Characterization of *Drosophila melanogaster* cytochrome P450 genes

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Cytochrome P450s form a large and diverse family of heme-containing proteins capable of carrying out many different enzymatic reactions. In both mammals and plants, some P450s are known to carry out reactions essential for processes such as hormone synthesis, while other P450s are involved in the detoxification of environmental compounds. In general, functions of insect P450s are less well understood. We characterized *Drosophila melanogaster* P450 expression patterns in embryos and 2 stages of third instar larvae. We identified numerous P450s expressed in the fat body, Malpighian (renal) tubules, and in distinct regions of the midgut, consistent with hypothesized roles in detoxification processes, and other P450s expressed in organs such as the gonads, corpora allata, oenocytes, hindgut, and brain. Combining expression pattern data with an RNA interference lethality screen of individual P450s, we identify candidate P450s essential for developmental processes and distinguish them from P450s with potential functions in detoxification.

detoxification genes | in situ hybridization | insecticide resistance | multigene family | RNAi

Cytochrome P450s form a diverse and important gene superfamily present in virtually all organisms. Genome sequencing projects have identified a large number of P450 sequences in eukaryotes (1–5). Originally identified as monooxygenases, P450 enzymes are now known to catalyze an extremely diverse range of chemical reactions important both in developmental processes and in the detoxification of foreign compounds (6–8).

P450s that perform essential functions in mammals and plants have been identified. For example, in humans, CYP17A1 and CYP19A1 catalyze steps in the production of androgens and estrogens (9). CYP26B1 metabolizes retinoic acid and is involved in the fate of germ cells in the testes of mice (10). CYP4F2 is involved in postabsorptive elimination of gamma-tocopherol forms of vitamin E (11). Mutations in human P450s also lead to various diseases (12, 13). Plant P450s are essential for catalyzing steps in the synthesis of many compounds, including phenylpropanoids, lipids, phytohormones, and carotenoids (14, 15). Roles for P450s in the detoxification of xenobiotics, for example P450-mediated drug metabolism in mammals (16), are also well characterized.

Compared to plants and mammals, much less is known about functions of the different insect P450 enzymes. One exception is the involvement of P450s in the biosynthesis of the major insect hormone 20-hydroxyecdysone (20H) from plant sterols, where in *Drosophila melanogaster* at least six P450s are involved (17). *Cyp302a1*, *Cyp306a1*, *Cyp315a1*, and *Cyp307a2* are all expressed in the prothoracic glands of late embryos and larvae and catalyze steps in the ecdysone biosynthetic pathway (18–21), while *Cyp314a1* (*shade*), responsible for catalyzing the conversion from ecdysone to 20H, is expressed in the midgut, Malpighian tubules, and the fat body (22). *Cyp307a1* (*spook*) has also been suggested to play a role in the synthesis of ecdysone, but it is not expressed in larvae (21). P450s involved in other important endogenous processes in *D. melanogaster* have also been identified, including *Cyp303a1*, required for the development and structure of sensory bristles (23), and *Cyp4g1*, an oenocyte-specific P450 required for correct triacylglycerol composition (24). P450s are also involved in behavioral phenotypes in *D. melanogaster*, with *Cyp6a20* associated with aggressive behavior in males (25, 26) and *Cyp4d21* necessary for efficient male mating (27). In the cockroach *Diploptera punctata*, *Cyp15a1* is involved in the biosynthesis of juvenile hormone III (28).

It has been suggested that the large complement of P450s in insect genomes (most insects possess around 100 different P450s) is necessary to protect the insect from the diverse array of harmful compounds in its environment (29). Insect P450s capable of metabolizing allelochemicals have been identified (30, 31). For example, CYP6B1v1 and CYP6B3v1 from the black swallowtail butterfly, *Papilio polyxenes*, can metabolize the furanocoumarins it is exposed to in its diet (32, 33). Numerous individual P450s have also been implicated in insecticide resistance in different insect populations (34–38).

Approaches, such as sex-specific expression of P450s, induction capacity of P450s by various exogenous compounds, and transcriptional profiling have been useful in characterizing P450 functions (39–42). In an effort to distinguish P450s with essential endogenous functions from those involved in detoxification, we characterized expression patterns of *D. melanogaster* P450s by in situ hybridization and performed a P450 RNA interference (RNAi) knockdown screen.

Results

The sequenced, isogenic *D. melanogaster* strain *y*; *cn bw sp* (43) was used in this study, as the complement of P450s in this strain has been annotated (4). In *y*; *cn bw sp*, most of the 86 P450 genes were experimentally distinguishable from paralogs, except for the recently described duplication of *Cyp12d1* (*Cyp12d1-d* and *Cyp12d1-p*), which only differ by 3 nucleotides in the coding region (www.flybase.org). We classified both *Cyp12d1-d* and *Cyp12d1-p* as *Cyp12d1*, bringing the number of P450s analyzed to 85. Using RT-PCR with P450 specific primers, 70 P450s were amplified from cDNA isolated from embryos and 81 P450s were amplified from cDNA isolated from third instar larvae, with 69 P450s amplified from both embryos and larvae. *Cyp6t3*, *Cyp308a1*, and *Cyp313a2* were the only P450s that could not be amplified from either embryos or larvae. *Cyp308a1* and *Cyp313a2* could not be amplified from cDNA isolated from embryos, larvae, or adults, while *Cyp6t3* could only be amplified from cDNA isolated from adults. Using DIG-labeled RNA probes for the 82 P450s amplified, in situ hybridization was performed on the different stages of embryogenesis, feeding third instar larval stage and wandering third instar larval stage.

EVOLUTION

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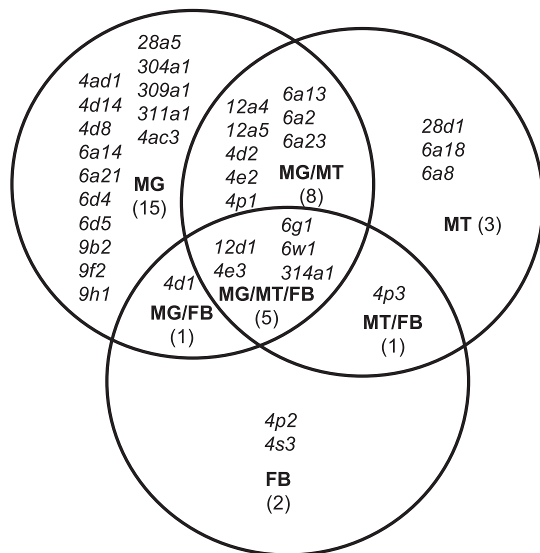

Fig. 1. Venn diagram representing unique and overlapping tissue expression of P450 genes expressed in the 3 key metabolic tissues of third instar larvae. FB, fat body; MG,midgut; MT, Malpighian tubules.

Expression patterns were obtained for 50 P450s during embryogenesis and 58 P450s during the third instar larval stages (Table S1).

Many P450s Are Expressed in the Larval Midgut, Malpighian Tubules, and Fat Body. Hybridization signals for 35 P450s were detected in combinations of the midgut, Malpighian tubules, and fat body during third instar larval stages (Fig. 1; see Table S1). This is consistent with a presumed function of P450s in detoxification processes, as the metabolism of both exogenous and endogenous compounds is likely to occur in these tissues (44, 45). Seventeen P450s were detected in the larval Malpighian tubules (see Fig. 1 and Table S1), which are suggested to be the primary organs of excretion in insects (46). It has recently been demonstrated that the Malpighian tubules are important for the metabolism and detoxification of xenobiotics, and might also be involved in immunity (44, 47). There are at least 5 defined domains in the Malpighian tubules (48). Of the 17 P450s detected in the Malpighian tubules, 15 are expressed in all whole tubules, except *Cyp28d1*, which is expressed only in the lower tubule and ureter, and *Cyp6w1* which is expressed only in the main segment and the transitional segment (Fig. 2*A*). Because of the initial segments of the tubules often being lost during dissection and in situ hybridization, expression in the initial segment was not characterized. In contrast to the relatively high number of P450s expressed in the larval tubules, only *Cyp6a8* and *Cyp6a18* are detected in Malpighian tubules of embryos (Fig. S1). Nine P450s are detected in the fat body, including the previously characterized *Cyp6g1*, *Cyp12d1*, and *Cyp314a1* (22, 40, 49). *Cyp4p2* and *Cyp4s3* are the only P450s detected exclusively in the fat body. *Cyp4d1* is alternatively spliced, giving rise to 2 different isoforms, *Cyp4d1*-PA and *Cyp4d1*-PB. The DIG-labeled RNA probe used in this study hybridizes to both isoforms and detected expression in the midgut and fat body. RT-PCR of individual tissues using exon-specific primers showed that *Cyp4d1-PA* is preferentially expressed in the midgut while *Cyp4d1-PB* is preferentially expressed in the fat body (Fig. S2).

The Larval Midgut Contains Different Regions of P450 Expression. The role of the *D. melanogaster* midgut in food digestion is well accepted (50) and contains at least 13 different distinct regions of gene expression, likely to be composed of multiple cell types

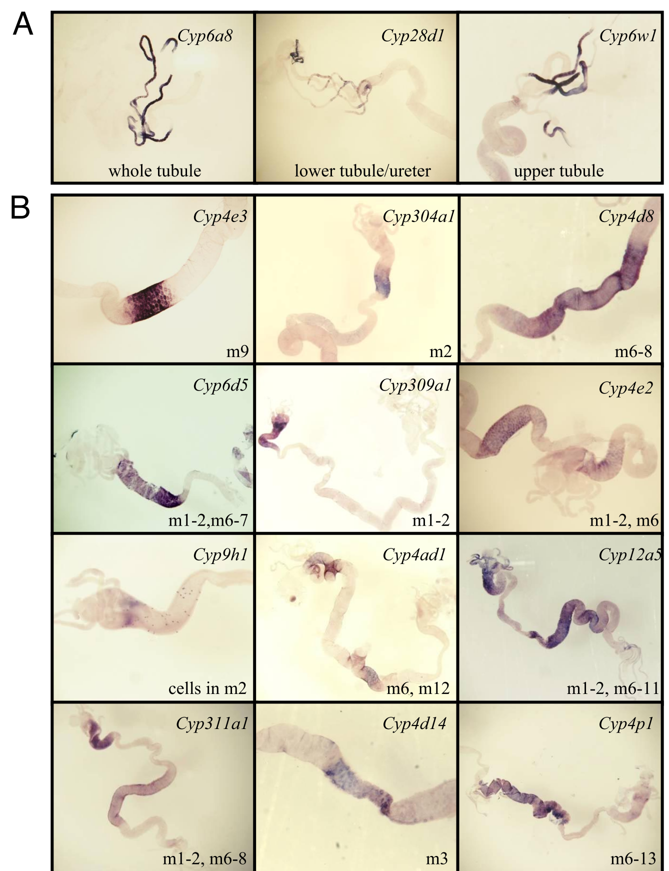

Fig. 2. P450s expressed in the midgut and Malpighian tubules are detected in specific compartments by in situ hybridization. (*A*) Spatial patterns of P450s expressed in Malpighian tubules. (*B*) Spatial patterns of P450s in the midgut.

(51). The anterior portion of the midgut (m1–4) is suggested to play some role in immunity (52, 53), although the functions of many midgut regions are not well established (54, 55). Using in situ hybridization, we detected expression of different P450s in a number of specific but different midgut regions in third instar larvae (Fig. 2*B*), with a predominance of P450s in the anterior (m1–m4) (55% of all midgut expressed P450s) and middle (m5–m10) midgut regions (49% of all midgut expressed P450s expressed).

While expression patterns for the majority of P450s is consistent between individual larvae, expression patterns for 2 P450s, *Cyp6d4* and *Cyp28a5*, are not. In different larvae reared under the same conditions, expression in different compartments of the midgut for these 2 genes was consistently observed. When the upstream regulatory region of *Cyp28a5* was cloned upstream of a GFP reporter [*p*-Stinger (56)] and transformed into flies (*SI Methods*), GFP expression was observed in different midgut regions in different larvae from an individual transgenic line, even when larvae of the same life stage grown under the same conditions were compared (Fig. S3). Specificity of P450 expression within in the embryonic midgut was also observed, with 15 P450s expressed in different regions of the midgut at different stages throughout embryogenesis (see Fig. S1).

Some P450s Have Specific Expression Patterns. A subset of P450s was found to have specific tissue-expression patterns. *Cyp302a1*, *Cyp306a1*, *Cyp307a2*, and *Cyp315a1* were detected in the prothoracic cells of the ring gland in embryos and larvae (Fig. 3*A*), as per previous studies (18, 19, 21, 57). Five P450s were detected in the hindgut (*Cyp6a17*, *Cyp301a1*, *Cyp4c3*, *Cyp49a1*, and

Fig. 3. (*A–I*) P450s with specific expression patterns in third instar larvae as determined by in situ hybridization.

Cyp4aa1) (Fig. 3*B*). *Cyp301a1*, *Cyp49a1*, *Cyp4aa1* are also expressed in the embryonic hindgut (see Fig. 3*B*). *Cyp6g2* is the only P450 detected in the corpora allata (Fig. 3*C*). *Cyp6d2* (Fig. 3*D*), and *Cyp4e3* are expressed in the tracheal system, although *Cyp4e3* is also expressed in other tissues. *Cyp28c1* is detected in the salivary glands (Fig. 3*E*). *Cyp310a1* is expressed in the larval wing disc (Fig. 3*F*), *Cyp4g15* expressed in the embryonic and larval brain and central nervous system (Fig. 3*G*), and *Cyp4g1* is expressed in the embryonic and larval oenocytes (Fig. 3*H*), confirming previous studies (24, 58, 59), Nine P450s were detected in the larval gonads (*Cyp305a1*, *Cyp28d2*, *Cyp309a2*, *Cyp312a1*, *Cyp4ad1*, *Cyp6a22*, *Cyp6t1*, *Cyp6u1*, and *Cyp6v1*) (Fig. 3*I*). These P450s were not detected in the embryonic gonads (data not shown) (60). Refer to Fig. S4 for complete P450 in situ hybridization patterns in embryos and larvae.

Some P450 Show Differential Expression Between Feeding and Wandering Stages of Third Instar Larvae. As a minor 20H peak thought to induce wandering behavior in third instar larvae had been shown to alter the expression of certain metabolic genes (61), in situ hybridization experiments were performed on both feeding and wandering larvae. A number of P450s were found to have expression changes between feeding and wandering larval stages. Four P450s expressed in the hindgut, *Cyp301a1*, *Cyp49a1*, *Cyp4aa1*, and *Cyp4c3* were only detected in feeding larvae. *Cyp6a2*, implicated in insecticide resistance (35), is expressed in the midgut and Malpighian tubules in feeding larvae and not detected in wandering larvae. Similarly, *Cyp4e3*, expressed in specific regions of the midgut, and *Cyp28a5*, expressed in the midgut in a dynamic fashion (see Fig. S3), are expressed only in feeding but not wandering larvae. Other P450s were more readily detected in wandering larvae. These include the P450s involved in the synthesis of ecdysone, *Cyp302a1*, *Cyp306a1*, *Cyp307a2*, *Cyp314a1*, and *Cyp315a1*. *Cyp6g1* is detected in the midgut and Malpighian tubules in both feeding and wandering third instar larvae, with additional expression detected in the fat body of wandering larvae (49). Expression for a subset of these P450s was confirmed by semiquantitative PCR (*SI Methods*; Fig. S5).

RNAi Knockdown Screen Indicates that Some P450s Are Essential for Viability. A conditional knockdown screen of P450s using RNAi was performed to identify P450s essential for viability. Using available strains from the Vienna Drosophila RNAi Centre (62), we assayed for viability after RNAi knockdown of individual P450s in all tissues using the binary GAL4/UAS system and a *tubulin*-GAL4 strain (63). Most RNAi strains selected do not have off-target matches [defined as one or more 19-mer match (62)]. We were conservative in our approach, and only selected strains with off-targets that were other P450s. Out of the 59 different P450s targeted, 9 were found to be lethal by RNAi. As expected, RNAi of P450s in the ecdysone biosynthetic pathway (*Cyp306a1* and *Cyp314a1*) resulted in lethality. RNAi of *Cyp4g1* also resulted in lethality, giving a similar phenotype to *Cyp4g1* knockout mutants (24). Six P450s (*Cyp4c3*, *Cyp4d2*, *Cyp6g2*, *Cyp28c1*, *Cyp309a1*, *Cyp311a1*) not as yet functionally characterized, were found to result in lethality by RNAi (Table S2). Phenotypic characterization of lethality revealed these P450s to be lethal at larval or pupal stages. Additional RNAi constructs targeting *Cyp4g1* and *Cyp4c3*, constructed using the *pWIZ* vector (*SI Methods*), were also lethal. Fifty-one P450s are viable when targeted by RNAi. Of these, 3 P450s (*Cyp318a1*, *Cyp4s3*, *Cyp4ac2*) had lower numbers of progeny emerge than expected, suggesting a potential fitness deficit may exist when these individual P450s are targeted (see Table S2). As RNAi silencing can be variable, we cannot conclude that all of these P450s are involved in nonessential processes (62). Along with inefficient silencing, some P450s may be involved in survival on alternate food sources to the standard laboratory media used.

Table 1. A higher Proportion of P450s lethal by RNAi are evolutionarily stable

	Stable P450s	Unstable P450s	Total
RNAi lethal P450s	8 (36.4%)	1 (2.6%)	9
RNAi sub-viable P450s	1 (4.5%)	2 (5.3%)	3
RNAi viable P450s	13 (59.1%)	34 (92.1%)	47
Total	22	38	59

Percentages refer to P450s in each class (stable or dynamic) for the 60 individual P450s assayed by RNAi.

Discussion

Insect P450s are a large gene family, with many P450s evolving rapidly. Few P450 orthologs are identifiable in insect genomes of species 250 million years diverged (5, 29, 64–66). We undertook a systematic approach to studying this gene family in the model organism *D. melanogaster*, characterizing expression patterns in embryos and larvae, and investigating function with an RNAi screen. Expression patterns for 68 out of 85 P450s were obtained from either embryos, larvae, or both life stages. We found that most P450s are expressed in the larval midgut, Malpighian tubules, and fat body, suggesting potential roles in detoxification processes and protection from harmful exogenous compounds. Other P450s were identified to have specific expression patterns (e.g., corpora allata, brain, salivary glands, hindgut, and oenocytes). These expression patterns are conserved between life stage, and these P450s potentially have specific biological functions within these tissues.

The number of P450s successfully detected by in situ hybridization is less than the number of P450 that was amplified by PCR from each life stage. This is likely because of limitations in the in situ hybridization technique. Tissues, such as the cuticle, are waterproof and would not allow the entry of reagents. P450s expressed in late embryos also escape detection because of deposition of the cuticle (67). Additionally, P450s expressed at very low levels, which are detected by PCR, may not be detected by the conventional in situ hybridization protocol, but development of fluorescence in situ hybridization techniques might improve detection sensitivity (68).

Identification of a P450 Expressed in the Corpora Allata. *Cyp6g2* is the only P450 detected in the corpora allata of embryos and larvae, the site of juvenile hormone (JH) biosynthesis in insects (69). Given this expression pattern, *Cyp6g2* might possibly be involved in the production of JH. There is precedent for the involvement of P450s in JH biosynthesis. *Cyp15a1* from the cockroach *D. punctata* is expressed selectively in the corpora allata, and encodes a P450 enzyme that catalyses the conversion of methyl farnesoate to JH III (28). No clear orthologue to *Cyp15a1* is identifiable in *D. melanogaster* (28). However, unlike in *D. punctata*, JH III bisepoxide (JHB_3) is thought to be the major JH produced in *D. melanogaster* (70). JHB_3 is synthesized by an alternate pathway possibly involving 2 epoxidation reactions catalyzed by a P450, converting farnesoic acid to JHB_3 (71). It is possible that this P450 is CYP6G2.

Temporal Expression Differences of P450 Between Embryos and Larvae. Out of the 68 P450s that we were able to detect by in situ hybridization, 9 are detected only in embryos, 15 are detected only in larvae, and the expression of another 32 P450s are not completely conserved between embryos and larvae (see Table S1). The P450s detected only in embryos are mostly expressed in structures such as the yolk cells, or are maternal transcripts, whereas the P450s detected in larvae only are mostly expressed in tissues involved in detoxification, such as the midgut, Malpighian tubules, and the fat body. *Cyp6g1* and *Cyp12a4*, which confer insecticide resistance when over-expressed (36, 38, 49), are expressed in the midgut and Malpighian tubules in feeding third instar larvae, but show no detectable expression in embryos. RNAi of these 2 genes showed no mortality under laboratory conditions. In contrast, *Cyp309a1* is expressed in the midgut in both embryos and larvae. RNAi of *Cyp309a1* results in a lethal phenotype, suggesting that it might have an essential endogenous function. Genes with known developmental functions, such as the ecdysone biosynthesis genes, *Cyp302a1*, *Cyp306a1*, *Cyp307a2*, and *Cyp315a1* are expressed in the prothoracic glands in both embryos and larvae. *Cyp4g1* is also expressed in the oenocytes in both embryos and larvae. These genes are essential for the viability of the fly (24, 72). It is possible that P450s expressed in the same tissue throughout development are more likely to be involved in essential endogenous processes, while P450s expressed at only larval or adult stages are more likely to be involved in detoxification processes, although an overlap of these processes cannot be ruled out.

Comparison with Microarray Results. Microarrays on dissected tissues detect a high number of P450s expressed in the larval midgut (54, 73), the larval Malpighian tubules, and the larval fat body (74). Comparing microarrays to our in situ hybridization results, we find a high number of P450s are detected using both methods. A microarray of mosquito (*Anopheles gambiae*) midgut regions show P450s to be highly enriched in the anterior midgut (75), consistent with our finding of a higher proportion of P450s in the anterior and middle midgut regions in *D. melanogaster.* A genome-wide study of embryonic gene expression patterns also characterized 30 P450s (67). Compared to the current study, expression pattern differences were found for 7 P450s. Any differences between datasets, microarray, and in situ hybridization, may be because of strain differences, detection methods, or rearing conditions.

Other microarray experiments performed by challenging *D. melanogaster* to a wide variety of conditions, such as exposure phenobarbital (40–42, 76), ethanol (77, 78), starvation (79), and looking at transcriptome changes in response to aging (80) identified a number of P450s that alter expression levels in response to variations in these parameters. We highlight 2 examples in which tissue-expression data can be used to extend microarray results. First, it has been shown that the transcription factor *dGATAe* is expressed in the Malpighian tubules and midgut, where it has roles in the development of the endoderm (81) and tissue-specific immune response (52). When *dGATAe* was misexpressed in the larval fat body, whole-genome microarray analysis revealed 291 genes to be up-regulated when compared to fat body of control larvae. This set of genes includes 21 P450s (52), 16 of which we have identified as being expressed in the larval midgut (Table S3). It is possible that *dGATAe* has a role in the regulation of these P450s in the larval midgut. Secondly, exposure of *y*; *cn bw sp* larvae to phenobarbital had shown that 21 P450s are induced by this regime (40). This list of 21 P450s includes 16 P450s, which are either expressed in the midgut or Malpighian tubules, suggesting that the induction response mainly occurs in these tissues (Table S4).

Evolutionary Dynamics of Drosophila P450s. Many insect P450s are rapidly evolving, with orthologs to *D. melanogaster* P450s being

difficult to identify outside of Drosophila (29, 64). Phylogenetic analysis of P450s in vertebrates has separated P450s into stable and unstable groups. Stable P450s have endogenous substrates, implying these P450 have essential functions. Most of the unstable P450s encode enzymes that function as xenobiotic detoxifiers (82). Evolutionary analysis of P450s found in 12 sequenced Drosophila genomes has also identified stable and unstable P450s across the species (83) (Lydia Gramzow, Robert Good and Charles Robin, unpublished data). For the 58 P450s with characterized expression patterns in third instar larvae, 80% of the P450s expressed in the midgut, Malpighian tubules, and fat body are classified as unstable, while only 27% of P450s expressed in other tissues are unstable, suggesting that the large complement of rapidly evolving P450s could be an evolutionary consequence of the wide variety of xenobiotics that *D. melanogaster* are exposed to. Our RNAi screen also shows that out of the 9 P450s with essential endogenous functions identified, 8 are categorized as evolutionarily stable (Table 1), suggesting that conserved P450s are likely to be have essential functions.

In this study, the combination of tissue expression analysis and RNA interference allowed us to infer the general functions of P450s in *D. melanogaster*. Further evolutionary and functional studies of this rapidly evolving gene family may provide insights into various developmental and habitat defining processes in insects.

Materials and Methods

Drosophila Strains. Strains were maintained on a glucose, semolina, and yeast medium at 25 °C with constant light. All strains were obtained from the Bloomington *Drosophila* Stock Center, Indiana, except strains used in RNAi experiments, which were obtained from the Vienna Drosophila RNAi Center. *y; cn bw sp* was used in all in situ hybridization experiments.

Synthesis of cDNA and in Situ Hybridization. Synthesis of cDNA was performed using standard techniques as reported previously (38), using total RNA extracted using TRIzol reagent (Invitrogen) from either mixed stage embryos or a pool of ≈30 feeding and 30 wandering third instar larvae. PCR were performed using primers spanning the complete ORF of each P450.

In Situ Hybridization. DIG-labeled RNA probes were synthesized as per standard protocol using the PCR products amplified from cDNA. In situ hybridization in embryos and larvae was performed as described previously (49, 84).

RNAi of P450s. UAS-RNAi strains were individually crossed to *tubulin*-GAL4/TM3 (63), resulting in RNAi knockdown in a ubiquitous pattern. Reciprocal crosses were performed at 29 °C. The sex and phenotype of emerging adults was scored. Stubble bristles were used to indicate the presence of the *TM3*, *Sb* chromosome in progeny, and therefore the absence of the *tubulin-GAL4* chromosome. CHI square analysis of sex and genotype of emerging progeny was performed. Strains where the RNAi knockdown resulted in lethality or reduced numbers of flies in both reciprocal crosses were characterized further by defining life-stage of lethality.

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