Supplementary Information for

Dissecting the insect metabolic machinery using twin ion mass spectrometry: A single P450 enzyme metabolizes the insecticide imidacloprid *in vivo*

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Table of Contents

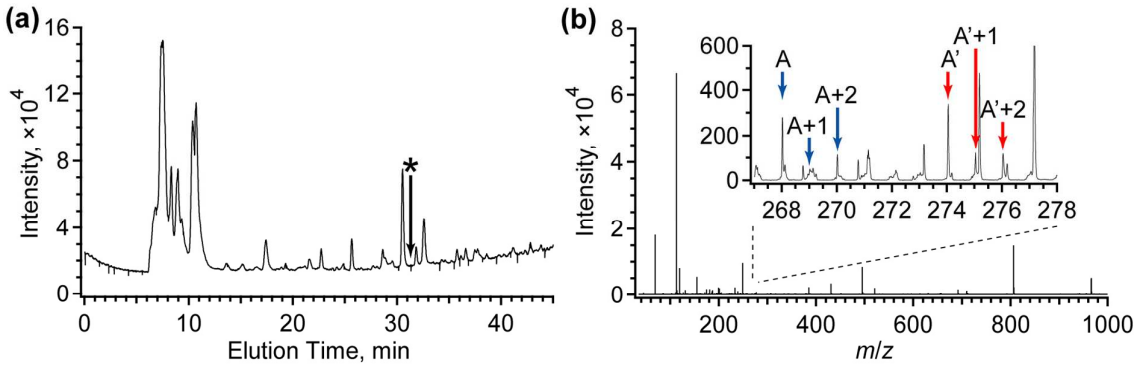

Figure S1 (a) Total ion chromatogram (TIC) of a metabolite extract that is obtained from 200 w^{1118} *D. melanogaster* larvae bodies that were exposed to 1:1 ratio of imidacloprid and [$^{13}C_6$]imidacloprid at a sublethal dosage for 6 hours. (b) Mass spectra obtained at an elution time of ~32 min (see "*" symbol in panel *a*). Inset is a twin ion signal corresponding to a metabolite of imidacloprid with an *m*/*z* value of 268 (neutral mass is 269 Da). Arrows indicate the *m*/*z* values of the unlabelled (A) and labelled (A') metabolite and the +1 Da (A+1, A'+1) and +2 Da (A+2, A'+2) isotopes.

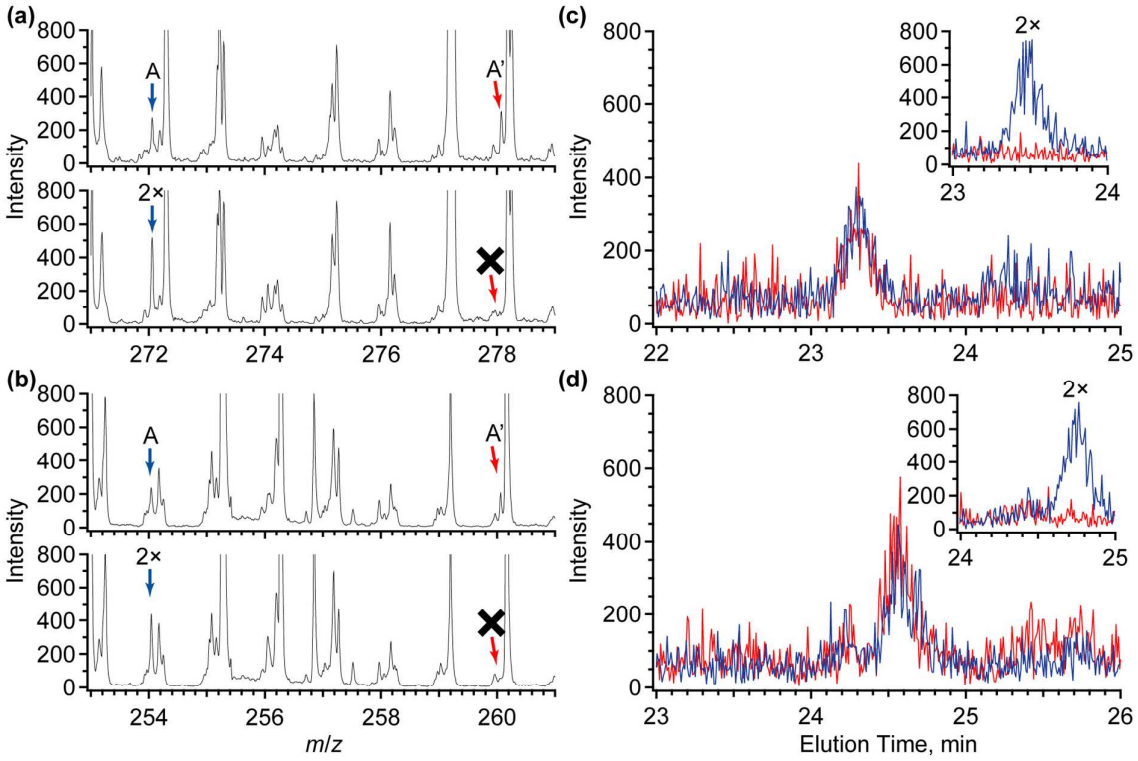

Figure S2 Twin ion metabolite mass spectra of the (**a**) hydroxy and (**b**) olefin metabolites that were detected in metabolite extracts obtained from the excreta of a single w^{1118} *D. melanogaster* larva that was exposed to 1:1 ratio (top spectra) or 2:0 ratio (bottom spectra) of imidacloprid and [$^{13}C_6$]imidacloprid at a sublethal dose for 6 h. Extracted ion chromatograms of A (unlabelled) and A' (labelled) *m*/*z* values for the (**c**) hydroxy and (**d**) olefin metabolites. Crosses indicate that signal for the labelled metabolite was not detected.

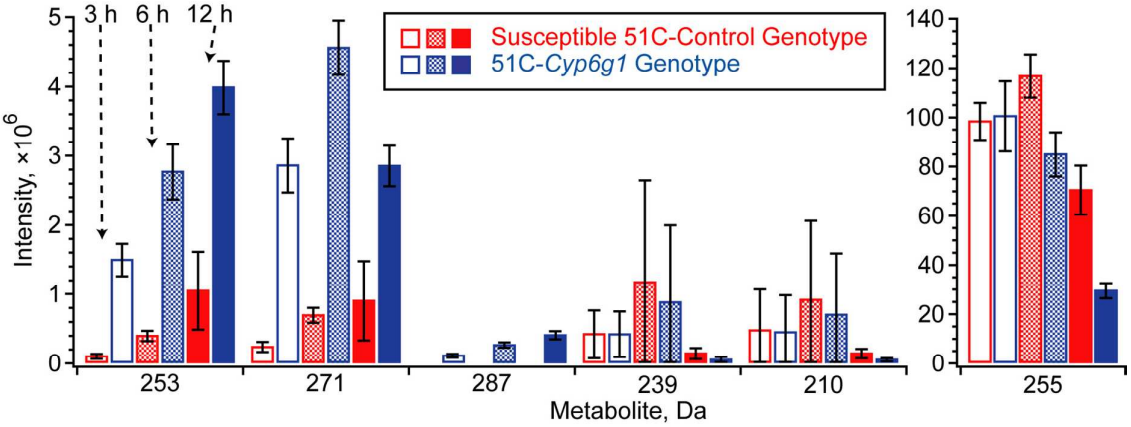

Figure S3 Absolute intensities of five metabolites and imidacloprid detected in the extracts obtained from the excreta of 200 transgenic larvae that selectively overexpress *Cyp6g1* exposed to imidacloprid (blue bars; 51C-*Cyp6g1*) compared to that for a control strain (red bars; 51C-Control) for imidacloprid exposure times of 3 (open bars), 6 (hatched bars) and 12 h (closed bars). Abundance values are obtained from the average of two biological replicates that were each analyzed in triplicate (6 total experiments). Error bars correspond to ±1 standard deviation.

Table S1. Target list of molecular formulae and relative molar mass (M_r) of imidacloprid metabolites that have been reported in other organisms and in the environment under various conditions.[1-7] Asterisks indicate the metabolites that were detected using the targeted twin ion method (see main text).

Molecular Formula	M_r
$C_9H_{10}N_5O_3Cl^*$	271.0472
$C_9H_{10}N_5O_4Cl^*$	287.0421
$C_9H_8N_5O_2Cl^*$	253.0367
$C_7H_8N_5O_2Cl^*$	229.0367
$C_9H_{10}N_5OCl^*$	239.0574
$C_9H_{11}N_4Cl^*$	210.0672
C_6H_6NOCl	143.0138
$C_6H_4NO_2Cl$	156.9931
$C_9H_{10}N_3OCl$	211.0512
$CH_4N_4O_2$	104.0334
$C_3H_4N_4O_2$	128.0334
$C_3H_6N_4O_2$	130.0491
$C_6H_5NO_3$	139.0269
$C_7H_7NO_2S$	169.0198
$C_8H_8N_2O_4$	196.0484
$C_8H_7N_2O_3Cl$	214.0145
$C_6H_5NO_6S$	218.9838
$C_9H_{12}N_5Cl$	225.0781
$C_9H_{10}N_2O_3S$	226.0412
$C_8H_8N_2O_7S$	276.0052
$C_{12}H_{12}N_5OCl$	277.0730
$C_{10}H_5N_9S$	283.0389
$C_{11}H_{12}N_2O_5S$	284.0467
$C_{12}H_{15}NO_8$	301.0798
$C_{12}H_{14}NO_7Cl$	319.0459
$C_{13}H_{17}NO_7S$	331.0726
$C_{13}H_{15}N_3O_6S$	341.0682
$C_{11}H_{22}N_2O_{10}S$	374.0995
$C_{12}H_{15}NO_{11}S$	381.0366
$C_3H_6N_4O$	114.0541
C_6H_4NOCl	140.9981
$C_6H_7N_2Cl$	142.0298
$C_7H_6N_3Cl$	167.0250

$C_8H_{12}N_3Cl$	185.0720
$C_7H_8N_3OCl$	185.0356
$C_9H_{11}N_4O_2Cl$	242.0570
$C_{15}H_{18}N_5O_9Cl$	447.0793
$C_7H_7N_2OCl$	170.0247
$C_8H_9N_2OCl$	184.0403
$C_7H_9N_4Cl$	184.0516
$C_9H_8N_3O_2Cl$	225.0305
$C_{12}H_{16}NO_6Cl$	305.0666
$C_{18}H_{26}NO_{11}Cl$	467.1195
$C_9H_9N_4Cl$	208.0516
$C_9H_{10}N_3O_3Cl$	243.0410
$C_{11}H_{12}N_2O_6S$	300.0416
$C_{18}H_{18}N_8OCl_2$	432.0980

Table S2. Unique molecular formulae and relative molar mass (M_r) of metabolites predicted for imidacloprid metabolism using the KEGG-based predictor and microbial-based software.[8-9] Asterisks indicate the metabolites that were detected using the non-targeted twin ion method (see main text).

KEGG-based predictor[a]	M_r	Enzyme Class: Reaction class	Microbial-based predictor	M_r
$C_2H_5N_5O$	143.0138	EC1: dealkylation	CH_3NO	45.0215
$C_7H_8N_5O_2Cl$ *	229.0367	EC4: C-N lyases to C=C	$C_2H_8N_2$	60.0687
$C_9H_{11}N_5O_3$	237.0862	EC3: Halide bond substitution	$C_2H_5NO_2$	75.0320
$C_9H_8N_5O_2Cl$ *	253.0367	EC1: dehydrogenation	$C_3H_6N_2O$	86.0480
$C_9H_{12}N_5O_2Cl$	257.0680	EC4: C-C lyases to (=O)	$C_3H_5NO_3$	103.0269
$C_{10}H_{12}N_5O_2Cl$[a]	269.0680	EC2: N-methylation	$C_3H_8N_2O_2$	104.0586
$C_9H_{10}N_5O_3Cl$ *	271.0472	EC1: C-H hydroxylation	$C_3H_5NO_4$	119.0219
$C_9H_{12}N_5O_3Cl$	273.0629	EC3:3.5.4	C_6H_4ClNO	140.9981
$C_{10}H_{11}N_6O_2Cl$	282.0632	EC2: Hydroxymethyl, Formyl 2.1.2	$C_6H_7ClN_2$	142.0298
$C_{10}H_{10}N_5O_3Cl$	283.0472	EC2: Hydroxymethyl, Formyl 2.1.2	$C_6H_4ClNO_2$	156.9931
$C_{10}H_{12}N_5O_3Cl$	285.0629	EC2:Hydroxymethyl and formyl transferases	$C_6H_7ClN_2O$	158.0247
$C_9H_{10}N_5O_4Cl$ *	287.0421	EC1: aromatic dioxygenase	$C_6H_4ClNO_3$	172.9880
$C_9H_9N_5O_2Cl_2$	289.0133	EC1: C-Cl oxidation	$C_8H_9ClN_2O$	184.0403
$C_9H_{12}N_5O_4Cl$	289.0578	EC1: Epoxidation & dihydoxylation	$C_8H_{12}ClN_3$	185.0720
$C_{11}H_{12}N_5O_3Cl$	297.0629	EC2: N-acylation	$C_8H_9ClN_2O_2$	200.0353
$C_{10}H_{11}N_6O_3Cl$	298.0581	EC2: 2.1.3&4	$C_8H_{12}ClN_3O$	201.0669
$C_{10}H_{10}N_5O_4Cl$	299.0421	EC6: C-C ligases	$C_9H_{10}ClN_3O$	211.0512
$C_9H_{10}N_5O_5ClS$	335.0091	EC2: Sulfotranferases	$C_8H_{11}ClN_2O_3$	218.0458
$C_9H_{11}N_5O_5ClP$	335.0186	EC2: Phosphotranferases	$C_9H_{12}ClN_3O_2$	229.0618
$C_{14}H_{19}N_5O_5Cl$	372.1075	EC2: Pentosyltransferases		

[a] This list does not include the 66 additional metabolites with unique molecular formulae that were predicted by using the metabolites detected using the primary predicted metabolites (predicted secondary metabolites). For the secondary metabolite list, $C_9H_8N_5O_3Cl$ (neutral M_r = 269.03157 Da), was routinely detected with a mass accuracy of <6 ppm (see main text). This metabolite is 135 ppm lower than $C_{10}H_{12}N_5O_2Cl$ (269.0680 Da) indicating that the detected twin ion corresponds to $C_9H_8N_5O_3Cl$.

Table S3. Accurate masses, relative abundances, and fragmentation assignments of the three major product ions formed upon CID of protonated 5-hydroxy and olefin imidaclorpid (authentic standards) vs. the hydroxy and olefin metabolites that are formed *in vivo*.

Authentic 5-hydroxy imidacloprid	***in vivo* hydroxy metabolite**	**Authentic olefin imidacloprid**	***in vivo* olefin metabolite**
191.0926 (100%) ($-NO_2Cl$)	191.0931 (100%) ($-NO_2$, -Cl)	205.0272 (100%) ($-H_3NO_2$)	205.0292 (100%) ($-H_3NO_2$)
225.0534 (77.45%) ($-HNO_2$)	225.053 (77.19%) ($-HNO_2$)	171.0663 (62.34%) ($-H_2NO_2Cl$)	171.0647 (63.49%) ($-H_2NO_2$)
228.0532 (67.12%) ($-N_2O$)	228.054 (57.95%) ($-N_2O$)	206.035 (60.64%) ($-H_2NO_2$)	206.0342 (83.29%) ($-H_2NO_2$)

Table S4. P-values obtained from statistical *t*-test to test whether the abundances of metabolites detected in 51C-*Cyp6g1* are statistically different than those detected in the control 51C strain (6 replicates; see Figures 4 and S3). P-values that are less than 0.05 indicate that these populations are generally considered to be statistically different.

	253 Da	**271 Da**	**287 Da**	**239 Da**	**210 Da**	**255 Da**
3 h	0.0	0.0	0.0	0.5	0.5	0.4
6 h	0.0	0.0	0.0	0.4	0.4	0.0
12 h	0.0	0.0	0.0	0.0	0.0	0.0

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