Reduced pro-insecticide activation by cytochrome P450 confers coumaphos resistance in the major bee parasite *Varroa destructor*

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Abstract

Varroa destructor is one of the main problems in modern beekeeping. The use of highly selective acaricides with low toxicity to bees are used internationally to control this mite. One of the key acaricides is the organophosphate (OP) pro-insecticide coumaphos, that becomes toxic after enzymatic activation inside *Varroa*. We show here that mites from the island Andros (AN-CR) exhibits high levels of coumaphos resistance. Resistance is not mediated by decreased coumaphos uptake, target-site resistance, or increased detoxification. Reduced pro-insecticide activation by CYP was the main resistance mechanism, a powerful and rarely encountered evolutionary solution to insecticide selection pressure. After treatment with sublethal doses of [^{14}C] coumaphos, susceptible mite extracts had substantial amounts of coroxon, the activated metabolite of coumaphos, while resistant mites had only trace amounts. This indicates a suppression of the cytochrome P450 (CYP) mediated activation step in the AN-CR mites. Bioassays with coroxon to bypass the activation step, showed that resistance was dramatically reduced. There are 26 CYPs present in the *V. destructor* genome. Transcriptome analysis revealed overexpression in resistant mites of *CYP4DP24* and underexpression of *CYP3012A6* and *CYP4EP4*. RNA interference of *CYP4EP4* in the susceptible population to mimic down-regulation seen in the resistant mites, prevented coumaphos activation, and decreased coumaphos toxicity.

Significance Statement

Honey bees pollinate the majority of crops but their survival is under threat. The bee parasitic mite Varroa destructor are a major cause of honey bee decline. The successful control of Varroa mites depends on a small number of in-hive acaricides. Species-specific activation of the chemical coumaphos is a powerful “pro-drug” approach to attain this selective toxicity and protect bees. We identified a coumaphos-resistant Varroa population that escapes toxicity by downregulating the activating enzyme, a P450 monooxygenase. Decreased activation and toxicity is a rare but evolutionarily powerful solution to achieve resistance. It demonstrates that the current pro-insecticide approach is under threat, as it can be circumvented, and highlights the need to restart the pipeline to develop selective pest control agents.

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Introduction

Beekeeping plays an important role in providing nutritional, economic and ecological security globally, with direct contribution to the economy valued >230 billion dollars per year (1). Bee colony collapse disorder (CCD) is a major problem internationally, leading to a loss of >10 million beehives in North America alone and causing dramatic economic losses. Several possible causes for CCD have been proposed, such as the impact and side effects of pesticides used in agriculture, although currently still under considerable debate (2). Other suggested causes include damage by pests and diseases, malnutrition, genetic factors, immuno-deficiencies, loss of habitat, and changing beekeeping practices. In reality a combination of factors are likely to be involved.

The major bee ectoparasitic mite *Varroa destructor* is the most important pest for apiculture (3, 4) and it has been directly implicated with major economic losses (5). In addition to its direct parasitism effects on bee physiology and sociability, *Varroa* transmits viruses and pathogens, likely associated with CCD (6, 7). Commercial beekeepers primarily use selective chemical acaricides with low bee toxicity s to control *Varroa* infestation (8, 9). Given the need for selectivity, very few acaricides can be used in-hive and common commercial use is restricted to the pyrethroids tau-fluvalinate and flumethrin, the formamidine amitraz and the organophosphorus (OP) coumaphos (10).

Coumaphos was synthesized 70 years ago for use in livestock to control insect ectoparasites, such as ticks, and helminths. Coumaphos is a phosphorothioate pro-insecticide (11) that requires *in vivo* bioactivation by cytochrome P450 (CYP) monooxygenases to its active phosphate metabolite coroxon (coumaphos-oxon), which is an irreversible inhibitor of acetylcholinesterase (AchE) (Figure 1).

The extensive use of acaricides against *Varroa* has selected resistant populations in several countries (12): pyrethroid (tau-fluvalinate and flumethrin) resistance has been reported in Europe and many other parts of the world (13); formamidine (amitraz) resistance has been

detected in Mexico (14) and Argentina (15); coumaphos resistance has been recorded in the USA and Argentina (12, 16).

Insecticide and acaricide resistance mechanisms are typically mediated either by changes in the target site making it insensitive, or by changes in penetration, activation, or detoxification, that alters the amount of insecticide that reaches the target-site. Mutations, such as the L925V, L925I and L925M in the voltage gated sodium channel (VGSC), the target site for pyrethroids, have been associated with pyrethroid resistance in *Varroa* (13), but the resistance mechanisms for amitraz and coumaphos are unknown. However, amitraz target-site resistance mutations in the octopamine receptor (17) and detoxification-based resistance of coumaphos due to enhanced metabolism of coroxon (Figure 1), have been reported for the tick *Boophilus microplus* (18, 19).

Characterisation of the resistance mechanisms in*Varroa* has been hampered by their biological cycle inside hives (20). Publication of the *Varroa* genome sequence has now made resistance characterization easier (21). The development of robust reverse genetic (RNAi) tools (22), also facilitates functional studies for the implication of specific genes in the resistant phenotypes. Using these tools and classical approaches we have now identified the underlying cause of coumaphos resistance in a *Varroa* population from a Greek Aegean island.

Results

Coumaphos resistance in *Varroa*

Among six populations tested, the AN-CR population from the Aegean island Andros, originated from a beekeeper who used coumaphos almost exclusively for many years exhibited substantially reduced mortality rates (7%) (Table S1). Dose response bioassays were subsequently conducted to determine the strength of the phenotype. Resistance Ratio (RR) scaled up to 217-fold in the AN-CR compared to the coumaphos susceptible ATH-S population (Table 1).

Penetration, detoxification and altered target-site are not involved in resistance

Acaricide uptake (penetration) was not significantly different between resistant and susceptible mites (Table S2). Analysis of detoxification enzyme activities with different substrates, more specifically carboxyl/choline esterase (CCE) activity measured with the substrates PNPA, 1-NA and 2-NA; GST activity measured with the substrate CDNB and; MO activity measured with the substrate 7-EC did not show any significant differences between the AN-CR and the ATH-S mites (Table 2). In contrast, a significant reduction of GST activity measured with the substrate MCB was recorded (Table 2). The isoelectric focusing polyacrylamide (IEF) profile of CCEs was identical between the ATH-S and AN-CR populations (Figure S1). No significant differences, in the total AChE activity, or in the inhibition of AChE by coroxon or malaoxon were observed between the ATH-S and AN-CR populations (Table 2).

Decreased coumaphos activation causes resistance

Analysis of whole body extracts of ATH-S and AN-CR *Varroa* mites after treatment with ^{14}C coumaphos, using normal phase TLC revealed, besides the parental substrate (Rf=0.83), two main known metabolites and some putative unknown secondary metabolites: the activated phosphate metabolite coroxon (R_f=0.75); the non-toxic metabolite chlorferon (R_f=0.50) and some more polar compounds at around R_f=0.25 and lower (Figure 2). The formation (detoxification rate) of the main coumaphos detoxification product (inactive metabolite) chlorferon was similar between the AN-CR and the ATH-S populations, as was the formation of the unknown metabolites. However, a significant difference in the formation of coroxon, the activated toxic organophosphate acaricide was observed. Coroxon was readily formed in the ATH-S population, in a time dependent manner (Figure 2). However, this metabolite was barely detected in the resistant AN-CR mites, even 18 h post-treatment with 200 mg a.i./L ^{14}C coumaphos (Figure 2).

To confirm that the failure to activate the pro-insecticide was the cause of resistance, we conducted bioassays directly with coroxon, thus by-passing the activation step inside the mite. The bioassays showed that the resistance phenotype was dramatically alleviated (resistance ratio dropped from 217-fold for coumaphos, to 25-fold for coroxon) (Table 1).

Cytochrome P450 monooxygenases are associated with coumaphos resistance

To investigate the transcriptional basis of coumaphos resistance in *Varroa*, we performed RNAseq on ATH-S, AN-CR and AN-CR pretreated with coumaphos (tAN-CR). After filtering the Illumina RNAseq dataset (Table S3) for the presence of viral sequences (Table S4), a differential gene expression analysis was performed between the AN-CR and the ATH-S population and between the coumaphos treated (tAN-CR) population and the AN-CR population. A total of 270 differentially expressed genes (DEGs) were identified in AN-CR compared to ATH-S (fold change (FC) > 2 and a Benjamini-Hochberg adjusted p-value < 0.05) (Table S5). Among them, only twelve encoded typical detoxification enzymes (P450s InterPro domain IPR001128), CCEs (IPR002018) and GSTs (IPR036282)) or proteins involved in xenobiotic transport (ABC transporters (IPR003439)) (Table S6). Three genes encoded CYPs: two were under-expressed (*CYP4EP4*: with TMM (trimmed mean of M values) normalized counts per million (CPM) between 288-594 in ATH-S vs 94-127 for AN-CR, i.e. relatively highly expressed; *CYP3012A6*: TMM normalized CPM between 14-25 in ATH-S vs 4-10 in AN-CR, i.e. lowly expressed) and one was over-expressed (*CYP4DP24*: TMM normalized CPM between 7-21 in ATH-S vs 27-59 in AN-CR). The coding sequence of all three CYP was identical in the TSA data of AN-CR and ATH-S.

Only one gene was differentially expressed in the pairwise comparison between the tAN-CR and AN-CR populations (Table S6), hence, in line with PCA analysis (Figure S2), the DEG profile of the AN-CR and the tAN-CR populations were highly similar, indicating that very limited gene expression changes are associated with exposure (induction) of the AN-CR to the apparently non activated coumaphos, inside these varroa mites.

We mined the RNAseq data of the ATH-S and AN-CR population for the presence of mutations in the *V. destructor Ace* genes. The protein encoded by the *V. destructor* gene LOC111243720 showed the best BLASTp hit with *T. urticae* AChE (E-value 0, 54% identity). This gene is the ortholog of the tick *Rhipicephalus* (*Boophilus) microplus* AChE 1 (23). The protein encoded by LOC111246636 was the second best BLASTp hit to *T. urticae* AChE (E-value of E^{-101}, 34% identity). This gene is the ortholog of *R. microplus* AChE 3 (23). 82.2 and 99.8 % of the CDS of LOC111243720 and 100% of the CDS of

LOC111246636 were covered by the RNA-seq consensus sequence of the ATH-S and AN-CR population, respectively. No single nucleotide polymorphisms were identified compared to the reference genomic sequence. Thus, in agreement with our biochemical data, we have no evidence of AChE target site resistance in *Varroa*.

CYPome of *Varroa destructor*

The curated CYPome of *V. destructor* consists of 26 full length sequences. This is one of the the smallest CYPomes of arthropods. It is much smaller than the 63 CYPs of the western orchard mite, *Metaseiulus occidentalis* and only half the size of the CYPome of the honey bee, the *Varroa* host. The 26 *Varroa* CYPs form four CYP clans (Figure 3, Suppl. File 1), as in the spider mite and most insects. *CYP307G4* of the CYP2 clan, and *CYP302A1*, 314A1 and 315A1 in the mitochondrial CYP clan are involved in ecdysteroid biosynthesis and are highly conserved. There was no CYP18 or CYP306 ortholog in *Varroa*, a situation similar to the spider mite *Tetranychus urticae*.

Silencing of the *CYP4EP4* prevents coumaphos activation and induces tolerance in susceptible *Varroa*

Having established that the down regulation of two CYPs (*CYP3012A6* and *CYP4EP4*) in AN-CR *Varroa* mites is associated with resistance, we silenced these CYPs, using RNA interference (RNAi), in the ATH-S susceptible population, aiming to mimic the down regulation in resistant mites. Silencing of CYP4EP4 significantly reduced mortality at the diagnostic dose by 35%, i.e. from 90.48% (95%CI = 84.92% – 96.04%) in the GFP injected control mites to 58.33% (95%CI = 48.16% – 68.50%) in the *dsCYP4EP4* treated mites (Figure 4A). In contrast, silencing of the *CYP3012A6* in ATH-S mites did not significantly reduce mortality at the diagnostic dose compared to the GFP control mites (Figure 4A). Silencing of CYP4EP4 but not of GFP or CYP3012A6 in the susceptible ATH-S mites was also associated with a substantial reduction of the activation of coumaphos to its active metabolite coroxon (Figure 4B). The silencing efficiency was verified by the statistically significant reductions in CYP4EP4 (28.7% SE = 10.3%, P = 0.032) and *CYP3012A6* (34.59% SE = 11.6%, P = 0.031) mRNA levels, respectively, compared to the GFP control (Figure 4C).

Discussion

An unusual case of CYP-mediated reduced pro-insecticide activation conferring very high levels of acaricide resistance in the major bee ectoparasitic mite *V. destructor* was identified and elucidated at the molecular level. OP compounds, which dominated the insecticide market in the 19770s-80s, are phosphorothioates (with a P=S moiety). These are pro-insecticides, with a 4-5 order of magnitude lower acetylcholinesterase inhibitionthan that of their activated metabolite, the corresponding phosphates (P=O). The activation step can lead to selective toxicity. For instance, malathion is rapidly inactivated by mammalian carboxylesterases, while insects preferentially activate it to malaoxon. The P=S to P=O activation step is catalyzed by CYPs, for instance house fly *CYP6A1* and *CYP12A1* (26, 27).

Here we show that in *Varroa* reduced CYP-mediated activation is involved in coumaphos resistance. Bioassays with coroxon, which bypassed the CYP activation step, reduced the AN-CR resistance from 217-fold for coumaphos, to 25-fold for coroxon. The CYPome in Varroa is limited (N= 26) which is usual in parasitic organisms. The human body louse has 37 CYP genes (28) and the crustacean salmon louse *Lepeophtheirus salmonis* just 21 (29). There was differential expression of three CYPs, one (*CYP4DP24* from the CYP4 clan) over- and two underexpressed (*CYP3012A6* and *CYP4EP4* from the mitochondrial and CYP4 clan, respectively) in the AN-CR compared to ATH-S mites. Gene expression changes after induction by sublethal doses of coumaphos in the resistant AN-CR population were negligible (only one gene).

Changes in CYP levels in both directions could in theory lead to resistance. This is because the CYP enzymes that attack the phosphorothioate (P=S) moiety of OP compounds (30) first generate a monooxygenated, unstable intermediate which can then result in two outcomes (see Figure 1 for coumaphos, showing the main radiolabeled metabolites). One outcome is ester cleavage and formation of the non-toxic metabolite chlorferon and the other outcome is desulfuration and formation of the activated, toxic acaricide coroxon. The ratio of the two products depends on the substrate and the CYP. For coumaphos, a CYP catalyzing a high coroxon/chlorferon ratio would favor activation over detoxification. Its underexpression may lead to resistance, and vice-versa (see schema – Figure S3).

Analysis of coumaphos metabolism indicated that there was no difference between the ATH-S and the AN-CR strains in the formation of chlorferon, or in the depletion of the parental coumaphos. However, coroxon formation was decreased in resistant mites. Thus, the underexpression of a CYP in the resistant population with a high ratio of coroxon formation is the most likely resistance mechanism.

Despite many attempts, we were not able to functionally express the differentially regulated CYP enzymes to directly verify our interpretation of the *in vivo* metabolism studies. Hence, weconfirmed the resistajce mechanism using reverse genetics of the two down regulated CYPs. RNA interference of the down regulated *CYP4EP4* in the susceptible strain decreased coumaphos activation and increased the tolerance to coumaphos. Thus, this reverse genetics manipulation mimics long term selection for selection for a down regulation mechanism of *CYP4EP4*, with similar outcome. Expression of *CYP4EP4* is ~20 times higher than *CYP3012A6*. Silencing *CYP3012A6* did not change the resistance phenotype, either because this CYP does not metabolize coumaphos or because its expression level is too low to affect the toxic outcome

Most studies on OP resistance have documented the overexpression of CYP genes which generate a high ester cleavage (detoxification)/ desulfuration (activation) ratio, such as *CYP6A1* in diazinon-resistant house flies (ratio 2.66, (26)). Examples of the reverse, i.e. decreased levels of enzymes with higher desulfuration / ester cleavage ratios have not been reported. However, methyl parathion resistance in a field strain of *Heliothis virescens* was associated with lower microsomal activation to methyl paraoxon, and this activation product had a significantly lower resistance factor (31). This situation is similar to the mechanism described here for coumaphos resistance in *Varroa*.

The possibility that additional mechanisms may also play a role in the coumaphos resistance can not be excluded. For example, the reduced GST activity with the substrate MCB and the two downregulated GSTs (LOC111244895 and LOC111253932, Table 3) in the AN-CR may have a direct or supportive role in the resistance phenotype.

Varroa infestations are associated with severe economic losses in apiculture and coumaphos is one of the few acaricides available for its control worldwide. The presence and frequency of this coumaphos resistance mechanism can now be tracked. If it is common, the Janus face of CYP metabolism, with different activation vs detoxification

profiles for different insecticides catalyzed by the same CYP, could potentially open up new options for Insecticide Resistance Management (IRM). For example, Adolfi et al. (2019) (32) showed that the overexpression of the *Anopheles AgCYP6M2* CYP confers permethrin resistance via detoxification, and increases the susceptibility to malathion, via bioactivating it to its more toxic metabolite malaoxon. Similarly pyrethroid-resistant *H. virescens* was more susceptible to the pro-insecticide chlorfenapyr (33). This negative cross-resistance between different insecticide/pro-insecticide classes, due to differential regulation of CYPs in resistant insects could be exploited in a push-pull strategy of IRM.

Materials and Methods

Chemicals and ^{14}C-labeled coumaphos

The following technical grade chemicals were used in the bioassays: coumaphos 98% purity (Sigma Aldrich, Greece), malaoxon, coumaphos-oxon 96% (LGC Standards, UK), 3-Chloro-7-hydroxy-4-methylcoumarin (chlorferon) 97% purity (Sigma Aldrich, Greece). All the other reagents were obtained by Sigma Aldrich, unless otherwise stated.

Radiolabeled [coumarin-4-^{14}C] coumaphos (Figure 1) of high chemical and radiochemical purity (>99%) was purchased from Institute of Isotopes Co., Ltd. (Izotop, Hungary). The specific activity of the [^{14}C] coumaphos was 2.024 MBq/mg (1MBq= 60x10^{6}dpm).

Mite populations

On the island of Andros, a population was collected from a site where coumaphos had been used almost exclusively for the last 14 years and where control failure was reported (hereafter named AN-CR, Andros Coumaphos Resistant). A *Varroa* population collected from beehives at the Agricultural University of Athens was used as a susceptible strain, as the bee colony has been maintained without chemical treatments for the last twenty years (hereafter named ATH-S, Athens Susceptible).

Bioassays

Chemicals dissolved in acetone were freshly prepared 0.5 mL of solutions was placed into individual 12-mL glass vials. Vials were rolled until the acetone evaporated. Batches of ten

(10) adult female mites were introduced into the coated vials, closed with holed parafilm, each time at 25 °C for 20 h. Dose response bioassays were conducted. For gene expression analysis described below, induction bioassays were conducted at the coumaphos LC_5 for the AN-CR and the treated mites were collected at the end of the bioassay (tAN-CR). After the treatment period, mites were considered dead in absence of leg movement when prodded with a fine paintbrush. Toxicity data were analyzed using PoloPC (LeoRA, Software, Berkeley, CA, USA). Resistance ratios were calculated by dividing the LC_{50} of a population with the LC_{50} of the ATH-S susceptible/reference population.

Detoxification enzyme activities and acetylcholinesterase inhibition

The enzymatic activity of carboxyl/choline esterases (CCEs), monooxygenases (MOs), Glutathione S-transferases (GSTs) and acetylcholinesterase (AChE) was determined according to Van Leeuwen et al (2005) (34) with slight modifications. Briefly, mass homogenates were prepared by crushing 1-10 freshly sampled adult mites in sodium phosphate buffer (0.1M, pH 7.2, 200µl) with a Teflon pestle. The homogenate was centrifuged at 5000g and 4^0C for 5 min and the resulting supernatant was used as an enzyme source. Approximately 10-20 µg crude protein extract was included in each assay, with at least 3-5 replicates for each population and assay. Fluorescence and absorbance were measured with a TECAN SpectraFluor microplate reader.

For the CCE assays, the substrates p-nitrophenol (PNPA) (kinetically for 10-min), 1-naphthyl acetate (1-NA) and 1-naphthyl acetate (2-NA) (end point after 30 minutes of incubation) were used. For the GST assays, the substrates 1-chloro-2,4-dinitrobenzene (CDNB) (kinetically, for 10-min) and monochlorobimane (MCB) (end point after 20 min) were used. For MO assays, the substrate 7-ethoxycoumarin was used to measure the O-deethylation activity kinetically.

For AChE activity and inhibition rates with the OP oxon analogues malaoxon and coroxon, the reaction was conducted in 0.2mL substrate-reagent solution containing 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) 0.5mM and acetylthiocholine (AcSCh) 1.25mM. Residual AChE activities, after 10-min incubation with malaoxon and coroxon (at concentrations 10^{-8}M – 10^{-4}M) were measured kinetically at 405nm. The IC_{50} was determined using GraFit Version 3.0, Erithacus Software Ltd., Staines, U.K (35).

Analysis of coumaphos metabolism

Mites exposed to [^{14}C] coumaphos vials, as described above, were washed and homogenized in 200µl methanol. Supernatant was collected after centrifugation (5 min at 5,000 rpm) and the pellet was resuspended in methanol. The supernatant was reduced with air flow to 10 µl. Thin Layer Chromatography (TLC) was prepared by saturating the chamber with chloroform/methanol (100:7) and allowing to equilibrate for 1 h. 10 µl of each sample was spotted onto a silica gel pre-coated plate. After chromatography in chloroform/methanol (100:7), the position of cold coumaphos, coroxon and chlorferon was marked and the plate wrapped in plastic foil. Radioactivity was recorded with a phosphorimager (GE Healthcare Typhoon™ FLA 7000), followed by calculation of the retention factor (R_f) as the ratio of migration distance of the compound of interest to that of the solvent front. CorelDRAW Home & Studend x7 software was used for image processing. Cold chemistry unlabeled UV-active controls was used to localize the ^{14}C determined spots, and link retention time.

Illumina RNAseq

Varroa mites were introduced in 12 mL glass vials coated with either acetone (ATH-S and AN-CR) or 200 mg a.i. coumaphos/L acetone (tAN-CR) and kept at 25°C for 20 h. Glass vials were coated as described in the Bioassays section. After 20 h, alive mites were collected in an Eppendorf tube (10-20 mites/tube), snap frozen with liquid nitrogen and stored at -80°C until RNA extraction. RNA was extracted, using the TRI reagent protocol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and treated with TURBO™ DNase I (ThermoFisher Scientific) to remove genomic DNA contamination. Illumina libraries were constructed from the RNA samples with the TruSeq Stranded mRNA Library Preparation Kit with polyA selection (Illumina, USA), and sequenced on an Illumina HiSeq 2500 to generate strand-specific paired reads of 2 x 125 bp (library construction and sequencing was performed at the High-Throughput Genomics Core of the Huntsman Cancer Institute, University of Utah, Utah, USA). All RNAseq data that was generated during the current study is available at the Gene-Expression Omnibus (GEO) repository with accession number GSE153472. The quality of the reads was verified using

FASTQC version 0.11.5 (36) and subsequently, viral contamination was removed (Table S3). A PCA was created as described by Love et al 2015 (37) (Figure S1). A differential expression (DE) analysis was performed using voom (version 3.34.9) (38) and the read count data obtained by HTSeq 0.6.0. Differentially expressed genes (DEGs, log2FC >1 and Benjamini-Hochberg adjusted p-value < 0.05) were determined for the following comparisons: AN-CR vs ATH-S, tAN-CR vs ATH-S and tAN-CR vs AN-CR).

The RNAseq data of both ATH-S and AN-CR was also used to search for mutations in *Ace*, the gene(s) encoding the target-site of coumaphos. First, *Varroa Ace* sequences were identified by a BLASTp search against the *Varroa* proteome (version of October 2017) and using *Tetranychus urticae Ace* as query (tetur19g00850, http://bioinformatics.psb.ugent.be/webtools/bogas/overview/Tetur). Next, a BAM-file for the 4 samples of the ATH-S population and for the 8 samples of the AN-CR population (both AN-CR and TAN-CR) was generated by merging the individual BAM-files of the ATH-S and (t)AN-CR samples that were created during the STAR alignment (see paragraph above), respectively. Subsequently, this merged BAM-file was used to generate an ATH-S and AN-CR *Ace* consensus sequences using the "mpileup" and "call" command of the samtools 1.4.1 and bcftools 1.3.1 software (39), respectively and the *Varroa* genome as reference (GCF_002443255.1). The resulting FASTQ file was then converted to a FASTA file using the seqtk 1.2 software (using default Illumina settings: "-aQ 64 -q 20" as suggested on https://github.com/lh3/seqtk). Last, we assembled a *de novo* transcriptome using CLC Genomics workbench 11 (Qiagen, Belgium) with default settings, and using 24 million random subsampled, bee virus filtered, reads (8 million/sample) from ATH-S2-ATH-S4 and 24 million random, bee virus filtered, reads (3.42 million/sample) from AN-CR1- AN-CR4 and tAN-CR1-tAN-CR3 (ATH-S1 and tAN-CR4 were not used for *de novo* assembly as these samples had less than ten million reads (Table S5)).

Cytochrome P450 (CYP) gene annotation

The predicted *V. destructor* CYP sequences from the NCBI annotation release 100 were manually curated for accuracy and completeness. Seven were corrected, including an artefactual fusion of three CYPs. In addition, one full length CYP was found in our

transcriptome assembly, and while not predicted in the NCBI RefSeq models, it was present in the genome as an intronless gene. This resulted in a CYPome of 26 full-length genes, for which official CYP names were obtained from Dr. D.R. Nelson (University of Tennessee). All CYP genes were found in the *de novo* transcriptome assembly (Dataset S1). Six partial CYP sequences and CYP pseudogenes were also found and were excluded from further analysis. Full-length CYP sequences were aligned by MUSCLE (40), a phylogeny obtained by PhyML at www.phylogeny.fr and the tree was drawn and annotated with FigTree v1.4.4. (http://tree.bio.ed.ac.uk/).

RNA interference functional validation assays

Reverse genetic experiments using submersion in dsRNA were performed according to Campbell et al., 2010 (22). Briefly, PCR was performed on cDNA from AN-CR mites using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) following the manufacturer's instruction., with specific primer pairs designed using NCBI Primer BLAST to produce a product with a length of 400–600 bp and a GC content of 20–50% that also introduce a T7 promoter sequence: dsGFP (control), ds*CYP4EP4* and ds*CYP3012A6* (Table S2). dsRNA was created by using the Megascript Kit from Ambion and the T7 RNA polymerase with a 16-h 37 °C incubation, following the manufacturer's instructions. The dsRNA was cleaned using a MegaClear Transcription Clear Up kit (Ambion). The resultant dsRNA product was analysed using a Nanodrop spectrometer (Nanodrop Technologies). Batches of 10 female *Varroa* mites were immersed into dsRNA (2.5 μg/ μl) for 6-12 h. Eight biological experiments were performed with each dsRNA. As a control, non-endogenous GFP dsRNA was used at the same concentration.

Quantitative Real time PCR

The efficiency of the dsRNA assays were validated by quantitative PCR (qPCR). RNA was extracted in a similar way as for Illumina RNAseq experiments and subsequently used for cDNA synthesis, using Superscript III reverse transcriptase and Oligo-dT 20 primers (Invitrogen, Athens, Greece). Amplification reactions of 10 μl final volume were performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and Kapa SYBR FAST qPCR Master Mix (Kapa-Biosystems), using the primers listed in Table S6. The thermal protocol consisted of a polymerase activation step at 95ºC

for 3 min and 40 cycles of denaturation and annealing/extension steps at 95ºC for 10 s and 60ºC for 45 s, followed by a melting curve analysis step. Two housekeeping genes, NADH (LOC111249888) and 18S rRNA (LOC111253957), were used as reference genes for normalization (41). A tenfold dilution series of pooled cDNA was used to assess the efficiency of the qPCR reaction for each gene specific primer pair. A no template control (NTC) was included to detect possible contamination and a melting curve analysis was done in order to check the presence of a unique PCR product. Experiments were performed with four biological replicates and two technical replicates for each reaction. Relative expression analysis was done according to Pfaffl et al., 2002 (42) and significance of calculated differences in gene expression was identified by a pair-wise fixed reallocation randomization test.

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FIGURE LEGENDS

Figure 1. Metabolism of [^{14}C]-coumaphos in *Varroa*. Radiolabeled coumaphos (* indicates the position of the label) is converted to two main metabolites, chlorferon and coroxon. Both compounds result from P450 monooxygenation of the P=S moiety of coumaphos that initially leads to an unstable product in the active site. Ester cleavage to the non-toxic chlorferon and desulfuration to the acetylcholinesterase inhibitor coroxon are the two outcomes of P450 metabolism. The ratio of the two outcomes is specific to each P450. See text for details.

Figure 2. Analysis of metabolites after *in vivo* treatment with [14C] coumaphos. Normal Phase TLC in chloroform/methanol (100/7) of whole body extracts of ATH-S and AN-CR mites (*V. destructor*) treated with [^{14}C] coumaphos. C1: parental coumaphos (R_f=0.83); C2: coumaphos oxon (R_f=0.75); C3: chlorferon (R_f=0.50); C4 (Rf=0.1) unknown metabolites. A. Reduced activation of coumaphos to coroxon associated with resistance: ATH-S and AN-CR mite homogenates 2 h after treatment with [^{14}C] coumaphos. B. Time dependent formation of coroxon in ATH-S but not in AN-CR Varroa mites. ATH-S mites after 2h, 6h and 18h *in vivo* treatment and AN-CR after 18 h treatment with [14C] coumaphos (vials coated with 200 mg a.i./L).

Figure 3. Maximum likelihood phylogeny of the 26 CYP sequences of *Varroa destructor*. The four CYP clans are marked with a different color: yellow: CYP2 clan; red: CYP4 clan;

black: CYP3 clan; blue: mitochondrial clan. The three differentially expressed CYPs are marked with an asterisk.

Figure 4. Silencing of the CYP4EP4 prevents coumaphos activation and induces tolerance in susceptible *Varroa destructor*. Mortality rates against coumaphos of ATH-S immersed for 6 h in dsRNA (GFP; CYP4EP4 and CYP3012A6) and tested with diagnostic coumaphos concentration (200 ppm) after 72 h. B. Normal Phase TLC of whole body extracts of ATH-S mites immersed for 6 h in dsRNA (GFP; CYP4EP4 and CYP3012A6) and treated with [^{14}C] coumaphos as in 4A. C. Validation of gene silencing efficacy by RNAi. Relative expression levels in dsRNA-submersed *Varroa* (GFP control, CYP4EP4and CYP3012A6). mRNA expression was calculated using the 2^-dCt formula and was normalized to housekeeping genes; * indicate statistical significance at the $P < 0.05$ level, calculated using independent samples t-test; Error bars represent SE of mean. AU: Arbitrary Units (×20 for CYP3012A6 expression to harmonise the scale).