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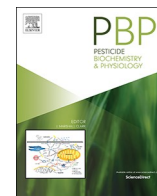
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ABSTRACT

Herbivorous insects encounter a variety of toxic environmental substances ranging from ingested plant defensive compounds to human-introduced insecticidal agents. Dietary antioxidants are known to reduce the negative physiological impacts of toxins in mammalian systems through amelioration of reactive oxygen-related cellular damage. The analogous impacts to insects caused by multigenerational exposure to pesticides and the effects on adaptive responses within insect populations, however, are currently unknown. To address these research gaps, we used *Drosophila* as a model system to explore adaptive phenotypic responses to acute dichlorodiphenyltri-chloroethane (DDT) exposure in the presence of the dietary antioxidant vitamin C and to examine the structural genomic consequences of this exposure. DDT resistance increased significantly among four replicates exposed to a low concentration of DDT for 10 generations. In contrast, dietary intake of vitamin C significantly reduced DDT resistance after mutigenerational exposure to the same concentration of DDT. As to the genomic consequences, no significant differences were predicted in overall nucleotide substitution rates across the genome between any of the treatments. Despite this, replicates exposed to a low concentration of DDT without vitamin C showed the highest number of synonymous and non-synonymous variants (3196 in total), followed by the DDT plus vitamin C (1174 in total), and vitamin C alone (728 in total) treatments. This study demonstrates the potential role of diet (specifically, antioxidant intake) on adaptive genome responses, and thus on the evolution of pesticide resistance within insect populations.

1. Introduction

In many instances, the repeated exposure of an arthropod population to an insecticidal agent has resulted in the selection of individuals with adaptive phenotypes that survive increased levels of xenobiotic exposure, known also as insecticide resistance (Melander, 1914). Across arthropods, resistance has evolved to most classes of chemical insecticides (Mota-Sanchez and Wise, 2018) and many plant-based allelochemicals (Dermauw et al., 2018). This evolution of resistance has become a concern and a threat to the sustainability of current crop and livestock production practices, where lack of control of resistant insect populations causes annual losses valued at several billion U.S. dollars across the agricultural sector in the United States (Gould et al., 2018). Furthermore, pesticide resistance within populations of disease-vectoring arthropods directly impacts the ability to manage the exposure of human populations to disease agents (Hemingway and Ranson, 2000; Nauen, 2007). Resistance to insecticides, like resistance to host-plant resistance factors, evolves from selection for adaptive phenotypes within insect populations (Alyokhin and Chen, 2017; Cheng et al., 2017; Dermauw et al., 2013; Dermauw et al., 2018; Erb and Robert, 2016; Strycharz et al., 2013). For example, plants often produce an array of compounds that cause antibiosis and/or antixenosis, which defend against herbivorous insect

feeding damage. Herbivorous insects have evolved corresponding mechanisms that avoid, sequester, or detoxify a broad spectrum of these plant defense compounds (Alyokhin and Chen, 2017; Dermauw et al., 2018; Erb and Robert, 2016). Among insects, prior selection of such defensive mechanisms may facilitate a pre-adaptation to pesticides that could increase the likelihood of resistance evolution in the field (Alyokhin and Chen, 2017; Cheng et al., 2017; Dermauw et al., 2013; Dermauw et al., 2018; Feyereisen, 2012; Li et al., 2007).

In addition to producing defensive compounds, many plants also produce various antioxidant secondary metabolites, such as plant polyphenols, polysaccharides, alkaloids, saponins, β-carotene, vitamin E, and L-ascorbic acid (vitamin C) (Jaydeokar et al., 2012). These antioxidants can effectively scavenge free radical-containing reactive oxygen species (ROS), thereby protecting cells from damage by ROS-mediated reactions such as peroxidation-mediated damage to proteins, lipids, DNA/RNA, and other important bio-macromolecules (Rietjens et al., 2002; White et al., 2014; Wojcik et al., 2010). Thus, antioxidant biosynthesis by plants and dietary acquisition by animals is essential for protection from cellular damage and for roles as enzyme cofactors and as regulators of gene expression and cell signaling (Kurutas, 2015). Antioxidants such as vitamin C have been implicated in the minimization of oxidative damage to DNA and may have a role in suppressing nucleotide mutation rates (Aly et al., 2010; Fedirko et al., 2010; Møller et al., 2004).

The organochlorine insecticide, dichlorodiphenyltrichloroethane (DDT), disrupts insect nervous system function by affecting ion channels in the nerve cell plasma membrane and causes cell depolarization, nervous system hyperexcitability, convulsion, and eventually paralysis (Davies et al., 2007; Silver et al., 2014). In addition, DDT is known to cause oxidative stress (Chen et al., 2016). DDT resistance has been extensively studied in the model organism *Drosophila melanogaster* (hereafter *Drosophila*) (Daborn et al., 2002; Le Goff et al., 2003; Pedra et al., 2004; Perry et al., 2011; Seong et al., 2017; Seong et al., 2018a; Seong et al., 2018b; Seong et al., 2019; Strycharz et al., 2013).

A relationship between oxidative DNA damage and pesticide exposure has been observed in mammals (Abdollahi et al., 2004). Antioxidants, including vitamins C and E, have been shown to alter the impacts of DDT and other toxins in mammalian systems (Abdollahi et al., 2004; Aly et al., 2010; Jin et al., 2014). However, the interaction between the protective effects of antioxidants and the increased levels of ROS generated within DDT-exposed insects remains unknown. Furthermore, the degree to which antioxidants protect against ROS-mediated mutagenesis and alter the impacts of pesticide selective pressures within an insect population remains a point of conjecture. To begin to address these questions, we explored the impact of a dietary antioxidant (vitamin C) on the evolution of DDT-resistant phenotypes and underlying genotypes within the *Drosophila* model system. Specifically, we demonstrate that dietary vitamin C impacted directional selection for both synonymous and non-synonymous variants in the genome and significantly decreased DDT resistance following multigenerational low-dose DDT selection.

2. Materials and methods

2.1. Drosophila strain and treatments

DDT (≥ 98% pure) and vitamin C (L-ascorbic acid, A5960, ≥ 99.0% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For selection, DDT was dissolved in 100% acetone, diluted to a concentration of 0.1 g/mL and used as a stock solution. Vitamin C was dissolved in distilled water to a concentration of 2 mM (Bahadorani et al., 2008). The DDT-susceptible *Drosophila* strain *Canton-S* was obtained from the Bloomington *Drosophila* Stock Center (BDSC, Bloomington, IN, USA) and reared on a commercially available blue diet medium (Carolina Biological Supply Company, Burlington, NC, USA) under 25 ± 2 °C, 55–58% relative humidity, and 14 h light /10 h dark cycle conditions without any insecticide

exposure. A single mated pair, consisting of a single virgin *Canton-S* female and a male, were used as the initial parents for all subsequent populations within this study. After two initial generations of expansion, 100 progeny (1:1 male/female ratio) were collected and frozen at −80 °C (hereafter called generation zero; G_0), and all remaining flies (about 100) were then divided into four numerically equal proportions for four replicates in four different bottles (20 flies each bottle; see *SI Appendix*, Fig. S1). After one generation of random mating, flies from each replicate (bottle) were further divided into four numerically equal proportions for four treatment groups and reared for 10 generations with feeding on (I) blue diet alone (Control), (II) blue diet with 2 mM incorporated vitamin C (Vitamin C), (III) blue diet with recurrent adult exposure to 0.1 μg DDT (DDT only), or (IV) blue diet containing 2 mM vitamin C and recurrent adult exposure to 0.1 μg DDT (DDT + Vitamin C), as described in detail below. Backcrosses were prevented in all treatment groups by removing adults of the previous generation from bottles as soon as next-generation pupae had developed. We would predict lower mortality as the result of higher numbers of variants and consequent adaptation to the toxic effects of DDT. In contrast, we would predict higher mortality as the result of lower numbers of variants and the lack of adaptation would support the premise that antioxidants are protective against xenobiotic-induced effects.

For each of the four replicates of the DDT-only and DDT + Vitamin C treatments, 15 virgin females and males (each 3 d old) were collected every generation and placed in a 20 mL glass scintillation vial coated with 0.1 μg DDT dissolved in 100% acetone (Sigma-Aldrich, St. Louis, MO, USA). The vial was then capped with cotton moistened with 1 mL of a 5% (*w/v*) sucrose solution. After 24 h, 10 male and 10 female survivors (1:1 male/female ratio) were transferred to a 60 mL bottle with blue diet (DDT-only treatment) or blue diet containing 2 mM vitamin C (DDT + Vitamin C treatment). For the Control and Vitamin C treatments, 15 each of female and male flies (1:1 male/female ratio) were placed in a vial pretreated with 100% acetone, and after 24 h, 10 surviving female and 10 male flies were transferred to 60 mL bottles containing blue diet (Control) or blue diet with 2 mM of vitamin C (Vitamin C treatment). All other rearing conditions were identical to those above. Mated flies were then removed from each bottle after 7 d to prevent backcross mating with their progeny. Flies from each replicate across the four treatment groups were subjected to additional identical rounds of selection for a total of 10 generations of selection. At the end of the tenth generation, the 10 pairs of parent flies were removed from each bottle after a 7 d mating period, their progenies (about 250) from each bottle (treatment-by-replicate combination) were collected for genome sequencing. The final collections for sequencing from treatments ($n = 4$) by replicates ($n = 4$) comprised 16 total pools. The newly merged flies from each bottle were expanded for two generations without selection and then used for bioassays as described below.

2.2. Log DDT concentration versus probit mortality-response bioassays

After the 10 generations of selection and subsequent population expansion, 20 virgin female flies (3 d post-emergence) each were placed into 20 mL glass scintillation vials coated with test concentrations of DDT (0.0 μg [control]), 0.25 μg, 0.5 μg, 1.0 μg, 1.5 μg, and 2.0 μg) and capped with a cotton ball moistened with 1 mL of a 5% (*w/v*) sucrose solution. Four biological replicates were performed at each dose level for each of the 16 treatment-by-replicate combinations from the selection experiment. Females were observed for 24 h and were considered dead when all movement and leg twitching had ceased. Log_{10} concentration versus percent mortality regression lines (probit) were generated to determine the median lethal concentration values (LC_{50}) for the treated flies by using SAS statistical software (SAS 9.1.3, SAS Institute, Inc., Cary, NC, USA). The significance of differences in maximum-log likelihood results comparing LC_{50} values estimated from mortality curves between treatments within replicates was determined on the basis of non-overlapping confidence intervals. Additionally, Bonferroni-corrected *p*-values were determined.

2.3. Genome sequencing and variant identification

Bulk genomic DNA was extracted from all progenies from 10 pairs of flies (about 250) from each of the replicates within each treatment group ($n = 16$) from the selection experiment and from the pool of flies collected at the G_0 generation ($n = 1$) (17 total) using the Qiagen DNeasy Plant Maxi Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Seventeen indexed genomic libraries were constructed by using the Illumina TruSeq Nano DNA Library Preparation Kit on a Perkin Elmer Sciclone G3 robot following the manufacturer's recommendations. All of the indexed libraries were pooled in equimolar quantities into a single sample and paired-end sequence read data were generated on a single lane of an Illumina HiSeq4000 at the Genomics Core at Michigan State University (East Lansing, MI, USA). Resulting fastq read data were trimmed of Illumina adaptor sequences, low-quality sequence (phred score of $q < 20$), read lengths < 64 bp, and those containing > 1 ambiguous nucleotide (N) by using the CLC Workbench v.8.5 (Qiagen) as described previously (Steele et al., 2014).

All trimmed reads from the 17 libraries were mapped to the *Drosophila melanogaster* genome assembly from FlyBase (http://www.flybase.org; December 2017). The mapping parameters were as follows: match score = 1, mismatch score = 3, insertion cost = 3, length fraction = 0.5, similarity fraction = 0.8, and no global alignment option. A two-pass local realignment tool from CLC was used to optimize the initial alignment. The Low Frequency Variant Detection tool in CLC was used to calculate potential variants at a significance level of 0.01 against sequencing error using realigned read mappings as inputs. The parameters for variant detection were minimal coverage = 10, minimal count = 2, and minimal frequency = 1. Variants from the G_0 population, regarded as background variation, were subtracted from each population by using the variant-filtering tool in CLC. Variants from the Control group, regarded as random genetic drift, were compared with those from other treatment groups.

One-way ANOVA procedures were used to compare the numbers of accepted variants among all treatments. When comparing each treatment against the initial G_0 group (which had three technical replicates), all four biological replicates were similarly used in comparisons (Li et al., 2010). Fisher exact tests ($p < .05$) were used to test for the significance of any difference in filtered variant frequencies for each accepted variant between the four replicates between control and other treatment groups. The selected variants in the variant output tables were mapped back to the reference genome to identify synonymous and non-synonymous mutations. Gene ontology (GO) terms were retrieved for genes impacted by non-synonymous (amino acid–changing) variant sites from FlyBase (http://www.flybase.org; last accessed December 06, 2017) (Dos Santos et al., 2014) using the Generic Gene Ontology (GO) Term Mapper (https://go.princeton.edu/cgi-bin/GOTermMapper).

3. Results

3.1. Log DDT concentration versus probit mortality response bioassays

After 10 generations of selection, log DDT concentration versus probit mortality-response curves were generated for all treatment groups (Fig. 1). The mean LC_{50} values of the four replicates of the DDT-only treatment group ranged from 0.99 to 1.41 μg/vial (Table 1), which were significantly higher ($p < .05$) compared to those for the other treatments on the basis of non-overlapping confidence intervals. The differences between LC_{50} values across treatments were also estimated using the NLEstimate macro in SAS. The LC_{50} values for the DDT-only treatment were significantly higher than those of the other three treatments for all four replicates after Bonferroni correction ($p < .0006$; *SI Appendix*, Table S1). Each Bonferroni-corrected p value was obtained by taking the uncorrected p value and multiplying by the number of comparisons (6). The LC_{50} estimates among replicates of the

DDT + Vitamin C treatment were significantly higher than those of the control in replicates 3 and 4 ($p \leq .0060$); but not in replicates 1 and 2 ($p \geq .0600$). The estimated LC_{50} values among flies from the Vitamin C treatment were not significantly different from those of the Control group ($p \geq .0625$) in any of the four replicates.

3.2. Genome sequencing and variant estimation

Illumina HiSeq4000 sequence data yielded 119,671,315 to 148,947,255 reads with lengths that ranged from 148.08 to 148.29 bp among the 17 replicate-by-treatment–specific libraries (*SI Appendix*, Table S2). The percent of reads aligned to the genome ranged from 81.91 to 86.66% for all 17 samples, with a resulting range of read depths from 98.6 to 144.3 across chromosome segments 2 L, 2R, 3 L, 3R, 4, and X chromosome. The corresponding mean coverages ranged from 15.6 to 34.8 among library-specific reads aligned to the Y chromosome and 1235 to 5718 for mitochondrial genome (*SI Appendix*, Table S3).

No statistical differences were observed among the four treatment populations and the G_0 in terms of predicted molecular variants (single-nucleotide variants [SNVs], InDels, or multi-nucleotide variants [MNV]) compared against the *Drosophila* reference genome by the ANOVA protocol in SAS ($p \geq .2266$; *SI Appendix*, Table S4). No statistically significant differences were observed among counts of predicted variants between treatments after subtracting those putatively arising from shared genetic variation compared to the consensus reference genome sequence assembly (subtraction of genetic background accounted for within the G_0 pool; $p \geq .7340$; *SI Appendix*, Table S4), from random genetic drift within the Control group ($p \geq .5390$; *SI Appendix*, Table S4), or combined variation due to background and drift ($p \geq .5360$; *SI Appendix*, Table S4). We further focused our analysis on both arms of chromosome 2 as this is where most of the DDT-resistance-associated traits have been located in past studies (Seong et al., 2019; Steele et al., 2014, 2015); no statistical difference was found among the treatments for the numbers of unique variants (generated by subtracting variants sequentially from the G_0 and Control groups) in chromosome arms 2 L ($p \geq .5540$) and 2R ($p \geq .5260$); *SI Appendix*, Table S5.

Next, we examined the frequencies of accepted variants (both synonymous and non-synonymous) within each of the treatments. Fisher exact tests ($p < .05$) for variant frequency differences between treatment groups compared to the Control group indicated that the DDT-only treatment had the highest number of accepted variants (3196; Table 2). The DDT + Vitamin C treatment had the second-highest number of accepted variants (1174), and the Vitamin C treatment had the fewest (728) (Table 2). Analogously, Fisher exact tests ($p < .05$) of variants after filtering to remove those shared with the G_0 in every treatment-by-replicate population showed that compared to the Control, the DDT-only treatment had the highest number of non-synonymous variants (53; Table 3; *SI Appendix*, Table S6). By comparison, the DDT + Vitamin C treatment had the second-highest number of variants (39; Table 3, *SI Appendix*, Table S7), and the Vitamin C treatment had the fewest (29; Table 3, *SI Appendix*, Table S8). Among the detected variants in the DDT-selected populations, several frameshift mutations predicted in a multiple drug resistance protein (MRP) coding gene were found within the DDT treatment group. Additionally, variants were predicted within three transcription factor genes (CAMTA, DPY and ZLD) in the DDT-only treatment group and within one transcription factor (Abd-B) in the DDT + Vitamin C treatment group (*SI Appendix*, Tables S6–S7; see Discussion).

When we examined the Gene Ontology (GO) terms from the molecular function ontology (https://go.princeton.edu/cgi-bin/GOTermMapper) analysis of the non-synonymous variants, the results showed that most of the genes, where we observed differences, were not functionally annotated. For example, only 19 non-synonymous variants (18 genes, one with two non-synonymous variants) in the comparison of the DDT-only group against the Control group were within coding regions of annotated genes (*SI Appendix*, Table S9). Similarly, only 12 non-synonymous sites differing between the
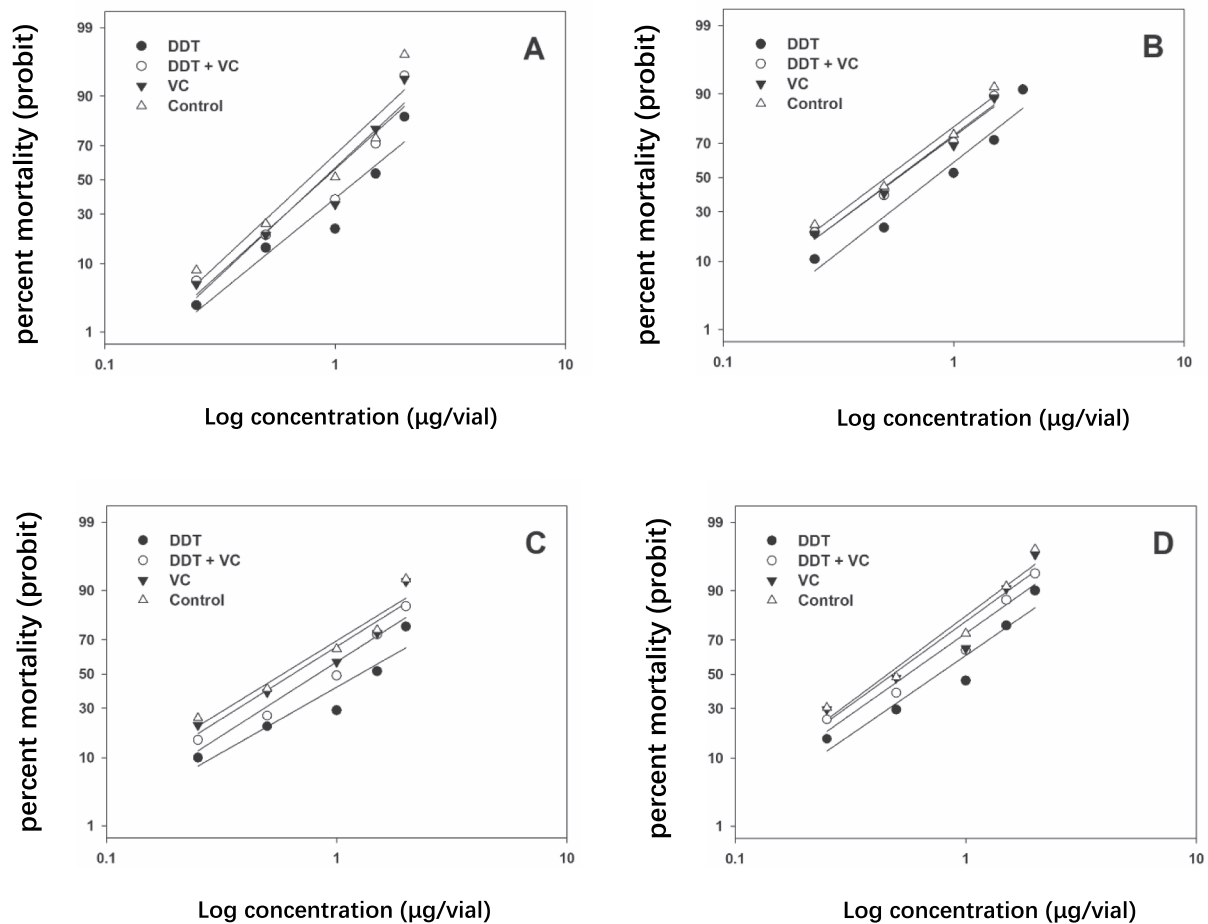
Fig. 1. Log-concentration versus percent probit mortality response curves to DDT in selected *Drosophila* populations. The selection experiment contained four replicates, each containing four treatments: (1) DDT only, (2) DDT plus dietary vitamin C (DDT + VC), (3) dietary vitamin C only (VC), and (4) Control; see *SI Appendix*, Fig. S1 for details. The log-concentration versus percent probit mortality response curves were determined for each replication of the four treatments following contact exposure to DDT (0–2 μg/vial) with a probit model. Each treatment–replicate combination from the selection experiment was represented by four biological replicates at each dose level. A: Replicate 1; B: Replicate 2; C: Replicate 3; D: Replicate 4.

DDT + Vitamin C group and the Control, and 16 non-synonymous sites differing between the Vitamin C group and the Control, were located within coding regions of annotated genes (*SI Appendix*, Table S10 and S11). When compared against the Control group, the non-synonymous variants in all three groups included ion-binding proteins as the most common classification (6 out of 32 for DDT vs control; 7 out of 27 for DDT + VC vs control and 5 out of 25 for VC vs control) according to the molecular function ontology analysis (*SI Appendix*, Tables S9–S11). Another common molecular function ontology of interest among the DDT-containing treatments involved the DNA-binding proteins in the DDT-only group versus the Control group (4 out of 32 for DDT vs control), and the RNA binding proteins (3 out of 27 for DDT + VC vs control) in the comparison between the DDT + Vitamin C group and the Control group.

Only one protein-coding gene, CG15635, was found in common among the three different treatment groups. This gene showed a functional GO annotation of being involved in the biological process of reproduction (Fig. 2; *SI Appendix*, Table S12). In total, however, six genes co-occurred when comparing either the DDT-only treatment group or the DDT + Vitamin C group to the Control group (Fig. 2; *SI Appendix*, Table S12). Two of these genes (CG31807 and dpy) show GO terms for the molecular function of ion binding.

4. Discussion

The adaptation of arthropod pests to insecticidal control measures has

implications for the sustainability of agricultural crop production and for the protection of human health from vectored disease. Understanding, developing, and applying processes that may suppress the accumulation of adaptive mutations in target insects could theoretically be used to increase the durability of insecticidal agents. Vitamins E, C, and B12 were previously reported to reduce the incidence of de novo DNA mutations under cellular stress conditions following exposure to insecticides, ROS, or pioglitazone (a diabetes drug to control high blood sugar) (Alzoubi et al., 2012; Lutsenko et al., 2002; Soloneski et al., 2003). Furthermore, supplementation with an antioxidant, such as vitamin C, can reduce genomic aberrations in human induced pluripotent stem cells (Ji et al., 2014).

Thus, the results of the current study are consistent with prior findings and support a hypothesis that the dietary antioxidant vitamin C may reduce the incidence of inherited genetic mutation putatively induced by exposure to insecticidal agents, such as DDT. Our analyses—showing the greatest number of synonymous and non-synonymous variants within the DDT-only treatment group and, correspondingly, the lowest prevalence among the Vitamin C-only treatment and Control groups—may support the premise that antioxidants are protective against xenobiotic-induced DNA damage (Tables 2 and 3). Moreover, our findings demonstrate that recurrent low-concentration DDT exposures consistently generated a lower number of nucleotide changes putatively under directional selection when the diet was supplemented with vitamin C. These observations support a hypothesis that treatment

Table 1
Effects of dietary vitamin C on DDT resistance in DDT-selected *Drosophila melanogaster* lines.

Replicate[a]	Treatment[a]	LC_{50}[b] (95% C.I.[c])	Separation test[d]	χ^2	Slope
1	DDT	1.41 (1.34–1.48)	A	287.49	1.49
	DDT + VC	1.12 (1.06–1.18)	B	331.39	1.64
	VC	1.10 (1.05–1.16)	B	346.22	1.73
	Control	1.00 (0.94–1.06)	B	320.08	1.64
2	DDT	1.04 (0.98–1.11)	A	292.35	1.43
	DDT + VC	0.70 (0.64–0.75)	B	267.00	1.76
	VC	0.71 (0.64–0.77)	B	267.32	1.69
	Control	0.63 (0.57–0.69)	B	246.23	1.75
3	DDT	1.28 (1.13–1.48)	A	199.22	1.07
	DDT + VC	0.82 (0.75–0.90)	B	235.24	1.18
	VC	0.67 (0.60–0.73)	C	211.81	1.14
	Control	0.59 (0.53–0.66)	C	200.17	1.12
4	DDT	0.99 (0.92–1.06)	A	259.18	1.29
	DDT + VC	0.73 (0.65–0.80)	B	240.55	1.33
	VC	0.62 (0.54–0.69)	BC	219.78	1.35
	Control	0.57 (0.48–0.64)	C	217.43	1.41

[a] Four separate selection experiments (replicates) were performed, each with four treatments: (i) DDT only (DDT), (ii) DDT and dietary vitamin C (DDT + VC), (iii) dietary vitamin C only (VC), and (iv) no selection (Control).
[b] Lethal Concentration (LC_{50}) (μg/vial; contact exposure) that killed 50% of the flies.
[c] 95% Confidence Interval.
[d] Different letters indicate that log DDT dose versus probit mortality regressions between treatments were significantly different ($p < .05$). Detailed means separation tests using maximum-log likelihood test are shown in Table S1.

of DDT exposed flies with dietary antioxidants, such as vitamin C, has both phenotypic (as per decreased LC_{50} values) and molecular consequences (as per decreased non-synonymous variants) in terms of slowing the evolution of insecticide resistance. Although other factors likely impact the accumulation of resistance traits in field populations (Georghiou and Taylor, 1986), our evidence suggests that antioxidants might reduce the number of genetic mutations using high cell stress conditions and potentially slow the evolution of insecticide resistance. An alternative hypothesis is that antioxidants can act as a protector, decreasing the damage induced by xenobiotics, which also relieves the selection pressure that would otherwise result in the evolution of resistance. Additional experiments are needed to further investigate these aforementioned or other potential alternative hypotheses.

Literature concerning antioxidant and xenobiotic resistance in insects has often focused on endogenous genes with antioxidant roles (Mueller et al., 2008); in mammals, long-established research has demonstrated the protective effect of antioxidants, such as vitamins, against damage to cells from pesticides (Møller et al., 2004; Fedirko et al., 2010; Aly et al., 2010). However, our work is not without precedent in insects, as vitamin E is known to antagonize the toxicity of the pyrethroid insecticide permethrin in susceptible and resistant insects with the knockdown resistant (*kdr*)-mutation in the voltage gated sodium channel (Scott, 1998); melatonin also has been shown to have a protective effect against oxidative stress damage induced by the pyrethroid cypermethrin in *Spodoptera litura* (Karthi and Shivakumar, 2015). An alternative hypothesis is that antioxidants can act as a protector, decreasing the damage induced by xenobiotics, which also relieves the selection pressure that would otherwise result in the evolution of resistance.

Diet supplementation with antioxidants may protect the mitochondria from damage by oxidative stress, preserving the genomic and structural integrity of these energy-producing organelles, and concomitant increase in functional life span (Miquel, 2002). For example, ascorbic acid supplementation extended the life span of wild-type fruit flies under normoxia (Bahadorani et al., 2008). Supplementation of vitamin C is also known to increase the lifespan of the cowpea bruchid *Callosobruchus maculatus* (Garg and Mahajan, 1994). Additionally, absorption and cellular levels of vitamin C declines with age in *Drosophila*, suggesting that decreased vitamin C may be an indicator of aging (Massie et al., 1991). Aspects of lifespan or other age-related effects of vitamin C supplementation on DDT resistance were not investigated in this study but warrant further investigations beyond the scope of the work presented here.

As per our observations, most of the genes impacted by non-synonymous variant sites located within protein-coding genes have not been annotated (listed in *SI Appendix*, Tables S6–S8). Among those that were annotated, genes with the GO molecular function term of ion binding were

Table 2
Total number of non-synonymous and synonymous variants within the *Drosophila melanogaster* lines in each treatment.

Treatment[a]	Variant type	Total variants	Variants by chromosome							
			2 L	2R	3 L	3R	4	X	Y	Mit[b]
DDT vs Control	Deletion	632	206	86	88	101	6	99	46	0
	Insertion	617	248	99	67	94	8	83	17	1
	MNV[c]	202	58	34	29	36	3	32	10	0
	Substitution	233	78	43	29	39	2	32	10	0
	SNV[d]	1512	341	257	216	264	68	122	244	0
	Total	**3196**	**931**	**519**	**429**	**534**	**87**	**368**	**327**	**1**
DDT + VC vs Control	Deletion	241	48	65	35	33	2	53	5	0
	Insertion	231	45	73	42	24	2	40	5	0
	MNV	87	21	18	19	12	0	14	3	0
	Substitution	115	21	30	18	20	1	23	2	0
	SNV	500	89	143	86	88	5	59	30	0
	Total	**1174**	**224**	**329**	**200**	**177**	**10**	**189**	**45**	**0**
VC vs Control	Deletion	176	28	25	34	37	0	48	4	0
	Insertion	146	27	20	28	38	2	29	2	0
	MNV	25	5	3	7	5	0	3	2	0
	Substitution	64	3	11	20	10	1	17	2	0
	SNV	317	40	60	53	94	2	31	37	0
	Total	**728**	**103**	**119**	**142**	**184**	**5**	**128**	**47**	**0**

Total number of non-synonymous and synonymous variants within the *Drosophila melanogaster* lines in each treatment ($p < .05$ using Fisher exact tests).
[a] All selection experiment populations (replicates) were combined within their respective treatments. Comparisons were made between the Control populations and (1) DDT only, (2) DDT and dietary vitamin C (DDT + VC), and (3) dietary vitamin C only (VC). The variants were accepted using the Fisher exact test at $p < .05$.
[b] Mit, mitochondrial genome.
[c] MNV, multi-nucleotide variation.
[d] SNV, single-nucleotide variation.

Table 3
Comparison of non-synonymous variants for *Drosophila melanogaster* lines in each treatment.

Treatment[a]	Type of variant	Total number of variants	Variants by chromosome							
			2 L	2R	3 L	3R	4	X	Y	Mit[b]
DDT vs Control	Deletion	15	8	1	2	1	0	3	0	0
	Insertion	4	2	0	0	2	0	0	0	0
	MNV[c]	8	6	1	0	1	0	0	0	0
	Substitution	1	0	0	1	0	0	0	0	0
	SNV[d]	25	12	3	3	2	0	2	3	0
	Total	**53**	**28**	**5**	**6**	**6**	**0**	**5**	**3**	**0**
DDT + VC vs Control	Deletion	12	2	8	1	1	0	0	0	0
	Insertion	4	0	0	2	0	1	1	0	0
	MNV	1	1	0	0	0	0	0	0	0
	Substitution	0	0	0	0	0	0	0	0	0
	SNV	22	7	5	3	4	0	3	0	0
	Total	**39**	**10**	**13**	**6**	**5**	**1**	**4**	**0**	**0**
VC vs Control	Deletion	2	1	0	0	0	0	1	0	0
	Insertion	3	1	0	0	0	2	0	0	0
	MNV	0	0	0	0	0	0	0	0	0
	Substitution	0	0	0	0	0	0	0	0	0
	SNV	24	4	8	1	9	0	2	0	0
	Total	**29**	**6**	**8**	**1**	**9**	**2**	**3**	**0**	**0**

Total number of non-synonymous and synonymous variants within the *Drosophila melanogaster* lines in each treatment ($p < .05$ using Fisher exact tests).

[a] All selection experiment populations (replicates) were combined within their respective treatments. Comparisons were made between the Control populations and (1) DDT only, (2) DDT and dietary vitamin C (DDT + VC), and (3) dietary vitamin C only (VC). The variants were accepted using the Fisher exact test at $p < .05$.

[b] Mit, mitochondrial genome.

[c] MNV, multi-nucleotide variation.

[d] SNV, single nucleotide variation.

DDT vs Control   **DDT+VC vs Control**

32 (36%)   5 (5.6%)   24 (27%)

1 (1.1%)

1 (1.1%)   1 (1.1%)
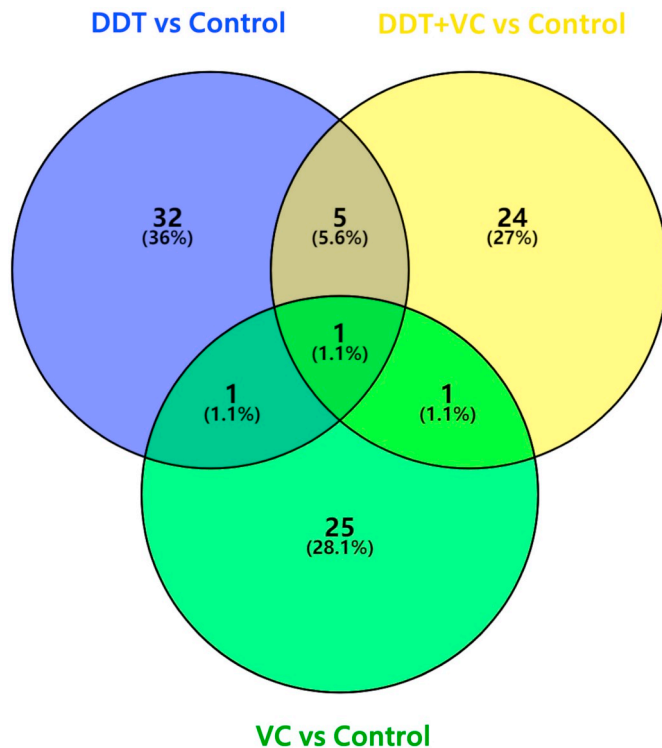
25 (28.1%)

VC vs Control

Fig. 2. Venn diagram representing the number of genes containing non-synonymous variants comparing the control to the three treatments: DDT only (DDT), vitamin C only (VC), and DDT plus vitamin C (DDT + VC).

most prevalent (see *SI Appendix*, Tables S9–S11). Additionally, a gene for MRP was among those impacted by changes at non-synonymous sites, but solely within the DDT-only treatment group (*SI Appendix*, Table S6). MRP is a member of the ABC C-type transporter superfamily, and *Drosophila* MRPs are functionally orthologous to human MRPs as high-capacity, ATP-dependent, vanadate-sensitive organic anion transporters (Tarnay et al., 2004; Prince et al., 2014; Chahine and O'Donnell, 2010). ABC

transporters, including MRP1, MDR49, MDR50, and MDR65, were previously implicated as playing important roles in DDT resistance mechanisms, presumably by facilitating xenobiotic efflux (Strycharz et al., 2013; Gellatly et al., 2015; Seong et al., 2016; Drummond et al., 2019). The impact of these predicted frameshift mutations, putatively causing non-functional protein products, on resistance levels in the DDT exposure group were not investigated in this study but would be a logical focus of future investigations.

Additionally, variants within several transcription factor genes were identified in both DDT-only (CAMTA, DPY and ZLD) and DDT + Vitamin C treatment group (Abd-B) (*SI Appendix*, Tables S6–S7), all of which encode transcription factors containing Zn-finger DNA-binding motifs or RNA binding motifs. Mutations in transcription factors that affect DNA–protein or protein–protein interactions at the promoter (or with enhancer) elements can cause changes in expression of physically unlinked genes. Therefore, genes participating in interconnected regulatory pathways may show a coordinated response to transcription factor mutations (Alzoubi et al., 2012). Interestingly, there was a putative in-frame 12 bp deletion within CAMTA (calmodulin-binding transcription activator) from the DDT only exposed treatment (Table S6). CAMTAs are a conserved group of co-activators of transcription with a variety of functions including cell proliferation and tumor suppression (Finkler et al., 2007) and neurological and behavioral function in *Drosophila*. Regardless, the role that these transcription factors may play in mediating increased DDT resistance is beyond the scope of this study but undoubtedly may be the focus of future investigations.

The work we present here is an initial test of the hypothesis that a dietary antioxidant can impact the evolution of pesticide resistance at both the genomic and phenotypic levels. Subsequent tests of this hypothesis, across other pesticides, pests, pesticide doses, and broader conditions will determine narrowness or broadness of the hypothesis and ideas presented here. Further research including components of population genomics is also needed to determine and assess how generalizable our observations are across different concentrations of dietary vitamin C and exposures to DDT, as well as across different classes of antioxidants and insecticidal agents in general. Any analogous interplay within herbivorous insects that consume host plant tissues high in antioxidants and the evolution of insecticide

resistance remains to be determined. For instance, there is anecdotal evidence for a current lack of insecticide resistance among *Drosophila suzukii*, which consumes, among other things, antioxidant-rich blueberries (Drummond et al., 2019).

Finally, beyond the scope of second-generation pesticides, it remains to be determined whether such interactions occur between plant defensive compounds and plant-produced antioxidants—in other words, whether some plants, or some of their tissues, produce high levels of antioxidants that alter the evolutionary path of resistance in herbivorous insects to those defensive compounds. Nevertheless, this work demonstrates that dietary alterations can impact the evolution of pesticide resistance at both the genomic and phenotypic levels.

Author contribution statement

JH and WS contributed equally. BRP conceived and WS and BRP designed research. JH and WS conducted the experiments and analyzed the data. JH, BRP, KMS and WS wrote the manuscript. JO, OM, BC, KP and JC critically edited manuscript and provided feedback and ideas on data analysis and interpretation. All authors read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2020.104631.

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