



# Multiple acaricide resistance and underlying mechanisms in *Tetranychus urticae* on hops

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## Abstract

The polyphagous pest *Tetranychus urticae* feeds on over 1100 plant species including highly valued economic crops such as hops (*Humulus lupulus*). In the key hop production region of the Pacific Northwest of the USA, *T. urticae* is one of the major arthropod pests. Over the years, *T. urticae* control has been dominated by the application of various acaricides. However, *T. urticae* quickly adapts to these acaricides by developing resistance. Here, we determined resistance ratios of *T. urticae* populations in hops to three acaricides: etoxazole, fenpyroximate, and spiroticlofen. The mechanisms underlying resistance to these and three other acaricides were investigated in 37 field-collected *T. urticae* populations using a comprehensive diagnostic approach. Our data showed that *T. urticae* populations exhibited complex adaptation patterns to acaricides. Resistance to abamectin, fenpyroximate, and spiroticlofen by enhanced target metabolic detoxification gene(s) was identified in 100%, 50%, and 20% of populations tested, respectively. Resistance to bifenthrin, bifenazate, and etoxazole by target site insensitivity was pervasive among tested populations. Our study provides new information in understanding the complexity of *T. urticae* adaptation to multiple acaricides, which will help in designing sustainable pest control strategies for *T. urticae* on hops and other economically valuable crops.

**Keywords** Acaricide resistance · Resistance ratio · Molecular markers · Target site insensitivity · Metabolic detoxification

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## Key message

1. IPM-compatible acaricides are mainstays for sustainable *Tetranychus urticae* management in crop systems, while resistance to these acaricides threatens effective control practices.
2. We developed a comprehensive strategy to examine phenotypic status and underlying mechanisms of adaptation to acaricides with molecular markers.
3. Our study revealed many *T. urticae* populations collected from hopyards exhibited evidence of resistance to multiple acaricides.
4. This robust proactive diagnostic tactic can help prevent the loss of effectiveness of acaricides in mite control.

## Introduction

Phytophagous mites pose a threat to global agricultural production through direct feeding or transmission of pathogens and viruses (Van Leeuwen et al. 2015). The two-spotted spider mite, *Tetranychus urticae* Koch is a ubiquitous agricultural pest of many major crops, including cotton, soybean, maize, vegetables, fruits, and ornamentals as well as numerous specialty crops such as hops (Grbic et al. 2011; Piraneo et al. 2015; Van Leeuwen et al. 2015). *T. urticae* has been reported to be one of the major arthropod pests in Pacific Northwest (PNW) hop fields, where it represents above 99% of the US hop acreage (O'Neal et al. 2015; Piraneo et al. 2015). The US hop industry is increasing in value annually, and it was estimated to be worth over \$500 million in 2016 (USDA NASS). Although various strategies such as biocontrol using natural enemies, cultural control and population monitoring have been adopted for *T. urticae* management (McMurtry and Croft 1997; O'Neal et al. 2015), the use of acaricides remains essential to prevent mite populations from reaching economic thresholds (O'Neal et al. 2015). Unfortunately, *T. urticae* has been documented to quickly develop resistance to all acaricides used for its control, irrespective of mode of action (Van Leeuwen et al. 2013). Development of acaricide resistance will lead to an even greater increase of acaricide application, raising the risk of chemical exposure to non-target beneficial arthropods and cause the complete failure of pest management strategies. Thus, there is a critical need to reveal the mechanisms underlying adaptation to acaricides, and to apply this knowledge for pest management practices in the field (Van Leeuwen et al. 2010; Ilias et al. 2014; Piraneo et al. 2015; Adesanya et al. 2018).

In urban and agro-ecosystems, rotation of multiple pesticides with diverse modes of action is a typical approach to reduce the development of pesticide resistance (Goodhue et al. 2011; Piraneo et al. 2015; Zhu et al. 2016a). According to spray records from PNW hopyards, the acaricides regularly used by growers to suppress *T. urticae* include abamectin (IRAC group 6), bifenthrin (IRAC group 3A), bifenazate (IRAC group 20D), hexythiazox and etoxazole (IRAC group 10A and 10B), fenpyroximate (IRAC group 21A), and the recently registered acaricides spiroticlofen (IRAC group 23), and acequinocyl (IRAC group 20B) (Piraneo et al. 2015) (Fig. S1). Possessing different modes of action, these chemicals are applied in rotation or combination during the hop growing season from May to August (Piraneo et al. 2015) (Fig. S1a). Our previous study investigated the resistance ratios (RRs) and mechanisms of resistance to abamectin, bifenazate, and bifenthrin in *T. urticae* populations collected from PNW

hopyards (Piraneo et al. 2015). However, the phenotypic and genotypic resistance status of *T. urticae* populations from hops to etoxazole, fenpyroximate, and spiroticlofen remains unknown.

Due to specific activities against spider mites compared to predatory mites and other non-target beneficial arthropods, etoxazole, fenpyroximate, and spiroticlofen have been used as valuable IPM tools for *T. urticae* management on hops and other crops (Kim et al. 2005). Etoxazole belongs to a class of acaricides known as mite growth inhibitors (MGIs), which inhibit chitin deposition during embryonic and immature stages of mites (Van Leeuwen et al. 2012; Demaeght et al. 2014). MGIs are highly specific against spider mites and show low or no toxicity to beneficial arthropods and vertebrates (Adesanya et al. 2018). The primary target site of MGIs is the chitin synthase 1 (*CHS 1*) (Van Leeuwen et al. 2012). Fenpyroximate is a mitochondrial electron transport inhibitor (METI), with the mode of action in inhibition of complex I of the respiratory chain at the ubiquinone site of NADH oxidoreductase (referred as METI-I) (Bajda et al. 2017). Fenpyroximate has a broad-spectrum activity against all life stages of many mite species but is relatively safe to beneficial insects and predatory mites (Motoba et al. 1992; Van Pottelberge et al. 2009). Spiroticlofen belongs to the group of spirocyclic tetrone acid derivatives (keto-enols) and targets on the acetyl-coenzyme A carboxylase (ACCase) in the lipid biosynthesis pathway (referred as LPI) (Van Leeuwen et al. 2009). Spiroticlofen is active against all developmental stages, but particularly against eggs, and shows high activity against female mites through decreasing their fecundity and fertility after tarsal contact (Nauen 2005).

Target site insensitivity is one of the most important mechanisms of acaricide resistance by *T. urticae* (Van Leeuwen et al. 2010; Van Leeuwen and Dermauw 2016). A non-synonymous mutation I1017F was identified in the non-catalytic domain of the *CHS 1* gene that is correlated with resistance to MGIs in *T. urticae* (Van Leeuwen et al. 2012; Demaeght et al. 2014). A recent study showed that the fenpyroximate resistance in *T. urticae* has been associated with a H92R mutation in the PSST homologue of complex I (Bajda et al. 2017). The cyclic keto-enol insecticide spirotetramat inhibits insect and spider mite ACCase by interfering with the carboxyltransferase partial reaction (Lümmen et al. 2014). However, so far there is no report of target site insensitivity associated with cyclic keto-enol insecticides resistance in *T. urticae*. In the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood), a glutamic acid substitution with lysine in position 645 (E645K) of the ACCase gene was linked to spiromesifen resistance (Karatolos et al. 2012). Two mutations, G323D and G326E were identified in the glutamate-gated chloride channel (*GluCl*) genes associated with abamectin resistance (Kwon et al. 2010a, b; Dermauw

et al. 2012). Studies reported that resistance to bifenthrin was tightly linked with mutations or mutation combinations in the mitochondrial cytochrome *b* (*cytb*) gene (Van Leeuwen et al. 2008; Van Nieuwenhuysse et al. 2009). Several amino acid substitutions in the voltage-gated sodium channel (*VGSC*) gene have been associated with pyrethroid resistance in *T. urticae* (Tsagkarakou et al. 2009; Kwon et al. 2010a, b). Besides target site insensitivity, enhanced metabolic detoxification by cytochrome P450s, glutathione S-transferases (GSTs), and esterases is another important mechanism responsible for resistance to many acaricides and insecticides (Feyereisen 1995; Stumpf and Nauen 2002; Liu et al. 2006; Demaeght et al. 2013; Zhu et al. 2013; Van Leeuwen and Dermauw 2016). For example, recent studies revealed that the P450 CYP392A16, and the GSTs TuGSTd10, TuGSTd14, and TuGSTm09 are associated with high levels of abamectin resistance in *T. urticae* (Riga et al. 2014; Pavlidi et al. 2015). Another P450, CYP392A11 was shown to be able to hydroxylate fenpyroximate (Riga et al. 2015). Functional characterization of CYP392E10 confirmed that it can metabolize spirotetramat and spirotetramat (Demaeght et al. 2013). The high level of spirotetramat resistance in *T. urticae* was also potentially linked with enhanced hydrolysis activity of the carboxylesterase CCE04 (Demaeght 2015). The mutations associated with acaricide resistance and enhanced expression of detoxification genes with known functions in acaricide metabolism potentially can be used as molecular markers for resistance monitoring in the field (Zhu et al. 2016a).

The current study examined the phenotypic resistance status within 37 field-collected *T. urticae* populations to three acaricides: etoxazole, fenpyroximate, and spirotetramat. Then, we extensively searched for any mutations responsible for resistance to abamectin, bifenthrin, bifenthrin, etoxazole, fenpyroximate, and spirotetramat among these populations. Lastly, we investigated the relative expression of several detoxification genes, including cytochrome P450s, GSTs and a carboxylesterase, that are associated with resistance to abamectin, fenpyroximate, and spirotetramat.

## Materials and methods

### Mites

The susceptible *T. urticae* population was initially collected from weeds in Montana in 1995 and has never been exposed to any pesticides (Piraneo et al. 2015; Morales et al. 2016; Adesanya et al. 2018). Thirty-seven *T. urticae* populations were collected from seven major locations of commercial hopyards in the Yakima Valley of Washington State from June to September, 2016 (Table S1). One population was collected from Grandview (46°15'13"N 119°54'36"W), nine

populations from Harrah (46°24'15"N 120°32'35"W), two populations each from Mabton (46°12'42"N 119°59'47"W) and Moxee (46°33'23"N 120°23'14"W), nine populations from Prosser (46°12'25"N 119°45'56"W), ten populations from Toppenish (46°22'44"N 120°18'43"W), and four populations from White\_swan (46°23'40"N 120°42'11"W) (Table S1). Hop's leaves from each hopyard were randomly sampled covering all the areas of the farm in an X-shaped pattern. Leaves were collected from the upper, middle, and lower parts of hop plants to ensure that each sample was representative of the actual mite population occurring in the hopyard. The leaves were kept in Ziploc® plastic bags in ice coolers and transported immediately to the laboratory, where the number of mites per leaf was counted under a dissecting light microscope. Mites were reared on lima bean plants (*Phaseolus lunatus* L.) for one generation under laboratory conditions (28 ± 2 °C, 70 ± 5 RH) and then used for subsequent experiments.

### Bioassays

Mites were subjected to leaf disk bioassays with three acaricides: etoxazole (Zeal®, 72 WP), fenpyroximate (Fujimite®, 5% SC), and spirotetramat (Envidor®, 240 g/L SC). Direct spray of chemicals on freshly laid eggs of *T. urticae* was found to be the most efficient bioassay method for assaying mite growth inhibitors (Adesanya et al. 2018) and was therefore used to calculate the toxicity of the MGI etoxazole. Bioassay arenas were created by placing a fresh lima bean leaf disk (2 cm in diameter) on water-saturated cotton in a clean petri dish. Six to eight gravid adult female mites were allowed to oviposit for 24 h. After oviposition, the adult female mites were removed. The fresh laid eggs were sprayed using a Potter precision spray tower (Burkard Manufacturing, Rickmansworth, Herts, UK) with 2 ml of etoxazole solution with varying concentrations, ranging from 0 (control with Millipore-filtered water only) to the field-recommended dose on hops, 300 ppm a.i. The Potter spray tower was calibrated to deliver 2.0 ± 0.1 mg/cm<sup>2</sup> of liquid under 1.1 kg/cm<sup>2</sup> of pressure. Each dose was replicated 3–5 times. After spraying, the total number of eggs laid was recorded using a light microscope. After approximately 5 days, the number of eggs that successfully hatched into larvae was counted. Female adults were used for the bioassay of fenpyroximate, as described by Piraneo et al. (2015). Larvae were used for the bioassay of spirotetramat, following the procedure similar as Van Pottelberge et al. (2009). Briefly, gravid adult female mites were allowed to lay eggs on fresh lima bean leaf disks (2 cm in diameter). The period of oviposition was less than 24 h to ensure synchronous hatching of eggs into larvae. The larvae (48 h after hatching) were subjected to varying doses of spirotetramat using the precision Potter spray system. After 48 h, the

mortality was scored for the sprayed adults or larvae. Mites were counted as dead if they did not respond to a gentle tap by a fine camel-hair brush. The dose–mortality response was adjusted to the control treatment using Abbot’s formula (Abbott 1925). Probit analysis was used to estimate  $LC_{50}$  values, slopes and 95% confidence intervals (POLO Probit 2014). The statistical comparison of  $LC_{50}$  values was performed according to non-overlapping of 95% CIs (Liu and Yue 2000).

### Detection of resistance-associated point mutations

gDNA was extracted from 100 to 150 individuals of susceptible and 36 field-collected *T. urticae* populations using the DNeasy Blood & Tissue kit (QIAGEN®). PCR were carried out in a ProFlex PCR system (Thermo Fisher Scientific, USA). Each PCR contained 1  $\mu$ L gDNA (100 ng/ $\mu$ L), 4  $\mu$ L PCR buffer (5 $\times$ ), 0.8  $\mu$ L dNTP mix (10 mM), 0.6  $\mu$ L forward and reverse primers (Table S5) for each target gene (stock 10  $\mu$ M), 0.8  $\mu$ L Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Pittsburgh, PA), and 12.8  $\mu$ L ddH<sub>2</sub>O. PCR was conducted under the following cycling parameters: 94 °C for 3 min 50 s, 35 cycles of 94 °C for 30 s, 50–58 °C for 30 s, and 72 °C for 1 min, with a final extension for 10 min at 72 °C. After the PCR, 2  $\mu$ L of the PCR product was run on agarose gels for evaluation of PCR product integrity. The raw PCR products were submitted to Functional Biosciences (Madison, WI) for purification and sequencing. The sequences obtained were analyzed using BioEdit 7.01 software (Ibis Biosciences, Carlsbad, CA). The presence or absence of mutations was determined by inspection of sequencing chromatographs. The frequency of resistance alleles was estimated by comparing the height of the wild-type and resistance allele peaks in chromatographs based on the methods in Bajda et al. (2017).

### RNA extraction, cDNA synthesis, and qRT-PCR

Total RNA was extracted from 250 female *T. urticae* adults with TRIZOL reagent (Invitrogen®) following the manufacturer’s protocol. Nanodrop spectrophotometer ND-2000 (Thermo Scientific, Pittsburgh, PA) was used to quantify and qualify the total RNA. The total RNA was treated with DNase I (Ambion Inc. Austin, TX) to remove the contaminating genomic DNA (gDNA). cDNA synthesis was then performed with M-MLV reverse transcriptase (Promega, Madison, WI) and 2  $\mu$ g of DNase I-treated total RNA as template. qRT-PCR was performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Each reaction contained 5  $\mu$ L iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.4  $\mu$ L forward and reverse gene specific primers (stock 10  $\mu$ M) (Table S5), 1.0  $\mu$ L cDNA, and 3.6  $\mu$ L ddH<sub>2</sub>O. The

optimized program used included an initial incubation of 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 30 s, followed by a final melting curve cycle of 95 °C for 10 s, 65 °C for 5 s, and 95 °C for 10 s. The reactions were set up in a 96-well full-skirted PCR plate (USA Scientific, Ocala, FL) with three technical replicates and at least three biological replicates. Three negative (no-template) controls were included in each plate. Two previously identified most stable housekeeping genes, *CycA* and *Rp49*, were used as the reference genes (Morales et al. 2016). Both PCR efficiency and correlation coefficient ( $R^2$ ) values were taken into consideration in evaluating the primers. Primer pairs with PCR efficiency higher than 94% or lower than 110% and  $R^2$  values > 0.99 were considered as qualified for further analysis. The  $2^{-\Delta\Delta CT}$  method was used to calculate the relative gene expression (Huggett et al. 2005). Student’s *t* test was used to test for significant difference in gene expression between susceptible and field-collected *T. urticae* populations.

## Results

### Phenotypic resistance levels of *T. urticae* populations on PNW hops

The toxicities of 3 registered acaricides, etoxazole, fenpyroximate, and spiroticlofen, were assessed on 31, 28, and 21 of total 37 field-collected *T. urticae* populations, respectively. The  $LC_{50}$  of a susceptible *T. urticae* population to etoxazole was 0.65 ppm a.i. (Table 1). The  $LC_{50}$ s of field *T. urticae* populations ranged from 16.0 to 217.4 ppm a.i., which are below the field dose of etoxazole (300 ppm a.i.). The RRs of the field populations to etoxazole ranged from 24.72 to 336.59 (Table 1). Based on RRs, 52% and 48% of the tested populations had moderate ( $100 > RR \geq 10$ ) and high ( $RR \geq 100$ ) resistance to etoxazole, respectively (Fig. 1). Three hopyards, Harrah\_1, Toppenish\_6, and Toppenish\_7, were sampled twice during the hops growing season (Table S1). The RR of Harrah\_1 increased slightly from 120.71 to 122.23 (Table 1). The RR of Toppenish\_6 decreased slightly from 120.09 to 112.69, while that of Toppenish\_7 increased 1.7-fold in the 2 months (Table 1; Table S1). In the etoxazole bioassay, the mortality at field dose ranged from 63.2% to 86.5%. The highest resistance level to etoxazole was recorded in the sample collected from Prosser\_1 in 2016 ( $RR = 336.59$ ) (Table 1). However, no Prosser population collected from 2015 showed high levels of etoxazole resistance (Adesanya et al. 2018).

The  $LC_{50}$  of the susceptible *T. urticae* population to fenpyroximate was 9.5 ppm a.i. (Table 2). The  $LC_{50}$ s of field *T. urticae* populations to fenpyroximate ranged from 25.0 to 228.5 ppm a.i.  $LC_{50}$ s in two populations, Toppenish\_2

**Table 1** Etoxazole toxicity in susceptible and field-collected *T. urticae* populations

Population	% Mortality <sup>a</sup>	N	LC <sub>50</sub> (ppm a.i.)	95% CI	Slope ± SEM	χ <sup>2</sup>	df	RR <sup>b</sup>
Susceptible	100.0	701	0.65	0.6–0.7	2.69 ± 0.21	9.74	21	1.00
Harrah_1	66.7	510	78.0	45.5–138.0	0.65 ± 0.09	13.19	18	120.71
Harrah_1_1	69.4	447	79.0	49.4–114.9	0.63 ± 0.08	9.60	18	122.23
Harrah_2	69.3	661	140.0	104.5–202.2	2.28 ± 0.26	44.50	18	216.72
Harrah_3	75.2	523	61.0	41.1–89.4	0.93 ± 0.12	4.10	18	94.46
Harrah_4	74.1	498	85.1	58.6–124.6	1.10 ± 0.15	7.30	13	131.73
Harrah_5	79.7	564	66.5	44.1–93.9	0.88 ± 0.12	18.17	18	102.94
Harrah_6	77.9	540	19.7	11.1–40.8	0.77 ± 0.12	39.25	18	30.50
Harrah_7	85.6	293	29.7	15.4–53.5	0.73 ± 0.07	10.95	13	45.90
Mabton_1	80.8	903	31.1	22.2–42.7	0.77 ± 0.06	15.33	18	48.07
Mabton_2	65.0	420	126.3	77.4–219.5	0.84 ± 0.15	2.46	13	195.43
Moxee_1	82.5	577	45.4	16.8–332.9	0.46 ± 0.06	46.34	13	70.29
Moxee_2	81.4	640	50.2	29.8–88.7	0.62 ± 0.07	20.07	18	77.65
Prosser_1	63.2	490	217.4	134.5–435.8	0.85 ± 0.14	8.46	18	336.59
Prosser_2	68.2	314	66.9	34.3–191.6	0.56 ± 0.10	7.60	13	103.61
Prosser_3	84.0	451	60.5	32.5–107.9	0.85 ± 0.13	17.76	13	93.61
Prosser_4	86.5	275	72.9	50.1–110.1	3.00 ± 0.49	26.83	13	112.83
Prosser_6	76.4	421	30.1	19.9–45.2	0.82 ± 0.09	15.28	18	46.63
Prosser_7	83.4	567	16.0	9.8–26.2	0.55 ± 0.06	15.10	18	24.72
Prosser_8	74.2	778	21.7	10.5–45.9	0.44 ± 0.05	25.89	18	33.56
Prosser_9	81.1	364	25.5	15.6–41.0	0.73 ± 0.08	4.20	13	39.54
Toppenish_1	66.2	309	34.4	11.1–92.3	0.67 ± 0.10	30.19	13	53.31
Toppenish_2	82.9	489	34.5	17.0–57.8	1.04 ± 0.12	29.55	18	53.42
Toppenish_3	67.3	452	90.7	27.6–198.4	0.40 ± 0.06	34.18	18	140.42
Toppensih_6	77.0	676	77.6	33.2–218.8	0.72 ± 0.11	46.82	18	120.09
Toppensih_6_1	75.1	403	72.8	38.7–149.2	0.64 ± 0.10	10.92	13	112.69
Toppenish_7	70.4	656	44.7	28.4–74.0	0.64 ± 0.06	20.16	18	69.24
Toppenish_7_1	72.5	427	75.2	47.8–127.0	0.83 ± 0.13	7.70	13	116.47
White_swan_1	67.6	622	92.6	64.9–144.3	0.67 ± 0.09	12.50	18	143.27
White_swan_2	66.1	505	89.1	45.0–275.5	0.38 ± 0.06	11.26	18	137.94
White_swan_3	67.1	573	95.0	64.6–162.9	0.66 ± 0.07	24.14	20	147.04
White_swan_4	72.6	368	86.2	57.0–137.3	0.94 ± 0.15	9.50	13	133.50

<sup>a</sup>% Mortality stands for the % mortality at field dose of etoxazole, which is 300 ppm a.i

<sup>b</sup>RR represents resistance ratio = LC<sub>50</sub> of field population/LC<sub>50</sub> of susceptible population

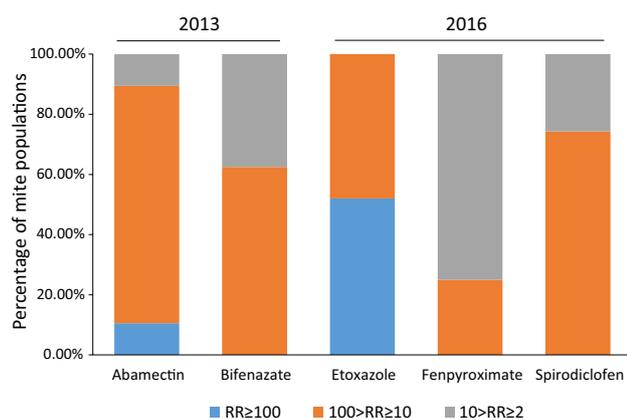
and Toppenish\_3, are above the field dose of fenpyroximate (180 ppm a.i.), suggesting the acaricide tested tends to lack general efficiency in these populations. Mortality at the field dose of fenpyroximate varied from 52% to 100%, though 100% mortality of mites was only observed in the Prosser\_7 population (Table 2). The RRs of field populations ranged from 2.63 to 24.05 (Table 2). Based on RRs, 75% and 25% of the populations had low and moderate resistance, respectively (Fig. 1). The highest resistance level to fenpyroximate was recorded in the samples collected from Toppenish\_3 (RR = 24.05) (Table 2).

The LC<sub>50</sub> of the susceptible *T. urticae* population to spirodiclofen was 1.9 ppm a.i. (Table 3). The LC<sub>50</sub>s of field *T. urticae* populations ranged from 6.0 to 83.8 ppm a.i., which are below the field dose of spirodiclofen (300 ppm a.i.)

Mortality at field dose ranged from 71% to 100%. One hundred percent mortality of mites at field dose of spirodiclofen was observed in the Mabton\_1 and Prosser\_7 populations. The RRs of field *T. urticae* populations to spirodiclofen varied from 3.16 to 44.11 (Table 3). Based on RRs, low and moderate levels of resistance were observed in 25.7% and 74.3% of tested populations, respectively (Fig. 1). The highest resistance level to spirodiclofen was recorded in the samples collected from Harrah\_2 (RR = 44.11) (Table 3).

### Distribution pattern of resistance-associated mutations in *T. urticae* populations from hops

The presence of 19 resistance-associated mutations in six acaricide target genes, *GluC11* and *GluC13* (targets of



**Fig. 1** Proportion of field-collected *T. urticae* populations with different resistance ratio (RR) of resistance in 2013 (Piraneo et al. 2015) and 2016. Low level of resistance,  $10 > RR \geq 2$ ; moderate resistance,  $100 > RR \geq 10$ ; high level of resistance,  $RR \geq 100$  (Piraneo et al. 2015)

abamectin), voltage-gated sodium channel (*VGSC*, target of bifenthrin), cytochrome *b* (*cytb*, target of bifenazate), *CHS 1* (target of etoxazole), *PSST* (target of fenpyroximate), and *ACCase* (target of spirodiclofen) in 36 *T. urticae* populations was evaluated by diagnostic PCR and sequencing analysis (Table S2). Populations were genotypically scored as wild-type, resistant, or mixed at each gene locus based on the sequencing chromatographs. The allele frequency of each resistant allele was also estimated by inspection of chromatographs (Tables S3 and S4). In total, five mutations were detected: M918L, F1534S, and F1538I in *VGSC*; G126S in *cytb*; and I1017F in *CHS 1* (Fig. 2). There were no mutations found in *GluCl1* and *GluCl3*, *PSST*, and *ACCase* (Table S4), indicating that target site insensitivity may not be important in *T. urticae* resistance to abamectin, fenpyroximate, or spirodiclofen in hopyards.

We sequenced three *VGSC* fragments containing regions of domain II, domain II-III interlinker, and domain III, and

**Table 2** Fenpyroximate toxicity in susceptible and field-collected *T. urticae* populations

Population	% Mortality <sup>a</sup>	<i>N</i>	LC <sub>50</sub> (ppm a.i.)	95% CI	Slope ± SEM	$\chi^2$	<i>df</i>	RR <sup>b</sup>
Susceptible	100.0	445	9.5	4.7–16.6	0.81 ± 0.14	27.97	32	1.00
Harrah_1	74.2	199	68.1	32.8–200.8	0.84 ± 0.25	2.70	13	7.17
Harrah_2	70.6	213	89.3	36.4–640.0	0.48 ± 0.15	6.10	13	9.40
Harrah_3	78.4	223	39.4	15.5–151.0	0.47 ± 0.13	12.20	13	4.15
Harrah_5	66.7	174	154.0	63.7–474.2	0.83 ± 0.20	6.00	13	16.21
Harrah_6	77.9	171	94.6	48.8–227.0	0.77 ± 0.20	11.20	13	9.96
Harrah_7	62.0	149	105.8	52.4–279.4	0.76 ± 0.20	1.60	13	11.14
Mabton_1	52.0	230	164.0	75.0–572.4	0.60 ± 0.20	1.60	13	17.26
Mabton_2	56.7	207	85.4	48.8–215.1	0.82 ± 0.18	3.18	13	8.99
Moxee_1	67.9	245	105.7	61.3–312.6	0.74 ± 0.14	12.43	13	11.13
Moxee_2	69.0	192	84.0	53.5–153.0	1.10 ± 0.20	9.50	13	8.84
Prosser_1	63.4	207	25.0	16.2–87.6	1.30 ± 0.46	1.80	13	2.63
Prosser_2	85.0	164	77.8	41.0–199.5	1.10 ± 0.23	17.10	13	8.19
Prosser_3	90.0	196	48.5	29.6–84.8	0.98 ± 0.17	10.00	13	5.11
Prosser_4	95.8	208	42.9	20.9–88.6	1.10 ± 0.18	24.60	13	4.52
Prosser_5	96.6	192	95.0	40.8–409.0	0.65 ± 0.21	3.60	13	10.00
Prosser_6	70.9	207	69.6	33.1–170.8	0.60 ± 0.17	7.30	13	7.33
Prosser_7	100.0	221	33.8	15.9–45.7	1.95 ± 0.46	14.00	14	3.56
Prosser_8	97.0	200	50.4	26.8–98.2	1.30 ± 0.19	24.00	13	5.31
Prosser_9	60.0	219	90.0	48.4–266.4	0.74 ± 0.16	3.60	13	9.47
Toppenish_1	65.7	229	90.7	48.6–255.7	0.68 ± 0.16	2.42	13	9.54
Toppenish_2	53.7	234	187.0	70.8–250.5	0.47 ± 0.14	3.50	13	19.68
Toppenish_3	59.0	179	228.5	125.8–913.7	1.19 ± 0.41	5.80	14	24.05
Toppenish_4	60.0	225	113.4	62.7–281.3	0.78 ± 0.16	2.62	13	11.94
Toppensih_6	69.0	191	90.8	40.9–256.1	1.20 ± 0.21	35.30	13	9.56
Toppensih_7	60.0	199	159.7	90.4–465.8	0.93 ± 0.20	4.60	13	16.81
White_swan_1	61.3	178	89.6	42.0–334.7	0.64 ± 0.18	4.00	13	9.43
White_swan_2	76.2	193	67.8	29.8–288.3	0.60 ± 0.16	7.00	13	7.14
White_swan_3	69.1	166	112.4	79.4–242.8	2.10 ± 0.60	3.00	13	11.83

<sup>a</sup>% Mortality stands for the % mortality at field dose of fenpyroximate, which is 180 ppm a.i

<sup>b</sup>RR represents resistance ratio = LC<sub>50</sub> of field population/LC<sub>50</sub> of susceptible population

**Table 3** Spirodiclofen toxicity in susceptible and field-collected *T. urticae* populations

Population	% Mortality <sup>a</sup>	N	LC <sub>50</sub> (ppm a.i.)	95% CI	Slope ± SEM	$\chi^2$	df	RR <sup>b</sup>
Susceptible	100.0	524	1.9	1.5–2.4	1.65 ± 0.16	6.20	22	1.00
Harrah_1	95.0	495	21.3	12.6–36.7	1.30 ± 0.08	55.70	18	11.21
Harrah_2	73.0	382	83.1	52.2–146.6	0.95 ± 0.10	20.80	17	43.74
Harrah_5	80.0	723	83.8	47.5–168.7	0.89 ± 0.60	77.30	22	44.11
Harrah_6	97.0	309	17.8	9.5–39.3	1.00 ± 0.10	32.30	16	9.37
Harrah_7	89.0	373	30.3	19.5–50.0	1.00 ± 0.08	19.70	16	15.95
Mabton_1	100.0	626	16.5	13.0–20.5	1.40 ± 0.09	14.10	17	8.68
Mabton_2	71.0	374	40.4	25.2–68.0	0.70 ± 0.07	9.40	16	21.26
Moxee_2	77.0	608	45.3	32.4–68.4	0.97 ± 0.07	10.40	16	23.84
Prosser_5	90.0	290	19.5	12.4–32.2	0.90 ± 0.09	10.40	16	10.26
Prosser_6	79.0	444	55.4	35.4–94.3	0.90 ± 0.08	25.10	20	29.16
Prosser_7	100.0	320	6.0	3.70–9.40	1.30 ± 0.11	20.90	16	3.16
Prosser_8	94.5	579	61.2	31.4–143.0	1.10 ± 0.08	87.10	17	32.21
Prosser_9	84.4	262	74.5	40.3–170.0	0.83 ± 0.10	13.40	16	39.21
Toppenish_3	95.0	360	17.2	11.10–26.3	1.10 ± 0.09	23.20	18	9.05
Toppenish_4	92.0	330	36.8	23.5–59.7	1.00 ± 0.09	19.90	17	19.37
Toppenish_6	92.3	459	30.3	19.3–49.8	0.80 ± 0.08	21.50	16	15.95
Toppenish_7	97.0	268	24.4	13.3–42.8	0.90 ± 0.09	20.40	16	12.84
White_swan_1	98.0	525	20.9	13.0–34.0	1.00 ± 0.07	49.00	22	11.00
White_swan_2	97.0	296	19.1	12.4–29.4	1.10 ± 0.10	17.40	16	10.05
White_swan_3	97.0	379	10.0	7.1–14.0	1.20 ± 0.08	8.50	18	5.26
White_swan_4	90.0	306	24.5	17.3–35.6	1.20 ± 0.11	14.60	16	12.90

<sup>a</sup>% Mortality stands for the %mortality at field rate of spiroadiclofen, which is 300 ppm a.i

<sup>b</sup>RR represents resistance ratio = LC<sub>50</sub> of field population/LC<sub>50</sub> of susceptible population

evaluated nine candidate pyrethroid resistance-associated loci (Table S2). Three mutations, M918L, F1534S, and F1538I were detected in 25% (9 populations), 28% (10 populations) and 83% (30 populations) of the 36 *T. urticae* populations collected from hops, respectively (Fig. 2; Table S3).

We also looked for 5 bifenthrin resistance-associated mutations (G126S, I136T, S141F, D161G, and P262T) in the mitochondrial *Cyt b* gene by performing PCR and sequencing an 828 bp fragment of the *T. urticae* *cyt b* gene (Table S2). The G126S mutation was detected in 72% (26) of 36 populations, which all were a mix of the resistant and susceptible alleles (Fig. 2; Table S4). The G126S mutation alone only results in low to moderate bifenthrin resistance and does not contribute to cross-resistance between bifenthrin and acequinocyl (Van Leeuwen et al. 2008; Van Nieuwenhuysse et al. 2009). Other *cyt b* mutations or mutation combinations, such as P262T, G126S + I136T, and G126S + S141F, that have been reported to confer high levels of bifenthrin resistance in *T. urticae* (Van Leeuwen et al. 2008; Van Nieuwenhuysse et al. 2009) were not observed in any of *T. urticae* populations that we tested.

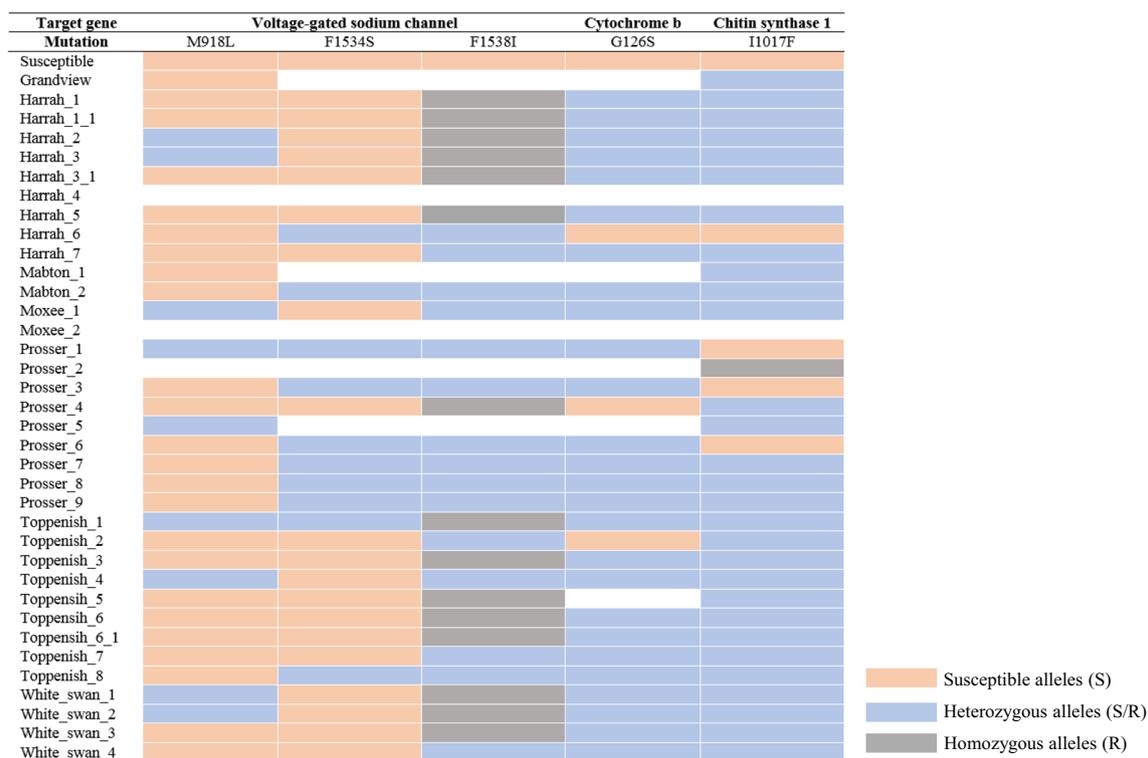
Resistance to MGIs (etoxazole, hexythiazox, and clofentezine) has been previously linked to a single non-synonymous mutation (I1017F) in the *CHS 1* gene (Van Leeuwen et al. 2012; Demaeght et al. 2014). Our initial survey

of four *T. urticae* populations collected from hops in 2015 found that this mutation was detected in one of these populations (Adesanya et al. 2018). Here, we found the I1017F mutation in 83% (30) of the 36 *T. urticae* populations we examined (Fig. 2).

### Expression pattern of resistance-associated metabolic genes

Recent functional studies have revealed that several P450s, GSTs and one esterase contribute resistance to abamectin (Riga et al. 2014; Pavlidi et al. 2015), fenpyroximate (Riga et al. 2015), and spiroadiclofen (Demaeght et al. 2013) in *T. urticae* (Table 4). These genes can serve as molecular markers to evaluate the acaricide resistance in *T. urticae* populations on hops. Thus, we randomly chose one to two population(s) from each of the hop production districts used in this study [Harrah\_3, Harrah\_8, Mabton\_2, Moxee\_1, Prosser\_1, Prosser\_9, Toppenish\_7, Toppenish\_7\_1, White\_swan\_1, and White\_swan\_4 (Table S1)] to examine transcription levels of these genes as possible mediators of acaricide resistance.

Overexpression of *CYP392A16*, two TuGSTs of the delta class (*TuGSTd10* and *TuGSTd14*), and one of the mu class (*TuGSTm09*) have been highly associated with abamectin



**Fig. 2** Multiple resistance-associated mutations on target site genes identified from *T. urticae* populations collected from hopyards. Pink fill indicates susceptible allele (S), gray fill indicates the resistant

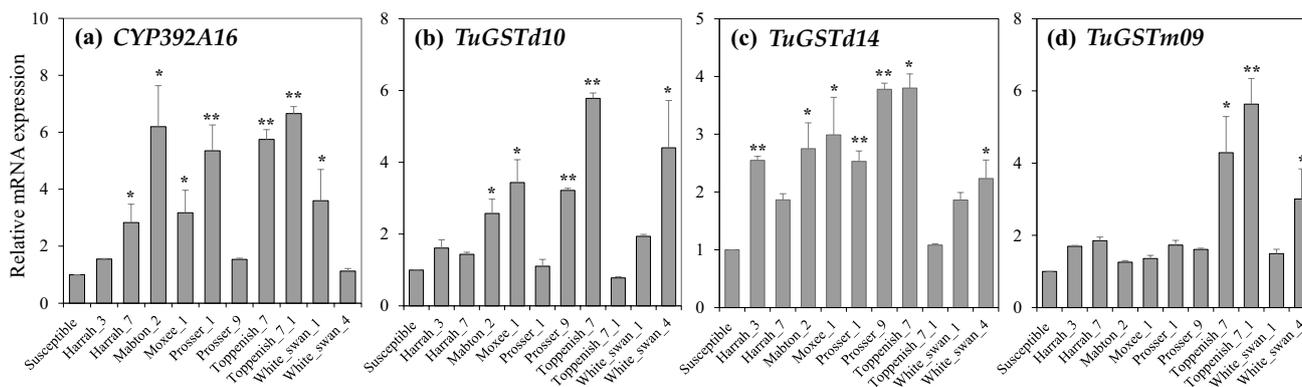
allele (R), and blue fill indicates both alleles were detected in the population (S/R). No fill indicates no data available. (Color figure online)

**Table 4** Genotypic resistance of nine *T. urticae* populations in hopyards to multiple acaricides

Populations	Acaricides						Number of acaricides resistant to
	Abamectin	Fenpyroximate	Spirodiclofen	Bifenthrin	Bifenazate	Etoxazole	
Susceptible	S <sup>a</sup>	S	S	S	S	S	0
Harrah_3	R <sup>b</sup>	S	S	R	R	R	4
Harrah_7	R	S	S	R	R	R	4
Mabton_2	R	S	S	R	R	R	4
Moxee_1	R	R	S	R	R	R	5
Prosser_1	R	R	S	R	R	R	5
Prosser_9	R	S	R	R	R	R	5
Toppenish_7	R	R	R	R	R	R	6
White_swan_1	R	R	S	R	R	R	5
White_swan_4	R	S	S	R	R	R	4
Molecular markers used in hopyards	<i>CYP392A16</i> , <i>TuGSTd10</i> , <i>TuGSTd14</i> , <i>TuGSTm09</i>	<i>CYP392A11</i>	<i>CYP392E10</i> , <i>CCE04</i>	F1538I M918L + F1538I F1534S + F1538I M918L + F1534S + F1538I	G126S	I1017F	Average = 4.7
Resistance mechanisms	Enhanced metabolic detoxification			Target site insensitivity			

<sup>a</sup>S stands for the susceptible genotype

<sup>b</sup>R stands for the resistant genotype



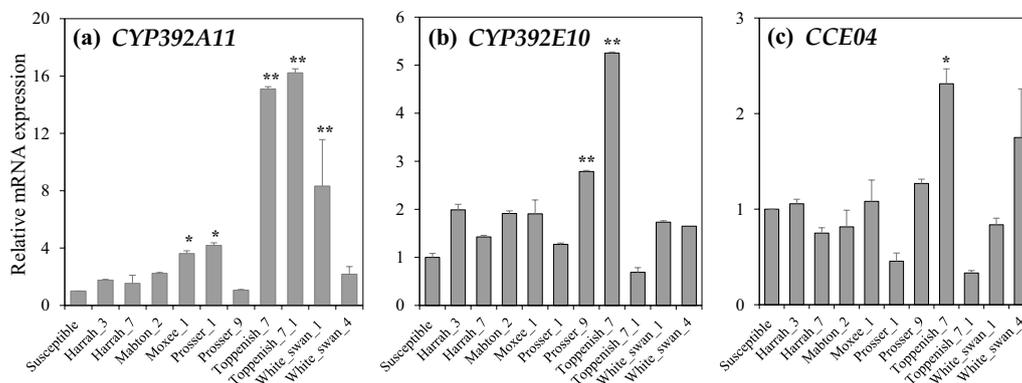
**Fig. 3** Relative expression of abamectin resistance-associated genes **a** *CYP392A16*, **b** *TuGSTd10*, **c** *TuGSTd14*, and **d** *TuGSTm09* in field-collected *T. urticae* populations compared to the susceptible strain. \*\* $P < 0.01$ , \* $P < 0.05$

or multiple acaricide resistance (Dermauw et al. 2013). Figure 3 displays the relative expression of these four abamectin-associated genes in 10 field-collected *T. urticae* populations compared to the susceptible one. In general, all of these four genes show significantly higher expression ( $\geq 2$ -fold,  $p$  value  $\leq 0.05$ ) than the susceptible strain in at least one of the field-collected *T. urticae* populations (Fig. 3), suggesting P450- or GST-associated abamectin resistance is common in *T. urticae* populations on hops. *CYP392A16* and *TuGSTd14* were significantly overexpressed ( $\geq 2$ -fold,  $p$  value  $\leq 0.05$ ) in seven out of the 10 tested *T. urticae* populations (Figs. 3a, c). *TuGSTd10* and *TuGSTm09* showed overexpression ( $\geq 2$ -fold,  $p$  value  $\leq 0.05$ ) in five and three of the *T. urticae* field populations, respectively (Fig. 3b, d).

A recent study revealed that a cytochrome P450, *CYP392A11* plays a role in fenpyroximate resistance in *T. urticae* (Riga et al. 2015). Therefore, we used *CYP392A11* as a molecular marker to evaluate the fenpyroximate resistance in *T. urticae* populations from hops. As shown in Fig. 4a, *CYP392A11* was significantly overexpressed ( $\geq 2$ -fold,  $p$  value  $\leq 0.05$ ) in five out of 10 tested *T. urticae* populations.

The highest overexpression levels of *CYP392A11* occurred in the Toppenish\_7 and Toppenish\_7\_1 populations, with about 16-fold overexpression relative to the susceptible strain (Fig. 4a), which is consistent with the moderate level of fenpyroximate resistance in these populations (Table 2).

Molecular analysis of spiroadiclofen resistance in *T. urticae* suggested a P450 (*CYP392E10*) and an alternative allele of carboxyl/choline esterase-4 (*CCE04*) were strongly associated with high levels of spiroadiclofen resistance (Demaeght et al. 2013; Demaeght 2015). These two genes can be used as molecular markers to evaluate P450 or esterase-associated spiroadiclofen resistance in the field. *CYP392E10* was only overexpressed ( $\geq 2$ -fold,  $p$  value  $\leq 0.05$ ) in the Prosser\_9 and Toppenish\_7 populations (Fig. 4b), both of which exhibited moderate levels of spiroadiclofen resistance (Table 3). *CCE04* was only overexpressed in the Toppenish\_7 population (Fig. 4c). This is despite our finding that 74.3% of 21 *T. urticae* populations from hops exhibited moderate levels of resistance to spiroadiclofen (Table 3), suggesting other mechanisms besides *CYP392E10*- and *CCE04*-mediated detoxification are responsible.



**Fig. 4** Relative expression of fenpyroximate or spiroadiclofen resistance-associated genes **a** *CYP392A11*, **b** *CYP392E10*, **c** *CCE04* in field-collected *T. urticae* populations compared to the susceptible strain. \*\* $P < 0.01$ , \* $P < 0.05$

## Multiple acaricide resistance in *T. urticae* populations

With the availability of molecular markers, we summarized the genotypic resistance status in nine randomly selected field-collected *T. urticae* populations to six commonly used acaricides in hopyards. As shown in Table 4, abamectin resistance by enhanced target P450 and/or GST gene(s)'s expression was observed in all nine populations tested. In addition, target site insensitivity-mediated resistance to bifenthrin, bifenazate, and etoxazole was identified in all nine populations tested as well. However, metabolic detoxification-associated resistance to fenpyroximate and spiroadiclofen only occurred in four and two out of nine populations, respectively (Fig. 4; Table 4). Among these nine populations, one population (Toppenish\_7) contains resistance-associated genotype to all six acaricides evaluated (Table 4). Taken together, all nine *T. urticae* populations simultaneously harbor resistance-associated genotypes to multiple acaricides (average = 4.7), raising the specter of “super mites” that possess resistance to multiple acaricides.

## Discussion

Narrow-spectrum and reduced-risk acaricides are mainstays for sustainable *T. urticae* management in crop systems. To retain the utility of these IPM-compatible acaricides, it is necessary to develop a comprehensive strategy to frequently examine the phenotypic status and underlying mechanisms of adaptation to acaricides in field populations of mites. Our study found that *T. urticae* populations in hopyards exhibit a moderate to high levels of resistance to etoxazole, but resistance to fenpyroximate and spiroadiclofen remains at low to moderate levels. Our data revealed a complex genetic acaricide adaptation pattern toward six commonly used acaricides in PNW hopyards. Resistance to abamectin, fenpyroximate, and spiroadiclofen by enhanced metabolic detoxification-associated gene(s) was detected. P450 and/or esterase gene(s)-mediated resistance to fenpyroximate and spiroadiclofen only occurred in a few populations. However, target P450 and/or GST genes-associated resistance to abamectin was ubiquitous. In addition, target site insensitivity-mediated resistance to bifenthrin, bifenazate, and etoxazole was identified in a wide range of *T. urticae* populations from hops. Many of these *T. urticae* populations collected from PNW hopyards exhibited evidence of resistance to multiple acaricides, indicating efforts need to be made to slow down the development of such resistance.

Because of their relative safety and unique mode of action, MGIs are widely used in field and greenhouses for *T. urticae* management (Zhu et al. 2016b). Etoxazole and hexythiazox have been used on hops since they were registered

in the 1980s and 2000s, respectively (Adesanya et al. 2018). Based on spray records we examined, hop farmers applied etoxazole or hexythiazox two to four times each year (Fig. S1a) (Piraneo et al. 2015). In 2016, approximately 60% of the hop farms in Washington were treated with etoxazole or hexythiazox (Fig. S1b). Moreover, cross-resistance between etoxazole and hexythiazox and from MGIs to bifenazate and bifenthrin had been reported (Adesanya et al. 2018). Frequent usage of MGIs and cross-resistance are likely responsible for the moderate to high levels of etoxazole resistance observed in the *T. urticae* populations in this study (Figs. 1, 2). Hence, farmers should limit the use of etoxazole and other MGIs to avoid control failure (Demaeght et al. 2014; Adesanya et al. 2018).

According to the spray records we obtained, Class I METI acaricides, which include fenpyroximate and pyridaben, were only applied to 16.5% of all hop farms in 2016 (Fig. S1b). Fenpyroximate was only sprayed once in the growing season (Fig. S1a) (Piraneo et al. 2015). It is possible that the reduced application of these acaricides is responsible for the lack of resistance we observed. LPIs including spiroadiclofen and spirotetramat are recently registered acaricides on hops. However, in 2016 approximately 83.8% of the hop farms in Washington were treated with LPIs (Fig. S1b). Therefore, monitoring of resistance plays an essential role in preventing the loss of effectiveness of LPIs in mite control.

Pyrethroids have been registered for use in hopyards for more than 20 years. Our previous study reported that one mutation, F1538I, was observed in 66.7% of tested *T. urticae* populations collected from hopyards in 2013 (Piraneo et al. 2015). Here, the mutation F1538I alone and the presence of two or multiple mutations of M918L + F1538I, F1534S + F1538I, and M918L + F1534S + F1538I were detected in all 30 tested populations with complete genotypic data (Fig. 2). This is the first study to report the presence of the M918L and F1534S mutations in *T. urticae* populations. The M918L mutation, which is a variant of the “super-*kdr*” (M918T) mutation, was originally identified from cypermethrin-resistant *Aphis gossypii* populations (Carletto et al. 2010), and then in *Hyaella azteca* populations (Weston et al. 2013). The “super-*kdr*” M918T was reported in the tomato red spider mite, *Tetranychus evansi*, in the absence of L1014F (*kdr*), and was associated with the pyrethroid resistance (Van Leeuwen et al. 2008). In the present study, the mutation M918L was observed with F1534S (in 2 out of 9 populations) and F1538I (in 8 out of 9 populations) (Fig. 2), suggesting a probably high level of pyrethroid resistance in these *T. urticae* populations. The mutation F1534C was first identified in pyrethroid resistant mosquito *Aedes aegypti* and *A. albopictus* (Kawada et al. 2009; Kasai et al. 2011). The functional properties of this mutation have been evaluated through in vitro expression in *Xenopus* oocytes. It was found that the phenylalanine (F) to cysteine

(C) change reduced sodium channel sensitivity dramatically to type I pyrethroids (e.g., bifenthrin) but not type II pyrethroids (e.g., deltamethrin) (Hu et al. 2011). Therefore, the presence of F1534S in 10 tested *T. urticae* populations from hops is likely to confer resistance to bifenthrin. Interestingly, the F1534S mutation was always found together with the F1538I mutation (Fig. 2). F1538I occurs in domain IIIS6 of the VGSC and was previously identified as one of the primary pyrethroid resistance mutations linked to high level resistance in multiple arthropod species, including *T. urticae* (Soderlund and Knipple 2003; Van Leeuwen et al. 2010). We detected F1538I in almost all field-collected *T. urticae* populations (30), except for six populations for which we lack complete diagnostic data. In fact, this mutation has been fixed in 15 of our collected *T. urticae* populations (Fig. 2). These results indicate that pyrethroid resistance of *T. urticae* populations in hopyards is increasing, which reflects the prolonged selection from the consistent usage of pyrethroids over the years. The use of pyrethroids, especially the type I pyrethroids, in hopyards should be reduced in order to avoid control failure and maintain the usefulness of these IPM-compatible acaricides.

Acaricides are one of the cornerstones of effective mite management efforts in agriculture worldwide. However, extensive usage of a limited number of acaricides for a prolonged period could cause the escalated accumulation of resistance-associated genes in populations. Therefore, it is not surprising that there are so many field populations exhibiting resistance genotypes to multiple acaricides. Compared with phenotypic bioassays alone, the comprehensive diagnostic approach we used here with both phenotypic bioassays and molecular markers provides a more accurate and proactive tactic for evaluating acaricide resistance and allowing efficient acaricide rotation, which can help prevent the loss of effectiveness of acaricides in mite control.

## Author contributions

DBW and FZ conceived and designed the experiments; MW, AWA, and MAM performed the experiments; MW, AWA, and FZ analyzed the data; DBW, LCL, MDL, and FZ contributed new reagents/analytic tools; MW, AWA, and FZ wrote the original draft; all authors contributed in revising and preparing the paper for submission.

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## Compliance with ethical standards

**Conflict of interest** The authors declare they have no conflicts of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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