Author/s:
Harrop, TWR;Denecke, S;Yang, YT;Chan, J;Daborn, PJ;Perry, T;Batterham, P

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Harrop Thomas (Orcid ID: 0000-0001-6557-0440)

Evidence for activation of nitenpyram by a mitochondrial cytochrome P450 in *Drosophila melanogaster*

Short title: Nitenpyram activation by a P450 in *D. melanogaster*

Thomas W.R. Harrop[a,1,2], Shane Denecke[1], Ying Ting Yang, Janice Chan, Phillip J. Daborn, Trent Perry, and Philip Batterham

Department of Genetics and Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Victoria 3010, Australia.

[a] Present address: Department of Biochemistry, The Laboratory for Evolution and Development, The University of Otago, Dunedin 9054, Aotearoa-New Zealand.

[1] These authors contributed equally to this work

[2] Corresponding author: twharrop@gmail.com

Abstract

BACKGROUND:

Nitenpyram is a member of the economically important neonicotinoid class of insecticides. The *in vivo* metabolism of nitenpyram is not well characterised, but cytochrome P450 activity is the major mechanism of resistance to neonicotinoids identified in insect pests, and P450s metabolise other neonicotinoids including imidacloprid.

RESULTS:

Here, we used the GAL4/UAS system to direct RNAi against the cytochrome P450 redox partners to interrupt P450 functions in specific tissues in *Drosophila*

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melanogaster. RNAi of the mitochondrial redox partner *dare* in the digestive tissues reduced nitenpyram mortality, suggesting an activation step in the metabolism of nitenpyram carried out by a mitochondrial P450. RNAi of the mitochondrial P450 *Cyp12a5*, which is expressed in the digestive tissues, resulted in the same phenotype, and transgenic overexpression of *Cyp12a5* increased nitenpyram sensitivity.

CONCLUSION:

These results suggest that *in vivo* metabolism of nitenpyram by the mitochondrial P450, CYP12A5, results in the formation of a product with higher toxicity than the parent compound.

Keywords

Cytochrome P450, neonicotinoids, nitenpyram, pesticide, pesticide metabolism

Highlights

- RNAi knockdown of the mitochondrial P450 redox partner, *dare*, decreased the toxicity of nitenpyram, while knockdown of the endoplasmic reticulum redox partner, *Cpr*, had no effect.
- RNAi of *Cyp12a5* resulted in the same phenotype as *dare* RNAi
- Overexpression of *Cyp12a5* increased nitenpyram susceptibility
- These data are consistent with a role for CYP12A5 in nitenpyram bioactivation

Introduction

Insect cytochrome P450 enzymes (P450s) perform diverse physiological functions. The genes encoding these enzymes form four phylogenetic clades, three encoding proteins that localise to the endoplasmic reticulum (ER) and one encoding proteins that localise to mitochondria.[1,2] Electrons for P450 reactions are supplied by obligatory redox partners, which differ for mitochondrial and ER P450s. In the ER, Cytochrome P450 reductase accepts two electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH) and transfers them to the P450.[3] In the mitochondria, Adrenodoxin reductase provides electrons to mitochondrial P450s by reducing Adrenodoxin using electrons from NADPH.[4] In *Drosophila melanogaster*, the ER and mitochondrial redox partners are encoded by *Cytochrome P450 reductase* (*Cpr*) and *defective in the avoidance of repellents* (*dare*), respectively.[5,6]

All vertebrate mitochondrial P450s characterized so far have developmental functions.[7] In contrast, some arthropod mitochondrial P450s have essential developmental roles in hormone synthesis and degradation,[8] but others act as xenobiotic-metabolising enzymes (XMEs) capable of transforming the structure of chemical pesticides.[1,9] Increased expression of P450s is often positively associated with xenobiotic resistance and is a common resistance mechanism to members of the neonicotinoid class of insecticides.[10] Individual P450s have been associated with neonicotinoid resistance by comparing transcript levels between resistant and susceptible strains or by genetic mapping,[11–17] and some candidates have been validated by transgenic overexpression in *D. melanogaster*.[12,13,18,19]

Nitenpyram was one of the first commercially released neonicotinoids, and has high water solubility and poor photostability compared to other neonicotinoids. Because of these properties it has been most commonly used in animal health *e.g.* for the control of ectoparasites such as cat fleas. Nitenpyram is thought to target specific insect nicotinic acetylcholine receptor (nAChR) subunits, because 13- and 70-fold resistance ratios were conferred in *Drosophila melanogaster* by mutations in the

Dα1 and Dβ2 subunits, respectively.[20,21] In mice, *in vivo* metabolites of nitenpyram resemble those formed from other neonicotinoids.[22] In insects, nitenpyram metabolism is not well characterised. In general, P450-mediated metabolism may lead to detoxification of neonicotinoids, but metabolism can also lead to compounds that bind target sites with equal or greater affinity than the parent compound.[23,24]

We investigated the genetics of nitenpyram toxicity by manipulating expression of P450 redox partners and individual P450s in *D. melanogaster*. Knockdown of the mitochondrial redox partner, *dare*, resulted in increased survival of larvae reared on nitenpyram. RNAi-mediated knockdown of a mitochondrial P450 gene, *Cyp12a5*, decreased nitenpyram toxicity, whilst transgenic overexpression increased susceptibility. These results suggest that CYP12A5 is negatively associated with resistance and may interact with nitenpyram to increase the toxicity of this insecticide.

Material and methods

Transgenic fly lines

Fly lines used in this study are listed in Supplementary table 1. To generate *Cpr* and *dare* RNA interference (RNAi) lines, we amplified two non-overlapping RNAi fragments from each gene (Supplementary figure 1) using the Expand High Fidelity Plus PCR System (Roche Applied Science), cloned them into pGEM-T Easy (Promega) and subcloned them twice in separate steps into the *Nhe*I and *Avr*II sites of pWIZ[25] so that the fragment was in the opposite direction in each site. The primers are listed in Supplementary table 1. The recombinant plasmids were transformed into the w^{1118} strain of *D. melanogaster* and transgenic progeny with autosomal insertions were selected for further characterisation.

We constructed *Cyp12a4* and *Cyp12a5* UAS lines by amplifying the open reading frames from the *y* ; *cn bw sp* reference strain of *D. melanogaster* using Q5 High-Fidelity DNA Polymerase (New England BioLabs), cloning them into pGEM-T Easy and subcloning them into the pUASt-attB vector[26] via the *Not*I sites. We injected the recombinant plasmids into a target strain of genotype y^1 M{vas-int.Dm}ZH-2A w^* ; P{CaryP}attP40 (attP40 background), carrying the X-chromosome from Bloomington stock 24482 and the autosomes from Bloomington stock 25709. We obtained strains from the Vienna Drosophila Resource Centre KK library for RNAi knockdown of *Cyp12a4* and *Cyp12a5* (VDRC stock numbers 107088 and 103961, respectively).

Cpr and *dare* RNAi phenotyping

We tested the lethality of ubiquitous RNAi knockdown of *Cpr* and *dare* by crossing male RNAi lines to virgin female 5′*tubulin* and 5′*Actin* driver lines (Bloomington stock numbers 5138 and 3954) and counted emergence of progeny after 15 days.

To determine the life stage mortality of flies expressing the *Cpr* and *dare* RNAi constructs in the prothoracic gland, RNAi lines were crossed to a 5′*phantom* driver.[27]

The driver line includes a balancer chromosome that constitutively expresses GFP, carries the *CyO* allele (encoding the Cy^1 phenotype) and does not produce *GAL4* (*CyO*-GFP). A cross between the driver and w^{1118} was used to control genetic background. We assayed viability by placing three replicates of 20 eggs from each cross in normal Drosophila food vials. Emerging adults were scored as Cy^1 (control) or Cy^+ (RNAi). For life stage mortality, larvae expressing GAL4 were collected at first instar larval stage using a SZX12 fluorescence stereomicroscope (Olympus). Life stage mortality was determined by individually raising non-GFP first instar larvae in 5 mL vials containing 1 mL of normal Drosophila medium (n = 40). Pulses of 8.7 μg of 20-hydroxyecdysone in a yeast paste wetted with 10 % v/v ethanol were administered after two and six days to half of the larvae. The remaining larvae were treated with yeast paste wetted with ethanol without 20-hydroxyecdysone. Vials were inspected daily to determine life stage and mortality.

To measure the lethality of *Cpr* and *dare* RNAi in the *D. melanogaster* digestive system, we crossed male RNAi lines to virgin females carrying the 5′*Cyp6g1*HR construct, which drives GAL4 production in the midgut, Malpighian tubules and fat body,[28] over a TM6b, Tb^1 balancer chromosome. Lethality was inferred by scoring the ratio of adults emerging with the balancer phenotype ($Antp^{Hu}$).

Insecticide bioassays

The homozygous 5′*Cyp6g1*HR-3a driver line[28] was used for all bioassays, and crosses with the recipient strain of the *Cpr* and *dare* or *Cyp12a4* and *Cyp12a5* RNAi constructs (w^{1118} or VDRC stock 60100, respectively) or the attP40 background with an empty pUASt-attB insertion (UAS-Null) were used to control genetic background. We exposed replicates of 20 four-day-old, mated, adult female progeny to discriminating doses of 2, 4 or 8 μg·vial^{-1} of DDT (Sigma) using 24 hour contact assays in glass scintillation vials.[29] We reared replicates of 25 or 50 first instar larvae on food containing discriminating doses of 1 or 1.5 × 10^{-4} % w/v nitenpyram or lufenuron (Novartis), and 0.9 or 1.1 × 10^{-6} w/v dicyclanil (Novartis) and counted emergence after 15 days. All bioassays for the RNAi constructs were performed on

standard semolina medium, while a soy/corn meal medium was used for all *Cyp12a4* and *Cyp12a5* overexpression bioassays. Corrected mortality was estimated using Abbott's formula,[30] and confidence intervals were calculated using formulae described by Rosenheim and Hoy.[31] Two genotypes were considered to respond differently if the 95% confidence intervals derived from this calculation did not overlap.[31]

Overexpression and RNAi quantification

To quantify the knockdown of *Cpr* and *dare*, we collected three biological replicates of five midguts dissected from first and third instar larvae and four-day-old adults. We homogenized tissues in TRIzol Reagent (Invitrogen) at 20.0 Hz for 4 minutes in a Mixer Mill MM 400 (Retsch), and isolated RNA from the homogenate by chloroform extraction and isopropanol precipitation. After testing RNA quality and yield by agarose gel electrophoresis and fluorometric assay (Qubit Fluorometer, Invitrogen), cDNA was synthesised with the SuperScript III First-Strand Synthesis System (Invitrogen) using random hexamer oligonucleotides. SYBR Green Master Mix (Applied Biosciences) was used for quantitative real-time RT-PCR (qRT-PCR) with a RotorGene RG-3000 (Corbett). Primers used for qRT-PCR are listed in Supplementary table 1. We validated the efficiency of our target gene primers and analysed the qRT-PCR results using the $2^{-\Delta\Delta C_T}$ method described by Livak and Schmittgen,[32] using *RpL11* as a reference gene.

Results

RNAi of *dare*, a mitochondrial P450 redox partner, reduces nitenpyram mortality

To detect phenotypes caused by disruption of cytochrome P450 functions in specific tissues, we generated an RNAi system to interrupt the functions of two P450 redox partners, *Cpr* and *dare*, under control of the GAL4-UAS system. Two non-overlapping UAS-RNAi constructs were tested for each redox partner (Supplementary figure 1). Ubiquitous RNAi with the 5′*tubulin* driver or specific RNAi in the prothoracic gland using the 5′*phantom* driver resulted in lethality (Table 1; Supplementary figure 2A). The second instar mortality caused by RNAi in the prothoracic gland was rescued by the addition 20-hydroxyecdysone in both *Cpr* RNAi lines, suggesting that RNAi of *Cpr* inhibits P450 functions. The low dose of 20-hydroxyecdysone did not rescue larval mortality of prothoracic gland RNAi of *dare*, although the number of days the third instar larvae survived increased for one construct (Figure 1A–B).

We then used the redox partner RNAi system to investigate phenotypes related to P450-based metabolism of insecticides in the insect digestive system. The redox partner RNAi lines were crossed to the 5′*Cyp6g1*HR-3a driver, which produces GAL4 in the midgut, Malpighian tubules and fat body. 60–80% removal of *Cpr* mRNA and 70–90% of *dare* mRNA was detected in midguts from first instar larval, third instar larval and adult progeny by qRT-PCR (Table 2). To assess the lethality of the redox partner RNAi system in these tissues, heterozygous flies carrying the driver transgene and a balancer chromosome (genotype 5′*Cyp6g1*HR-3a / *TM6B*, *Tb*^{1}) were crossed to the *Cpr* and *dare* RNAi lines. The balancer chromosome was detected in adult progeny by scoring the dominant *Antp*^{Hu} phenotype. There was no reduction in emergence of *Antp*$^{+}$ progeny (Supplementary figure 2B), suggesting that RNAi of the P450 redox partners in these tissues does not cause developmental lethality.

To assess the effect of *Cpr* and *dare* knockdown in the *D. melanogaster* digestive tissues, we reared flies expressing the UAS-RNAi constructs under control of the 5′*Cyp6g1*HR-3a driver on nitenpyram or dicyclanil, or exposed them to DDT as adults. Mortality of flies expressing either of the *dare* RNAi constructs reared on two different concentrations of nitenpyram was lower compared to both control crosses and both crosses expressing *Cpr* knockdown constructs (Figure 2A). No difference in DDT or dicyclanil mortality was detected (Supplementary figure 3). This suggests that metabolism of nitenpyram by one or more mitochondrial P450s expressed in the midgut, Malpighian tubules or fat body is negatively associated with resistance.

Modulation of *Cyp12a5* expression level affects nitenpyram mortality

Four mitochondrial P450s were detected by *in situ* hybridisation in the *y* ; *cn bw sp* strain of *D. melanogaster*,[33] and a further three were detected at low levels in RNA sequencing of midguts from transgenic flies in the w^{1118} background.[34] Because two of these P450s, *Cyp12a4* and *Cyp12a5*, are arranged in tandem on chromosome 3R and *Cyp12a4* overexpression decreases toxicity of the insect growth regulator lufenuron,[35] we tested them for a role in nitenpyram resistance. First instar larvae expressing RNAi constructs against *Cyp12a4* and *Cyp12a5* under control of the 5′*Cyp6g1*HR-3a driver were reared on nitenpyram medium. Mortality was lower than the control cross for *Cyp12a5* flies, but not for *Cyp12a4* flies (Figure 2B). This result indicated that *Cyp12a5* is a candidate for activation of nitenpyram in the *D. melanogaster* digestive tissues.

To test the effect of constitutive overexpression of *Cyp12a4* and *Cyp12a5*, we crossed transgenic UAS lines containing the open reading frame from each gene (UAS-*Cyp12a4* and UAS-*Cyp12a5*) to the 5′*Cyp6g1*HR-3a driver and reared first instar larval progeny on nitenpyram. Flies overexpressing *Cyp12a5* had increased mortality compared to the control cross, whereas no change in mortality was detected for *Cyp12a4* (Figure 2C). These results support the negative correlation between *Cyp12a5* expression and nitenpyram resistance detected by RNAi.

Because resistance to lufenuron maps to the *Cyp12a4*/*Cyp12a5* region,[35] we also reared first instar larvae overexpressing *Cyp12a4* or *Cyp12a5* on lufenuron. Mortality of UAS-*Cyp12a4* progeny decreased on lufenuron compared to the control cross, but no change was observed for UAS-*Cyp12a5* progeny (Supplementary figure 5), in concordance with Bogwitz *et al.*[35]

Discussion

Knockdown of *Cpr* or *dare* either ubiquitously or more specifically in the prothoracic gland, where the moulting hormone ecdysone is synthesised from cholesterol,[36] resulted in mortality. This was expected, because knockdown or mutation of individual P450s involved in ecdysone production is also lethal.[33,37–42] In our experiments, the partial rescue of *Cpr* RNAi mortality by the addition of 20-hydroxyecdysone indicates that redox partner RNAi interrupts P450 function. The manipulation of *Cpr* expression has also been used to provide evidence for P450 functions in xenobiotic metabolism and hydrocarbon biosynthesis. Transgenic RNAi of *Cpr* in *D. melanogaster* oenocytes resulted in a decrease in cuticular hydrocarbon, consistent with the role of *Cyp4g1* in that tissue.[43] Injection of double-stranded *CPR* RNA increased permethrin sensitivity in *Anopheles gambiae*,[44] and a similar technique increased susceptibility to deltamethrin in *Cimex lectularius*.[45]

Crossing the *Cpr* and *dare* RNAi constructs into resistant *D. melanogaster* backgrounds or injecting *Cpr* and *dare* dsRNA into pest species may yield results analogous to the use of P450 inhibitors and be useful for detecting P450-based metabolism of insecticides and other P450 functions, and for testing a broader range of insecticide classes for P450 metabolism. Despite the clear role of *Cyp6g1* in DDT metabolism,[13,23,46] we did not detect a change in DDT sensitivity in *Cpr* RNAi lines. Different effects of *Cyp6g1* RNAi on DDT mortality have been reported,[47,48] possibly associated with the resistance status of the strain used. The w^{1118} strain we used in our RNAi experiments is naïve to insecticides and may have low constitutive tolerance to DDT, making it difficult to detect increased DDT sensitivity. Alternatively, the knockdown of *Cpr* in some cases may not have resulted in rate-limiting levels of CPR protein.

Cpr and *dare* knockdown in the digestive tissues (the Malpighian tubules, midgut and fat body) did not cause mortality, but RNAi of *dare* resulted in a nitenpyram resistance phenotype, indicating the involvement of a mitochondrial P450 in

nitenpyram metabolism. Transgenic manipulation of expression of the mitochondrial P450 gene *Cyp12a5* mirrored these results, although the decrease in toxicity was greater in *dare*-RNAi larvae than in *Cyp12a5*-RNAi larvae. This may suggest that *dare*-RNAi had a greater impact on CYP12A5 activity than *Cyp12a5*-RNAi, or that other mitochondrial P450s affected by *dare* knockdown also influence nitenpyram toxicity. Mitochondrial P450s from mammalian genomes have not generally been found to metabolise xenobiotics.[7] In contrast, although some insect P450s from the mitochondrial clade function in the synthesis of ecdysone,[49] others are involved in the metabolism of xenobiotics. *M. domestica* CYP12A1 converts diazinon to diazoxon *in vitro*,[4] and lufenuron resistance is conferred by overexpression of *Cyp12a4*.[35] Furthermore, *Cyp12d1* is overexpressed in strains resistant to DDT,[50,51] and transgenic expression confers resistance to DDT and dicyclanil.[9] Despite the functions of mitochondrial P450 in xenobiotic metabolism, activation of neonicotinoids by mitochondrial P450s has not been reported.

P450 overexpression is a major mechanism of resistance to neonicotinoids,[10] and transgenic overexpression of two other *D. melanogaster* P450 genes, *Cyp6g1* and *Cyp6g2*, suggests that P450-based metabolism of nitenpyram can confer resistance.[9] Similarly, constitutive overexpression of P450s is associated with resistance in field populations of pests including *Bemisia tabaci*, *Myzus persicae* and *Nilaparvata lugens*.[11,12,14–17] In contrast, the correlation between *Cyp12a5* expression and nitenpyram mortality that we report suggests that CYP12A5 activity increases the toxicity of nitenpyram *in vivo*. This may be a result of CYP12A5 activity towards nitenpyram or a metabolite of nitenpyram. Other P450s have been suggested to produce metabolites which are potentially more toxic than their parent compounds. Both heterologous expression and *in vivo* overexpression of the *Cyp6g1* gene of *D. melanogaster* correlate with increased production of the toxic olefin metabolite of imidacloprid.[23,24] This metabolite has a lower LC_{50} and higher affinity for nAChRs than imidacloprid.[52] However, the production of the olefin metabolite may be restricted to the peripheral tissues,[53] and overexpression of *Cyp6g1* results in a

higher rate of olefin excretion, which explains the resistance phenotype of *Cyp6g1* overexpression.[24] The mammalian P450 CYP3A4 is also proposed to activate imidacloprid,[54] while thiamethoxam, another neonicotinoid insecticide, appears to be activated by insects or plants to the more toxic clothianidin.[55] However, individual P450s that are involved in neonicotinoid modifications have not been reported to increase susceptibility *in vivo*. Possible explanations of the increase in nitenpyram toxicity correlated with *Cyp12a5* expression observed here include that (*i*) one or more metabolites of nitenpyram have a higher affinity for target nAChRs than nitenpyram does or (*ii*) toxic metabolites of nitenpyram are less readily excreted than nitenpyram. These possibilities are not mutually exclusive. One barrier to testing these hypotheses is that, in contrast to the neonicotinoid imidacloprid, pathways of nitenpyram metabolism have not been defined in insects. Nitenpyram metabolites have been described in plants and mammals,[56,57] but it is not known whether the same metabolites are formed in insects or if the metabolites have insecticidal properties. Our data suggest that a detailed investigation of nitenpyram metabolism in insects would be worthwhile, and it will be of interest to see where CYP12A5 acts in this pathway.

Finally, given their well-defined role in conferring insecticide resistance, it is easy to assume that a P450 implicated in a resistance phenotype has a role in detoxification. Our data and other examples discussed suggest that the metabolism of insecticides by P450s needs to be examined in greater detail to understand mechanisms underlying detoxification resistance in cases where the metabolites produced are more toxic.

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Tables

Table 1. Number of adults emerged from 20 eggs expressing *Cpr* and *dare* RNAi constructs in the prothoracic gland (mean ± SEM, $n = 5$). No Cy^+ adults emerged from crosses with the RNAi lines, indicating that RNAi of *Cpr* or *dare* in the prothoracic gland is lethal.

Table 2. Quantification of *Cpr* and *dare* knockdown in the midgut.

Figure legends

Figure 1. Effect of *Cpr* and *dare* RNAi in the prothoracic gland. (A) Number of days at each developmental stage. *Cpr*-RNAi1 flies died before the 3rd instar larval stage, whereas *Cpr*-RNAi2 and *dare* RNAi flies died before the pupal stage. Addition of two pulses of 20-hydroxyecdysone (20HE) rescued the first or second instar mortality of *Cpr*-RNAi1 flies and increased the number of days *dare*-RNAi2 flies survived. (B) Percentage survival between developmental stages. 20HE rescued the 1st to 3rd instar survival of *Cpr* RNAi flies to wild-type levels but did not affect survival later in development. The genotypes indicated on the *x*-axes are the male parents that were crossed to virgin 5′*phantom* females, which produces *GAL4* in the prothoracic gland.[27]

Figure 2. Nitenpyram bioassays. Mortality of (A) *dare* RNAi flies and (B) *Cyp12a5* RNAi flies was reduced, and mortality of (C) flies overexpressing *Cyp12a5* was increased. The error bars show 95% confidence interval for corrected mortality from 10 replicates at each dose.

Supporting information

Supplementary figure 1. Non-overlapping regions targeted by *Cpr* and *dare* RNAi constructs. Each panel shows the FlyBase 6.16 transcripts and coding regions (CDS) and the regions targeted by the RNAi construct. *Cpr* has four annotated transcripts, and the *Cpr* RNAi constructs target shared exons. *dare* has a single transcript with overlapping transcripts from neighbouring genes *CG18335* and *CG30033*. The *dare* RNAi constructs target the *dare* transcript but not transcripts from *CG18335* and *CG30033*.

Supplementary figure 2. Lethality of *Cpr* and *dare* RNAi with ubiquitous and digestive tissue GAL4 drivers. (A) Number of female and male progeny emerging from a single cross between male UAS-RNAi lines and female 5′*tubulin* driver line (Bloomington stock 5138). Sb^{1} progeny inherited the balancer chromosome and do not produce *GAL4*. (B) Average number of progeny emerging from reciprocal crosses between UAS-RNAi lines and heterozygous flies carrying the 5′*Cyp6g1*HR-3a driver and a balancer chromosome. $Antp^{Hu}$ progeny inherited the balancer chromosome and do not produce *GAL4*. Crosses were repeated at 22°C and 29°C.

Supplementary figure 3. DDT and dicyclanil bioassays. There was no change in DDT mortality, even in *Cpr* RNAi flies, which are expected to have interrupted *Cyp6g1* activity in the digestive tissues.

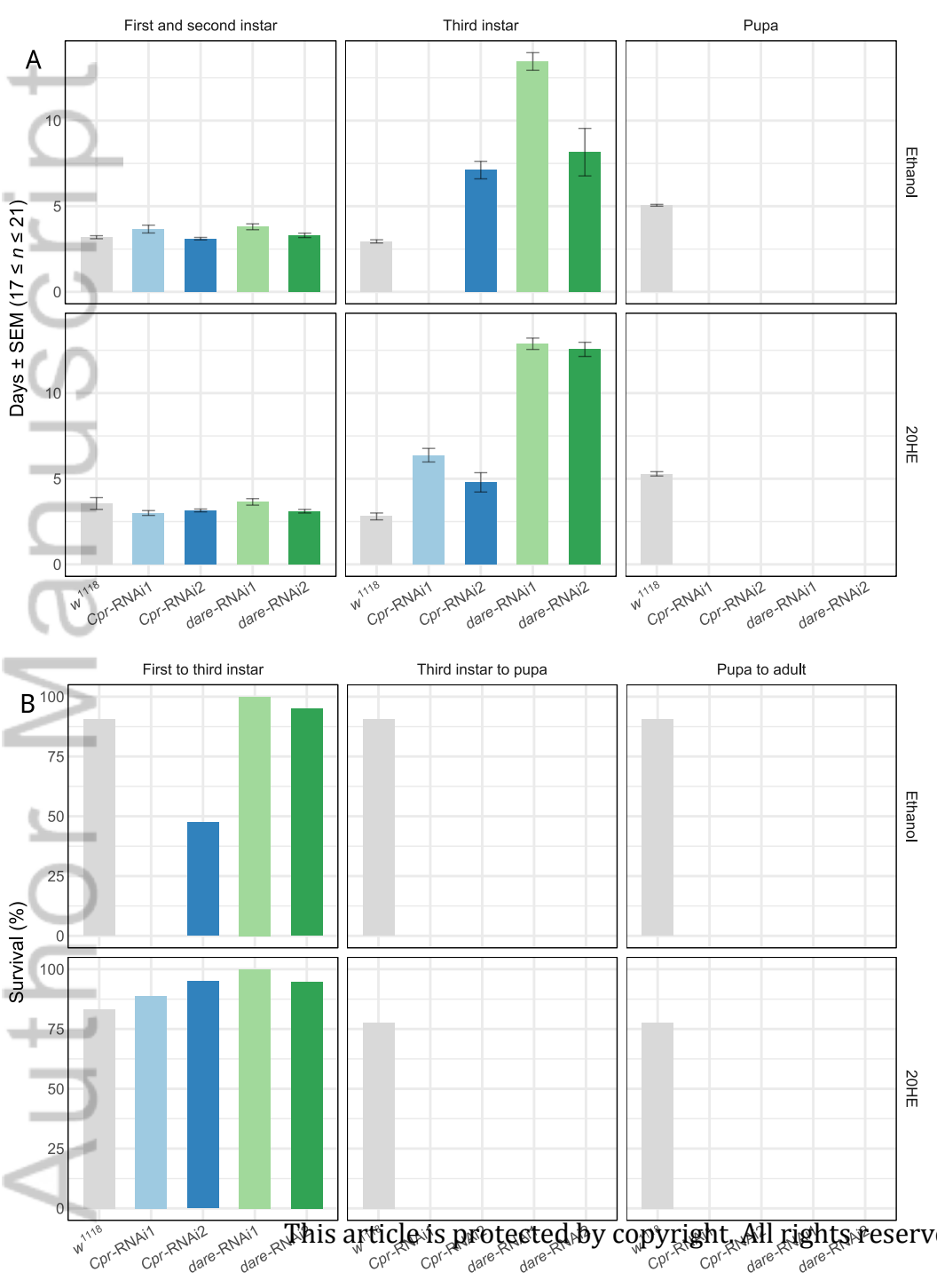
Supplementary figure 4. Expression of mitochondrial P450s in the midgut.

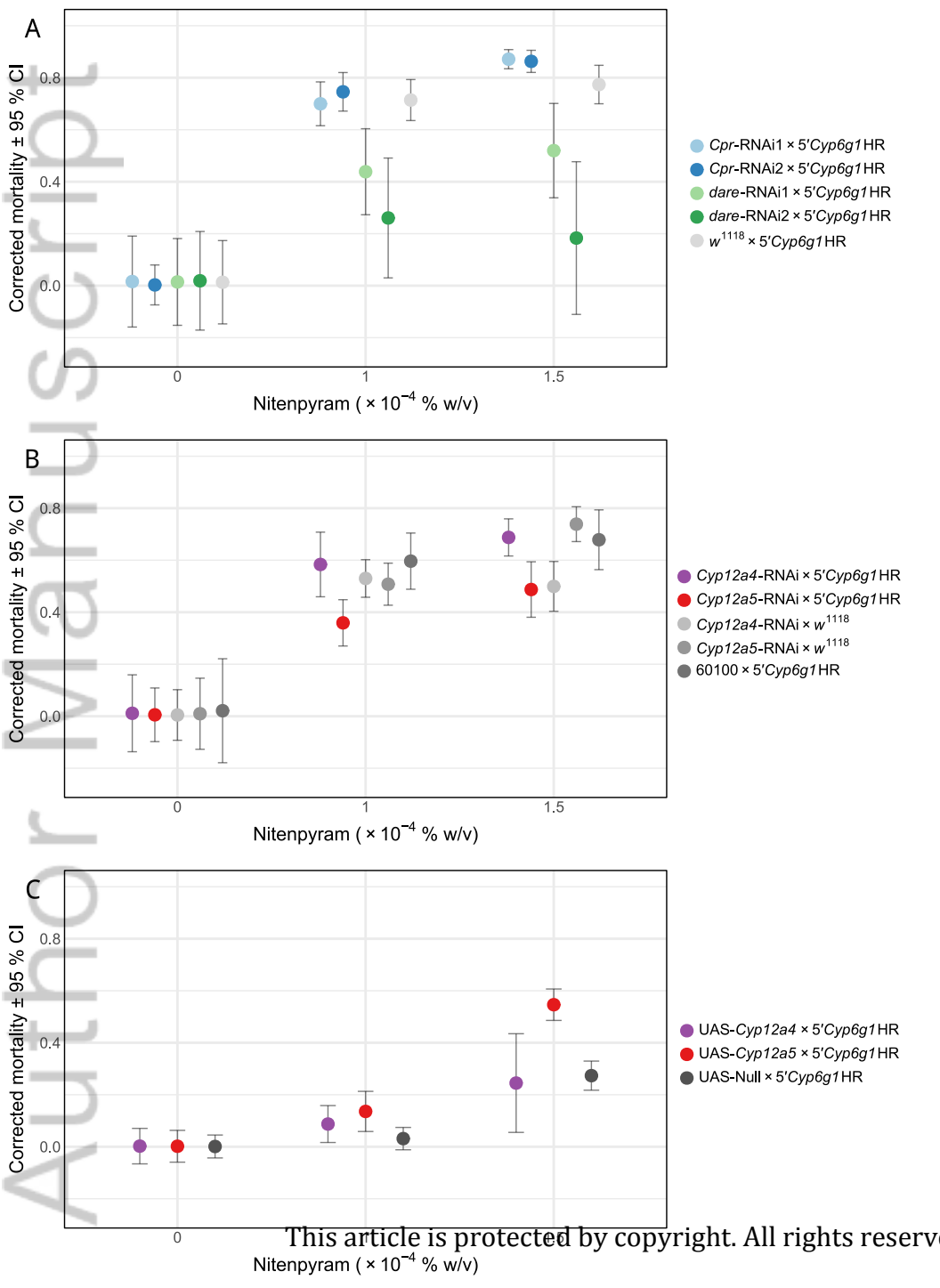
Supplementary figure 5. *Cyp12a4* and *Cyp12a5* overexpression lufenuron bioassays.

Supplementary table 1. Fly lines used in this study.

Supplementary table 2. Primers used in this study.

Graphical abstract





Evidence for activation of nitenpyram by a mitochondrial cytochrome P450 in *Drosophila melanogaster*

Short title: Nitenpyram activation by a P450 in *D. melanogaster*

Thomas W.R. Harrop[a,1,2], Shane Denecke[1], Ying Ting Yang, Janice Chan, Phillip J. Daborn, Trent Perry, and Philip Batterham

Department of Genetics and Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Victoria 3010, Australia.

[a] Present address: Department of Biochemistry, The Laboratory for Evolution and Development, The University of Otago, Dunedin 9054, Aotearoa-New Zealand.

[1] These authors contributed equally to this work

[2] Corresponding author: twharrop@gmail.com

Abstract

The *in vivo* metabolism of nitenpyram is not well characterised, but cytochrome P450 activity is the major mechanism of resistance to neonicotinoids identified in insect pests, and P450s metabolise other neonicotinoids including imidacloprid.

Figure 1

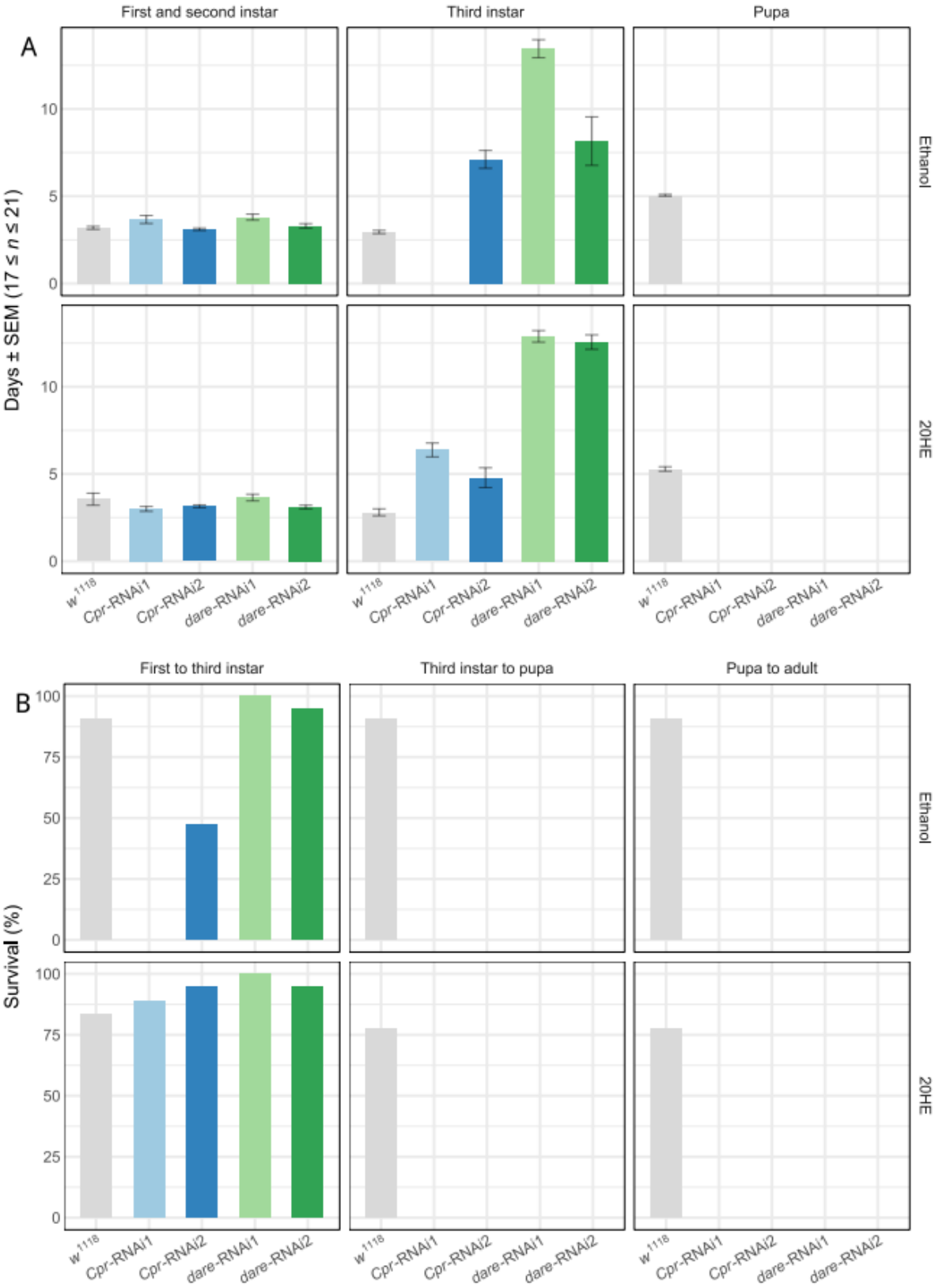


Figure 2

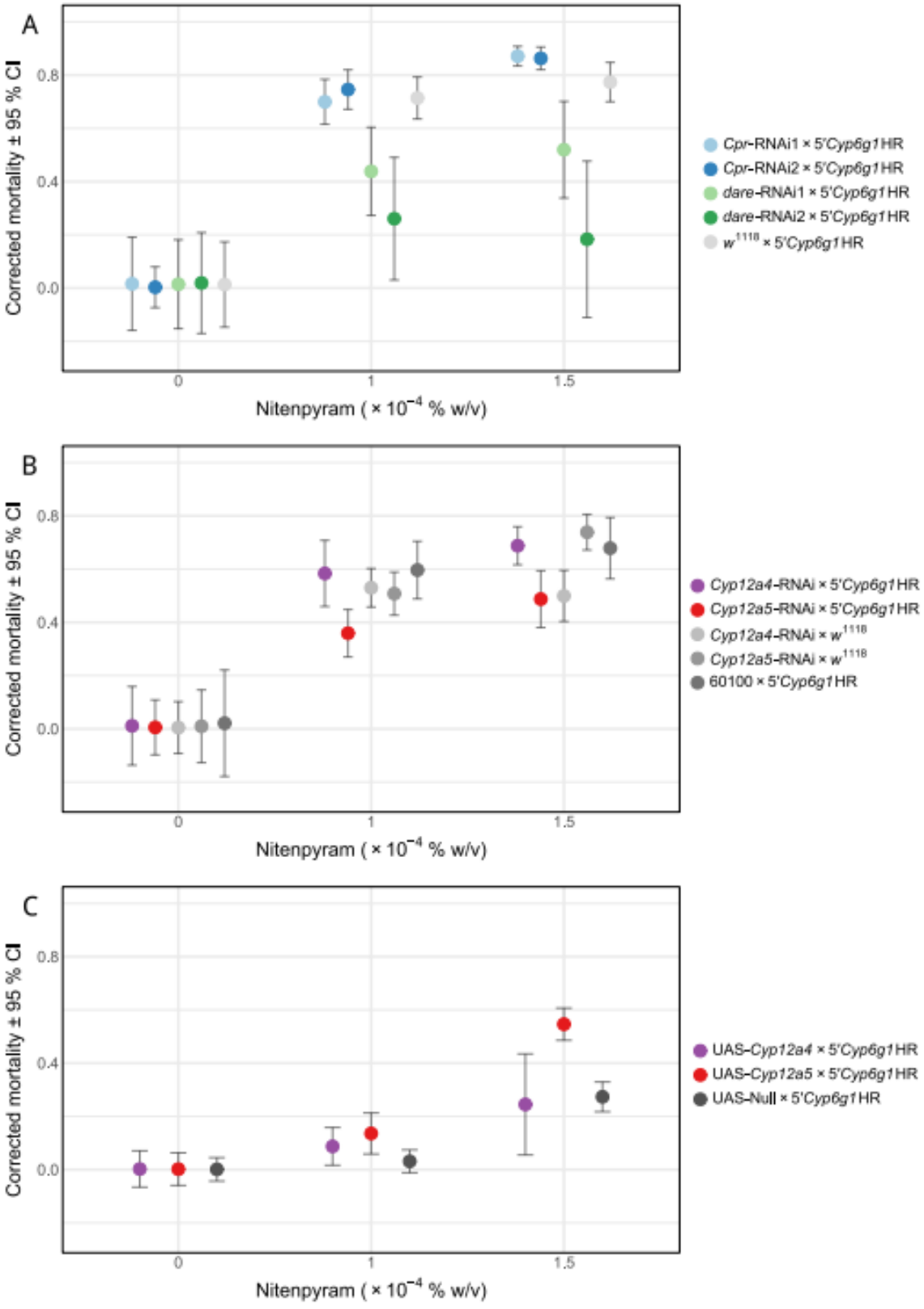


Figure S1

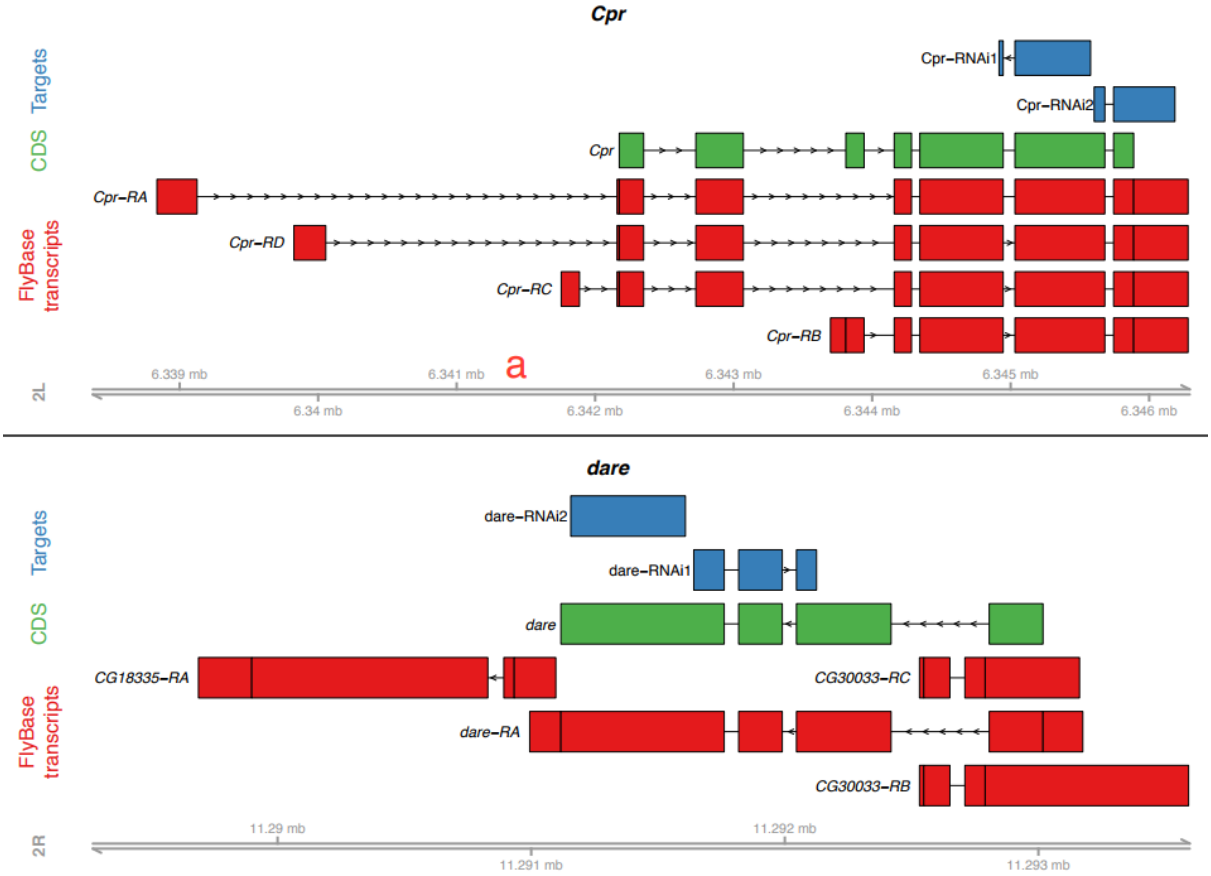
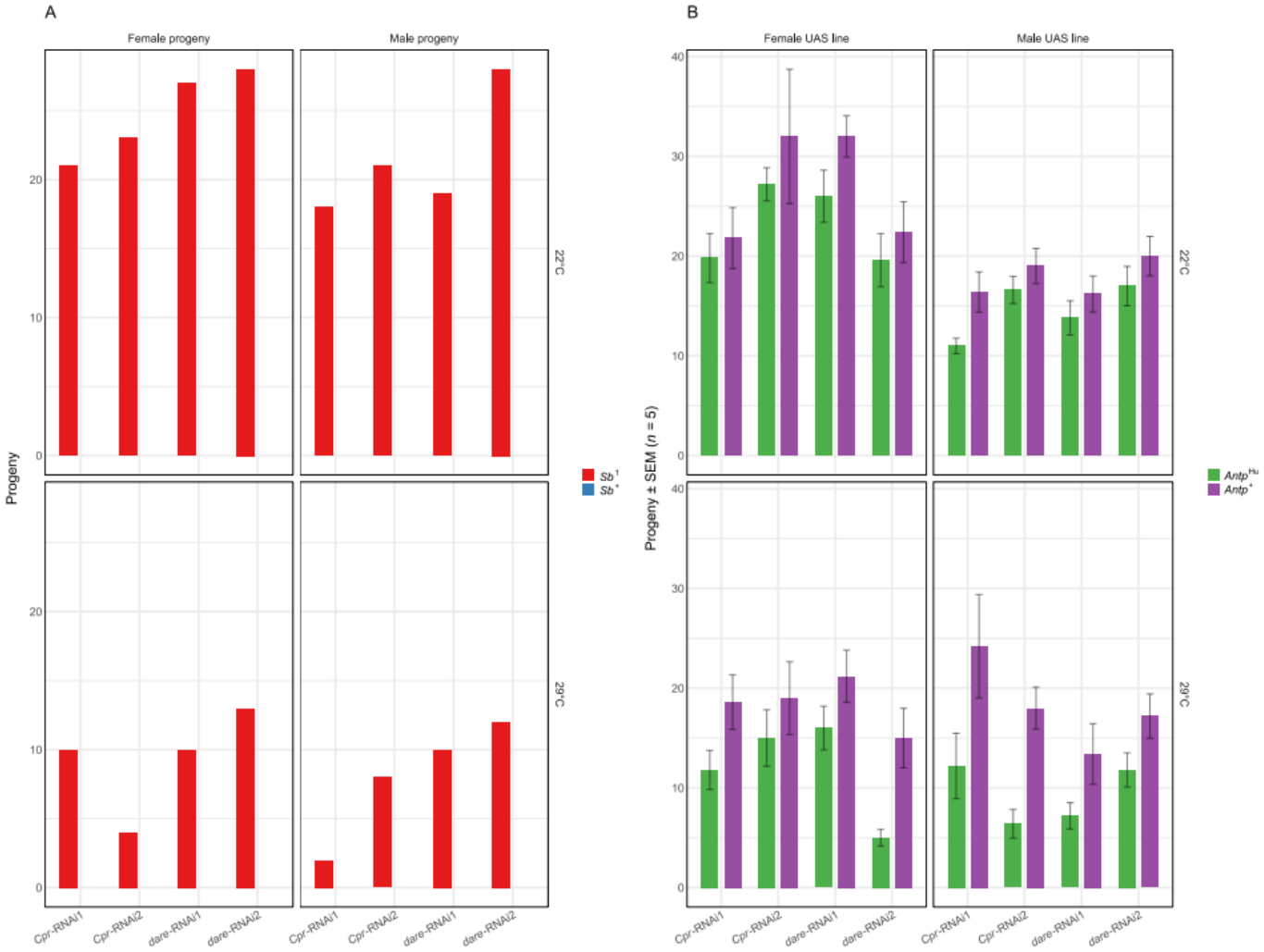


Figure S2



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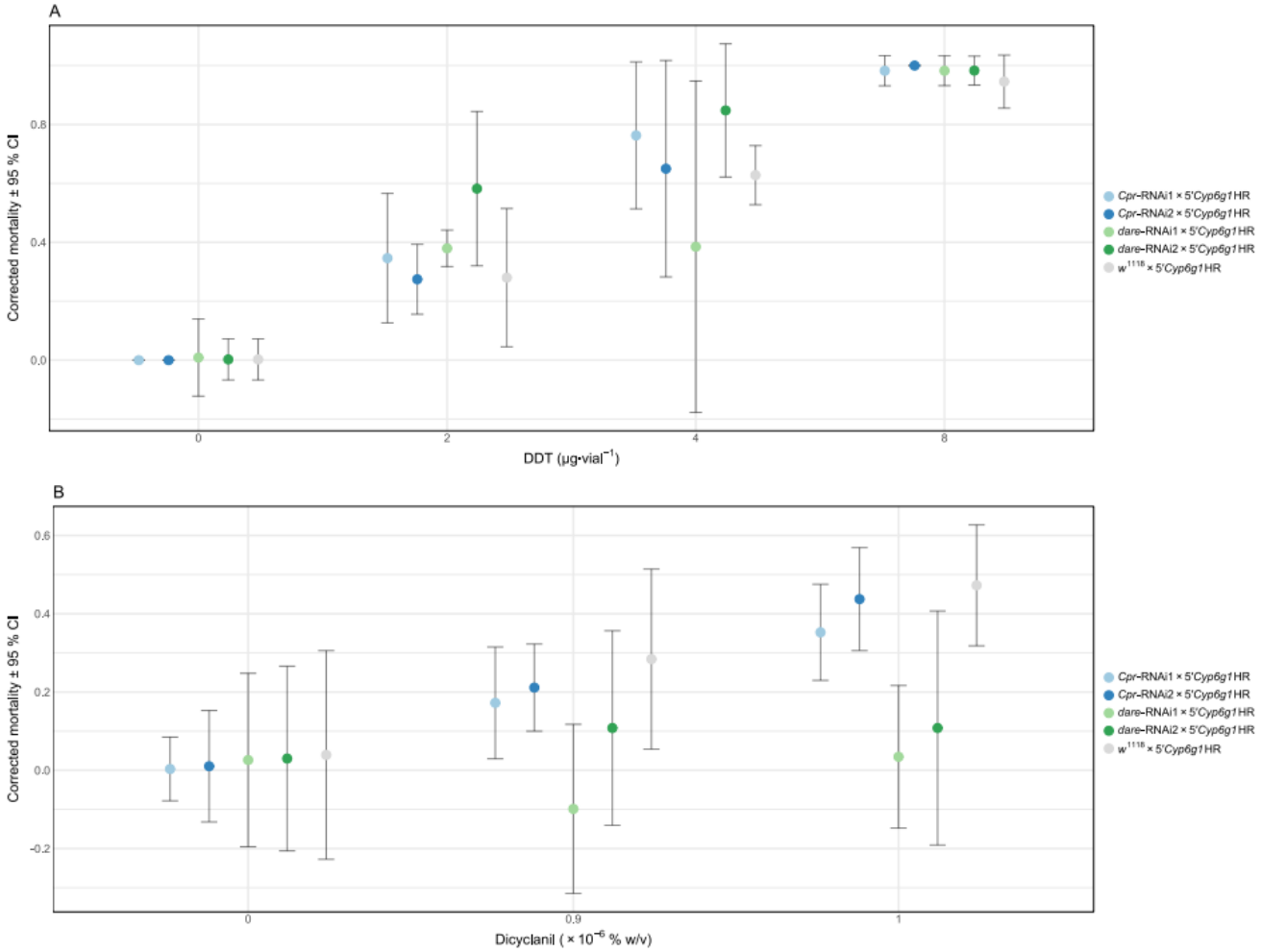

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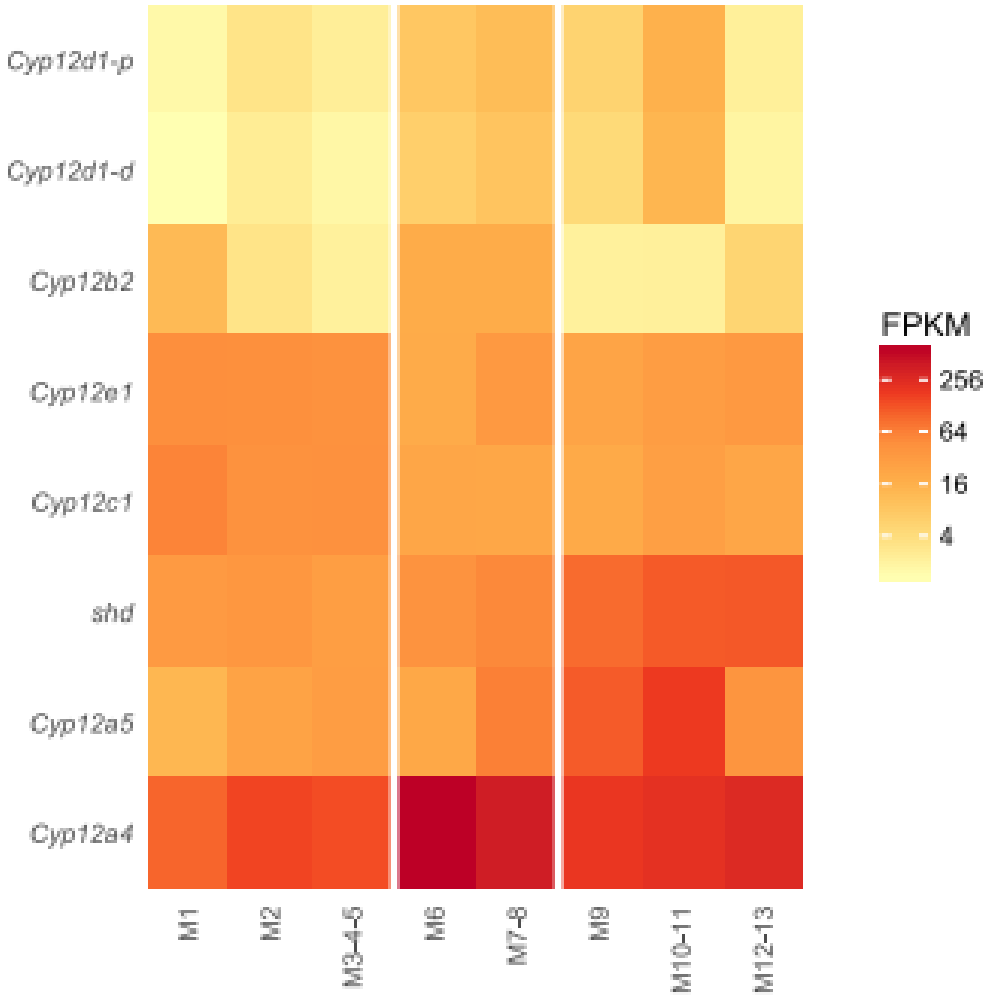

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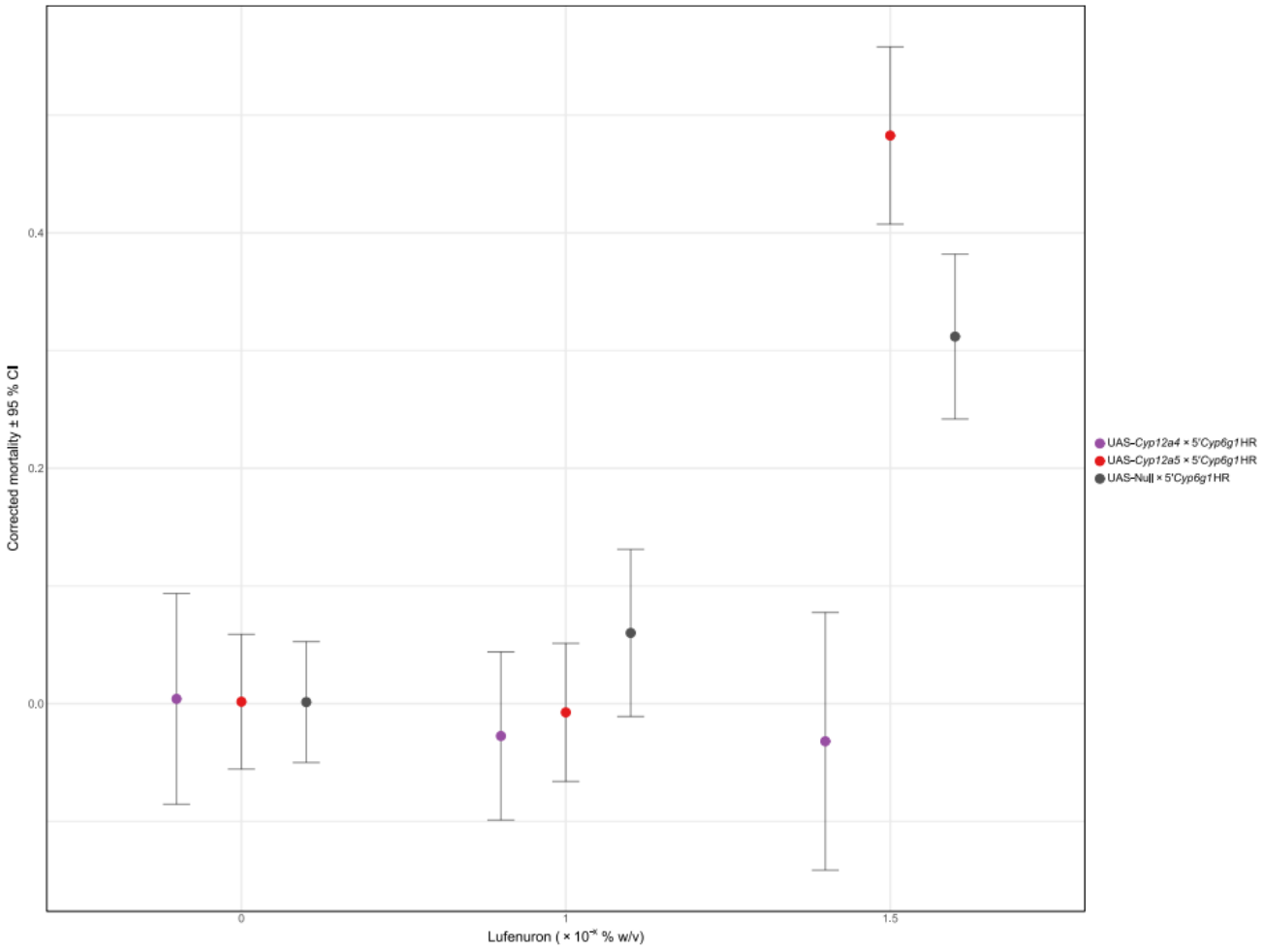

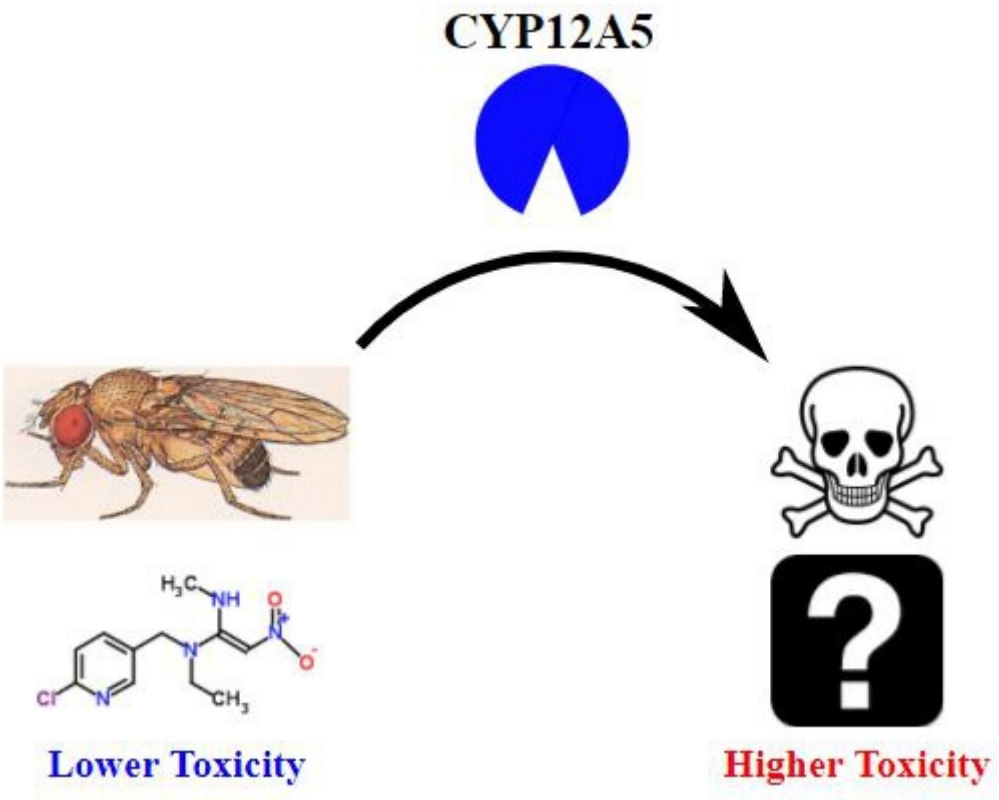

JanCapture.JPG

Cross	*Cy*^{1}	*Cy*$^{+}$
w^{1118} × 5′*phantom*	3.8 ± 1	3.6 ± 1.2
Cpr-RNAi1 × 5′*phantom*	3.4 ± 0.81	0 ± 0
Cpr-RNAi2 × 5′*phantom*	6 ± 0.95	0 ± 0
dare-RNAi1 × 5′*phantom*	4 ± 0.71	0 ± 0
dare-RNAi2 × 5′*phantom*	5 ± 2.3	0 ± 0

Table 1. Number of adults emerged from 20 eggs expressing *Cpr* and *dare* RNAi constructs in the prothoracic gland (mean ± SEM, $n = 5$). No *Cy*$^{+}$ adults emerged from crosses with the RNAi lines, indicating that RNAi of *Cpr* or *dare* in the prothoracic gland is lethal.

	First instar	Third instar	Adult
Cpr-RNAi1 × 5′*Cyp6g1*HR	32.8 ± 7.4	24.6 ± 2.1	38.3 ± 1.6
Cpr-RNAi2 × 5′*Cyp6g1*HR	42.3 ± 11.4	22.4 ± 4.5	40 ± 5.7
dare-RNAi1 × 5′*Cyp6g1*HR	27.5 ± 6.8	10.6 ± 3.2	15.1 ± 1.6
dare-RNAi2 × 5′*Cyp6g1*HR	21.2 ± 6.4	20 ± 2.6	18.3 ± 4.7

Table 2. Relative quantity of *Cpr* and *dare* mRNA remaining in midguts from RNAi progeny (mean % ± SEM, n = 3).