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Profiling of *Dermanyssus gallinae* genes involved in acaricide resistance

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ABSTRACT

The poultry red mite (PRM), *Dermanyssus gallinae*, is a major threat for the poultry industry worldwide. Chemical compounds have been extensively used for PRM control, leading to selection of resistant mites. Molecular mechanisms of resistance have been investigated in arthropods, showing the role of target-site insensitivity and enhanced detoxification. Few studies are available about those mechanisms in *D. gallinae*, and none have yet focused on the expression levels of detoxification enzymes and other defense-related genes through RNA-seq. We tested PRM populations from Italy for their susceptibility to the acaricidal compounds phoxim and cypermethrin. Mutations in the voltage-gated sodium channel (*vgsc*) and in acetylcholinesterase (*AChE*) were investigated, detecting mutations known to be associated with acaricide/insecticide resistance in arthropods, including M827I and M918L/T in the *vgsc* and G119S in the *AChE*. RNA-seq analysis was performed to characterize metabolic resistance in fully susceptible PRM and in cypermethrin-resistant PRM exposed and unexposed to cypermethrin as well as phoxim resistant PRM exposed and unexposed to phoxim. Detoxification enzymes (including P450 monooxygenases and glutathione-S-transferases), ABC transporters and cuticular proteins were constitutively overexpressed in phoxim and cypermethrin resistant mites. In addition, heat shock proteins were found constitutively and inductively upregulated in phoxim resistant mites, while in cypermethrin resistant mites esterases and an aryl hydrocarbon receptor were constitutively highly expressed. The findings suggest that acaricide resistance in *D. gallinae* is due to both target-site insensitivity and overexpression of detoxification enzymes and other xenobiotic defense-related genes, which is mostly constitutive and not induced by treatment. Understanding the molecular basis of resistance could be useful to screen or test PRM populations in order to select targeted acaricides and to avoid the abuse/misuse of the few available compounds.

1. Introduction

One of the main challenges for the poultry industry worldwide is infestation by the poultry red mite (PRM), *Dermanyssus gallinae* (De Geer, 1778). This hematophagous ectoparasite has serious impacts on hen health and welfare and infestation results in heavy economic losses, estimated up to 231 million euros per year in Europe (Sigognault Flochlay et al., 2017). Besides the direct effects on bird health (Sparagano et al., 2020), several infectious agents are associated with *D. gallinae* (Schiavone et al., 2022), with evidence the mite is able to transmit *Salmonella enterica* serovars Gallinarum (Cocciolo et al., 2020) and Enteritidis (Valiente Moro et al., 2007) and avian influenza virus (Sommer et al., 2016). For many years PRM infestations have been controlled using chemical compounds such as organophosphates (OPs), carbamates (CBs), and synthetic pyrethroids (SPs), some of which are not specifically labelled for their use with poultry (Abbas et al., 2014).

Abbreviations: PRM, poultry red mite; OP, organophosphate; CB, carbamate; SP, synthetic pyrethroid; P450, cytochrome P450 monooxygenases; GST, glutathione-S-transferase; CCE, carboxyl/cholinesterase; ABC, ATP-binding cassette; CP, cuticle protein; HSP, heat shock protein; Ahr, aryl hydrocarbon receptors; vgsc, voltage-gated sodium channel; AChE, acetylcholinesterase; EC, efficacy class; R, resistant; MR, moderately resistant; I, intermediate; S, susceptible; HS, highly susceptible; FS, fully susceptible; PR, phoxim resistant unexposed; PRExp, phoxim resistant exposed; CR, cypermethrin resistant unexposed; CRExp, cypermethrin resistant exposed; FDR, false discovery rate; DEG, differentially expressed gene; RIN, RNA integrity number; PCA, principal component analysis; GO, gene ontology; BP, biological process; MF, molecular function.

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Many of these acaricides have been withdrawn from market or even banned in European countries due to their associated health risks (Sigognault Flochlay et al., 2017). The acaricides affected by these measures include the OPs (dichlorvos, fenitrothion, chlorpyrifos, diazinon), CBs (carbaryl, methomyl, propoxur) and SPs (cyhalothrin) (Sigognault Flochlay et al., 2017). Starting from the early 2000s, specific compounds have been authorized for the use in poultry farms while hens are present, such as the OP phoxim ("Byemite", Bayer), spinosad ("Elector", Elanco) and, most recently, fluralaner ("Exzolt", MSD Animal Health).

Phoxim, the first acaricide labelled for the control of PRM, is widely used by commercial producers and is a preferred choice of chemical treatment due to its high efficacy and a zero day withholding period for eggs (Meyer-Kühling et al., 2007). Other acaricides, not specifically authorised for use in poultry farms, are widely used to control PRM. Examples include the SPs cypermethrin, deltamethrin, λ-cyhalothrin or permethrin, which are used for control of the house fly *Musca domestica* in poultry-rearing premises and for the control of PRM in empty poultry houses (Zdybel et al., 2011; Koç et al., 2022; Guerrini et al., 2022). As these acaricides are not authorised for PRM control, there are no standardised operative procedures and therefore these chemical treatments may be used sub-optimally (Guerrini et al., 2022). In such a framework, the improper use of these pesticides (e.g., underdosage, repeated treatments, spot applications inside the poultry house), has contributed to the selection of resistant *D. gallinae* populations, which have been reported in different countries (Zeman and Železný, 1985; Marangi et al., 2009; Zdybel et al., 2011; Abbas et al., 2014; Pugliese et al., 2019; Katsavou et al., 2020; Wang et al., 2021; Koç et al., 2022; Guerrini et al., 2022).

In other arthropods, resistance to acaricides/insecticides arises through genetic changes in the arthropod population that leads to modifications in the target site (genetic resistance), increased metabolism of the active chemical (metabolic resistance) and/or their reduced penetration (Van Leeuwen and Dermauw, 2016). Metabolic resistance to acaricides/insecticides is often associated with transcriptional induction of detoxification enzymes including cytochrome P450 monooxygenases (P450s), glutathione-S-transferases (GSTs), and carboxyl/cholinesterases (CCEs) (Van Leeuwen et al., 2010). Other xenobiotic defence mechanisms reported in resistant arthropod species include reduced penetration of acaricides/insecticides through their efflux from cells by means of ATP-binding cassettes (ABCs) transporters, as well as cuticular thickening due to increased cuticle protein (CP) expression (Van Leeuwen et al., 2010; Mamidala et al., 2012). In addition, protective stress-responsive factors, such as heat shock proteins (HSPs), have been shown to be involved in acaricide/insecticide resistance (De Marco et al., 2017; Dumas et al., 2019). Moreover, transcription factors as aryl hydrocarbon receptors (Ahr) have been suggested to act as sensors of chemicals, promoting the transcription of genes involved in detoxification, such as GSTs and P450s (Peng et al., 2017; Zhang et al., 2019).

Previous studies have shown genetic resistance in PRM towards SPs and OPs is associated with point-mutations in their target sites, which includes voltage-gated sodium channel (*vgsc*) and acetylcholinesterase (*AChE*), respectively (Katsavou et al., 2020; Koç et al., 2022). The mode of action of SPs and OPs differs, with OPs inhibiting AChE (Abbas et al., 2014), while SPs binds and inhibits insect and mite vgsc (Field et al., 2017). In addition, in resistant PRM populations, metabolic resistance is likely mediated through detoxification enzymes that include GSTs (Bartley et al. 2015, Wang et al., 2021) and P450s (Wang et al., 2020). In our current study, we extend this analysis by investigating the genetic and metabolic mechanisms of resistance to phoxim (an OP) and cypermethrin (a SP) in PRM populations from Italy. First, we identified populations with varying levels of susceptibility to both classes of acaricides and used differential RNA-seq analysis between susceptible and resistant populations to identify differentially expressed genes that are likely to be associated with acaricide resistance. In addition we used target sequencing of *AChE* and *vgsc* to identify mutations associated with resistance to phoxim and cypermethrin, respectively.

2. Materials and methods

2.1. Mite populations

Eleven field populations of *D. gallinae* were collected from laying hen farms located in different provinces of the regions Apulia and Campania (Italy) (Fig. 1 and Table 1). Once collected, the mites were starved at room temperature in 5 % CO_2 for 5 days, and then used for the laboratory bioassays.

2.2. Efficacy test

Mite populations were tested against phoxim (Sebacil, Bayer Animal Health, Germany) and cypermethrin (Bio-Revanol, Sandokan, Italy). The mites were exposed to a two-fold concentration of the recommended dose; 8 g/L for phoxim and 2000 ppm of cypermethrin in distilled water.

Drug efficacy was evaluated with a filter paper assay, as previously described (Pugliese et al., 2019). Briefly, mite "cells" were obtained with two squares of filter paper attached each on a plastic plate with a hole cut in its centre. The filter paper was impregnated with 200 μL of acaricide solution or distilled water for the control cells, and 20 adult mites were placed in the center of the square. The plastic layers were then sealed together using vinylic glue along the edge of the central hole. Once prepared, the "cells" were incubated for 24 h at 20 °C. Subsequently, they were opened, and the mites were observed, counting the number of live and dead individuals in each "cell".

The percent efficacy of the acaricides was assessed according to Abbott's formula (Abbott, 1925):

$$E = \frac{(\%\text{living mites in control cells} - \%\text{living mites in test cells})}{\%\text{living mites in control cells x } 100}$$

For each population and drug, efficacy data was grouped in five efficacy classes (EC), namely resistant (R, 0–20 % mortality), moderately resistant (MR, 21–40 %), intermediate (I, 41–60 %), susceptible (S, 61–80 %), and highly susceptible (HS, 81–100 %), as previously described (Pugliese et al., 2019).

2.3. Total RNA extraction and RNA-seq analysis

2.3.1. Mite population selection

Among the populations tested, three were further selected for RNA-seq analysis, based on the results of the efficacy tests: fully susceptible (BA1), phoxim resistant (LE4), and cypermethrin resistant (CAS). In total, from those 3 populations, 5 sub-groups were obtained, including: fully susceptible (FS = BA1), LE4 phoxim resistant unexposed (PR), LE4 phoxim resistant exposed (PRExp), CAS cypermethrin resistant unexposed (CR) and CAS cypermethrin resistant exposed (CRExp). Specifically, at least 5 biological replicates (n = 5) with 50–100 specimens each, were obtained for each group and stored at −80 °C prior to RNA extraction.

2.3.2. RNA extraction

Total RNA was extracted from 5 biological replicates from each of the 5 groups, for a total of 25 samples. Mite samples were homogenized in 600 μL of RLT buffer (Qiagen, Hilden, Germany) containing β-mercaptoethanol with sterile pestles, and RNA extracted using a RNeasy mini kit (Qiagen) with the optional on-column DNase digest. Total RNA was eluted in 30 μL nuclease-free water, according to the manufacturers protocol. The extracted total RNA was quantified using a Multiskan SkyHigh Microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and stored at −80 °C.

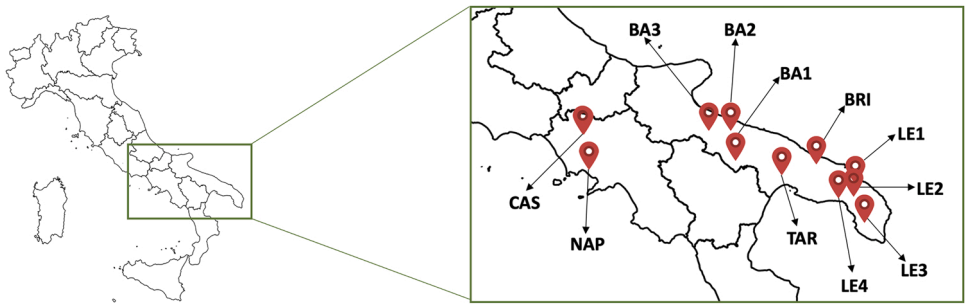

Fig. 1. *Dermanyssus gallinae* sampling sites. Map of Italy showing the location of the eleven sampling sites that were used for PRM collection from which populations of phoxim and cypermethrin resistance mites were identified.

Table 1
Geographic origin and collection dates of the 11 *D. gallinae* populations used in this study.

Isolate	Origin	Housing system	Collection date
CAS	Caserta, Campania, Italy	Aviary	Feb. 2022
NAP	Naples, Campania, Italy	Cage	Feb. 2022
BA1	Bari, Apulia, Italy	Cage	Feb. 2022
LE1	Lecce, Apulia, Italy	Cage	Mar. 2022
BRI	Brindisi, Apulia, Italy	Cage	Mar. 2022
LE2	Lecce, Apulia, Italy	Cage	Apr. 2022
LE3	Lecce, Apulia, Italy	Free-range	Apr. 2022
BA2	Bari, Apulia, Italy	Cage	Apr. 2022
BA3	Bari, Apulia, Italy	Free-range	Apr. 2022
TAR	Taranto, Apulia, Italy	Cage	Mar. 2022
LE4	Lecce, Apulia, Italy	Cage	Mar. 2022

2.3.3. RNA-seq analysis

All library synthesis and sequencing were performed by BGI Tech Solutions (Hong Kong). Only one sample was considered not valid for RNA-seq analysis, due to low quality of the RNA sample. Therefore, a total of 24 libraries were constructed (Supplementary Table 1). In brief, dual-indexed, strand-specific RNA-seq libraries were constructed from submitted total RNA sample. The barcoded individual libraries were pooled and sequenced on a DNBSEQ platform (Paired-end, 2 × 150 bp sequencing), generating on average 80 million reads per sample.

2.3.4. Bioinformatic analysis

Following sequencing, adaptors were trimmed from raw data and low-quality sequences removed using SOAPnuke software developed by BGI (with the following parameters: -n 0.001 -l 20 -q 0.4 –adaMR 0.25 –ada_trim –minReadLen 150) (Chen et al., 2018). Sequence reads were checked for quality using FastQC v0.11.7. Reads were pseudo-aligned to the draft *D. gallinae* transcriptome ($n = 14{,}608$ coding sequences) (Burgess et al., 2018) using Kallisto v0.46.2 with default settings (Bray et al., 2016) resulting in an average mapping rate of 44.20 % matched reads. Read abundance generated by Kallisto as transcripts per million (TPM) was exported as a read count matrix, which was trimmed, removing genes with read sum counts <100 TPM. Gene expression data were analysed by principal component analysis (PCA) using pcaExplorer v2.23.0 R/Bioconductor package (Marini and Binder, 2019). For RNA-seq analysis, Kallisto generated estimated count data was imported into the DESeq2 package using the tximport package (Soneson et al., 2015) and then normalised within the DESeq2 package using the median of ratios method "estimateSizeFactors()"(Love et al., 2014). Differential expression analysis was made by performing six pairwise comparisons between the following datasets: fully susceptible (FS) v phoxim resistant unexposed (PR); FS v phoxim resistant exposed (PRExp); PR v PRExp; FS v cypermethrin resistant unexposed (CR); FS v cypermethrin resistant exposed (CRExp); CR v CRExp and also included false discovery rate (FDR) correction for multiple testing comparisons (Benjamini and Hochberg, 1995). Genes were considered differentially expressed with log2 fold change >±2 and an FDR adjusted p-value <0.05. For annotation, the obtained differentially expressed genes (DEGs) datasets were imported into OmicsBox v.2.2.0 (Biobam, Spain) and annotated using BLAST2GO. To identify shared DEGs between treatments, lists of DEGs for phoxim resistant and cypermethrin resistant mites were visualised using a three-way Venn/Euler diagram, generated using InteractiVenn (Heberle et al., 2015).

2.4. Screening of target-site mutations

2.4.1. In vitro feeding

Adult female mites from LE4 and CAS resistant isolates, which had survived after treatment with phoxim or cypermethrin, respectively, were collected and blood-fed in vitro. Briefly, chicken blood samples were collected into heparin tubes (APTACA, Italy), with a final concentration of 20 units/mL blood. Feeding devices were constructed using a Baudruche membrane (Preservation Equipment Ltd, Diss, UK), as previously described (Nunn et al., 2020). Up to 50 adult females were transferred into each device, with 250 µL of heparinized blood in the upper reservoir, and the devices were incubated in the dark at 39 °C and 85 % relative umidity (RH) for 3 h. Fully engorged mites, distinguishable by the presence of internalized blood, were transferred in a 48-well tissue culture plate (APTACA) sealed with a breathable AeraSeal tape (Sigma-Aldrich Co. Ltd., Dorset, UK) and incubated at 30 °C and 85 % RH for 5 days. After incubation, the protonymphs generated by the adult mites were collected and stored at −80 °C prior to RNA extraction.

2.4.2. RNA extraction and cDNA synthesis

Total RNA was extracted from the protonymphs ($n = 25$ for CAS, $n = 3$ for LE4) and additional mite samples obtained during the efficacy tests (Supplementary Table 2) using the RNeasy mini kit (Qiagen), as described in Section 3.2. For cDNA synthesis, 10 µL of each RNA sample was reverse transcribed using the SuperScript IV Reverse Transcriptase (Invitrogen, Waltham, MA, United States) with an oligo$(dT)_{18}$, according to the manufacturers protocol.

2.4.3. Amplification and sequencing of vgsc and AChE

The regions of the domains IIS4-IIS5 (145 bp) and IIIS6 (237 bp) of the *vgsc* were amplified using primer pairs previously described (Katsavou et al., 2020). Primers for *AChE* (1580 bp) were designed based on the results of alignment of *AChE* from *D. gallinae* (DEGAL3446g00020, accessible at https://bioinformatics.psb.ugent.be/orcae/overview/Degal), *Varroa destructor* (Accession no. XP_022645444.1), *Varroa jacobsoni* (Accession no. XP_022690113.1), *Tropilaelaps mercedesae* (Accession no. OQR75606.1) and *Kampimodromus aberrans* (Accession no. CCV19958.1). Regions that were conserved across all species in the alignment were used for primer design (Supplementary Table 3).

PCR reactions were performed in a total volume of 50 µL containing 5 µL cDNA, 2.5 µL of both forward and reverse primers (final concentration 0.5 µM), 27 µL nuclease-free water, 10 µL 5X Phusion GC Buffer, 1.5 µL DMSO, and 0.5 µL Phusion High-Fidelity DNA Polymerase

(Thermo Fisher Scientific). Cycling conditions were as follows: 98 °C for 30 s; 35 cycles of 10 s 98 °C, 30 s 66.5 °C, 8 s 72 °C for *vgsc* IIS4-S6, 35 cycles of 10 s 98 °C, 30 s 66.5 °C, 8 s 72 °C for *vgsc* IIIS6, and 40 cycles of 10 s 98 °C, 30 s 67 °C, 90 s 72 °C for *AChE*; a final hold of 10 min at 72 °C. Amplification products were analyzed on a 1 % (*w/v*) agarose/TAE gel to determine size. PCR products were cloned into pJET1.2 using the CloneJET PCR Cloning kit (Thermo Fisher Scientific) and transformed into chemically competent JM109 *Escherichia coli* cells (Promega) and selected on Luria-Bertani (LB) agar plates with 100 µg/mL ampicillin, incubated overnight at 37 °C. Colony PCR was performed on up to 5 randomly selected colonies for each sample using pJET1.2-F (5′-CGACTCACTATAGGGAGAGCGGC-3′) and pJET1.2-R (5′-AAGAACATCGATTTTCCATGGCAG-3′). Each 50 µL PCR reaction contained template DNA (100 ng), 1 U Platinum™ Taq DNA Polymerase (Thermo Fisher Scientific), 10X PCR Buffer, 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.2 µM of each primer. Cycling conditions were: 94 °C for 5 min; 20 cycles of 45 s at 94 °C, 30 s at 55 °C, 45 s at 72 °C; 5 min at 72 °C. PCR products were analysed on a 1 % (*w/v*) agarose/TAE gel and colonies containing the expected insert size were grown overnight in 10 mL LB broth with 100 µg/mL ampicillin at 37 °C. Plasmid DNA was isolated from each clone using Wizard® Plus SV Minipreps DNA Purification System (Promega). A total of 60 individual clones were sequenced at Eurofins Genomics Germany GmbH with pJET1.2-F and pJET1.2-R primers. Due to the large size of the *AChE* amplicon (1580 bp), an additional primer pair was used for sequencing, including: AChE_fwd_seq (5′-ATGGACCAGGTGATGGCGC-3′) and AChE_rev_seq (5′-CTCGAATATGATTGCCTCATTCACC-3′) (606 bp). Sequence data were analysed using CLC Genomics Workbench v22 (Qiagen).

2.4.4. VGSC and AChE sequences from RNA-seq data

Sequence reads from one RNA sample for each condition were mapped against the reference genes of *AChE* (DEGAL3446g00020) and *vgsc* (DEGAL3828g00150) of *D. gallinae*, using Minimap2 v2.22-r1101 (Li, 2018). The mapped reads were extracted with SAMtools v1.14 (Danecek et al., 2021) and gene sequence variations were detected using BCFtools v1.15.1 (Danecek et al., 2021).

3. Results

3.1. Efficacy test

PRM were collected from 11 regions from southern Italy (Fig. 1 and Table 1) and analysed for their sensitivity to phoxim and cypermethrin. Among the PRM isolates analyzed for their susceptibility to phoxim, no isolates were found to be highly resistant to that drug (0 %), while one isolate was moderately resistant (9 %), 2 isolates were intermediate (18 %), 4 isolates susceptible (36 %) and 3 isolates highly susceptible (27 %) (Table 2). In contrast, most of the isolates were resistant to cypermethrin (54 %), one isolate was moderately resistant (9 %), 2 isolates were intermediate (18 %), no isolates were susceptible (0 %) and 2 isolates were highly susceptible (18 %) (Table 2).

Table 2
Percent efficacy and efficacy classes (EC) of phoxim and cypermethrin on the analyzed isolates of Dermanyssus gallinae. EC are resistant (R), moderately resistant (MR), intermediate (I), susceptible (S) and highly susceptible (HS).

	Phoxim			Cypermethrin		
Isolate	n^a	% Efficacy	EC	n^a	% Efficacy	EC
CAS	60	59	I	620	17	R
NAP	60	78	S	60	25	MR
BA1	340	82	S	200	98	HS
LE1	80	77	S	60	41	I
BRI	60	100	HS	60	55	I
LE2	60	82	HS	60	18	R
LE3	60	92	HS	60	7	R
BA2	1580	75	S	60	44	R
BA3	60	31	MR	60	55	R
TAR	220	70	S	60	100	HS
LE4	900	56	I	40	0	R

[a] Number of mites tested.

3.2. Transcriptional analysis of susceptible and resistant mites

The RNA integrity numbers (RIN) for the 24 samples used for RNA-seq ranged from 7.1 to 8.9, with an average value of 8 (Supplemetary Table 1). Expression profiles of the 24 mite samples were initially analysed by principal component analysis (PCA) (Fig. 2). In summary, biological replicates (n = 4 – 5) from each treatment group clustered together (Fig. 2). Principal component 1 (PC1) accounted for 49.82 % of the variance in the dataset, and separated mites on their exposure to acaricide, with phoxim resistant mites (both exposed and unexposed) clustering separately from cypermethrin resistant mites (both exposed and unexposed). In PC1, fully susceptible mites formed a distinct cluster, that was close to, but distinct from, cypermethrin resistant mites (Fig. 2). Principal component 2 (PC2), accounted for 23.97 % of the variance in the dataset and separated phoxim resistant mites on their exposure to acaricide, with phoxim exposed and unexposed mites clustering separately. In contrast, PC2 did not separate cypermethrin resistant mites that were either exposed or unexposed to acaricide (Fig. 2).

To assess whether acaricide resistant PRM populations show different gene expression levels compared to susceptible PRM, we used differential RNA-seq analysis to perform a series of pairwise comparisons between resistant and susceptible populations. In total, we ran 6 pairwise comparisons, these included: fully susceptible (FS) v phoxim resistant unexposed (PR) [996 DEGs identified]; FS v phoxim resistant exposed (PRExp) [711 DEGs identified]; PR v PRExp [396 DEGs identified]; FS v cypermethrin resistant unexposed (CR) [814 DEGs identified]; FS v cypermethrin resistant exposed (CRExp) [412 DEGs identified]; CR v CRExp [58 DEGs identified] (Fig. 3). A complete list of the identified DEGs is presented in Supplementary File 1.

To identify genes that were potentially involved in resistance to phoxim and cypermethrin, we searched each list of DEGs for genes that were: i) upregulated in resistant mites relative to fully susceptible mites

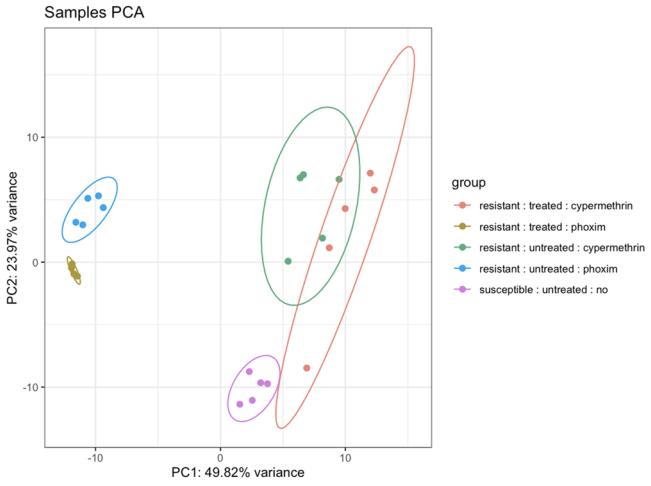

Fig. 2. Principal component analysis (PCA). The PCA plot shows the distribution of the most variant genes along the analyzed groups. The first (PC1) and the second (PC2) principal components represent the two uncorrelated directions along which the variation of the data is maximal. The sample groups are indicated in the key and include: mites resistant to cypermethrin surviving after exposure to the drug (CRExp; red), mites resistant to phoxim surviving after exposure to the drug (PRExp; olive green), mites resistant to cypermethrin not exposed to acaricides (CR; green), mites resistant to phoxim not exposed to acaricides (PR; blue), mites fully susceptible to the tested drugs (FS; purple). Ellipses indicates 95 % confidence intervals for each group.

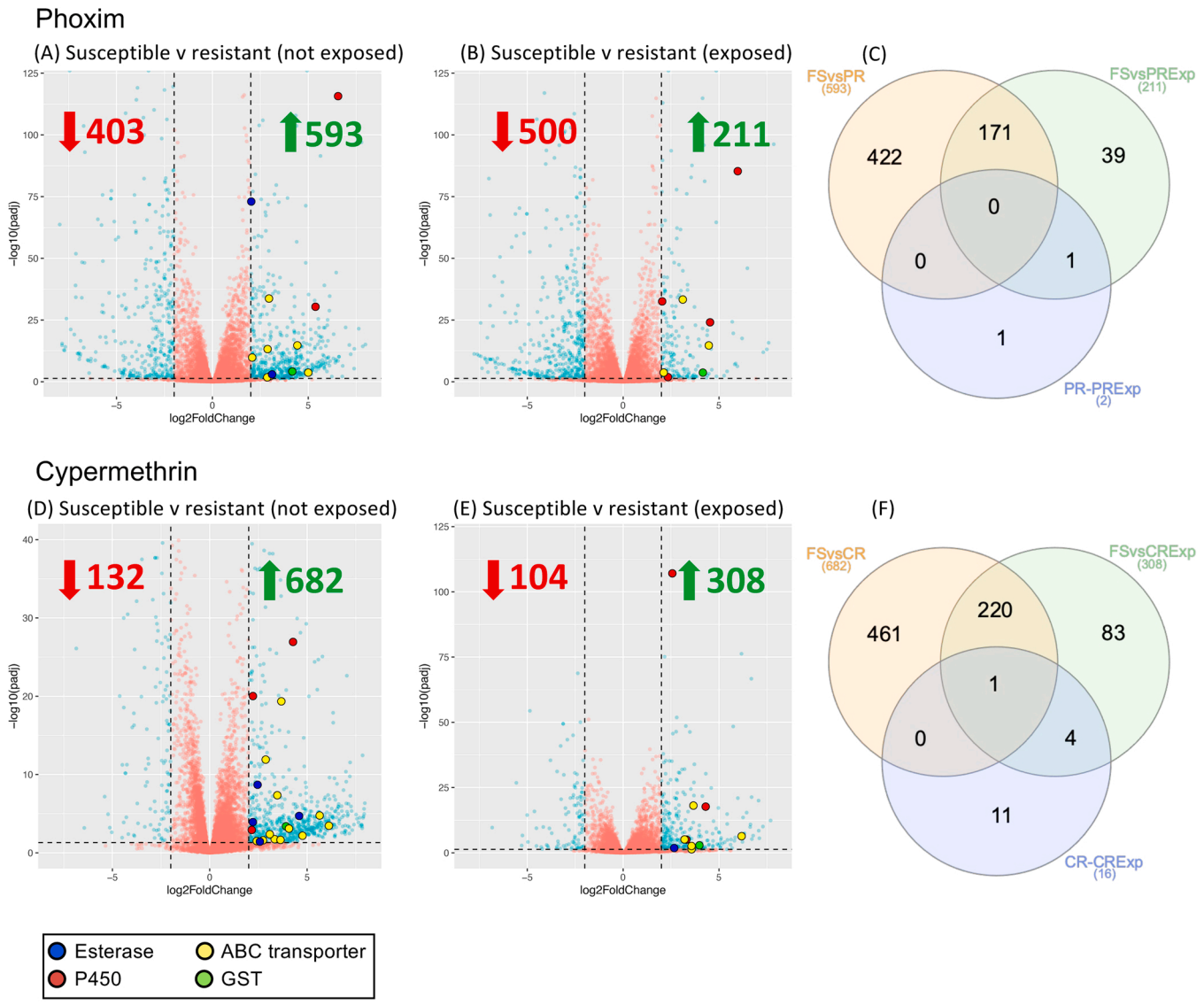

Fig. 3. Differentially expressed genes in susceptible and resistant *Dermanyssus gallinae* populations. (A and B) Volcano plot showing differentially expressed PRM genes from: (A) fully susceptible (FS) v phoxim resistant not exposed to acaricides (PR) and (B) fully susceptible (FS) v phoxim resistant exposed to acaricides (PREXp). (C) Three-way Venn/Euler diagram of the upregulated DEGs in FS v PR; FS v PREXp and PR v PREXp (D and E) Volcano plot showing differentially expressed mite genes from: (D) fully susceptible (FS) v cypermethrin resistant not exposed to acaricides (CR) and (E) fully susceptible (FS) v cypermethrin resistant exposed to acaricides (CREXp). (F) Three-way Venn/Euler diagram of the upregulated DEGs in FS v CR; FS v CREXp and CR v CREXp. In each volcano plot DEGs are *P*-value <0.05 and Log2 fold change >2 and the number of up- and down-regulated DEGs are indicated. Identified DEGs classified as potential detoxification enzymes are highlighted.

or upregulated in response to acaricide treatment and ii) are likely to be involved in acaricide detoxification (i.e., genes that encode P450s, GSTs, CCEs and ABC transporters) or stress-protection (i.e., genes that encode CPs and HSPs). In summary, comparison of fully susceptible mites with phoxim resistant mites identified up regulation of the following detoxification genes in resistant mites: P450 (DEGAL6570g00040; DEGAL3713g00020; DEGAL2439g00050; DEGAL3402g00020); GST (DEGAL6027g00010); CCEs (DEGAL5617g00040; DEGAL5617g00080) and ABC transporters (DEGAL425g00060; DEGAL1782g00320; DEGAL1782g00340; DEGAL279g00010; DEGAL279g00020; DEGAL2038g00040; DEGALg2038g00050; DEGAL2489g00020) (Fig. 3 and Supplementary Table 4). In addition we identified three HSPs and fourteen CPs that are potentially involved in stress-protection in phoxim resistant mites (Supplementary Table 4). To identify genes that are upregulated on exposure to acaricide we compared phoxim resistant mites that were either unexposed or treated with acaricide (PR v PREXp). This comparison identified two genes that were upregulated in response to acaricide treatment, which included: voltage-dependent calcium channel gamma-7 subunit-like (DEGAL4321g00060) and heat shock 70 kDa protein cognate 4 (DEGAL5756g00010) (Supplementary File 1).

A similar set of comparisons were performed for cypermethrin resistant mites. Comparison of fully susceptible mites with cypermethrin resistant mites identified upregulation of the following genes detoxification genes in resistant mites: P450 (DEGAL3713g00020; DEGAL3402g00020; DEGAL4935g00010; DEGAL4935g00030); GST (DEGAL6027g00010); CCE (DEGAL5617g00040; DEGAL2755g00080; DEGAL5617g00080; DEGAL2755g00090) and ABC transporters (DEGAL425g00060; DEGAL1782g00340; DEGAL279g00020; DEGAL2038g00040; DEGAL1782g00320; DEGAL2038g00050; DEGAL2489g00020; DEGAL755g00010; DEGAL2489g00030; DEGAL2357g00010; DEGAL3857g00010; DEGAL4055g00040; DEGAL6449g00010) (Fig. 3 and Supplementary Table 4). In addition we identified two HSPs, eight CPs and a single Ahr gene that may be

involved in stress-protection in cypermethrin resistant mites (Supplementary Table 4). To identify genes that are upregulated on exposure to acaricide we compared cypermethrin resistant mites that were either unexposed or treated with acaricide. This analysis identified induction sixteen transcripts, none of which encoded P450s, GSTs, CCEs, ABC transporters, HSPs, CPs or Ahr (see Supplementary File 1).

To identify gene that are associated with resistance we conducted a Venn analysis of the gene lists derived from each of the pairwise comparisons to identify genes that were either expressed at high levels in resistant mites (independent of acaricide exposure) or were induced in response to acaricide exposure (Fig. 3, panel C and F). In phoxim and cypermethrin resistant mites, 171 genes and 220 genes were upregulated relative to fully susceptible mites, respectively (Fig. 3). Exploration of these gene sets using GO analysis reveals "cellular macromolecule catabolic process" and "transmembrane transport" are the top GO terms for biological process (BP) in phoxim and cypermethrin resistant mites, respectively. The top 30 GO terms for BP and molecular function (MF) in phoxim and cypermethrin resistant mites is shown in Supplementary Figure 1. Furthermore, this analysis identified P450s, GSTs, ABC transporters, HSPs and CPs that are constitutively highly expressed in phoxim resistant mites, independent of acaricide exposure (Fig. 3), while the upregulation of a HSP gene was induced by exposure to acaricide. Likewise, in cypermethrin resistant mites P450s, GSTs, CCEs, ABC transporters, CPs and a Ahr were constitutively highly expressed, while no detoxification enzymes or defensive factors were present among the four drug induced genes. This suggests that phoxim and cypermethrin resistant PRM are not inducing detoxification enzymes in response to acaricide exposure, but instead that resistant mites express detoxification enzymes and other protective/stress-related genes at higher levels relative to drug sensitive mites.

3.3. Screening of target-site mutations

Target-site mutations were screened in the *vgsc* and *AChE* regions amplified by PCR from the isolates examined during the efficacy tests. Additionally, the composite sequences obtained by mapping the RNA-seq reads from 3 isolates to the entire reference sequences of *vgsc* and *AChE* were aligned. In total, six PRM isolates and 14 conditions were analysed for *vgsc*, including adults and protonymphs, while 6 isolates and 10 conditions were considered for *AChE*, with only adult mites. Several variable nucleotide sites, including inter- and intra-isolate polymorphisms, were found in the tested PRM isolates, resulting in a total of 12 amino acid substitutions in *vgsc* and 5 in *AChE* (Fig. 4, Tables 3 and 4).

In the *vgsc*, of the analysed PRM isolates, 12 mutations were identified overall: A70S, I249M, M827I, I874V, M918L/T, L925V, Q1387H, N1420H, L1439P, V1529L, F1537L, F1538L (*M. domestica vgsc* numbering). To summarise the data presented in Table 3, mites that were resistant to cypermethrin (EC code, R, resistant) are associated with mutations in domains II and III of the *vgsc* gene that are known to confer acaricide resistance. Other novel mutations were identified in cypermethrin resistant mites (for example, V1529L, F1537L), but it

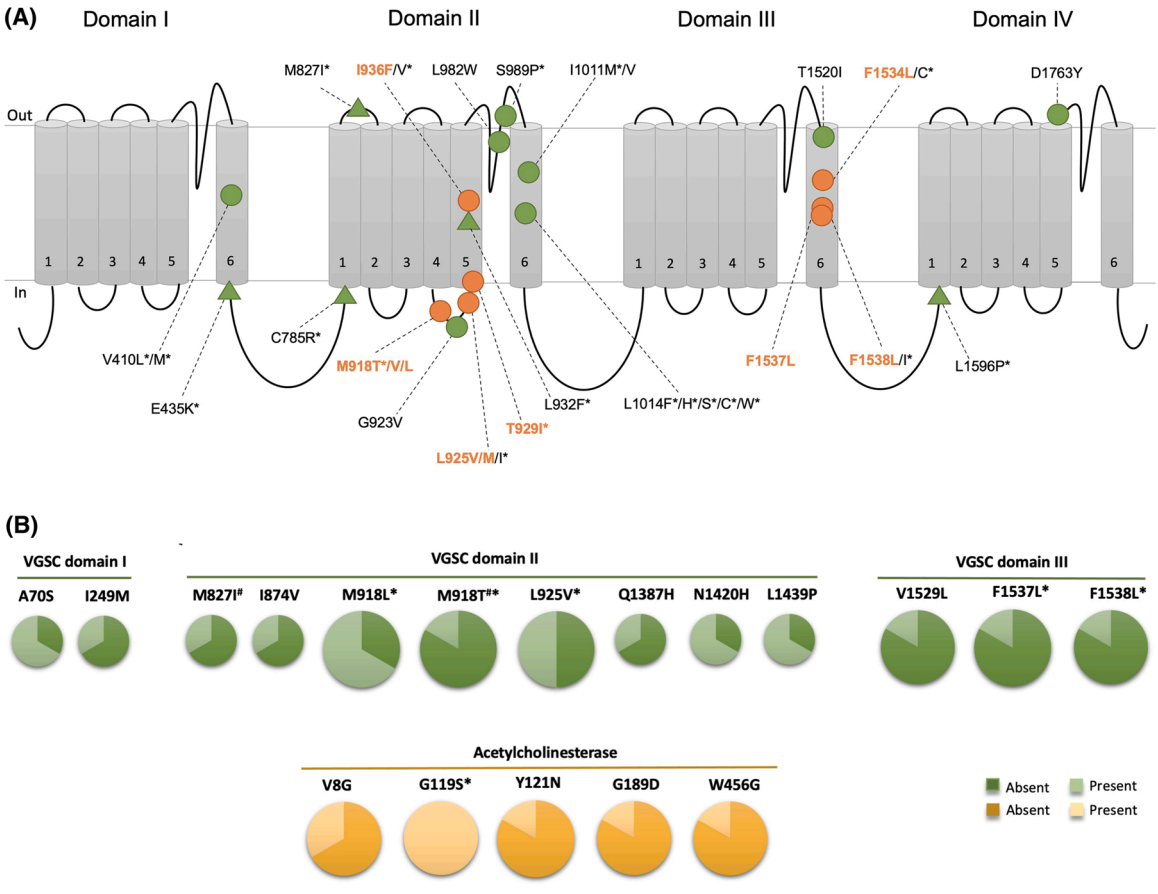

Fig. 4. Mutations in voltage-gated sodium channel and acetylcholinesterase. (A) Mutations associated with pyrethroid resistance in arthropod species. Orange circles and text indicate mutations previously detected in *Dermanyssus gallinae* (Katsavou et al., 2020; Koc et al., 2021). Green circles indicate mutations found in arthropods other than *D. gallinae* (triangles for only one species) (Dong et al., 2014; Du et al., 2016). Substitutions functionally validated in *Xenopus* oocytes are marked by an asterisk. (B) Mutations detected in voltage-gated sodium channel (vgsc) and acetylcholinesterase of 11 *Dermanyssus gallinae* isolates analyzed in this study. The size of the pie charts reflects the number of isolates investigated; the positions in larger charts include sequences obtained from targeted PCRs and RNA-seq data (6 isolates) and in smaller charts those from RNA-seq data only (3 isolates). *Mutations previously described in resistant *Dermanyssus gallinae* isolates (Katsavou et al., 2020; Koc et al., 2022). #Mutations associated with knockdown resistance in arthropod species, functionally confirmed in *Xenopus* oocytes (Dong et al., 2014).

Table 3
Amino acid mutations in the voltage-gated sodium channels (*vgsc*) between reference (OrcAE database: DEGAL3828g00150) and tested isolates of *Dermanyssus gallinae*. Numbers refer to the position in *vgsc* of *Musca domestica* (accession number: AAB47604.1), except for Q1387H, N1420H and L1439P, whose number is referred to the position in *vgsc* of *D. gallinae*. The non-wild type amino acid is indicated with bold underlined font.

Isolate	Condition	EC[a]	Domain I		Domain II							Domain III		
			A70S	I249M	M827I[b]	I874V	M918L/T*[b]	L925V[c]	Q1387H	N1420H	L1439P	V1529L	F1537L	F1538L[c]
CAS	Adults not exposed to acaricides	R	A	**M**	M	**V**	**L**	L	**H**	N	L	V	F	F
	Adults survived after exposure to cypermethrin	R	**S**	I	M	I	**L**	**V**	Q	**H**	**P**	V	F	F
	Protonymphs from adults exposed to cypermethrin	R	-	-	-	-	**L**	**V**	-	-	-	V	F	F
BA1	Adults not exposed to acaricides	HS	A	I	M	I	M	L	Q	N	L	V	F	F
	Adults survived after exposure to phoxim	HS	-	-	-	-	M	**V**	-	-	-	**L**	F	F
BA2	Adults not exposed to acaricides	R	-	-	-	-	**L**	L	-	-	-	V	F	F
	Adults survived after exposure to phoxim	R	-	-	-	-	**L**	L	-	-	-	V	F	F
BA3	Adults not exposed to acaricides	R	-	-	-	-	**L/T**	L	-	-	-	V	F	**F/L**
	Adults survived after exposure to phoxim	R	-	-	-	-	**L**	L	-	-	-	V	F	F
TAR	Adults not exposed to acaricides	HS	-	-	-	-	M	L	-	-	-	V	F	F
	Adults survived after exposure to phoxim	HS	-	-	-	-	M	L	-	-	-	V	F	F
LE4	Adults not exposed to acaricides	R	A	I	**I**	I	**L**	L	Q	N	L	V	**F/L**	F
	Adults survived after exposure to phoxim	R	**S**	I	M	I	**L**	**V**	Q	**H**	**P**	V	F	F
	Protonymphs from adults exposed to phoxim	R	-	-	-	-	**L**	L	-	-	-	V	F	F

[a] Efficacy classes (EC) are resistant (R), moderately resistant (MR), intermediate (I), susceptible (S) and highly susceptible (HS).
[b] Mutations associated with knockdown resistance in arthropod species, functionally confirmed in *Xenopus* oocytes (SupYoon et al., 2008; Dong et al., 2014).
[c] Mutations previously described in *Dermanyssus gallinae* isolates resistant to pyrethroids (Katsavou et al., 2020; Koc et al., 2022)

Table 4
Amino acid mutations in the acetylcholinesterase between reference (OrcAE database: DEGAL3446g00020) and tested isolates of *Dermanyssus gallinae*. Numbers refer to the position in mature AChE1 of *Torpedo californica* (accession number: CAA27169) (Massoulié et al., 1992). Numbers in brackets refer to the position in *D. gallinae* AChE1 (DEGAL3446g00020). The non-wild type amino acid is indicated with bold underlined font.

Isolate	Condition	EC[a]	Acetylcholinesterase				
			V8G (*48*)	G119S (*160*)[b]	Y121N *(230)*	G189D (*170*)	W456G (*494*)
CAS	Adults not exposed to acaricides	I	V	**S**	**N**	G	W
	Adults survived after exposure to cypermethrin	I	V	**S**	Y	G	W
BA1	Adults not exposed to acaricides	S	V	**S**	Y	G	W
BA2	Adults not exposed to acaricides	S	V	**S**	Y	G/**D**	W
	Adults survived after exposure to phoxim	S	V/**G**	**S**	Y	G	W/**G**
BA3	Adults not exposed to acaricides	MR	V	**S**	Y	G	W
	Adults survived after exposure to phoxim	MR	V	**S**	Y	G	W
TAR	Adults survived after exposure to phoxim	S	**G**	**S**	Y	G	W
LE4	Adults not exposed to acaricides	I	V	**S**	Y	G	W
	Adults survived after exposure to phoxim	I	V	**S**	Y	G	W

[a] Efficacy classes (EC) are resistant (R), moderately resistant (MR), intermediate (I), susceptible (S) and highly susceptible (HS).
[b] Mutation previously detected in *Dermanyssus gallinae* (Koc et al., 2022).

remains to be determined if these are associated with resistance.

In the *AChE* of the three examined PRM isolates five amino acid substitutions were detected (V8G, G119S, Y121N, G189D, W456G) (*Torpedo californica AChE* numbering) (Fig. 4 and Table 4). The substitution G119S was present in all tested isolates and has been previously reported in *D. gallinae* as well as other arthropods resistant to OPs and CBs. We also detected four novel mutations in PRM *AChE* (including: V8G, Y121N, G189D, W456G) (Fig. 4 and Table 4). Although we only sampled a small number of mites, the mutations we observed are present in both susceptible (EC code S) and resistant mites (EC codes: MR,

moderately resistant and I, intermediate), thus further studies are needed to understand if these are associated with resistance.

4. Discussion

Poultry red mite isolates resistant to phoxim and cypermethrin were found among the 11 populations tested in this study, in agreement with previous reports of resistance in PRM (Zdybel et al., 2011; Thomas et al., 2018; Pugliese et al., 2019). Since its approval for use in poultry in 2006, phoxim showed a high efficacy against PRM, but in recent years its efficacy has started to decrease with the emergence of resistant mites (Zdybel et al., 2011; Thomas et al., 2018; Pugliese et al., 2019). Resistance to SPs in PRM has been repeatedly reported in many countries, this is likely due to SPs being the first class of acaricides adopted in poultry farms, as well as the most widely used class of chemical for mite control (Zeman, 1987; Beugnet et al., 1997; Marangi et al., 2009; Thomas et al., 2018; Pugliese et al., 2019; Katsavou et al., 2020).

The molecular mechanism of resistance to both classes of acaricides include genetic changes to the target sequence (target-site insensitivity) and diverse mechanisms resulting in decreased exposure (reduced penetration, increased metabolism, or sequestration of the molecules before they reach the target site) (Van Leeuwen et al., 2010). The molecular mechanisms of PRM resistance to both classes of acaricides is discussed below.

4.1. Resistance to cypermethrin: target site insensitivity

Several natural and synthetic neurotoxins, such as dichlorodiphenyltrichloroethane (DDT) and SPs (including cypermethrin), bind to and open arthropod voltage-gated sodium channels, leading to the so-called "knockdown", with paralysis and death of the target species (Soderlund, 2008; Field et al., 2017). Many pest species have developed resistance to SPs, mainly due to a knockdown resistance (kdr) mechanism, linked to mutations at specific points of the *vgsc* gene (Dong et al., 2014; Vlogiannitis et al., 2021). The first mutations reported to decrease the efficacy of pyrethroids were L1014F in IIS6 (kdr) and M918T in IIL45 (super-kdr) (Williamson et al., 1996), and others have been found associated with SPs resistance in arthropods, some of them functionally validated in *Xenopus* oocytes (Dong et al., 2014; Du et al., 2016) (Fig. 4). The super-kdr M918T has previously been detected at a low rate in *D. gallinae* populations from Europe (8 %) (Katsavou et al., 2020) and Turkey (10 %) (Koç et al., 2022). Our analysis of cypermethrin resistant PRM populations identified mutations in the mite *vgsc* that have previously been associated with acaricide resistance (Table 3). All highly sensitive (HS) and sensitive (S) populations tested were wild-type at the M918L/T site (Table 3), whereas all resistant populations (including CAS, BA2, BA3 and LE4) encoded the M918L/T mutation that has been functionally characterised to confer cypermethrin resistance (Katsavou et al., 2020; Koc et al., 2022). The BA3 isolate contained the super-kdr mutation (M918T). In addition, we also identified the M827I mutation in isolate LE4 (a resistant population), that has previously been characterised in *Pediculus humanus capitis* and functionally shown to confer acaricide resistance (SupYoon et al., 2008; Dong et al., 2014). Further mutations were detected in PRM *vgsc*, but it is currently unknown whether they contribute to acaricide resistance.

4.2. Resistance to phoxim: target site insensitivity

Target-site insensitivity has been also observed for AChE, the target of OPs and CBs, which hydrolyses acetylcholine, preventing the transmission of nervous impulses at synapses and neuromuscular junctions (Li et al., 2021). These classes of acaricide/insecticide irreversibly bind to AChE, resulting in continuous nerve discharges, with paralysis and death (Abbas et al., 2014). Mutations in *AChE* might reduce the action of OPs and CBs, leading to resistance to those pesticides, as reported for several resistant arthropod species, such as *M. domestica*, *Tetranychus urticae*, *Aedes aegypti* (Fournier, 2005). The only mutation found in OP-resistant *D. gallinae* populations is G119S and its variant G119A (Koç et al., 2022), which are located in the active site of AChE, critical for its catalytic activity. Accordingly, all the PRM isolates analysed herein hosted the G119S substitution irrespective of their susceptibility to phoxim, while the variant G119A was not detected. G119S reduces the catalytic efficiency or the stability of the enzyme, resulting in a fitness cost in arthropods carrying these mutations (Koç et al., 2022). This drawback can be compensated with the introduction of additional counteracting mutations resulting in hyperactivity, as seen in *Drosophila melanogaster* and *T. urticae* (Shi et al., 2004; Lee et al., 2015). Additional mutations throughout the *AChE* were detected in the tested isolate, not previously reported in arthropods (i.e., V8G, Y121N, G189D and W456G). Considering that moderately resistant (BA3) and intermediate (LE4) isolates hosted only the G119S, while almost all the susceptible populations also had other mutations, it cannot be excluded that some of these additional substitutions might contribute to mitigate the impact of G119S.

4.3. Metabolic resistance to phoxim and cypermethrin

Acaricide resistance may also be attributed to other mechanisms, such as the overexpression of detoxification enzymes and/or the decreased exposure through increased drug efflux from cells and reduced penetration, as seen in many other resistant arthropod species, such as: the two-spotted spider mite, *T. urticae*; the scabies mite, *Sarcoptes scabiei*; the hard tick, *Rhipicephalus bursa* and the predatory mite, *Metaseiulus occidentalis* (Pittendrigh et al., 2014; Wu and Hoy, 2016). Here, we examined differentially expressed genes (DEGs) between the fully susceptible (FS) versus the phoxim-resistant and cypermethrin-resistant PRM populations to identify genes associated with detoxification and xenobiotic-defence.

Our results suggest that detoxification enzymes (including P450s, GSTs, CCEs) are not induced in response to acaricide exposure in resistant mites, but instead are constitutively overexpressed in resistant strains. Similar mechanisms have been characterised in other arthropods, including ticks, mites and insects. For example, in the aphid *Bemisia tabaci*, insecticide resistance is associated with the constitutive overexpression of genes *GST, CYP6CX4, CYP6DW3, CYP6DZ6* and *CYP9F* (Zhang et al., 2016). Here we report constitutive overexpression of GST, P450 and CCEs in phoxim and cypermethrin resistant mites (Fig. 3).

Previous studies in *D. gallinae* have shown that P450 genes are induced and constitutive up-regulated, as previously reported in pyrethroid-resistant *D. gallinae* populations (Wang et al., 2020). In addition, GSTs have been implicated in acaricide resistance in resistant *D. gallinae* populations. Three GST genes have previously been characterised in *D. gallinae* (*Deg*-GST-1, *Deg*-GST-2 and *Deg*-GST-3) and shown that they bind different classes of acaricide (Bartley et al., 2015; Wang et al., 2021; Koç et al., 2022). Among the three GSTs, only *Deg*-GST-2 was able to directly metabolise phoxim, while none were able to metabolise beta-cypermethrin (Wang et al., 2021). In the PRM populations tested here, an analogue of *Deg*-GST-2 (DEGAL6027g00010) was constitutively up-regulated in both phoxim and cypermethrin-resistant populations (FS-PR and FS-PRExp; FS-CR and FS-CRExp), in agreement with previous findings in pyrethroid-resistant *D. gallinae* (Wang et al., 2021). These results may be related to the ability of *Deg*-GST-2 to directly metabolise phoxim, thus increasing its expression level in phoxim-resistant mites, irrespective of exposure to the acaricide. Therefore, overproduced *Deg*-GST-2 might play a key role in acaricide resistance, by increasing the efficiency of drug metabolism.

In addition, a gene encoding an aryl hydrocarbon receptor was constitutively overexpressed in the cypermethrin resistant mites. This transcription factor has been found upregulated in *Anopheles gambiae* after exposure to the SP permethrin (De Marco et al., 2017) and its role in promoting the expression of detoxification enzymes, such as P450s

and GSTs, has been demonstrated in other arthropods (Peng et al., 2017; Zhang et al., 2019), suggesting that Ahr may have a role in acaricide/insecticide resistance.

The differential RNA-seq analysis presented here identified several ABC transporters that are constitutively overexpressed in both phoxim and cypermethrin resistant mites, implicating their role in removal of xenobiotics from cells. Indeed, ABC transporters are crucial for the efflux of substances from the cells and they were found to be overexpressed in pesticide-resistant arthropods, such as *P. humanus capitis*, *S. scabiei*, *D. melanogaster*, *Plutella xylostella* and *A. gambiae* (You et al., 2013; Dermauw and Van Leeuwen, 2014). Further studies are necessary to determine their role in acaricide resistance in PRM.

Cuticle protein genes were constitutively upregulated in phoxim and cypermethrin resistant mite populations from this study, similarly to what reported in resistant *Culex pipiens pallens* (Fang et al., 2015) and *Cimex lectularius* (Mamidala et al., 2012). Increased expression of CPs can be associated with cuticular thickening, which contributes to reduce xenobiotic penetration, aiding drug resistance. Additionally, ABC transporters are involved in the transcuticular translocation of cuticular components, and a link between their overexpression and increased cuticle thickness in resistant arthropods has previously been suggested (Dermauw and Van Leeuwen, 2014; Pignatelli et al., 2018).

In the mite populations examined here, overexpressed HSPs were found only in the phoxim resistant mites, both constitutively expressed and induced following exposure to acaricide. Similar results were observed in the silkworm *Bombyx mori*, in which HSPs were found to be upregulated in response to phoxim exposure (Gu et al., 2015; Fang et al., 2021). HSPs are stress-responsive factors and are considered as potential resistance-related genes, although their role has not been fully elucidated (Li et al., 2017).

5. Conclusions

Taken together, the results from this study confirm that resistance to phoxim and cypermethrin is widespread in Italian *D. gallinae* populations. Furthermore, resistance to both classes of acaricides in *D. gallinae* is related to both target-site insensitivity and constitutive overexpression of detoxification enzymes and other defence-related genes in resistant mites. Our data suggests that resistant mites constitutively overexpress detoxification enzymes and may not necessarily increase expression of these classes of enzymes upon exposure to acaricide. Considering the short life cycle of the red mite, the surviving resistant sub-populations can rapidly spread within the poultry houses, especially under the selective pressure of repeated treatments. Therefore, understanding the molecular bases of resistance could be useful to set up novel methods to screen or test mite populations and use targeted acaricides, in order to avoid the abuse and misuse of the few available choices. Considering that OPs and SPs have different molecular targets, and that specific mutations may contribute to resistance to the respective acaricide, those findings may pave the way for the development of focused diagnostic tests directed toward the resistance-associated mutations, in order to rapidly verify the suitability of an acaricide treatment. Indeed, these compounds are becoming less effective due to resistance, including the relatively new compound, fluralaner. In this framework, future perspectives about the control of *D. gallinae* should rely on the appropriate use of acaricides, the development of novel target compounds and future development of a vaccine for more sustainable control of PRM.

CRediT authorship contribution statement

Antonella Schiavone: Investigation, Formal analysis, Visualization, Data curation, Writing – Original draft; **Daniel R.G. Price**: Methodology, Investigation, Data curation, Formal analysis, Resources, Writing – Review & Editing; **Nicola Pugliese**: Conceptualisation, Methodology, Writing – Review & Editing; **Stewart T.G. Burgess:** Methodology, Validation, Formal analysis, Writing – Review & Editing; **Ifra Siddique**: Investigation; **Elena Circella**: Data curation, Writing – Review & Editing; **Alasdair Nisbet**: Supervision, Resources, Validation, Project administration, Writing – Review & Editing; **Antonio Camarda**: Supervision, Conceptualisation, Project administration, Resources, Funding acquisition, Writing – Review & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.109957.

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