Met (B) were shown to bind JH III competitively against [³H]JH III. (C) The ability of PsMet and BgMet to bind [³H]JH III was substantially hindered by T316Y and T324Y mutations, respectively. Each data point represents the mean ±SD from three replicates.

4   DISCUSSION

4.1   The number of active JHags in cell-based experiments contrasts with that observed *in vivo*

Although the HTS was based on the *D. melanogaster* Kc167 cell line and the effect of JHags was verified in the two-hybrid assay with *D. melanogaster* Gce-Tai and all compounds were found to activate JH signaling, only the two JHags induced response *in vivo* (Fig. 6). The live animals were treated either topically by JHags in acetone (induction of *Kr-hlα*) or incorporating the JHags dissolved in ethanol into food (pupal lethality). In the latter the JHags might have entered the larvae from the food in which the larvae live, or as the larvae swallowed the food. Using such approach, we should bypass the inability of JHags to penetrate the cuticle. The barrier that does not play any role in cell-based experiments. Also, we presented that JHag8 binds DmGce in vitro (Fig. 9B), albeit the JHag8 caused neither increased pupal lethality nor *Kr-h1α* induction. These data suggest that the inactivity of JHags in *D. melanogaster* may be caused by metabolism that breaks down the compounds. This hypothesis is in line with data from *gce*-overexpressing flies that exhibited only more intense response but did not react to different compounds than wild type flies (Fig. 6).

The differences between data from two-hybrid assay and from *in vivo* experiments were also found in *T. castaneum*. Seven JHags induced the Met-Tai interaction in the two-hybrid assay by at least 40% relative to S-methoprene (Tab. 5), while 6 JHags caused induction of *Kr-h1* relative to S-methoprene in treated pupae (Fig. 7C). Out of those 6 JHags, only 2 mimicked the specific JH effect by causing a formation of second pupa (Fig. 7B). These results indicate that effective JH agonists selected in the cell-based assay may turn out to be inactive in live animals, as they might lack abilities either to penetrate the cuticle or avoid detoxification by insect metabolism. This observation corresponds with studies reporting that changes in cuticle permeability and metabolism of xenobiotics contribute to insecticide resistance (Balabanidou et al. 2018; Yahouédo et al. 2017; Zimmer et al. 2018).

Unlike in holometabolous *D. melanogaster* and *T. castaneum*, in *P. apterus* the *in vivo* data (Fig. 8) correlate with those obtained in two-hybrid assay (Tab. 5). However, the data corroborating the effect of JHags on JH signaling is missing. To date, there has not been any

evidence that hemipteran Met binds JH analogue. Therefore, future research should address the impact of JHags on JH signaling.

4.2 The species-selectivity of JHags in examined insect models

Comparison of the results from bioassays on individual species implies that some JHags could function in a species-selective manner. Unlike S-methoprene, no JHag proved to effectively disrupt the development in all three species. While the JHag3 affected JH signaling *D. melanogaster*, it influenced the development of neither *T. castaneum* nor *P. apterus*. Such results are consistent with findings from two-hybrid assay (Tab.5). JHag12 proved to be selectively effective for *T. castaneum*. Although the treatment with JHag12 did not lead to the formation of the second pupa it caused the lethal pharate-adult phenotype. The results from measuring the induction of *Kr-h1* suggest that the second pupa phenotype is connected with higher induction of *Kr-h1* and lethal pharate-adult phenotype correlates with lower *Kr-h1* induction (Fig. 7). However, the zero effect on pupal lethality and Kr-h1α induction is inconsistent with the results from two-hybrid assay that show the JHag12 is active in *D. melanogaster* (Tab5). Causing no effect in *D. melanogaster* and *T. castaneum*, JHag9 and JHag14 function as selective JH agonists for *P. apterus*. JHag8 and JHag11 were found to disrupt development in both *T. castaneum* and *P. apterus*, but not in *D. melanogaster*. As in the case of JHag12, the results from in vivo experiments differ from those in two-hybrid that show the both JHag9 and JHag14 induce interaction not only of *P. apterus* Met-Tai but also of *T. castaneum* and *D. melanogaster* (Gce-Tai) (Tab. 5). This discrepancy illustrates the necessity to validate compounds *in vivo*.

In general, these data suggest that at least some JHags act as selective JH agonists in examined species. In combination with further derivatization, it could be possible to develop truly species-selective compounds, harmless for non-target species. The possibility of such compounds is implied by the existence of insect growth regulators selective only to species in the Pyrrhocoridae family (Babu and Sláma 1972; Sláma and Williams 1965; Zaoral and Sláma 1970).

4.3 Hemimetabolous JHR specifically binds JH III

Until now, only JHR from holometabolous species proved to specifically bind JH III. In this thesis, we report the first example of Met from hemimetabolous species to bind JH III (Fig. 11).

Whilst the dissociation constant (K_d) was determined for TcMet (K_d=2.9 nM), DmMet (K_d=5.3 nM), DmGce (K_d=19.3 nM), and AaMet (K_d=4.4) (Charles et al. 2011; Jindra et al. 2015b; Li et al. 2014; Miura et al. 2005), the affinity of [^{3}H]JH III for PsMet and BgMet remains to be measured. However, from the dose-response curve, we can estimate the order of PsMet and BgMet Kd in nanomolar concentration that corresponds to the physiological range of JH titers (Sevala et al. 1999; Treiblmayr et al. 2006). In previous studies examining the JH-binding ability of insect JHRs, conserved residues in the PAS-B domain pivotal for JH-binding were identified (Fig.2). When mutated to a bulkier tyrosine residue, threonine T254 in TcMet (T272 in DmGce, T406 in DmMet, and T403 in AaMet) prevented ligand-binding (Charles et al. 2011; Jindra et al. 2015b; Li et al. 2014). We mutated the corresponding threonine to tyrosine in PsMet and BgMet and observed similar results showing the importance of T316 in PsMet and T324 in BgMet for the ligand-binding capacity (Fig. 11C). Interestingly, out of all species in this thesis, only Met of *C. lectularius* has serine instead of threonine at that position (Fig. 2). As the results from the two-hybrid assay demonstrate, no JHag was able to reach at least 30% relative to the activation of ClMet-Tai interaction by a reference compound (Tab. 5). Therefore, it remains a question what impact of this serine residue on JH-binding by ClMet is and how it could be exploited for further design of JH agonists.

5   CONCLUSION

In this thesis, JHags yielded by a HTS using JHRE-luc reporter assay in Kc167 cell line were examined in vivo using three insect species from distinct orders. Our results show that JHags mimic the effect of JH on insect development. Evidence that JHags affect insect development by induction of JH target gene *Kr-h1* was obtained for *D. melanogaster* and *T. castaneum*. Moreover, it was demonstrated that one of the selected compounds JHag8 binds DmGce and TcMet *in vitro*, confirming its JH agonist activity. It was shown that some JHags tend to disrupt the development of some insect species while being inactive in others. We also prepared a new insect system for either further examination of new JHags and unbiased two-hybrid assay-based HTS. Finally, the first evidence of direct JH-binding to Met of hemimetabolous insect species was provided.
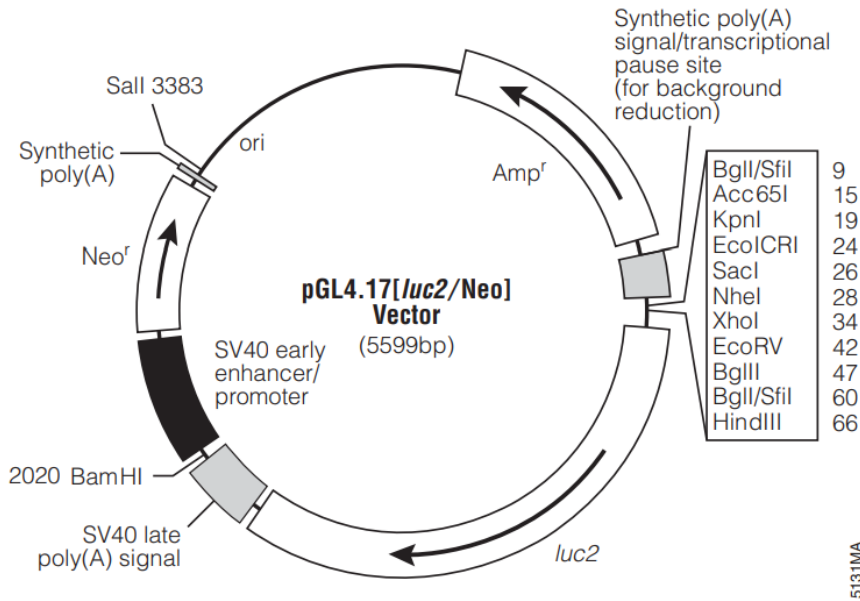
6   VECTOR MAPS

Figure 12: Vector circle map of pGL4.17 used for JHRE-luc reporter assay. The JHRE-luc reporter was inserted into multiple cloning site upstream of *luc2*. Adapted from Promega.
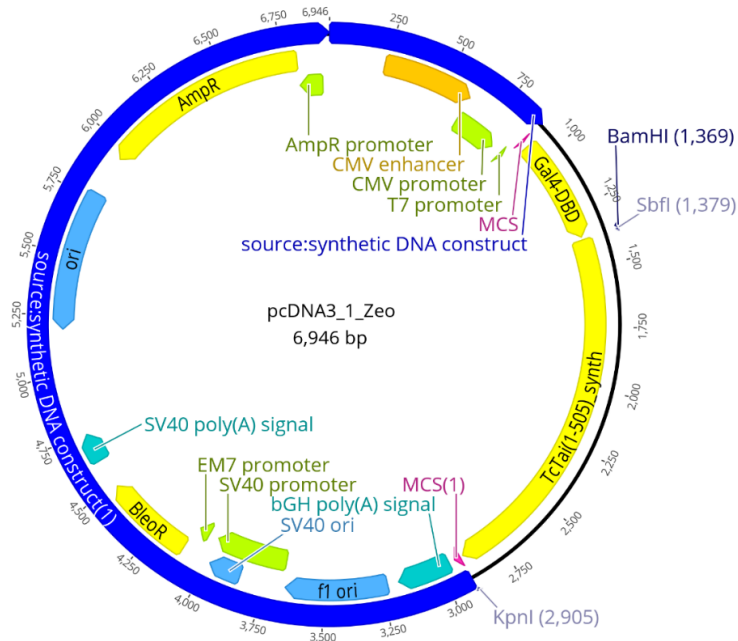

Figure 13: Vector circle map of pcDNA3.1 plasmid. TcTai sequence was replaced by Tai from different species using indicated restriction sites. This plasmid was used for the selection of stabile cell line harboring sequences encoding GAL4-Tai fusion protein.

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