
Sabina A. Bajda¹*, Patrick De Clercq¹, Thomas Van Leeuwen*¹

¹ Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, 9000, Ghent, Belgium.

* Corresponding authors
Abstract

BACKGROUND: Acaricide application remains an integral component of IPM for the two-spotted spider mite *Tetranychus urticae*. Species and strains of phytoseiid predatory mites can vary significantly in their response to acaricides. For the success of IPM, it is imperative to identify the determinants of selectivity and molecular stress responses of acaricides in predatory mites.

RESULTS: The three classical acaricides bifenazate, cyflumetofen, and fenbutatin oxide did not affect survival and fecundity of *Phytoseiulus persimilis* regardless of the route of exposure. Selectivity of the orange oil- and terpenoid blend-based botanical acaricides was low via a combination of direct exposure, acaricide-laced diet, and residual exposure but improved when limiting exposure only to the diet. To gain insights into the molecular stress responses, the transcriptome of *P. persimilis* was assembled. Subsequent gene expression analysis of predatory mites orally exposed to fenbutatin oxide and orange oil yielded only a limited xenobiotic stress response. In contrast, *P. persimilis* exhibited target-site resistance mutations, including I260M in SdhB, I1017M in CHS1, and kdr and super-kdr in VGSC. Extending the screen using available Phytoseiidae sequences uncovered I136T, S141F in cytB, G119S in AChE, and A2083V in ACC, well-known target sites of acaricides.

CONCLUSION: Selectivity of the tested botanical acaricides to *P. persimilis* was low but could be enhanced by restricting exposure to a single route. Differential gene expression analysis did not show a robust induced stress response after sub-lethal exposure. In contrast, this study uncovered target-site mutations that may help explain the physiological selectivity of several classical acaricides to phytoseiid predators.
Keywords: acaricide selectivity, Phytoseiidae, *Phytoseiulus persimilis*, physiological selectivity, ecological selectivity, IPM
1. Introduction

Predatory mites, mainly from the family Phytoseiidae, are key Biological Control Agents (BCAs), reducing the populations of a range of agricultural pests, including spider mites, thrips, and whiteflies. *Amblyseius swirskii* Athias-Henriot, *Phytoseiulus persimilis* Athias-Henriot, *Neoseiulus californicus* McGregor, and *N. cucumeris* Oudemans are among the most economically important arthropod BCAs used in augmentative biological control.

Due to its economic importance and global distribution, *P. persimilis* is the species attracting the most interest and has been the focus of the largest number of studies among Phytoseiidae predators. *P. persimilis* is a specialist that feeds primarily on the herbivorous Tetranychidae spider mites and has been employed worldwide to control spider mite populations infesting various crops.

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), a major polyphagous pest in greenhouses and field crops, is the main target of *P. persimilis*. *T. urticae* is characterized by a tremendous reproductive potential, short generation time, and an arrhenotokous mode of reproduction, allowing unfertilized females to lay eggs. *Phytoseiulus persimilis* mediated strategies successfully keep *T. urticae* damage below the economic threshold level, in a range of greenhouse crops. However, chemical control remains an integral component of spider mite management. Spider mite outbreaks can often only be contained by the tandem use of acaricides with distinctive modes of action (MoAs). It is clear that exposure of naive *P. persimilis* to acaricides may reduce the efficacy of augmentative control and, consequently, the success of an integrated pest management (IPM) program.

The global acaricide portfolio recommended for spider mite control continues to evolve. As a general trend, older chemistries like organophosphates and carbamates are gradually replaced...
with more selective compounds. Among classical acaricides, electron transport inhibition at the mitochondrial respiratory chain has been a remarkably successful MoA. Several commercial acaricides target these processes at different sites. Mitochondrial electron transport Inhibitors (METI) III, like bifenazate inhibiting cytochrome b at the Q0-site in complex III (IRAC group 20)\textsuperscript{13,14}, METI II including cyflumetofen specifically inhibiting complex II and most likely interacting with succinate dehydrogenase subunits B (SDhB) and C (SDhC) (IRAC group 25)\textsuperscript{15} and acaricides that directly interfere with ATP synthesis at complex V (IRAC group 12), such as the organotin compound fenbutatin oxide\textsuperscript{5} are known for their excellent efficacy in controlling spider mites\textsuperscript{12}. Surprisingly, despite their conserved mode of action in the mitochondria, many of these compounds are fairly selective, not only between insects and mites but also between Tetranychidae and Phytoseiidae\textsuperscript{5,16–18}.

A number of botanical extracts with often non-specified MoA, have been recently included in the IRAC MoA classification scheme as part of the 'biologics' group\textsuperscript{19}. Botanicals have gained considerable attention in agriculture, addressing the need for inexpensive, easily sourced, and biodegradable alternatives to classical pesticides\textsuperscript{20}. Prev-Am and Requiem are novel botanical products recommended to manage soft-bodied arthropod pests, including spider mites. Prev-Am (60 g/L active ingredient 'orange oil') consists of a naturally occurring essential oil extracted from orange peel rich in D-limonene, the main constituent of the terpenoid fractions\textsuperscript{21}. Requiem (153 g/L active ingredient 'terpenoid blend') is a synthetic terpenoid blend composed of D-limonene, p-cymene, and α-terpinene, mimicking the terpene composition of Mexican tea, \textit{Chenopodium ambrosioides}\textsuperscript{22}.

Selective toxicity is an essential quality for acaricides, particularly the ones used in IPM programs. Selectivity can be attained either on the ecological or physiological level\textsuperscript{23}. Ecological selectivity builds on the bio-ecology of a given arthropod and exploits its habitat characteristics allowing for
the accommodation of compounds essential for plant protection that are otherwise non-selective.

The physiological selectivity accounts for similarities and differences in toxicokinetics (TK) and toxicodynamics (TD) pathways. The TK mechanisms encompass physiological pathways that determine if and how much of the pesticide reaches the target site and include penetration, activation, metabolism, transport, and excretion processes. TD mechanisms describe a toxicant’s interactions with its target site and the biological consequences of this interaction \(^{24}\). In addition to the intrinsic differences between arthropod species, exposure to pesticides and selection leads to the development of resistance in both pests and BCAs, which is generally caused by decreased exposure due to quantitative or qualitative changes in major detoxification enzymes and transporters (TK) and/or mechanisms that decrease sensitivity due to changes in target site sequence (TD) \(^{24,25}\).

Many studies have previously addressed the issue of compatibility between *P. persimilis* and acaricides, some of them simultaneously investigating compounds from different MoA groups, including classical and botanical acaricides \(^{26-28}\). Although most studies test only a single population of a certain predatory mite species, it becomes apparent that different strains of *P. persimilis* vary in their susceptibility to a given MoA \(^{26,29,30}\) and are capable of developing acaricide resistance as a result of selection pressure \(^{25,31,32}\).

Compatibility achieved by acquiring heritable insecticide/acaricide resistance has been a highly desirable characteristic for commercial BCAs \(^{25,33,34}\). However, the molecular mechanisms behind varying susceptibility to acaricides in Phytoseiidae mites remain largely unknown. In several phytoseiid species, the mechanism of acaricide detoxification has been studied exclusively via biochemical assays and with acaricide synergists, and target site resistance has been studied to an even lesser degree \(^{23,35}\). Transcriptomic studies on phytoseiid mites rarely address the molecular mechanism of acaricide adaptation \(^{36}\). The general lack of genomic and transcriptomic
resources for Phytoseiidae impedes the development of functional genetic tools and ecotoxicological biomarkers assessing acaricide effects and the health status of a predator. This study attempted to explore the prospect of increasing ecological selectivity and simultaneously investigated the molecular basis of physiological selectivity of three classical selective acaricides, fenbutatin oxide, cyflumetofen, bifenazate, and two plant-based botanical acaricides, Prev-Am and Requiem, towards the predatory mite *P. persimilis*. The survival and fecundity of *P. persimilis* were assessed following acaricide administration via different routes: direct contact, pesticide-laced diet (prey), or a combination of diet, direct and residual contact. The transcriptomic changes affiliated with oral exposure to sublethal doses of fenbutatin oxide and Prev-Am were explored. Further, the transcripts encoding known target sites of acaricides routinely used against *T. urticae* were identified in the search for single nucleotide substitutions (SNP) potentially associated with acaricide target-site based selectivity in *P. persimilis*. Lastly, a comprehensive search of NCBI databases was performed to estimate the incidence of acaricide-target site mutations across predatory mite species within the family Phytoseiidae.

2. Materials and Methods

2.1. Chemicals

Commercial formulations of the mitochondrial complex III electron transport inhibitors bifenazate (Floramite® 240 g L\(^{-1}\) SC) and cyflumetofen (Scelta® 20 g L\(^{-1}\) SC) were purchased from Intergrow (Aalter, Belgium). Fenbutatin oxide (Torque® 550 g L\(^{-1}\)) was originally bought from Fyto Vanhulle (Belgium). The commercial formulation of orange oil (Prev-Am® Plus 60 g L\(^{-1}\) SL) was purchased from Intergrow (Aalter, Belgium). Orange oil is a common name for an extract from the rind of *Citrus aurantium* of which D-limonene is the active substance at a content of 94.5 – 96.5 %. Bayer CropScience (Diegem, Belgium) provided an experimental sample of terpenoid blend QRD 460
Terpenoid blend QRD 460 consists of three components: \( \alpha \)-terpinene, p-cymene and D-limonene. The nominal concentration of each technical grade component in the active substance as manufactured is as follows: \( \alpha \)-terpinene 59.7% w/w, p-cymene 22.4% w/w, D-limonene 17.9% w/w. Both 'orange oil' and 'terpenoid blend QRD 460' are registered and approved active ingredients in the EU (https://ec.europa.eu/food/plants/pesticides/eu-pesticides-database_en).

2.2. Predatory mite and spider mite populations

A stock culture of *P. persimilis* was obtained from Koppert Biological Systems (Berkel en Rodenrijs, the Netherlands) and reared on detached *T. urticae*-infested bean leaves resting on a moist cotton sheet in a water-filled plastic tray. The London strain of *T. urticae* was used to maintain the *P. persimilis* stock culture. The London strain was also used as a diet for *P. persimilis* in orange oil and terpenoid blend tests. In the experiments with bifenazate, cyflumetofen, and fenbutatin oxide, *T. urticae* strains BR-VL, Tu008R, and MR-VL, known for their high resistance levels to the respective compounds, were used as a food for *P. persimilis*.

2.3. Survival and fecundity of acaricide treated *Phytoseiulus persimilis*

The impact of acaricides on *P. persimilis* fecundity and survival was investigated via three delivery routes (Figure 1A): (1) via direct contact (SCC), (2) via the diet (CSC), and (3) a combination of direct contact, residual contact, and diet (SSS). All treatments consisted of spraying 0.8 ml of spray fluid at 1 bar pressure in a Cornelis spray tower (1.5 ± 0.06 mg aqueous acaricide deposit cm\(^2\)).

Acaricide solutions were applied at the field rate recommended for spider mite management. These were 96 mg L\(^{-1}\) active ingredient (a.i.) bifenazate, 200 mg L\(^{-1}\) a.i. cyflumetofen, 247 mg L\(^{-1}\) a.i. fenbutatin oxide, 0.4% of commercial formulation orange oil, and 0.6% of commercial...
formulation terpenoid blend. A water-sprayed control accompanied each acaricide treatment (CCC).

Acaricide bioassays were performed in experimental units consisting of a 3 cm diameter plastic dish with a lid having a circular opening secured with mite-proof mesh. A 4 cm² bean leaf square was placed on cooled but not yet solidified 1% agarose. The abundance of prey for the control (CCC), direct (SCC), and combined (SSS) treatments was ensured by allowing five adult females of *T. urticae* to propagate on the arenas three days before the start of the experiment. Prey for the acaricide-laced diet-treatment (CSC) was sprayed on a separate full-leaf arena and brushed onto clean experimental arenas after drying. Any emerging adult females of *T. urticae* were removed from the arenas to prevent the predatory mites from feeding on unsprayed eggs. The impact of an acaricide via direct contact (SCC) was investigated by spraying adult females of *P. persimilis*, which were then moved to clean arenas with unsprayed *T. urticae* prey. The combined effect of direct, residual contact and diet was investigated by directly spraying the experimental arena with *P. persimilis* and *T. urticae* (SSS).

Once acaricides were administered via one of three routes, single, two-day-old *P. persimilis* females were allowed to feed on the same *T. urticae*-populated arena for four days (Figure 1B). Predatory mite eggs were counted daily and removed from the arena, and the mortality of the predators was recorded. The experiment was kept in a climatically controlled chamber (PHCBI MLR-352H-PE) at 25 ± 0.5 °C, 70 ± 5% RH, and 16/8 h (light/dark) photoperiod. For practical reasons, experiments were performed in 2-4 time blocks.

Fecundity data were checked for normality and homoscedasticity before statistical analysis and analyzed with a linear mixed-effects model (lmer of the package “lme4”) with acaricide-delivery-mode (CCC, CSC, SCC, SSS) as a fixed factor and time block as a random factor. The contrast among treatments was assessed using Tukey’s HSD (package “multcomp”, 42). Mortality data were
analyzed with a Cox proportional hazards model from package "survival" to test whether differences in *P. persimilis* survival depended on the acaricide delivery mode. Function pairwise_survdiff (package "survminer") was used to perform pairwise comparisons between the different delivery modes. P-values were Benjamini-Hochberg adjusted. Statistical analysis was performed in R, version 3.6.3.

2.4. Acaricide treatment for differential gene expression studies

Fenbutatin oxide and orange oil were used to investigate gene expression patterns in predatory mites exposed to *T. urticae* diet sprayed with the field rate of acaricide (247 mg L⁻¹ and 0.4% respectively) vs. water (Figure 1C, CSC). *T. urticae* prey was obtained from heavily infested bean leaves. They had been mostly abandoned by adult spider mites for their lack of nutrients but were still infested by other life stages. By eliminating adult females, we limited the number of unsprayed eggs in the diet. *T. urticae* infested bean leaves were sprayed until run-off with a hand atomizer and allowed to air-dry. An excess of either acaricide- or water-sprayed *T. urticae* mites was brushed into a plastic container (9.5 cm x 7 cm) with a tightly fitted lid. The lid had a 4 cm diameter opening secured with mite-proof netting. A moist piece of cotton was placed inside the container to maintain humidity. Approximately one hundred two-day-old female *P. persimilis* were added. Experimental units were kept in a climatically controlled chamber at 25 ± 0.5 °C, 70 ± 5% RH, and 16/8 h (light/dark) photoperiod. Live predatory mites were collected after a two-day and four-day exposure to orange oil and fenbutatin oxide, respectively. Sampling time was chosen based on the daily fecundity values to maximize the chance of capturing transcriptomic changes related to the sublethal acaricide exposure (Figure 1C, 2A, 2E). A water control accompanied each acaricide treatment to account for the difference in age between the orange oil, and fenbutatin oxide treated samples (Figure 1C). The experiment was repeated four times.
2.5. RNA extraction, sequencing, and quality control

Total RNA was isolated from 80 live adult females of *P. persimilis* using the RNEasy Plus mini kit (Qiagen, Belgium). RNA quality and quantity were estimated using a DeNovix DS-11 spectrophotometer (DeNovix, U.S.A.) and running an aliquot on a 1% agarose gel. Illumina libraries were constructed following a standard protocol with size fractionation (Illumina, USA). The resulting libraries were sequenced on an Illumina NovaSeq 6000, and strand-specific paired reads (2 × 100 bp) were generated. Library construction and sequencing were performed at the Fasteris sequencing facility (Geneva, Switzerland). Before read-mapping, the quality of the reads was verified using FASTQC version 0.11.8.

2.6. De novo transcriptome assembly and annotation

The *de novo* assembly, transcript annotation, and differential expression analysis were performed according to Alavijeh et al., 2020, with minor deviations from the pipeline. In an attempt to estimate the number of reads sufficient to create a complete assembly of the *P. persimilis* transcriptome, we subsampled a random selection of 2.5 and 5 million reads per replicate, totaling 40 and 80 million reads. *De novo* assemblies were created using CLC Genomics Workbench 10. Next, long open reading frames (ORFs) were extracted from the resulting assemblies using Transdecoder v. 5.5.0 with a minimum ORF length of 100 amino acids. CD-HIT-EST was used to filter extracted ORFs at the identity threshold of 98% (−c 0.98) and word size of 10 (−n 10). The transcripts to which the filtered ORFs belonged were retained for further analysis. The transcripts of the two assemblies were then loaded into OmicsBox 1.3.11 and used as a query in a CloudBlast, BLASTx search, using an E-value threshold of E<sup>−3</sup> and the "fast" setting, against the NCBI non-redundant (nr) protein database (the version of 23<sup>rd</sup> June 2020). Blast2GO was subsequently used to map and annotate gene ontology (GO) terms to transcripts based on the sequences retrieved by the BLASTx search. Finally, the InterProScan pipeline was run, and InterPro identified GO
terms were merged to the Blast2GO annotated GO terms. Subsequently, the transcripts having a
first BLASTx hit with viruses, bacteria, protozoa, fungi, or *T. urticae* were excluded from the
assemblies. Other sequences were removed from the assembly if the mean sequence similarity
value was ≥ 98% with members of the genus *Tetranychus*. Based on the overall mapping success
rate against an assembly (before removing contamination) and the total amount of sequences
with a blast hit (after removing contamination), the "40 million reads assembly" was selected for
further analysis, Table S1.

2.7. Expression quantification, principal component analysis (PCA)

Once contaminating sequences were removed from the final "40 million read assembly", paired-
end sequences were pseudo-aligned with kallisto (100 bootstraps) to create abundance estimates
51. Differential expression analyses were performed in R version 4.0.2 using sleuth 0.30.0 running
on the default settings and an additional package, gridExtra. The Wald test in sleuth was used to
analyze the kallisto bootstrap estimates 52, while the transformation function log2(x + 0.5) 53
option in the sleuth_prep command was used to calculate the effect size (β value) as log2-based
fold changes (log2FC). Transcripts with log2FC > 0 and a q-value (Benjamini-Hochberg multiple
testing corrected p-value) ≤ 0.05 between the fenbutatin oxide and orange oil samples and their
respective controls were considered differentially expressed. For each comparison’s differentially
expressed transcripts (DETs), a GO enrichment analysis was performed using Fisher’s exact test in
the functional analysis toolbox of OmicsBox 1.3.11. Only the GO categories with an FDR adjusted
p-value of <0.05 were considered significantly enriched. Finally, a PCA was created using the
interactive sleuth visualization package, Shiny 1.4.0.2.

2.8. Identification of target-site mutations in Phytoseiidae

The *P. persimilis* transcriptome assembled in this study, the nucleotide collection (nr/nt), and
Transcriptome Shotgun Assembly (TSA) databases available from NCBI, were screened to identify
orthologues of acaricide target genes across the family Phytoseiidae. These included known target sites of acaricidal compounds frequently used against *T. urticae*: PSST subunit of Mitochondrial Complex I, cytb, Succinate dehydrogenase, subunits B (SdhB) and C (SdhC), ATP synthase, subunits a and c, Chitin Synthase 1 (CHS1), Glutamate-gated Chloride channel (GluCl), Acetyl CoA carboxylase (ACC), Acetylcholinesterase (AChE), and Voltage-Gated Sodium Channel (VGSC). Only well-documented substitutions, located in the protein most conserved regions across different species, were considered. Although there are no identified target site mutations in arthropods against inhibitors of mitochondrial ATP synthase, conserved domains within a and c subunits were also screened for SNPs.

The *P. persimilis* transcriptome and NCBI databases were mined for contigs encoding target-sites of acaricides using tBLASTn (E-value cutoff < 1E⁻⁵) and *T. urticae* protein sequences as a query. When obtaining a complete and continuous target site sequence was impossible, we made sure to identify *P. persimilis* ORFs extending over the regions carrying a known target-site mutation. Cytochrome b sequence was not present in the final 40 million read assembly and was instead identified in an earlier version of this assembly with tBLASTx (E-value cutoff < 1E⁻⁵, File S1). Open reading frames were extracted using Transdecoder v. 5.5.0 with TransDecoder.LongORFs. A minimum ORF length of 100 amino acids was set for AChE, GluCl, and VGSC. These genes belong to multimember gene families of Carboxy/Cholinesterases (CCEs), Cys-loop ligand-gated channels, and voltage-gated channels, respectively, and their identification was facilitated by phylogenetic analysis. For the remaining target sites, there was no minimum ORF length specified. To further maximize sensitivity for capturing ORFs that may have functional significance, candidate peptides were screened for homology using BLASTp against *T. urticae* amino acid (aa) sequence (Table 1, E-value cutoff < 1E⁻⁵). Subsequently TransDecoder.Predict was used to predict the likely coding regions, and the best hit per sequence was retained with function –
single_best_only. Amino acid sequences were aligned using MAFFT v 7.475 \(^{55}\), and misaligning sequences were removed.

The phylogenetic analysis of cys-loop channels was performed on *P. persimilis* (this study), *Metaseiulus occidentalis* (Nesbitt), and *A. swirskii*. Protein sequences of *T. urticae* and *Drosophila melanogaster* (Meigen) were used as a reference. For the phylogenetic analysis of CCEs, a reference set of *T. urticae* CCE protein sequences was included in the alignment with *P. persimilis* (this study), *M. occidentalis*, *A. swirskii* and *Kampimodromus aberrans* (Oudemans), and representatives of insect CCEs; *D. melanogaster*, *Apis mellifera* L. and *Bombyx mori* L. \(^{56}\). As N- and C- termini of CCEs are highly variable, divergent regions were trimmed according to Claudianos et al., 2006. Phylogenetic analysis on voltage-gated channels was performed with sequences of *P. persimilis* (this study), *A. swirskii*, *M. occidentalis*, and *Neoseiulus barkeri* (Hughes). VGSC protein sequences of *T. urticae* (tetur34g00970) and *Musca domestica* L. (Q25439) were used as a reference. Phylogenetic trees were built using IQ-TREE v 2.0.3 with 1,000 ultrafast bootstrap (-B 1,000) combined with automatic model finding through ModelFinder Plus (-m MFP) \(^{58}\). The resulting trees were midpoint rooted and edited with MEGA v 10.2.4 software \(^{59}\). Only bootstrap support values \(\geq 85\%\) were shown in the tree.

**3. Results**

**3.1. Survival and fecundity of *P. persimilis* after acaricide treatment**

Survival and fecundity of predatory mites treated with the acaricides bifenazate, cyflumetofen, bifenazate, fenbutatin oxide, and botanicals terpenoid blend and orange oil, either directly (SCC), only via acaricide-treated diet (CSC) or by a combination of exposure routes (SSS) (Figure 1A), were scored daily for four days (Figure 1B, 2). Figure 3 presents a graphical overview of total fecundity and survival for all acaricide treatments.
### 3.1.1. Daily and total fecundity

Except for the first day of cyflumetofen treatment, when the fecundity of the water sprayed control (CCC) in this setup was significantly lower than in the combined acaricide treatment (SSS) (Figure 2C), average daily fecundity did not differ between different acaricide delivery routes for the classical acaricides (fenbutatin oxide, bifenazate, and cyflumetofen) and the respective control (Figure 2A, B and C, respectively).

However, the negative effect of consuming terpenoid blend-sprayed *T. urticae* (CSC) on daily fecundity became significant on the first day of scoring and remained so throughout the experiment (Figure 2D). The combination of diet, direct and residual contact (SSS) also had immediate and even more pronounced consequences for daily fecundity (Figure 2D). Direct spraying with terpenoid blend (SCC) had a negligible effect on daily fecundity (Figure 2D). Consuming orange oil-treated prey (CSC) had little influence on the fecundity of the predator during the first two days but became significant with time. The direct contact (SCC) and combined treatment (SSS) immediately and acutely affected predator fecundity (Figure 2D). The results of the variance analysis for the daily fecundity are listed in Table S2.

There was no significant difference in total fecundity after four days of egg-laying between the different delivery modes and the control for bifenazate ($F_3=0.93$, $p>0.05$, Figure S1A), cyflumetofen ($F_3=1.2$, $p>0.05$, Figure S1B) and fenbutatin oxide ($F_3=1.71$, $p>0.05$, Figure S1C). Total fecundity differed significantly between the water sprayed control (CCC), terpenoid blend sprayed diet (CSC), and the combined treatment (SSS), and between the direct contact (SCC) and combined treatment (SSS) ($F_3=12.68$, $p<0.05$, Figure S1E). All comparisons except SSS vs. SCC for orange oil yielded a significant difference in the total number of eggs laid ($F_3=95.50$, $p<0.05$, Figure S1D).
3.1.2. Survival

A significant difference in the survival rate of *P. persimilis* mites could not be observed between water sprayed control (CCC) and the treatments involving different routes of acaricide delivery (CSC, SCC, SSS), for either of the classical acaricides, fenbutatin oxide ($\chi^2 = 0.64$ on 3 df, $p>0.05$), bifenazate ($\chi^2 = 0.09$ on 3 df, $p>0.05$) or cyflumetofen ($\chi^2 = 0.14$ on 3 df, $p>0.05$).

On the contrary, the survival rate of predatory mites sprayed with a combination treatment of terpenoid blend (SSS) differed significantly from all the other treatments (CCC, CSC, SCC) ($\chi^2 = 8.14$ df=3, $p<0.05$). All comparisons except SSS vs. SCC for orange oil yielded a significant difference in the survival rate ($\chi^2 = 59.02$ on 3 df, $p<0.05$). P-values for the log-rank pairwise comparison between survival rates for orange oil and terpenoid blend can be found in Table S3.

3.2. *Phytoseiulus persimilis* transcriptome and transcriptional responses

RNA from adult *P. persimilis* females exposed via diet (sprayed immature *T. urticae*) to orange oil and fenbutatin oxide and a water-control was isolated and Illumina-sequenced. RNA from predators exposed to orange oil was sampled after two days of acaricide exposure, whereas fenbutatin oxide-exposed predators were sampled after four days. The sampling time was chosen based on the daily fecundity data (Figure 2 A, E) in the hope of detecting transcriptomic changes preceding the statistically significant drop in *P. persimilis* fecundity. Illumina sequenced short reads of each treatment are available in the Gene-Expression Omnibus (GEO) repository with accession number ([SRA submission in progress, submission number, SUB10153985](#)). Two transcriptome assemblies were generated, and detailed comparisons of the" 80 million" and" 40 million" assemblies along the preprocessing and annotation pipeline can be found in Table S1. The" 40 million" and" 80 million" assemblies initially consisted of 19,696 (file S2) and 25,076 (file S3) transcripts (Table S1). The average pseudo-alignment mapping rate of reads against the 19,696 and 25,076 transcripts were 87.9% and 86.8%, respectively. A Blast2GO analysis revealed that for
17

the 40 million read assembly" 16,410 (83.5%) of the 19,696 transcripts had a BLASTx hit, and 3,395 (17.2%) transcripts were either protozoan, viral, bacterial, *T. urticae*, or other members of the genus *Tetranychus*, showing a mean sequence similarity value ≥ 98% (Table S4). Consequently, after the 3,395 transcripts were removed, 12,969 (79.6%) reads out of the final assembly of 16,301 reads (file S4) had a blast hit (Table S5). For the" 80 million read assembly", 20,100 (80.2%) of the 25,076 transcripts had a BLASTx hit, and 7,286 (29.1%) transcripts were either protozoan, viral, bacterial, fungal, *T. urticae*, or other members of the genus *Tetranychus*, showing a mean sequence similarity value ≥ 98% (Table S6). After removing the 7,286 transcripts, 12,755 (71.7%) reads out of 17,790 (file S5) had a blast hit (Table S7). Based on the pseudo alignment mapping rate and the absolute number of transcripts with a blast hit, the" 40 million read assembly" was chosen for further analysis (Table S1). The reads were pseudo-aligned against the remaining 16,301 transcripts using kallisto. A principal component analysis (PCA) across all RNAseq samples revealed that 35.0% of the total variation could be explained by PC1, while 16.7% could be explained by PC2 (Figure 4). Clustering by treatment was not apparent, indicating that the treatment is not the primary source of variation. Fenbutatin oxide treated replicates occupied an area mainly overlapping with the control. For the orange oil-treated samples, however, all but one replicate clustered away from the control. We used sleuth to perform a differential transcript expression analysis (*logfc2 > 0, q-value ≤ 0.05*). Transcript expression levels were pairwise compared between acaricide treatment (orange oil and fenbutatin oxide) and the relevant control (orange oil_c and fenbutatin oxide_c). Unsurprisingly only two transcripts were found differentially regulated between fenbutatin oxide and fenbutatin oxide_c. Contig_22124 and contig_24988 were upregulated in fenbutatin oxide (log2FC= 3.04 and 3.24, respectively), and neither contig had a BLASTx hit against the NCBI nr protein database at the threshold of E^-3. We found only 34 DETs between orange oil and orange oil_c, with fold changes being overall modest. Among the
34 DETs, 27 were up-regulated, and seven were down-regulated. Eleven DETs did not have a BLASTx hit with the nr protein database. The remaining 23 annotated transcripts are listed in Table S8.

Contigs upregulated after treatment with orange oil coded for proteins involved in DNA integration and binding, including tigger transposable element (contig_20133, contig_15792, contig_9434), mRNA binding, processing and splicing factors (contig_1302, contig_1223, contig_7063, contig_7578, contig_10832, contig_226), and endoplasmic reticulum activity (ER) (contig_7415). Apart from one transcript blasting with M. occidentalis cytochrome P450 1A1 (contig_5327) sequence, no representatives of other gene families typically involved in xenobiotic metabolism were found among DETs. There were no significantly enriched GO-terms in orange oil vs. orange oil_c nor fenbutatin oxide vs. fenbutatin oxide_c.

### 3.3. Presence of target site substitutions in Phytoseiidae

The transcriptome of P. persimilis allowed for successfully identifying sequences coding for CHS1, PSST subunit of Complex I, ACCase, AChE, SdhB and SdhC, cytb, GluCl, ATP synthase subunit c, and a and partial sequence of VGSC (File S6). The IDs of P. persimilis contigs coding for the target site sequences can be found in Table S9.

Except for cytb, which sequence is routinely used to discriminate between phylogenetically related species, and for which 303 sequences of 51 Phytoseiidae genera could be screened, tBLASTn searches across Phytoseiidae performed on NCBI servers yielded a modest overall outcome with M. occidentalis and A. swirskii being often the only identified species (Table 1, Table S10).

Subsequent phylogenetic analysis identified Phytoseiidae orthologues of AChE (Figure S2), VGSC (Figure S3), and GluCl (Figure S4) within multigene families of cholinesterase, voltage-gated channels, and cys-loop channels, respectively. In many cases, protein sequences were incomplete, preventing screening for target site mutations at know conserved sites (Table 1).
We identified an I1017M substitution in the *P. persimilis* transcript coding for the CHS1 gene (Figure 5, Table 1, File S6) \(^{60,61}\). NCBI search resulted in 13 hits. Two of these belonged to *M. occidentalis* and 11 to *A. swirskii*. Substitution at position 1017 (*T. urticae* numbering) was absent from all the examined sequences (Table 1, Table S10).

The H92R substitution associated with Mitochondrial Electron Transport Inhibitors I (METI I) resistant phenotype in *T. urticae* was not detected in the *P. persimilis* transcript, nor were the other mutations reported to debilitate the function of the respiratory Complex I in *Yarrowia lypolitica* \(^{62-66}\), and Human, \(^{67,68}\). NCBI search identified three orthologous sequences for *M. occidentalis* and five of *A. swirskii* that could be efficiently aligned. None of the mutations were present in the sequences of *M. occidentalis*. Substitutions found in NQO6 (PSST orthologue in bacteria) of *Y. lypolitica* V88I and E140D were identified in *A. swirskii* GHIT01033895.1, GHIT01065208.1, GHIT01035679.1 (Table 1, Table S10.).

Phylogenetic analysis allowed for high fidelity identification of AChE genes of *M. occidentalis*, *A. swirskii*, *P. persimilis* and *K. aberrans* (Figure S2, Table S10). We did not identify any of the mutations previously associated with resistance to organophosphates and carbamates in the AChE sequence of *P. persimilis* (File S6) \(^{70}\). An NCBI search revealed that both *M. occidentalis* sequences (XM018642151.1 and XM018638971.1) and a sequence of chlorpyrifos resistant strain of *K. aberrans* (HF934044.1) carry mutation G119S \(^{71,72}\) (Table 1).

The recently identified A2083V in a highly conserved region of the carboxyltransferase domain (CT) in ACC of a *Bemisia tabaci* (Gennadius) resistant to spiromesifen and spirotetramat \(^{73}\) was not found in the *P. persimilis* orthologue (File S6). Nineteen hits belonging to *A. swirskii* (12 sequences) and *M. occidentalis* (7 sequences) were found via the NCBI. Among five sequences long enough to span the CT domain, three GHIT01022503.1, GHIT01028753.1, GHIT01031955.1 of *A. swirskii* carried A2083V. (Table 1, Table S10).
Screening for VGSC mutations (reviewed in Rinkevich et al., 2013) resulted in finding kdr (L1014F) and super-kdr (M918V) mutations (M. domestica numbering) in the transcript of *P. persimilis* (Figure 5, Table 1, File S6). Phylogenetic analysis identified five sequences of *A. swirskii*, and a single sequence for *N. barkeri* and *M. occidentalis* (Fig S3, Table 1). In *N. barkeri* KT768110.1, Methionine at super kdr (918) was substituted with Leucine (Table 1, Table S10). The phylogenetic analysis allowed the identification of GluCl genes of *M. occidentalis*, *A. swirskii* and *P. persimilis* supported by high bootstrap values (Fig S4). Contig_4086 of *P. persimilis* (this study) and accession numbers: GHIT01011578.1, GHIT01010960.1, GHIT01043426.1 of *A. swirskii*, and XM_029110899.1 of *M. occidentalis* clustered with GluCl sequences of *T. urticae* and *D. melanogaster* (Figure S4). Contig_4086 of *P. persimilis* (this study), transcripts XM_029110899.1 of *M. occidentalis*, and GHIT01011578.1 of *A. swirskii* had sequences long enough to screen for the three target site mutations. However, substitutions G314D, G326E, and I321T previously associated with low resistance levels to abamectin in *T. urticae* were not present (File S6, Table S10). Resistance mutations in highly conserved cd1 and ef helices of cytb were not found in the *P. persimilis* orthologue of the Koppert strain analyzed in this study (File S6). Screening of cytb orthologues within the Phytoseiidae family did not reveal the presence of any of the known mutations in ef helix (Table S10). Substitutions in cd1 helix at the positions corresponding to *T. urticae* mutations were present in *Neoseiella littoralis* (Swirski & Amitai) (G126A, GU938141.1), several species of genus *Typhlodromus*: *T. pyri* (Scheuten) (A133G, JF279255.1, JF279271.1, JF279279.1, JF279280.1), *T. aestivalis* (Athias-Henriot) (S141T, A MK014109.1, MK014110.1), *T. verrucosus* (Wainstein) (I136T, MK014112.1), *T. rhenanoides* (Athias-Henriot) (S141F, MK014067.1, MK014068.1, MK014070.1, MK014073.1), *T. ilicis* (Athias-Henriot) (S141F,V,D, MK014095.1), *T. laurae* (Arutunjan) (S141A, MK014141.1), *T. exhilaratus* (Ragusa) (S141F, A, MK014144.1, MK014141.1, MK014148.1), *T. setubali* (Dosse) (G132E, MK014116.1), genus *Typhlodromalus*, *T. aripo* (De Leon)
(S141T, KU318210.1, KU318209.1, KX610079.1), genus *Amblydromalus*, *A. limonicus* (Garman & McGregor) (S141T in KU318220.1 and KU318219.1) and genus *Euseius*, *E. fustis* (Pritchard & Baker) (S141T in KU318220.1 and KU318219.1), (Table 1, Table S10).

*P. persimilis* I260 in SdhB of Complex II was substituted with Methionine (File S6, Figure 5).

Similarly, I260M substitution was found in both *A. swirskii* orthologues, GHIT01048799.1 and GHIT01059435.1. Serine substituted the residue R256 in the *P. persimilis* orthologue found in this study (Figure 5, Table 1, File S6), and a proportion of *M. occidentalis* (JL035473.1, JL014348.1, JL012205.1), and *A. swirskii* (GHIT01060452.1) sequences found via NCBI. The S56L in SdhC causing cyenopyrafen resistance in *T. urticae* was not detected in *P. persimilis* and other Phytoseiidae orthologues (File S6). However, residue R74 (*T. urticae* numbering) was substituted by Serine (Table 1, Table S10, File S6). The same substitution was found in both (GHIT01023969.1 and GHIT01063425.1) orthologue s of *A. swirskii*, identified via NCBI (Figure 5, Table 1, File S6).

We have identified V166L, A195S, A213S (*T. urticae* numbering) in the otherwise conserved residue of ATP synthase subunit a of *P. persimilis* (File S6). NCBI search within Phytoseiidae yielded 14 sequences that could be effectively aligned against *T. urticae* tetur01g06130. No other sequence carried A195S SNP, but V166L and A213S coincided in a proportion of *A. swirskii* sequences (GHIT01019070.1, GHIT01047891.1) and all available sequences of *M. occidentalis* (XM_003741056.1, JL040066.1, JL020987.1, JL020984.1 - not long enough to confirm the presence of A213S) (Table 1, Table S10). Substitution R109S (*T. urticae*, tetur06g03780) was detected in the conserved C-terminal domain of the ATP synthase subunit c (File S6). Out of 21 sequences, the same substitution has been found at the corresponding position in ATP synthase subunit c of *M. occidentalis* orthologue JL013409.1, JL013411.1, JL013412.1, JL028982.1, JL013416.1, JL013415.1, JL021266.1, JL013413.1, and GHIT01045723.1 of *A. swirskii* (Table 1, Table S10).
4. Discussion

This study finds that the classical acaricides targeting different complexes of the mitochondrial electron transport chain, bifenazate (Complex III), cyflumetofen (Complex II), and fenbutatin oxide (complex V) did not adversely affect the fecundity or survival of adult *P. persimilis* in our experimental setup, regardless of whether predators were exposed via diet, by direct contact or a combination of direct contact, residual contact, and diet (Figure 2, Figure 3, Figure S1). The impact of the three acaricides on herbivorous spider mites and predatory mites has been studied extensively.\(^5,17,18,23,27,39\) While very effective against *T. urticae*, there have been conflicting reports of their safety to key phytoseiids.\(^26\) Particularly for fenbutatin oxide, prolonged surveillance of treated predatory mites saw increased mortality in the laboratory tests\(^18\) and fewer *P. persimilis* in the field experiments.\(^82\) Despite that, and in line with the results of this study, the risk of acute toxicity of bifenazate, cyflumetofen, and fenbutatin oxide is generally low, and these MoAs are often recommended for the control of *T. urticae*.\(^17,18,26,83–86\)

Surprisingly, in contrast with the classical acaricides, the botanical acaricides containing orange oil and terpenoid blend as active ingredients were harmful to *P. persimilis*. When tested in an identical setup, their impact varied depending on their route of administration (Figure 2, 3, S1). Upon contact, lipophilic monoterpene constituents of orange oil and terpenoid blend exert their toxic properties by directly altering epicuticular waxes' components and entering arthropod bodies via cuticle or respiratory tracheoles.\(^87\) D-limonene is efficient as a contact acaricide.\(^88–90\) Its high content in orange oil may explain the acute contact toxicity towards *P. persimilis*. Indeed, orange oil and terpenoid blend-based acaricides are thought to act mainly by physical contact, but their target sites can be broad-spectrum from a physiological perspective. Monoterpene mode of action inside the arthropod's body is unclear but most likely relies on interference with
biological membrane properties. Monoterpenes are also suspected of sharing some of the intracellular target sites with classical insecticides/acaricides acting on the insect nervous system. The sublethal effect of providing *P. persimilis* with orange oil and terpenoid blend laced-diet could be mediated by the repellent properties of monoterpenes, particularly D-limonene. Orange oil and terpenoid blend-based acaricides may offer little physiological selectivity towards non-target organisms, interfering with arthropods' essential biochemical and physiological functions. However, our results suggest that the utility of these acaricides in the integrated control of *T. urticae* can be preserved by smart management practices that increase their selectivity on the ecological level.

*Phytoseiulus persimilis* females surviving ingestion of orange oil-laced diet showed diminished fecundity (Figure 2E and S1D). Although the same could not be seen for fenbutatin oxide in our setup (Figure 2A and S1C), prolonged exposure to this acaricide was harmful to BCAs elsewhere. We thus selected fenbutatin oxide and orange oil to investigate differences in gene expression profiles of *P. persimilis*, attempting to identify genetic signatures associated with ingestion of sublethal doses of classical and botanical acaricides. The clustering by treatment was not readily apparent, indicating that the treatment did not cause large reproducible shifts in gene expression (Figure 4, Table S8). In support of the empirical data, differential gene expression in fenbutatin oxide treated samples was negligible, with only two unidentified transcripts being differentially regulated between the treatment and the control. Treatment with orange oil resulted in the 23 annotated DETs (log2FC>0, qvalue<0.05). None but one downregulated contig encoded a member of a classical detoxification gene family, P450 1A1. The remaining differentially regulated transcripts may suggest increased transcriptional regulation, modulation of proteome diversity by alternative splicing, and enhanced protein production and export as a response to orange oil treatment (Table S8). However, overall, the data also suggests that molecular
markers of stress responses are difficult to identify in *P. persimilis*, especially when the phenotype resulting from the treatment is subtle. Similarly, Paspati *et al.*, 2019 have found only 39 DETs in *A. swirskii* exposed to a challenging host, tomato vs. pepper, suggesting that mild transcriptomic response towards synthetic and natural xenobiotics may characterize Phytoseiidae mites. Phytoseiidae mites, including *P. persimilis*, belong to Parasitiformes, while *T. urticae* belongs to Acariformes. These two lineages of *Acari* separated approximately 400 mln years ago 100,101. Therefore, differences in target sites sequences that might affect the response to acaricide application could result from a phylogenetic distance and be naturally present. However, direct acaricide selection might act in some populations. Different life histories (herbivory versus carnivory) might have also selected for differences in detoxification machinery 102. The prevalence of nucleotide differences (mutations) in target-site sequences potentially involved in natural tolerance or evolved resistance was screened for in the assembled here *P. persimilis* transcriptome and sequences of other Phytoseiidae species available via public sequence repositories. We encountered a limited availability of genetic data that prevents a realistic estimate of the frequency of mutations in acaricide target sites in Phytoseiidae. However, even in the small sequence pool, we identified SNPs previously validated for their role in resistance to at least six out of nine acaricide/insecticide MoA groups considered in this study (Table 1, Figure 5). In several cases, a target site substitution was present in a proportion of all sequences available for a given Phytoseiidae species, suggesting that these SNPs could be the result of acaricide selection in some populations (Table 1).

In pro-acaricides such as bifenazate and cyflumetofen that must undergo metabolic conversion before becoming pharmacologically active 40,103, differential metabolism can result in selectivity 104. As the screening of the cytb did not reveal any SNPs associated with resistant phenotype in *P. persimilis*, the excellent selectivity of bifenazate observed in this study (Figure 2, Figure 3) might
rel on the inability to convert the pro-acaricide to a biologically active metabolite. Interestingly, the NCBI searches found bifenazate target site mutations in 12 other Phytoseiidae species. Mutation of a conserved and strongly validated residue S141 in the cd1 helix of cytb was especially ubiquitous across the genera. In the genus Typhlodromus, S141 is substituted with Phenylalanine as in *T. urticae*. But substitutions with Alanine, Valine, and Aspartic acid could also be found (Table 1). All the identified cytb sequences of *T. aripo*, *A. limonicus*, and *E. fustis* carried S141T (Table 1). Whether different substitutions of S141 are reflected in bifenazate resistance levels is not known.

Cyflumetofen, cyenopyrafen, and pyflubumide are relatively novel compounds with a new mode of action in the arthropods as inhibitors at Complex II (succinate dehydrogenase; Sdh) in the mitochondrial electron transport chain (METI II)\(^{39,105}\). The genetic basis of METI II resistance in *T. urticae* was clarified using quantitative trait locus (QTL) analysis, revealing that cyflumetofen and pyflumubide resistance was associated with I260T and I260V in SdhB, respectively. In contrast, cyenopyrafen resistance was mapped to SdhC carrying S56L substitution\(^ {15,106}\). We have identified the substitution I260 (I260M) in SdhB of *P. persimilis*, which may explain the high selectivity of cyflumetofen seen in this study (Figure 2, Figure 3, File S6). The same substitution has been found in sequences of *A. swirskii* available via NCBI (Table 1). The cyenopyrafen resistance mutation, S56L in SdhC was absent in all the examined Phytoseiidae species, but residue R74 (*T. urticae* numbering) known to bind ubiquinone at the Q2-site in *Escherichia coli*\(^ {81}\) was substituted with Serine in *P. persimilis*, and *A. swirskii*, suggesting that exact residues interacting with METI II acaricides may differ between *T. urticae* and *Phytoseiidae* species (Figure 5).

Newly discovered SNPs in ATP synthase subunits a (A195S, V116L and A213S, *T. urticae* numbering), and c (R109S, *T. urticae* numbering) of *P. persimilis*, *A. swirskii* and *M. occidentalis* are present at the conserved positions within proteins previously identified as binding sites of
organotin compounds. They could potentially decrease the efficiency of organotin binding, leading to resistance. However, their contribution to fenbutatin oxide insensitivity in *P. persimilis* must be verified beyond this article’s scope.

In mites and insects, mutations in CHS1 were previously reported and validated to be responsible for cross-resistance between acaricides Mite Growth Inhibitors (IRAC 10) and insecticides Chitin Synthase Inhibitors (IRAC 15) in *T. urticae* (I1017F), *P. xylostella* (I1017M) and *Culex pipiens* L. (I1043L,M,F). We identified an I1017M substitution in *P. persimilis* transcript coding for the CHS1 gene, the first report of this SNP in Phytoseiidae (Figure 5). However, no SNP was found at the corresponding positions in *M. occidentalis* and *A. swirskii* CHS1 sequences found via NCBI.

The kdr (L1014F) and super-kdr (M918V,L,T) found in VGSC of *P. persimilis* are well-known pyrethroid resistance mutations present in a wide range of insects. Within Tetranychidae, only *T. evansi* is known to carry the super-kdr, but no kdr mutation in *T. urticae*, instead of kdr and super kdr, target-site resistance relies on the presence of L1024V or F1538I. *P. persimilis* used in this study carried both insect kdr (L1014F) and super-kdr (M918V) instead of the mutations typical for pyrethroid-resistant *T. urticae* (Figure 5). Interestingly Benavent-Albarracin et al., 2020 have reported commercial strains of *P. persimilis* with super-kdr M918L but no kdr. Instead, L925V identified in *Varroa destructor* Anderson & Truema and previously not described mutations A1536T, S1539T, were present. VGSC sequences identified via NCBI belonging to *A. swirskii* were free of known pyrethroid-resistance mutations, but super-kdr substitution M918L was found in VGSC of a fenpropathrin-resistant *N. barkeri* (Table 1).

Interestingly, we have not found any known AChE mutations in the *P. persimilis* orthologue. However, a common organophosphate/carbamate resistance mutation, G119S, was identified in AChE sequences belonging to *M. occidentalis* and *K. aberrans* resistant to the carbaryl-organophosphate-sulfur and chlorpyrifos, respectively (Table 1). The lack of
organophosphate/carbamate resistance mutations in *P. persimilis* is surprising considering the widespread use of organophosphates/carbamates that has continued since the 1950s. It is perhaps a consequence of modern agricultural practices, increasingly relying on more selective compounds.\textsuperscript{11} NCBI searches resulted in identifying two interesting SNPs in a Spanish commercial Koppert strain of *A. swirskii* that were absent in this study's *P. persimilis* strain. A2083V in ACC and V88I in the PSST subunit of mitochondrial respiratory Complex I are reported for the first time in Chelicerata and Eukaryota, respectively (Table 1). The A2083V substitution was previously identified in Spanish field populations of *B. tabaci* and caused resistance to spirotetramat, which was thoroughly validated.\textsuperscript{73} Given that *A. swirskii* is often used to control whitefly populations and hence exposed to the same insecticides, the presence of the A2083V in both species is probably reflecting strong selection. In *T. urticae*, resistance to METI I acaricides depends partly on the presence of H92R in the PSST subunit.\textsuperscript{116,117} Although H92R was not identified in any Phytoseiidae species, residing four residues away, V88I makes a plausible candidate for the METI I target site resistance in *A. swirskii*. The residue is located in a highly conserved aa stretch previously photoaffinity labeled with METI I derivatives and has undergone extensive site-directed mutagenesis in *Y. lipolytica*, revealing its crucial role in quinone/inhibitor binding.\textsuperscript{62,63}

To conclude, bifenthrate, cyflumetofen and fenbutatin oxide, the representatives of acaricide classes targeting Complex III, II, and V of the mitochondrial electron transport chain, appear safe for the predatory mite *P. persimilis* irrespective of the route of exposure. In contrast, the botanical acaricides, orange oil and terpenoid blend seem much more toxic. Acute and sublethal effects were present via direct exposure, diet, and a combination of direct exposure, diet, and residual contact. Orange oil appears to be particularly harmful to *P. persimilis*. The transcriptomic analysis of selected combinations of acaricides and exposure routes revealed only a limited molecular
stress response, impeding the development of ecotoxicological biomarkers to detect signs of failing biological control. However, transcriptome analysis also allowed the identification of *P. persimilis* orthologues coding for acaricide target sites of acaricide classes frequently used to control *T. urticae*. Surprisingly, many known insecticide/acaricide target site mutations were readily identified in the studied *P. persimilis* strain, potentially explaining its resilience to the tested acaricides. The search for target-site mutations was extended by the Phytoseiidae sequences available via public databases, revealing that an insensitive target site may be a common resistance mechanism in predatory mites of agricultural importance. Several target site mutations reported here have not been previously identified in Phytoseiidae and await functional validation.

Acknowledgments

We thank Nicky Wybouw for his help in analyzing predatory mite transcriptome and Wannes Dermauw for sharing his insights into the phylogenetic analysis of CCEs. SB is a post-doctoral researcher supported by ERANET, C-IPM, grant no. 618110. TVL is supported by the Research Council (ERC) under the European Union’s Horizon 2020 research and innovation program [grant 772026–POLYADAPT and 773902–SUPERPEST].

Conflict of Interest Statement

The authors state no conflict

**Figure legends**

Graphical abstract-text


Sabina A. Bajda, Patrick De Clercq, Thomas Van Leeuwen
Acaricide selectivity towards Phytoseiidae may be achieved by limiting exposure routes and exploiting differences in acaricides’ molecular targets.

Figure 1. An overview of the experiments performed in this study. The predatory mite *Phytoseiulus perimilis* and *Tetranychus urticae* spider mites are depicted by red and green arthropods, not drawn to scale. A field dose of either bifenazate, fenbutatin oxide, cyflumetofene, orange oil or terpenoid blend was sprayed on two-day-old *P. persimilis* directly (SCC), or on *T. urticae* mites serving as a diet to the predator (CSC), or the whole experimental arena was sprayed while *P. persimilis* and *T. urticae* mites were residing on it (SSS). A water control accompanied each acaricide treatment (CCC). B. Fecundity and survival of individual predatory mites was monitored over four consecutive days. C. Orange oil and fenbutatin oxide were chosen to investigate differential gene expression in response to acaricide treatment. Two-day-old *P. persimilis* mites were fed with an acaricide-laced diet for two (orange oil) or four days (fenbutatin oxide). Subsequently, RNA was isolated from acaricide, and water-treated samples, and Illumina-sequenced. We assembled the transcriptome of *P. persimilis*, and differential gene expression analysis was performed between the acaricide and its relevant water-spray control.

Figure 2. Mean daily fecundity per female of *Phytoseiulus persimilis*, depending on acaricide treatment. A- fenbutatin oxide, B- bifenazate, C- cyflumetofen, D- terpenoid blend, E- orange oil, and the route of exposure, yellow – water sprayed control (CCC), gray – exposure via contaminated diet (CSC), green – direct exposure (SCC), brown – combined diet, direct and residual exposure (SSS). The numbers of eggs per day were compared between different delivery routes. Error bars represent standard error. Means labeled with the same letter are not significantly different.

Figure 3. Mean total fecundity of *Phytoseiulus persimilis* females vs. % surviving females after four days of exposure for all acaricides and acaricide delivery modes tested in this study. Circle-

Figure 4. PCA plot of gene expression levels of *Phytoseiulus persimilis* mites feeding on spider mite prey laced with fenbutatin oxide and orange oil and their corresponding water-treated controls. For each treatment, convex hulls were added for a more straightforward visual interpretation.

Figure 5. Comparison of partial amino acid sequences of acaricide target site proteins. CHS1: *Phytoseiulus persimilis* (contig_1573), *Tetranychus urticae* (tetur03g08510), *Musca domestica* (AFP61787.1), *Anopheles gambiae* (AAL23627.1), *Saccharomyces cerevisiae* (NP_009579.1), *Tribolium castaneum* (NP001034491.1), *Plutella xylostella* (BAF47974.1); VGSC: *M. domestica* (ATZ81482.1), *T. urticae* (tetur34g00970), *Metaseiulus occidentalis* (XP_028966827.1), *Bos taurus* (DAA30006.1), *Homo sapiens* (AAA18895.1), *P. persimilis* (contig_4927), *A. gambiae* (CAM12801.1); SdhB: *T. urticae* (tetur01g15710), *P. persimilis* (contig_236), *Escherichia coli* (REE06640.1), *B. taurus* (NP_001035573.1), *Homo sapiens* (NP_002991.2), *D. melanogaster* (NP_477101.1), *S. cerevisiae* (GAX71644.1); SdhC: *T. urticae* (tetur30g00210), *P. persimilis* (contig_7732), *H. sapiens* (CAG33383.1), *B. taurus* (NP_787008.1), *D. melanogaster* (NP_001262472.1), *S. cerevisiae* (AAT93043.1), *E. coli* (VWQ01513.1). Universally conserved residues are marked in bold. The position of the I1017M in CHS1, kdr and super-kdr in VGSC, R256S and I260M in SdhB, R74S in SdhC in *P. persimilis* are indicated in gray. Resistance mutations in SdhB and SdhC against fungicidal SDH inhibitors reported in a previous study are indicated by asterisks. Solid squares indicate the residues in SdhB and SdhC that may bind with ubiquinone at the Q2-site in *E. coli*. 
Open squares indicate resistance mutations previously reported in *T. urticae*[^15][^60]. The insect Kdr and super-kdr are indicated with open and closed circles, respectively[^74].

Figure S1. The total number of eggs laid by female predatory mites over four days, depending on the acaricide they were exposed to. A- bifenazate, B- cyflumetofen, C- fenbutatin oxide, D- orange oil, E- terpenoid blend, and the route of exposure. Values in each graph represent means, and error bars represent the standard error. Means labeled with the same letter are not significantly different.

Figure S2. Phylogenetic Identification of Phytoseiidae putative acetylcholinesterase sequences (AChE). Maximum likelihood tree of *Kampimodromus aberrans*, *Amblyseius swirskii*, *Metaseiulus occidentalis*, *Phytoseiulus persimilis*, and *Tetranychus urticae*, *Drosophila melanogaster*, *Apis mellifera*, *Bombyx mori* carboxylesterase (CCE) protein sequences. The phylogenetic tree was built using IQ-TREE v 2.0.3 with 1,000 ultrafast bootstrap combined with automatic model finding through ModelFinder Plus[^58]. The resulting trees were midpoint rooted and edited with MEGA v 10.2.4 software. Only bootstrap support values > 85% were indicated in the tree. Colour and shape codes are as follows: *K. aberrans*, blue circle, *A. swirskii*, purple circle, *M. occidentalis*, yellow circle, *P. persimilis*, green circle, *T. urticae*, red square, *D. melanogaster*, pink diamond, *A. mellifera*, black diamond, *B. mori*, gray diamond. The tree branch drew in green contains identified AChE sequences. Accession numbers and IDs of sequences that appear in the tree can be found in Table S10.

Figure S3. Phylogenetic Identification of Phytoseiidae putative voltage-gated sodium channels (VGSC). Maximum likelihood tree of *Neoseiulus barkeri*, *Amblyseius swirskii*, *Metaseiulus occidentalis*, and *Phytoseiulus persimilis* VGSC protein sequences. VGSC sequences of *Tetranychus urticae* and *Musca domestica* were included as a reference. The phylogenetic tree was built using IQ-TREE v 2.0.3 with 1,000 ultrafast bootstrap combined with automatic model
finding through ModelFinder Plus. The resulting trees were midpoint rooted and edited with MEGA v 10.2.4 software. Only bootstrap support values > 85% were indicated in the tree. Colour and shape codes are as follows: *N. barkeri*, brown circle, *A. swirskii*, purple circle, *M. occidentalis*, yellow circle, *P. persimilis*, green circle, *T. urticae*, red square, *M. domestica*, black diamond shape. Black arrow points at the branch with identified VGSC sequences. Accession numbers and IDs of sequences that appear in the tree can be found in Table S10.

Figure S4. Phylogenetic Identification of Phytoseiidae putative glutamate-gated chloride channels (GluCls). Maximum likelihood tree of *Amblyseius swirskii*, *Metaseiulus occidentalis*, *Phytoseiulus persimilis*, and *Tetranychus urticae* cys-loop ligand-gated ion channel protein sequences. The phylogenetic tree was built using IQ-TREE v 2.0.3 with 1,000 ultrafast bootstrap combined with automatic model finding through ModelFinder Plus. The resulting trees were midpoint rooted and edited with MEGA v 10.2.4 software. Only bootstrap support values > 85% were indicated in the tree. Colour and shape codes are as follows: *D. melanogaster*, pink diamond shape, *A. swirskii*, purple circle, *M. occidentalis*, yellow circle, *P. persimilis*, green circle, *T. urticae*, red square. Black arrow points at the branch with identified GluCl sequences. Accession numbers and IDs of sequences that appear in the tree can be found in Table S10.

Tables

Table 1. Occurrence and frequency of acaricide target site mutations in Phytoseiidae. Only SNPs that occur in at least one of the examined predatory mite species were listed. Mutations previously validated for their role in arthropod resistance to insecticides/acaricides are marked in bold.

Table S1. Comparison of the two *Phytoseiulus persimilis* transcriptome assemblies.

Table S2. Variance analysis of the daily fecundity data.
Table S3. P values of the log-rank pairwise comparisons between survival rates after treatment with orange oil and terpenoid blend. P values are Benjamini-Hochberg adjusted.

Table S4. List of contigs having a BLASTx hit (E^-3) with protozoa, viruses, bacteria, Tetranychus urticae, and other members of genus Tetranychus showing a mean sequence similarity value ≥ 98%, removed from the "40 million reads assembly".

Table S5. Annotation of the "40 million reads assembly" of Phytoseiulus persimilis.

Table S6. List of contigs having a BLASTx hit (E^-3) with protozoa, viruses, bacteria, Tetranychus urticae, and other members of genus Tetranychus showing a mean sequence similarity value ≥ 98%, removed from the "80 million reads assembly".

Table S7. Annotation of the "80 million reads assembly" of Phytoseiulus persimilis.

Table S8. Transcripts differentially expressed in Phytoseiulus persimilis females (log2FC>0, qvalue<0.05) after two days of consuming orange oil treated Tetranychus urticae mites, compared to the water-sprayed control (orange oil_c).

Contig names written in bold indicate upregulated transcripts, while contig names written with regular letters indicate the downregulated transcripts.

Table S9. Target sites of acaricides commonly used against Tetranychus urticae, their orthologues in Phytoseiulus persimilis, and the IRAC acaricide classes that target these proteins.

Table S10. Accession numbers and IDs of arthropod acaricide target-site proteins included in the alignments and phylogenetic analysis.

Files

File S1. Preprocessed "40 million assembly" of Phytoseiulus persimilis used to identify cytb sequence. Requires FASTA file reader.
File S2. "40 million reads" assembly of *Phytoseiulus persimilis* transcriptome before removing the contaminating sequences. Requires FASTA file reader

File S3. "80 million reads" assembly of *Phytoseiulus persimilis* transcriptome before removing the contaminating sequences. Requires FASTA file reader

File S4. Final "40 million reads" assembly of *Phytoseiulus persimilis* transcriptome. Requires FASTA file reader

File S5. Final "80 million reads" assembly of *Phytoseiulus persimilis* transcriptome. Requires FASTA file reader


References


42. Hothorn, T., Bretz, F. & Westfall, P. Simultaneous inference in general parametric models.


Figure 1.
Figure 2.
Figure 3.
Figure 4.
**Figure 5.**

<table>
<thead>
<tr>
<th>IRAC group</th>
<th>Target gene</th>
<th>SNP</th>
<th><em>P. persimilis</em></th>
<th>NCBI species (spanning the region/total seqs)</th>
<th>No of seq. With SNP, SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>Mitochondrial complex</td>
<td>PSST</td>
<td>V88M, L, F&lt;sub&gt;62-64&lt;/sub&gt;</td>
<td><em>M. occidentalis</em> (2/2)</td>
<td>1, V88I</td>
</tr>
<tr>
<td></td>
<td>I electron transport inhibitors</td>
<td>E140Q&lt;sup&gt;65&lt;/sup&gt;</td>
<td>-</td>
<td><em>M. occidentalis</em> (1/3)</td>
<td>1, E140D</td>
</tr>
<tr>
<td>25</td>
<td>SdhB</td>
<td>I260T/V</td>
<td>I260M</td>
<td>M. occidentalis (1/3)</td>
<td>A. swirskii (4/5)</td>
</tr>
<tr>
<td>----</td>
<td>------</td>
<td>---------</td>
<td>-------</td>
<td>----------------------</td>
<td>------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3, R145K</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>I260M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R256P</td>
<td>R256S</td>
<td>M. occidentalis (4/8)</td>
<td>A. swirskii (4/5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3, R256S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SdhC</td>
<td>R74S</td>
<td>R74S</td>
<td>M. occidentalis (2/4)</td>
<td>A. swirskii (2/2)</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2, R74S</td>
</tr>
<tr>
<td>20</td>
<td>cytb</td>
<td>G126S</td>
<td>-</td>
<td>N. litoralis (5/5)</td>
<td>1, G126A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A133T</td>
<td>-</td>
<td>T. pyri (22/36)</td>
<td>4, A133G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I136T</td>
<td>-</td>
<td>T. verrucosus (2/2)</td>
<td>1, I136T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S141F</td>
<td>-</td>
<td>T. aripo (3/3),</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G132A</td>
<td>-</td>
<td>T. setubali (9/9)</td>
<td>1, G132E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>AChE</td>
<td>G119S</td>
<td>-</td>
<td>M. occidentalis (2/2)</td>
<td>2, G119S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K. aberrans (2/2)</td>
<td>1, G119S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A. swirskii (1/1)</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>Inhibitors of acetyl CoA carboxylase</td>
<td>ACC</td>
<td>A2083V&lt;sup&gt;73&lt;/sup&gt;</td>
<td>-</td>
<td>M. occidentalis (2/7)</td>
</tr>
<tr>
<td>10</td>
<td>Mite growth inhibitors affecting CHS1, 15 Inhibitors of chitin biosynthesis affecting CHS1</td>
<td>CHS1</td>
<td>I1017L,M,F&lt;sup&gt;60,61,109&lt;/sup&gt; I1017M</td>
<td>A. swirskii (2/11)</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Sodium channel modulators</td>
<td>VGSC</td>
<td>L1014F (kdr)&lt;sup&gt;74&lt;/sup&gt; L1014F</td>
<td>M. occidentalis (0/1)</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Inhibitors of mitochondrial ATP synthase</td>
<td>ATP synthase subunit c</td>
<td>R109S</td>
<td>A. swirskii (8/8)</td>
<td>1, R109S</td>
</tr>
</tbody>
</table>