Identification and CRISPR-Cas9 validation of a novel β-adrenergic-like octopamine receptor mutation associated with amitraz resistance in *Varroa destructor*

Emre İnak[1,2], Sander De Rouck[1], Nafiye Koç-İnak[3], Esengül Erdem[4], Mustafa Rüstemoğlu[4], Wannes Dermauw[1], Thomas Van Leeuwen[1*]

[1] Laboratory of Agrozoology, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000, Ghent, Belgium

[2] Department of Plant Protection, Faculty of Agriculture, Ankara University, Diskapi, 06110, Ankara, Türkiye

[3] Department of Parasitology, Faculty of Veterinary Medicine, Ankara University, Altindag, 06070, Ankara, Türkiye

[4] Plant Protection Department, Faculty of Agriculture, Şırnak University, Şırnak, Türkiye

* Corresponding author: Thomas Van Leeuwen

Emre İnak	Email: einak@ankara.edu.tr	ORCID: 0000-0003-0411-4198
Sander De Rouck	Email: sander.derouck@ugent.be	ORCID: 0009-0001-6322-1121
Nafiye Koç-İnak	Email: nafiyekoc@ankara.edu.tr	ORCID: 0000-0003-2944-9402
Esengül Erdem	Email: eozdemir@sirnak.edu.tr	ORCID: 0000-0002-8274-282X
Mustafa Rüstemoğlu	Email: mustafa.rustemoglu@gmail.com	ORCID: 0000-0002-1298-1702
Wannes Dermauw	Email: wannes.dermauw@ugent.be	ORCID: 0000-0003-4612-8969
Thomas Van Leeuwen	Email: thomas.vanleeuwen@ugent.be	ORCID: 0000-0003-4651-830X

Abstract

Varroa destructor is widely recognized as a significant contributor to colony collapse disorders. Chemical acaricides, such as amitraz, have been extensively used for *Varroa* control due to their selectivity within beehives. However, the increasing number of cases of amitraz resistance across global *V. destructor* populations poses a significant challenge. In this study, we conducted a comprehensive molecular screening of the β-adrenergic-like octopamine receptor (Octβ2R), the target-site of amitraz, across 66 Turkish and 63 Belgian *V. destructor* populations. Although previously reported resistance mutations were not detected, the screening revealed a novel Y337F mutation located within transmembrane 7 (TM7) of Octβ2R in Turkish *Varroa* populations. Notably, this mutation was identified in the last residue of the highly conserved NPxxY motif associated with the activation of G-protein coupled receptors (GPCR). Among the 66 *Varroa* samples from Türkiye, twenty harbored the Y337F mutation, with eight samples exhibiting fixation of the mutation. Subsequent bioassays revealed over 8-fold resistance to amitraz in populations that contain the Y337F mutation. Genotyping of mites after exposure to 10 mg a.i./L amitraz demonstrated that all surviving mites were homozygous for the Y337F mutation, whereas dead mites carried susceptible alleles, providing genetic linkage between mutation and phenotype. Further, we used CRISPR-Cas9 editing to introduce the Y337F mutation in the orthologous Octβ2R of the model organism *Tetranychus urticae*. Crispants exhibited over threefold resistance to amitraz. In conclusion, this study identified and validated a novel amitraz resistance mutation. Additional research is required to further evaluate the phenotypic strength of Y337F in the context of operational resistance with current treatment strategies.

Keywords: octopamine receptor, Octβ2R, acaricide resistance, amitraz, target-site mutation, Y337F, SYNCAS

1 Introduction

The honey bee *Apis mellifera* contributes significantly to agriculture, pollinating a large number of crops worldwide, hence ensuring global food security (Calderone, 2012; Hung et al., 2018; Khalifa et al., 2021). Additionally, the many products honeybees produce such as honey, wax and propolis have diverse applications, ranging from culinary and medicinal to industrial uses (Topal et al., 2021). However, in the past decade honeybee populations have declined worldwide with increasing reports of colony losses reported by beekeepers. Colony collapse disorder (CCD) is a phenomenon where the worker population suddenly decrease from tens of thousands of individuals to several hundred or fewer over a time period from one to several weeks and was first reported in the USA in 2006 (Evans and Chen, 2021).

Although various biotic and abiotic factors are responsible for the honeybee population decline and occurrence of CCD, one of the most threatening stressors is the *Varroa* mite, *Varroa destructor* (Warner et al., 2023). *Varroa* mites are ectoparasites mainly feeding on the honeybee's fat body and hemolymph, with dietary preferences varying according to the life stage of the host (Han et al., 2024; Ramsey et al., 2019). Infections with *Varroa* can adversely affect the immune system of honey bees, negatively changing bee behavior and altering their responses to abiotic factors (Aronstein et al., 2012; Frizzera et al., 2023; Muijres et al., 2020; Zanni et al., 2017). Most importantly, *Varroa* mites can transmit multiple bee viruses including the well-known Deformed Wing Virus (DWV), which is the most prevalent virus disease in honeybees worldwide (Martin and Brettell, 2019).

Varroa infections without control practices can result in the entire collapse of colonies (Rosenkranz et al., 2010). Organic acids, as well as essential oils such as thymol, are widely used to control *Varroa* mites due to their environmentally friendly nature (Jack and Ellis, 2021). However, chemical acaricides are still one of the most common tools for *Varroa* management in many regions (Brodschneider et al., 2023). *Varroa* control using acaricides can be challenging, as a high level of in-hive selectivity is required. This prerequisite, together with the risks of pesticide residues in honey or related products,

has resulted in a limited number of acaricides available for *Varroa* control. Subsequent use of these acaricides has led to failures in chemical control, mainly due to the development of resistance (De Rouck et al., 2023; Mitton et al., 2022).

Amitraz is a formamidine pesticide widely used against mites and ticks, with a market value of 7 million dollars (Sparks et al., 2020). It acts as an octopamine receptor agonist (IRAC Group 19), leading to hyperexcitation (Davenport et al., 1985; Kita et al., 2017). Octopamine is a biogenic amine, and its role in multiple physiological events as a neuromodulator, neurotransmitter, and neurohormone is well known (Farooqui, 2007). It is often referred to as the 'insect adrenaline,' mediating the 'fight or flight' response in arthropods (Roeder, 2005). Octopamine receptors belong to the G protein-coupled receptors (GPCR), and three main subgroups have been identified: α-adrenergic-like octopamine receptors (αAOR: Octα1R and Octα2R), β-adrenergic-like octopamine receptors (βAOR: Octβ1R, Octβ2R, Octβ3R), and octopamine/tyramine receptors (OCT/Tyr: Tyr1R, Tyr2R, and Tyr3R) (Evans and Maqueira, 2005). Although the exact target site was unknown for many years, more and more studies suggest that the main target site of DPMF (N^2-(2,4-dimethylphenyl)-N^1-methyformamidine) - the active metabolite of amitraz - is Octβ2R (Cai et al., 2023; Guo et al., 2021; Kita et al., 2017; Li et al., 2022; Takata et al., 2020). Binding of DPMF to βAOR results in an increase of cAMP levels, which in turn results in altered cellular signaling, and eventually death of the animal (Ismail and Matsumura, 1989; Ohta and Ozoe, 2014). Of particular note, chlordimeform is another formamidine acaricide historically used for *Varroa* control and, for arthropods, its active metabolite - desmethylchlordimeform (DCDM) - was shown to act on the same receptor as DPMF (Chen et al., 2010; Ezzat Ghazy El-Kholy, 2010; Huang et al., 2010; Ismail and Matsumura, 1989).

Acaricide resistance can occur via pharmacokinetic and pharmacodynamic mechanisms. The former includes quantitative and qualitative changes in detoxification enzymes/transporters, resulting in a decreased amount of active compound reaching its target site (Amezian et al., 2024; De Rouck et al., 2023). On the other hand, pharmacodynamic mechanisms are mainly based on target-site mutations

altering the protein structure and binding kinetics, as well as changes in copy number and/or expression of the target-site (Feyereisen et al., 2015). In the case of resistance to amitraz, involvement of detoxification enzymes such as cytochrome P450 monooxygenases has been reported in amitraz resistance in spider mites (Vandenhole et al., 2024) and ticks (de La Canal et al., 2021), although such metabolic resistance has not been reported for *Varroa* mites so far. On the other hand, target-site mutations N87S and Y215H (N92S and Y218H in *Bombyx mori* numbering) in Octβ2R were previously uncovered in *Varroa* mites (Hernández-Rodríguez et al., 2022). A recent study demonstrated the absence of correlation between the N87S mutation and amitraz resistance (Marsky et al., 2024), while the significance of Y215H was further confirmed in a follow-up study (Rinkevich et al., 2023). A mutation in the 5' untranslated region (UTR) of Octβ2R was uncovered in the citrus red mite *Panonychus citri* and the frequency of the mutation was correlated with amitraz resistance, although it is not clear whether the mutation is causal, or present by linkage (Yu et al., 2021). Several target-site resistance mutations were also reported in ticks, however, only the role of an I45F mutation (*B. mori* numbering) was further supported (Corley et al., 2013; Jonsson et al., 2018; Takata et al., 2020).

In the present study, we performed a comprehensive molecular screening for genetic variation in Octβ2R of previously obtained *V. destructor* populations from Türkiye and Belgium (Erdem et al., 2024; Koç et al., 2021; Vlogiannitis et al., 2021). We uncovered a novel mutation (Y337F, *V. destructor* numbering) in a conserved region in transmembrane 7 (TM7) of Octβ2R in *V. destructor* (Fig. 1). The role of the mutation was further evaluated by genotyping mites after exposure to amitraz. Finally, we introduced the mutation into a strain of another mite species, *Tetranychus urticae,* using SYNCAS CRISPR-Cas9 gene editing (De Rouck et al., 2024) to validate its role in amitraz resistance.

2 Materials and Methods

2.1 Molecular screening of target-site mutations in Octβ2R in Turkish and Belgian *Varroa* populations

Varroa samples collected in previous studies, (Erdem et al., 2024; Koç et al., 2021; Vlogiannitis et al., 2021), were used for molecular screening of *Octβ2R* target-site mutations. A total of 114 *Varroa* populations (66 from Türkiye and 45 from Belgium) were screened. Since an individual DNA extraction was conducted for the Belgian samples (Vlogiannitis et al., 2021), 10 individual DNA samples were pooled per population and used as template for mutation screening. For the Turkish samples, DNA was obtained using pools of ten adult mites (Koç et al., 2021).

The primers used to amplify the *Octβ2R* gene of *V. destructor* are presented in Table S1. PCR reactions were performed in a TAdvanced thermal cycler (Biometra, Germany) using the GoTaq® Flexi kit (Promega, USA) in a total volume of 50 µL, containing 1 µL of dNTP, 10 µL of 5X Buffer, 3 µL of MgCl, 2.5 µL of each primer, 0.25 µL of Taq DNA polymerase, and 2 µL of template (70-130 ng μL^{-1}). The temperature cycling procedure consisted of an initial denaturation step at 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 2 minutes.

PCR amplicons were purified using the E.Z.N.A. Cycle-Pure kit (Omega Biotek, USA) and Sanger sequenced at the LGC Sequencing Service (Berlin, Germany). The obtained sequences were analyzed with BioEdit 7.0.5 software (Hall, 1999). The presence and frequencies of mutations were determined based on the peaks in sequencing chromatograms at the mutation site, as previously described by (Van Leeuwen et al., 2008).

2.2 Amitraz bioassays on Turkish *Varroa* populations and Y337F screening

After the molecular screening, bioassays were conducted to reveal the role of Y337F in amitraz resistance. For this purpose, three *Varroa* populations were collected from Turkish apiaries located in Mardin (Vd1), Diyarbakır (Vd2) and Ankara (Vd3) with a history of acaricide treatment (including

amitraz) in 2023, directly from hives during spring. A putatively susceptible *Varroa* population was collected from an apiary in Şırnak (Türkiye) with no prior chemical acaricide treatment for several years. Mites, along with bee broods, were transferred to the laboratory for toxicity assays.

Toxicity assays were performed according to Vlogiannitis et al. (2021). Briefly, technical grade amitraz (Sigma Aldrich; CAS No.: 33089-61-1) was dissolved in acetone and gradually decreasing concentrations were prepared using the stock solution. Next, 1 ml of dissolved amitraz was added into 10 ml glass vials which were then rolled until acetone evaporated. After transferring 10 female adult mites, glass vials were closed with a wet cotton to ensure high humidity and then kept at 25°C for 20h. Bioassays were performed with three replicates and five concentrations. Mites that could not move their legs after 20h were considered dead. Probit analysis was used to assess LC_{50} values and their 95% confidence limits with PoloPC (LeOrA Software, Berkeley, CA). Aceton was used in control groups. In case of the putatively susceptible strain, only a single concentration (0.25 mg/L) with three replicates could be tested due to the limited number of mites obtained during the sampling time.

DNA extraction for three populations used in bioassays was performed from pools of 10 mites using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. 100 µl elution buffer was used to elute the DNA in the final step. The purity and quantity of genomic DNA was evaluated using a NanoDrop 2000 (Thermo Scientific) spectrophotometer. DNA extracts were stored at -20 °C until used. Analysis of the target gene was performed as described earlier.

2.3 **Genotyping after amitraz exposure**

In addition to the amitraz bioassays, an assay was conducted using the highest concentration (10 mg a.i./L) from the bioassay for further genotyping of surviving mites individually. For each population, 20 *Varroa* mites were used for diagnostic concentration assays. After amitraz exposure, viability was assessed and DNA from individual mites (both dead and alive) was extracted following the method used by Alissandrakis et al. (2017). Genotyping was performed as described earlier. A Fisher's exact test was used to compare the survival rates of the homozygous mutant mites (Y/Y) with homozygous

wild type (Y/Y) and heterozygous (Y/F) mites for each of the evaluated populations. The link of the Y337F substitution with amitraz resistance was considered significant for p values below 0.05.

2.4 **CRISPR-Cas9 gene editing in *T. urticae***

The genetic model organism *T. urticae* was used to assess the *in vivo* role of Octβ2R Y337F in amitraz resistance. *T. urticae* Octβ2R (*tetur08g04980*, accessible at https://bioinformatics.psb.ugent.be/orcae/overview/Tetur) also has a tyrosine at the residue (419, *T. urticae* numbering) corresponding to Y337 in *V. destructor* Octβ2R and phylogenetic analysis showed a 1:1 orthology of Octβ2R between these two organisms (Fig. S1). The German Susceptible Strain (GSS) of *T. urticae* was used in CRISPR-Cas9 experiments using the highly efficient and recently developed SYNCAS method (De Rouck et al. 2024). GSS is a reference susceptible population that has not been exposed to any pesticides for more than 50 years (Stumpf et al., 2001). The target region of GSS was amplified and the presence of tyrosine at position 419 was confirmed. PCR was performed as described earlier using primer pair "Tu_diag " (Table S1).

Recombinant *Streptococcus pyogenes* Cas9 protein (Alt-R® S.p. Cas9 Nuclease V3) at a custom concentration of 50 µg/µl, sgRNAs (Alt-R™ CRISPR-Cas9 sgRNA), ssODN repair template (Alt-R™ HDR Donor Oligo) and enhancer V2 were purchased from Integrated DNA Technologies (Leuven, Belgium). BAPC was purchased from Phoreus Biotech whereas saponin was obtained from Sigma Aldrich (cat#558255) and were dissolved in water at a concentration of 50 µg/µl and 2.6 µg/µl respectively. The sgRNA targeting *Octβ2R* (5'-TTAAAGTACGCATAGATGAC-3') was designed using the CRISPOR website (Concordet and Haeussler, 2018), where the guide closest to the mutation site was selected, and was dissolved in TE buffer at a concentration of 20 µg/µl. Repair template (File S1) was dissolved in nuclease free water at a concentration of 1 mM.

Gene editing was performed as described earlier (De Rouck et al., 2024). Briefly, the CRISPR-Cas9 injection mix was prepared combining 1.8 µl Cas9 (50 µg/µl), 1.8 µl sgRNA (20 µg/µl), 0.5 µl ssODN (1000 µM), 0.5 µl BAPC (50 µg/µl) and 0.2µl saponin (2.6 µg/µl). About 1,500 virgin female mites (4

192 days post adult emergence) were aligned on agarose gel and were injected with 3 nl of CRISPR mix
193 using a Nanoject III (Drummond Scientific). All mites were injected a second time with 1 nl of Enhancer
194 V2 (100 µM) immediately after all mites had been injected with the CRISPR mix.

195 **2.5 Establishment of homozygous lines**

196 Injected mites were transferred to clean leaf discs and allowed to lay eggs for 24h. Next, females were
197 transferred to a new leaf and laid eggs for another 24 hours. Since the injected mothers remained
198 unfertilized, all eggs developed into haploid males as a result of the arrhenotokous reproduction
199 system of spider mites. Then a 100 mg a.i./L of commercial formulation of amitraz (Mitac EC; 20 g /L,
200 Arysta Life Science) was sprayed to larval stages of mites in order to eliminate individuals with
201 susceptible genotypes. This diagnostic concentration (100 mg a.i./L) was approximately twice the LC_{99}
202 value for the larval stages of mites in the GSS population.

203 Survivors of amitraz treatment were transferred to new square-cut leaf discs along with three virgin
204 susceptible females and allowed to mate for 2-3 days. Subsequently, females were individually
205 transferred to new square-cut leaf discs, while males were genotyped to ascertain the occurrence of
206 the intended CRISPR event. At least 24 F1 daughters, resulting from the preceding mating, were in turn
207 mated with their first-born son. These first-born males were genotyped, and subsequent generations
208 originating from pairs with mutant males were retained. Then, resulting F2 daughters (either
209 heterozygous or homozygous mutant) were mated with their sons once more, allowing them to lay
210 eggs. Finally, these F2 daughters were genotyped after a number of eggs were laid and homozygous
211 females were identified based on the Sanger chromatogram. The resulting line was further expanded
212 for toxicity assays (Fig. 2)

213 2.6 **Toxicity assays on CRISPRed lines of *T. urticae***

214 Bioassays on homozygous lines of *T. urticae* were conducted according to İnak et al (2022). Briefly, 25-
215 30 adult female mites were transferred onto square-cut leaf discs (9 cm^{-2}) placed on wet cotton. Each
216 leaf disc was sprayed with 0.7 ml of a dilution of the commercial formulation of amitraz (Mitac EC; 20

g/L; from Arysta Life Science, Ethiopia) using a Cornelis spray tower, resulting in 1.5 ± 0.05 mg cm^{-2} acaricide deposit. Control groups were only sprayed with distilled water. Bioassays were performed using at least five concentrations with four replicates. Mortality was assessed after 72 hours, with mites that did not move upon probing with a fine brush, being considered as dead. The obtained data was subjected to probit analysis to assess LC_{50} values using POLOPC (LeOra software, USA). If the 95% confidence intervals of resistance ratios (RRs) did not include or overlap 1, then the LC_{50} values were considered significantly different (Robertson et al., 2017).

3 Results

3.1 A novel Y337F mutation in a conserved region of Octβ2R

Molecular screening of 129 Turkish and Belgian *Varroa* populations did not reveal any of the previously reported amitraz resistance mutations (N87S and Y215H). However, an amino acid substitution - tyrosine (Y) to phenylalanine (F) - at residue 337 (*V. destructor* numbering; 7.53 according to Ballesteros-Weinstein numbering; [Ballesteros and Weinstein, 1995]) in a conserved fragment of TM7 of Octβ2R (Fig. 3), was detected in 20 out of 66 Turkish *Varroa* samples, and for 8 of these 20 samples the mutation was fixed (Table S2), while this mutation was absent in all screened Belgian populations. The spatial distribution of the Y337F mutation in Türkiye is illustrated in Fig. 4. For the sampling sites of the Belgian *Varroa* samples, see Vlogiannitis et al. (2021).

3.2 Amitraz toxicity on *Varroa* populations

Over three independent replicates, only 6.6% (±3.3) of *Varroa* mites from the putatively susceptible population survived exposure to 0.25 mg a.i./L of amitraz, indicating an LC_{50} value lower than 0.25 mg a.i./L, and hereby confirming its status as susceptible. On the other hand, *Varroa* populations with a preceding amitraz exposure history exhibited over 8.4 to 12.1 fold resistance (LC_{50} values between 2.1-3.0) to amitraz compared to their susceptible counterpart (Table 1).

Table 1: LC_{50} values and resistance ratios (RRs) of *V. destructor* populations

Population	Slope	LC_{50} (mg a.i./L) (95% CL)	χ^2 (df)	RR
Susceptible		<0.25		
Vd1	1.82±0.27	2.10 (1.54 - 2.82)	2.6 (13)	>8.4
Vd2	1.84±0.28	2.81 (2.10 - 3.83)	1.4 (13)	>11.2
Vd3	1.64±0.27	3.02 (2.20 - 4.30)	1.5 (13)	>12.1

3.3 Determination of Y337F mutation in bioassay populations and genotyping after amitraz exposure

The *Varroa* populations exhibiting resistance in the bioassays harbored the Y337F mutation, but this mutation was not fixed (Fig. S2, File S2).T Genotyping of individuals after exposure to 10 mg a.i./L amitraz revealed that all *Varroa* mites that survived exposure had the Y337F mutation in a homozygous state. Conversely, the sequencing chromatograms obtained from dead mites indicated they were either homozygous for the wild-type allele or were heterozygous for the mutation (Table 2).

Table 2: Genotyping of single *Varroa* mites after 10 mg a.i./L amitraz exposure. Between brackets are the number of mites in each group. P-values were calculated using a Fisher's exact test comparing the homozygous 337F mites (F/F) with homozygous 337Y (Y/Y) and heterozygous (Y/F) mites. In all three populations, a significant link ($p < 0.05$) between the F/F genotype and amitraz resistance is present.

Populations	Alive/Dead	# Y/Y	# Y/F	# F/F	P-value
Vd1	Alive (2)	-	-	2	0.0048
	Dead (18)	17	1	-	
Vd2	Alive (3)	-	-	3	0.0009
	Dead (17)	15	2	-	
Vd3	Alive (3)	-	-	3	0.0009
	Dead (17)	17	-	-	

3.4 CRISPR-Cas9 experiments and susceptibility of homozygous lines to amitraz

Since the Y337 residue is located in a highly conserved region in TM7 (Fig. 3) and CRISPR-Cas9 gene editing is not yet available for *V. destructor*, an attempt was made to introduce the Y337F mutation in the Octβ2R orthologue of *T. urticae*, the phylogenetically closest species amendable to genetic manipulation.

Despite multiple trials, a clean knock-in (KI) mutant could not be obtained after injecting over 1,500 virgin *T. urticae* females (Table S3). In the resulting offspring, only two males survived after amitraz

exposure. The genotypes of these two males are available in File S2. For the first male, although the Y337F substitution was successfully introduced, an error occurred during homology directed repair causing a part of the ssODN template to be incorporated in its entirety after the target site (Fig. S3), causing an early stop codon in the reading frame resulting in the loss of the last 30 C-terminal amino acids of the receptor (Fig. 3). In the case of the second (chimeric) male, a deletion of an amino acid (isoleucine) immediately before Y337 was detected. Although the initial mating event produced F1 heterozygous females, no subsequent F2 males with the deletion could be identified among the 50 genotyped sons, most likely due to the deleterious effect of this deletion on the fitness of the mites.

From the first male, exhibiting the Y337F mutation in a truncated version of Octβ2R, we were able to establish two independent homozygous lines originating from the same CRISPR event (Tu1 and Tu2). Toxicity assays revealed that the CRISPRed lines exhibited 3-3.7 fold resistance ratios (RRs) compared to the GSS background used for gene editing.

Table 3. LC_{50} values of susceptible wild type GSS and Y337F KI lines. LC_{50} values of which the 95% confidence intervals of the resistance ratio (RRs) do not include or overlap 1, are considered significant.

Populations	**Slope ± SE**	**LC_{50} (mg/L) (95% CI)**	**χ^2 (df)**	**RR (95% CI)**
GSS	3.72±0.51	25.29 (20.65 - 29.62)	10.7 (22)	-
Tu1	2.23±0.21	76.75 (62.52 - 91.25)	6.8 (17)	3.0 (2.3-3.9)
Tu2	2.29±0.17	94.62 (83.49 - 107.06)	6.0 (18)	3.7 (3.0-4.6)

4 Discussion

Amitraz resistance is believed to be less widespread compared to resistance to other chemical acaricides in *V. destructor* populations worldwide. However, several resistance cases have been reported in different continents (Mitton et al., 2022), with the first report dating back more than 20 years ago (Elzen et al., 2000). Amitraz has been used in Türkiye for many years (Öder, 1983), and its widespread use continues to the present day (Erdem et al., 2024; Girisgin et al., 2019; Koç et al., 2021).

Target-site mutations have been suggested to be a major mechanism in amitraz resistance in *Varroa* as well as ticks (Corley et al., 2013; Hernández-Rodríguez et al., 2022; Jonsson et al., 2018; Rinkevich et al., 2023). More specifically, N87S and Y215H mutations were reported in Octβ2R of French and American populations of *V. destructor*, respectively (Hernández-Rodríguez et al., 2022). In the present study, over 110 Turkish and Belgian *V. destructor* populations were screened for the presence and distribution of Octβ2R target-site mutations. Notably, none of the Belgian *Varroa* samples exhibited any mutations. This observation aligns with the limited use of amitraz among Belgian beekeepers (Brodschneider et al., 2023). Although previously reported mutations were also absent, nearly 30% of Turkish *Varroa* samples from different geographical regions harbored a novel Y337F mutation in a conserved fragment of TM7 of Octβ2R. On the contrary to Belgian apiaries, amitraz together with flumethrin, are among the most commonly used acaricides (Erdem et al., 2024; Koç et al., 2021).

The relatively high prevalence of the mutation specifically in the Turkish populations, coupled with its highly conserved position, suggests a functional role in amitraz resistance. Initially, bioassays revealed *Varroa* populations exhibiting over an 8-fold resistance to amitraz. However, this is only an (under)estimate, as the exact LC_{50} value of the susceptible population could not be determined and is clearly much lower than the assayed concentration of 0.25 mg a.i./L. Genotyping of resistant populations revealed the presence of a tyrosine (Y) to phenylalanine (F) substitution at position 337. However, this mutation was not fixed and thus the effect on toxicity is also probably underestimated. Because we used DNA of pooled mites, and the mutation was not fixed in the pooled mites, a direct link between mutation and resistance needs further validation. To further corroborate the significance of this mutation a genetic linkage experiment was conducted. *Varroa* mites were genotyped following exposure to amitraz and it was demonstrated that all surviving mites harbored the Y337F mutation in a homozygous state, whereas dead mites exhibited the wild-type Y337 allele or were heterozygous. Considering the susceptibility of heterozygous mites, the Y337F mutation most likely inherits as a recessive mutation.

After establishing genetic linkage, further validation of the Y337F mutation in *V. destructor* Octβ2R was undertaken using CRISPR-Cas9 and *T. urticae* as a model chelicerate species (De Rouck et al., 2024). Gene-editing via CRISPR-Cas9 in *T. urticae* resulted in two different CRISPR events. One of the CRISPR-events caused a deletion of residue I336 and attempts to generate a homozygous line for the deletion were made. Although heterozygous F2 daughters could be obtained from the initial cross, all F2 sons were wild type, indicating the deletion is most likely detrimental for receptor functioning and lethal in the homozygous state. The original male harboring this deletion might have survived due to chimerism with tissue that has a functional wildtype Octβ2R. The observed lethality is not unexpected given the highly conserved nature of I336, being part of the NPxxY motif (Fig. 3).

The second CRISPR-Cas9 event introduced the Y337F mutation correctly, but resulted in a premature stop, leading to a protein with a truncation of 30 AA. The mutation was successfully fixed in two stable lines that were used in toxicity assays. Homozygous *T. urticae* lines harboring the mutation were more than 3-fold resistant compared to original susceptible GSS strain used for gene editing, validating the effect of the mutation on amitraz toxicity. However, the Octβ2R in these two CRISPRed lines lacked the last 30 AA. Although these residues are not conserved across Acari (Fig. 3), it cannot be ruled out that this might affect amitraz toxicity, especially given that this was the only KI mutant found after injecting 1,500 females. This is a low transformation rate compared to previous KI experiments performed in *T. urticae*, where mutations were introduced at similar distance from the Cas9 cleavage (De Rouck et al., 2024; İnak et al., 2024). Perhaps the effect of Y337F alone on amitraz resistance is not as strong as it is in *Varroa*, and hence the applied selection dose of amitraz on the offspring, resulting from injected females, was simply too high. Nevertheless, given the apparent lack of the mutation on viability, it is likely that the truncation does not influence the receptor's functioning.

In summary, although we could not unambiguously link resistance with the mutation alone, the CRISPR-Cas9 data is still in line with the genetic linkage and bioassay data on *Varroa* populations. In addition, the CRISPRed lines do confirm that Octβ2R is the true target of amitraz, as genetic

modification lead to a clear effect in susceptibility. This is most likely caused by decreased binding, whether caused by the mutation, truncation, or a combination of these genetic events. However, the relative contribution of the substitution or the loss of the last 30 amino acids to resistance remains unclear.

Similar to other GPCRs, octopamine receptors, including Octβ2R, have seven transmembrane domains (Evans and Maqueira, 2005; Hill et al., 2018). The binding of endogenous GPCR ligands including octopamine is mediated by polar and hydrophobic contact residues located in TM3-5-6-7 (Chen et al., 2011; Rosenbaum et al., 2009). The orthosteric active metabolite of amitraz, DPMF, is reported to bind to Octβ2R (Takata et al., 2020), followed by activation of specific G proteins, which leads to transient changes in concentrations of intracellular second messengers. In contrast to previously reported mutations (N87S and Y215H), the newly uncovered Y337F mutation was located in a highly conserved NPxxY motif, which is conserved in 94% of all A class GPCR (Oliveira et al., 1999; Rosenbaum et al., 2009; Wang et al., 2020), at the cytoplasmic end of TM7 of Octβ2R. The NPxxY motif does not have a direct interaction with the bound G protein, however, it plays a crucial role in stabilizing the active conformation (Rosenbaum et al., 2009). More specifically, contact of tyrosine (7.53; Y326 in the human beta-2 adrenoreceptor) within the NPxxY motif with another conserved tyrosine in TM5 (5x58) via a hydrogen bond through water molecules, is of critical importance to preserve the active state of the receptor. This coupling promotes an outward shift in the cytoplasmic terminus of TM6, enhancing the affinity for G protein binding and facilitating intracellular signaling (Barak et al., 1994; Ragnarsson et al., 2019). The substitution of the amino acid from Y to F is a favored mutation according to Russel et al. 2003, with the only difference being the absence of the reactive hydroxyl group in the ortho-position on the benzene ring for phenylalanine (Betts and Russell, 2003). Previous studies have shown that the Y337F$^{7.53}$ mutation in various GCPRs, including β2-adrenoceptors, resulted in the disruption of the Tyr$^{7.53}$ -Tyr$^{5.58}$ hydrogen bond, causing a reduction in lifetime of active state and decreased G protein activation (Gabilondo et al., 1996; Goncalves et al., 2010; Ragnarsson et al., 2019). In addition, high-affinity binding of agonists was also reported to be reduced in human Y326F beta-2

adrenoreceptors (Gabilondo et al., 1996; Ragnarsson et al., 2019) Hence the resistance towards amitraz-induced Octβ2R activation by Y337F in *Varroa* mites could be explained by a similar mechanism.

In conclusion, a novel mutation, Y337F, was identified at the target site of amitraz, Octβ2R, located within a highly conserved fragment of TM7. The observed decrease in sensitivity within *Varroa* populations, genetic linkage, coupled with the widespread distribution of this target-site mutation, underscores the importance of favoring rotational use of amitraz in the country. Crispant *T. urticae* lines harboring Y337F exhibited decreased susceptibility towards amitraz. Further studies are warranted to elucidate the exact role and phenotypic strength of the mutation in conferring amitraz resistance in *Varroa*.

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579

Figure captions

Fig. 1: Snake plot of the β-adrenergic-like octopamine receptor from *V. destructor* generated using "Protter" (Omasits et al., 2014) showing the seven transmembrane regions (I-VII). Residue Y337 Is marked in yellow. Numbers indicate residue positions. Molecular structures of the pro-acaricide amitraz and its orthosteric active metabolite DPMF, which binds to the octopamine receptor, are shown in the upper right corner.

Fig. 2: Graphical representation of the generation of CRISPR KI mutants in *T. urticae.*

Fig. 3: ClustalW alignments of β-adrenergic-like octopamine receptors. Residues are colored according to their physicochemical properties: aliphatic/hydrophobic (ILVAM) – teal; aromatic (FWY) – yellow; positive (KRH) – blue; negative (DE) - red; hydrophilic (STNQ) – green; conformationally special (PG) - purple, cysteine (C) - orange. Numbers flanking the alignment indicate respective beginning and ending position of the residues in the full protein sequence of the organism. A) Partial alignment of Octβ2R displaying TM7 and beyond from various organisms. 50% threshold was used for identity shading. Orange triangles indicate the highly conserved NPxxY motif associated with activation of G protein coupled receptors. A black star indicates residue Y337 that was investigated in this study. Accession numbers of the protein sequences used in the alignment are as follows: XP_022664697.1 (*Varroa destructor*), tetur08g04980 in ORCAE (*Tetranychus urticae*), XP_053214250.1 (*Panonychus citri),* derpt01g01960 (*Dermatophagoides pteronyssinus*), XP_028966328.1 (*Galendromus occidentalis*), DEGAL2972g00010 in ORCAE (*Dermanyssus gallinae*), AFC88978.1 (*Rhipicephalus microplus*), KAE8743276.1 (*Frankliniella occidentalis*), NP_001280501.1 (*Tribolium castaneum*), NP_001034049.1 (*Drosophila melanogaster*), CCO13923.1 (*Apis mellifera*), BAJ06526.1 (*Bombyx mori*). B) Full alignment of Octβ2R from *V. destructor* and *T. urticae* demonstrating a high degree of conservation. Amino acids in red font were lost in the CRISPRed *T. urticae* line due to incorrect repair (Fig. S3) and replaced with two valine residues.

Fig. 4: A map showing the spread and frequency of the Y337F substitution in *Varroa destructor* populations from Türkiye. Corresponding data used to generate the graphs is shown in Table S2. Sampling locations of Belgian *Varroa* mites are reported in Vlogiannitis et al. (2021).