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ORIGINAL PAPER

There is more than chitin synthase in insect resistance to benzoylureas: molecular markers associated with teflubenzuron resistance in *Spodoptera frugiperda*

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Abstract

Chitin synthesis inhibitors are successfully used in pest control and an excellent option for integrated pest management programs due to their low non-target effects. However, field-evolved resistance of lepidopteran pests to chitin synthesis inhibitors and the selection of laboratory-resistant strains to these products has been already reported. Therefore, to support efficient resistance management programs it is necessary to expand the knowledge on the resistance mechanisms and potential molecular markers that detect resistant alleles. Teflubenzuron is a chitin synthesis inhibitor used to control the world widely distributed fall armyworm, *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae). Here, we report the selection and inheritance characterization of *S. frugiperda* strain resistant to teflubenzuron. We also evaluated the cross-resistance to other chitin-synthesis inhibitors and identified single nucleotide polymorphisms (SNPs), which can be used as molecular markers for monitoring the evolution of resistance of *S. frugiperda* to teflubenzuron. The resistance of the selected strain of *S. frugiperda* to teflubenzuron was characterized as polygenic, autosomal, and incompletely recessive. The resistance ratio observed was nearly 1,365-fold. Teflubenzuron-resistant strain showed cross-resistance to lufenuron and novaluron, but not to chlorfluazuron. We also detected a set of 72 SNPs that could support monitoring of the resistance frequency to teflubenzuron in field populations. Our data contribute to the understanding of the resistance mechanisms and the inheritance of resistance of *S. frugiperda* to benzoylureas. We also contribute with candidate markers as tools for monitoring the emergence and spread of teflubenzuron resistance in *S. frugiperda*.

Keywords Detoxification · Resistance markers · Insecticide resistance · Insecticide resistance management · Resistance evolution · Genetic markers

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Key message

- *S. frugiperda* resistance to teflubenzuron is polygenic, autosomal, and incompletely recessive.
- No cross-resistance to chlorfluazuron was observed.
- Resistance of *S. frugiperda* to teflubenzuron is not associated to chitin synthase mutation.
- We detected a set of SNPs that could support monitoring of *S. frugiperda* resistance to teflubenzuron.

Introduction

Insect resistance evolution to insecticides and *Bacillus thuringiensis (Bt)*-genetically modified crops is of great concern to biologists, farmers, industry, and government agencies. The strong selection pressure impinged both by numerous insecticides sprays and the wide adoption of *Bt*-crops increased resistance frequency in many agroecosystems, especially in the successive crop systems used in the central Cerrado savanna region in Brazil (Carvalho et al. 2013; Farias et al. 2014; Nascimento et al. 2016; Okuma et al. 2018; Bolzan et al. 2019; Lira et al. 2020).

The fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) is a polyphagous species native to American tropical regions which gained worldwide distribution after invading Africa a couple of years ago (Goergen et al. 2016). FAW is a serious pest of several economically important crops, such as maize (Cruz 1995), cotton (Santos 2007), and soybean (Bueno et al. 2011). Currently, *Bt* crops and insecticides are the main tactics in use for FAW management in the world (Assefa 2018).

Insecticides from the benzoylurea group, which were introduced in the early 1970s, act as chitin synthesis inhibitors (van Daalen et al. 1972). These insecticides have been successfully applied to control several pest species in the field. They have high acaricidal and insecticidal activity, exhibiting activity against immatures lepidopterans, coleopterans, hemipterans, and dipterans (Yu 2015). Moreover, the low non-target effects of benzoylureas allow their use in association with other control strategies within well-designed integrated pest management programs (Beeman 1982; Oberlander and Silhacek 1998; Post and Vincent 1973).

Initial studies on the evolution of resistance to chitin synthesis inhibitors under laboratory conditions failed in selecting resistant populations even after 20 generations of directed-selection pressure (Perng et al. 1988). However, their broad use increased the frequency of resistance, leading to evolution of field-evolved resistance of *Plutella xylostella* (Lepiodptera: Plutellidae) in China and *Spodoptera litura* (Lepidoptera: Noctuidae) in Pakistan (Ahmad et al. 2008; Lin et al. 1989), and the selection of resistant strains of *Spodoptera frugiperda* and *Cydia pomonella* (Lepidoptera: Tortricidae) under laboratory conditions (Sauphanor and Bouvier 1995; Nascimento et al. 2016).

The evolution of resistance in field conditions indicates that the selection pressure and the resistance frequency are high enough to allow the selection of resistant phenotypes, resulting in the complete failure of the management strategies taken in place. The implementation of suitable management plans is required in order to maintain benzoylureas available as a technology for pest control in areas where the resistance frequency is still manageable. The development of reliable and successful resistance management requires the adoption of pro-active strategies and the understanding of the resistance mechanism and its heritability in ways that could contribute to resistance monitoring programs.

Benzoylureas act as chitin synthesis inhibitors by interfering with the synthesis or deposition of chitin in the exoskeleton and other chitinous structures of insects (Merzendorfer and Zimoch 2003; Merzendorfer 2006). The exactly mode of action of benzoylureas has been debated as they were thought to indirectly affect chitin biosynthesis upon binding to sulfonylurea receptors, resulting in vesicle trafficking alterations; however, the role of the ABC transporter sulfonylurea receptor in chitin synthesis was arguable (Abo-Elghar et al. 2004; Meyer et al. 2013). The use of bulk segregants mapping analysis to investigate the resistance mechanism of *Tetranychus urticae* (Acari: Tetranychidae) to the mite chitin synthesis inhibitor etoxazole led to the characterization of resistance of field populations as monogenic and recessive (Van Leeuwen et al. 2012). The authors of that study also identified a single nonsynonymous mutation (I1017F) in chitin synthase 1 as the resistance mechanism, demonstrating the direct effect of this acaricide on chitin synthase (Van Leeuwen et al. 2012). Their proposition that is benzoylurea with insecticide activity could also target chitin synthase due to the similarities with etoxazole and led to the identification of a mutation at the same position for the isoleucine residue in the chitin synthase 1 in different insect species resistant to chitin synthesis inhibitors (Douris et al. 2016; Fotakis et al. 2020).

Currently, benzoylureas as chlorfluazuron, diflubenzuron, lufenuron, flufenoxuron, novaluron, triflumuron and teflubenzuron are used to control insects in soybean, cotton, and maize crops in Brazil (MAPA 2020). The high selection pressure caused by this group of insecticides has decreased the susceptibility of *S. frugiperda* to benzoylureas (Schmidt 2002). For instance, FAW populations in central Brazil evolved resistance to lufenuron, carrying a high resistance

ratio, and an autosomal and polygenic inheritance of resistance (Nascimento et al. 2016).

Teflubenzuron, 1-(3,5-Dichloro-2,4-difluorophenyl)-3-(2,6-difluorobenzoyl) urea, has been used to control lepidopterans, coleopterans, and dipterans larvae (Yu 2015), since the ovicidal and larvicidal activities of this product were first demonstrated (Ascher and Nemny 1984). Despite the efficacy of this insecticide in controlling insect pests, there are reports of insect resistance to teflubenzuron as early as in the 1980′s, such as for *P. xylostella* (Iqbal and Wright 1997; Lin et al., 1989; Perng et al. 1988) and *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Ishaa and Klein 1990). In Brazil, the increased number of control failures with pyrethroids, organophosphates, and benzoylureas (mainly lufenuron) early in 2000s stimulated the use of teflubenzuron to control *S. frugiperda* in cotton, maize, and soybean crops.

In this study, we characterized the genetic basis of resistance of *S. frugiperda* to teflubenzuron. By selecting a resistant strain, we (1) characterized the inheritance of resistance, (2) evaluated the cross-resistance to other chitin-synthesis inhibitors, and (3) used a genome scanning approach to identify genomic regions and single nucleotide polymorphisms (SNPs) associated to teflubenzuron resistance for further using them as molecular markers to monitor resistance evolution in field conditions.

Material and methods

Insects

The susceptible *S. frugiperda* strain (Sf-ss) has been maintained on an artificial diet based on bean, wheat germ and casein (Kasten Junior et al. 1978) in the Arthropod Resistance Laboratory (University of São Paulo, campus ESALQ, Brazil) without insecticide selection for 25 years. The resistant strain (Tef-rr) was selected from field-collected larvae from maize fields from the state of Mato Grosso, Brazil (13°25′35.9″S; 58°38′17.84″W), during the 2014–2015 crop season.

Selection of teflubenzuron-resistant *Spodoptera frugiperda* strain

Selection for teflubenzuron resistance was carried out using the F_2 screen method proposed by Andow and Alstad (1998), since this method increases the likelihood of obtaining a resistant genotype. About 1,000 field-collected larvae were reared under controlled laboratory conditions (25 ± 2 °C; 60 ± 10% RH; 14 h photophase) up to pupation. From the field-collected larvae, we isolated 33 individual couples (families). The progeny obtained for each family was reared under the same controlled conditions described before until adult emergence. Adults originating from these families, which correspond to a F_1 generation, were allowed to sib-mate. All eggs laid from each family were collected. Then, 120 larvae from each sib-mated line (i.e., from the F_2 generation) were used in insecticide bioassays. We used a common diet-overlay bioassay to select for teflubenzuron resistance at the diagnostic concentration of 10 µg mL^{-1} of teflubenzuron (Nomolt® 150, teflubenzuron 150 g/L, BASF S.A., São Paulo, Brazil), based on the concentration–response of the susceptible strain (Sf-ss) to this insecticide. The artificial diet (Kasten Junior et al. 1978) was poured into 24-well acrylic plates (Costar®, Corning®), and 30 µL/well of the diagnostic concentration of teflubenzuron in a water solution (v:v) containing 0.1% Triton X-100 was applied on the diet surface. The control diet was treated only with distilled water and 0.1% Triton X-100. Plates were kept under a laminar flow hood for drying the diet surface, and each well was inoculated with one *S. frugiperda* third instar larva. We allowed larvae to feed for five days under controlled conditions (25 ± 2 °C; 60 ± 10% RH; photophase of 14 h). After five days, we collected and transferred the surviving *S. frugiperda* larvae to plastic cups (100 mL) containing 50 mL of artificial diet for their rearing until pupation. All surviving individuals from the insecticide treatment were combined and used for successive selection rounds with increasing concentration of teflubenzuron from 10 to 560 µg mL^{-1} of teflubenzuron for seven generations until the establishment of the teflubenzuron-resistant strain (Tef-rr).

Characterization of *Spodoptera frugiperda* resistance to teflubenzuron

The susceptible (Sf-ss) and resistant (Tef-rr) strains of *S. frugiperda* to teflubenzuron were subjected to concentration–response assays with five to 12 logarithmically spaced concentrations between 0.1 and 3,200 µg mL^{-1} of teflubenzuron. Larval bioassays were conducted using the diet overlay assays earlier described. The lethal concentration 50 (LC_{50}) was estimated with Probit analysis (Finney 1971, 1949) using the POLO software (Robertson et al. 2007). The resistance ratio of Tef-rr was calculated by dividing the LC_{50} of the Tef-rr by that of the Sf-ss strain.

Estimation of dominance levels

Newly emerged adults from the susceptible (Sf-ss) and resistant (Tef-rr) strains were reciprocally crossed: Tef-rr males × Sf-ss females (RC-1) and Tef-rr females × Sf-ss males (RC-2). Adults (10 couples/cage) were kept in PVC cages (20 cm high × 15 cm diameter) lined with paper to serve as substrate for egg laying. Adults were fed with a 10% honey solution that was replaced every other day. The progenies obtained from each reciprocal cross (F_1) were reared on

artificial diet until the third instar. Afterwards, third-instars from the reciprocal crosses were exposed to teflubenzuron using the diet-overlay assay explained before.

We estimated the dominance level of resistance from (Bourguet et al. 2000),

$$D = \frac{M_{RS} - M_{SS}}{M_{RR} - M_{SS}} \quad (1)$$

where M_{SS}, M_{RR}, and M_{RS} are the mortalities of the Sf-ss, Tef-rr, and heterozygous strains, respectively, exposed to different concentrations of teflubenzuron. Values of D close to zero ($D=0$) represent completely recessive inheritance, and values close to 1 ($D=1$) represent completely dominant inheritance.

We also estimated dominance level by applying equation [2] (Stone 1968), where D is the degree of dominance and X_F, X_R, X_S are the LC_{50} values, respectively, for the heterozygote (offspring from reciprocal cross RC1 or RC2), Tef-rr and Sf-ss.

$$D = \frac{2X_F - X_R - X_S}{X_R - X_S} \quad (2)$$

Genetic inheritance associated with teflubenzuron resistance in *Spodoptera frugiperda*

We used the method proposed by Roush and Daly (1990) and Tsukamoto (1983) to test the hypothesis that a single gene is responsible for teflubenzuron resistance of *S. frugiperda*. We backcrossed the offspring resulting from the twenty-mating pairs Tef-rr♂ × Sf-ss♀ (heterozygous) with individuals from the resistant strain Tef-rr. We performed diet-overlay bioassays, using eight concentrations of teflubenzuron as earlier described.

The possibility of monogenic inheritance was calculated by using the Chi-square test (Eq. 3) (Sokal and Rohlf 1969), where *Ni* is the mortality observed in backcrossed larvae at each concentration and *ni* is the number of individuals tested; *q* is the expected survival, and *p* is the expected mortality calculated from the Mendelian model (Eq. 4) (Georghiou, 1969), where *a* is the mortality obtained for the parental strain Tef-rr, and *b* is the mortality of the heterozygote derived from the reciprocal crosses (Tef-rr♂ x Sf-ss♀). The hypothesis of monogenic inheritance is rejected when the calculated Chi-square is equal or higher than the tabulated Chi-square value, with 1 degree of freedom.

$$X^2 = \frac{(N_i - pni)^2}{pqni} \quad (3)$$

$$p = \frac{a+b}{2} \quad (4)$$

Cross-resistance to other benzoylurea insecticides

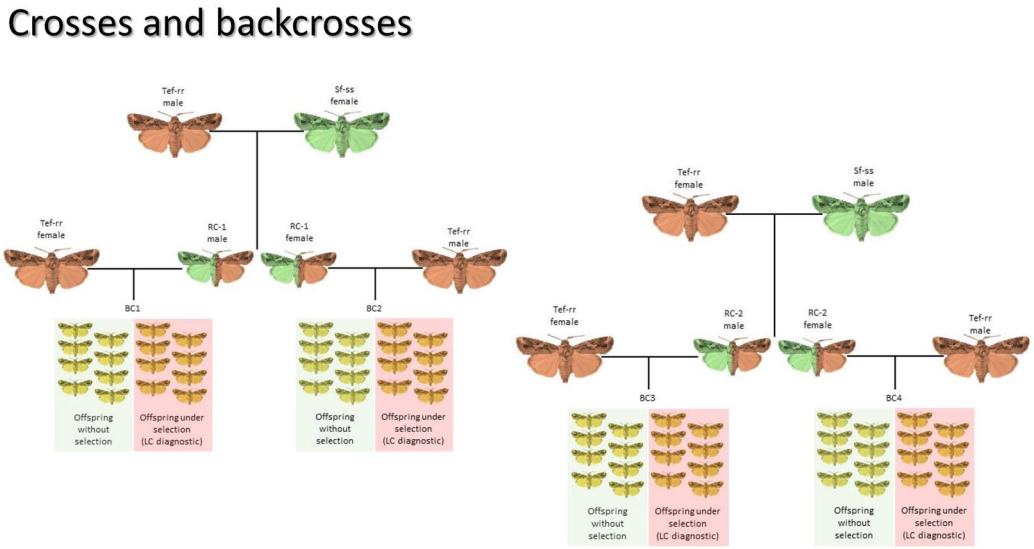
Cross-resistance assays of the Tef-rr strain with three other benzoylureas were carried out using the diet-overlay bioassay earlier described utilizing commercial formulations of lufenuron (Match®, 50 g/L, Syngenta, Basel, Switzerland), novaluron (Rimon Supra®, 100 g/L, Syngenta), and chlorfluazuron (Atabron, 50 g/L, ISK Biosciences). For each insecticide, we performed concentration–response bioassays for Tef-rr and Sf-ss as already described. Larval mortality was assessed five days after treatment, and larval mortality was characterized by larval unresponsiveness to stimulation with a fine brush or the occurrence of body malformations. LC_{50} values were estimated using the POLO software (Robertson et al. 2007).

Genetic crossings and sample selection for gDNA pool sequencing

The pool sequencing was designed to highlight potential markers associated with resistance. We established backcrosses with the resistant strain (Tef-rr) and the offspring from the reciprocal crosses described above (RC-1 and RC-2), e.g., BC1 = (RC-1♂ x Tef-rr♀), BC2 = (RC-1♀ x Tef-rr♂), BC3 = (RC-2♂ x Tef-rr♀), BC4 = (RC-2♀ x Tef-rr♂). Each backcross was split in two groups of insects: (1) individuals randomly collected (BC-random) and (2) individuals that survived the selection pressure by exposure to the diagnostic concentration of 10 µg teflubenzuron/mL (BC-selected) (Fig. 1).

DNA extraction and sequencing

DNA was extracted from nine larvae from each parental line, Tef-rr (resistant), and Sf-ss (susceptible), and from both groups of each backcross BC1, BC2, BC3, and BC4. Larval genomic DNA was obtained with the modified CTAB method (Doyle 1991). Briefly, 50 mg of tissue from each individual larva was macerated in 650 µL of extraction buffer containing 2% cetyltrimethyl ammonium bromide (CTAB), 1.4 M NaCl, 100 mM tris(hydroxymethyl) aminomethane (Tris–HCl) at pH 8.0, 20 mM ethylenediaminetetraacetic acid (EDTA) at pH 8.0, 1% polyvinylpyrrolidone, 0.2% β-mercaptoethanol, and 20 µL of proteinase K (0.1 $\mu g \cdot mL^{-1}$). Samples were incubated at 55 °C for 1 h, added with 650 µL of chloroform: isoamyl alcohol (24:1), and mixed until emulsion. Samples were centrifuged (14,000 g × 5 min × 4 °C), and then the supernatant was collected and transferred to new tubes, where 200 µL of the same extraction buffer (minus the β-mercaptoethanol and proteinase K) was added followed by the addition of the same volume of chloroform: isoamyl alcohol (24:1). The emulsion was thoroughly vortexed, centrifuged



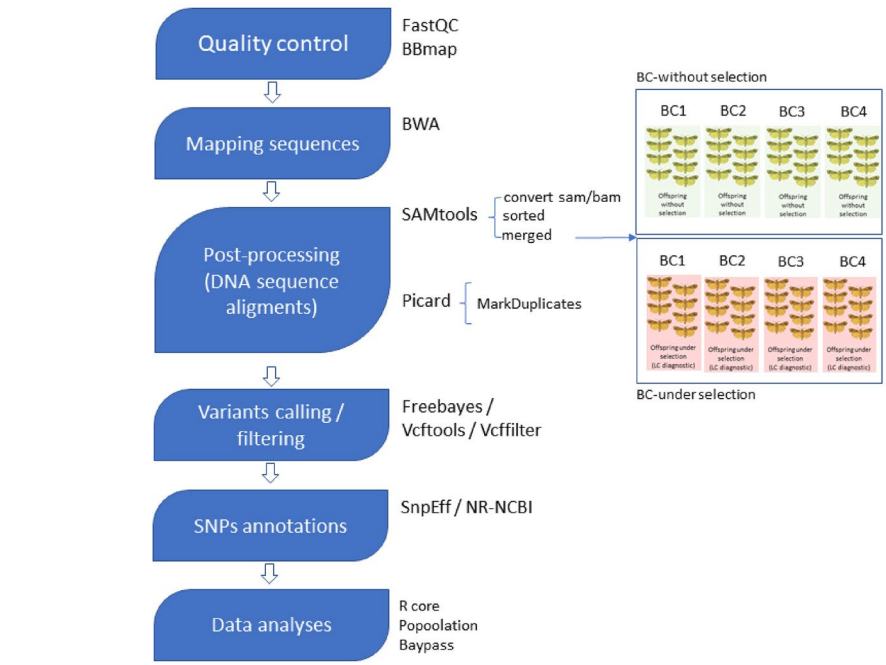


Fig. 1 Experimental design, pool sequences, and snp-calling pileline

(14,000 g × 5 min × 4 °C), and the supernatant collected; we repeated this process 3 times. Samples were combined with 650 µL of cold isopropanol and incubated at −20 °C overnight before centrifugation (14,000 g × 5 min × 4 °C). The pelleted DNA was washed twice with 1 mL of 70% ethanol. The pellet was dried at room temperature, resuspended in 40 µL TE and Rnase A (10 µg mL^{-1}), and stored at −20 °C until further analyses. Genomic DNA was evaluated quantitatively with a Qubit fluorometer (Thermo Fisher Scientific, USA) and checked for degradation with agarose gel electrophoresis. Finally, 7 ng of DNA from each one of nine larvae were combined into a single tube for each treatment.

Briefly, we sheared total pooled DNA into ~ 300–400 bp fragments in an ultrasonicator and used it to build sequencing libraries with the NEBNext Ultra DNA library prep kit (New England Biolabs), according to the manufacturer's instructions. The whole genome (WGS) for each pool was sequenced in a Miseq platform (Illumina, Inc., San Diego, CA, USA) at the Molecular and Cellular Imaging Center at the Ohio State University.

Sequencing data processing

The quality of raw paired-end reads was assessed using FastQC (Andrews et al. 2015), and reads were filtered using BBmap (http://jgi.doe.gov/data-and-tools/bbtools) by excluding nucleotides with a Phred quality score < 30 from subsequent analyses. Afterwards, the filtered reads were mapped against the *S. frugiperda* pseudo-genome assembly available at BIPAA—Bioinformatics Platform for Agroecosystem Arthropods (https://bipaa.genouest.org/sp/spodoptera_frugiperda_pub/), using the BWA-MEM (Li and Durbin 2010). Alignment files were converted to SAM/BAM files using SAMtools (Li 2011). Alignment in BAM format from the BC1-random, BC2-random, BC3-random, and BC4-random were combined on a single BAM file (BC-random), whereas BC1-selected, BC2-selected, BC3-selected, and BC4-selected were combined on a single BAM file (BC-treated). Read alignments with PCR duplicates were removed using the *MarkDuplicates* from Picard software (https://broadinstitute.github.io/picard/), and SNP calling was performed using freebayes (Garrison and Marth 2012). SNPs called were subject to quality filters (quality score > 20 and depth > 10) using the programs Vcftools (Danecek et al. 2011) and Vcffilter (Müller et al. 2017).

Analyses

The vcfR package was used to visualize and manipulate the vcf format. The global F_{ST} was calculated for all SNPs using the R package PoolFstat. Tajima's π and *D* were calculated for each pooled DNA sample in a 5 kb sliding window with a 5 kb step size for each comparison group using Popoolation v.1.2.2 (Kofler et al. 2011).

Candidate SNPs associated with the resistance of *S. frugiperda* to teflubenzuron were identified using a population genomics-based approach, which uses the genetic differentiation between the pools to identify genomic regions potentially targeted by selection (Pool-GWAS).

For the population genomics-based approach, SNP count data were analyzed using two different implementations of the bayesian hierarchical models available in the Baypass version 2.2 (Gautier 2015). First, we applied the core model (Coop et al. 2010; Nicholson et al. 2002) to identify loci with significant allele frequency differences. This method is equivalent to the methods that search for loci with higher intra-locus F_{ST}. However, in this model a variance–covariance matrix of population allele frequencies (Ω matrix) that works as a kinship matrix is used to control for population structure. Controlling for population structure reduces the likelihood of spurious association between the marker and the phenotype. This method is covariate-free and was expanded to include the calibration of the XtX statistics as proposed by Günther and Coop (2013). Second, we employed the STD model representing an extension of core model, which allows the evaluation of the association of SNP allele frequency with covariates (Gautier 2015). For the covariate model, we conducted two independent analysis: (1) using the LC_{50} as covariate, and (2) using the mortality obtained with the diagnostic concentration for each bulk (susceptible, resistant, and the BC-random and BC-treated) as a covariate. Because we performed the analysis with the two backcrosses-derived and the parental pools, both genome scans (the F_{ST}-like method and the covariate association method) were insensitive to the identification of false positive associations between the markers and the phenotype. These methods are prone to the identification of the strongest signal that might highlight higher differences mostly associated with demography and drift (because the resistant and the susceptible populations share a common ancestor many generations ago), not with selection. The identification of true positives can be done with the identification of SNPs in linkage disequilibrium with the causal gene in the pool that was subjected to selection (BC-treated), and in loci that present allele frequency differences between the BC before and after selection.

Functional annotation and identification of putative markers associated with the resistant phenotype

Annotation of loci associated with SNPs was proposed using the *gff* file available for the *S. frugiperda* genome (https://bipaa.genouest.org/sp/spodoptera_frugiperda_pub/) using SnpEff (Cingolani et al. 2012). Genes with no functional annotation in the available genome were annotated after

heuristic search using the BLASTx algorithm against the non-redundant protein database available at the NCBI.

Results

Characterization of teflubenzuron resistance in *Spodoptera frugiperda*

Eleven out of the 33 lines of the F_2 generation subjected to selection yielded survivors and were considered to carry resistance traits. The high resistance traits selected in the teflubenzuron-resistant strains were observed by comparing the concentration response curves of the Tef-rr against the Sf-ss and their backcrosses (Fig. 2), and the obtained values of LC_{50}. The LC_{50} for the resistant Tef-rr (641.47 µg mL^{-1}; IC = 213.05–2,748.81 µg mL^{-1}) was nearly 1,365-fold the LC_{50} for the susceptible Sf-ss strain (0.47 µg mL^{-1}; IC = 0.35–0.63 µg mL^{-1}). Both Sf-ss ($P = 0.02$) and Tef-rr ($P = 0.03$) showed no evidence of distortion at $\chi^2 > 0.01$, indicating a good fit to the probit inheritance model of resistance (Table 1).

Bioassays with progenies of the two reciprocal crosses showed no significant differences, since there was an overlap of 95% CI of the LC_{50} values (Table 1). Therefore, the hypothesis of parallelism was not rejected ($P = 0.247$, $df = 1$). The overlap of the confidence intervals indicated that inheritance of teflubenzuron resistance of *S. frugiperda* is autosomal, and not related to maternal effects or sex linked.

The dominance values for the reciprocal crosses of the offspring estimated following Stone (1968) were 0.32 (Tef-rr♂ × Sf-ss♀) and 0.29 (Tef-rr♀ × Sf-ss♂). The dominance level estimated using the Bourguet-Genissel-Raymond method showed decreased dominance with increased teflubenzuron concentrations (Fig. 3). In both cases, teflubenzuron resistance of *S. frugiperda* was shown to have an incompletely recessive inheritance.

The direct hypothesis test for monogenic inheritance of the teflubenzuron resistance of *S. frugiperda* based on larval mortality of the F_1 × Tef-rr backcross was significant ($P < 0.01$) for concentrations between 1 and 10 µg mL^{-1}.

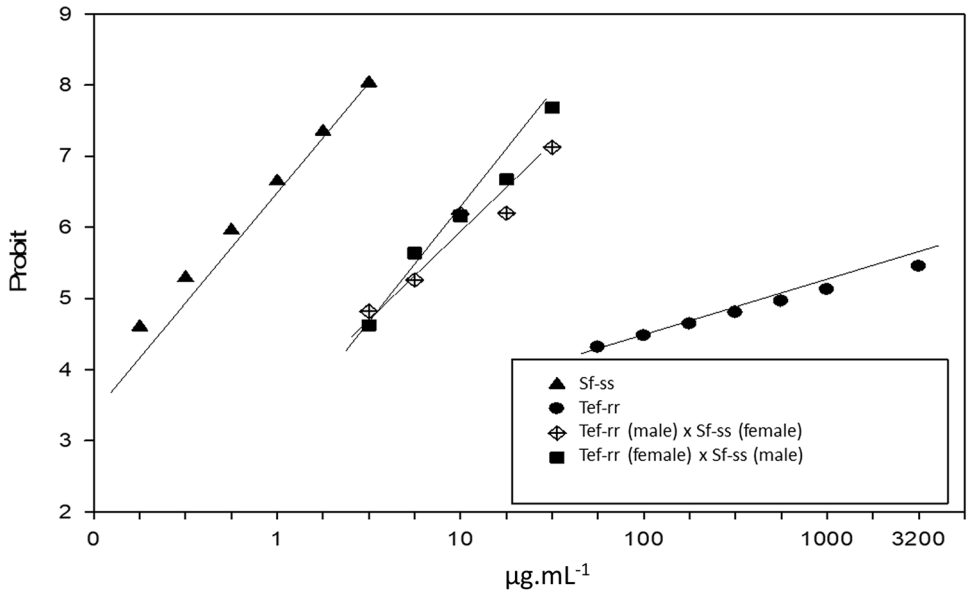


Fig. 2 Log concentration–probit of susceptible (Sf-ss) and resistant (Tef-rr) *S. frugiperda* strains and progenies of reciprocal crosses between susceptible and resistant strains

Table 1 Concentration—mortality to teflubenzuron of susceptible (Sf-ss) and resistant (Tef-rr) *S. frugiperda* strains and progenies of reciprocal crosses between Sf-ss and Tef-rr strains

Strains	*n*	Slope ± SE*	LC_{50} (95% CI) (µg AI mL^{-1})	χ^2	*df***	RR***
Sf-ss	715	3.07 ± 0.24^a	0.47(0.35–0.63)	11.630	4	–
Tef-rr	840	0.64 ± 0.09^c	641.47 (213.05–2,748.81)	10.590	4	1,364.83
Tef-rr♂ vs Sf-ss♀	936	2.05 ± 0.14^b	4.88 (3.58–6.31)	8.054	5	10.38
Tef-rr ♀ vs Sf-ss ♂	983	2.29 ± 0.17^b	3.94 (3.13–4.78)	7.604	6	8.38

*Slopes followed by the same letter in the columns do not differ significantly for test of parallelism

***df* = degrees of freedom

***Resistance ratio (RR) = LC_{50} of resistant strain/LC_{50} of susceptible strain

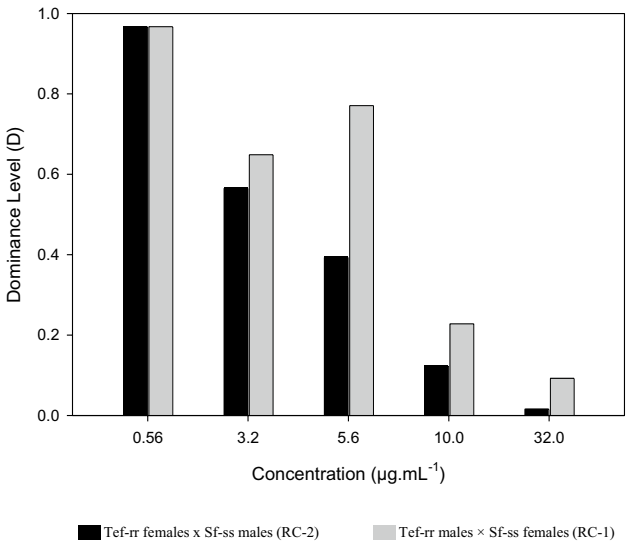


Fig. 3 Level of dominance of *S. frugiperda* resistance as a function of teflubenzuron concentration

Table 2 Chi-square analysis of the mortality data from backcross between the progeny of reciprocal cross (Tef-rr♂ x Sf-ss♀) and Tef-rr *S. frugiperda* strain (F1 progeny) exposed to different concentrations of teflubenzuron

Concentration µg AI mL^{-1}	Expected mortality	Observed mortality	χ^2 (df = 1)	*P*
1	1.042	5.042	18.47	< 0.00001*
3.2	21.677	9.574	8.10	0.0044*
10	44.167	22.222	14.06	0.0001*
32	49.167	48.958	0.001	0.9674
100	64.600	68.750	0.72	0.3951
320	70.795	78.125	2.49	0.1142
1000	80.530	82.291	0.19	0.6629

This result allows the rejection of the hypothesis that teflubenzuron resistance in the selected strain is monogenic (Table 2).

Cross-resistance

Both susceptible and teflubenzuron-resistant strains were tested against chlorfluazuron, lufenuron, and novaluron (Table 3). Teflubenzuron-resistant strain showed some cross-resistance to lufenuron (121.7-fold) and novaluron (75.8-fold), but no cross-resistance of the Tef-rr strain to chlorfluazuron (fourfold) was detected (Table 3).

Genome-scan of Tef-rr and Sf-ss strains of *Spodoptera frugiperda*

The sequencing of the pooled WGS libraries generated 220 million high-quality paired-end reads after adapter removal and quality trimming. The read mean length was 229 bp and the maximum length was 300 bp, resulting in paired-end fragments with an average of 429 bp in length, with an estimated coverage of 108-fold (Table S1). After mapping and filtering the reads against the *S. frugiperda* reference genome, 890,209 SNPs were called.

The global genetic differentiation among the resistant, the susceptible and the two backcrosses samples were moderate and significant ($F_{ST} = 0.10169$ with 95% CI of 0.10138–0.10205). The pairwise measurements between BC-random and BC-treated showed that both backcross pools were virtually identical ($F_{ST} = -0.01454$ 95% CI of −0.0146–0.01442). However, the pairwise F_{ST} between Sf-ss and Tef-rr was high ($F_{ST} = 0.37581$ with 95% CI of 0.37489–0.37684). Genetic differentiation was also high between Sf-ss and BC-random ($F_{ST} = 0.20461$ with 95% CI of 0.20394–0.20532), and Sf-ss and BC-treated ($F_{ST} = 0.21186$ with 95% CI of 0.21116–0.21253) (Figure S2).

Table 3 Cross-resistance of *S. frugiperda*-resistant strain (Tef-rr) to benzoylphenylureas insecticides

Strains	Insecticide	*n*	Slope ± SE	LC_{50} (95% CI) (µg AI mL^{-1})	χ^2	*df*[a]	RR[b]
Sf-ss	Teflubenzuron	715	3.07 ± 0.24	0.47(0.35–0.63)	11.63	4	–
	Lufenuron	963	1.99 ± 3.13	0.23 (0.17–0.29)	11.94	4	–
	Novaluron	696	2.60 ± 0.30	0.35 (0.23–1.73)	9.33	3	–
	Chlorfluazuron	1029	1.20 ± 0.07	0.15(0.11–0.20)	11.91	6	–
Tef-rr	Teflubenzuron	840	0.64 ± 0.09	641.47 (213.05–2748.81)	10.59	4	1,364.8
	Lufenuron	739	2.36 ± 0.16	28.01 (14.00–45.05)	40.65	5	121.7
	Novaluron	659	2.05 ± 0.24	26.53 (21.96–31.70)	2.51	4	75.8
	Chlorfluazuron			0.63 (0.24–1.04)	0.84	4	4.2

[a] *df* = degrees of freedom

[b] Resistance ratio (RR) = LC_{50} of resistant strain/LC_{50} of susceptible strain

The variation within the sliding window estimates for π resulted in nucleotide diversities of 0.0192, 0.0285, 0.0336, and 0.0337, respectively, for the Sf-ss and Tef-rr strains and their backcrosses. We identified genomic regions with reduced diversity when comparing the Sf-ss and Tef-rr strains. Large genomic regions with reduced diversity were observed when comparing the resistant and the susceptible strains (Tajima's $\pi < 0.002$), including 600 scaffolds from *S. frugiperda* genome. Regions of low nucleotide diversity can be observed in Table S3.

The total number of SNPs called was narrowed to 9,161 after the application of *XtX* statistics (*XtX P*-value < 0.001) with evidence of divergent selection. We also identified 4,120 SNPs with eBF > 3 db, supporting a moderate evidence of association. Four SNPs exceeded the threshold for strong evidence of association with LC_{50} values according to the Jeffreys' rule, whereas 38 SNPs supported moderate evidence of association with mortality using the diagnostic concentration as variable. Finally, 537 SNPs overlapped with both the neutrality model and the association with environmental variables model (Figure S4), distributed in 232 scaffolds.

Scaffold annotation indicated that most of the variants under selection were located in intergenic (47%) and regulatory regions (downstream regions—18%; upstream regions—18%), with 2% presenting missense effect (Figure S5). GO terms distribution demonstrated an impressive number of variants on processes related to primary metabolism, metabolism of organic compounds, and components of membrane (Figure S4). We identified 19 SNPs with non-synonymous effects (Table 4), distributed in 19 scaffolds. Most of the variants are in regulatory regions (Table 5).

Discussion

In the present study, we selected a strain highly resistant (≈ 1,365-fold) to teflubenzuron from a field-collected population of *S. frugiperda* in the state of Mato Grosso, Brazil. Teflubenzuron resistance was found to be polygenic, incompletely recessive with an autosomal mode of inheritance. *Spodoptera frugiperda* resistant to teflubenzuron showed evidence of cross-resistance to lufenuron and novaluron, but not to chlorfluazuron.

The pattern of genetic inheritance of *S. frugiperda* resistance to teflubenzuron is similar and common to lepidopteran species resistant to several insecticides and *Bt* toxins, e.g., Dipel resistance and Cry1Ab resistance in *Ostrinia nubilalis* (Lepidoptera: Crambidae) (Huang et al. 1999), and in *S. frugiperda* resistance to lufenuron (Nascimento et al. 2016),

Table 4 Missense mutation under selection in *S. frugiperda* resistant to teflubenzuron

Scaffold	Position	Nucleotide modification	Protein modification	Gene	Description
pseudoscaff_1708	178,922	A2228G	Asn743Ser	GSSPFG00030930001	GATA zinc finger domain-containing protein 14-like
pseudoscaff_3427	17,439	T1358C	Val453Ala	GSSPFG00031119001.2	Cytochrome CYP340AB1
pseudoscaff_3907	6057	C616T	Pro206Ser	GSSPFG00030391001	Nose resistant to fluoxetine 6-like
pseudoscaff_653	4817	C764T	Ser255Leu	GSSPFG00012677001	Cuticle CPG4855
pseudoscaff_957	60,700	T731A	Met244Lys	GSSPFG00023674001	Probable serine threonine- kinase kinX
pseudoscaff_1765	469,701	T644G	Leu215Arg	GSSPFG00027555001	Serine protease
pseudoscaff_3391	127,787	C2146T	Pro716Ser	GSSPFG00003324001	Uncharacterized protein LOC110384046 isoform X1
pseudoscaff_3689	183,291	C518T	Thr173Met	GSSPFG00005692001.1	Uncharacterized protein LOC110370494
scaffold_168	131,785	C1496T	Pro499Leu	GSSPFG00028493001	UPF0061 Pfl01_0444-like
pseudoscaff_2533	256,404	G200C	Trp67Ser	GSSPFG00005566001	Unknown
pseudoscaff_3278	1521	C971A	Thr324Asn	GSSPFG00015719001	rho GTPase-activating 11A-like
pseudoscaff_1247	66,548	C203T	Thr68Ile	GSSPFG00033321001.3	Chemosensory ionotropic receptor IR40a
pseudoscaff_1409	38,337	G160A	Ala54Thr	GSSPFG00025253001	sid 3
pseudoscaff_1573	23,130	T428G	Leu143Trp	GSSPFG00009731001	Peptidyl-tRNA hydrolase mitochondrial-like
pseudoscaff_2560	3374	C2099T	Thr700Met	GSSPFG00009517001.3	Ubiquitin carboxyl-terminal hydrolase 36 isoform X1
superscaffold_605	216,090	C341T	Pro114Leu	GSSPFG00011282001	Alpha-1,3/1,6-mannosyltransferase ALG2
pseudoscaff_3041	7075	C1067T	Ala356Val	GSSPFG00001156001	Serpin B8-like
pseudoscaff_2143	20,100	G833A	Arg278Lys	GSSPFG00012527001	Eukaryotic initiation factor 4A-III
pseudoscaff_3248	90,010	T754C	Cys252Arg	GSSPFG00017635001	Spindle assembly abnormal 6 homolog

Table 5 Candidate SNPs on downstream, upstream, intronic, and splice regions

Scaffold	Nucleotide modification	Region of variant	Functional description
pseudoscaff_1297	A22203C	Downstream	Protein charybdis-like
pseudoscaff_1307	A232656G	Downstream	Insulin-like precursor polypeptide 2
pseudoscaff_1362	G67863C	Downstream	U4 tri-snRNP-associated 1 isoform X2
pseudoscaff_1648	C137023A	Downstream	Alpha-tubulin *N*-acetyltransferase-like isoform X2
pseudoscaff_1648	C137251T	Downstream	Alpha-tubulin N-acetyltransferase-like isoform X2
pseudoscaff_1775	T199512C	Downstream	Transcription initiation factor IIA subunit 2
pseudoscaff_2258	G33649A	Downstream	Methyltransferase-like 26
pseudoscaff_2598	C12345T	Downstream	Allatostatin-A receptor
pseudoscaff_2606	G19017A	Downstream	Acyl-desaturase
pseudoscaff_2896	T22770C	Downstream	Insulin-like growth factor-binding complex acid labile subunit isoform X3
pseudoscaff_3278	C1521A	Downstream	F-box only 11
pseudoscaff_3604	T173487C	Downstream	homeobox orthopedia-like isoform X2
pseudoscaff_3799	A16059G	Downstream	E3 ubiquitin-protein ligase MARCH1-like isoform X1
pseudoscaff_4151	G20990A	Downstream	STAGA complex 65 subunit gamma-like
pseudoscaff_4151	C21023T	Downstream	STAGA complex 65 subunit gamma-like
scaffold_168	G131785A	Downstream	DNA repair and recombination RAD54-like
superscaffold_161	T93320C	Downstream	Beclin 1-associated autophagy-related key regulator
pseudoscaff_1108	C68071T	Intron	Gamma-aminobutyric acid type B receptor subunit 1-
pseudoscaff_1494	T585C	Intron	Paraplegin
pseudoscaff_1648	A135286C	Intron	Alpha-tubulin *N*-acetyltransferase-like isoform X2
pseudoscaff_1648	A126799G	Intron	Alpha-tubulin N-acetyltransferase-like isoform X2
pseudoscaff_1877	C65279T	Intron	EH domain-containing 3
pseudoscaff_2418	T62036G	Intron	G-coupled receptor Mth2-like
pseudoscaff_2486	C31733G	Intron	scm-like with four MBT domains 2
pseudoscaff_2598	G6055A	Intron	Allatostatin-A receptor
pseudoscaff_26	T40740A	Intron	Guanylate cyclase
pseudoscaff_4046	A194050T	Intron	Probable 3-hydroxyacyl-dehydrogenase isoform X2
pseudoscaff_838	T36912C	Intron	UDP-glucuronic acid decarboxylase 1 isoform X1
pseudoscaff_10	G53445T	Splice_region	Eukaryotic translation initiation factor 3 subunit
pseudoscaff_1095	G68217A	Upstream	BTB/POZ domain-containing protein 6-B
pseudoscaff_1230	G44162C	Upstream	Coup transcription factor
pseudoscaff_1244	T24415C	Upstream	Small GTPase Rab4b
pseudoscaff_1282	A22175T	Upstream	Cytochrome P450 337B3v2
pseudoscaff_1394	A541334T	Upstream	Cytochrome P450 CYP321A9
pseudoscaff_1456	C16151G	Upstream	Probable phosphatase 2C
pseudoscaff_1648	T166815C	Upstream	Tyrosine-kinase
pseudoscaff_165	A42337G	Upstream	Mannose-1-phosphate guanyltransferase beta
pseudoscaff_1745	T20426C	Upstream	Pancreatic triacylglycerol lipase-like
pseudoscaff_2012	G288294A	Upstream	Aldo-keto reductase
pseudoscaff_2143	G20100A	Upstream	DDB1- and CUL4-associated factor 10 isoform X2
pseudoscaff_215	T100348A	Upstream	Disks large 1 tumor suppressor isoform X6
pseudoscaff_2153	A41682T	Upstream	Chromatin modification-related eaf-1-like isoform X1
pseudoscaff_2411	A307493G	Upstream	ADP-ribosylation factor 6-interacting 4
pseudoscaff_3028	G39454A	Upstream	Exocyst complex component 8
pseudoscaff_3790	A99724T	Upstream	UDP-glucose 4-epimerase-like
pseudoscaff_3799	A16059G	Upstream	Eukaryotic translation initiation factor 6
pseudoscaff_4013	A27061G	Upstream	ras-related Rab-39B
pseudoscaff_4148	C20818T	Upstream	Triosephosphate isomerase
pseudoscaff_597	T129616A	Upstream	Eukaryotic translation initiation factor 2A

Table 5 (continued)

Scaffold	Nucleotide modification	Region of variant	Functional description
pseudoscaff_597	T58764A	Upstream	MRN complex-interacting
pseudoscaff_838	T36912C	Upstream	Multidrug resistance-associated 4-like
scaffold_15106	T1653C	Upstream	Steroidogenic acute regulatory-like isoform X1
scaffold_4023	A1781G	Upstream	E3 ubiquitin-ligase RNF13 isoform X3

spinosad (Okuma et al. 2018), and spinetoram (Lira et al. 2020).

The inheritance mechanism of teflubenzuron resistance was influenced by insecticide concentration. At lower concentrations, resistance inheritance assumes incompletely dominant features, but at higher concentrations it becomes incompletely recessive. The higher concentration is close to the recommended concentration currently used in field applications for *S. frugiperda* control. In resistance management, the level of dominance is a variable feature, resulting not only from the genetic background, but also from the interaction between phenotypes and environmental conditions (Bourguet et al. 2000). The level of dominance is one of the most important features for successful IRM (Lenormand and Raymond 1998), since the frequency of resistant insects can be related to the level of dominance. But if resistance inheritance is recessive, the evolution of resistance is delayed because the resistant phenotype is present only in homozygotes, and the alleles that confer resistance are rare (Ffrench-Constant 2013). However, the use of lower concentrations than the level recommended for field application helps to maintain heterozygous individuals in the system and increases the frequency of the resistant alleles within the population. Thus, the continued exposure to the selection pressure (teflubenzuron applications) favors the rapid increase of individual resistance, leading to a concomitant increase in the likelihood of heterozygous mating, and an ultimate production of resistant homozygotes.

The significant deviation between the observed and expected mortalities for the offspring of the resistant–susceptible backcrosses in three concentrations of teflubenzuron indicates that resistance is anchored on more than one gene. Multiple genes with additive and quantitative effects can also lead to the generation of resistance features, and it has been difficult to identify a specific gene or genetic marker associated with such evolutionary process in these cases. The polygenic nature of resistance of *S. frugiperda* to teflubenzuron agrees with the resistance characterized for another strain of this insect to the benzoylurea lufenuron (Nascimento et al. 2016).

The cross-resistance of teflubenzuron-resistant insects to lufenuron and novaluron may be related to strong selection of insects with overexpression of the detoxification genes, such as cytochrome P450 (CYP), glutathione S-transferases (GSTs), UDP-glucosyltransferases (UGTs), and esterases (CCEs) (Nascimento et al. 2015). These genes are largely associated with detoxification of xenobiotics in several lepidopteran species. Therefore, selection of these genes within these superfamilies may be responsible for the evolution of resistance to different insecticide compounds within the same IRAC group.

The lack of cross-resistance detected to chlorfluazuron as compared to the high cross-resistance levels observed toward lufenuron and novaluron agrees with data available on cross-resistance among different benzoylurea compounds. Cross-resistance to benzoylureas with other chemical compounds was also reported to *C. pomonella* (Sauphanor and Bouvier 1995; Sauphanor et al. 1998, 2000; Stará and Kocourek 2007). Chlorfluazuron cross-resistance to other benzoylureas, when detected, is very low (cross-resistance to teflubenzuron = 9.9-fold) (Fahmy et al. 1991). The lack of cross-resistance of other benzoylureas to chlorfluazuron is likely linked to the higher toxicity and delayed excretion of chlorfluazuron (Gazit et al. 1989; Guyer and Neumann 1988), but mostly due to the structural nature of this compound. Insect resistance to benzoylureas has been reported to occur due to the overexpression of cytochrome P450 monooxygenases (P450) (Bogwitz et al. 2005a, b) or site mutations in the chitin synthase gene (Douris et al. 2016; Fotakis et al. 2020; Suzuki et al. 2017). In one particular case, resistance of a natural population of *Drosophila melanogaster* to the benzoylurea lufenuron due to the overexpression of a P450 (*Cyp6g1*) has evolved as a result of cross-resistance to chemical compounds this fly commonly encounters in nature (Daborn et al. 2002; Wilson and Cain 1997). But chlorfluazuron resistance of a highly resistant (resistance ratio > 2,000-fold) strain of *P. xylostella* was not affected when using the synergist piperonyl butoxide (PBO), a P450 inhibitor (QingJun et al. 1997). Although PBO had been previously shown to reduce the resistance ratio of a chlorfluazuron selected strain of *P. xylostella* (resistance ratio = 50-fold) to 4.3-fold, it was suggested the microsomal enzymes acting on this pesticide might be different from other benzoylurea compounds (Ismail and Wright, 1992). In fact, glutathione-S-transferases were later suggested to play a role in *P. xylostella* resistance to chlorfluazuron (Sonoda and Tsumuki 2005). However, other evolutionary events, such as gene duplication, could be alternative or additional

adaptations routes, resulting in the overexpression and/or neofunctionalization of cytochrome P450s (Zimmer et al. 2018).

Several candidate SNPs showed signals of strong positive selection, supporting the polygenic nature of the resistance of *S. frugiperda* to teflubenzuron. Although resistance of insects to benzoylureas has been associated with site mutations in *chitin synthase* (Douris et al. 2016; Fotakis et al. 2020; Suzuki et al. 2017), the resistant population of *S. frugiperda* analyzed did not display any missense variants in this gene. Missense variants were identified in two other genes. One is up-regulated in a chlorantraniliprole-resistant strain of *S. exigua*, the cytochrome P450 monooxygenase *Cyp340AB1* (Wang et al. 2018). The other is the cuticular protein *CPG4855*, a gene that participates in the formation of the larval and pupal endocuticle in *S. exigua* (Jan et al. 2017). Although P450 enzymes have been implicated in the metabolization of several pesticides, including benzoylureas, as earlier discussed, we do not believe the resistance mechanism of *S. frugiperda* to teflubenzuron would be associated with a point mutation in the *Cyp340AB1* gene, a P450 belonging to the CYP4 clan. The *Cyp* genes mostly commonly involved in the metabolization of xenobiotics, including insecticides, belongs to the family *Cyp6* of the CYP3 Clan of P450 enzymes (Feyereisen 2012), as the *Cyp6g1* gene previously reported in lufenuron-resistant *D. melanogaster* (Daborn et al. 2002; Wilson and Cain 1997). On the other hand, the site mutation in the cuticular protein CPG4855 could have a close association with the resistance mechanism observed in *S. frugiperda* to teflubenzuron, once RNAi experiments targeting cuticular proteins demonstrated association of these genes and insects resistant to insecticides (Fang et al. 2015; Huang et al. 2018).

The resistance of *S. frugiperda* to teflubenzuron was found to be polygenic, and as such much more likely to involve mechanisms of regulation of gene expression, as reported to other benzoylurea-resistant insects. In fact, several polymorphic SNPs were detected upstream (within 5 Kb of the start codon) and downstream (within 5 Kb of the stop codon) of gene regions, including intronic regions of the genome. These SNP variations might be responsible for modifications leading to regulation of gene expression and protein function. In humans, GWAS analysis demonstrated that more than 90% disease-associated SNPs were located up and downstream (promoter and enhancer regions) gene regions or even in non-coding regions of the genome (Hindorff et al., 2009; Hrdlickova et al. 2014; Ricaño-Ponce and Wijmenga 2013).

Annotation allowed the identification of several upstream, downstream, and intron variants in genes associated with several biological processes besides biological regulation and regulation of cellular processes. These annotation results point to multiple gene interactions and regulation playing decisive roles in insecticide resistance.

Thus, the high number of SNPs in genomic regions involved in mechanisms that interfere with the regulation of gene expression of a number of genes involved in processes of metabolization and excretion of xenobiotics are thought to serve as candidate molecular markers for monitoring the polygenic teflubenzuron-resistant phenotypes in the field. These candidates involve the multidrug resistance-associated protein 4 or ATP-binding cassette subfamily C member 4 (ABCC4), a transmembrane protein involved in the efflux of organic compounds from cells (Hardy et al. 2019), including xenobiotics in insects (Labbé et al. 2011). ABCCs can be functionally diverse, but they are capable to translocate a range of organic xenobiotics including insecticides, and have been involved in insecticide resistance mechanisms in insects and drug resistance in humans (Dermauw and Van Leeuwen 2014). Moreover, ABCC transporters act in synergy with glutathione-S-transferases (GSTs) and UDP-glucosyltransferases (UGTs) which are enzymes acting in phase II of detoxification. In humans, the synergy of ABCCs, GSTs, and UGTs confer resistance to drugs and carcinogens (Dermauw and Van Leeuwen 2014).

G-protein-coupled receptors (GPCRs) play a central role in cell signaling as receptors of neuromodulators, neurotransmitters, hormones and neuropeptides. GWAS analysis for the identification of SNPs in the teflubenzuron-resistant strain of *S. frugiperda* identified variants in genomic regions that can interfere with gene expression of a GPCR belonging to the *methuselah* (*mth*) subfamily of the *secretin* family. *Methuselah* is a secretin receptor reported to be insect-specific. *Mth* play a role in several biological processes in insects, such as stress response, regulation of fluid and ion secretion, and longevity, among others (Araújo et al. 2013). An exploration of GPCRs in *Tribolium castaneum* indicated *mth* is also important for larval molt and metamorphosis (Bai et al. 2011). Moreover, *mth* gene mutation led *D. melanogaster* to become tolerant to dichlorvos (Pandey et al., 2015), but insect response to insecticide exposure was also altered with changes in the levels of *mth* expression (Cao et al. 2019; Li et al. 2015; Lucas et al. 2019; Ma et al. 2020). Similarly to the early discussed synergism of ABCCs and phase II conjugation enzymes in insect response to insecticide exposure, *mth* is co-expressed with the phase I detoxification P450 enzymes, contributing to insect resistance to insecticides (Cao et al. 2019; Li et al. 2015).

The selection of a strain of *S. frugiperda* highly resistant to teflubenzuron, the identification of cross-resistance to lufenuron and novaluron, and the use of genome wide association analysis led us to identify several candidate molecular markers for monitoring resistance evolution to benzoylureas. Several SNPs identified in association with the teflubenzuron-resistant strain indicates that the polygenic

mechanism of resistance selected in the resistant strain of *S. frugiperda* is based on a dense and intricate network of co-expressing genes, of which many are important regulatory genes. The variation in the levels of cross-resistance observed for the different benzoylureas assayed against the teflubenzuron-resistant strain of *S. frugiperda* provides us with additional tools to investigate and understand the particular differences in the target sites of the many structural compounds sharing chitin synthesis inhibition as a mode of action. The well-defined mutation in the *chitin synthase* gene related to target site mutation resistance to benzoylureas in arthropods (Van Leeuwen et al. 2012) and the several molecular marker candidates we added as sources of metabolic resistance to benzoylureas can be strategically used for monitoring the resistance of *S. frugiperda* and for future implementation of successful insecticide resistance management strategies.

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Author Contributions ARBN, FLC, AM, and CO conceived the study. ARBN and JGR collected the data. CO and AM provided reagents and sequencing financing. ARBN, VACP, and KLSB performed the analysis. ARBN and FLC wrote the main manuscript. All authors contributed to writing and editing the manuscript.

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Data Availability Statement Illumina data will be publicly available at the NCBI BioProject PRJNA678657.

Declaration

Conflicts of interest The authors declare they have no conflicts of interest.

Ethical approval This research did not involve humans or vertebrates.

References

Abo-Elghar GE, Fujiyoshi P, Matsumura F (2004) Significance of the sulfonylurea receptor (SUR) as the target of diflubenzuron in chitin synthesis inhibition in *Drosophila melanogaster* and *Blattella germanica*. Insect Biochem Mol Biol 34:743–752. https://doi.org/10.1016/j.ibmb.2004.03.009

Ahmad M, Sayyed AH, Saleem MA, Ahmad M (2008) Evidence for field evolved resistance to newer insecticides in *Spodoptera litura* (Lepidoptera: Noctuidae) from Pakistan. Crop Prot 27:1367–1372. https://doi.org/10.1016/j.cropro.2008.05.003

Andow DA, Alstad DN (1998) F2 screen for rare resistance alleles. J Econ Entomol 91:572. https://doi.org/10.1093/jee/91.3.572

Andrews S, Krueger F, Seconds-Pichon A, et al (2015) FastQC. A quality control tool for high throughput sequence data. https://www.bioinformatics.babraham.ac.uk/projects/fastqc. Accessed 26 June 2018

Araújo AR, Reis M, Rocha H, et al (2013) The *Drosophila melanogaster methuselah* Gene: a novel gene with ancient functions. PLoS One 8. https://doi.org/https://doi.org/10.1371/journal.pone.0063747

Ascher KRS, Nemny NE (1984) The effect of CME 134 on *Spodoptera littoralis* eggs and larvae. Phytoparasitica 12:13–27. https://doi.org/10.1007/BF02980794

Assefa F (2018) Status of fall armyworm (*Spodoptera frugiperda*), biology and control measures on maize crop in Ethiopia: a review. Int J Entomol Res 6:75–85. https://doi.org/https://doi.org/10.33687/entomol.006.02.2498

Bai H, Zhu F, Shah K, Palli SR (2011) Large-scale RNAi screen of G protein-coupled receptors involved in larval growth, molting and metamorphosis in the red flour beetle. BMC Genomics 12. https://doi.org/https://doi.org/10.1186/1471-2164-12-388

Beeman RW (1982) Recent advances in mode of action of insecticides. Annu Rev Entomol 27:253–281. https://doi.org/10.1146/annurev.en.27.010182.001345

Bogwitz MR, Chung H, Magoc L et al (2005a) Cyp12a4 confers lufenuron resistance in a natural population of *Drosophila melanogaster*. Proc Natl Acad Sci USA 102:12807–12812. https://doi.org/10.1073/pnas.0503709102

Bogwitz MR, Chung H, Magoc L et al (2005b) Cyp12a4 confers lufenuron resistance in a natural population of Drosophila melanogaster. Proc Natl Acad Sci USA 102:12807–12812. https://doi.org/10.1073/pnas.0503709102

Bolzan A, Padovez FEO, Nascimento ARB et al (2019) Selection and characterization of the inheritance of resistance of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to chlorantraniliprole and cross-resistance to other diamide insecticides. Pest Manag Sci 75:2682–2689. https://doi.org/10.1002/ps.5376

Bourguet D, Genissel A, Raymond M (2000) Insecticide resistance and dominance levels. J Econ Entomol 93:1588–1595. https://doi.org/10.1603/0022-0493-93.6.1588

Bueno RCOF, De Freitas BA, Moscardi F et al (2011) Lepidopteran larva consumption of soybean foliage: Basis for developing multiple-species economic thresholds for pest management decisions. Pest Manag Sci 67:170–174. https://doi.org/10.1002/ps.2047

Cao C, Sun L, Du H et al (2019) Physiological functions of a methuselah-like G protein coupled receptor in *Lymantria dispar* Linnaeus. Pestic Biochem Physiol 160:1–10. https://doi.org/10.1016/j.pestbp.2019.07.002

Carvalho RA, Omoto C, Field LM et al (2013) Investigating the molecular mechanisms of organophosphate and pyrethroid resistance in the fall armyworm *Spodoptera frugiperda*. PLoS ONE 8:e62268. https://doi.org/10.1371/journal.pone.0062268

Cingolani P, Platts A, Wang LL et al (2012) A program for annotating and predicting the effects of single nucleotide polymorphisms,

SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. Fly (Austin) 6:80–92. https://doi.org/10.4161/fly.19695

Coop G, Witonsky D, Di Rienzo A, Pritchard JK (2010) Using environmental correlations to identify loci underlying local adaptation. Genetics 185:1411–1423. https://doi.org/10.1534/genetics.110.114819

Cruz I (1995) Manejo Integrado de pragas de milho com ênfase para o controle biológico. http://ainfo.cnptia.embrapa.br/digital/bitstream/item/43838/1/Manejo-integrado-2.pdf. Accessed 11 November 2020

Daborn PJ, Yen JL, Bogwitz MR et al (2002) A single P450 allele associated with insecticide resistance in *Drosophila*. Science 297:2253–2256. https://doi.org/10.1126/science.1074170

Danecek P, Auton A, Abecasis G et al (2011) The variant call format and VCFtools. Bioinformatics 27:2156–2158. https://doi.org/10.1093/bioinformatics/btr330

Dermauw W, Van Leeuwen T (2014) The ABC gene family in arthropods: comparative genomics and role ininsecticide transport and resistance. Insect Biochem Mol Biol 45:89–110. https://doi.org/10.1016/j.ibmb.2013.11.001

Douris V, Steinbach D, Panteleri R et al (2016) Resistance mutation conserved between insects and mites unravels the benzoylurea insecticide mode of action on chitin biosynthesis. Proc Natl Acad Sci USA 113:14692–14697. https://doi.org/10.1073/pnas.1618258113

Doyle J (1991) DNA Protocols for Plants. In: Hewitt GM, Johnston AWB, Young JPW (eds) Molecular Techniques in Taxonomy. NATO ASI Series(Series H: Cell Biology), vol 57. Springer, Berlin, pp 283–285. https://doi.org/10.1007/978-3-642-83962-7_18

Fahmy AR, Sinchaisri N, Miyata T (1991) Development of chlorfluazuron resistance and pattern of cross-resistance in the diamondback Moth, *Plutella xylostella*. J Pestic Sci 16:665–672. https://doi.org/10.1584/jpestics.16.665

Fang F, Wang W, Zhang D, et al (2015) The cuticle proteins: a putative role for deltamethrin resistance in *Culex pipiens pallens*. Parasitol Res 114. https://doi.org/https://doi.org/10.1007/s00436-015-4683-9

Farias JR, Horikoshi RJ, Santos AC, Omoto C (2014) Geographical and temporal variability in susceptibility to Cry1F toxin from *Bacillus thuringiensis* in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations in Brazil. J Econ Entomol 107:2182–2189. https://doi.org/10.1603/EC14190

Feyereisen R (2012) Insect CYP Genes and P450 Enzymes. Insect Mol Biol Biochem 236–316. https://doi.org/https://doi.org/10.1016/B978-0-12-384747-8.10008-X

Ffrench-Constant RH (2013) The molecular genetics of insecticide resistance. Genetics 194:807–815. https://doi.org/10.1534/genetics.112.141895

Finney DJ (1949) the Adjustment for a natural response rate in Probit analysis. Ann Appl Biol 36:187–195. https://doi.org/10.1111/j.1744-7348.1949.tb06408.x

Finney DJ (1971) Probit analysis, 3rd edn. Cambridge University Press, London

Fotakis EA, Mastrantonio V, Grigoraki L et al (2020) Identification and detection of a novel point mutation in the chitin synthase gene of *Culex pipiens* associated with diflubenzuron resistance. PLoS Negl Trop Dis 14:1–10. https://doi.org/10.1371/journal.pntd.0008284

Garrison E, Marth G (2012) Haplotype-based variant detection from short-read sequencing. arXiv Prepr arXiv12073907

Gautier M (2015) Genome-wide scan for adaptive divergence and association with population-specific covariates. Genetics 201:1555–1579. https://doi.org/10.1534/genetics.115.181453

Gazit Y, Ishaaya I, Perry AS (1989) Detoxification and synergism of diflubenzuron and chlorfluazuron in the red flour beetle *Tribolium castaneum*. Pestic Biochem Physiol 34:103–110. https://doi.org/10.1016/0048-3575(89)90147-8

Georghiou GP (1969) Genetics of resistance to insecticides in houseflies and mosquitoes. Exp Parasitol 26:224–255. https://doi.org/10.1016/0014-4894(69)90116-7

Goergen G, Kumar PL, Sankung SB, et al (2016) First report of outbreaks of the fall armyworm *Spodoptera frugiperda* (J E Smith) (Lepidoptera, Noctuidae), a new alien invasive pest in West and Central Africa. PLoS One 11. https://doi.org/https://doi.org/10.1371/journal.pone.0165632

Günther T, Coop G (2013) Robust identification of local adaptation from allele frequencies. Genetics 195:205–220. https://doi.org/10.1534/genetics.113.152462

Guyer W, Neumann R (1988) Activity and fate of chlorfluazuron and diflubenzuron in the larvae of Spodoptera littoralis and *Heliothis virescens*. Pestic Biochem Physiol 30:166–177. https://doi.org/10.1016/0048-3575(88)90050-8

Hardy D, Bill RM, Jawhari A, Rothnie AJ (2019) Functional expression of multidrug resistance protein 4 MRP4/ABCC4. SLAS Discov 24:1000–1008. https://doi.org/10.1177/2472555219867070

Hindorff LA, Sethupathy P, Junkins HA et al (2009) Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. Proc Natl Acad Sci U S A 106:9362–9367. https://doi.org/10.1073/pnas.0903103106

Hrdlickova B, de Almeida RC, Borek Z, Withoff S (2014) Genetic variation in the non-coding genome: Involvement of micro-RNAs and long non-coding RNAs in disease. Biochim Biophys Acta Mol Basis Dis 1842:1910–1922. https://doi.org/10.1016/j.bbadis.2014.03.011

Huang F, Buschman LL, Higgins RA, McGaughey WH (1999) Inheritance of resistance to *Bacillus thuringiensis* toxin (dipel ES) in the European corn borer. Science 284(80):965–967. https://doi.org/https://doi.org/10.1126/science.284.5416.965

Huang Y, Guo Q, Sun X, et al (2018) *Culex pipiens pallens* cuticular protein CPLCG5 participates in pyrethroid resistance by forming a rigid matrix. Parasit Vectors 11. https://doi.org/https://doi.org/10.1186/s13071-017-2567-9

Iqbal M, Wright DJ (1997) Evaluation of resistance, cross-resistance and synergism of abamectin and teflubenzuron in a multi-resistant field population of *Plutella xylostella* (Lepidoptera: Plutellidae). Bull Entomol Res 87:481–486. https://doi.org/10.1017/s0007485300041341

Ishaa Ya I, Klein M (1990) Response of susceptible laboratory and resistant field strains of *Spodoptera littoralis* (Lepidoptera: Noctuidae) to teflubenzuron. J Econ Entomol 83:59–62. https://doi.org/10.1093/jee/83.1.59

Ismail F, Wright DJ (1992) Synergism of teflubenzuron and chlorfluazuron in an acylurea-resistant field strain of *Plutella xylostella* L. (lepidoptera: Yponomeutidae). Pestic Sci 34:221–226. https://doi.org/10.1002/ps.2780340306

Jan S, Liu S, Hafeez M, et al (2017) Isolation and functional identification of three cuticle protein genes during metamorphosis of the beet armyworm, *Spodoptera exigua*. Sci Rep 7. https://doi.org/https://doi.org/10.1038/s41598-017-16435-w

Kasten P Jr, Precetti AACM, Parra JRP (1978) Dados biológicos comparativos de *Spodoptera frugiperda* em duas dietas artificiais e substrato natural. Rev Agric 53:68–78. https://doi.org/10.37856/bja.v53i1-2.3948

Kofler R, Orozco-terWengel P, de Maio N et al (2011) Popoolation: A toolbox for population genetic analysis of next generation sequencing data from pooled individuals. PLoS ONE 6:e15925. https://doi.org/10.1371/journal.pone.0015925

Labbé R, Caveney S, Donly C (2011) Genetic analysis of the xenobiotic resistance-associated ABC gene subfamilies of the Lepidoptera. Insect Mol Biol 20:243–256. https://doi.org/10.1111/j.1365-2583.2010.01064.x

Lenormand T, Raymond M (1998) Resistance management: the stable zone strategy. Proc R Soc B Biol Sci 265:1985–1990. https://doi.org/10.1098/rspb.1998.0529

Li H (2011) A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics 27:2987–2993. https://doi.org/10.1093/bioinformatics/btr509

Li H, Durbin R (2010) Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26:589–595. https://doi.org/10.1093/bioinformatics/btp698

Li T, Cao C, Yang T et al (2015) A G-protein-coupled receptor regulation pathway in cytochrome P450-mediated permethrin-resistance in mosquitoes, *Culex quinquefasciatus*. Sci Rep 5:1–13. https://doi.org/10.1038/srep17772

Lin JG, Hung CF, Sun CN (1989) Teflubenzuron resistance and microsomal monooxygenases in larvae of the diamondback moth. Pestic Biochem Physiol 35:20–25. https://doi.org/10.1016/0048-3575(89)90098-9

Lira EC, Bolzan A, Nascimento ARB et al (2020) Resistance of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to spinetoram: inheritance and cross-resistance to spinosad. Pest Manag Sci 76:2674–2680. https://doi.org/10.1002/ps.5812

Lucas ER, Rockett KA, Lynd A, et al (2019) A high throughput multilocus insecticide resistance marker panel for tracking resistance emergence and spread in *Anopheles gambiae*. Sci Rep. https://doi.org/https://doi.org/10.1038/s41598-019-49892-6

Ma Z, Zhang Y, You C, et al (2020) The role of G protein-coupled receptor-related genes in cytochrome P450-mediated resistance of the house fly, *Musca domestica* (Diptera: Muscidae), to imidacloprid. Insect Mol Biol 29. https://doi.org/https://doi.org/10.1111/imb.12615

MAPA (2020) Sistema de Agrotóxicos Fitossanitários. Ministério da Agricultura, Pecuária e Abastecimento. http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons. Accessed 11 November 2020

Merzendorfer H (2006) Insect chitin synthases: a review. J Comp Physiol B Biochem Syst Environ Physiol 176:1–15. https://doi.org/10.1007/s00360-005-0005-3

Merzendorfer H, Zimoch L (2003) Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J Exp Biol 206:4393–4412

Meyer F, Flötenmeyer M, Moussian B (2013) The sulfonylurea receptor Sur is dispensable for chitin synthesis in *Drosophila melanogaster* embryos. Pest Manag Sci 69:1136–1140. https://doi.org/10.1002/ps.3476

Müller H, Jimenez-Heredia R, Krolo A et al (2017) VCF.Filter: interactive prioritization of disease-linked genetic variants from sequencing data. Nucleic Acids Res 45:W567–W572. https://doi.org/10.1093/nar/gkx425

Nascimento ARB, Farias JR, Bernardi D et al (2016) Genetic basis of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) resistance to the chitin synthesis inhibitor lufenuron. Pest Manag Sci 72:810–815. https://doi.org/10.1002/ps.4057

Nascimento ARB, Fresia P, Cônsoli FL, Omoto C (2015) Comparative transcriptome analysis of lufenuron-resistant and susceptible strains of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). BMC Genomics 16. https://doi.org/10.1186/s12864-015-2183-z

Nicholson G, Smith AV, Jónsson F et al (2002) Assessing population differentiation and isolation from single-nucleotide polymorphism data. J R Stat Soc Ser B Stat Methodol 64:695–715. https://doi.org/10.1111/1467-9868.00357

Oberlander H, Silhacek DL (1998) Mode of action of insect growth regulators in lepidopteran tissue culture. Pestic Sci 54:300–302. https://doi.org/10.1002/(sici)1096-9063(1998110)54:3%3c300::aid-ps830%3e3.0.co;2-8

Okuma DM, Bernardi D, Horikoshi RJ et al (2018) Inheritance and fitness costs of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) resistance to spinosad in Brazil. Pest Manag Sci 74:1441–1448. https://doi.org/10.1002/ps.4829

Pandey A, Khatoon R, Saini S et al (2015) Efficacy of methuselah gene mutation toward tolerance of dichlorvos exposure in *Drosophila melanogaster*. Free Radic Biol Med 83:54–65. https://doi.org/10.1016/j.freeradbiomed.2015.02.025

Perng FS, Yao MC, Hung CF, Sun CN (1988) Teflubenzuron resistance in diamondback moth (Lepidoptera: Plutellidae). J Econ Entomol 81:1277–1282. https://doi.org/10.1093/jee/81.5.1277

Post LC, Vincent WR (1973) A new insecticide inhibits chitin synthesis. Naturwissenschaften 60:431–432. https://doi.org/10.1007/BF00623561

QingJun W, GuoRen Z, JianZhou Z, Xing Z, Xiwu G (1997) Resistance selection of *Plutella xylostella* (L.) by chlorfluazuron and patterns of cross-resistance. Acta Entomol Sin 41:34–41

Ricaño-Ponce I, Wijmenga C (2013) Mapping of immune-mediated disease genes. Annu Rev Genomics Hum Genet 14:325–353. https://doi.org/10.1146/annurev-genom-091212-153450

Robertson JL, Savin NE, Savin NE, Preisler HK (2007) Bioassays with arthropods, 2nd ed. CRC Press, Boca Raton. https://doi.org/10.1201/9781420004045

Roush RT, Daly JC (1990) The role of population genetics in resistance research and management. Pestic Resist Arthropods 97–152. https://doi.org/https://doi.org/10.1007/978-1-4684-6429-0_5

Santos WJ (2007) Manejo das pragas do algodão com destaque para o cerrado brasileiro. In: Freire EC (ed) Algodão no cerrado do Brasil. ABAPA, Brasilia, p 403–478

Sauphanor B, Bouvier JC (1995) Cross-resistance between benzoylureas and benzoylhydrazines in the codling moth, *Cydia pomonella* L. Pestic Sci. https://doi.org/https://doi.org/10.1002/ps.2780450412

Sauphanor B, Bouvier JC, Brosse V (1998) Spectrum of insecticide resistance in *Cydia pomonella* (Lepidoptera: Tortricidae) in Southeastern France. J Econ Entomol. https://doi.org/10.1093/jee/91.6.1225

Sauphanor B, Brosse V, Bouvier JC, et al (2000) Monitoring resistance to diflubenzuron and deltamethrin in French codling moth populations (*Cydia pomonella*). Pest Manag Sci. https://doi.org/https://doi.org/10.1002/(SICI)1526-4998(200001)56:1<74::AID-PS96>3.0.CO;2-C

Schmidt FB (2002) Linha básica de suscetibilidade de *Spodoptera frugiperda* (Lepidoptera: Noctuidae) a lufenuron na cultura do milho. Dissertação, Escola Superior de Agricultura Luiz de Queiroz: Universidade de São Paulo. https://doi.org/10.11606/D.11.2002.tde-04112002-171009

Sokal RR, Rohlf F (1969) Biometry. The principles and practice of statistics in biological research. Freeman and Co., San Francisco

Sonoda S, Tsumuki H (2005) Studies on glutathione S-transferase gene involved in chlorfluazuron resistance of the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). Pestic Biochem Physiol 82:94–101. https://doi.org/10.1016/j.pestbp.2005.01.003

Stará J, Kocourek F (2007) Insecticidal resistance and cross-resistance in populations of *Cydia pomonella* (Lepidoptera: Tortricidae) in Central Europe. J Econ Entomol. https://doi.org/https://doi.org/10.1603/0022-0493(2007)100[1587:IRACIP]2.0.CO;2

Stone BF (1968) A formula for determining degree of dominance in cases of monofactorial inheritance of resistance to chemicals. Bull World Health Organ 38:325–326. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2554319/. Accessed 12 June 2020

Suzuki Y, Shiotsuki T, Jouraku A et al (2017) Benzoylurea resistance in western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae): the presence of a point mutation in chitin synthase 1. J Pestic Sci 42:93–96. https://doi.org/10.1584/jpestics.D17-023

Tsukamoto M (1983) Methods of genetic analysis of insecticide resistance. Pest Resist to Pestic 71–98. https://doi.org/https://doi.org/10.1007/978-1-4684-4466-7_3

van Daalen JJ, Meltzer J, Mulder R, Wellinga K (1972) A selective insecticide with a novel mode of action. Naturwissenschaften 59:312–313. https://doi.org/10.1007/BF00593360

Van Leeuwen T, Demaeght P, Osborne EJ et al (2012) Population bulk segregant mapping uncovers resistance mutations and the mode of action of a chitin synthesis inhibitor in arthropods. Proc Natl Acad Sci USA 109:4407–4412. https://doi.org/10.1073/pnas.1200068109

Wang X, Chen Y, Gong C et al (2018) Molecular identification of four novel cytochrome P450 genes related to the development of resistance of *Spodoptera exigua* (Lepidoptera: Noctuidae) to chlorantraniliprole. Pest Manag Sci 74:1938–1952. https://doi.org/10.1002/ps.4898

Wilson TG, Cain JW (1997) Resistance to the insecticides lufenuron and propoxur in natural populations of *Drosophila melanogaster* (Diptera: Drosophilidae). J Econ Entomol 90:1131–1136. https://doi.org/10.1093/jee/90.5.1131

Yu SJ (2015) The Toxicology and Biochemistry of Insecticides, 2nd ed. CRC Press, Boca Raton. https://doi.org/10.1201/b18164

Zimmer CT, Garrood WT, Singh KS, et al (2018) Neofunctionalization of duplicated p450 genes drives the evolution of insecticide resistance in the brown planthopper. Curr Biol. https://doi.org/10.1016/j.cub.2017.11.060