Final Report

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Student Project No. CP 162

Combating insecticide resistance in the tomato leafminer, *Tuta absoluta*

Charles Grant
University of Exeter
Penryn Campus
Penryn
Cornwall TR10 9FE

Supervisors: Professor Chris Bass, Professor Richard ffrench-Constant, Martin Williamson and Dr. Rob Jacobson

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CONTENTS

1. Industry Summary

- **Resistance to Conserve and Coragen in UK populations of *T. absoluta*?**

T. absoluta collected from the UK showed high-level resistance (up to 480-fold) to Conserve (Spinosad). This data suggests Conserve will no longer be an effective control measure in UK glasshouses. Resistance to Coragen (Chlorantraniliprole) was low at all sites tested but still higher than in susceptible strains. Exposure of laboratory populations to Coragen showed they could evolve high levels of resistance (4167-fold). These results show that Conserve is still an effective compound at controlling *T. absoluta* however populations are capable of evolving high levels of resistance.

- **What are the underlying mechanisms of this resistance?**

The nerve receptor that Conserve targets had undergone structural alteration of the translated gene rendering the target site of the compound redundant - explaining the high levels of resistance observed. An second, less severe alteration was also detected and was associated with the moderate resistance. Therefore, *T. absoluta* can evolve multiple independent mechanisms of resistance to Conserve. Analysis of the target site of Coragen showed no clear signs of any alteration before selection but after prolonged exposure to the pesticide a mutation arose that inhibited the binding of the pesticide resulting in the 4167-fold resistance. Together these finding explain the resistance observed in the UK and highlight biomarkers capable of being utilised for molecular diagnostics - rapidly identifying resistant populations informing pest management strategies.

- **Does *T. absoluta* possess the capacity to detoxify pesticides?**

Further research into Coragen resistance showed the *T. absoluta* could also alter its gene expression to detoxify the compound. It could express high levels of detoxification enzymes specific to Coragen mitigating the effect of pesticide. This finding is beneficial for pesticide research as it identifies metabolic pathways that can be targeted by pesticide manufacturing companies to enhance pesticide formulations that maintain the efficacy of the pesticide.

- **Can asexual reproduction in *T. absoluta* overcome mating disruption?**

T. absoluta that were collected after the introduction of the mating disruptor Isonet T showed a small but significant increase in the rate of parthenogenesis associated with the use of Isonet T. Marked differences in several other life history traits associated with reproduction including longevity were also observed in these populations. The low rate of parthenogenetic reproduction observed in this study is unlikely to result in loss of efficacy of mating disruption. However, the observed changes in longevity and egg laying may allow *T. absoluta* to persist for longer within the crop.

2. Introduction

Introduction

The tomato leafminer *Tuta absoluta* was detected in tomato glasshouses in the UK in 2009 and immediately caused massive economic loss to tomato growers. Shortly after its arrival, a succesful integrated pest management (IPM) strategy was implemented which resulted in a good level of control of the pest. The strategy utilised the pesticides Conserve and Coragen along with the biocontrol agent *Macrolophus pygmaeus.* Conserve was applied early in the growing season to supress early populations of *Tuta absoluta.* This allowed *Macrolophus pygmaeus* to establish effective population sizes and control *T. absoluta* populations through predation of the larvae. If *T. absoluta* populations were unable to be controlled by *M. pygmaeus* then a second round of chemical control was applied in the form of Coragen. This IPM strategy was successful at mitigating economic losses by the pest. Shortly after, however, growers in the UK were reporting loss in efficacy from the IPM strategy. It was suspected that resistant populations of *T. absoluta* had evolved. It was vital for growers to find out whether this was the case, and of great interest to agronomists to understand the mechanisms of resistance - informing future IPM and enabling rapid identification of resistance in populations of previously susceptible *T. absoluta.*

In 2017, the development and roll-out of a new control measure, a mating disrupter under the trade name of Isonet T, proved successful at eradicating *T. absoluta* infestations. The mating disruptor utilised a synthetic version of *T. absoluta* female sex pheromone inundating glasshouses with the compound - preventing male moths from successfully finding females and inhibiting reproduction. Research conducted by Megido and Verheggen (2012) however, raised caution about the long-term effectiveness of this strategy as it highlighted the capacity for females to reproduce asexually potentially rendering the mating disruption redundant. This concern was further elevated after some glasshouses in the UK reported a reduced level of efficacy by the control measure. This incentivised my research project to investigate if asexual reproduction was occurring in glasshouses in the UK and if this was contributing to a reduced effect of Isonet T.

The objectives of this research were to identify:

1. **If resistance to Conserve and Coragen is present in UK populations of *T. absoluta*?**
2. **What are the underlying mechanisms of this resistance?**
3. **Can *T. absoluta* rapidly evolve resistance under selection?**
4. **Does the genetic background of *T. absoluta* possess the capacity to detoxify and neutralise the effects of pesticide through modification or increased expression of detoxification genes?**
5. **Can *T. absoluta* adapt its mating strategy to over come mating disruption?**
6. **What would be the effects of any/all of these adaptations to current and future IPM?**

3. Materials and methods

3.1. Methods

3.1.1. Insects.

T. absoluta were collected in 2015 from four commercial tomato glasshouse sites across the UK. ~200 larvae were collected from each site They have been coded as (EVH), (NY), (LAN) and (IOW). A second strain was collected from EVH in 2016 (EVH2016) after Conserve had not been used for a growing season and *T. absoluta* had become re-established in the crop. A Conserve susceptible strain was acquired from Rothamsted Research, Hertfordshire (TA1). Insects were housed in controlled environment rooms at 25°C, 60% R.H., 16:8 light-dark cycle and fed *ad libitum* on tomato plants (var. Money Maker).

3.1.2. *Leaf dip assays*.

Pooled replicates of larvae were subjected to a range of pesticide concentrations. The pesticide was diluted in Triton X-100 (0.2 g L^{-1}) to make a range of concentrations expected to induce 0-100% mortality in *T. absoluta*. Five replicates of fresh tomato leaves (var. Money Maker) were immersed in each of the varying concentrations of pesticide solutions and allowed to dry for ~ 2 hours. Once dried, each individual leaf was placed in a Petri dish on a moist sheet of filter paper. 250 L2-L3 larvae were collected from the relevant culture and 10 larvae were placed on each leaf. The larvae were left in CT rooms at 25°C, 60% R.H., 16:8 light-dark cycle for 72 hours. After 72 hours mortality was scored. Probit analysis was used to assess LC_{50} values and confidence intervals using R (Burgess , King, and Geden 2020). Resistance ratios (RR) were calculated by dividing the LC_{50} value of the target population by the LC_{50} of the susceptible strain.

3.1.3. *Coragen selection*

T. absoluta were selected with Coragen over a period of ~ 12 months. Selection chambers consisted of 2 containers placed inside each other with petiole sized holes in the bottom of the inside container and water in the bottom of the outside container. Fresh tomato leaves were submerged in Coragen solution (at 1ppm) and their petioles placed through the holes of the inside container. A coarse mesh was secured over the selection chamber. Infested tomato leaves were extracted from culture (~500 L3 larvae) and placed on the coarse mesh with a fine mesh placed over that. Larvae exited the mines and repelled down to the fresh treated leaves. Once larvae started to pupate the contents of the chamber was transferred to a bug dome (W60 x D60 x H60 cm, MegaView science co.) with a fresh tomato plant to bulk up population numbers over 1-2 generations. This was repeated for Coragen

concentrations of 5 mg L^{-1}, 10 mg $L^{-1}$and 50 mg L^{-1}. The population was then bioassayed to assess resistance.

3.1.4. *RNA extraction and cDNA synthesis*

RNA extractions were carried out on pools of 10 individuals using ISOLATE II RNA Mini Kit (Bioline, London, UK) following the manufacturer's instructions. cDNA was then synthesized from 1µg of total RNA using Maxima H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, US) according to the manufacturer's protocol.

3.1.5. *Assessment of conserve target site, the Taα6 from resistant strains of T. absoluta*

Total RNA was extracted using a RNA Mini Kit (Bioline, London, UK) and cDNA synthesized from 1µg of total RNA using Maxima H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, US). From this cDNA a 1585 bp region of the nAChR *Taα6* sub unit was amplified using primers listed in table 1 designed in Geneious (v8.1.9, Biomatters Ltd) based on the previously published *Taα6* (Berger et al. 2016). A nested PCR protocol was employed using 1 µl (10 µM) of each primer, 10 µl of DreamTaq Green PCR master mix (Thermo Fisher Scientific, Waltham, US), 10ng (1µl) of cDNA as a template in the first round and (1µl) of a 1:10 dilution of the product used in the second reaction, made up to a total volume of 20 µl with sterile distilled water. Temperature cycling conditions were the same for both reactions and comprised of 3 min at 95°C followed by 35 cycles of 95°C for 30s, 54.5 for 30s and 72°C for 60s, and a final extension step of 72°C for 6 min. The first round reaction utilized primers 1R and 2R. Three second round reactions utilized primer combinations 3F and 4R, 3F and 6R and 3F and 4R (Figure 1).

Table 1. Primers and positions of primers for nAChR Taα6 amplification and sequencing

Primer	Sequence 5'-3'
1F	GCATGTATCGCTGCTAGCGCAAC
2R	TCATTGCACGATGATGTGTGGG
3F	TGGTCCACTACCTCGCAGTGC
4R	CCACCGTTGCGATGATCGTGAA
5F	TCCGGGCATATTCAAGAGCACATG
6R	GTCGTGACATCCTCAAGATCCATG

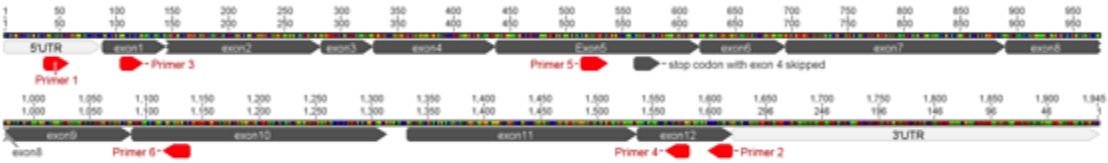


Figure 1. Position of primers (Table 1) on nAChR α6 sub unit

3.1.6. *Association study*

Analysis of the *Taα6* sub unit exposed a triplet deletion present in the LAN strain corresponding to position F238. To examine the association of this candidate conserve resistance mutation (F238del) with phenotypic resistance a phenotype-genotype association experiment was conducted using the LAN strain as it was heterogeneous for the mutation (see results). Twenty replicates of ten larvae (L2-L3) of this strain were placed on tomato leaves treated with 150 ppm of conserve. As a control five replicates of ten larvae were placed on a tomato leaf treated only with the insecticide dilutant. Mortality was scored after 72 h and all larvae were snap-frozen in liquid nitrogen and stored at −80 ◦C. DNA was subsequently extracted from over 200 individual larvae using DNAzol following the manufacturer's protocol. A TaqMan SNP genotyping assay was designed using the Custom TaqMan Assay Design Tool (Applied Biosystems Foster City, US) and used to score the genotypes of dead and alive individuals generated above for the F238del mutation. Primers and minor groove binder (MGB) probes are detailed in Table 2. Each probe was labeled with a 5' reporter dye specific to both wild-type and mutant alleles. The VIC reporter dye was used to detect the wild-type allele and 6FAM to detect the mutant allele. Each probe also had a 3' non-fluorescent quencher and a minor grove binder at the 3' end. The minor grove binder enhances allelic discrimination of the probe by increasing melting temperature (TM) between matched and mismatched probes. Dissociation of the reporter dye from the probe during the PCR results in its spatial separation from the non-fluorescent quencher resulting in the emission of a photon of wavelength specific to the reporter dye used. This produced a unique and detectable fluorescent signal during the qPCR for each allele.

Each PCR reaction contained 2 µl genomic DNA, extracted from individual insects using DNAzol reagent following the manufacturer's protocol, 7.5 µl of SensiFAST SYBR No-ROX kit (Bioline), 800nM of each primer and 200nM of each probe. Samples were run on CFX96 system (Bio-Rad) using the temperature cycling conditions of 10 minutes at 95°C followed by 40 cycles of 95°C for 10 s and 60°C for 45 s. The increase in fluorescence of the two probes VIC and FAM was monitored in real time. In all runs, at least one control for each genotype (mutant homozygous, heterozygous and wild-type homozygous) was included. Homozygous resistant individuals produced a signal from the FAM probe only, homozygous susceptible individuals produced a signal from the VIC probe and heterozygous individuals emitted a signal from both probes. To analyze the individual interactions between phenotype and genotype post-hoc analyses were conducted with adjusted residuals converted to a Chi-square score and compared to a Bonferroni adjusted α. Chi-square and post-hoc analyses were completed in SPSS (ver. 22, IBM Corp. Armonk, US).

Table 2. List of TaqMan assay primers and probes. Highlighted nucleotides represent triplet deletion.

Primer/probe	Sequence
6FAM	ACGATCAGGTTGAAATA
VIC	ATCAGGTTGAAGAAATA
Forward primer	CAGAATCAGAAGACGGACGCTATAC
Reverse Primer	GCCATGGAGGAAATCAGAACACAT

3.1.7. *Sequencing of Coragen target site, the ryanodine receptor (RYR)*

To analyse the structure of the *T. absoluta* RyR gene a 1353 bp region (encompassing the C-terminal domain) containing sites of previously reported Coragen resistance-conferring mutations (Kato et al. 2009; Troczka et al. 2012) was amplified by PCR. The standard PCR protocol was used with the primers listed in table 3.

Table 3. List of primers for the amplification of the RyR C-terminal domain.

Primer	Sequence
RyR I4970 and G4946 forward	AAGGTTCAGGACTTGGAGAC
RyR I4970 and G4946 reverse	GCTGTATAAAGGTGCGTGTC
RyR I4970 forward	GAGCATGTCATCAACATAGCG
RyR I4970 reverse	GGAGTCGAAGTCATATGTCTC
RyR G4946 forward	GAGACATATGACTTCGACTCC
RyR G4946 reverse	GGTGATGTCGAAGATGATGC

3.1.8. *Analysis of G4903V proximity to coragen binding site*

To implicate target site alterations with the coragen binding site of the RyR (Lin et al. 2020) the 3D protein structure of the rabbit RyR (PDB ID:5J8V) was downloaded from N.B.C.I. To validate the use of the rabbit RyR as a model, the conserved transmembrane region was also downloaded for several other species including mammals and insects and aligned in Geneious v10.2.6 (Biomatters Ltd.) to show consensus. G4903V and the coragen binding site were then mapped to the rabbit RyR to highlight physical association.

3.1.9. *Genomic and transcriptomic resources resources*

Genome assembly was completed by Kumar Singh. All *T. absoluta* transcriptomes were acquired from Syngenta (Switzerland) that were collected from commercial glasshouses in Spain (Ssus and Sres). Ssus was susceptible to Coragen and Sres was highly resistant. Sus and Mur were collected

from Spain by Pablo Bielza and sequenced at the Earlham Institute. Sus and Mur were susceptible and moderately tolerant to Coragen.

3.1.10. *Assessment of target-site modifications*

Assessment of sequence reads mapped to the RyR, the coragen target-site, was conducted to ensure that no target-site alterations were present in the sequenced strains. For this transcriptomes were mapped to the RyR gene sequence for all strains using Geneious mapper with default parameters (Geneious v10.2.6, Biomatters Ltd.) Alignments were checked for the presence of mutations and indels.

3.1.11. *Candidate resistance gene selection*

Candidate resistance genes were selected based on i) their presence in comparisons involving both resistant strains, ii) the literature of resistance to coragen and iii) the level of observed overexpression. The sequences of candidate genes for each strain were also examined by manual inspection of RNAseq data mapped to each gene, to ensure each gene was a viable candidate and check for intra-population sequence variation. Consensus sequences from transcript alignments for each population were then extracted and aligned to assess inter-strain variation.

3.1.12. *Functional validation of candidate gene*

3.1.13. *Gene synthesis*

UGT g995 sequence was edited *in silico* to include restriction enzyme sites ECOR1 and Xba1 and the 5' and 3' sites respectively. The sequence was codon optimised for *Drosophila melanogaster* and synthesised by TWIST bioscience (San Francisco, California, US) in a pTwist Amp high copy vector.

3.1.14. *Plasmid digestion*

The gene was excised from the pTwist Amp high copy vector in a 20 µl digestion reaction. 10 µl of DNA synthesised vector was combined with 2 µl of CutSmart buffer (New England Biolabs), 1 µl of EcoR1, 1 µl of Xba1 and 5 µl of water. The solution was incubated at 37 °C for 1 hour. The fragments were then separated using gel electrophoresis using a standard protocol for 75 V for 30 min. The smaller fragment (~1.5 kb) containing the UGT was extracted using QIAquick Gel Extraction Kit (Qiagen, Inc.) and tested for concentration using spectrophotometry (NanoDrop, Thermo Fisher Scientific, Waltham, US).

3.1.15. *pUAST ligation*

For injection into fly embryos the gene was inserted into a pUAST plasmid. The pUAST vector was digested in a 20 µl reaction containing 2 µl of CutSmart buffer (New England Biolabs, Massachusetts, US), 2 µl of pUAST plasmid, 1 µl of EcoR1, 1 µl of Xba1 and 14µl of water at 37 °C for 1 hour. The

UGT was then inserted into the vector in a 20 µl ligation reaction. 50 ng (1 µl) of the vector was added to 150ng (3.9 µl) of the UGT, 10 µl of reaction buffer, 1 µl of T4 DNA ligase (Invitrogen) and 4.1 µl of water. The reaction was left for 30 min at room temperature.

3.1.16. *Cloning*

1 µl of the ligation was added to 50 µl of competent cells and incubated on ice for 30 min. The cells were then exposed to a heat shock of 42°C for 45 seconds and then returned to ice. 250 µl of S.O.C. recovery medium was added and the solution was place in a shaker incubated at 37°C for 1 hour. 75 µl of bacterial cells were deposited on LB-ampicillin plates and incubated at 37°C for 16 hours. 25 µl colony PCR reactions and gel electrophoresis were used to assess the presence of the UGTs in the plasmids of viable colonies. 1 µl of forward and 1 µl of reverse primers were added to 12.5 µl of PCR master mix and 10.5 µl of water. Pipette tips were used to transfer cells from selected colonies to the solution. Cycling conditions comprised 5 min at 95°C followed by 35 cycles of 95°C for 20s, 50°C for 30s and 72°C for 2 mins, and a final extension step of 72°C for 6 min. Gel electrophoresis identified colonies that had taken up the plasmid. The remainder of the colonies that displayed gel bands were extracted from the plates pipette tips and deposited into falcon tubes containing 4ml of LB and 4 µl of ampicillin. The falcon tubes were placed in shakers and incubated overnight at 37°C. The cells were then centrifuged as 6000 rcf fir 15 min at 15°C and the tip and supernatant were removed. Transformed plasmids were then extracted using QIAprep Spin Miniprep Kit (see methods 2.14) and eluted in water in preparation for embryo injection.

3.1.17. *Embryo injection*

Fly embryos were injected with UGT transformed plasmids by the fly facility at the University of Cambridge. 30 µl of 675 ng µl $^{-1}$ plasmids were submitted for injection into 200 phiC31; attP embryos.

3.1.18. *Fly crossing*

Three fly strains were required for the crossing experiment for the expression of UGT in the fly lines. A strain containing GAL4 yeast transcription factor (GAL4), a strain containing a pUAS-attB element with the candidate UGT (UAS-UGT) and a control strain (C) with the same genetic background and an attP docking site but no pUAS-attB element. Fly strains were kept in Flystuff 8oz Round Bottom. Each bottle contained 4.8 g of Nutri-Fly Food ® mixed in 25ml of water, 0.125 ml of propanoic acid and 0.125 ml of 10% nipagin in EtOH. Fly stocks were bulked up prior to crosses. When populations were sufficient, 6 replicates of 30 virgin females from GAL4 and 10 males from UAS-UGT were set up. Six replicates were also set up for the control with 30 virgin females of the GAL4 line and 10 males

from the C line. After eclosion 600 females were collected from each treatment group (UAS-UGT and C) for bioassay.

3.1.19. *Fly insecticide bioassays*

Coragen was diluted in 50% acetone to generate a range of 5 concentrations (1 mg L^{-1}, 10 mg L^{-1}, 100 mg L^{-1}, 1000 mg L^{-1} and 10000 mg L^{-1}). 100 µl of each pesticide dilution was pipetted into Genesee Scientific fly tubes containing 2ml of sugar agar ensuring the surface was completely covered, and allowed to dry. This was repeated for the control with 50% acetone minus the pesticide. 20 female flies were added to each tube and all treatments were replicated 5 times for both the UAS-UGT and the C crosses. Flies were scored for mortality after 48 hours. LC_{50} and full dose-response curves were calculated using R (Burgess, King, and Geden 2020). Both control groups from UAS-UGT and C crosses were snap frozen in liquid nitrogen and stored at -80 for downstream molecular analysis.

3.1.20. *QPCR*

To verify expression of the transgene, flies were collected from the bioassay control treatment group for both UAS-UGT and C. Flies were divided into four samples of 25 flies from each line, snap frozen in liquid nitrogen and stored at -80 °*C.* cDNA was generated from total RNA and diluted to 20 ng/µl. Primers were designed in Geneious v10.2.6 (Biomatters Ltd.) (Table 4) to amplify a 310 bp region of UGT g995 expressed in UAS-UGT. Each biological replicate was quantified twice and compared to the house keeping genes RPL and SDHA to normalise for any differences in cDNA levels of each sample. Each 25 µl reaction consisted of 12.5 µl of SYBR green master mix 1 µl each of forward and reverse primer at 10 µM and 10.5 µl of cDNA (210 ng total). Initial denaturation was at 94 °C for 2 min followed by 40 cycles of 95 °C for 14 seconds followed by 60 °C for 1 min. Analysis was conducted according to the ΔΔCT method (Pfaffl 2001) and normalised to previously published housekeeping genes RPL32 and SDHA (Vandesompele et al. 2002).

Table 4. qPCR primers for the amplification of a 310 bp region of T. absoluta RyR and D. melanogaster housekeeping genes.

Name	Sequence 5' - 3'
DmUGTF1	GAATCACTCCCGTTTTGGAA
DmUGTR1	CCAGGTGAAGGATAGGGTGA
SDHAF	CACGACCCTCCATGATCTCG
SDHAR	CGGATGTCTCATCACCGAGG
RPL32F	GCGCTTGTTCGATCCGTAAC
RPL32R	GCCCAAGGGTATCGACAACA

3.1.21. *Assessment of life history traits of virgin female* T. absoluta

Sexing of pupae was confirmed with the use of a microscope according to differences in morphology described by Genc (2016). 100 female pupae were placed in individual chambers. A plastic beaker with a small hole in the bottom was placed in another plastic beaker containing 100ml of water and fertilizer. The petiole of a tomato leaf was inserted through the hole with any leftover space blocked with cotton wool. A 1.5ml Eppendorf containing a sugar water solution and bunged with cotton wool was placed in the chamber as a food source for the adult moth. The chambers were covered with a double layer of fine cloth mesh. Longevity of females was assessed, the numbers of eggs produced were counted daily and leaves were monitored for mining. Any offspring surviving to the pupal stage were sexed and placed in new chambers as described above and the same metrics recorded.

3.1.22. *Statistical analysis*

All statistical analyses were conducted in R. Data were tested for normality using Shapiro-Wilks normality test. As groups were not normally distributed the Wilcoxon rank sum test was used to test significance. In all comparisons, two tailed tests with $p \leq 0.05$ were utilized to determine significance and reject the null hypothesis that no difference exists. Where outcomes were categorical X^2 tests were used to detect differences in the populations and where assumptions of X^2 were not met Fishers exact test was used.

4. Results

Sensitivity of* T. absoluta *to Conserve

The sensitivity of UK strains of *T. absoluta* to Conserve and Coragen was compared to a susceptible strain (TA1) from Spain by leaf dip bioassays. For Conserve, the LAN strain showed moderate levels of resistance (28.7-fold). The most potent resistance was observed in the EVH (165-fold), EVH2016 (>96-fold) and NY2016 (> 480.8-fold) strains (Table 5).

For Coragen, only NY and EVH2016 showed tolerance to Coragen with EVH, LAN and IOW showing no differences in sensitivity compared to TA1 (Table 6). EVH2016 showed an 8-fold decrease in susceptibility and NY a 27-fold decrease compared to TA1. Furthermore in the NY strain, the LC_{50} value was higher than the lower recommended field application rate of 27-53 mg L^{-1}. In EVH2016 the upper confidence limit (18.1 mg L^{-1}) was close to the lower field application rate and its LC_{50} was 79-fold higher than the EVH population from the previous year. Selection of the NY strain with Coragen failed to rapidly result in a strain with higher levels of resistance. However, progressive selection of the EVH2016 strain with Coragen over just 12 generations resulted in a strain with potent resistance to this compound (LC_{50} of >5000 mg L^{-1}).

Table 5. Strain names, derived locations and bioassay results (CL = confidence limits and RR = resistance ratio) from populations collected from the UK and a known susceptible strain acquired from Rothamsted research

Strain	Origin	LC_{50} (ppm)	Chi-square	95% CLs	RR
TA1	Italy	5.2	N/A	3.1 - 7.3	1.0
EVH	UK	860	0.03	484-2114	165.4
EVH2016	UK	>500	N/A	N/A	>96
NY2016	UK	>2500	N/A	N/A	> 480.8
LAN2016	UK	149	0.13	31-5385	28.7
IOW2016	UK	8.6	0.14	5.3	1.7

Table 6. Leaf dip bioassay results for the LC_{50}s of coragen for UK strains of *T. absoluta*. CL = confidence limits and RR= resistance ratios.

Strain	Supplier	Origin	LC 50 (ppm)	Chi-square	95% CLs	RR
TA1		Italy	1.23	0.02	0.095-5	1.0
EVH2016		UK	9.53	0.02	5.025 - 18.09	7.94
NY2016		UK	33	<0.001	11.2 - 97.9	27.5
EVH2016sel		UK	>5000	N/A		>4166.67
LAN2016		UK	4.75	<0.001	1.7 - 12.8	3.96
IOW2016		UK	4.75	0.25	1.7 - 12.8	3.96

***Assessment of Conserve target site, the Taα6 from resistant strains of* T. absoluta**

Amplification and sequencing of the nAChR *Taα6* cDNA prepared from the UK strains and TA1 revealed the presence of two alterations in the four resistant strains that were not observed in the susceptible strains TA1 and IOW. The first of these, observed in all *Taα6* transcripts of the NY and EVH strains, was a deletion of 109 base pairs. This deletion results the introduction of a premature termination codon (PTC) in the gene (Figure 2). Amplification and sequencing of the Taα6 gene from genomic DNA of the NY and EVH strains revealed that these 109 base pairs were present and unaltered in both strains confirming that the loss of this region in mRNA results from RNA modification processes known as ‘exon skipping’ rather than a mutation resulting in the deletion of the genomic DNA.

The second alteration, observed only in LAN strain, was a triplet deletion resulting in the loss of an amino acid at position 238 (Figure 2). The ‘F238del’ mutation occurs in the transmembrane domain (TM1) of the *Taα6* subunit. A comparison of insect nAChR α6 sequences from a range of insect species (Figure 2) demonstrate that this region is highly conserved across the insects. This mutation was also observed in the *Taα6* gene when amplified from genomic DNA, and at a frequency of 0.57 as determined by genotyping >100 individuals. To examine the association of the F238del mutation with conserve resistance the LAN strain was treated with a discriminating dose of conserve and alive, dead and affected larvae genotyped. Of the 200 individuals exposed to 150 mg/L of Conserve 108

died, 30 were affected (alive but were unable to respond to stimuli in a coordinated manor), 55 survived the treatment and 7 escaped. To rapidly genotype these individuals, and to provide a diagnostic tool for future resistance monitoring studies, a TaqMan SNP genotyping assay was designed. This assay showed excellent discrimination of the resistant and susceptible alleles using two fluorescently labeled probes when tested with sequence verified DNA samples (Figure 3). 175 individuals from the discriminating dose assay were successfully genotyped using this assay with 31 individuals scored as homozygous for the wild-type allele, 56 as homozygous for the mutant allele and 88 heterozygous. A significant association was observed between the individual's genotype and its response to Conserve exposure (χ^2 (4) = 78.50, P<0.001). Post-hoc analysis revealed that none of the 'insecticide affected' group showed a statistically significant deviation from the proportion of genotypes expected by chance. However, individuals that died in the assay had a lower than expected proportion of the RR (F238del/F238del) genotype (P < 0.001) and a greater than expected proportion of SS (P < 0.001) and RS genotypes (P < 0.001). In contrast, individuals surviving the assay had a higher than expected proportion of the RR genotype (P < 0.001) and a lower proportion of SS (P < 0.001) and RS (P < 0.001) genotypes (Figure 4).

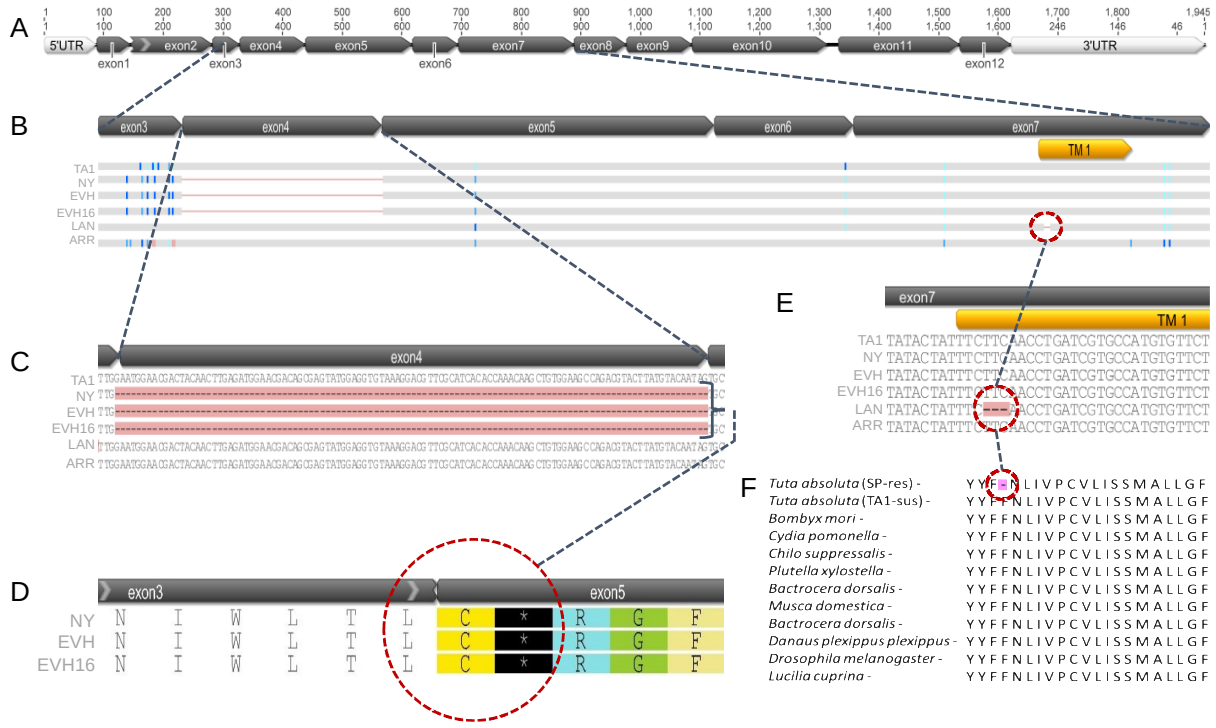
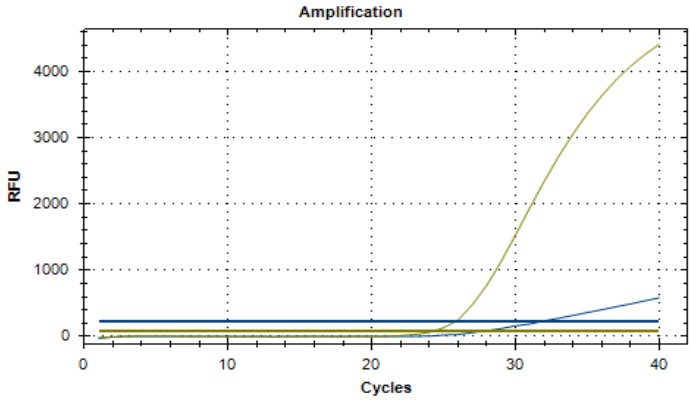
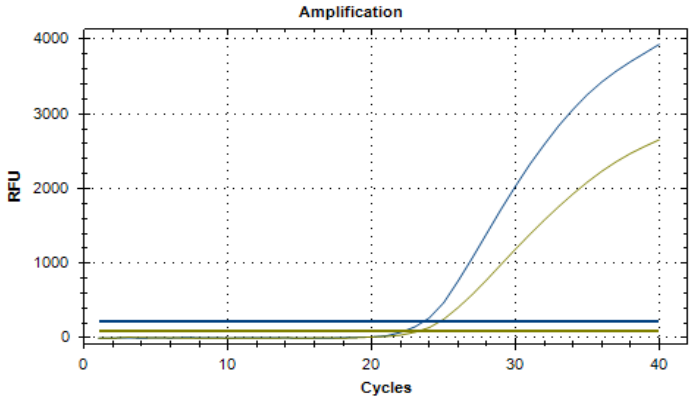


Figure 2. Diagram of mutations identified in *Tαα6* transcript of UK strains of *T. absoluta.* (A) Structure of the α6 sub-unit. B-D Highlight the skipping of exon 4 in three of the conserve-resistant strains and show the introduction of a premature stop codon in exon 5. (E,F) Triplet deletion in highly conserved transmembrane region of the LAN2016 strain.

A.



B.



C.

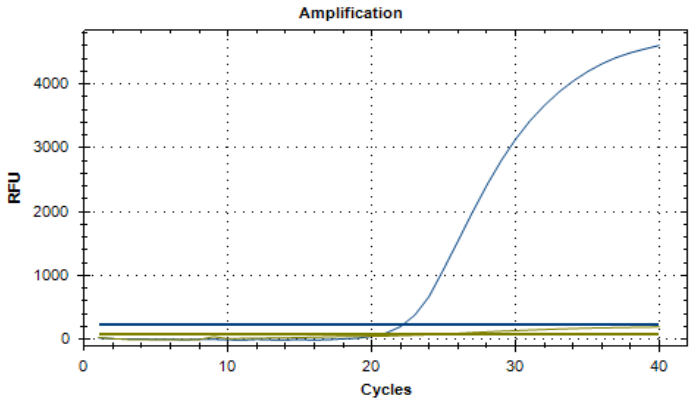


Figure 3. Fluorescence of MGB probes (VIC = brown, wild-type genotype 6FAM = blue F238del mutation) in TaqMan assay of different genotypes A) Homozygous wild type, B) heterozygote, C) F238del homozygote.

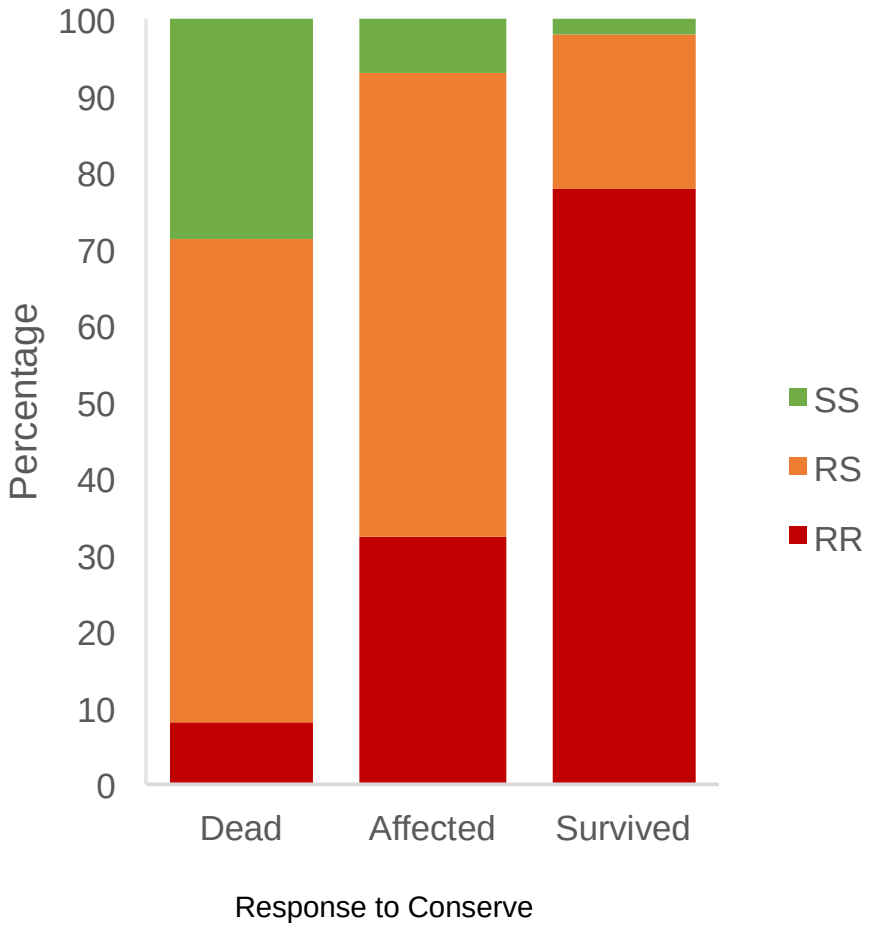


Figure 4. A graph showing the percentage of Dead, Affected and Survived individual for each genotype when exposed to 150ppm of Conserve. RR = Homozygous (F238del) resistant, RS = Heterozygotes (F238/F238del) and SS = Homozygous susceptible (F238/F238).

Sequencing of Coragen target site, the ryanodine receptor (RYR)

The sequenced 1353 bp region of the RyR C-terminal domain encompassing the transmembrane regions and sites of mutations (G4946E, I4790M, G4903V and I4746T) previously shown to confer resistance to Coragen in *T. absoluta* (Roditakis et al. 2017) and *P. xylostella* (Troczka et al. 2012) was amplified from the Coragen selected line (EVH2016sel), the parent strain from which it was derived (EVH2016), NY and TA1. The sequence obtained from pooled samples of NY and TA1 encoded the wild-type amino acid at all of the positions detailed above, however, in EVH2016 a very small secondary peak was observed in the sequence chromatogram at position 4903 that suggested the G4903V substitution is present at very low frequency in this strain (figure 5). This finding was corroborated when the RyR sequence from the selected line was examined, which revealed fixation of the mutation leading to this amino acid substitution in this strain.

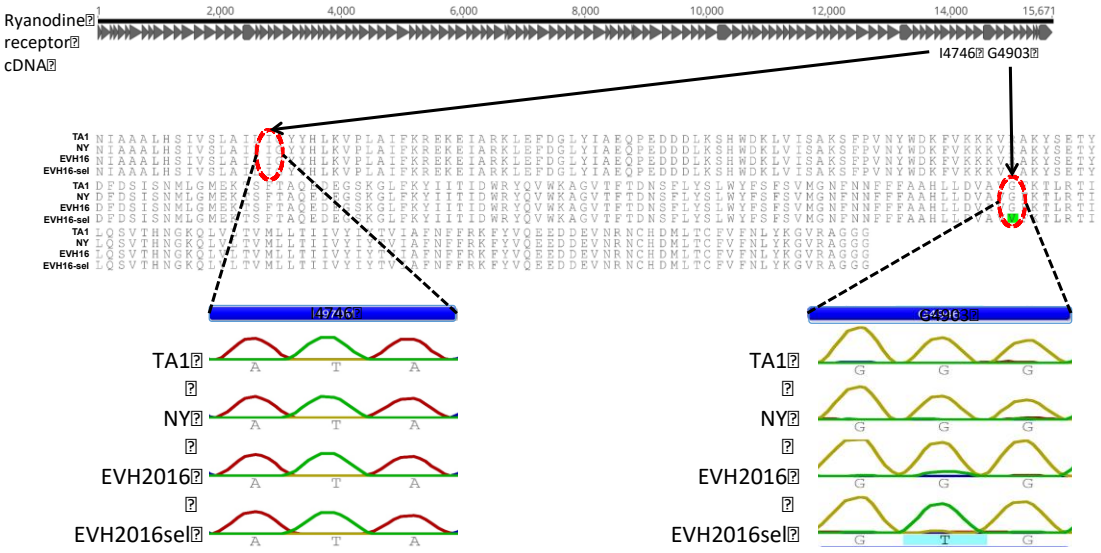


Figure 5. Alignment of RyR sequences (TA1, NY, EVH2016 and EVH2016sel) showing genotypes at Coragen resistance mutation sites.

Physical association of G4903V with Coragen binding

Mapping G4903V to the 3D structure of the rabbit RyR showed it was positioned in the transmembrane region in the proposed Coragen binding site (figure 6)

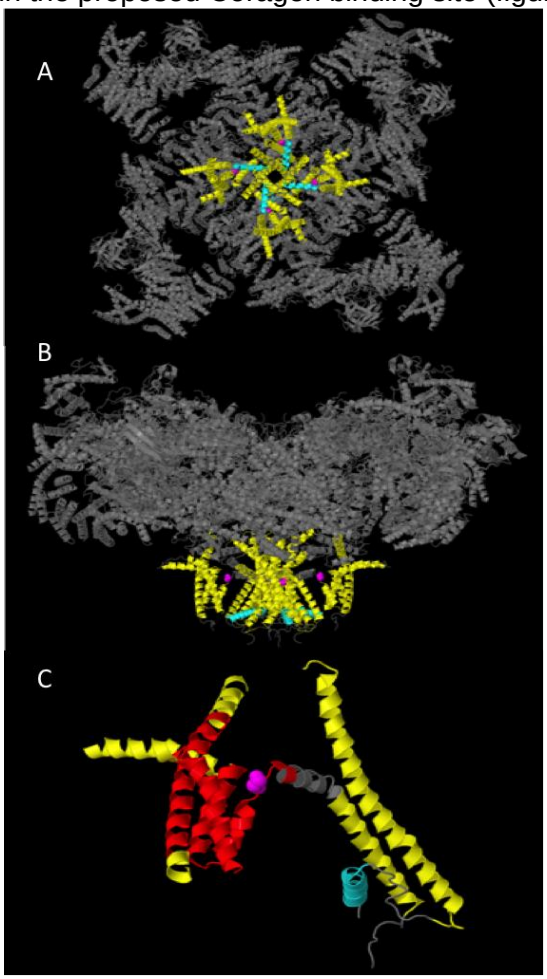


Figure 6. Image of a rabbit RyR (PDB ID:5J8V) with homologous *Plutella xylostella* transmembrane (TM) regions S1-S6 in yellow, pore helix in cyan (Steinbach et al. 2015) and G4903V in magenta (A)

from underneath showing the central ion pore. (B) From the side showing TM regions and (C) a close up view of the TM region from one subunit highlighting suggested Coragen binding regions in red as described by Lin et al. (2020).

Non target site resistance to Coragen in non-UK strains.

Bioassay results

T. absoluta strains were also acquired from Spain to aid research into resistance mechanisms, specifically resistance to Coragen, as the insects lacked any target site resistance. Leaf dip bioassays of these strains showed high susceptibility in Ssus and Sus with LC_{50} values of 0.088 and 0.15. Tolerance in Mur was 18.2-fold higher than the most susceptible strain Ssus. High levels of resistance (44613.6-fold) were identified in Sres with an LC_{50} of 3926 (Table 7).

Table 7. Leaf dip assay results for four Spanish strains of *T. absoluta*.

Strain	Location	LC_{50} (PPM)	RR
Sus	Murcia, Spain	0.15	1.7
Mur	Murcia, Spain	1.6	18.2
Ssus	Spain	0.088	1
Sres	Spain	3 926	44613.6

Analysis of the RyR

To facilitate this line of research the *T. absoluta* genome was sequenced and libraries of expressed genes were generated for all strains. Mapping of the expressed gene data to the *T. absoluta* RyR gene (Figure 7) revealed no nonsynonymous mutations in the whole gene for Sus and Mur and were 100% wild type for the RyR amino acid sequence. Sres showed low-level sequence variance occurring in the previously reported sites of the resistance mutations G4946E (0.4%) and I4790L (0.6%). Ssus also had the G4946L mutation at very low frequency (0.4%).

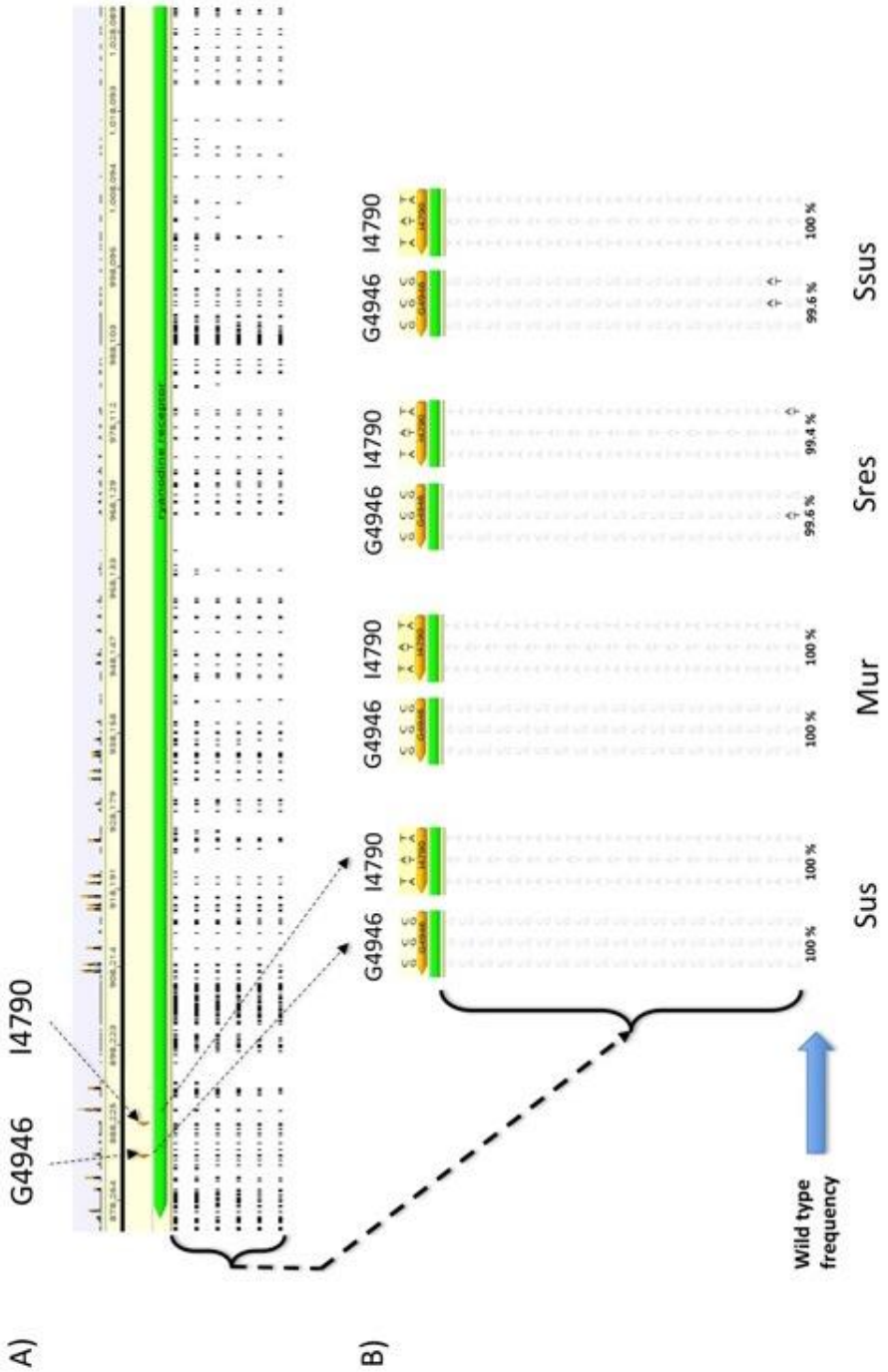


Figure 7. (A) Transcripts (black dashes) mapped to the RyR (green bar) with previously reported resistance mutation sites highlighted. (B) Close up subset of mapped transcripts for all four strains at the resistance mutation sites showing wild type frequency.

Differential gene expression

Analyses of differentially expressed genes revealed that 228 genes were upregulated (Figure 8) in the comparison of the Sus and Mur strains. Between Sus and Sres, 2632 genes were upregulated. A total of 4128 upregulated genes were identified in the comparison of the Ssus and Mur strains. Lastly, between Ssus and Sres 1794 genes were upregulated. Cross referencing of genes upregulated in all the comparisons of resistant and susceptible strains (Figure 8) generated a short list of 20 upregulated genes (Table 8).

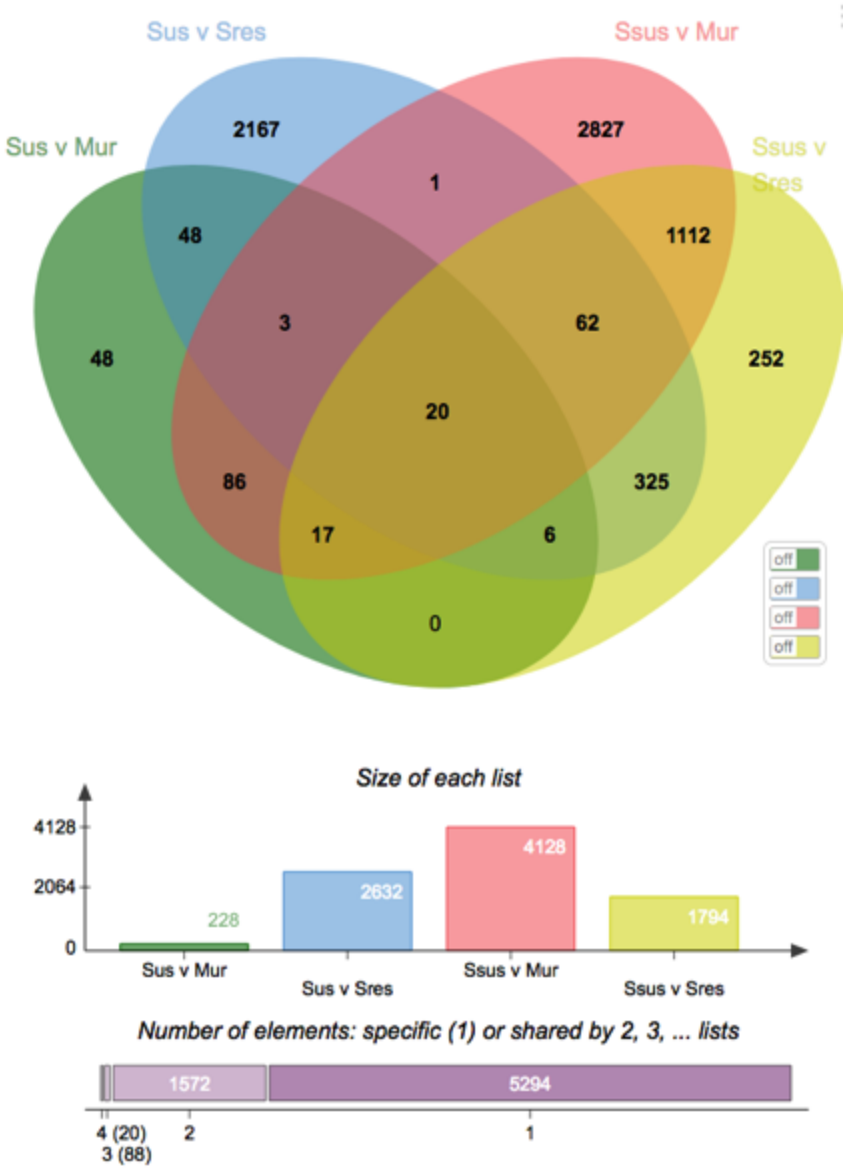


Figure 8. Venn diagram showing genes commonly upregulated in comparisons of Coragen susceptible and resistant strains.

Table 8. Short list of upregulated genes across all comparisons of coragen susceptible and resistant strains, with gene number from genome annotation, sequence description from BLAST annotation and fold increase for each comparison. Colours represent level of expression with green being the most highly expressed and red having the lowest expression.

Gene	Sequence.Description	SpainSus v Mur	SpainSus v SpainRes	Sus v Mur	Sus v SpainRes
g995	UDP-glucosyl transferase	22.6	149.1	20.6	116.9
g24845	uncharacterized protein LOC113226571 isoform X2	22.8	26.0	36.0	12.4
g23043	succinate dehydrogenase [ubiquinone] flavoprotein subunit, mi	21.4	24.4	42.0	13.2
g24846	PREDICTED: uncharacterized protein LOC106129042	6.1	15.4	12.7	7.0
g24840	PREDICTED: uncharacterized protein LOC106129042	33.6	12.4	10.7	35.7
g6220	hypothetical protein B5X24_HaOG214996, partial	12.7	9.2	5.9	19.8
g20158	---NA---	5.4	9.1	5.4	8.6
g18600	hypothetical protein B5V51_11225	2.5	7.2	5.2	3.4
g26926	U3 small nucleolar ribonucleoprotein protein MPP10	12.3	6.3	5.0	15.2
g33152	bromodomain-containing protein 8 isoform X1	3.4	5.7	2.5	6.9
g6681	Alpha-endosulfine	3.7	5.2	4.8	4.0
g6361	uridine diphosphate glucose pyrophosphatase-like	2.8	4.6	2.8	4.4
g33632	cytochrome P450 9e2-like	7.1	4.4	7.2	4.2
g29380	SOSS complex subunit B homolog	4.9	4.0	3.7	5.2
g2862	---NA---	29.3	3.4	6.5	16.8
g662	hypothetical protein g.8432	2.3	3.3	2.9	2.6
g21016	guanine nucleotide-binding protein G(s) subunit alpha isoform	39.8	3.3	11.0	11.3
g4624	---NA---	3.4	2.6	2.0	4.4
g26402	protein arginine N-methyltransferase 1	3.6	2.3	2.2	3.7
g7901	uncharacterized protein LOC113398902 isoform X2	12.0	2.3	2.9	9.2

Candidate resistance gene

From the gene tables, one gene stood out as the strongest resistance candidate, based on its level of upregulation and sequence identity to a known resistance associated gene family - UGT g995. This gene was 149.1-fold up regulated in the Sres vs Ssus comparison and 116.9-fold up regulated in the Sres vs sus comparison. Expression was lower in Mur strain (but still 20-fold overexpressed) when compared to the two susceptible strains, but this correlates with the lower level of resistance observed in this strain.

Functional validation of UGT g995 using transgenic *D. melanogaster*

Pesticide bioassays of *D. melanogaster* expressing the Sres variant of UGT g995 generated an LC_{50} value of 29.04 mg L^{-1} compared to 2.69 mg L^{-1} in the control line (Table 9). Thus, the expression of UGT g995 in D. melanogaster resulted in a 10.8-fold increase in tolerance to Coragen.

Table 9. Bioassay results for transgenic *D. melanogaster* expressing UGT.

Treatment	LC mg L-1	Lower confidence limit	Upper confidence limit
Expressed UGT	29.04	13.24	63.62
Control	2.69	0.57	7.11

***Reproductive traits of virgin* T. absoluta**

There was no statistically significant difference between the numbers of females eclosing from the pupal stage between the populations collected before and after the deployment of Isonet T (X^2= 2.6,

df = 1, p-value = 0.10). Ninety-two females eclosed successfully in the EVH2016 strain and 98 eclosed successfully in the EVH2019 strain. There was no significant difference between the numbers of individuals that laid eggs in each group (X^2 = 1.9, df = 1, p-value = 0.17) with 84 females laying eggs in the EVH2016 population compared to 82 females in EVH2019.

There was a significant difference in the number of eggs laid between the two groups (W = 6149.5, p < 0.005) with EVH2016 laying 1313 eggs with an average of 14.27 eggs per individual and EVH2019 laying 604 eggs with an average of 6.16 eggs per individual. Figure 9 shows the differences between average daily increase in egg number across the two populations. Differences between the populations were also observed in lifespan (W = 1682, p-value < 0.005) with EVH2016 having an average lifespan of 21.28 days compared to 32.71 days in EVH2019 (Figure 10). Differences were observed in the time it took females to start laying eggs (W = 3155.5, p-value < 0.005), on average EVH2016 started laying after 6.96 days compared to 10.32 days for EVH2019. No significant differences were observed between the populations between the total number of days the females laid eggs (W = 4929.5, p-value = 0.27) with the average range being 8.42 and 7.39 for EVH2016 and EVH2019.

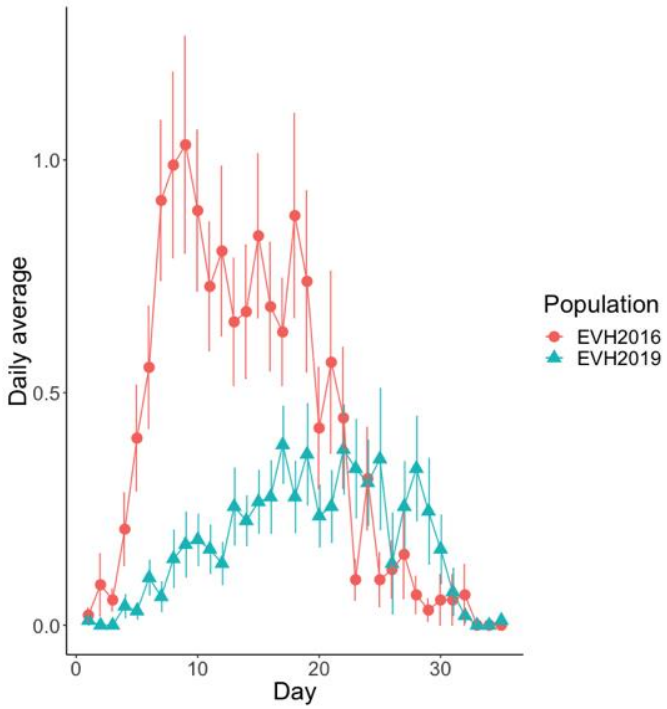


Figure 9. Differences in average daily egg production by virgin females of the EVH2016 and EVH209 strains over 35 days (W = 806, p-value = 0.02). Bars represent ± SEM.

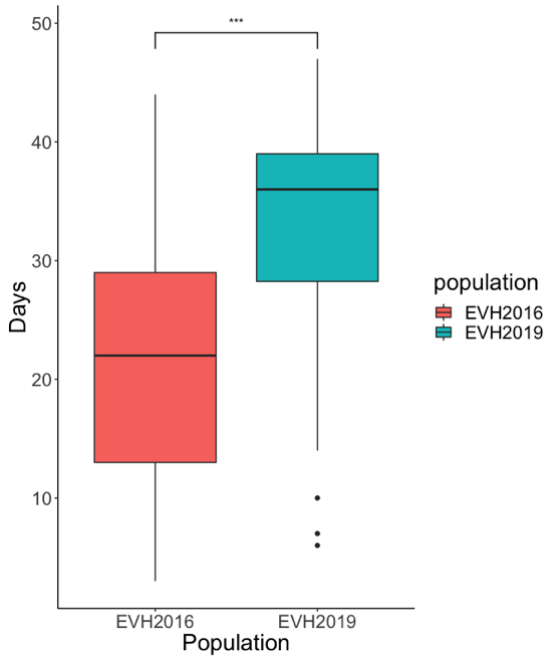


Figure 10. Box plots showing range of lifespan of virgin females from EVH2016 and EVH2019 strains (W = 1682, p-value < 0.001).

Parthenogenesis

Six virgin females (7%) of the EVH2016 strain laid viable eggs, from which 14 larvae were detected (1% of eggs laid from the population). Five larvae survived to pupation with a sex ratio of 2:3 (males/females). Of these, 3 females and one male eclosed. These females laid 27, 57 and 8 eggs and survived for 16, 27 and 31 days, respectively. No F1 virgins produced viable eggs. Eight virgin EVH2019 females laid viable eggs (8%) from which 15 larvae were detected (2.5% of all eggs), 10 of these developed into pupae (67%) with a sex ratio of 3:7 (males/females). From these 6 females and 2 males eclosed. These females laid 26, 0, 0, 7, 1 and 11 eggs and survived for 21, 11, 14, 13, 17 and 12 days respectively. No F1 virgin females laid viable eggs.

There was no significant differences in the number of active larvae, pupae or adults produced parthenogenetically by the two F0 female populations, however, there were significant differences between the populations when the likelihood of larvae, pupae and adults emerging from F1 eggs was compared. There was a 2.3-fold increase in the proportion of larvae (X^2 = 4.666, df = 1, p-value = 0.03), a 4.3-fold increase in proportion of pupae (p-value < 0.01) and a 4.3-fold increase in proportion of adults (p-value = 0.01) in the EVH2019 strain compared to the EVH2016 strain (Figure 11).

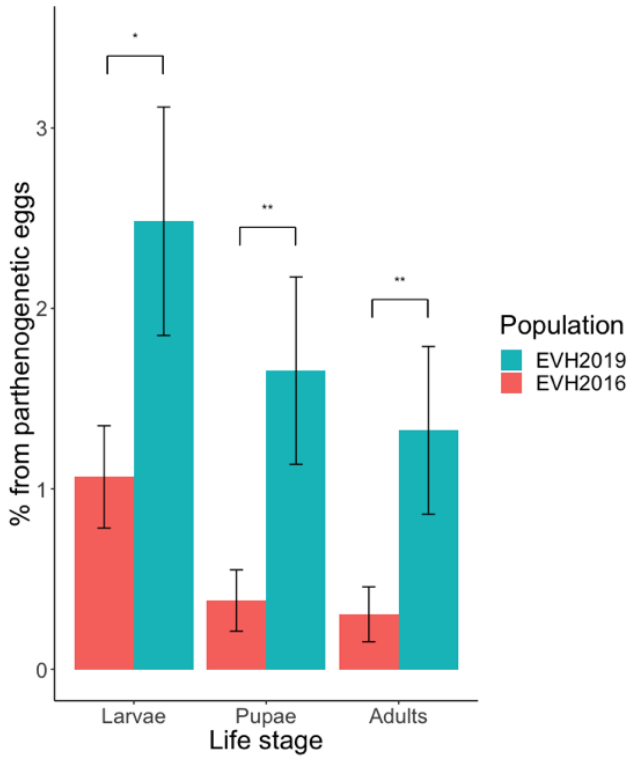


Figure 11 Percentage differences in larvae, pupae and adults from total number of eggs laid by EVH2016 and EVH2019 (* $p \leq 0.05$, ** $p \leq 0.01$).

5. Discussion

Conserve resistance

The research presented in this report confirmed resistance at three sites in the UK. Leaf dip bioassays showed moderate to high levels of resistance (149 ppm - >2500 ppm). Investigation into the cause of this resistance revealed two distinct resistance mechanisms that had not previously been described for *T. absoluta.* Moderate resistance was detected in Lancashire and was associated with an F238 deletion in exon 7 of the nAChR α6 subunit. This modification altered the structure of a highly conserved transmembrane region believed to influence binding of Conserve based on sequence homology to pesticide binding sites on glutamate-gated chloride channel, a protein with similar structure to nAChR (Hibbs and Gouaux 2011). Furthermore, physical proximity to Conserve associated mutations G275E in *F. occidentalis*, *T. palmi* and *T. absoluta* (Puinean et al. 2013; Bao et al. 2014; Silva et al. 2016) add support to the claim F238del influences Conserve binding. Finally association studies using TaqMan assays showed a significant correlation between genotype and the resistant phenotype. The assay also quickly, easily and cheaply identified resistance allele frequency within the population.

The second alteration was present in the highly resistant strains. The excision of exon 4 from mRNA resulted in a frame shift and premature termination codon at the start of exon 5 resulting in a massively

truncated protein (31% of its original size). Exon 3 skipping has been previously described in *T. absoluta* in the context of Conserve resistance, however this alteration didn't result in a frame shift or the introduction of a PTC. In Conserve resistant strains of *B. doralis,* α6 transcripts were non functional and expressed PTC in exon7 (Hsu et al. 2012). Conserve resistance mechanisms appear to fall into one of two categories, either target-site alterations that impede the binding of Conserve (G275E of F238del) or alterations that render the whole α6 subunit redundant (such as exon skipping events or PTCs). The latter processes would likely inhibit the incorporation of the α6 subunit into the pentameric receptor protein. This could occur through transcript hydrolysis by non-sense mediated decay, before translation could take place, or through excisions of regions that are integral to pentamer formation. This would prevent inclusion of α6 in the functional receptor as the intact cystine loops are necessary for complete nAChR assembly (Green and Wanamaker 1997). Does *T. absoluta* replace the truncated α6 with a different subunit? How does the fitness of the restructured nAChR compare to the wild type configuration and the F238del genotype? Thus further experiments under field realistic conditions are required to investigate this further.

The fact that F238del and exon 4 skipping alterations have not been previously described in resistant populations of *T. absoluta* suggests they evolved in the UK and also that the modifications rose to sufficient frequency to confer high resistance in a relatively short time (2014-2016). The effects of these resistance alterations are detrimental to many growers. The lack of efficacy of Conserve resulted in withdrawal of its use in some glasshouses, however the pesticide was still effective in IOW. These results and those of previous resistance mechanisms should be used to diagnose all outbreaks in the IOW regardless of the effect of Conserve in the population. The use of the pesticide in conjunction with other control measures would mask the true resistance to the compound. Therefore detection of resistant alleles, even at frequencies that would permit protection from Conserve would be invaluable in the decision to weigh the short-term gain of crop protection in the immediate growing season from the application of Conserve to the long term gain of maintaining the efficacy of Conserve and thus its role in IPM. For growers currently with resistant populations these detection techniques would also be valuable. If there were a fitness cost to Conserve resistance, selection for wild-type alleles in the absence of Conserve molecular diagnostics would allow for the monitoring of the return of susceptibility.

Coragen resistance

At the start of this PhD*,* Coragen was still a highly effective compound in supressing *T. absoluta* pest populations. Coragen was used as a second line of defence if Conserve and the biocontrol failed to fully supress *T. absoluta.* Therefore selection pressures for resistance were lower than that of Conserve. With the increasing resistance to Conserve however the reliance on Coragen increased, and consequently so has selection for resistance. In 2016, two growers reported reduced efficacy of Coragen in some of their glasshouses (R. Jacobson, personal communication), glasshouses that had

previously reported loss of control to Conserve. These reports were confirmed in this thesis through leaf dip bioassays that showed that levels of resistance were 7.9-fold and 27.5-fold elevated.

This research found evidence of G4903V at low frequency in one of the populations, EVH2016. Selection of this population with increasing concentrations of the pesticide drove the resistant allele to fixation. This correlated with LC_{50} values of >5000 which was 525-fold higher the parental strain collected after the reported control failure. Considerable evidence is reported in the literature of this sites functional contribution to Coragen resistance (Richardson et al. 2020). My findings are important for direction of the appropriate use of Coragen in the control of *T. absoluta* in current IPM. The fact that Conserve resistance is present in the UK means Coragen is the only remaining chemistry in the current IPM compatible with biocontrol and pollinators. The fact that resistance is low, but alleles to Coragen are present in UK populations, means careful monitoring of populations must take place to retain the compounds efficacy. Efforts should be made to assess RyR mutation frequency across the UK to better understand the distribution of resistance alleles among glasshouses.

Metabolic resistance of Coragen

Metabolic resistance was identified as a second, independent mode of Coragen resistance. Spanish populations of *T. absoluta* showing no target-site alterations in the RyR had 44614-fold resistance to Coragen. With the importance of Coragen increasing with the loss of efficacy of Conserve, gaining a comprehensive understanding of resistance mechanisms is of great interest to growers and agronomists. Assessment of four transcriptomes sequenced from Spanish strains with a range of resistance profiles, together with a new reference genome for *T. absoluta*, allowed genome-wide assessment of gene expression. The up regulation of genes, especially those associated with xenobiotic resistance, is a key indicator of metabolic involvement in detoxification. This study's identification of a commonly upregulated UGT g995 (149.1-fold increase in expression) in resistant strains compared to susceptible strains was a clear candidate for explaining the resistance observed. UGT's are part of a complex detoxification pathway and are known to facilitate excretion of toxins via conjugation with modified sugar molecules enhancing pesticide solubility. This process had been associated with Coragen in other pest species and was functionally validated for *T. absoluta* in this study through expression in transgenic lines of *D. melanogaster* resulting in Coragen resistant flies.

The value of this work goes beyond the identification and functional validation of UGT mediated resistance in these *T. absoluta* strains by creating three resources for future research on the detection of resistance mechanisms and enhancement of control measures. Firstly it provides a screening tool for pesticide/synergist assays by clearly demonstrating that *D. melanogaster* can express *T. absoluta* UGT's and that these genes are functional in the genetic background of *D. melanogaster.* The second resource made available by my research is the curation of the entire UGTome. Having this resource allows the expression of all UGT's to be tested in resistant *T. absoluta* populations with simple, quick

and cheap qPCR experiments. This could identify candidate detoxification genes in resistant populations without the expensive and lengthy process of full transcriptome analysis. Finally, I was able to contribute to the creation of a new draft genome assembly for *T. absoluta* that can enable or accelerate research into a range of topics on this species including methods for its control. This would include curating all genes families associated with insecticide resistance including ABC's (Dermauw and Van Leeuwen 2014), P450s (Scott 1999), esterases (Montella, Schama, and Valle 2012) and GSTs (Enayati, Ranson, and Hemingway 2005). It would also facilitate research into novel control strategies such as RNAi, a post-transcriptional gene silencing mechanism. The genome can be used to identify genes sequence in important developmental pathways which can be used as targets for RNAi resulting in supressed expression and mortality (Swevers and Smagghe 2012; Yoon et al. 2018). In the case of gene drives, identification of genes necessary for female development could be targeted. Only male offspring would survive reproductive events and they would be carriers of the gene drive system. That fatal gene and the drive system would proliferate until the population was supressed (Scott et al. 2018; Kyrou et al. 2018).

Bioassays on *D. melanogaster* lines expressing *T. absoluta* UGTs would highlight their contribution to pesticide detoxification before upregulation has occurred in the field through selection. Any fly lines expressing UGTs characterised as candidate detoxification genes could then facilitate the search for pesticide synergists before resistance has evolved. The use of commercial synergists is limited but has great potential to facilitate the maintenance of pesticide efficacy in the field by overcoming resistance. The identification of successful synergists would not only be invaluable for extending the life of current pesticides but may also provide opportunity for the reintroduction of older pesticides classes to populations with previously described metabolic resistance, such as organophosphates (Barati, Hejazi, and Mohammadi 2018) and spinosyns (Campos et al. 2015). The value of such synergists would spread far beyond *T. absoluta* and potentially offer renewed pesticide efficacy in any pest species expressing metabolic resistance, depending on the specificity of the synergist.

Parthenogenesis

The inclusion of Isonet T-based mating disruption to the IPM strategy was vital in maintaining control of *T. absoluta*. Glasshouses had started to lose control due to Conserve resistance and the addition of this technology not only restored control but also relived selection pressure on the second line of defence – Coragen. However, soon after the introduction of Isonet T glasshouses reported slight loss of control where the mating disruption had previously been successful. This was of great concern to tomato growers especially in light of reports coming out of Tunisia that *T. absoluta* could reproduce through parthenogenesis (Abbes and Chermiti 2014). My research directly addressed the question: Can *T. absoluta* 'resist' mating disruption through alternative mating strategies and can these adaptations be selected for?

I conducted a longitudinal study of populations of *T. absoluta* that were collected before and after mating disruption was implemented. It showed that both populations of *T. absoluta* could reproduce asexually and produce viable male and female offspring. There was no significant difference between the two populations in the number of larvae, pupae or adults produced through parthenogenesis. There was however, a significant reduction in total eggs laid by the population that had been exposed to mating disruption over a three-year period. Therefore, the frequency of parthenogenesis had significantly increased. These results suggest two processes were at play in an environment with limited mates. Firstly, adaptation in frequency of parthenogenesis driven by a selective advantage to females that could lay a higher proportion of parthenogenetic eggs and secondly adaptation of life history traits - increased longevity would increase the chance of a mating event. It is possible that in this population a trade-off occurred that increased longevity at the cost of reduced reproductive output.

These results suggest that while evolution may be occurring it is not likely the cause of reduced efficacy of Isonet T as the increased frequency of parthenogenetic offspring was offset by a reduction in eggs laid. Also, no parthenogenetic offspring managed to lay viable eggs through parthenogenesis in this study. This suggests the maintenance of populations via this reproductive strategy beyond a single generation is limited. The data does however support the possibility of there being an inherited genetic component that could be further selected under conditions of mate limitation. This research was limited to the one glasshouse and so extending this research to glasshouses across the UK is vital in building support for this hypothesis. In the psychid moth evolution of parthenogenesis seems to have occurred several times (Grapputo, Kumpulainen, and Mappes 2005) and in the facultative parthenogenetic *Dahlica triquetrella,* reproductive output was shown to equal that of sexually reproducing individuals (Kumpulainen, Grapputo, and Mappes 2004), suggesting a possible route of resistance to Isonet T. My results show that the post Isonet T deployment populations can persist for longer within the crop, allowing re-establishment should environmental concentrations of the pheromone drop below the critical threshold. Beneficially, however, increased life span and delayed egg laying may act synergistically with the biocontrol agent *M. pygmaeus.* It would permit more time for the bug to establish within the crop, meaning predator/prey ratios would be higher - reducing damage to the crop by persistent *T. absoluta* infestations.

In the short period of time since the implementation of the current IPM strategy, *T. absoluta* has managed to evolve two independent mechanisms of resistance to Conserve, and, resistance genes have been detected in the field for Coragen. These results also show *T. absoluta* has a large pre-existing suite of detoxification genes (UGTs) and can upregulate them to produce adaptive resistant phenotypes. Lastly while mating disruption is still an effective control measure populations have persisted in these glasshouses and are potentially evolving strategies to overcome this. The fact that multi insecticide resistant strains can readily evolve and are viable show how adaptable *T. absoluta* is in the face of strong and diverse selection pressures.

Research summary

1. UK *T. absoluta* populations are resistant to Conserve through the evolution of two novel mechanisms - the skipping of exon 4 and the F238 deletion in the nAChR α6 subunit.
2. Alleles conferring resistance to Coragen via a G4903V substitution in the RyR exist in UK populations and can be selected to fixation producing highly resistant, viable populations.
3. *T. absoluta* is capable of evolving Coragen resistance through upregulation of UGT metabolic detoxification genes.
4. *T. absoluta* express's 40 UGT genes many of which share close phylogeny to genes associated with resistance to a broad range of pesticides for a variety of species.
5. Increased rate of parthenogenesis does not likely contribute to loss of control of Isonet T but evolution in the presence of mating disruption may allow infestations to persist in crops for extended periods of time.

IPM recommendations

1. Applications of Conserve should be stopped to prevent fixation of resistance alleles in the population.
2. Coragen should be used as a last line of defence to prevent elevated frequency of pre-existing resistance alleles.
3. Populations should be diagnosed seasonally for resistant allele frequency to provide resistance profiles to inform appropriate application of pesticides
4. *T. absoluta* infestations in glasshouses employing Isonet T should be reported and monitored to assess potential mechanisms of 'resistance' to mating disruption.

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