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Molecular Evolution of Juvenile Hormone Signaling

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1. Introduction

Insect development proceeds through a series of discrete developmental stages called instars. During hexapod evolution, the development of complete metamorphosis introduced a novel mechanism for separating feeding and reproductive stages (Truman & Riddiford, 2002), facilitating the tremendous evolutionary success of holometabolous insects. In contrast to hemimetabolous insects, which progress through a series of instars that appear as smaller iterations of the adult form, holometabolous insects proceed from egg to adult through a progression of isomorphic larval instars and a pupal transitory stage. In each case, the physical boundary for growth during an instar is established by a chitinous exoskeleton, which must be periodically shed. This molting process is under the control of two counteracting hormones.

Toward the end of an instar, a pulse of the insect molting hormone, 20-hydroxyecdysone (20E) initiates a transcriptional cascade that carries the molt to a subsequent instar. However, it is the interaction of 20E and the sesquiterpenoid juvenile hormone (JH) that governs the developmental outcome of each molt. During larval development, an elevated JH titer and 20E directs the sequential progression through larval development until the final larval instar, when the JH titer substantially declines. The removal of circulating JH facilitates a 20E-directed developmental switch that initiates the metamorphic molt. Thus, it was proposed that JH can modulate 20E activity, maintaining the status quo during pre-adult development.

1.1 JH and JHAs: Insecticidal use of hormone agonists

Since the first chemical analysis resolved the sesquiterpenoid structure of endogenous JH (Röller *et al.,* 1967), several homologs have been identified, each bearing opposing, terminal epoxide and methyl ester functions. Variation in the degree and identity of alkyl group substitution at C3, C7, and C11 along the carbon skeleton defines the homologs. The evolutionary importance of multiple JH homologs is unclear. JH 0, I, II, and III have all been isolated from lepidopteran insects, whereas JH III, the presumed evolutionary precursor to the higher homologs, is found in all insects. JH bisepoxide (JHB3) has been identified as a product of the corpus allatum (CA) in higher Diptera including *Drosophila melanogaster* and *Sarcophaga bullata* (Richard *et al.,* 1989; Bylemans *et al.,* 1998). Nearly identical in structure to JH III, JHB3 is distinguished by an additional epoxide group spanning C6-C7.

The major JHs and some juvenile hormone analogs (JHAs) are presented in Figure 1.

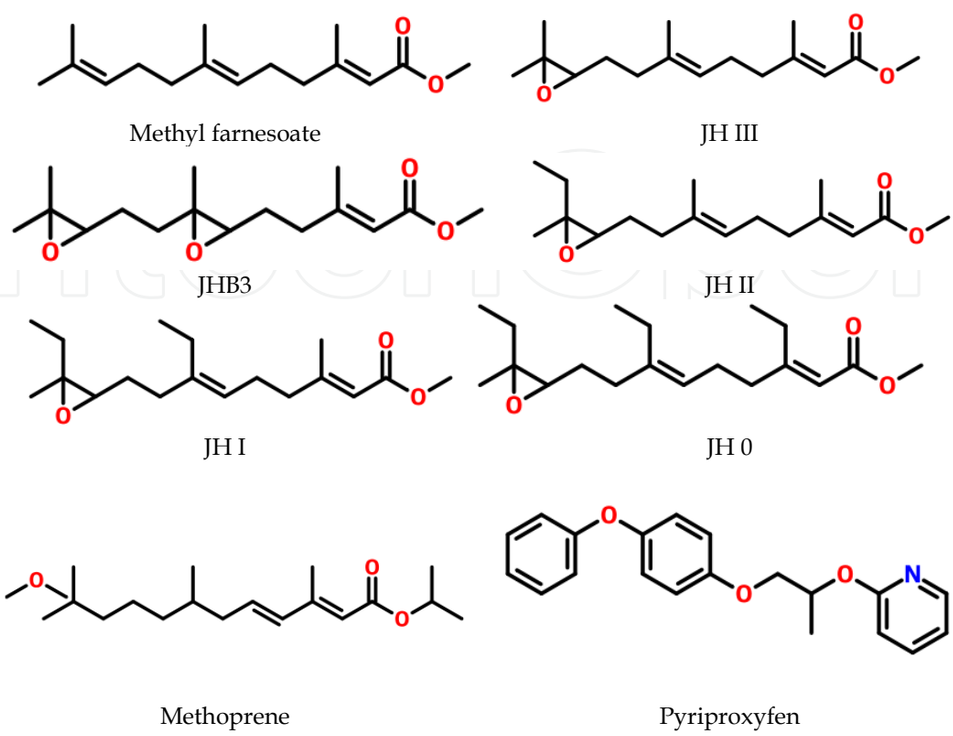

Fig. 1. Structures of endogenous JH molecules and two synthetic JHAs, methoprene and pyriproxyfen.

The physiology and chemistry of JH prompted intense research into the synthesis and commercial-scale production of JH analogs, or juvenoids, for agricultural use. The allure of these compounds was at least twofold. First, juvenoids exhibit extremely low non-target (in particular, mammalian) toxicity. Second, it was originally thought that insect resistance to JHAs would be unlikely, since an insect was not likely to become refractory to an endogenous hormone (Williams, 1967). Methoprene, a juvenoid structurally similar to endogenous JH, has enjoyed success in the management of larval mosquito populations. However, JHAs need not mimic the chemical structure of endogenous JH, as exemplified by the pyridine-based pyriproxyfen, whose activity exceeds JH by two orders of magnitude in dipteran white puparial and larval assays (Riddiford and Ashburner, 1991).

Exogenous JH exposure can elicit classic antimetamorphic activity in both Lepidoptera and Coleoptera (Srivastava & Srivastava, 1983; Konopova & Jindra, 2007), extending larval development through one or more supernumerary instars. Also in these insects, exposure to exogenous JH or to its chemical analogs (JHA) can result in the deposition of a second pupal cuticle (Zhou & Riddiford, 2002). Thus, in Lepidoptera and Coleoptera, JH exposure at an inappropriate time inhibits 20E-directed developmental progression.

In Diptera, treatment with exogenous JH produces dose-dependent lethality at the pharate adult stage. All adult structures arise from imaginal discs in flies, and these discs are insensitive to JH during development, unlike Lepidoptera and Coleoptera, in which the

polymorphic larval epidermis gives rise to pupal and adult structures. When flies are challenged with JHAs, the adult structures that differentiate from imaginal discs remain unaffected (Postlethwait, 1974). In *D. melanogaster*, only the abdominal histoblasts are JH sensitive; diagnostic (sublethal) doses of methoprene disrupt abdominal bristle formation in female flies (Madhavan, 1973).

2. Molecular mechanism of JH signal transduction

The molecular events underlying 20E signaling are relatively well understood. Ecdysone released from the prothoracic glands is converted to its active metabolite 20E in target tissues, where it regulates transcription through a heterodimeric receptor complex comprised of *Ecdysone receptor* (EcR) and *Ultraspiracle* (USP) proteins. When bound with 20E, ECR-USP recognizes and binds ecdysone response elements located in the promoter region of target genes, inducing transcription of a hierarchical network of early and late genes. The early genes either repress their own expression or induce expression of late genes (Ashburner *et al.,* 1974). In this manner, the expression of genes involved in the 20E transcriptional cascade is tightly controlled. In contrast, the nature of JH signal transduction has been difficult to elucidate, largely due to the enigmatic nature of the JH receptor. A body of ever-increasing experimental evidence strongly supports the product of the *Methoprene tolerant* (*Met*) gene as the prime candidate for a JH receptor component (Wilson & Fabian, 1986; Konopova & Jindra, 2007; Yang *et al*., 2011).

Met was originally discovered by screening progeny of ethyl methanesulfonate (EMS)-mutagenized *D. melanogaster* for resistance to methoprene (Wilson & Fabian, 1986). *Met* mutants show dramatically enhanced (~100 fold) resistance to both the toxicity and morphogenetic defects caused by methoprene exposure, but not to other classes of insecticides (Wilson & Fabian, 1986). Such resistance is not restricted to compounds with high structural similarity to JH; *Met* mutants are also resistant to the more potent, structurally distinct JHA pyriproxyfen (Riddiford & Ashburner, 1991).

Cloning and sequence analysis identified *Met* as a member of the basic Helix-Loop-Helix *Period Ahr Sim* (bHLH PAS) family of transcriptional regulators (Ashok *et al.,* 1998). PAS proteins function as dimers in a diverse array of functions in development, xenobiotic binding, and detection of environmental signals (Crews, 1993). Both the bHLH domain and the PAS repeats (PAS A and B) facilitate dimerization between PAS proteins (Huang, *et al*., 1993). Additionally, the PAS domains function in small molecule ligand binding and target gene specificity. Each dimerization partner recognizes and binds one half of a palindromic E-box consensus sequence CANNTG in the promoter region of target genes via the stretch of basic residues immediately N-terminal to the HLH motif. Examples of PAS proteins with ligand binding activity include the bacterial *photoreactive yellow protein* (*PYP*), and the vertebrate *aryl hydrocarbon receptor* (*Ahr*).

Genetic and biochemical data show that MET binds JH with nanomolar affinity (Shemshedini & Wilson, 1990) and that MET product is present in the nuclei of several known JH target tissues, including ovary, MAG, and larval fat body (Pursley *et al.,* 2000). In addition, MET can drive the expression of a reporter gene in a JH-sensitive manner (Miura *et al.,* 2005). All of the above data satisfy criteria for a hormone receptor.

Analysis of the Met^{27} null allele provided the first demonstration of insecticide resistance due to the absence of a target macromolecule (Wilson & Ashok, 1998). Even though Met^{27} flies are viable, *Met* deficiency carries reproductive consequences, namely substantially

reduced oogenesis (~20% compared to Met^{+}), consistent with a role for JH in this physiology. However, since absence of a JH receptor is expected to preclude normal development, the viability of Met^{27} flies challenged the notion of *Met* as a *bona fide* JH receptor. Some evidence supports alternative mechanism(s) of JH signaling (see Flatt *et al.*, 2008; Riddiford *et.al.*, 2010). In this chapter, we review data that support the notion of *germ cell expressed* (*gce*), the paralog of *Met* in higher Diptera, as conferring at least partial functional redundancy.

3. *Met* homologs across holometabola

Reports of methoprene resistance in mosquito populations (Dame *et al.*, 1998; Cornel *et al.*, 2000; Cornel *et al.*, 2002) led us to investigate the *Met* orthologs of three mosquito species: *Aedes aegypti*, *Culex pipiens*, and *Anopheles gambiae*. Using a combination of degenerate RT-PCR and genomic database mining, we isolated a single *Met* homolog from each of these mosquitoes. Sequence analysis of these genes showed that they share high identity with both *Met* and the closely related *gce* from *Drosophila*, as expected. However, a comparison of the genomic structures among *DmMet*, *Dmgce*, and the three putative mosquito *Met* genes revealed higher structural conservation between each mosquito *Met* and *Dmgce*. Importantly, the intron number of these genes is more consistent with that of *Dmgce* than *DmMet* (from six to nine, *versus* one in *DmMet*). Furthermore, several introns in each mosquito gene are positionally conserved with those in *Dmgce*. This led to our proposal that the *Met* gene of higher Diptera originated via retrotransposition of a basal, *gce*-like gene of lower Diptera (Wang *et al.*, 2007).

Retrotransposition, or retroposition is a mechanism of gene duplication that proceeds through an mRNA intermediate. Following post-transcriptional splicing, the parental message is reintegrated into the genome. Ultimately, for the duplicate copy to escape the fate of becoming a pseudogene, it must reintegrate with associated regulatory elements intact or incorporate into a suitable transcriptional environment elsewhere in the genome. Following duplication, the increase in copy number of the parental gene affords a relaxation of selective constraint, facilitating functional divergence. This may manifest as subfunctionalization, in which a modification of the parental function evolves, or neofunctionalization, which refers to attainment of a novel function (MacCarthy & Bergman, 2007). *DmMet* retains a strong diagnostic feature of retroposition: a paucity of introns relative to *gce*, which is consistent with splicing and genomic reintegration of an ancestral *gce*-like transcript.

A conserved *gce*-like gene appears to be conserved across holometabolan genomes, including the red flour beetle, *Tribolium castaneum*, and the honeybee, *Apis mellifera*. An independent gene duplication within the Lepidoptera has given rise to two *Met*-like proteins, presently called Methoprene tolerant proteins I and II, whose functions are currently under investigation (i.e. Li *et al.*, 2010). Despite a demonstrated sequence conservation favoring the *Met*-like genes of more primitive Holometabola as ancestral to *gce*, we will continue to refer to these genes as *Met*-like in this text.

3.1 *Met* and *gce* within the genus *Drosophila*

When the genomes of 12 representative *Drosophila* species became available (Ashburner, 2007), we chose to examine the molecular evolution of *Met* and *gce* within this genus of flies. Both paralogs are conserved in each species, indicating that the origin of *Met* predates that

of the genus *Drosophila,* some 63 million years ago (Tamura *et al*., 2004). The architecture of these genes is generally conserved in each species, with a few notable exceptions. A single conserved intron is present in *Met* in the PAS B domain of 11 species. In addition to this conserved intron, independent intron gains have occurred in the lineages leading to *D. simulans* and *D. willistoni*. A single *Met* ortholog exists in each *Drosophila* genome examined, but *D. persimilis* harbors two separate, consecutive loci on the X chromosome, currently called GL13106 and GL13107, that align to distinct regions of *DmMet*. The 5′ putative gene GL13106 contains a complete PAS A domain followed by a severely truncated PAS B domain. We performed RT-PCR across these two genes and failed to obtain a single PCR product, suggesting that GL13106 and GL13107 indeed code for two distinct open reading frames. Eleven of the 12 representative *gce* orthologs contain at least six conserved introns, with independent intron gains evident in the lineages leading to *D. melanogaster*, *D. pseudoobscura*, and *D. mojavensis*, whereas a substantial deletion in *D. persimilis gce* has eliminated the central portion of this gene, including the PAS repeats.

In addition to the bHLH, PAS, and PAC domains, putative transactivation domains (TAD) are evident in *Met* and *gce* orthologs. TADs are glutamine and/or aspartic acid-rich motifs whose amino acid sequences are broadly defined and generally reside in the C-terminal region of PAS proteins (Ramadoss & Perdew, 2005). *Met* homologs show Q- and D-rich motifs between the PAS B and PAC domains, while alignments of *gce* homologs indicate a D-rich region C-terminal to the PAC domain. Miura *et al*. (2005) suggest the presence of a C-terminal TAD in recombinant MET protein, but this region has yet to be functionally defined.

Using *DmMet* and *Dmgce* as query sequences, we conducted homology searches under tBLASTx criteria (translated nucleotide query to search a translated nucleotide database) against the publicly available EST library of *Glossina morsitans*, the tsetse fly. Our search recovered several clones, which were imported into the Sequencher program to produce two independent contigs. These composite nucleotide sequences were used to infer a gene tree with other holometabolan *Met* and *gce* orthologs, including those of two representative *Drosophila* species (Figure 2). This preliminary analysis reveals the presence of distinct *Met* and *gce* orthologs in the *G. morsitans* genome, indicating that the origin of *Met* predates the divergence of the Aschiza and Schizophora. These two taxonomic groups, which are estimated to have diverged more than 85 million years ago (Bertone & Wiegmann, 2009), reside within the brachyceran infraorder Muscomorpha.

3.2 Evidence for differential selective constraint imposed on *Met* and *gce*

Based on an *a priori* hypothesis that *Met* and *gce* were subject to differential post-duplication selective constraint, we performed analyses of nonsynonymous-to-synonymous (dN/dS) substitution ratios on codon alignments of these *Drosophila* paralogs. Datasets were analyzed using the DataMonkey tool (Kosakovsky-Pond & Frost, 2005), a web-based implementation of the HyPhy package (Kosakovsky Pond *et al*., 2005). dN/dS analyses can be used to infer the relative selective pressure along entire coding sequences or in a site-specific manner. A substantially depressed dN/dS ratio (i.e. zero or close to zero) implies purifying (negative) selection. That is, nonsynonymous changes are stringently selected against. In contrast, when dN/dS is nearly one, neutral evolution is inferred. A dN/dS value far in excess of one implies positive selection, or adaptive evolution. In this case, nonsynonymous substitutions confer a selective advantage.

The results of our dN/dS analyses showed dramatic dissimilarity in the relative selective pressures that have shaped the coding sequences of *Met* and *gce*. In the case of *Met*, dN/dS was generally suppressed along the entirety of the coding sequence, indicating strong selection against nonsynonymous codon substitution. This is perhaps surprising, since MET deficiency has no effect on viability (Wilson & Ashok, 1998). Possibly, mutations that alter amino acid identities are selected against in *Met* due its involvement in reproduction. In the absence of methoprene selection, *Met* mutants are quickly out-competed by wild type flies despite the seemingly slight fitness cost of *Met* loss (Minkhoff III & Wilson, 1992). In contrast, dN/dS values close to one dominate the C-terminal half of *gce*, indicating a substantial relaxation of selective constraint in this region. The N-terminal region of this gene, containing the canonical bHLH and PAS functional domains, shows a strongly depressed dN/dS. Based on functional data from other PAS proteins, this region is assumed to harbor DNA and ligand binding activity, whereas the C-terminal region contains putative TADs. C-terminal degeneracy was shown to confer differential target gene specificity between the *Ahr* homologs of mice and humans (Ramadoss & Perdew, 2005; Flaveny *et al.*, 2010). Similarly, the disparate selective constraints evident in the C-terminal regions of *Met* and *gce* may partially define these genes' functions.

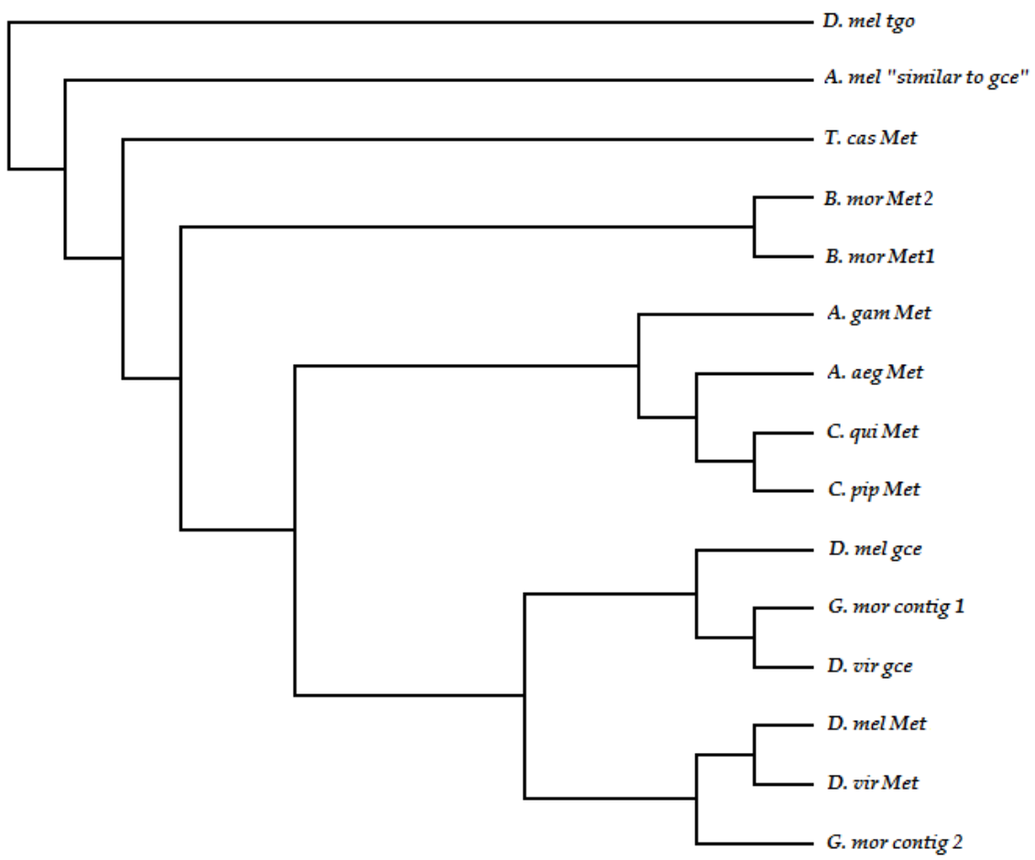

Fig. 2. A gene tree of some holometabolous *Met*-like genes, showing placement of two distinct *G. morsitans* sequences as putative *Met* and *gce* orthologs. *D. melanogaster Tango* (*tgo*), the homolog of the vertebrate *Aryl hydrocarbon receptor* (*Ahr*), is used as an outgroup sequence.

4. Toward a functional definition of *Dmgce*

A functional characterization of *gce*, named for its expression in a subset of embryonic germ cells (Moore *et al.*, 2000), is in its infancy. Column pulldown assays showed MET, in addition to forming homodimers, forms heterodimers with GCE, and addition of JH or either of two JHAs significantly impaired these interactions (Godlewski *et al.*, 2006). It is unknown whether GCE forms homodimers, like MET, or whether GCE can bind JH or its analogs. *GAL4/UAS*-driven (Brand & Perrimon, 1993) overexpression of Met^{+} from *actin* or *tubulin* promoters results in larval lethality in the absence of methoprene (Barry *et al.*, 2008), perhaps by upsetting the stoichiometry of MET and GCE dimers, favoring MET homodimerization at inappropriate times or in inappropriate tissues. Recently, JH was shown to inhibit MET and GCE in *D. melanogaster* by preventing caspase-driven programmed cell death (PCD) and histolysis of the larval fat body. DRONC and DRICE, evolutionarily conserved caspase genes involved in this physiology at the onset of metamorphosis, were shown to be downregulated in *Met* and *gce* deficient flies (Liu *et al.*, 2009). Similarly, methoprene interferes with caspase-driven midgut remodeling in *A. aegypti* (Nishiura *et al.*, 2003; Wu *et al.*, 2006) and *T. castaneum* (Parthasarathy *et al.*, 2008; Parthsarathy *et al.*, 2009), showing that this mechanism of JH action is evolutionarily conserved. It is noteworthy that recombinant MET can repress reporter gene expression in the absence of JH (presumably, MET forms homodimers in this system; Miura *et al.*, 2005); transcriptional repression has previously been reported in other PAS proteins (Dolwick *et al.*, 1993). Therefore, the JH-dependent, stage-specific formation of alternative MET/GCE dimers may have unique regulatory consequences on distinct suites of target genes.

4.1 *Dmgce* substitution for *DmMet*

To evaluate the notion that *gce* might confer viability to *Met* null flies, we manipulated *gce* expression using a binary *UAS/GAL4* system to drive either a *gce* cDNA or an RNAi construct designed to target *gce* transcript. We carried these experiments out in a variety of genotypic contexts in order to examine the effect of *gce* transcript abundance on several methoprene conditional and non-conditional phenotypes (Baumann *et al.*, 2010b). First, we explored the effect of *gce* over- and under-expression on a *Met*-specific non-conditional phenotype that manifests as a variable number of grossly malformed posterior facets of the compound eye (Figure 3). This phenotype is visible in Met^{27} and Met^{w3} flies, and is enhanced in the latter genotype. In our experiments, we found that *gce* overexpression in a Met^{w3} genetic background can rescue the *Met*-specific eye phenotype, suggesting functional overlap of *gce* and *Met*. Notably, when *gce* was overexpressed in a Met^{27} background from the *GawB}dan[AC116]* promoter, targeting transgene expression to the compound eye, the eye phenotype was completely rescued (Baumann *et al.*, 2010b).

The Met^{27} phenotype mimics a set of defects resulting from genetic ablation of the JH-producing corpus allatum (CAX), including a heterochronic shift in EcR-B1 expression in the optic lobe (Riddiford *et.al.*, 2010). Exogenous JH application rescues the entire suite of defects in CAX prepupae, while JH provision to Met^{27} flies rescues only a subset of these defects, suggesting an alternate mechanism of JH signal transduction (Riddiford *et al.*, 2010). Based on our findings that *gce* can substitute for *Met* in the compound eye, further study of GCE involvement in eye development may provide a link between these phenomena. For instance, GCE may partially substitute for MET as a ligand binder to mediate JH signaling when this hormone is supplied in excess.

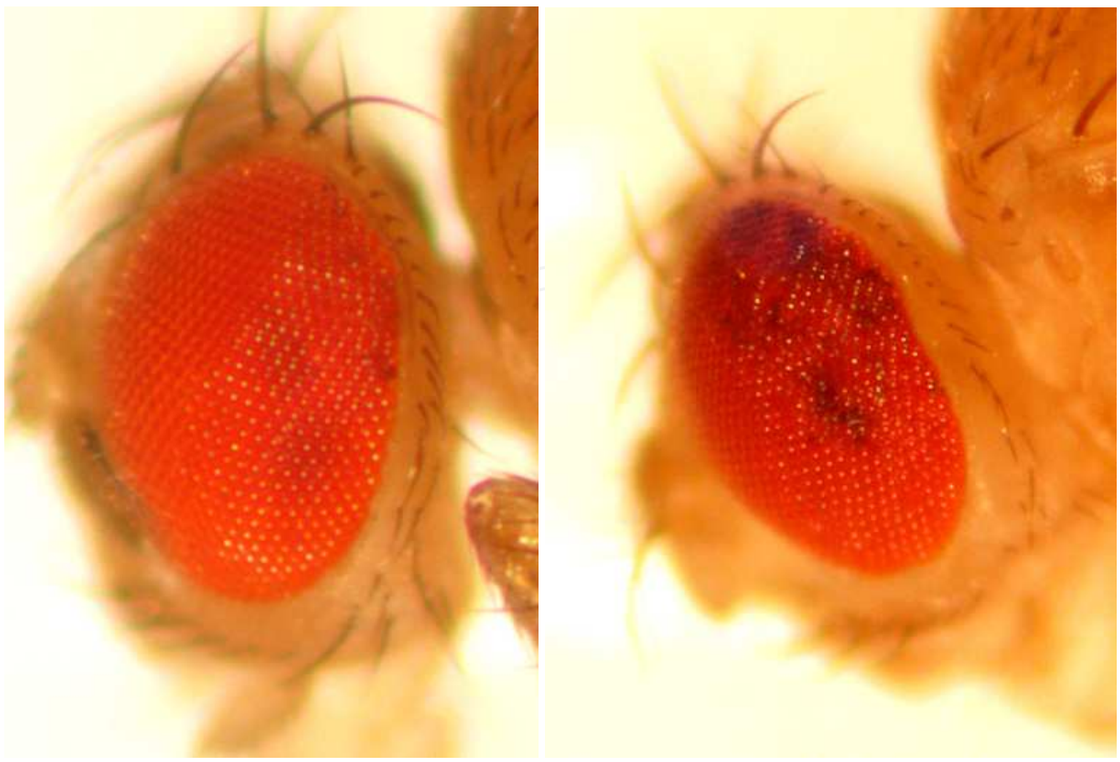

Fig. 3. Left: malformed facets in the posterior compound eye of Met^{w3} flies appear dark under light microscopy. Right: EMS-induced production of an unidentified enhancer gene dramatically intensifies the Met^{w3} phenotype (T.G.W., unpublished).

We also explored the effect of *gce* overexpression on several methoprene-conditional phenotypes. Overexpressed *gce* rescued both the diagnostic malrotation of male genitalia and sensitivity to the toxic effects of methoprene exposure. Sublethal doses of methoprene can induce malrotation of the male genital disc in *D. melanogaster*, resulting in terminalia that are improperly oriented for copulation (Bouchard & Wilson, 1987). Met^{27} males are resistant to this phenotype. We found that global *gce* overexpression in a Met^{27} background rescues blockage of the malrotation phenotype in *Met^{27}; UAS-gce/ tubulin-GAL4* flies. When these flies were exposed to methoprene, we observed malrotation close to levels seen in Met^{+} flies (Baumann *et al.*, 2010b).

Met and *gce* are generally co-expressed in JH target tissues, but we detected insignificant amounts of *gce* transcript in late third instar larval fat body. When *gce* was expressed from a construct targeting expression to this tissue, partial rescue of JH-induced pupal lethality was achived, perhaps as a result of supplying *gce* to a tissue in which its expression is normally depressed at this time in development. *gce* expression in the larval fat body was unable to rescue either the eye phenotype or to prevent methoprene-induced malrotation of the male genitalia, indicating that *gce* substitution for *Met* is tissue specific (Baumann *et al.*, 2010b).

4.2 Functional partitioning of *DmMet* and *Dmgce* in *D. melanogaster* reproduction

Following metamorphosis, the interaction of 20E and JH is crucial in insect reproduction. JH was first isolated in large quantities from the MAG of *Hyalophora cecropia* (Williams,

1956), suggesting a role in male reproductive biology. In *D. melanogaster*, JH controls MAG protein accumulation (Yamamoto *et al.,* 1988) and male *apterous* (*ap*) mutants court females less vigorously than wild-type flies (Tompkins, 1990). In females, the activity of these counteracting hormones is critical for ovarian development and oocyte maturation. Development of the *D. melanogaster* oocyte is under the control of JH through previtellogenic stages 8-9. Female *D. melanogaster apterous*^{4} mutants are sterile owing to reduced levels of JH synthesis (Bownes, 1989); provision of exogenous JH rescues vitellogenic oocyte development in *ap* females (Postlethwait & Weiser, 1973). In *A. aegypti*, JH also controls previtellogenic ovarian development (Clements, 1992). In this case, JH signaling is necessary to promote 20E competence in the fat body, the site of post-blood meal vitellogenin synthesis. In contrast, vitellogenesis is retarded by JH treatment in the gypsy moth, *Lymantria dyspar* (Davis *et al.,* 1990). Thus, there is variation in hormonal control in insects.

GCE clearly compensates for MET deficiency in preadult development (Baumann *et al*., 2010b). In our experiments, over-expressed *gce* failed to rescue both the documented behavior of reduced courtship in *Met*^{27}*; UAS-gce/tubulin-GAL4* males and the reduction in oocyte development and oviposition in these females. Therefore, it appears that excess *gce* cannot compensate for *Met*-induced reduction of reproductive capacity. This result suggests that the functional roles for MET and GCE are incompletely partitioned between preadult development and reproduction in adults.

In *A. aegypti*, Aa*Met* regulates the transcription of several JH target genes in newly eclosed, previtellogenic adult females (Zhu *et al*., 2010). Presumably, the MET-like gene product in lower Diptera serves an analogous function both MET and GCE in JH signaling, but through the action of a single gene. This is perhaps accomplished by virtue of its modular architecture of *DmMet*- and *Dmgce*-specific domains. Higher sequence identity exists between the bHLH and PAS B of *Dmgce* and more primitive holometabolous *Met*-like genes, while the PAS A and PAC domains share higher sequence identity with *DmMet*. These domains may confer a discriminating *Met*-like function that may partially underlie the functional divergence of *Met* and *gce* in higher Diptera.

4.3 *Dmgce* is a vital gene

Overepxression studies demonstrated that *gce* can substitute for *Met* in a tissue specific manner to rescue several preadult *Met* mutant phenotypes. Hence, our results empirically support the notion of functional redundancy between *Met* and its paralog *gce*. To further explore the relationship between *Met* and *gce* in JH signaling, we carried out underexpression studies in *Met*$^{+}$ and *Met* mutant backgrounds by driving the expression of a *gce* RNAi construct.

First, we examined the consequence of *gce* deficiency in a *Met* mutant background under the justification that, if *gce* is responsible for *Met*^{27} viability, then concomitant reduction of *Met* and *gce* could result in lethality. Interestingly, *Met*^{27}*; UAS-gce-dsRNA/tubulin-GAL4* flies died as early pupae (0-2 days), whereas expression of the dsRNA construct from an *actin-GAL4* promoter caused lethality in the pharate adult stage. Next, we assessed the effects of *gce* reduction in *Met*$^{+}$ flies. Surprisingly, *Met*$^{+}$*; UAS-gce-dsRNA/tubulin-GAL4* flies died as pharate adults, indicating that even in the presence of functional MET, *gce* is a vital gene. Driving the transgene from an *actin-GAL4* promoter allowed some degree of adult survival, but these adults were clearly affected by insufficient *gce*, dying within two to three days.

Differential intensity of transgene expression from *actin* and *tubulin* promoters was previously reported in our lab (Barry *et al*., 2008).
gce underexpression had no observable effect on embryonic development, a stage during which no role for JH has been demonstrated. We have shown that *gce* transcription begins after about eight hours in early embryos, in contrast to *Met*, which is supplied as a maternal message (Baumann *et al*., 2010a). The importance of such divergence in temporal expression profiles is unclear.

5. Evolutionary conservation of JH signaling mechanisms

Numerous JH target genes have been identified throughout Holometabola. Importantly, many of these genes are known components of the early 20E response. Table 1 lists some representative JH-inducible genes.

Symbol	Gene name	Molecular function	Reference
JhI-1	JH inducible protein 1	Endoribonuclease	Dubrovsky *et al*., 2000
JhI-26	JH inducible protein 26	Unknown	
Br	Broad-Complex (BR-C)	BTB POZ zinc finger transcription factor	Zhou *et al*., 1998; Zhou & Riddiford, 2002
mnd	Minidisks	Amino acid transmembrane transporter	Dubrovsky *et al*., 2002
JhI-21	JH inducible protein 21	Amino acid transmembrane transporter	
JHE	JH esterase	JH-specific esterase	Kethidi *et al*., 2005
E75A	Ecdysone-induced protein 75B	Heme binding	Dubrovsky *et al*., 2004
E74B	Ecdysone-induced protein 74EF	RNA polymerase II transcription factor activity	Beckstead *et al*., 2007
pepck	Phosphoenolpyruvate carboxykinase	Phosphoenolpyruvate carboxykinase (GTP) activity	
CG14949	CG14949	Unknown	

Table 1. Representative JH-inducible genes. Many of these genes have evolutionarily conserved roles in JH signaling in holometabolous insects. In addition, several are known components of the 20E transcriptional cascade.

The majority of the work done in our lab has been carried out on *D. melanogaster*, in which *DmMet* clearly plays a role in JH signaling: its absence both interferes with methoprene toxicity (Wilson & Fabian, 1986) and hinders JH-driven reproductive physiology (Wilson, 1992; Wilson *et al*., 2003). However, *Met* involvement in metamorphosis has been difficult to demonstrate in *Drosophila* (Riddiford, 2008). As previously stated, JH exposure has no effect on dipteran entry into metamorphosis, unlike other insects (Williams, 1961; Zhou & Riddiford, 2002). In recent years, researchers have turned to the model coleopteran, *T.*

castaneum. These beetles are both amenable to genetic manipulation and gene knockdown owing to the dramatic effects of systemic RNAi, and the larvae of this species are very sensitive to JH, unlike *D. melanogaster* larvae. Exposure to JH or a number of its chemical analogs precipitates supernumerary larval instars, similar to the effects of JH on the model lepidopteran, *Manduca sexta* (Parthasarathy & Palli, 2009). *T. castaneum*, like mosquitoes, has a single *Met-like* gene. In their seminal paper, Konopova and Jindra (2007) demonstrated that RNAi-mediated knockdown of *TcMet* results not only in a methoprene resistance phenotype, but also in the precocious metamorphosis of early instar larvae. A long sought-after result, the genetic reduction of *TcMet* provided the phenotype frustratingly absent in *D. melanogaster*: metamorphic disruption. Reproductive roles for *TcMet* have also been shown; *TcMet* knockdown results in a substantial decrease in vitellogenin transcription, (Parthasarathy, *et al*., 2010) consistent with *Met* deficiency in *D. melanogaster* females (Wilson & Ashok, 1998). These results demonstrate that the single *Met*-like genes in primitive Holometabola function in both development (metamorphosis) and reproduction. Further functional characterization of *TcMe*t (and the single *Met*-like gene of lower Diptera) could lead to a better understanding of how *DmMet* has apparently co-opted reproductive functional roles from a *gce*-like ancestor in higher Diptera

5.1 JH regulation of the E-20 transcriptional cascade

The molecular networks that link JH and 20E signaling pathways form the foundation of multiple aspects of insect physiology, as evidenced by the criticality of both hormones in development, reproduction, and diapause (Zhou & Riddiford, 2002; Soller *et al.,* 1999; Denlinger, 1985). *Broad Complex* (*Broad* or *BR-C*) is an early gene in the 20E cascade that encodes a family of alternatively spliced zinc finger transcription factors (four in *D. melanogaster*, Z1-Z4) fused to a common core protein. Certain *Broad* alleles phenocopy the morphogenetic defects incurred by methoprene exposure in *D. melanogaster*. Wilson *et al* (2006) showed phenotypic synergism in *Met* and *broad* double mutants, demonstrating JH-sensitive MET and BROAD interaction (BROAD protein accumulation is comparable to that of wild type flies, suggesting physical interaction with, rather than transcriptional regulation by *Met*), and providing a link between JH and 20E signaling (Wilson *et al*., 2006).

In a hemimetabolous insect, *Oncopeltus fasciatus*, continuous *Broad* expression directs progressive development through nymphal instars (Erezyilmaz *et al.,* 2006). In Holometabola, *Broad* expression is confined to the prepupal stage, acting as a pupal specifier (Zhou & Riddiford, 2002). Loss of *Broad* expression, characteristic of the *npr1* mutant (*non-pupariating*; a deletion of the entire complementation group), results in the namesake phenotype of failure to enter the pupal program. Consequently, a restriction of *Broad* expression during this developmental stage may have contributed to the evolution of complete metamorphosis. During larval development in *D. melanogaster*, JH represses *broad*. At pupariation, exogenous JH induces a second wave of *broad* expression in the abdominal epidermis, resulting in the deposition of a second pupal cuticle (Zhou & Riddiford, 2002), demonstrating that the networks underlying these signaling mechanisms are complex.

In *T. castaneum*, methoprene exposure induces *Broad* expression and this upregulation is ablated upon *TcMet* knockdown. Therefore, *TcMet* is upstream of *Broad* in JH signaling in these beetles (Konopova & Jindra, 2008). *Krüppel homolog 1* (*Kr-h1*) is upstream of *Broad* in *D. melanogaster* JH signaling, where its expression in abdominal epidermis produces sternal bristle disruption similar to that seen following low dose JHA exposure (Minakuchi *et al*.,

2008). Genetic suppression of *TcKr-h1* induces precocious metamorphosis, similar to *TcMet* deficiency; *TcMet* knockdown in combination with JHA treatment demonstrated that *TcKr-h1* exists downstream of *TcMet* and upstream of *TcBroad* (Minakuchi *et al*., 2009). Similarly, *Kr-h1* upregulation in newly eclosed *A. aegypti* females depends on *AaMet* expression (Zhu *et al*., 2010). Therefore, the relationships among these genes are generally conserved within holometabolan evolution.

While *Kr-h1* also has demonstrated roles in JH-influenced social behavior of honeybees, it has not been reported whether *AmKr-h1* is under the transcriptional control of an *A. mellifera Met*-like protein. However, there is evidence for conservation in the sets of genes regulated by JH between flies and bees. This is perhaps unsurprising given the deep evolutionary conservation of these genetic mechanisms; *Kr-h1* and *Broad* expression profiles in two species of hemimetabolous thrips, whose life histories involve pupa-like, quiescent or non-feeding stages, are compatible with the expression profiles of *Broad* and *Kr-h1* in holometabolous insects (Minakuchi *et al*., 2011).

Microarray data from *D. melanogaster* and *A. mellifera* identified a subset of conserved, JH-inducible genes (Li *et al*., 2007). In the promoter region of 16 of the *D. melanogaster* orthologs, a conserved JH response element (JHRE) was identified. RNAi-driven reduction of the expression of two proteins identified as JHRE binders, *FKBP39* and *Chd64*, inhibits JHIII-induced expression of a reporter construct, suggesting their involvement in JH-dependent transcriptional machinery. Bitra and Palli (2009) demonstrated physical interaction of MET with both ECR and USP. Furthermore, column pulldown assays showed FKBP39 and CHD64 as binding partners of *D. melanogaster* ECR, USP, and MET, providing a more robust framework for a protein complex involving constituents of both JH and 20E signaling pathways (Li *et al*., 2007). *FKBP39*, which is present at the onset of metamorphosis (Riddiford, 2008), is an inhibitor of autophagy in *D. melanogaster*; *FKBP39* overexpression precludes the developmental autolysis of larval fat body cells in wandering third instar larvae (Juhász *et al*., 2007), a physiology shown to be partially dependent on MET/GCE regulation of caspase gene expression (Liu *et al*., 2009). A role for GCE in any of these protein complexes has yet to be reported. *Chd64* is expressed during larval molts, but not in the third instar or during metamorphosis (Riddiford, 2008). Accordingly, putative regulatory complexes consisting of different combinations of these elements may assemble in a stage- or tissue-specific manner. Assembly of differential protein complexes in response to JH, 20E, or both could be a strategy for the tight regulation of the activities of these counteracting hormones.

The *Met*-like genes of *Tribolium* and *Drosophila* appear to act in similar genetic environments to regulate the expression of members of the 20E induced transcriptional cascade, including EcR (Riddiford *et al*., 2010) and USP (Xu *et al*., 2010), the heterodimeric components of the ecdysone receptor, various orphan nuclear receptors involved in 20E activity, and 20E-induced caspase genes involved in PCD (Liu *et al*., 2009). Knockdown of seven nuclear receptors (E75, HR3, EcR, USP, SVP, FTZ-F1, and HR4) results in a significant reduction of vitellogenin prouction in *T. castaneum* (Xu *et al*., 2010), a phenotype similar to that obtained via *TcMet* knockdown (Parthasarathy & Palli, 2009). The data presented in this section therefore strongly support for the action of *Met*-like genes as crucial to 20E/JH crosstalk.

5.2 Discovery of an evolutionarily conserved *Met* binding partner

Recent biochemical data from *A. aegypti* indicate that *AaMet* binds another bHLH PAS gene, *AaFISC*, and that this interaction requires a high JH titer. FISC is a coactivator of EcR/USP

(Chen, J.D., 2000; Zhu, *et al*., 2006), providing yet another link between 20E and JH signaling. The authors also report that coexpression of *DmMet* or *Dmgce* with *DmTaiman* (the *D. melanogaster AaFISC* ortholog) in the presence of JH III induced reporter gene expression in L57 cells (Li *et al*., 2011). Furthermore, this interaction has also been demonstrated in *T. castaneum* between *TcMet* and the FISC/TAI homolog, *TcSRC* (Steroid receptor coactivator; Zhang *et al*., 2011). This gene has previously been implicated in metamorphic activity. *T. castaneum* larvae treated with SRC RNAi fail to achieve critical weight and consequently die before the larval-pupal transition (Bitra *et al*., 2009). Therefore, MET interaction with FISC/SRC/TAIMAN underpins key transcriptional events of JH signaling throughout holometabolous insects.

Structure-function analyses performed using site-directed mutagenesis identified regions of MET that are necessary for homodimerization and GCE binding. Point mutations in the bHLH and PAS A domains (Met^{1} and Met^{w3} alleles, respectively) had no effect on partner binding, whereas N- and C-terminal truncations, deletions in the HLH or PAS A domains, and a point mutation in the PAS B domain (Met^{128} allele) all inhibited dimerization (Godlewski, *et al.,* 2006). Structure-function data for AaMET and AaFISC binding illustrates that the criticality of PAS domains for protein-protein interaction. Interestingly, two-hybrid assays showed that MET/FISC interaction increased when AaMET lacked a bHLH domain (Li *et al*., 2011). Therefore, this domain is unnecessary for MET-FISC interaction, suggesting that the sole function of the *AaMet* bHLH may be in DNA binding. In contrast, deletion of the bHLH domain in FISC hindered the JH-induced interaction with AaMET.

Mixespression of *DmTaiman* in a variety of *Met*/*gce* genetic backgrounds will be valuable from both physiological and evolutionary perspectives. How do these proteins interact in the context of hormonal control of *D. melanogaster* development? Presumably, during larval development JH secreted from the CA inhibits MET and GCE interaction while promoting MET and GCE binding with TAIMAN. Are MET:TAI and GCE:TAI dimers functionally congruent in *D. melanogaster* or do these complexes preferentially regulate disparate target genes? Is the *Met*-like gene in *A. aegypti* and *T. castaneum* functionally analogous to *Met*/*gce* or are other, unidentified proteins involved? How has the interaction of these proteins changed during dipteran evolution following the origin of *Met*?

6. Conclusions

RT-PCR analysis with degenerate primers identified a single *Met*-like homolog in the genome of each of the three mosquito species, *Aedes aegypti, Anopheles gambiae*, and *Culex pipiens*. Likewise, a single *Met*-like ortholog exists in the beetle, *T. castaneum*, (Konopva & Jindra, 2007). Phylogenetic analysis and comparison of intron number and position in each of the identified mosquito genes indicates that the mosquito *Met* orthologs share higher sequence identity with *Dmgce* than *DmMet*, suggesting that *DmMet* arose from the duplication of an ancestral, *gce*-like gene in lower Diptera. To examine the evolutionary history of *Met* and *gce* within the Diptera, we mined the public *G. morsitans* EST library, recovering unique putative *Met* and *gce* orthologs in this fly, showing conservation of a *Met* homolog within the Schizophora. We also recently isolated a putative *gce* homolog from a Bombyliid, *Bombylius major* (A.A.B., unpublished). Taxonomically, this group of flies exists in the Asilomorpha, a paraphyletic sister taxon to the Muscomorpha within the dipteran infraorder, Brachycera. While this study is in its preliminary stages, only a single *Met*-like gene has thus far been obtained from this fly using degenerate PCR with cDNA and

genomic DNA templates, suggesting the possibility that the *Met/gce* duplication occurred within the Brachycera. *Met* function is evolutionarily conserved in Diptera; consistent with independent reports (Zhu *et al*., 2010) we observed that RNAi-driven reduction of *AaMet* results in concomitant reduction of JH-inducible genes (Figure 4).

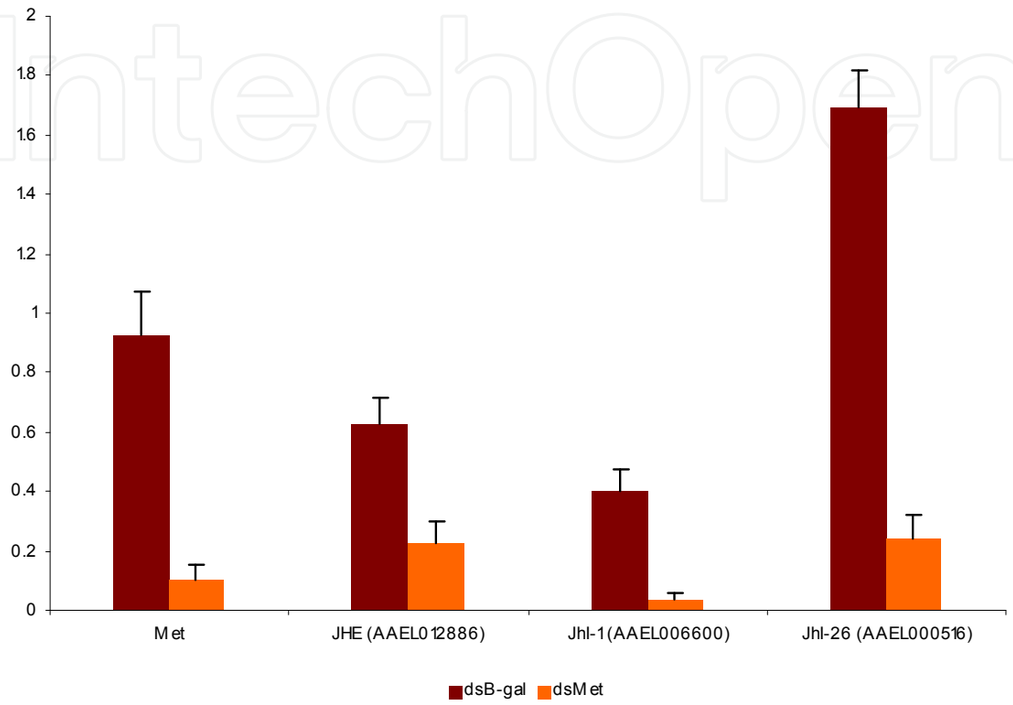

Fig. 4. Expression of three JH-inducible genes following RNAi-induced knockdown of *AaMet* (ds*Met*: orange bars) vs. controls (ds*B-gal*: red bars). *AaMet* reduction produced concomitant suppression of the *A. aegypti* homologs of *DmJHE*, *DmJhI-1*, and *DmJhI-26*.

Analysis of the nonsynonymous-to-synonymous substitution ratios (dN/dS) of *Met* and *gce* orthologs within the genus *Drosophila* indicates a substantial relaxation of selective constraint on the C-terminal half of *gce*, downstream of the functional domains. Conversely, nonsynonymous substitutions in the N-terminal half are stringently selected against. Depressed dN/dS values across the *Met* coding sequence indicate strong selective constraint over the entire open reading frame (Baumann *et al*., 2010b).

RT-PCR analysis of selected *D. melanogaster* tissues shows that *gce* is generally co-expressed with *Met* in known JH target tissues, including ovary and MAG. Overexpression of *gce* in a *Met* mutant background results in a dramatic enhancement of methoprene-conditional toxic and morphogenetic defects, similar to those seen in wild type (Met^{+}) flies after methoprene exposure. *Met* mutant flies overexpressing *gce* show rescue of a non-conditional adult phenotype, that of defective development of posterior facets in the compound eye. Our results therefore support the notion of functional redundancy that has been hypothesized to account for Met^{27} viability flies. On the other hand, we have also shown that these paralogs have undergone evolutionary subfunctionalization since their origin; *gce* overexpression

fails to rescue the phenotypes of deficient oogenesis or reduced male courtship characteristic of *Met* adults, showing that *Met* has co-opted therole as the mediator of JH-regulated reproductive functions in *Drosophila*.

RNAi-driven reduction of *gce* expression from either an *actin* or *tubulin* promoter demonstrates that unlike *Met*, *gce* is a vital gene. G*ce* underexpression in both *Met*$^{+}$ and *Met* genetic backgrounds results in lethality. *Met*$^{+}$ *; UAS-gce-dsRNA / tubulin-GAL4* homo-/hemizygotes do not survive to adulthood, and die primarily at the pharate adult stage, while the same *gce* RNAi construct expressed in a *Met* mutant background shifts lethality to early pupae (Baumann *et al*., 2010a).

6.1 Directions

Previously, USP was proposed as a candidate JH receptor (Jones, *et al*., 2001). Yet, USP only binds JH with micromolar affinity, requiring a hormone concentration that exceeds endogenous titers by orders of magnitude (Bownes & Rembold, 1987). It is now known that USP binds methyl farnesoate (MF), a precursor in the biological synthesis of JH III (Figure 1), with nanomolar affinity both in *D. melanogaster* and in *A. aegypti* (Jones *et al*., 2006; Jones *et al*., 2010). Recent studies on natural farnesoid derivatives including MF, JH III, and JHB3 (the main farnesoid secretion product of dipteran ring glands cultured *in vitro*) have teased out the relative activities of each of these compounds during development in a series of biological assays. Two recent studies have demonstrated that the activity series of these three compounds changes during development. Dietary MF and JH III (MF > JH III) were both more active than JHB3 in delaying larval attainment of the wandering stage. In contrast, JH III applied to prepupae (white puparial assay; Riddiford & Ashburner, 1991) showed much higher activity than MF or JHB3 in blocking adult eclosion (Jones *et al*., 2010; Harshman *et al*., 2010).

Topical application or dietary provision of these compounds adds to endogenous hormone titers. Therefore, just as USP binds JH III at concentrations exceeding physiological levels, it is possible that MET nonspecifically binds MF or JHB3 under these conditions. It is an intriguing proposition that MET and USP, which interact both with each other and JHRE binding proteins (Bitra & Palli, 2009), may partner in a stage-specific manner throughout development in response to a fluctuating mélange of methyl farnesoids. Does GCE participate in the assembly of the molecular machinery that facilitates the crosstalk between JH and 20E signaling? This protein has been largely ignored in studies regarding the molecular interaction of these hormones. Further, it is unknown whether GCE binds any of the farnesoid products of the CA. There appears to be a correlation between the presence of paralogous *Met*-like genes and multiple JH isoforms in higher Diptera. If each of the farnesoids JH III, MF, and JHB3 indeed has a unique receptor protein, the possibility arises that GCE fills the role of JHB3 binder. Or perhaps in MET/GCE dimers, MET is the sole ligand binder, while GCE and MET are both necessary for target gene transcription. Clearly, further functional characterization of GCE is necessary to unravel the mechanisms through which JH signaling has evolved from the basal holometabola to the most evolutionarily diverged insects, the higher Diptera.

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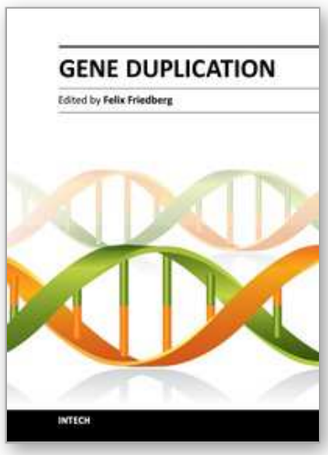

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The book Gene Duplication consists of 21 chapters divided in 3 parts: General Aspects, A Look at Some Gene Families and Examining Bundles of Genes. The importance of the study of Gene Duplication stems from the realization that the dynamic process of duplication is the "sine qua non" underlying the evolution of all living matter. Genes may be altered before or after the duplication process thereby undergoing neofunctionalization, thus creating in time new organisms which populate the Earth.

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