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Overexpression of *Methoprene-tolerant*, a *Drosophila melanogaster* gene that is critical for juvenile hormone action and insecticide resistance

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Abstract

The *Methoprene-tolerant* (*Met*) gene of *Drosophila melanogaster* is involved in both juvenile hormone (JH) action and resistance to JH insecticides, such as methoprene. Although the consequences of *Met* mutations on development and methoprene resistance are known, no studies have examined *Met*$^+$ overexpression. *Met*$^+$ was overexpressed in transgenic lines with various promoters that drive overexpression to different levels. Flies expressing either genomic or cDNA *Met*$^+$ transgenes showed higher susceptibility to both the morphogenetic and toxic effects of methoprene, consistent with the hormone-binding property of MET. Both the sensitive period and lethal period were the same as seen for non-overexpressing *Met*$^+$ flies. However, continual exposure of high-overexpressing *Met*$^+$ larvae to borderline-toxic or higher methoprene doses advanced the sensitive period from prepupae to first instar and the lethal period from pharate adults to larvae and early pupae. When expression of transgenic *UAS-Met*$^+$ was driven to high levels by either an *actin-GAL4* or *tubulin-GAL4* promoter, larvae showed high mortality in the absence of methoprene, indicating that high MET titer is lethal, perhaps resulting from expression in an inappropriate tissue. Adults overexpressing *Met*$^+$ did not show enhanced oogenesis, ruling out MET as a limiting factor for this hormone-driven physiology.

Keywords

Methoprene; *Met* gene; insecticide resistance; hormone action

1. Introduction

Insect development and reproduction are hormonally regulated by the steroid hormone 20-hydroxyecdysone and the sesquiterpenoid juvenile hormone (JH). JH is essential for a diverse array of functions, including maintenance of larval development (Riddiford, 1994; Riddiford, 1996), adult oogenesis, and male accessory gland function (Wyatt and Davey, 1996). Roles for JH, specifically JH III (which will be referred to in this paper as “JH”), in *Drosophila melanogaster* are best understood in adults, especially during reproduction (Bownes, 1989; Riddiford, 1993; Soller et al., 1999; Dubrovsky et al., 2002; Flatt et al., 2005). JH has been shown to be necessary for the endocytotic uptake of vitellogenin by oocytes (Postlethwait and

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Weiser, 1973; Kambysellis and Heed, 1974; Wilson, 1982; Soller et al., 1999) and is involved in female receptivity (Kerr et al., 1997). In male *D. melanogaster*, JH promotes the synthesis of accessory gland protein (Yamamoto et al., 1988; Shemshedini et al., 1990). Examples of other roles for JH in adults include an acceleration of the autolysis of the larval fat body during the first 2 days of adult life (Postlethwait and Jones, 1978; Wilson, 1982) and an intriguing involvement in life-span (Tatar et al., 2001). The hormone acts to regulate gene expression at the transcriptional level in various insects, including *D. melanogaster*, but only a few genes that are directly regulated have been identified (Dubrovsky et al., 2000; Li et al., 2007). Over the past four decades, insecticidal JH analogs (JHAs), such as methoprene, have appeared (Staal, 1975; Dhadialla et al., 1998) and shown to be effective against certain insects, including *D. melanogaster*. It is clear from numerous studies that methoprene and perhaps all JHAs act as JH agonists (Wilson, 2004). When applied to *D. melanogaster* at the onset of metamorphosis, these insecticides act to disrupt metamorphosis of both external and internal tissues (Ashburner, 1970; Madhavan, 1973; Postlethwait, 1974; Wilson and Fabian, 1986; Riddiford and Ashburner, 1991; Restifo and Wilson, 1998). Metamorphic disruption may result from misexpression of secondary-response genes, resulting in lethality or morphogenetic defects of sternal bristle patterns and malrotation of the male genitalia during pupal development (Restifo and Wilson, 1998; Zhou and Riddiford, 2002).

The *Methoprene-tolerant* (*Met*) gene was identified from a screen of mutagenized *D. melanogaster* as conferring resistance to both the toxic and morphogenetic effects of methoprene (Wilson and Fabian, 1986). *Met* was shown to confer resistance to application of the endogenous hormone, JH III (Wilson and Fabian, 1986), and to a more powerful JHA, pyriproxyfen (Riddiford and Ashburner, 1991), but not to other classes of insecticides (Wilson and Fabian, 1986). Judging from the ability of MET to bind JH (Shemshedini and Wilson, 1990; Miura et al., 2005; Konopova and Jindra, 2007) and the involvement of *Met* in JH-regulated physiology, such as male accessory gland development or oogenesis (Shemshedini et al., 1990; Ashok et al., 1998; Wilson and Ashok, 1998; Miura et al., 2005), we believe that *Met* participates in the action of endogenous and exogenous JH III, possibly as a component of a JH receptor. Recently, *Tribolium castaneum* was shown to display precocious metamorphosis following RNAi suppression of expression of the *Met* ortholog of this beetle (Konopova and Jindra, 2007), further demonstrating the involvement of this gene in JH action during molting.

Cloning and sequence analysis of *Met* identifed the gene as a member of the bHLH-PAS transcription factor family. bHLH-PAS proteins in both vertebrates and invertebrates play important roles during development and as transcriptional regulators in response to environmental signals such as light, sundry environmental chemicals, and hypoxia (Gu et al., 2000). For example, the dioxin receptor partners AHR and ARNT act as a ligand-activated heterodimeric transcription factor to regulate gene expression in response to xenobiotic chemicals (Hankinson, 1995). Likewise, MET has been hypothesized (Ashok et al., 1998) and shown (Miura et al., 2005) to be capable of transcriptional regulation. Thus, *Met* is of interest both as a gene with probable involvement in JH action and also in JH-insecticide resistance.

Isolation and characterization of Met^{27}, a null allele, showed the consequences of loss of MET from flies. In addition to JH resistance, oogenesis in Met^{27} females is reduced to about 20% of wild-type (Wilson and Ashok, 1998). Males have reduced protein accumulation in the male accessory glands, and they court and mate wild-type females much less avidly than do Met^{+} or Met^{27}; Met^{+} transgenic males (Wilson et al., 2003). Thus, the Met^{27} mutation has provided insight into the roles of both Met^{+} and JH in this insect.

Studies with *D. melanogaster* having overexpressed Met^{+} should give insight into the consequences of excess MET in the physiology of this insect. Therefore, genetic construction

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of transgenic *Met*$^+$ flies showing overexpression driven by different promoters was carried out. Analysis of the resultant strains showed enhanced sensitivity to methoprene. High overexpression resulted in larval death, and in the presence of methoprene, dramatically advanced both the methoprene-sensitive period and resultant lethal period in development. However, oogenesis was not appreciably affected, suggesting that MET is not a limiting factor in this physiology in wild-type flies.

2. Materials and methods

2.1. Drosophila strains

Isolation and molecular analysis of the *Met* alleles have been previously described (Wilson and Ashok, 1998; Wilson et al., 2006a). The *w; p[cDNA]* and *w; p[EN 71]* are *Met*$^+$ transgenic strains carrying either a *Met*$^+$ cDNA or *Met*$^+$ genomic fragment (termed *EN 71*) whose construction has been described (Ashok et al., 1998).

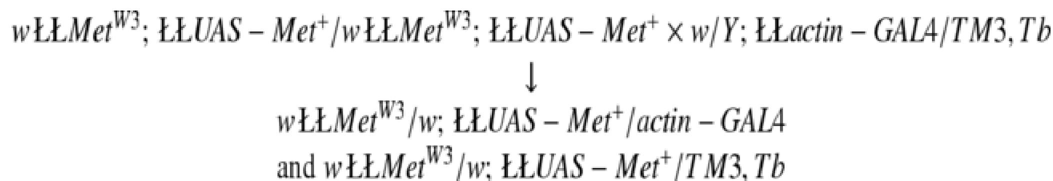
Two novel *Met*$^+$ transgenic stocks, *w; p[V2]* and *w; UAS-Met*$^+$, were constructed. Genomic DNA was extracted from Oregon-RC flies (Qiao and Raymond, 1995). Genomic *Met*$^+$ was amplified by PCR following synthesis of a forward primer 5′-AGG CGC CAA TTA AAG GGG AA-3′, located 92 bp upstream of the ATG start codon, and a reverse primer 5′-GTG AGC TAC CAA TTA CGT CCA-3′, located 66 bp downstream from the TGA stop codon. The amplification procedure was as follows: The initial denaturation step was at 94°C for 3 min. Amplifications were achieved through 35 cycles at 94°C for 30 s, 55°C for 45 s, and 72° C for 150 s. A final extension reaction was carried out for 10 min at 72°C. The obtained 2.3kb PCR-fragment was cloned into the pCR2.1-TOPO vector (Invitrogen), sequenced and inserted into *Eco*RI sites (since *Met* does not contain an *Eco*RI site) of the pUAST transformation vector (Brand and Perrimon, l993). Proper orientation of the fragment in the vector was verified by restriction site analysis and sequencing.

Germline transformation of *w* or *w Met*^{W3} embryos with purified plasmid together with "wings clipped" helper plasmid *pπ25.7* (Rubin and Spradling, 1982) in a ratio of 2-3:1 was carried out by injection into dechorionated embryos. Transformant flies were recognized by partial restoration of eye color and (if *w* embryos were transformed) crossed into a *w Met*^{W3} line to test for functionality of the *Met*$^+$ transgene, which rescues the eye defect phenotype (Wilson et al., 2006a) produced by this *Met* allele. 18 independent transformant lines were isolated and tested for *Met*$^+$ function. When grown at 25 °C, most of the lines weakly expressed *Met*$^+$, which was not unexpected since the *pUAST* vector is designed for *GAL4* expression and has only a weak *hsp70* promoter (Brand and Perrimon, l993). However, one of the transformant lines, *p [V2]*, strongly expressed *Met*$^+$ independent of a *GAL4* driver, presumably because the *Met*$^+$ transgene inserted downstream of a strong promoter of an unknown gene.

For RT-PCR, total RNA was extracted from whole flies using TRIzol (Invitrogen) and treated with RNase-free DNase-I (Invitrogen). Aliquots of 5 μg total RNA were reverse transcribed with 200 U/ml of MMLV reverse transcriptase (Invitrogen) and 1 μg random hexamers (Invitrogen) in a 25 μl reaction mixture. The cDNA was amplified by PCR in a 25 μl reaction mixture consisting of 2.5 μl of 10X PCR buffer, 1 μl *Taq* DNA polymerase (Invitrogen), 1 μl of 10 mM dNTP mix, 1μl of 50 mM mM $MgCl_2$, 1 μl each of 10 mM primer, 1 μl of cDNA, and 16 μl of H_20. PCR primers used were for *Met* were 5′-CAGAGCAGCAGTCCCGATTT and 3′-CCATCGTCCATTAGGCTTTCCA. The primers for *Rp49* were 5′-CCGCTTCAAGGGACAGTATC and 3′-ATCTCGCCGCAGTAAACG. Ten μl of each PCR product was electrophoresed on a 1% agarose gel and stained with ethidium bromide. An image of the gel was capture with the ImageQuant 400 (GE Healthcare) and then analyzed using the ImageQuant TL software (Amersham Biosciences). PCR products were cloned into TOPO vector (Invitrogen) and sequenced with M13 forward primer.

2.2. Met+ overexpression in GAL4-UAS transgenic strains

The *GAL4-UAS* system in *D. melanogaster* allows directed expression of a *UAS*-linked transgene by a specific selectable promoter that is ligated to the yeast *GAL4* transcriptional regulator (Brand and Perrimon, l993). Simple genetic crosses serve to bring the two transgenes together to control expression. The four independent *Met*+ transformant lines were made homozygous for the *UAS Met*+ transgene. Each of these lines represents independent insertions of the transgene into an autosome. Each line was crossed with flies carrying either an *actin-GAL4* or *tubulin-GAL4* transgene heterozygous to a *TM3* or *TM6* balancer chromosome carrying either a *Tubby (Tb)* or *Stubble (Sb)* dominant mutation, allowing ready identification of adults (*Sb*) or larvae (*Tb*) carrying the balancer chromosome. Both *tubulin* and *actin* genes are expressed widely in *D. melanogaster* tissues, so either *GAL4* transgene should drive *UAS-Met*+ expression throughout development in perhaps all tissues.



A similar cross with *tubulin-GAL4/TM6, Sb* allows this promoter to drive *UAS-Met*+ expression. Note that F_1 progeny carrying the *TM3* balancer chromosome lack *actin-GAL4* and therefore serve as control flies.

2.3. Methoprene resistance

For determination of the median effective doses (ED_{50}), cultures were assayed for methoprene sensitivity on each of 4 or 5 doses of methoprene (isopropyl-(2*E*,4*E*)-11-methoxy-3,7,11-tri-methyl-2,4-dodecadienonate, ChemService, PA), applied as ethanolic solutions (25 µl) to the surface of each culture (food surface area 3.8 cm^2) in diagnostic amounts consisting of 0.27, 0.54, 2.7, 5.4, and 26.8 µg/vial. Mortality occurs typically during the pharate adult stage; survivors were examined for methoprene-induced morphogenetic defects commonly seen in dipteran insects consisting of malrotated male genitalia and defective sternal bristles, particularly on the posterior sternites (Wilson and Fabian, 1986). The survival data were used to calculate the ED_{50} values using the PriProbit program (Sakuma, 1998).

2.4. Oviposition

Females were isolated within 8 h after eclosion and allowed to age for 6 days in the presence of wild-type (Oregon-RC) males and baker's yeast sprinkled on the food surface to maximize oogenesis. They were then placed individually in 28 × 95-mm plastic food vials (Capitol, Fonda, NY) in the continued presence of yeasted food and males. Eggs were counted during the next 4 days, a time period when the ovipositional rate reaches a maximal steady-state value and allows strain comparison (Wilson and Ashok, 1998). Egg fertility was noted, and vials having unfertilized eggs were discarded because unmated females are less fecund.

3. Results

3.1. Verification of transgenic stocks

Three transgenic stocks were created in *w Met*^{W3} genetic backgrounds following *P*-element-mediated transformation with the *Met*+ constructs. The first construct, termed *p[EN71]*, includes a genomic fragment carrying sequence comprising the *Met*+ 5.5 kb transcript plus 0.3 kb of upstream and 1.7 kb of downstream sequence and showing autonomous expression, as expected (Ashok et al., 1998). Two *Met*+ cDNA transgenic stocks, termed *p[V2]* and *p*

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[cDNA], also showed autonomous expression, probably resulting from insertion of each transgene into an unidentified promoter region. All three stocks were verified as expressing functional *Met*$^+$ by (1) loss of resistance following methoprene treatment of cultures (Ashok et al., 1998) and (2) rescue of the non-conditional *Met* eye phenotype readily expressed in the *Met*^{W3} allele (Wilson et al., 2006a).

3.2. Expression of Met$^+$ in w; p[Met$^+$] transgenic strains

To maximize *Met*$^+$ expression, the *Met*$^+$ transgene in each strain was genetically inserted into a *w* genetic background by backcrosses with *w* flies, which express a *Met*$^+$ allele (Ashok et al., 1998) and therefore contribute endogenous *Met*$^+$ transcript in addition to that expressed by the transgene. Following the creation of *Met*$^+$ transgenic homozygotes by standard chromosomal manipulation, *Met*$^+$ transcript levels in each stock were analyzed by RT-PCR. As shown in Table 1 and Figure 1, both *w; p[EN71]* and *w; p[cDNA]* express levels as high as 3.4-fold that of *w*, and *w; p[V2]* shows higher levels, 5.9-fold in larvae and 3.0-fold in adults. Therefore, the transgenic stocks offer a range of expression levels to evaluate overexpression of both genomic and cDNA *Met*$^+$.

3.2. Evaluation of Met$^+$ transgenic strain phenotypes

Resistance to toxicity—Each of the three transgenic strains was evaluated for levels of methoprene susceptibility following exposure to various amounts of methoprene applied to the surface of the larval diet, previously shown to be an effective method of exposure (Wilson, 1996). *w; p[EN71]* and *w; p[cDNA]* showed higher susceptibility than either *w* or the wild-type strain Oregon-RC, and, in agreement with the *Met*$^+$ transcript levels, *w; p[V2]* showed the highest level (Table 2). The increases in susceptibility were reflected not only by the survival values but also by the increased fraction of adults from cultures exposed to a lower sublethal dose that showed methoprene-induced morphogenetic defects, such as sternite bristle defects or malrotated male genitalia. The susceptibility value for *w; p[V2]* represents the highest susceptibility seen in our laboratory or reported for any *D. melanogaster* strain. Therefore, the overexpression of *Met*$^+$ transcript by these strains is also reflected by overexpression of functional *Met*$^+$ gene product.

Preadult development—The effect of *Met*$^+$ overexpression on preadult development was examined in the transgenic strain showing the highest *Met*$^+$ levels, *w; p[V2]*. Egg hatch was good for each strain (Table 3), showing little effect of *Met*$^+$ overexpression on embryonic development. Newly hatched (0–2 hrs) larvae from cultures of either *w* or *w; p[V2]* were carefully transferred to new food vials, and survival and time-to-pupariation measured for each strain. Although a significant difference was found between the two strains for development time, no differences were evident for survival (Table 3). Therefore, this high level of *Met*$^+$ overexpression does not appear to be markedly detrimental to preadult development.

Previous studies have shown that the sensitive period in dipteran insects for applied methoprene or JH III is a 12-hr window centered at pupariation, but the morphogenetic and toxic effects are not evident until the end of pupal development, 4–5 days later at the pharate adult stage (Postlethwait, 1974). To determine if *Met*$^+$ overexpression can alter this period of sensitivity and/or response to applied hormone, newly hatched (0–2 hrs) *w; p[V2]* larvae were exposed continuously to one of three diagnostic doses of methoprene that produced typical pharate adult lethality in control *w* animals treated in an identical manner. Cultures of *w* showed good larval survival and pupariation, and death at the pharate adult stage, as expected. However, *w; p [V2]* showed poorer larval survival, especially at the 26.8 μg/vial methoprene dose (Figure 2). Larval death was not rapid; some larvae died within a day of transfer as small larvae on the vial wall, whereas other larvae fed and increased in size for several days, then began dying as third-instar size. At all three doses, pupariation of surviving larvae occurred, but they usually

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did not survive to the pharate adult stage as did *w* pupae. At the 26.8 μg dose, prepupae were often misshaped and failed to pupate. At 2.7 μg, 25–30% of the pupae developed into abnormal-appearing pharate adults at this lowest methoprene dose. Therefore, when Met^+ is grossly overexpressed, not only are the flies more sensitive to methoprene, but at higher doses the sensitive period for methoprene pathology is also altered, resulting at the 26.8 μg dose in larval death beginning within 1 day following the onset of exposure and continuing until late third-instar. At a lower lethal methoprene level (2.7 μg), pupariation usually occurred, and death was seen in both early pupae and pharate adults.

3.3. Met⁺ overexpression in GAL4 transgenic strains

Although Met^+ is overexpressed in the three transgenic strains described above, the temporal and tissue specificity of expression is unknown and uncontrollable. The *GAL4-UAS* system allows expression of a *UAS* transgene by the specified promoter carried in a *GAL4* transgene (Brand and Perrimon, l993). By the choice of promoter-*GAL4* line that is genetically crossed to the *UAS*-transgene line, the temporal and/or tissue-specific expression of the *UAS*-transgene can be controlled. Three independent *UAS*-Met^+ transgenic strains were constructed and crossed individually with lines carrying either *actin* or *tubulin* promoter *GAL4* transgenic lines to express the *UAS*-Met^+ transgene. Both *actin* and *tubulin* genes are widely expressed in tissues, thus their promoters should drive Met^+ expression throughout development in perhaps all tissues.

Progeny from crosses of each line were examined for abnormal development that could be attributed to overexpressed Met^+. In the *w; UAS*-Met^+/*tubulin-GAL4* progeny, egg hatch was good (84%), but larvae began dying a day later and rarely pupariated and survived to adulthood. Dead larvae were seen either on the vial walls (younger larvae) or on the surface of the food in older larvae. Three-to-four day-old *w; UAS*-Met^+/*tubulin-GAL4* larvae were identified by their non-*Tubby* phenotype, carefully transferred to new food vials, and observed daily for 3–4 days. They grew little and generally died within 2 days. Each of the three *w; UAS*-Met^+ lines, as well as *w; p[V2]*, responded in a similar manner when crossed with *w; tubulin-GAL4* (data not shown). Control *w; UAS*-Met^+/*TM3* siblings transferred in a similar manner developed to typical third-instar size, pupariated, and eclosed with good survival, ruling out a transfer problem with *w; UAS*-Met^+/*tubulin-GAL4* larvae.

Overexpression was examined in a similar manner by crossing *w; UAS*-Met^+/flies to flies carrying an *actin-GAL4* transgenic driver. Survival to adulthood of larvae was found at low levels (<5%) in each of the three lines, indicating that the *actin* promoter does not drive expression of *UAS*-Met^+ as strongly as the *tubulin* promoter. Since the *w; p[V2]* strain has the Met^+transgene in the *pUAS vector*, these females were crossed with *w;/actin-GAL4* males to examine overexpression driven by *actin-GAL4* in this strain. No pupae were found in cultures of *w; p[V2]/actin-GAL4*progeny, indicating that these larvae responded more strongly to the *actin-GAL4* driver than did the other *w; UAS*-Met^+strains.

To examine functionality of the *UAS*-Met^+ transgene expressed in this manner in adults, *w* Met^{W3}; UAS-Met^+ females from each of the three strains were crossed with *w; actin-GAL4/TM3* males, and non-*TM3* F1 progeny males (hemizygous for the Met^{W3} mutation) examined for rescue of the eye phenotype. Each *w* Met^{W3}; UAS-Met^+/*actin-GAL4* male failed to display the eye phenotype shown by *w* Met^{W3}; UAS-Met^+/TM3 sibling males, demonstrating that functional MET, driven by *actin-GAL4*, was produced in the flies.

3.4. Oviposition

JH is necessary for vitellogenic oocyte development in *D. melanogaster* (Postlethwait and Weiser, 1973; Postlethwait and Handler, 1978). Previously, *Met* was shown to be involved in

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oogenesis as reflected by depressed oviposition and oogenesis in the *Met*^{27}null mutant but not in transgenic *Met*^{27}; *Met*$^{+}$ sibs (Wilson and Ashok, 1998). To determine if elevated MET results in altered, perhaps elevated oogenesis, oviposition was measured in *w; p[V2]* females during a 4-day period beginning 8 days after eclosion and compared with that of *w* females. No significant differences could be detected between the strains (Table 4). To determine if the transgenic females were producing extra oocytes but accumulating instead of ovipositing them, ten 8-day-old females were dissected from each strain and the stage-14 (mature) oocytes censused (Table 4). Again, no significant differences between the strains could be found, showing no enhancement of oogenesis in the *Met*$^{+}$ overexpressing strains.

Likewise, exceptional survivor *w; UAS-Met*$^{+}$*/actin-GAL4* males and females were isolated shortly after eclosion and found to be fecund and fertile when set up on fresh food.

4. Discussion

This work has examined the effects of overexpression of the *Met*$^{+}$ gene in *D. melanogaster*. This gene is involved in two related processes: action of endogenous JH, possibly as a component of a JH receptor (Shemshedini and Wilson, 1990; Miura et al., 2005), and resistance to JH analog insecticides (Wilson and Fabian, 1986; Konopova and Jindra, 2007). MET has been shown to bind JH (Shemshedini and Wilson, 1990; Miura et al., 2005), to be expressed in known JH target tissues (Pursley et al., 2000), and to have a structure (a bHLH PAS transcription factor) that is compatible with hormone action (Ashok et al., 1998). *Met*$^{+}$ transcriptional control of a reporter gene has been demonstrated (Miura et al., 2005). A homologous gene, *germ cell expressed* (*gce*) has about 70% homology to *Met* and appears to be the sole *Met*-like gene in lower Diptera (Wang et al., 2007) and perhaps other insects. MET physically binds with GCE in *D. melanogaster* cells (Godlewski et al., 2006), suggesting a functional heterodimer. The *Met*^{27} null mutant does not show the lethality that one might expect from a JH receptor mutant (Wilson and Ashok, 1998); either *gce* (or some other gene) can rescue the lethality as a redundant gene or JH is not required for vital functions in *D. melanogaster*.

Recently, it was demonstrated in *D. melanogaster* L57 cells that MET interacts with two proteins, FKB39 and Chd64, that bind to a *D. melanogaster* JH response element (Li et al., 2007). It was further demonstrated that these proteins interact with USP and EcR, the component proteins of the ecdysone receptor. MET may facilitate cross-talk between these two hormonal signaling pathways through the ecdysone receptor. Perhaps the larval death seen when *Met* is overexpressed reflects the corruption of proper stoichiometric amounts of the signaling complex components necessary for molting or other larval physiology.

Since MET can bind JH (and presumably JH analog insecticides such as methoprene), the increase in methoprene susceptibility seen in the overexpressed strains is perhaps not surprising. The extent of this increase was somewhat proportional to the overexpression level in the various strains, and it reached a level of about 10-fold over *w* in the *w; p[V2]* strain. We believe that the toxicity and morphogenetic effects of JH agonist insecticides result from mis-expression of certain genes of unknown identity during metamorphosis (Wilson et al., 2006b). JH is present in low levels in *D. melanogaster* pupae (Bownes and Rembold, 1987; Sliter et al., 1987), and application of JH or methoprene serves to disrupt regulation of one or probably several genes at this time, resulting in the pathology, both vital and morphological, seen when it is exogenously applied. Since a JH receptor must be involved in the regulatory effect, then increasing the titer of a JH-binding component increases the susceptibility to the disruptive influence of JH. Another possible explanation relies on the observation of both MET:GCE and MET:MET dimer formation in *D. melanogaster* cells (Godlewski et al, 2006). Perhaps an increased titer of MET drives the equilibrium to form homodimers instead

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of heterodimers that may be required at a certain time(s) in development or disrupts the ecdysone-JH signaling complex proposed by Li et al (2007), and the consequences are detrimental to development and survival.

JH has been shown to be involved in oogenesis in many insects, including *D. melanogaster*. Oogenesis and oviposition in the Met^{27} mutant is decreased to about 20% of wild-type (Wilson and Ashok, 1998), reflecting the role of *Met* in these processes. A change in oogenesis (enhancement) in the overexpressing females might be anticipated, given the effect seen on methoprene susceptibility. Since little change was seen, then MET must not be the limiting factor in oogenesis. This physiology is complex, so it is perhaps not surprising that overexpressing only one component does not enhance the whole process.

The alteration of the methoprene pathology seen in the *w; p[V2]* strain at the diagnostic doses used differs from the methoprene sensitivity/pathology characteristic of wild-type (Postlethwait, 1974). The stage of sensitivity appears to be early first-instar larvae as opposed to the late larval/prepupal stage of sensitivity seen in wild-type. If newly hatched *w; p[V2]* larvae are allowed to feed on regular food for 24 hours, then transferred to methoprene food, they do not display the late larval-early pupal lethality seen for newly hatched larval exposure in this strain (data not shown). Newly hatched *w; p[V2]* larvae exposed to the lower, but still lethal, 2.7 μg dose of methoprene begins to show lethality at the pharate adult stage (Figure 2) that is characteristic of non-over-expressing strains. Therefore, the combination of a higher dose of methoprene coupled with Met^{+} overexpression alters the sensitive/lethal stage of development. Possibly, a similar toxicology hypothesized for the usual methoprene effect is occurring: the high titer of MET coupled with a relatively high methoprene dose (5.4–26.8 μg) is resulting in mis-expression of a vital gene(s) early in larval development that affects larval or early pupal development.

Overexpression using a *tubulin*/*actin-GAL4* driver resulted in lethality during larval development even in the absence of methoprene. It is possible that the toxicity resulted from very high MET titers, which could not be accurately measured in this study due to the moribund condition of the larvae. Another possibility is that the *actin*/*tubulin* gene promoters resulted in Met^{+} overexpression in one or more tissues where it was especially toxic, perhaps in the presence of endogenous JH. The *w; p[V2]* strain also shows high overexpression, but without toxicity (unless driven by the *tubulin-GAL4* transgene); perhaps Met^{+} is overexpressed only in noncritical tissues in this strain. Clearly, though, there is a limit to overexpression, either in quantity or tissue location, of Met^{+} that can be tolerated by the animal.

This work examined overexpression of an insecticide resistance gene. Insect overexpression of resistance genes encoding detoxifying proteins, such as a cytochrome P450, have long been shown to result in increased resistance to the insecticide (Feyereisen, 1995; ffrench-Constant et al., 2004). The present work shows that overexpression of an insecticide-binding protein can result in the opposite effect of higher sensitivity. Future agrochemical work that focuses on novel chemistries to overexpress insecticide target-site proteins in pest insects could result in increased susceptibility and lower insecticide usage to control these pests.

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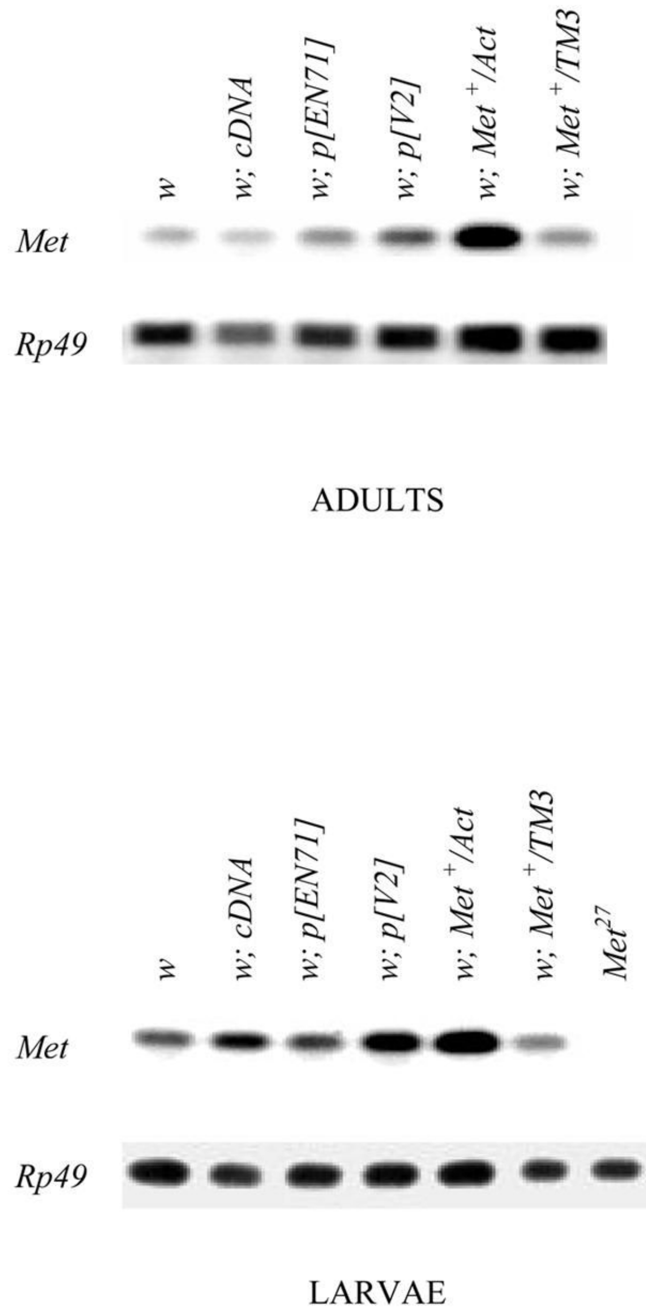

Figure 1.

RT-PCR determinations of *Met* transcripts from late third-instar larvae and adults of the *Met*+ overexpressing strains. The strain labeled *w; Met*+*/actin* expresses the *UAS-Met*+ transgene driven by *GAL4-actin*, and the strain labeled *w; Met*+*/TM3* is a sibling *TM3*-balancer control.

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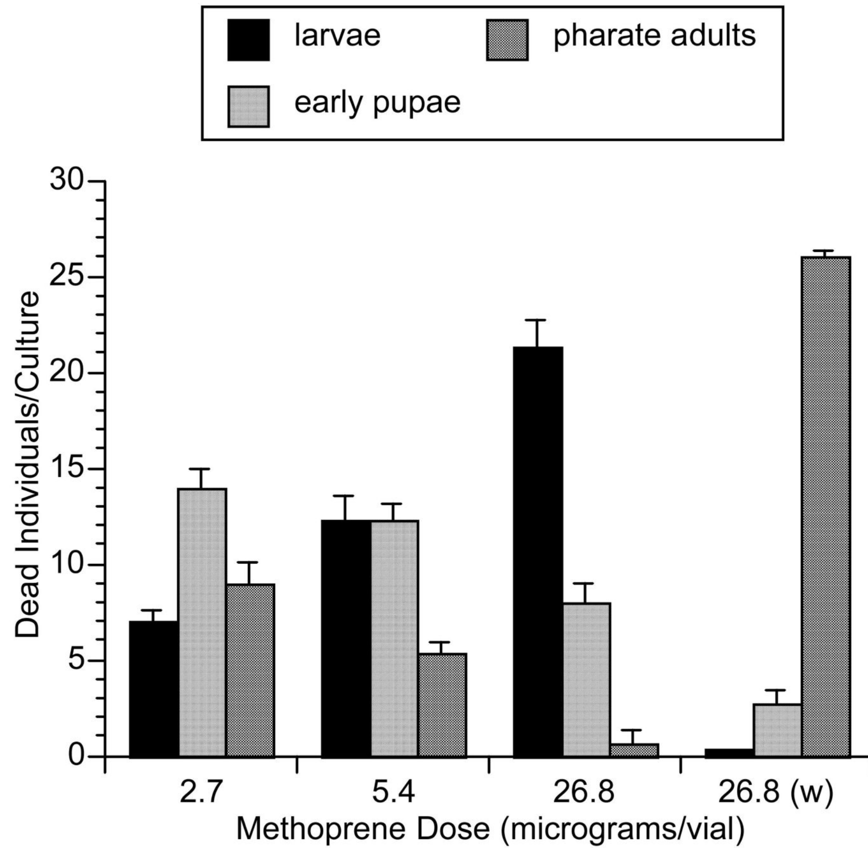

Figure 2.
Stage-specific death of *w; p[V2]* individuals raised on one of three lethal doses of methoprene compared with *w* raised on 26.8 μg/vial. Values for larval death were determined by subtracting the number of dead pupae from the total individuals in each of 3 separate cultures of 30. The predominant pharate adult stage of death of *w* is typical of the effect of methoprene on non-overexpressing Met^{+} strains. Error bars represent SEM values.

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Table 1

RT-PCR values for *Met* and *Rp49* transcript levels in each indicated strains. Each value is normalized to that of *w* larvae or adults set at the value 1. The strain *UAS-Met*$^+$*/actin* expresses *UAS-Met*$^+$ transgene driven by *GAL4-actin*, and the strain *UAS-Met*$^+$*/TM3* is a *TM3*-balancer control. ND=not determined; NA=not applicable.

Strain Genotype	Larval			Adult		
	Met	**Rp49**	**Met/Rp49**	**Met**	**Rp49**	**Met/Rp49**
w	1	1	1	1	1	1
w;p[cDNA]	2.4	0.7	3.4	0.8	0.6	1.3
w; p[EN71]	1.7	1.1	1.5	3.4	1.1	3.1
w; p[V2]	7.1	1.2	5.9	3.6	1.2	3.0
UAS-Met$^+$*/actin*	12.5	1.1	11.4	13.4	1.1	12.2
UAS-Met$^+$*/TM3*	1.1	0.8	0.8	3.1	1.0	3.1
w; Met^{27}	0	0.9	NA	ND	ND	NA

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Table 2

Effect of methoprene on the viability and morphology of *Met* overexpressing strains. The ED_{50} values are given in μg/food vial. Morphogenetic defects in posterior female sternite bristle morphology/patterning and male genitalia rotation were scored as either normal or abnormal (Wilson and Fabian 1986) after examination of 25 male and 25 female survivors at a low dose (0.054 μg/vial) chosen to demonstrate differential responses of the fly lines.

Genotype	Methoprene ED_{50} (95% fiducial limits)	Progeny with bristle/male genitalia defects (%)
Oregon-RC	1.4 (1.1–1.7)	4
w	0.92 (0.76–1.1)	8
w; p[EN71]	0.19 (0.16–0.23)	88
w; p[cDNA]	0.11 (0.086–0.14)	76
w; p[V2]	0.081 (0.030–0.36)	92
w; Met^{27}	18 (13–33)	0

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Table 3

Developmental parameters of the *w; p[V2]* strain compared with *w*. Egg hatch was determined for 50 eggs from each culture, and the remaining values were determined from 100 0–2 hour-old larvae carefully transferred to food vials without added yeast. Standard error of mean (SEM) given in parenthesis. Larval development time means compared by a Mann-Whitney test gave p=0.0104, a significant difference between the two strains. Chi-square tests for the egg hatch and survival values showed no difference (0.05 significance level, 1 df) between the two strains: χ= 0.044 for egg hatch, 0.05 for survival to pupariation, and 0.37 for survival to adult.

Genotype	Egg Hatch (%)	Time of Larval Developmental (days)	Survival to Pupariation (%)	Survival to Adult (%)
w	92	5.3 (0.066)	94	90
w; p[V2]	88	5.6 (0.082)	91	82

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Table 4

Comparison of oviposition and oogenesis between *w* and *w; p[V2]* females. Eggs were censused from each of 25 8-day-old females over a 4-day period. Stage 14 oocytes (most mature) were censused from each of ten 8-day-old females. SEM values given in parentheses. Comparison of mean values of either oviposition or stage 14 oocytes did not differ significantly between the two strains (p= 0.42 for oviposition and 0.27 for stage 14 oocytes/female using an unpaired t-test).

Genotype	Oviposition (eggs laid/female/4 days)	Stage 14 oocytes/female
w	143.7 (4.09)	13.3 (1.72)
w; p[V2]	138.8 (4.39)	10.9 (1.19)