2021

The insulin signaling pathway in *Drosophila melanogaster*: A nexus revealing an "Achilles' heel" in DDT resistance

Can Zhang
Guangdong Eco-Engineering Polytechnic

Keon Mook Seong
Kyungpook National University

Weilin Sun
Michigan State University

Omprakash Mittapalli
University of Kentucky

Baoli Qiu
South China Agricultural University

See next page for additional authors

Recommended Citation

Zhang, Can; Seong, Keon Mook; Sun, Weilin; Mittapalli, Omprakash; Qiu, Baoli; Clark, John M.; and Pittendrigh, Barry R., "The insulin signaling pathway in *Drosophila melanogaster*: A nexus revealing an "Achilles' heel" in DDT resistance" (2021). *Pesticide Biochemistry and Physiology*. 15.
https://doi.org/10.1016/j.pestbp.2020.104727

Pesticide Biochemistry and Physiology 171 (2021) 104727

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

The insulin signaling pathway in *Drosophila melanogaster*: A nexus revealing an "Achilles' heel" in DDT resistance

Can Zhang[a,b], Keon Mook Seong[b,c], Weilin Sun[b], Omprakash Mittapalli[d], Baoli Qiu[e], John M. Clark[f], Barry R. Pittendrigh[b,*]

[a] Department of Eco-Engineering, Guangdong Eco-Engineering Polytechnic, Guangzhou 510520, China
[b] Department of Entomology, Michigan State University, East Lansing, MI 48824, USA
[c] Department of Applied Biology, College of Ecology and Environment, Kyungpook National University, Sangju, Republic of Korea
[d] Department of Entomology, University of Kentucky, Lexington, KY 40506, USA
[e] Department of Entomology, South China Agricultural University, Guangzhou 510640, China
[f] Department of Veterinary and Animal Sciences, University of Massachusetts-Amherst, Amherst, MA, USA

ARTICLE INFO

Keywords:
Drosophila melanogaster
IIS (insulin/insulin-like growth factor signaling pathway)
DDT resistance
Pest control

ABSTRACT

Insecticide resistance is an ongoing challenge in agriculture and disease vector control. Here, we demonstrate a novel strategy to attenuate resistance. We used genomics tools to target fundamental energy-associated pathways and identified a potential "Achilles' heel" for resistance, a resistance-associated protein that, upon inhibition, results in a substantial loss in the resistance phenotype. Specifically, we compared the gene expression profiles and structural variations of the insulin/insulin-like growth factor signaling (IIS) pathway genes in DDT-susceptible (*91-C*) and -resistant (*91-R*) *Drosophila melanogaster* (*Drosophila*) strains. A total of eight and seven IIS transcripts were up- and down-regulated, respectively, in *91-R* compared to *91-C*. A total of 114 nonsynonymous mutations were observed between *91-C* and *91-R*, of which 51.8% were fixed. Among the differentially expressed transcripts, phosphoenolpyruvate carboxykinase (PEPCK), down-regulated in *91-R*, encoded the greatest number of amino acid changes, prompting us to perform PEPCK inhibitor–pesticide exposure bioassays. The inhibitor of PEPCK, hydrazine sulfate, resulted in a 161- to 218-fold decrease in the DDT resistance phenotype (*91-R)* and more than a 4- to 5-fold increase in susceptibility in *91-C*. A second target protein, Glycogen synthase kinase 3β (GSK3β-PO), had one amino acid difference between *91-C* and *91-R*, and the corresponding transcript was also down-regulated in *91-R*. A GSK3β-PO inhibitor, lithium chloride, likewise reduced the resistance but to a lesser extent than did hydrazine sulfate for PEPCK. We demonstrate the potential role of IIS genes in DDT resistance and the potential discovery of an "Achilles' heel" against pesticide resistance in this pathway.

1. Introduction

Piperonyl butoxide may represent the only commercial compound available to suppress metabolic insecticide resistance in insects, primarily through the inhibition of cytochrome P450 monooxygenases (P450s) (Cross et al., 2017). To our knowledge, very few reports have described approaches for identifying other resistance-inhibiting compounds (Hardstone et al., 2015). Pittendrigh et al. (2008, 2014) have proposed that "omics" tools could be used to identify potential "Achilles' heel" resistance traits (i.e., resistance-related proteins that, when inhibited, would result in the reduction or loss of the pesticide resistance phenotype). Pittendrigh et al. (2008, 2014) predicted that such an approach would involve determining pathways that both contribute to resistance and, if inhibited, may render the insect incapable of maintaining the resistance phenotype (or, as a corollary, may make susceptible insects hypersusceptible to pesticides).

Drosophila melanogaster (hereafter referred to as *Drosophila*) has been used over the last half-century as a tool to explore the mechanisms leading to insecticide resistance and the consequences of pesticide exposure. The laboratory-selected DDT-resistant *Drosophila* strain *91-R* was established over 60 years ago and has received intermittent but intense DDT selective pressure since then (Merrell and Underhill, 1956; Merrell, 1960, 1965). Its counterpart strain, *91-C*, was derived from the same progenitor population as *91-R*, but has not been selected with DDT in the laboratory. Using these *Drosphila* strains, Pedra et al. (2004, 2005) had shown genomic differences between DDT-resistant and -susceptible strains in energy-related pathways. For example, both the down-regulation of glycolytic enzyme transcripts and the up-regulation

* Corresponding author at: Center for Integrated Plant Systems, 578 Wilson Rd. Room 203, East Lansing, MI 48824, USA.
E-mail address: pittendr@msu.edu (B.R. Pittendrigh).

https://doi.org/10.1016/j.pestbp.2020.104727
Received 6 August 2020; Received in revised form 28 September 2020; Accepted 10 October 2020
Available online 18 October 2020

of acyl-CoA dehydrogenase and isocitrate dehydrogenase transcripts in a DDT-resistant strain suggest that lipid metabolism and the Krebs cycle may increase DDT detoxification (Pedra et al., 2004). Subsequently, a proteomics approach validated that DDT resistance involved a number of energy-related pathways including glycolysis, gluconeogenesis, the pentose phosphate shunt, the Krebs cycle, and fatty acid oxidation (Pedra et al., 2005). Thus, this pair of fly strains represents a logical system for testing the Achilles' heel resistance trait concept.

As one of the key regulatory pathways in energy metabolism, the insulin/ insulin-like growth factor (IGF)-like signaling (IIS) pathway, associated with insulin resistance, is evolutionarily conserved across invertebrates and vertebrates, and inhibitors for some of the proteins in this pathway are commercially available. In *Drosophila*, the IIS pathway plays important roles in growth and development (Underwood et al., 1994; Edgar, 2006; Engelman et al., 2006) and is also related to lifespan regulation, metabolism of xenobiotics, and stress resistance (Hwangbo et al., 2004; Saltiel and Kahn, 2001; Slack et al., 2011; Tatar et al., 2003). To date, the energy-related adverse effects of DDT have been extensively studied in higher animals such as rodents (Kacew and Singhal, 1974; Yau and Mennear, 1977) and humans (Everett et al., 2007; Cox et al., 2007; Lee et al., 2017), but, to the authors' knowledge, not in DDT-resistant *Drosophila*. Nevertheless, DDT increased the activity of the pentose phosphate pathway in DDT-susceptible *Drosophila* by increasing the activity of two key regulatory enzymes, which increased the overall fitness of the flies (Bijlsma and Kerver, 1983).

In this study, we (i) performed genomic and transcriptomic analyses of the IIS pathway using the DDT-susceptible *91-C* strain and the highly DDT-resistant *91-R* strain, (ii) compared life-history parameters (lifespan, starvation resistance, and impacts of dietary sucrose on longevity) and glycogen levels between the two strains, and (iii) used these insights to identify and test for Achilles' heel resistance traits by using inhibitors of IIS pathway proteins. The level of DDT resistance in this strain is high (~1500-fold) and is due to multiple energy-requiring mechanisms, including reduced penetration, increased metabolism, and enhanced efflux (Strycharz et al., 2013), involving the up-regulation of at least nine genes (Gellatly et al., 2015; Kim et al., 2018). Thus, the maintenance of such a high level of resistance through so many energy-requiring mechanisms undoubtedly places a large energy demand on *91-R* flies. Given the importance of the IIS pathway in energy regulation, the alterations identified in genes found in this pathway may promote our understanding of how insects cope with long-term insecticide exposure and compensate for the fitness cost of maintaining a highly resistant phenotype.

2. Materials and methods

2.1. Drosophila melanogaster strains

In this study, we used the highly DDT-resistant *Drosophila* strain *91-R* and its corresponding non-DDT-selected control strain, *91-C*, which were established previously (Merrell, 1960, 1965; Merrell and Underhill, 1956; Seong et al., 2017, 2018). These two fly strains were reared in separate colonies on a commercially available medium (Jazz-Mix *Drosophila* Food; Fisher Scientific, Cat. No. AS153) under conditions of 26 ± 1 °C, 50% relative humidity, and a 14-h light /10-h dark cycle. The *91-R* strain has been continually exposed to DDT (98%; Sigma-Aldrich) by maintaining the flies in a colony bottle in the presence of a filter paper disk with 150 mg DDT (Seong et al., 2018), whereas *91-C* has been maintained without any exposure to DDT. Flies were transferred to a bottle with fresh diet every 3 weeks.

2.2. Differential expression of IIS pathway genes

All RNA-seq read data sets were previously generated from *91-C* and *91-R* in triplicate (Seong et al., 2017) and, for this study, were retrieved from the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) database (accession numbers: SRX2611754, SRX2611759).

CLC Genomics Workbench version 9.5 (Qiagen) was used to analyze the transcriptomic data. After removing Illumina adapters and filtering out low-quality reads, the trimmed data were obtained from the raw reads. Gene sequences of the IIS pathway were downloaded from the FlyBase database (http://flybase.org; dos Santos et al., 2015 and then imported to CLC Genomics Workbench software as a reference gene set. The six sets of trimmed sequence reads were mapped to the reference genome and realigned.

Expression levels of the IIS pathway genes were compared between *91-C* and *91-R* using the number of reads per kilobase per million (RPKM) of mapped RNA-seq reads. The FDR method was used to determine the threshold *p*-values in order to establish the differential expression of genes in the IIS pathway. An FDR of < 0.05 and a $\log_2$ fold change of $\geq |1|$ were used as thresholds to determine significant differences in gene expression.

2.3. Reverse transcriptase–quantitative PCR (RT-qPCR) validation

RT-qPCR validation was carried out on IIS pathway genes for validation of RNA-seq estimated differences between the *91-C* and *91-R* strains. Both the RT-qPCR and first-strand cDNA synthesis methods were similar to those previously described by Seong et al. (2017). The RT-qPCR primers are shown in *SI Appendix*, Table S6, and ribosomal protein 49 (*rp49*) was used as the reference gene. The reaction for RT-qPCR included 10 μL of SYBR Select Master Mix (Applied Biosystems Inc., USA), 0.3 μM of each primer, approximately 100 ng of cDNA, and sterilized water to a total volume of 20 μL. The thermocycler program was as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. All amplification reactions were performed with the StepOnePlus Real-Time PCR system (Applied Biosystems Inc.) with three biological replicates. The mean of the threshold cycle (Ct) values from the three replicates of each strain were determined and the relative expression levels calculated using the formula ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001).

2.4. Comparison of predicted amino acid sequence of IIS pathway transcripts between the 91-C and 91-R strains

After separately mapping RNA-seq reads from *91-C* and *91-R* flies to the *Drosophila* IIS pathway reference genes, consensus sequences were obtained by using the "Map Reads to Reference" tool of the CLC Genomic Workbench 9.5 (Qiagen) software package. Related mapping parameters were set as follows: minimum length fraction = 0.9, minimum similarity fraction = 0.8, insertion/deletion cost = 3, and mismatch cost = 3. The consensus sequences of all IIS pathway genes were translated by using CLC Genomics Workbench. Alignments of the putative amino acid sequences were performed with Geneious 11.0.2 software (http://www.geneious.com) (Kearse et al., 2012). The consensus base frequency of mapped reads compared to the reference sequence was generated as previously described by Seong et al. (2018). All mutations found from RNA-seq data were verified by PCR amplification.

2.5. Lifespan determination under fed or starvation conditions

For the lifespan assay under fed conditions, virgin females and males from the *91-C* and *91-R* strains were collected and reared separately on a commercially available diet (Jazz-Mix *Drosophila* Food, FisherSci.com, Cat. No. AS153). To make sure the adults were of similar age, the newly emerged flies were collected from rearing bottles that had been emptied 5 h previously. Adults of the same sex were kept at a density of 30 per vial. Flies were transferred to fresh diet every 4 d. Mortality was monitored every 2 d. Six replicates were performed for each treatment.

Survival of *91-C* and *91-R* flies was measured in vials with 8 ml of 1% agar (starvation conditions), which allowed flies access to water but not nutrient as previously described by Broughton et al. (2005). Each vial contained 20 flies of the same sex, and females and males from both *91-C* and *91-R* were tested. Mortality was recorded every 4 h. For the starvation test, flies 3–4, 5–6, and 9–10 d old were used in each experiment. Three replicates were performed for each treatment.

2.6. Longevity assay at different levels of excess sucrose in the diet

Twenty virgin female and male flies from the *91-C* and *91-R* strains were collected and reared separately on blue diet (formula 4–24®; carolina.com) made with three different sucrose solutions in water: 5.13% sucrose for low sucrose (LS), 17.1% for medium sucrose (MS), and 34.2% for high sucrose (HS). Assuming no sucrose already present in the blue diet mix, which has a proprietary formula, these percentages represent the final sucrose percentage in the diet. To prevent bacterial and mold growth, propionic acid (0.5%) and nipagin solution (1% Nipagin M [Tegosept M], *p*-hydroxybenzoic acid) were added to all sucrose solutions used for making blue diets. There were four biological replicates for each treatment, starting on different dates. Flies were provided with fresh diet every 2 weeks to prevent bacterial and mold growth and desiccation of diet. Mortality was recorded every 2 d.

2.7. Glycogen assay

The glycogen contents of *91-C* and *91-R* female and male flies were measured at 0, 24, and 48 h after starvation according to the method described in Tennessen et al. (2014). Data (μg glycogen/mg fresh weight) were expressed relative to the fresh body weight of each fly. Three replicates were performed for each treatment.

2.8. Inhibitor treatment and DDT bioassay

Female or male flies 1 to 2 d old from each strain were placed into a plastic vial (28 mm × 95 mm height) and fed with inhibitors mixed in 5% sucrose solution. The inhibitors used were hydrazine sulfate (Hys; PEPCK inhibitor, Sigma-Aldrich, 10 mM) and lithium chloride (LiCl; GSK3β inhibitor, Sigma-Aldrich, 20 mM) (Hussain et al., 2017; Mudher et al., 2004). A cotton ball (FisherSci.com, Cat No. 22–456-883, USA) was soaked in an inhibitor solution in a 1.5-ml centrifuge tube and subsequently placed into a 100-ml plastic vial (Choi et al., 2017). After feeding on inhibitor solution for 2 d, 20 females or males from either the *91-C* or *91-R* strain were used in DDT bioassays.

To examine the effect of inhibitors under different rearing regimes, flies were reared either under optimal or suboptimal rearing conditions. Under optimal rearing conditions, 100 mating pairs of flies were maintained in bottles containing 50 ml of standard medium and allowed to lay eggs for 4 d. All adults were then removed to ensure the optimal density of larva. For suboptimal rearing conditions, adults were not removed until the adult emergence of the next generation. Under suboptimal conditions, flies were maintained in the same bottle on the same medium (diet) for at least three generations, so the 2nd-, 3rd-, and 4th-generation flies were fed on left-over diet as well as on excretions from previous generations. Thus, optimal rearing conditions provided sufficient food for flies, whereas under suboptimal rearing conditions, flies faced diminishing food quantity and quality.

Mortality bioassays using DDT were conducted following the method of Strycharz et al. (2013). Briefly, various concentrations of DDT dissolved in acetone were transferred into 20-ml transparent glass vials. Each vial was then rolled on its side in a fume hood till the acetone evaporated. To test flies raised under optimal dietary rearing conditions, the DDT working concentration was serially diluted by up to 256-fold from the stock concentration (64 mg/ml) for *91-R*, and diluted by 40–400 fold from the stock concentration (800 μg/ml) for *91-C*. To test flies raised under suboptimal dietary rearing conditions, the DDT working concentration was serially diluted by 2–1600 fold from the stock concentration (8 mg/ml) for *91-R* and by 20–8000 fold from the stock concentration (400 μg/ml) for *91-C*. After all the acetone evaporated, 20 flies of different strain/sex combinations were placed into the vials. Vials were capped with cotton plugs moistened with a 5% sucrose solution in distilled water. The number of dead flies in each vial was recorded after 24 h.

2.9. Statistical analyses

For RT-qPCR, statistical analysis was performed using Student's *t*-test to compare the results between *91-C* and *91-R*. For glycogen assays and body weight, one-way ANOVA was used, and means comparisons were made by using Student's *t*-test ($p < 0.05$) calculated using SPSS software (Version 19.0; SPSS Inc., USA). The median lifespans under fed, starvation, or excess sucrose conditions were all calculated using the probit function in SPSS (Version 19.0). For mortality bioassays, probit analysis was conducted by using SPSS software (Version 19.0).

3. Results

3.1. Differential gene expression between 91-C and 91-R

Fifteen transcripts from the IIS pathway were predicted via RNA-seq to be differentially regulated: eight and seven transcripts were respectively up- and down-regulated in *91-R* compared to *91-C* (false discovery rate (FDR) < 0.05 and $\log_2$ fold change $\geq |1.0|$; *SI Appendix*, Table S1, Fig. S1). The RT-qPCR validation of the predicted significant quantitative differences in expression levels among seven of the eight up-regulated transcripts and four of the seven down-regulated transcripts showed a similar trend between the results of RNA sequencing and those obtained by RT-qPCR ($R^2 = 0.6793$, $p < 0.01$), confirming the predicted differential expression between strains based on Pearson's correlation coefficient test (Fig. 1). The largest up-regulations in *91-R* relative to *91-C* were seen for *Lipin-PL*, *ACC-PA*, and *GlyS-PA*, and the largest down-regulations were observed for *PEPCK-PA*, *GSK3β-PO*, and *CCHa2-PA* (Fig. 1).

3.2. Nonsynonymous variation in IIS pathway genes differentially regulated between 91-C and 91-R

Nucleotide variations in IIS pathway genes were detected between *91-C* and *91-R* (Table 1; *SI Appendix*, Table S2). A total of 114 nonsynonymous nucleotide changes (i.e., predicted to encode amino acid changes) were found within the open reading frames (ORFs) of transcripts from 34 of the 80 IIS pathway genes examined, and no amino acid sequence differences were found in the predicted products of the remaining 46 genes. Out of the 114 nonsynonymous changes, 51.8% were fixed for different alleles in *91-C* and *91-R*, whereas the remaining 48.2% were segregating (unfixed) within one of the two strains.

Among the 15 differentially expressed transcripts identified by RNA-seq in *91-R* vs *91-C*, three up-regulated transcripts (*Lpin-PL*, *Hex-C-PA*, and *ERK (rl)-PH*) and three down-regulated transcripts (*PEPCK-PA*, *GSK3β-PO*, and *Lpin-PE*) together showed a total of 12 nonsynonymous mutations in *91-R* (Table 1). Among the down-regulated transcripts in *91-R*, six nonsynonymous mutations were found in *PEPCK-PA* at bp 200, 415, 1009, 1010, 1612, and 1870, which respectively led to amino acid changes V67G, M139L, R337K (resulting from the mutations at both 1009 and 1010 bp), E538K, and A624P (Table 1; *SI Appendix*, Fig. S2). Additionally, one fixed insertion starting at bp 1755 led to a single amino acid insertion at position 586 in *PEPCK-PA* in *91-R* (*SI Appendix*, Table S3, Fig. S2). Only one mutation in the transcript for *GSK3β-PO* (bp 922, which led to amino acid change T308A) and two in the transcript for *Lpin-PE* (bp 788 and 3134, which respectively led to amino acid changes T260P and S1045F) were predicted, and all three were classified as fixed (Table 1). For the three up regulated transcripts

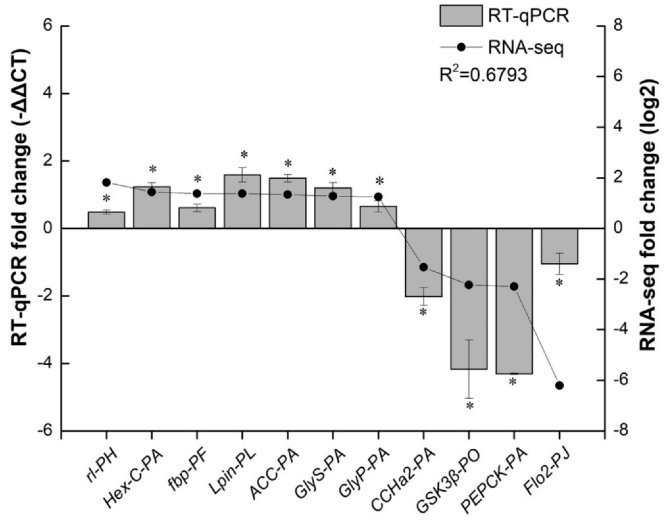

Fig. 1. Reverse transcription quantitative PCR (RT-qPCR) validation of transcripts in insulin signaling pathways that are shown by RNA-seq to be differentially expressed between DDT-resistant *91-R* and DDT-susceptible *91-C*. The *y*-axis on the left indicates the relative gene expression level of *91-R* versus *91-C* based on RT-qPCR results; that on the right corresponds to relative gene expression based on RNA-seq. *rl-PH*, rolled; *Hex-C-PA*, Hexokinase; *fbp-PF*, Fructose-1,6-bisphosphatase I; *Lpin-PL*, Lipin; *ACC-PA*, Acetyl-CoA carboxylase/biotin carboxylase 1; *GlyS-PA*, Glycogen synthase; *GlyP-PA*, Glycogen phosphorylase; *CCHa2-PA*, CCHamide-2; *GSK3β-PO*, Glycogen synthase kinase 3 beta; *PEPCK-PA*, Phosphoenolpyruvate carboxykinase; *Flo2-PJ*, Flotillin. Error bars indicate SEM for RT-qPCR results. Asterisks indicate the difference of expression between *91-C* and *91-R* is significant at $p < 0.05$.

(*Lpin-PL*, *Hex-C-PA*, and *ERK (rl)-PH*), each was predicted to have one mutation differing between the two strains. The mutations were predicted at bp 778 for *Lpin-PL* (T260P; fixed), bp 1049 for *Hex-C-PA* (K350R; fixed), and bp 443 for *ERK (rl)-PH* (which introduced a premature stop codon; unfixed) (Table 1). Moreover, six putative insertion/deletion polymorphisms between *91-R* and *91-C* were detected in four transcripts and validated by Sanger sequencing of the DDT-resistant *91-R* strain; among the four, only *PEPCK-PA* was differentially expressed. Among the six polymorphisms, five (including the one in *PEPCK-PA*) were fixed and only one was unfixed (*SI Appendix*, Table S3).

Among the 39 transcripts (encoded by 31 IIS pathway genes) that were not differentially expressed, 102 nonsynonymous nucleotide changes were found (*SI Appendix*, Table S2). Out of these nonsynonymous changes, 50% were fixed for different alleles in *91-C* and *91-R*, whereas 50% were unfixed within one of the two strains. Five fixed single-nucleotide polymorphism (SNP) mutations were found at bp locations 320, 423, 430, 532, and 1810 in *GSK3β-PM*, which respectively led to amino acid changes P107R, D141E, S144T, T178A, and T603A (*SI Appendix*, Fig. S3, Table S2).

3.3. Fitness cost: Lifespan of 91-C and 91-R under normal and starvation conditions

To determine the fitness costs resulting from resistance to DDT, the lifespan and effect of starvation of females and males of *91-C* and *91-R* were investigated. Under optimal rearing conditions, the DDT-resistant *91-R* strain showed a significantly longer lifespan than *91-C* for both females and males ($p < 0.05$ Fig. 2).

Three different adult age groups (3–4, 5–6, and 9–10 d old) were examined to investigate survival rates of *91-C* and *91-R* flies after starvation (Fig. 3). Under starvation conditions, *91-C* females survived longer than *91-R* females ($p < 0.05$), *91*-C males survived longer than *91-R* males ($p < 0.05$), and female flies survived longer than male flies ($p < 0.05$; Fig. 3).

3.4. Glycogen contents after starvation

The glycogen contents before starvation (0 h) of *91-C* and *91-R* females were 17.41 ± 1.03 and 14.33 ± 4.0 μg glycogen/mg fresh weight, respectively (Fig. 4). Nonstarved *91-R* females showed a 17.7% reduction in glycogen content compared to nonstarved *91-C* females. After starvation for 24 h, the average glycogen content for *91-C* females decreased to 12.99 ± 0.92 μg/mg fresh weight (a 25.4% reduction compared to nonstarved), which was significantly higher than that of *91-R* females (9.66 ± 0.25 μg/mg fresh weight, a 32.6% reduction compared to nonstarved). However, no difference in glycogen content between females of *91-C* and *91-R* was observed after 48 h of starvation. For males, there was no significant difference in glycogen content between *91-C* and *91-R* at any of the three time points tested.

Table 1
Nonsynonymous nucleotide changes and associated amino acid changes for differentially expressed insulin signaling pathway genes in the DDT-resistant *91-R* strain versus the DDT-susceptible *91-C* strain.

Gene	Isoform	Expression (*91-R* vs *91-C*)	Gene location	CDS length	SNP position	Allelic variant (frequency)			Amino acid change	Fixation
						91-C	*91-R*	*Can-S*		
PEPCK	*PEPCK-PA*	Down	2R:18,527,430–18,535,749	1917	200	T(1.0)	A(1.0)	T	V67G	Fixed
					415	A(1.0)	T(1.0)	A	M139L	Fixed
					1009	C(0.80)/A(0.20)	A	A	R337K	Unfixed
					1010	G(0.80)/A(0.20)	A	A		Unfixed
					1612	G(1.0)	A(0.84)/G(0.16)	G	E538K	Unfixed
					1870	G(1.0)	C(1.0)	G	A624P	Fixed
GSK3β	*GSK3β-PO*	Down	X:2,633,952–2,679,553	1326	922	A(1.0)	G(1.0)	A	T308A	Fixed
Lpin	*Lpin-PE*	Down	2R:8,136,986–8,156,654	3138	778	A(1.0)	C(1.0)	A	T260P	Fixed
					3134	C(1.0)	T(1.0)	C	S1045F	Fixed
	Lpin-PL	Up	2R:8,136,986–8,156,654	2979	778	A(1.0)	C(1.0)	A	T260P	Fixed
Hex-C	*Hex-C-PA*	Up	2R:15,218,837–15,220,329	1365	1049	A(1.0)	G(1.0)	A	K350R	Fixed
ERK (rl)	*ERK (rl)-PH*	Up	2R:1,071,462–1,125,927	800	433	G(1.0)	G(0.73)/T(0.27)	G	G210*	Unfixed

CDS, coding sequence; SNP, single-nucleotide polymorphism; *Can—S*, *Canton-S* strain.
* Stop codon.

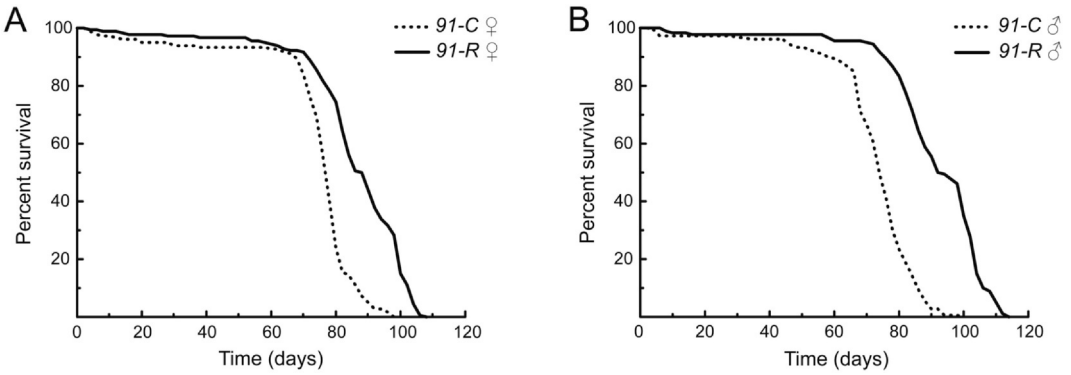

Fig. 2. Lifespan of *91-C* and *91-R* fly lines under optimal rearing conditions. (*A*) Lifespan of females of *91-C* and *91-R*. Median lifespan for *91-C* ♀ and *91-R* ♀ is 72.2 d (95% CI, 67.4–77.3 d) and 85.3 d (95% CI, 80.5–90.8 d), respectively. (*B*) Lifespan of males of *91-C* and *91-R*. Median lifespan for *91-C* ♂ and *91-R* ♂ is 70.6 d (95% CI, 64.5–77.1 d) and 91.8 d (95% CI, 85.8–99.4 d), respectively.

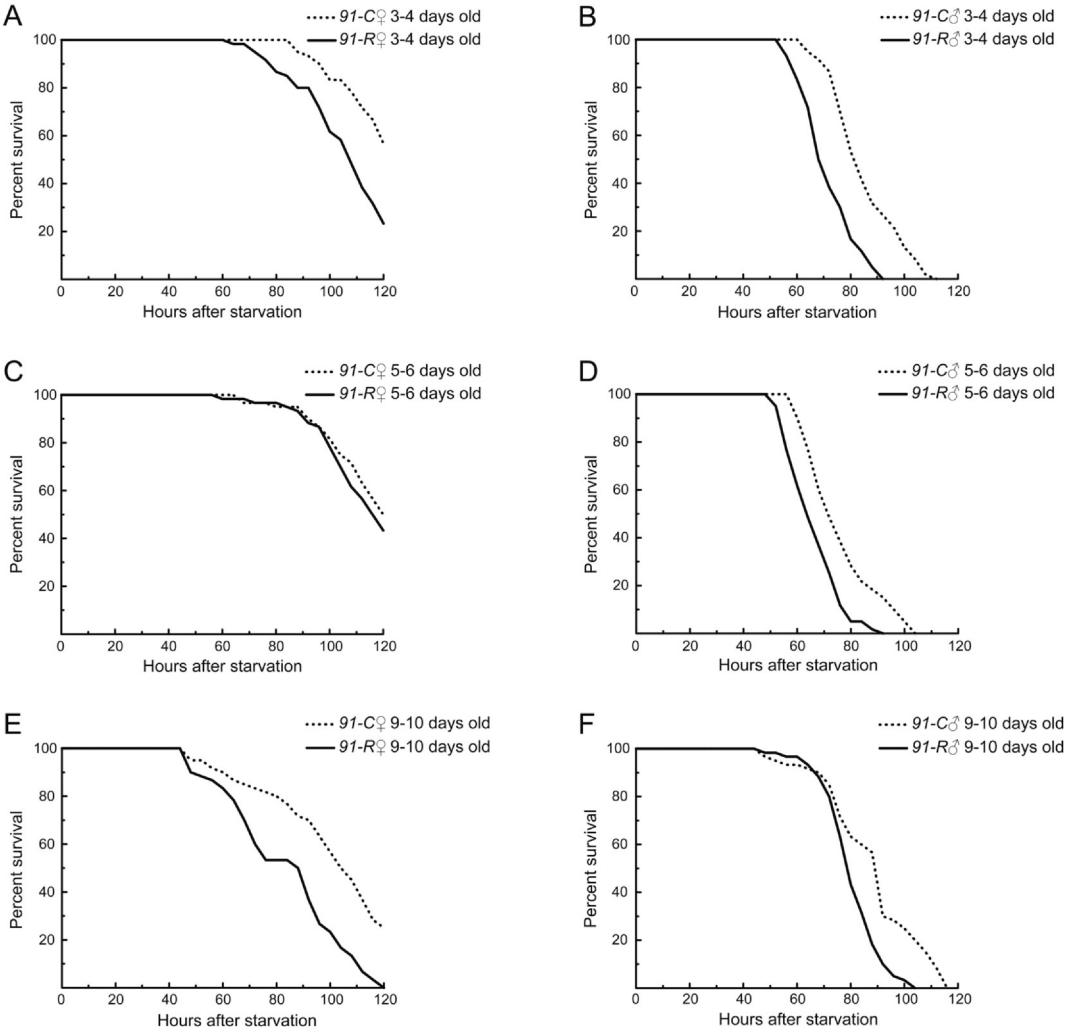

Fig. 3. Survival of *91-C* and *91-R* females and males after starvation. (*A*) Survival of 3- to 4-d-old females of *91-C* and *91-R*. The median lifespan was 122.3 h (95% CI: 119.5–125.8 h) and 106.4 h (95% CI: 104.7–108.2 h), respectively. (*C*) Survival of 5- to 6-d-old females of *91-C* and *91-R*. The median lifespan was 120.3 h (95% CI: 117.5–123.8 h) and 116.3 h (95% CI: 114.0–119.1 h), respectively. (*E*) Survival of 9- to 10-d-old females of *91-C* and *91-R*. The median lifespan was 102.8 h (95% CI: 100.7–105.2 h) and 82.0 h (95% CI: 80.3–83.7 h), respectively. (*B*) Survival of 3- to 4-d-old males of *91-C* and *91-R*. The median lifespan was 83.9 h (95% CI: 82.8–84.9 h) and 70.1 h (95% CI: 69.2–71.0 h), respectively. (*D*) Survival of 5- to 6-d-old males of *91-C* and *91-R*. The median lifespan was 74.8 h (95% CI: 73.4–76.2 h) and 64.7 h (95% CI: 64.0–65.8 h), respectively. (*F*) Survival of 9- to 10-d-old males of *91-C* and *91-R*. The median lifespan was 86.9 h (95% CI: 85.7–88.2 h) and 78.9 h (95% CI: 78.0–79.9 h), respectively.

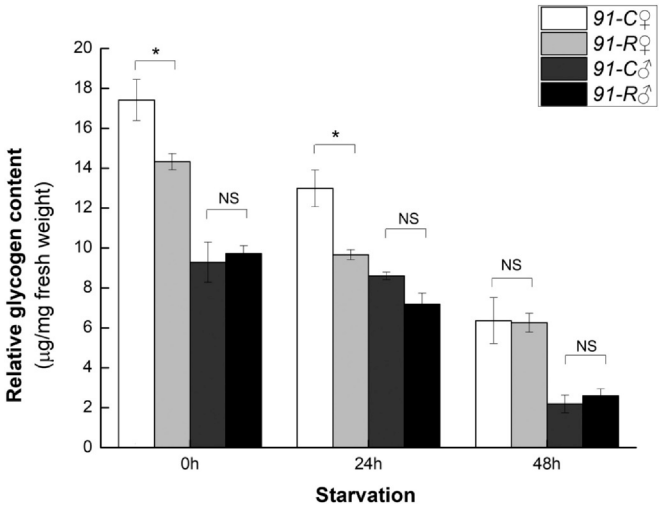

Fig. 4. Glycogen content of *91-C* and *91-R* flies per mg of fly (fresh weight) after starvation. Data are shown as means ± SEM. *, difference significant at $p < 0.05$; NS, no significant difference.

3.5. Impact of rearing conditions on adult weights and LC_{50} values

Flies under two different rearing conditions showed significantly different body weights for both genotypes and sexes ($p < 0.0001$ for all groups) (*SI Appendix*, Table S4). Compared with flies under optimal rearing conditions, flies under suboptimal rearing conditions showed weight decreases. Rearing conditions also impacted the LC_{50} of each strain-by-sex combination, both in the presence and in the absence of dietary inhibitors (Tables 2 and 3; *SI Appendix*, Figs. S4 and S5). Flies under optimal rearing conditions were consistently more resistant to DDT than those under suboptimal rearing conditions, and in some cases these differences were dramatic. For example, the LC_{50} of *91-C* females under optimal rearing conditions was 51.6 μg DDT/vial and decreased to 6.8 μg DDT/vial under suboptimal rearing conditions (a reduction of 7.6-fold). These differences were even more dramatic for the *91-R* strain. For females, an LC_{50} of 136,883 μg DDT/vial was observed under optimal rearing conditions and 523.3 μg DDT/vial under suboptimal rearing conditions (a reduction of 262-fold). For males, an LC_{50} of 66,073 μg DDT/vial was observed under optimal rearing conditions and 143.7 μg DDT/vial under suboptimal rearing conditions (a reduction of 460-fold).

3.6. Effect of excess sucrose in diet on longevity

The *91-C* strain responded differently from the *91-R* strain when reared on commercial diet prepared with excess sucrose (*SI Appendix*, Fig. S6, Table S5). The median lifespan (LT_{50}) for *91-C* females was 49, 34, and 28 d when reared on commercial diet supplemented with low, medium, and high excess sucrose, respectively, while *91-C* males had a LT_{50} of 26, 32, and 23 d, respectively. Female *91-R* flies fed with supplementary low, medium, and high excess sucrose had a median lifespan of 14, 19, and 16 d, respectively, indicating that *91-R* females have a shorter lifespan than *91-C* flies of either gender at comparable sucrose levels ($p < 0.05$). *91-R* females also had shorter lifespans than *91-R* males, which had an LT_{50} of 20, 24, and 24 d when reared on diet supplemented with low, medium, and high excess sucrose, respectively. On diet supplemented with low and medium excess sucrose, *91-R* males had a significantly shorter lifespan than *91-C* males ($p < 0.05$).

3.7. Test for Achilles' heel resistance traits

The aim of this study was to alter the level of resistance to DDT in flies fed inhibitors of selected proteins/enzymes in biochemical pathways involved in energy metabolism. Oral ingestion of either a PEPCK or GSK3β inhibitor (hydrazine sulfate [Hys] or lithium chloride [LiCl], respectively) decreased the LC_{50} values of females and males for both the *91-C* and *91-R* strains when exposed to a range of DDT concentrations. Under optimal rearing conditions (Table 2), oral exposure to Hys reduced the level of DDT resistance in *91-R* females by 161.2-fold and in *91-R* males by 218.1-fold. Oral exposure to LiCl reduced the level of DDT resistance in *91-R* females and males by 1.6- and 20.5-fold, respectively. For the susceptible *91-C* strain, feeding with Hys reduced DDT resistance by 5.3- and 4.0-fold for females and males, respectively. Oral exposure to LiCl reduced DDT resistance by 4.2- and 2.8-fold for *91-C* females and males, respectively. Hys + LiCl feeding reduced DDT resistance levels by 253.9- and 186.0-fold for *91-R* females and males, respectively. The combined treatment decreased the level of DDT resistance of *91-C* females and males by 9.7- and 4.7-fold, respectively. Combined exposure to Hys and LiCl significantly increased sensitivity to DDT in *91-C* females compared with Hys alone. Significant combinatory effects over Hys alone were not observed in *91-C* males and in *91-R* females or males, although the combination substantially reduced the

Table 2
LC_{50} values of *91-C* and *91-R* in the presence of DDT without (control) and with inhibitor feeding of hydrazine sulfate (Hys), lithium chloride (LiCl), or both under optimal dietary rearing conditions.

Strain by sex	Inhibitors	LC_{50} (μg/vial)	Slope	(95% C.I.)		Synergism ratio	χ^2
				Lower	Upper		
91-C ♀	Control	51.6	2.0	39.3	80.1	1.0	35.7 (< 0.0001)
	Hys	9.8	3.7	7.9	11.9	5.3	7.0 (< 0.0001)
	LiCl	12.2	7.6	11.2	13.1	4.2	88.4 (< 0.0001)
	Hys + LiCl	5.3	8.1	3.5	7.4	9.7	10.1 (0.0015)
91-C ♂	Control	19.8	3.1	17.4	22.4	1.0	82.9 (< 0.0001)
	Hys	5.0	3.1	4.0	6.1	4.0	73.1 (< 0.0001)
	LiCl	7.2	4.9	6.5	8.0	2.8	109.5 (< 0.0001)
	Hys + LiCl	4.2	6.7	3.8	4.6	4.7	80.7 (< 0.0001)
91-R ♀	Control	136,883.0	2.3	86,582.0	561,499.0	1.0	13.1 (0.0003)
	Hys	849.0	1.1	531.1	1235.0	161.2	39.1 (< 0.0001)
	LiCl	83,375.0	0.4	13,558.0	1.27E+16	1.6	5.0 (< 0.0252)
	Hys + LiCl	539.2	1.3	373.7	713.9	253.9	63.9 (< 0.0001)
91-R ♂	Control	66,073.0	1.9	49,796.0	118,998.0	1.0	19.1 (< 0.0001)
	Hys	302.9	1.7	206.5	397.4	218.1	63.2 (< 0.0001)
	LiCl	3224.0	1.0	2305.0	5077.0	20.5	31.1 (< 0.0001)
	Hys + LiCl	355.3	2.2	273.9	433.7	186.0	67.1 (< 0.0001)

Synergism ratio = LC_{50} (DDT control) of the indicated strain and sex / LC_{50} (DDT + inhibitor) of the indicated strain and sex.
See *SI Appendix*, Table S4 for the representative weights of adult *Drosophila* used in these bioassays.

Table 3
LC_{50} values of *91-C* and *91-R* in the presence of DDT without (control) and with inhibitor feeding of hydrazine sulfate (Hys), lithium chloride (LiCl), or both under suboptimal dietary rearing conditions.

Strain by sex	Inhibitors	LC_{50} (μg/vial)	Slope	(95% C.I.)		Synergism ratio	χ^2 (*p* value)
				Lower	Upper		
91-C ♀	Control	6.8	1.38	5.0	10.0	1.0	97.1 (< 0.0001)
	Hys	0.7	2.19	0.5	0.8	9.7	96.6 (< 0.0001)
	LiCl	3.0	1.44	2.0	5.1	2.3	46.5 (< 0.0001)
	Hys + LiCl	0.7	2.09	0.6	0.9	9.7	84.1 (< 0.0001)
91-C ♂	Control	2.1	2.03	1.5	3.4	1.0	38.7 (< 0.0001)
	Hys	0.2	3.21	0.1	0.3	10.5	20.0 (< 0.0001)
	LiCl	0.9	1.95	0.8	1.6	2.3	132.5 (< 0.0001)
	Hys + LiCl	0.2	3.37	0.1	0.2	10.5	16.9 (< 0.0001)
91-R ♀	Control	523.3	0.81	256.0	941.8	1.0	9.0 (< 0.0001)
	Hys	98.9	0.96	72.3	135.5	5.3	74.7 (< 0.0001)
	LiCl	157.6	1.85	97.6	215.5	3.3	41.3 (< 0.0001)
	Hys + LiCl	158.1	0.86	98.9	280.7	3.3	35.7 (< 0.0001)
91-R ♂	Control	143.7	1.29	84.7	210.0	1.0	27.8 (< 0.0001)
	Hys	12.9	1.27	9.2	17.0	11.1	96.9 (< 0.0001)
	LiCl	126.7	1.20	88.6	167.5	1.1	78.4 (< 0.0001)
	Hys + LiCl	9.6	0.88	5.3	14.4	15.0	60.1 (< 0.0001)

Synergism ratio = LC_{50} (DDT control) of the indicated strain / LC_{50} (DDT + inhibitor) of the indicated strain. The ratios were calculated using unrounded values. See *SI Appendix*, Table S4 for the representative weights of adult *Drosophila* used in these bioassays.

level of DDT resistance in *91-R* females.

A similar trend was observed under suboptimal rearing conditions (Table 3). Feeding with Hys reduced DDT resistance by 9.7- to 10.5-fold in *91-C* flies, and by 5.3- to 11.1-fold in *91-R* flies. By comparison, LiCl significantly decreased DDT resistance (by 3.3-fold) only in *91-R* females as the effects in *91-C* females, *91-C* males, and *91-R* males were not significant. The combination of the two inhibitors did not significantly increase the effect over the effect of Hys alone, although the largest change in resistance level was observed in *91-R* males (15.0-fold reduction).

4. Discussion

In *Drosophila*, a high-sugar diet resulted in a number of adverse symptoms including obesity, hyperglycemia, triglyceride accumulation and insulin resistance (as determined by small size and developmental delay in larvae and reduced lifespan in adults; Musselman et al., 2011). This diet also caused up-regulation of genes for enzymes involved in lipogenesis, gluconeogenesis, and β-oxidation (Musselman et al., 2011). These findings are consistent with those in *Caenorhabditis elegans*, mouse, and human and suggest a common, evolutionarily conserved mechanism leading to insulin resistance. Further, it is well established that chronic DDT exposure results in insulin resistance in vivo (Lee et al., 2011; Kim et al., 2014) and in vitro (Kim et al., 2017; Xiao et al., 2017). Given these findings, it is plausible that, during the long-term selection of the highly DDT-resistant *91-R* strain from the unselected *91-C* strain, the selected *91-R* flies may have initially become insulin-resistant but then evolved compensatory mechanisms allowing them to cope with fitness disadvantages caused by maintaining energetically demanding multiple resistance mechanisms resulting in their highly DDT-resistant phenotype. Consistent with this hypothesis, the pattern of differentially transcribed genes in the IIS pathway of *91-R* flies (Fig. 1) was for most part opposite of that found in insulin-resistant mammalian models or flies fed a high-sugar diet. In those studies, hexokinase, lpin, and GlyS were significantly down-regulated and PEPCK, GSK3β, and CCHa2 were significantly up-regulated (Musselman et al., 2011; Yao-Borengasser et al., 2006; Jope and Johnson, 2004; Saltiel and Kahn, 2001).

The impact of the changes in the IIS pathway gene expression levels and nonsynonymous mutation patterns seen in *91-R* is most clearly evident in their response to nutritional status. *91-R* flies only outlived *91-C* flies under optimal feeding conditions: their longevity was generally less than that of *91-C* flies under starvation, and excess sucrose conditions. It is interesting that the lifespan of *91-R* flies reared on excess sucrose was reduced compared with that of similarly treated *91-C* flies, a finding that mimics that found in insulin-resistant *Drosophila* fed a high-sugar diet (Musselman et al., 2011). It is also worth noting that in both high-sugar-fed and DDT-treated *Drosophila,* the genes for two key regulatory enzymes in the pentose phosphate pathway, glucose-6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase, were significantly up-regulated (Bijlsma and Kerver, 1983; Musselman et al., 2011). Because both treatments resulted in insulin resistance, a role for the pentose phosphate pathway is implied, with increased NADPH levels driving increased fatty acid synthesis and reduced glutathione (GSH) production as possible compensatory mechanisms (Bijlsma and Kerver, 1983; Musselman et al., 2011).

Consistent with the central roles of the IIS pathway in xenobiotic and stress responses, lifespan, and energy metabolism (Ikeya et al., 2002; Tatar et al., 2003; Kenyon, 2005; Giannakou and Partridge, 2007), our study demonstrates a role of this pathway in the maintenance of the DDT resistance phenotype in *91-R* flies. Inhibitors for the two most differentially expressed genes, *PEPCK* and *GSK3β* (Fig. 1), reduced the levels of DDT resistance in both *91-R* and *91-C*. However, the level of impact of the inhibitors was dependent on the nutritional status of the flies, with the greatest reductions occurring in optimally fed (heavier) flies of the *91-R* strain (Tables 2 and 3; *SI Appendix*, Table S4). Thus, the IIS pathway appears to be both an important component associated with the maintenance of DDT resistance in *91-R* and an exploitable vulnerability (i.e., an Achilles' heel) in the phenotype.

Both expression differences and nonsynonymous mutational differences were found in genes associated with longevity and energy metabolism. A number of genes in the IIS pathway with observed expression level differences between *91-R* and *91-C*, such as those encoding *Drosophila* insulin-like peptides (Dilps), and glycogen phosphorylase (GlyP), are known to be associated with lifespan (Suh et al., 2008; Bai et al., 2012). Of the two genes that were most highly down-regulated in *91-R*, namely those encoding GSK3β and PEPCK, both are associated with energy metabolism. GSK3β, an isoform of GSK3, regulates glycogen synthesis and multiple cellular processes including proliferation, differentiation, motility, and survival (Luo, 2009). PEPCK catalyzes the first committed and rate-limiting step in gluconeogenesis and plays an essential role in glucose metabolism, converting proteins and fat to glucose (Burgess et al., 2007). Additionally, PEPCK had the greatest number of amino acid differences identified between *91-R* and

91-C, both fixed and unfixed, among the screened proteins within the IIS pathway.

There has been no prior report indicating a direct relationship between DDT resistance and GSK3β, although this multifunctional serine/threonine kinase has been shown to be affected by pesticides, causing pathogenesis and neurodegeneration (Songin et al., 2011; Kaytor and Orr, 2002; Martinez et al., 2002). It is well established in diabetic mammals and humans with type 2 diabetes that GSK3 activity is increased, which inhibits glycogen synthesis by phosphorylating glycogen synthase and glucose uptake by phosphorylating insulin-receptor-coupled protein IRS-1, resulting in hyperglycemia (Jope and Johnson, 2004; Saltiel and Kahn, 2001). In addition, inhibitors of GSK3 have been used to treat insulin resistance and type 2 diabetes by reversing its effect on glycogen synthesis and glucose uptake (Dokken and Henriksen, 2006). Thus, the down-regulation of GSK3β in *91-R* may attenuate the insulin resistance caused by DDT, accelerating glycogen synthesis by up-regulating GlyS and increasing glucose uptake by allowing IRS-1 to be unphosphorylated. It should be noted that genes for both glycogen synthase and GlyP (which releases glucose-1-phosphate from glycogen) were significantly up-regulated in *91-R*. Consistent with previous reports (Lee et al., 2017; Dokken and Henriksen, 2006) and our observations of relevant expression, our glycogen assay indicated that *91-R* females stored less energy in the form of glycogen than did *91-C* females and consumed a larger percent of their stored glycogen during the early stages of starvation (Fig. 4), indicating the importance of this process in the survival of *91-R* females.

Given the above, the use of LiCl to inhibit GSK3β should have made the *91-R* flies more resistant to DDT, not less as seen in our current study. However, since the transcription of *GSK3β-PO* is already significantly down-regulated in *91-R*, its additional inhibition by LiCl likely has reduced the functions of this enzyme to levels where it is no longer capable of carrying out it vital physiological roles. Additionally, other cellular pathways besides glucose and glycogen transport under the regulation of GSK3β, such as protein synthesis, gene transcription, and cell differentiation, were affected and caused the increased susceptibility of the *91-R* flies to DDT in the presence of LiCl (Dokken and Henriksen, 2006; Jope and Johnson, 2004). It has been shown also that inhibition of GSK3 increases TNFα-induced cytotoxicity (Jope and Johnson, 2004), an effect that is consistent with the increased DDT sensitivity seen in LiCl-treated *91-R* flies. Taken together, these findings demonstrate that either too little or too much GSK3 activity can result in increased cell death and mortality.

Oxidative stress is also known to impact the IIS pathway, including altering energy utilization, inducing insulin resistance/diabetes, and increasing PEPCK gene expression (Ito et al., 2006; Evans et al., 2005; Valenti et al., 2008). DDT is also a known oxidative stressor (Hassoun et al., 1993; Barros et al., 1994) and inducer of insulin resistance (Arroyo-Salgado et al., 2017), and it has been shown to increase PEPCK activity, augmenting the production of hyperglycemia (Kacew et al., 1972). The reduced expression of *PEPCK* seen in our study suggests that gluconeogenesis in *91-R* is lower than in *91-C*. *PEPCK* is, however, overexpressed in all models of diabetes and is usually used as an indicator of gluconeogenic flux changes (Veneziale et al., 1983; Chakravarty et al., 2005) and hyperglycemia. The down-regulation, which reduces the production of glucose and hyperglycemia, and amino acid changes in PEPCK seen in *91-R* may be associated with selection for mechanisms that alleviate oxidative stress caused by prolonged DDT exposure and the stress of insulin resistance (such as the activation of key regulatory enzymes in the pentose phosphate pathway resulting in increased levels of NADPH, leading to increased levels of reduced glutathione) or possibly as a compensatory response to the hyperglycemic state associated with an insulin-resistance-like state) or both. These previous studies, coupled with our inhibitor experiments, suggest a role of PEPCK in the maintenance of the DDT resistance phenotype in *91-R*. Nonetheless, as with *GSK3β-PO*, the transcription of *PEPCK-PA* is significantly reduced in *91-R* and its further inhibition by hydrazine sulfate likely reduced the function of this enzyme to levels where it is no longer capable of carrying out it vital physiological role.

Although the down-regulation of both *GSK3β-PO* and *PEPCK-PA* is consistent with a possible compensatory mechanism against hyperglycemia resulting from insulin resistance caused by long-term DDT exposure in *91-R* flies, it may also present a problem of glucose availability for glycolysis and for providing Krebs cycle substrates used in energy production. DDT treatment of *Drosophila* has also been shown to increase enzymes involved in the pentose phosphate pathway, resulting in fatty acid synthesis, and in β-oxidation of fatty acids (an energy-producing process). The coordinated up regulation of *Hex-C-PA*, *lpin-PL*, and *ACC-PA* in the *91-R* strain is consistent with the possibility that fatty acid oxidation is an alternative energy-providing mechanism in this DDT-resistant strain.

The nutritional status of the flies also influenced the toxicity of DDT and impacted the effectiveness of hydrazine sulfate and LiCl on reducing resistance levels in the tested fly strains. Flies under suboptimal rearing conditions were lighter, more sensitive to DDT, and often less sensitive to the PEPCK and GSK3β inhibitors than were flies under more optimized rearing conditions. Our results are consistent with those of Way (1954) and Buhler and Shanks (1970), who showed that DDT in insects and vertebrates, respectively, is more toxic to lighter/smaller individuals; thus, DDT toxicity and the impact of the PEPCK and GSK3β inhibitors on such resistance/tolerance appears to be directly linked to energy metabolism and fly weight.

In the present study, we have (i) provided evidence for a role of the IIS pathway in maintaining a highly resistant phenotype against DDT and (ii) identified an Achilles' heel resistance trait in a DDT-resistant *Drosophila* population. This work, however, represents only a prove of concept of the Achilles' heel resistance trait, as DDT is not broadly in use, the IIS pathway is evolutionarily conserved across insects and mammals and may not provide the selectivity needed for safe implementation, and *Drosophila* is a model system that likely does not mimic many other insects. A considerable amount of work remains to be done to determine whether practical compounds for inhibiting resistance can be identified and how broadly applicable such an approach may be across insecticide classes, insect species, and resistant strains. Regardless of such potential applications, the *91-R* strain represents a unique model (due to its high resistance to DDT, well established resistance mechanisms, and many “omics” tools) for understanding the interplay between xenobiotic stress, energy metabolism, and longevity within the context of the insulin signaling pathway.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2020.104727.

Acknowledgments

This work was primarily supported by MSU Foundation Professor and AgBioResearch funds provided to BRP and partially funded by the China Scholarship Council (No: 201708440271).

References

Arroyo-Salgado, B., Olivero-Verbel, J., Guerrero-Castilla, A., 2017. Direct effect of *p,p'*-DDT on mice liver. Braz. J. Pharm. Sci. 52 (2), 287–298.

Bai, H., Kang, P., Tatar, M., 2012. *Drosophila* insulin-like peptide-6 (dilp6) expression from fat body extends lifespan and represses secretion of *Drosophila* insulin-like peptide-2 from the brain. Aging Cell 11, 978–985. https://doi.org/10.1111/acel.12000.

Barros, S.B., Pimente, R., Simizu, K., Azzalis, L.A., Costa, I.S., Junqueira, V.B., 1994. Dose-dependent study of liver lipid peroxidation related parameters in rats treated with pp'-DDT. Toxicol. Lett. 70, 33–38. https://doi.org/10.1016/0378-4274(94)90141-4.

Bijlsma, R., Kerver, J.W., 1983. The effect of DDT on the polymorphism at the G6pd and Pgd loci in *Drosophila melanogaster*. Genetics 103, 447–464.

Broughton, S.J., Piper, M.D., Ikeya, T., Bass, T.M., Jacobson, J., Driege, Y., Martinez, P., Hafen, E., Withers, D.J., Leevers, S.J., Partridge, L., 2005. Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands. Proc. Natl. Acad. Sci. U. S. A. 102, 3105–3110. https://doi.org/10.1073/pnas.0405775102.

Buhler, D.R., Shanks, W.E., 1970. Influence of body weight on chronic oral DDT toxicity in coho salmon. J. Fish. Res. Board Can. 27 (2), 347–358.
Burgess, S.C., He, T., Yan, Z., Lindner, J., Sherry, A.D., Malloy, C.R., Browning, J.D., Magnuson, M.A., 2007. Cytosolic phosphoenolpyruvate carboxykinase does not solely control the rate of hepatic gluconeogenesis in the intact mouse liver. Cell Metab. 5, 313–320. https://doi.org/10.1016/j.cmet.2007.03.004.
Chakravarty, K., Cassuto, H., Reshef, L., Hanson, R.W., 2005. Factors that control the tissue-specific transcription of the gene for phosphoenolpyruvate carboxykinase-C. Crit. Rev. Biochem. Mol. Biol. 40, 129–154. https://doi.org/10.1080/10409230590935479.
Choi, M.Y., Tang, S.B., Ahn, S.J., Amarasekare, K.G., Shearer, P., Lee, J.C., 2017. Effect of non-nutritive sugars to decrease the survivorship of spotted wing *drosophila, Drosophila suzukii*. J. Insect Physiol. 99, 86–94. https://doi.org/10.1016/j.jinsphys.2017.04.001.
Cox, S., Niskar, A.S., Narayan, K.M., Marcus, M., 2007. Prevalence of self-reported diabetes and exposure to organochlorine pesticides among Mexican Americans: Hispanic health and nutrition examination survey, 1982-1984. Environ. Health Perspect. 115, 1747–1752. https://doi.org/10.1289/ehp.10258.
Cross, A., Bond, C., Buhl, K., Jenkins, J., 2017. Piperonyl Butoxide General Fact Sheet. National Pesticide Information Center, Oregon State University Extension Services.
Dokken, B.B., Henriksen, E.J., 2006. Chronic selective glycogen synthase kinase-3 inhibition enhances glucose disposal and muscle insulin action in prediabetic obese Zucker rats. Am. J. Physiol. Endocrinol. Meta B 291https://doi.org/10.1152/ajpendo.00628.2005. E207-213.
dos Santos, G., Schroeder, A.J., Goodman, J.L., Strelets, V.B., Crosby, M.A., Thurmond, J., Emmert, D.B., Gelbart, W.M., FlyBase, C., 2015. FlyBase: Introduction of the *Drosophila melanogaster* Release 6 reference genome assembly and large-scale migration of genome annotations. Nucleic Acids Res. 43https://doi.org/10.1093/nar/gku1099. D690-697.
Edgar, B.A., 2006. How flies get their size: genetics meets physiology. Nat. Rev. Genet. 7, 907.
Engelman, J.A., Luo, J., Cantley, L.C., 2006. The evolution of phosphatidylinositol 3-kinase as regulators of growth and metabolism. Nat. Rev. Genet. 7, 606.
Evans, J.L., Maddux, B.A., Goldfine, I.D., 2005. The molecular basis for oxidative stress-induced insulin resistance. Antioxid. Redox Signal. 7, 1040–1052. https://doi.org/10.1089/ars.2005.7.1040.
Everett, C.J., Frithsen, I.L., Diaz, V.A., Koopman, R.J., Simpson Jr., W.M., Mainous 3rd, A.G., 2007. Association of a polychlorinated dibenzo-p-dioxin, a polychlorinated biphenyl, and DDT with diabetes in the 1999-2002 National Health and Nutrition Examination Survey. Environ. Res. 103, 413–418. https://doi.org/10.1016/j.envres.2006.11.002.
Gellatly, K.J., Yoon, K.S., Doherty, J.J., Sun, W., Pittendrigh, B.R., Clark, J.M., 2015. RNAi validation of resistance genes and their interactions in the highly DDT-resistant 91-R strain of *Drosophila melanogaster*. Pestic. Biochem. Physiol. 121, 107–115. https://doi.org/10.1016/j.pestbp.2015.01.001.
Giannakou, M.E., Partridge, L., 2007. Role of insulin-like signaling in *Drosophila* lifespan. Trends Biochem. Sci. 32 (4), 180–188.
Hardstone, M.C., Strycharz, J.P., Kim, J., Park, I.K., Yoon, K.S., Ahn, Y.J., Harrington, L.C., Lee, S.H., Clark, J.M., 2015. Development of multifunctional metabolic synergists to suppress the evolution of resistance against pyrethroids in insects that blood feed on humans. Pest Manag. Sci. 71, 842–849. https://doi.org/10.1002/ps.3856.
Hassoun, E., Bagchi, M., Bagchi, D., Stohs, S.J., 1993. Comparative studies on lipid peroxidation and DNA-single strand breaks induced by lindane, DDT, chlordane and endrin in rats. Comp. Biochem. Physiol. C 104, 427–431. https://doi.org/10.1016/0742-8413(93)90013-b.
Hussain, R., Shaukat, Z., Khan, M., Saint, R., Gregory, S.L., 2017. Phosphoenolpyruvate carboxykinase maintains glycolysis-driven growth in *Drosophila*. Tumors Sci. Rep. 7, 11531. https://doi.org/10.1038/s41598-017-11613-2.
Hwangbo, D.S., Gershman, B., Tu, M.P., Palmer, M., Tatar, M., 2004. Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature 429, 562–566. https://doi.org/10.1038/nature02549.
Ikeya, T., Galic, M., Belawat, P., Nairz, K., Hafen, E., 2002. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. Curr. Biol. 12, 1293–1300. https://doi.org/10.1016/s0960-9822(02)01043-6.
Ito, Y., Oumi, S., Nagasawa, T., Nishizawa, N., 2006. Oxidative stress induces phosphoenolpyruvate carboxykinase expression in H4IIE cells. Biosci. Biotechnol. Biochem. 70, 2191–2198. https://doi.org/10.1271/bbb.60135.
Jope, R.S., Johnson, G.V., 2004. The glamour and gloom of glycogen synthase kinase-3. Trends Biochem. Sci. 29, 95–102. https://doi.org/10.1016/j.tibs.2003.12.004.
Kacew, S., Singhal, R.L., 1974. Role of cyclic adenosine 3':5'-monophosphate in the action of 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane (DDT)on hepatic and renal metabolism. Biochem. J. 142, 145–152. https://doi.org/10.1042/bj1420145.
Kacew, S., Singhal, R.L., Ling, G.M., 1972. DDT-induced stimulation of key gluconeogenic enzymes in rat kidney cortex. Can. J. Biochem. 50, 225–229. https://doi.org/10.1139/o72-030.
Kanehisa, M., Goto, S., 2000. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28, 27–30. https://doi.org/10.1093/nar/28.1.27.
Kaytor, M.D., Orr, H.T., 2002. The GSK3 beta signaling cascade and neurodegenerative disease. Curr. Opin. Neurobiol. 12, 275–278. https://doi.org/10.1016/s0959-4388(02)00320-3.
Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649. https://doi.org/10.1093/bioinformatics/bts199.
Kenyon, C., 2005. The plasticity of aging: insights from long-lived mutants. Cell 120, 449–460. https://doi.org/10.1016/j.cell.2005.02.002.
Kim, K.S., Lee, Y.M., Kim, S.G., Lee, I.K., Lee, H.J., Kim, J.H., Kim, J., Moon, H.B., Jacobs Jr., D.R., Lee, D.H., 2014. Associations of organochlorine pesticides and polychlorinated biphenyls in visceral vs. subcutaneous adipose tissue with type 2 diabetes and insulin resistance. Chemosphere 94, 151–157. https://doi.org/10.1016/j.chemosphere.2013.09.066.
Kim, J., Park, M.Y., Kim, Y., Yoon, K.S., Clark, J.M., Park, Y., Whang, K.Y., 2017. 4,4′-Dichlorodiphenyltrichloroethane (DDT) and 4,4'-dichlorodiphenyldichloroethylene (DDE) inhibit myogenesis in C2C12 myoblasts. J. Sci. Food Agric. 97, 5176–5185. https://doi.org/10.1002/jsfa.8399.
Kim, J.H., Moreau, J.A., Zina, J.M., Mazgaeen, L., Yoon, K.S., Pittendrigh, B.R., Clark, J.M., 2018. Identification and Interaction of Multiple Genes Resulting in DDT Resistance in the 91-R Strain of *Drosophila melanogaster* by RNAi approaches. Pestic. Biochem. Physiol. 151, 90–99. https://doi.org/10.1016/j.pestbp.2018.03.003.
Lee, D.H., Steffes, M.W., Sjodin, A., Jones, R.S., Needham, L.L., Jacobs Jr., D.R., 2011. Low dose organochlorine pesticides and polychlorinated biphenyls predict obesity, dyslipidemia, and insulin resistance among people free of diabetes. PLoS One 6, e15977. https://doi.org/10.1371/journal.pone.0015977.
Lee, Y.M., Ha, C.M., Kim, S.A., Thoudam, T., Yoon, Y.R., Kim, D.J., Kim, H.C., Moon, H.B., Park, S., Lee, I.K., Lee, D.H., 2017. Low-dose persistent organic pollutants impair insulin secretory function of pancreatic beta-Cells: human and in vitro evidence. Diabetes 66, 2669–2680. https://doi.org/10.2337/db17-0188.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.
Luo, J., 2009. Glycogen synthase kinase 3beta (GSK3beta) in tumorigenesis and cancer chemotherapy. Cancer Lett. 273, 194–200. https://doi.org/10.1016/j.canlet.2008.05.045.
Martinez, A., Castro, A., Dorronsoro, I., Alonso, M., 2002. Glycogen synthase kinase 3 (GSK-3) inhibitors as new promising drugs for diabetes, neurodegeneration, cancer, and inflammation. Med. Res. Rev. 22, 373384. https://doi.org/10.1002/med.10011.
Merrell, D.J., 1960. Heterosis in Ddt Resistant and Susceptible Populations of *Drosophila melanogaster*. Genetics 45, 573–581.
Merrell, D.J., 1965. Competition involving dominant mutants in experimental populations of *Drosophila melanogaster* Genetics 52:165–189.
Merrell, D.J., Underhill, U.C., 1956. Selection for DDT resistance in inbred, laboratory and wild stocks of *Drosophila melanogaster*. J. Econ. Entomol. 49 (3), 300–306.
Mudher, A., Shepherd, D., Newman, T.A., Mildren, P., Jukes, J.P., Squire, A., Mears, A., Drummond, J.A., Berg, S., MacKay, D., Asuni, A.A., Bhat, R., Lovestone, S., 2004. GSK-3beta inhibition reverses axonal transport defects and behavioural phenotypes in *Drosophila*. Mol. Psychiatry 9, 522–530. https://doi.org/10.1038/sj.mp.4001483.
Musselman, L.P., Fink, J.L., Narzinski, K., Ramachandran, P.V., Hathiramani, S.S., Cagan, R.L., Baranski, T.J., 2011. A high-sugar diet produces obesity and insulin resistance in wild-type *Drosophila*. Dis. Model. Mech. 4, 842–849. https://doi.org/10.1242/dmm.007948.
Pedra, J.H., McIntyre, L.M., Scharf, M.E., Pittendrigh, B.R., 2004. Genome-wide transcription profile of field- and laboratory-selected dichlorodiphenyltrichloroethane (DDT)-resistant *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 101, 7034–7039. https://doi.org/10.1073/pnas.0400580101.
Pedra, J.H., Festucci-Buselli, R.A., Sun, W., Muir, W.M., Scharf, M.E., Pittendrigh, B.R., 2005. Profiling of abundant proteins associated with dichlorodiphenyltrichloroethane resistance in *Drosophila melanogaster*. Proteomics 5, 258–269. https://doi.org/10.1002/pmic.200400914.
Pittendrigh, B.R., Margam, V.M., Sun, L., Huesing, J.E., 2008. Resistance in the post-genomics age. In: Onstad, D.W. (Ed.), Insect Resistance Management: Biology, Economics and Prediction. Academic Press, pp. 39–68.
Pittendrigh, B.R., Margam, V.M., Walters Jr., K.R., Steele, L.D., Olds, B.P., Sun, L., Huesing, J., Lee, S.H., Clark, J.M., 2014. Understanding resistance and induced responses of insects to xenobiotics and insecticides in the age of “omics” and systems biology. In: Onstad, D.W. (Ed.), Insect Resistance Management: Biology, Economics, and Prediction, 2nd edn. Academic Press, pp. 55–98.
Saltiel, A.R., Kahn, C.R., 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414, 799–806. https://doi.org/10.1038/414799a.
Seong, K.M., Coates, B.S., Sun, W., Clark, J.M., Pittendrigh, B.R., 2017. Changes in neuronal signaling and cell Stress response pathways are associated with a multigenic response of *Drosophila melanogaster* to DDT selection. Genome Biol. Evol. 9, 3356–3372. https://doi.org/10.1093/gbe/evx252.
Seong, K.M., Coates, B.S., Berenbaum, M.R., Clark, J.M., Pittendrigh, B.R., 2018. Comparative CYP-omic analysis between the DDT-susceptible and -resistant *Drosophila melanogaster* strains 91-C and 91-R. Pest Manag. Sci. 74, 2530–2543. https://doi.org/10.1002/ps.4936.
Slack, C., Giannakou, M.E., Foley, A., Goss, M., Partridge, L., 2011. dFOXO-independent effects of reduced insulin-like signaling in *Drosophila*. Aging Cell 10, 735–748. https://doi.org/10.1111/j.1474-9726.2011.00707.x.
Songin, M., Ossowska, K., Kuter, K., Strosznajder, J.B., 2011. Alteration of GSK-3beta in the hippocampus and other brain structures after chronic paraquat administration in rats. Folia Neuropathol. 49, 319–327.
Strycharz, J.P., Lao, A., Li, H., Qiu, X., Lee, S.H., Sun, W., Clark, J.M., 2013. Resistance in the highly DDT-resistant *91-R* strain of *Drosophila melanogaster* involves decreased penetration, increased metabolism, and direct excretion. Pestic. Biochem. Physiol. 107 (2), 207–217.
Suh, Y., Atzmon, G., Cho, M.O., Hwang, D., Liu, B., Leahy, D.J., Barzilai, N., Cohen, P., 2008. Functionally significant insulin-like growth factor I receptor mutations in centenarians. Proc. Natl. Acad. Sci. U. S. A. 105, 3438–3442. https://doi.org/10.1073/pnas.0705467105.

Tatar, M., Bartke, A., Antebi, A., 2003. The endocrine regulation of aging by insulin-like signals. Science 299, 1346–1351. https://doi.org/10.1126/science.1081447.

Tennessen, J.M., Barry, W.E., Cox, J., Thummel, C.S., 2014. Methods for studying metabolism in *Drosophila*. Methods 68, 105–115. https://doi.org/10.1016/j.ymeth.2014.02.034.

Underwood, L.E., Thissen, J.P., Lemozy, S., Ketelslegers, J.M., Clemmons, D.R., 1994. Hormonal and nutritional regulation of IGF-I and its binding proteins. Horm. Res. 42, 145–151. https://doi.org/10.1159/000184187.

Valenti, L., et al., 2008. increased expression and activity of the transcription factor FOXO1 in nonalcoholic steatohepatitis. Diabetes 57, 1355–1362. https://doi.org/10.2337/db07-0714.

Veneziale, C.M., Donofrio, J.C., Nishimura, H., 1983. The concentration of P-enolpyruvate carboxykinase protein in murine tissues in diabetes of chemical and genetic origin. J. Biol. Chem. 258, 14257–14262.

Way, M.J., 1954. The effect of body weight on the resistance to insecticides of the last-instar larva of *Diataraxia oleracea* L., the tomato moth. Ann. Appl. Biol. 41 (1), 77–87.

Xiao, X., Clark, J.M., Park, Y., 2017. Potential contribution of insecticide exposure and development of obesity and type 2 diabetes. Food Chem. Toxicol. 105, 456–474. https://doi.org/10.1016/j.fct.2017.05.003.

Yao-Borengasser, A., Rasouli, N., Varma, V., Miles, L.M., Phanavanh, B., Starks, T.N., Phan, J., Spencer 3rd, H.J., McGehee Jr., R.E., Reue, K., Kern, P.A., 2006. Lipin expression is attenuated in adipose tissue of insulin-resistant human subjects and increases with peroxisome proliferator-activated receptor gamma activation. Diabetes 55, 2811–2818. https://doi.org/10.2337/db05-1688.

Yau, D.T., Mennear, J.H., 1977. The inhibitory effect of DDT on insulin secretion in mice. Toxicol. Appl. Pharmacol. 39, 8188. https://doi.org/10.1016/0041-008x(77)90179-x.