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

Submitted by Charles Edward Grant to the University of Exeter

as a thesis for the degree of

Doctor of Philosophy in Biological Sciences

In March 2021

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: 

Abstract

BACKGROUND: The tomato leafminer, *Tuta absoluta* is a damaging pest of tomato crops worldwide. In the UK, *T. absoluta* is controlled using an integrated pest management (IPM) strategy that includes the pesticides spinosad and chlorantraniliprole, the biocontrol agent *Macrolophus pygmaeus* and pheromone-based mating disruption. Some growers have reported a loss of efficacy of this technology. There are concerns that *T. absoluta* may have evolved resistance to these applied chemistries as well as undergone adaptations in its capacity to reproduce asexually. In this thesis I investigate whether pesticide resistance is present in UK populations and identify the molecular mechanisms for this resistance. I will also investigate the capacity *T. absoluta* to reproduce asexually through parthenogenesis in the absence of males.

RESULTS: I demonstrate that UK populations of *T. absoluta* are highly resistant to spinosad and identify two novel mechanisms by which resistance has evolved. Analysis of messenger RNA encoding the target site of spinosad, the nicotinic acetylcholine receptor (nAChR) α6 subunit, revealed resistant strains lack exon 4 resulting in a highly truncated protein. In a second resistant strain the deletion of three amino acids is detected in the transmembrane domain of the nAChR - predicted to be the binding site of spinosad. I identify low levels of tolerance to chlorantraniliprole in UK populations and show this resistance can be selected for to produce highly resistant populations. Analysis of the target site of chlorantraniliprole, the

ryanodine receptor, identified amino acid substitution G4903V that has been strongly linked to diamide resistance in a range of lepidopteran species including *T. absoluta*. With regards asexual reproduction, I observed a small but significant increase in the rate of asexual reproduction. This allows persistence of the pest in the presence of the mating disruptor, Isonet T. Marked differences in several other life history traits associated with reproduction were also observed in these populations including increased longevity further allowing *T. absoluta's* persistence within the crop.

CONCLUSION: My findings show that the evolution of resistance has rendered spinosad redundant at most sites in the UK. The mechanisms identified are unique to UK populations and so have likely evolved under selection in the UK. Chlorantraniliprole remains effective, however our findings of resistance at low frequency suggest that continued use of this pesticide must be monitored carefully. The low overall occurrence of asexual reproduction observed in this study is unlikely to result in loss of efficacy of mating disruption as reproductive rate remained low. However, the observed changes in longevity and egg laying may allow *T. absoluta* to persist for longer within the crop, and, together with the increased frequency of parthenogenesis, may reflect selection from the use of Isonet T. Thus, regular monitoring of the reproductive capacity of UK populations should be conducted, along with continual assessment of resistance allele frequencies of pesticides to inform resistance management strategies.

Acknowledgements

I would like to thank my supervisor Professor Chris Bass for his invaluable guidance and continued support throughout the entirety of this project

A special thank you to my industry consultant Dr. Rob Jacobson for his involvement in this work and being an instrumental link between the academic and industrial side of this project. I would like to also thank my co-supervisors Professor Richard ffrench-Constant and Martin Williamson.

I would like to thank Christoph Zimmer for getting me up and running with the project, and to Bartek Troczka for helping me along the way. Thanks to Kumar Singh for his work generating the *T. absoluta* genome. Thanks also to the entire Bass laboratory group for their daily support.

I would also like to thank the members of the British Tomato Growers' Association for their support and co-operation throughout the study, especially R and L Holt. I would like to thank the AHDB and the University of Exeter for funding this project.

Finally I would like to thank my parents for their support throughout this PhD and to my partner Lauren Jackson for her patience along the way.

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Definitions

ABC's = ATP-binding cassette proteins

AChE = Acetylcholinesterase

BUSCO = Benchmarking Universal Single Copy Orthologs

Ca2+ = Calcium ion

CCE = Carboxyl/choline esterase

cDNA = Complimentary deoxyribonucleic acid

DEseq2 = Differential gene expression sequencing analysis 2

-COOH = Carboxylic group

CYPome = All P450s

DNA = Deoxyribonucleic acid

dNTPs = Deoxyribo-nucleoside triphosphates

EdgeR = Empirical analysis of digital gene expression data

EDTA= Ethylenediaminetetraacetic acid

EtBr = Ethidium Bromide

FastQC = Fast quality control

g = Gram

GABA = Gamma-Aminobutyric acid

gDNA = Genomic DNA

GSTs = Glutathione S transferases

GSH = Glutathione

h = Hour

IPM = Integrated pest management

IRAC = Insecticide Resistance Action Committee

Kdr = Knock-down resistance

Kg = Kilogram

L = Litre

LB = Lysogeny broth

LC50 = Lethal concentration for 50% of insects in a population

min = Minute

mg = Milligram

ml = Milliliter

N.C.B.I. = National center for biotechnology information

nAChR = Nicotinic acetylcholine receptor

ng = Nanogram

nm = Nanometer

-NH2 = Amino group

-OH = Hydroxyl group

P450s = Cytochrome P450s

PBO = Piperonyl butoxide

PCR = Polymerase chain reaction

QPCR = Quantitative polymerase chain reaction

rbp1 = RNA-binding protein 1

RNA = Ribonucleic acid

Ry = Ryanodine

RyR = Ryanodine receptor

s = Second

sKdr = Super knock down resistance

SNP = Single-nucleotide polymorphism

Taq polymerase = *Thermus aquaticus* polymerase

Tm = Melting temperature

TM = Transmembrane domain

UGT = Uridine 5'-diphospho-glucuronosyltransferase

µl = Microlitre

µM = Micro molar

1. General Introduction

1.1 Food security

Food production and security is one of the grandest challenges facing the global community, especially with the exponential increase of the global population, expected to hit 9.7 billion by 2050 (United Nations 2015). It is estimated that this growth will require a 60% increase in food production to sustain the population (Alexandratos and Bruinsma 2012). The number of undernourished people in 2019 was an estimated 687.8 million and projections, including the effects of covid-19, predict the number to rise to 909 million in the next 10 years (FAO 2020). Secure and stable food production is vital for three main reasons: (1) Nutritional health; malnutrition is the largest contributor to disease in the world causing more than one-third of child deaths worldwide (Abate et al. 2019). (2) Economic security; in 2016 2.5 billion people were dependant on agriculture for their livelihoods (FAO 2016) and the 2011 global agriculture output was valued at $2.4 trillion (Alston and Pardey 2014). (3) Social stability; increases in food prices have been linked to civil unrest, as observed during the ‘food price spike’ of 2008 (Bellemare 2015). The fact that approximately 50% of agricultural production is lost each year highlights one area where farmers and scientists can collectively focus efforts to increase productivity. Research into mitigating food losses should be further incentivised when considering limited land resources for agricultural expansion and increasing negative effects of climate change. The latter

include rises in temperature, extreme heat, droughts, wildfires, heavy downpours, shifts in crop pest ranges and disruption to agricultural productivity, all of which impact crop yields and quality - threatening sustainable food security and price stability (USGCRP 2017).

1.2 Food loss

Up to 50% of food produce is lost each year (Lundqvist, de Fraiture, and Molden 2008). 80% of this lost produce, depending upon the crop, is attributed to crop pests (Oerke 2006). Pests are organisms that feed on, compete for resources with, or transmit disease within crops. Weeds potentially contribute to most of the losses caused by pests (up to 34%) followed by animal pests (up to 18%) and pathogens (up to 16%) (Oerke 2006). Since the agricultural revolution, about 10,000 years ago, the cultivation of monoculture crops has resulted in a greater 'reward' for the exploitation of these resources by pest herbivores. This resulted in a strong selection on pests to overcome plants natural defences. Overcoming this evolutionary barrier was made easier by the intensive process of selective breeding. Specifically, plant defences were traditionally, indirectly, selected against in a trade-off with nutritionally desirable traits such as fruit size. Larger fruits etc. would require greater resource allocation due to increased energetic costs of production, resources typically reallocated from other traits including plant defences (Rosenthal and Dirzo 1997; Chen, Gols, and Benrey 2015). Furthermore, any natural adaptive evolutionary processes by crops in response to herbivory would be slowed down through erosion of genetic

diversity as a result of the strong selection from breeding programs. Larger selected fruit/seed phenotypes would typically be maladapted in a wild setting due to these fitness related costs, which is why alleles for these traits are only present at low frequencies in wild populations (Tang, Sezen, and Paterson 2010). As humans developed an increased reliance on farmed crops for civilization, they sought means to prevent the herbivory of these crops, for example, through the application of compounds toxic to pests. The first recorded use of pesticides was the application of sulphur by the Mesopotamians 4500 years ago, a compound that is still used today to combat fungal disease. By the 17th century plant extracts were being utilised by humans for pesticides, such as nicotine sulphate, extracted from tobacco plants. Today most pesticides used commercially are synthetic compounds, however, many of these are based on the chemical structures of natural plant secondary metabolites.

Crop losses from pests have led to massive investment in the development of chemical control through the commercialisation of pesticides. 5.7 million tonnes of pesticides are exported each year with a value of $36.7 billion (FAOSTAT 2020). Despite investment in chemical defences new pest outbreaks and resurgences of ‘old’ pests still occur. These are attributed to numerous factors including: the global transport of commodities, rapid evolution of resistance mechanisms, accelerated climate-driven shifts in host ranges, increased dependence on monoculture farming and limited knowledge of pest biology. This means outbreaks and epidemics continue to have sporadic and devastating effects on crop production.

1.3 Tuta absoluta

T. absoluta is an economically important pest species of tomatoes. It spread from South America to Spain in 2006, and subsequently has radiated throughout Europe, Africa and the Middle East (Desneux et al. 2011a; Martins et al. 2018). Global tomato production exceeds 182 million tonnes and has a current gross production value of $47.7 billion and a constant gross production value from 2014-2016 of $93.9 billion (FAOSTAT 2020). In many tomato producing regions, including those in which tomatoes are a staple food such as Nigeria, *T. absoluta* has reached epidemic status destroying whole crops and in some cases inflating market prices by 400% (Toesland 2016). This has resulted in this pest being sensationally referred to as 'tomato ebola' (France-Presse 2016) and the 'tomatopocalypse' (Parker 2016). The devastating effect of *T. absoluta* is felt by commercialised and subsistence growers throughout its expanding range. *T. absoluta* is a micro lepidopteran species belonging to the Gelechiidae family which consists of about 500 genera and 4700 species (Zhang 2011). It was endemic to South America, first described in Peru in 1917 (Meyrick 1917) and genetic studies reveal that it's expansion throughout Europe, Africa and the Middle East came from a single introduction into Eastern Spain from central Chile in 2006 (Guillemaud et al. 2015). *T. absoluta is* a multivoltine oligophagous herbivore of plants in the Solanaceae family. The lifecycle of this species is holometabolous with 5 instar phases (L1-L5) throughout larval development, during which time damage to the plant takes place. Larvae mine through the leaf cuticle and feed on the parenchyma creating characteristic galleries in the process

(Figure 1.1). The development times of *T. absoluta* from egg to adult are dependant on host plant, cultivar (Krechemer and Foerster 2017) and temperature (Krechemer and Foerster 2015) (Table 1.1).

Reproduction is primarily sexual in *T. absoluta,* although it has also been reported to reproduce parthenogenetically (Megido, Haubruge, and Verheggen 2012). From the first day of eclosion into maturity, female *T. absoluta* attract males through the emission of a volatile pheromone (3E,8Z,11Z)-3,8,11-tetradecatrien-l-yl acetate (Attygalle et al. 1996). Calling females unfold their antennae and remain continuously immobile, while elevating their abdomen over the wings and exposing their ovipositor (Lee, Albajes, and Eizaguirre 2014), as well as their intersegmental glandular membrane, positioned at the tip of their abdomen, where the pheromone is released (Attygalle et al. 1996). Both males and females mate no more than once a day, during which a spermatophore is passed from the male to the bursa copulatrix of the female (Lee, Albajes, and Eizaguirre 2014). Most females lay the same day as mating and are capable of mating 13 days consecutively; although more staggered mating patterns are the norm. Longevity and fecundity is highest in polyandrous females with 67% of females mating more than once. Oviposition peaked at 2-3 days with 72% of eggs being laid in the first 7 days. On average 74.2 eggs were produced per female with egg viability at 87.17 % (Lee, Albajes, and Eizaguirre 2014). With a short lifecycle, high reproductive output and suitable niche *T. absoluta* are capable of rapid population booms over short periods of time.

Figure 1.1 Tomato leaf showing *Tuta absoluta* larvae inside a gallery - the area of consumed parenchyma between the upper and lower epidermis.

Table 1.1. Development times for life stages of *T. absoluta* on different tomato cultivars at 20°C ± 2, on the tomato cultivar Santa clara at different temperatures and on different plants of the Solanaceae family at 25°C ± 5 (Krechemer and Foerster 2015; Krechemer and Foerster 2017).

		Number of days			
Developmental condition	**Variable**	**Egg**	**Larvae**	**Pupae**	**Egg-adult**
Tomato cultivars	Cherry	5.8	14.9	9.9	24.8
	Cordilheira	5.9	18.3	9.8	28.2
	Giuliana	6.9	16.8	10.3	26.8
	Nemoneta	5.9	17.3	9.9	27.2
	Paron	6.9	16.7	10.3	27.2
	Santa Clara	6.9	17.8	10.2	27.8
Temperatures (°C)	10	*24.4*	*56.4*	*36.8*	*115.4*
	15	11.9	34.1	18.4	63.9
	20	6.9	17.8	10.2	34.8
	25	4.5	11.0	8.4	23.5
	30	2.5	10.4	5.4	18.3
Plant Variety	Potato (*Solanum tuberosum*)	4.3	8.5	7.6	20.4
	Bitter sweet (*solanum dulcamara)*	3.8	11.8	9.2	24.8
	Tobacco (*Nicotiana rustica)*	4	22.7	7	33.7
	Matrimony vine (*lycium barbarum*)	4	19	6.7	29.7

T. absoluta females are attracted to host plants, especially tomato species, through their release of aromatic volatiles. This attraction seems to be initiated by either mating or oviproduction as virgin females show no attraction response to tomato plants (Proffit et al. 2011). Females can detect plant suitability showing a preference for cultivated varieties over wild types. Wild tomato varieties have higher trichome density (fine hair like protrusions from

the leaf with a diverse array of functions including defence through secretion of secondary metabolites), and there is a negative correlation between trichome number, and oviposition rate and larval survival (Bitew 2018). Increased trichome density results in heightened levels of tridecan-2-one, a secondary metabolite that slows larval development and acts as an ovipositioning and feeding deterrent (Maluf, Barbosa, and Santa-Cecília 1997). Plant age also increase trichome density, with *T. absoluta* larvae exhibiting higher mortality on older plants (Leite et al. 2001).

1.4 Pest control

1.4.1 Overview of pest control

Current strategies for controlling crop pests employ integrated pest management (IPM), and this combinatory approach has three basic components: (1) Monitoring pest populations in the field for changes in density. (2) Focusing on economic injury levels and (3) integrating multiple control strategies. These practices can provide crop protection whilst reducing pesticide use, slowing the development of resistance (IRAC, 2007). Insecticide resistance is an inevitable evolutionary consequence of strong selection pressure applied by chemical agents targeting pest populations. To slow the evolution of resistance, selection pressure from pesticides must be reduced by appropriate application and alternative/alternating control methods. Such strategies also minimise application rates, reduce pesticide costs, and bring additional benefits, as more environmentally friendly farming practices have been shown to increase surrounding biodiversity. This in turn

can provide additional ecosystem services to agro-systems such as increased pollination services (Goulson et al. 2015) and natural pest control (Mills et al. 2016).

1.4.2 Monitoring

Monitoring is the first important component of IPM. It is key to making decisions on when control measures should be applied, and is commonly based on numbers of pests and natural enemies. It also prevents unnecessary applications. Monitoring should also be used after application to assess the efficacy of control. The threshold value for a population density where control should be used (economic injury level) can be defined as the numbers of a pest that cause economic yield losses of a greater value than the cost of the pesticide plus its application. Once pest densities reach this threshold an integrated approach to control is implemented. This includes good farming practices, biological control agents such as predators, parasites and pathogens, use of resistant crop varieties, chemical attractants/deterrents and the judicious use of pesticides.

1.4.3 Good farming practices

Good farming practices are practical ‘common sense’ approaches that limit persistence and transmission of pests. For *T. absoluta* these can include; education of manual workers for signs of infection, stripping mined leaves from plants when harvesting tomatoes, ensuring that distribution companies aren’t also working with growers with infestation problems, quarantine of distribution packaging when it arrives on site to ensure sterility, wearing

appropriate protective clothing in infested glasshouses to prevent transfer of pests between sites, ensuring waste plant material, once removed, is disposed of properly to prevent reinfection and, extensive end of season clean up to prevent old infestations re-establishing in new crops.

1.4.4 Chemical attractants and deterrents

Pheromones are vital for insect communication and synthetic or natural versions of these compounds are being increasingly utilised as an ecologically friendly approach to pest control. Pheromones are species specific and the majority are not known to be toxic to animals, however, they are not always as effective as pesticides. Progress in pheromone-based product development has been slow and there are few examples where control is achieved through pheromone technologies alone. Despite this, more than 20 million pheromone lures are produced each year for the purpose of monitoring and mass trapping (Witzgall, Kirsch, and Cork 2010). Pheromone pest control of is achieved through either annihilating the pest of preventing reproduction through mating disruption. Annihilation involves attracting the pest to a trap or a pesticide target. In the chickpea leaf miner *Liriomyza cicerina* mass trapping was used as an alternative to pesticide. 2000 traps per ha were applied to the crop resulting in an 18% reduction in pest infestation compared to a 20% reduction with pesticide treatment. Yield was 0.8 kg per m^2 for the pesticide treatment and the mass trapping, compared to 0.21 kg per m^2 for the control (Soltani, Amri, and Mediouni-Ben Jemâa 2018). In *T. absoluta* mass trapping has been shown to have a significant effect in field tests in Kenya (Wafula, Waceke, and Macharia 2018), however in Tunisia, trap effects were only detected in

conjunction with pesticide use (Cherif et al. 2018) and in Bulgaria no correlation was found between the number of trapped moths and the level of damage of leaves and fruits (Mohamedova et al. 2016). In the UK traps are only used for crop monitoring but use of pheromones in mating disruption has become a major part of IPM. A synthetic version of *T. absoluta* sex pheromone was approved for use in the UK at the start of 2017 and sold under the trade name Isonet T ("Isonet T - New Product - Fargro" 2017). Mating disruption techniques involve the synthesis and release of female sex pheromones, this confuses the males and prevents them finding a mate and reproducing. One of the first pests to be controlled by mating disruption was the pink bollworm *Pectinophora gossypiella* (Miller and Gut 2015) a pest of cotton that was difficult to control with pesticides. The application of rope pheromone dispensers in the field reduced populations by 97% compared with fields treated with conventional insecticides (Staten et al. 1987). The efficacy of pheromones is enhanced if the environment is enclosed such as in glasshouses. In Italian tomato glasshouses infested with *T. absoluta* the implementation of mating disruption reduced infestations by 57% to 85% and reduced the damaged fruits by 62% – 89% (Cocco, Deliperi, and Delrio 2013). Pheromone control is typically most effective when used in conjunction with other control measures but can be highly effective on its own if the conditions in which it is applied are suitable, and the biology of the pest is compatible.

1.4.5 Biocontrol control

Biological control is the use of predators, parasites or pathogens to supress pest populations and is broadly grouped into three categories; classical,

augmentative and conservation (Bale, Van Lenteren, and Bigler 2008). Classical control is the repression of invasive pests though the use of natural enemies that are native in the pests original host range and they are introduced to its new range. Over 2384 insect biological control agents have been introduced for the control of 588 pest species in 148 countries (Cock et al. 2016). The introduction of foreign organisms can however, have detrimental effects to non-target organisms and in some cases disastrous ramifications for ecosystems as a whole so must be carefully risk assessed (Barratt et al. 2010). Augmentative biocontrol is the periodic inundation of pest populations with natural enemies. These are applied to the crop after pest infestation and supress or eradicate the pest, typically in one generation, then often die out. Reapplication may be required later on in the season. This method of biological control requires mass production of control agents and can be costly (Bale, Van Lenteren, and Bigler 2008). Conservation bio control utilises predators and parasites that are present in the crops native host range. This system is most effective on outdoor crops where planting of hedgerows and wild flower borders to fields can enhance natural biodiversity and increases natural enemy populations (Bale, Van Lenteren, and Bigler 2008).

A vast range of natural enemies have been identified for the potential control of *T. absoluta* including approximately 70 species of predators and 100 species of parasitoids (Ferracini et al. 2019), as well as many parasites and pathogens. Of these the mirid predators, *Macrolophus pygmaeus* and *Nesidiocoris tenuis*, the entomopathogenic nematode *Steinernema feltiae,* the

bacteria *Bacillus thuringiensis* and the parasitoid, *Trichogramma achaeae* are commercially available in Europe. Both mirid bugs have been observed predating *T. absoluta* in the field (Arnó et al. 2009), and in the lab were capable of preying on over 30 eggs or ~ 2 L1 and ≤ 1 L2-4 larvae per day with females consuming significantly more than males (Urbaneja, Montón, and Molla 2009). *S. feltiae* were showed to cause a 92% larval mortality rate in leaf dip bioassays and 95% larval mortality under greenhouse conditions (Batalla-Carrera, Morton, and García-del-Pino 2010). *T. achaeae* were shown to parasitize 74% of eggs in laboratory bioassays and in field experiments, the number of galleries mined by *T. absoluta* larvae were reduced 4-fold compared to control groups (Zouba and Mahjoubi 2010). In addition to the advantages of using biocontrol agents to prevent yield loss they can also be more environmentally friendly than pesticides, they actively seek out the pest and are often pest-specific so have little impact on off-target organisms. However, the ecology of the systems must be understood, natural enemies can be slow to take effect and if pest densities are too low or at the wrong life stage then they can damage the crop. *Macrolophus pygmaeus* are zoophytophagous and if densities of *T. absoluta* are too low they will feed directly on plant tissue causing significant fruit damage (Moerkens et al. 2016).

1.4.6 Pesticides

The last line of defence in IPM is chemical control. Pesticides typically target pathways in the insect's nervous systems preventing coordinated muscular movement. They should be employed on their specificity, as broad-spectrum

pesticides may have off-target effects on beneficial insects such as pollinators or natural enemies. Care must also be taken that they are applied in accordance with the manufactures guidance and during the life stage the pest is most vulnerable. Furthermore, to minimise resistance, alternations of pesticides that have different modes of action (i.e. target different molecular sites within the pest) should be used in order to reduce overall selection pressure at each target-site. At the end of the growing season, it is important to destroy any crop residue where pests may persist. This will minimise pest establishment in the next growing season and is especially important where any remaining pests possess resistance alleles leading to the proliferation of new populations with resistance genes.

1.5 Resistance

1.5.1 Evolution of resistance

Application of pesticide creates strong selection pressure for evolution of resistance in pests and is a prime example of evolution in action. Proliferation of resistant phenotypes can occur in very short periods of time due to the immense selection pressures pesticides impose (Palumbi 2001). Standing genetic variance is ubiquitous among natural populations (Barrett and Schluter 2008) meaning individuals within a population will respond differently to environmental selection pressures. Phenotypes incurring lower fitness costs from novel selection pressures - such as the introduction of pesticides to the environment - will contribute alleles at a higher frequency to the next generation of offspring compared to individuals less suited to the environment.

This generational shift in allele frequency results in a higher proportion of the population possessing those alleles and thus a higher proportion expressing the favourable phenotype. While alleles that are beneficial under novel selection pressures can pre-exist in populations they can also arise *de novo.* Mutations can occur in alleles after the introduction of novel selection pressures (application of pesticide), producing novel phenotypes (resistance), which can then be amplified through the population by strong selection (Messer and Petrov 2013).

Insecticide resistant phenotypes are highly pervasive due to the incredibly strong selection pressures pesticides incur, with all modes of action having some level of insect resistance recorded (Nauen 2007). By economic value, 85% of pesticides sold act on the insect neuro–muscular system, targeting pathways that interfere with neuro-signalling and muscular contraction (Sparks and Nauen 2015). Insect resistance to pesticides occurs via one, or a combination, of the following mechanisms; behavioural avoidance, reduced penetration, enhanced insecticide metabolism, or structural alteration of the insecticide target site.

1.5.2 Behavioural resistance

Behavioural resistance results from alterations to an insect's behaviour that reduces their contact with the pesticide. Insects have a natural ability to avoid eating toxic plants through the stimulation of phagodeterrent cells in the mouthparts, preventing uptake of detrimental quantities of plant secondary metabolites (Chapman 2003). Insects can also feed selectively on specific

parts of plants or at specific developmental stages that express low levels of secondary metabolites (Nealis and Nault 2005). This ability to identify toxic compounds also applies to synthetic xenobiotics, and appears to be accompanied with a physiological tolerance to the compound, they have to assimilate the toxin to detect it before they can avoid it (Hostetler and Brenner 1994; Nansen et al. 2016). This chemosensory detection resulting in a behavioural response would allow pests to return to crops after pesticide degradation or to attack regions of the plant subject to insufficient pesticide application. This could result in yield loss and the necessity for reapplication. Furthermore, exposure to sub-lethal doses of pesticides could induce a plastic resistance response through hormetic priming (Rix, Ayyanath, and Cutler 2016), or may even accelerate evolution of resistance mechanisms through increased mutation rate (Gressel 2011).

1.5.3 Penetration resistance

Penetration resistance is an increased ability to physically slow down or inhibit pesticide assimilation by the insect, and occurs through cuticular thickening and/or modification. The multifunctional properties of the cuticle provide shape and support, interact with muscles to provide movement, prevents desiccation, provides waterproofing, acts as a food store and as a barrier to parasitism, disease and xenobiotic penetration (Vincent and Wegst 2004). In the mosquito *Anopheles gambiae,* absorbance of deltamethrin was slower in a resistant strain that possessed a thicker cuticle and increased cuticular hydrocarbon content. This process likely works in conjunction with metabolic resistance by alleviating the workload of the detoxification enzymes through

the reduction in somatic concentration (Balabanidou et al. 2016). Cuticular composition also impacts resistance to pesticides. An upregulation of laccases was observed in resistant mosquitoes *Culex pipiens.* Laccases are enzymes involved in cuticular tanning, a process that hardens the cuticle and is believed to reduce xenobiotic penetration in resistant strains (Pan, Zhou, and Mo 2009). Upregulation of cuticular transport proteins (ABC transporters) are also associated with resistant phenotypes in insects, however, as they function in both xenobiotic transport and transport of cuticular lipids in the epidermis, their exact role is unclear (Balabanidou, Grigoraki, and Vontas 2018)

1.5.4 Metabolic resistance

Metabolism is the entirety of biochemical reactions within the body. These include conversion of food to energy or somatic growth and repair, as well as the breakdown and elimination of unwanted/harmful substances. The elimination of unwanted/harmful substances is not only concerned with excretion of toxic nitrogenous waste products from reactions vital for life but also with the biotransformation and excretion of xenobiotics assimilated from the environment. This metabolic detoxification acts as a chemical defence inhibiting toxic effects from both biotic and synthetic compounds such as pesticides. Metabolic resistance arises through an enhancement of this pre-existing allelochemical detoxification system and allows for a broad range of specific and general molecular responses. This diversity is generated by gene duplication events, posttranscriptional modification, increased gene expression, or by mutations in the enzyme resulting in a conformational

change in the catalytically active site, enhancing activity or enabling novel substrate specificity.

Metabolic detoxification is typically a stepwise process that includes transport, modification and excretion. The transport of xenobiotics within organisms is often carried out by ATP-binding cassette (ABC) proteins, a superfamily of transport proteins present in all living organisms (Dassa and Bouige 2001). Insects usually have somewhere between 40-70 ABC genes arranged in eight subfamilies (ABCA to ABCH) (Merzendorfer 2014a). They have a highly conserved structure which consists of a pair of ATP binding domains and two sets of transmembrane domains (Dean, Hamon, and Chimini 2001). This allows them to act as efflux pumps, transporting of a wide range of molecules across lipid membranes. These include lipophilic compounds such as pesticides as well as metabolites from detoxification reactions (Dean, Hamon, and Chimini 2001). ABC's can expel toxins directly with other excretory products, transport insecticides for downstream processing by phase 1 and 2 detoxification enzymes as well as transport metabolites resulting from phase 1 and 2 reactions to the excretory system. (Merzendorfer 2014a)

Xenobiotic metabolism reactions fall into one of two classes, defined by type of compound modification. Phase 1 detoxification processes alter the chemical structure of the pesticide adding or exposing polar regions of the molecule (Westhouse and Car 2007). This can reduce or increase its toxicity by making the compound more reactive. Some compounds can be excreted directly after phase 1 processing but many are passed to the phase 2

enzymes. Phase 2 metabolism involves conjugation reactions. The bonding of endogenous molecules to the polarised xenobiotic from phase 1 reactions, increasing the compounds hydrophilicity facilitating excretion (Liska 1998). Some xenobiotics already containing -OH, -NH2 or -COOH groups can bypass phase1 and be processed by phase 2 metabolisers directly (Phang-Lyn and Llerena 2020).

The two main families of phase 1 metabolism are cytochrome P450's and esterase's. Like ABC's, P450's are distributed amongst virtually all-living organisms, are highly diverse, and one of the most abundant superfamilies of genes present in eukaryotic genomes (Feyereisen 2012). CYPs have been described as 'environmental response genes' due to their high diversity, proliferation by duplication events, rapid rates of evolution, occurrence in gene clusters and tissue/temporal–specific expression (Berenbaum 2002). In excess of 300 000 P450's have been sequenced from 70 families with 127 subfamilies (Nelson 2018). Their diversity and abundance is attributed to recurrent gene duplication (Werck-Reichhart and Feyereisen 2000) driven by functional importance in catalysing reactions including steroids, eicosanoids, fatty acids, lipid hydroperoxides, retinoids, lipid metabolites, and xenobiotics such as drugs, alcohols, procarcinogens, antioxidants, organic solvents, anaesthetics, dyes, odorants, flavorants and pesticides (Coon et al. 1992). Insect P450's have been divided into four main clades, CYP2, CYP3, CYP4 and mitochondrial CYPs each. Each clade is then divided into families and subfamilies (Feyereisen 2006). The CYPome size varies greatly between insect species from 36 genes in the head louse, *Pediculus humans* (Lee et al.

2010) to 170 genes in the mosquito *Culex quinquefasciatus* (Arensburger et al. 2010). CYP3 is the most numerous clade in insects. Within this clade is the family of CYP6's most of which are linked to the metabolism of insecticides (Feyereisen 2006).

P450 proteins are heme-thiolite enzymes that have a large amount of variation in their structure, but are typically conserved around the cysteinal-thiolate ligand. When the reduced form of this ligand is bound to carbon monoxide it results in a characteristic spectral absorbance peak at 450 nm giving P450's their name (Omura and Sato 1964). P450's are membrane bound enzymes found in the sarco/endoplasmic reticulum (apart from mitochondrial P450's) and distributed throughout insects with especially high density in the mid gut, fat bodies and malpighian tubules (Hodgson 1983). They also differ in expression throughout development of most insects. In the aquatic midge *Chironomus tentans* P450's were not detected at low levels in eggs, are detected at increasing levels throughout each instar phase and drop-off in expression in pupae and adults (Tang et al. 2017).

P450's primarily act as monooxygenases but other catalytic functions also include oxidases, reductases, desaturases, isomerases etc. Monooxygenases activity is involved in many process within the body including growth and development, feeding, degradation of hormones and pheromones, tolerance to plant toxins and resistance to pesticides (Scott 1999). Research into Human P450's has shown they metabolise 75% of drugs used (Wienkers and Heath 2005; Williams *et al.* 2004). P450s can be substrate specific, have a

broad range (>20) of targets and different P450s can overlap in substrate specificity (Rendic and Carlo 1997). Novel resistant phenotypes can arise from *cis*-mutations resulting in altered compound specificity or *trans*-mutations which can effect the expression P450 genes or gene clusters (Feyereisen 2005). The structural diversity, metabolic flexibility and the ability to respond 'rapidly' to environmental changes through mutation and differential expression, combined with selective pressure to metabolise xenobiotics has resulted in proliferation of CYPs within the genome and the reason they have been linked to the evolution of insecticide resistance to all classes of pesticide among most resistant insect species (Feyereisen 2005).

The second major class of phase 1 metabolic enzymes are the esterases. These are a structurally and functionally diverse superfamily of enzymes and were vital in the very earliest forms of life for essential processes such as nucleic acid and lipid metabolism. With the evolution of metazoans, adaptations to esterases allowed metabolism of lipid nutrients and xenobiotics (Oakeshott et al. 1999). The majority of esterases belong to the carboxyl/cholinesterases and are part of the alpha/beta hydrolase fold protein super family (Montella, Schama, and Valle 2012). The alpha/beta hydrolase fold is the catalytically active region of the enzyme and modify compounds in a two-step process based on a Ser-His-Glu triad brought into close proximity to each other due to the tertiary structure of the protein (Oakeshott et al. 1999). The esterase reaction is defined as the hydrolysis of an ester to its component alcohol and acid. These include the hydrolysis of carboxylic, thio-, phospho-, and other ester substrates (Oakeshott et al. 2019). Esterase

mediated insecticide resistance seems to be limited to the carbamates and organophosphates classes of insecticide and are metabolised by the carboxylic/cholinesterases, the most diverse family of esterases. Carboxylesterases are primarily localised in the endoplasmic reticulum (Hakamata et al. 2014). Their main function is to modify xenobiotics containing esters and have been linked to resistance of carbamates and pyrethroids in several insect species (Wheelock, Shan, and Ottea 2005)

Cholinesterases natural function is involved with the hydrolysis and termination of acetylcholine (Pohanka 2011). Release of acetylcholine at the synapse excites acetylcholine receptors at the interface between the nervous and muscular systems. This allows influx of ions producing intracellular ionic signals, driving coordinated and controlled muscle contraction (Dani 2001). The conformational change to the receptor is a result of the hydrolysis of bound acetylcholine by cholinesterase resulting in its breakdown into acetic acid and choline (Pohanka 2011). OP's and carbamates phosphorylate or carbamoylate cholinesterases. This inhibits termination of the neuro-signalling resulting in prolonged excitation of acetylcholine receptors - preventing coordinated signalling (Colovic et al. 2013). Three major mechanisms of resistance to this neuro-signalling inhibition are (1) mutations in the cholinesterases molecules preventing inhibition by the insecticide (target site resistance) (Mutero et al. 1994). (2) Up regulation of wild-type carboxylesterases (Li et al. 2020). While these have poor hydrolytic efficiency of OP's they can act to sequester it reducing cellular concentrations. (3)

Mutations in carboxylesterases can improve hydrolytic kinetics making them efficient at breaking down OPs (Oakeshott et al. 2019).

Once compounds have been modified by phase 1 they can either be excreted or passed to phase 2 metabolism for further biotransformation. This process typically involves uridine glucosyl-transferases (UGT) and glutathione-S-transferases (GST). GST's are another large family of multifunctional enzymes found in all eukaryotes (Kim et al. 2010). Insects GSTs fall into one of six classes, zeta, theta, sigma, omega, delta and epsilon. Delta and epsilon are typically only found in arthropods and appear to be most numerous (Ketterman, Saisawang, and Wongsantichon 2011). They account for the majority of GST's present in most insect orders analysed with the exception of certain species of hymenoptera which appear to have no GST's in the epsilon class (You et al. 2015). Total GST's range from 8 in the honey bee *Apis mellifera* to 37 in *D. melanogaster* (Ketterman, Saisawang, and Wongsantichon 2011). GSTs' primary function is detoxification through catalysing the conjugation of glutathione with electrophilic compounds which increases hydrophilicity aiding excretion (Armstrong 1997). They can also carry out a broad range of additional process within the cell including removal of reactive oxygen species, regeneration of S-thiolated proteins, catalysis of conjugations with endogenous ligands and metabolic activity in pathways unrelated to detoxification (Enayati, Ranson, and Hemingway 2005).

Detoxification occurs when reduced glutathione (GSH) is conjugated with a xenobiotic forming a thioester. The xenobiotic binds to the GST activating of

the thiol group of glutathione resulting in nucleophilic attack by the anionic GSH on the bound hydrophobic compound (Atkins et al., 1993). This conjugation reaction neutralizes the electrophilic sites of the lipophilic substrate and protects the cellular components, especially the nucleophilic oxygen and nitrogen of DNA from electrophilic attack of nucleophiles. Conjugation also renders the product more water soluble and therefore more readily execrable from the cell (Enayati, Ranson, and Hemingway 2005). GST's are homo/hetero dimers and although highly diverse in sequence, there is conservation of 3 dimensional structures in two of their domains. An N-terminal domain formed from consisting of four β-sheets with three α-helices, this site binds glutathione (Sheehan et al. 2001). The second, more variable C-terminus domain consists of five to six α–helices. This region interacts with the hydrophobic second substrate and contributes a highly conserved aspartic acid residue to the glutathione binding site (G-site) (Sheehan et al. 2001). The last domain, that adopts an α-helix structure, contains a portion of the G-site and a great part of the binding site for hydrophobic electrophiles (H-site) (Ruzza and Calderan 2013). The variance C-terminal domain is thought to result in the diversity in substrate specificity (Wilce and Parker 1994). GST detoxification has been associated with resistance to all major classes of insecticide (Enayati, Ranson, and Hemingway 2005).

The second superfamily of phase 2 metabolic enzymes is UGTs. They are ubiquitous in all free-living organisms and have a variety of functions within insects including, olfaction (Lazard et al. 1991), endobiotic modulation (Bock

2003), sequestration (Ahn et al. 2011), cuticle formation (Hopkins and Kramer 1992) and insecticide detoxification through conjugation reactions (Zhao et al. 2019). UGT families and subfamilies are defined by amino acid sequence identity (40% or greater and 60% or greater respectively) (Mackenzie et al. 1997). Insect UGT's are divided into several distinct families. The true number of families and the diversity within them remains largely unknown due to lack of research conducted into them. The number of UGTs in different insect species varies greatly ranging from 12 in *A. mellifera* to 58 in the pea aphid, *Acyrthosiphon pisum* (Ahn, Vogel, and Heckel 2012). Phylogenetic analysis of insect UGTs showed one group, UGT50, was common to all insects (except *A. pisum*) and some were order specific like UGT33 for Lepidoptera. In *Chilo suppressalis* 11/24 UGTs were in UGT33 and UGT40 families (Zhao et al. 2019). In *Plutella xylostella* 9/23 UGTs were present in UGT33 and UGT40 (Li et al. 2018). Similar distribution of UGTs among gene families was observed for in *Helicoverpa armigera, Bombyx mori* (Ahn, Vogel, and Heckel 2012) and *Spodoptera exigua* (Hu et al. 2019). Lineage specific divergence of UGT33 suggests independent gene expansions within the different species. Variation of UGT33 is driven by diversity in the N-terminal substrate binding domain which likely allows for broad substrate specificity and the detoxification of a wide range of compounds (Ahn, Vogel, and Heckel 2012).

UGT's are membrane bound proteins found in endoplasmic reticulum with enzymatic function being highest in the localised tissues of the midgut, fatbodies and malpighian tubules (Ahmad and Hopkins 1993). UGTs are typically around 530 residues long and comprise of two functional regions, an

amino (N-) and a carboxyl (C-) terminal domain. The N terminal domain is located in the ER lumen. It comprises about 95% of the polypeptide chain (Mackenzie 1986) and it the site of catalytic activity, responsible for glucuronidation of phenols in the human liver (Ouzzine et al. 1994). The C-terminal domain includes the transmembrane and cytoplasmic regions (Ouzzine et al. 2000). UGT's can function independently or associate to form homo/heterodimers. Due to the fluidity of the phospholipid bilayer, UGT's can also reposition them selves to interact with P450's directly facilitating efficient phase 1 to phase 2 processing (Radominska-Pandya et al. 2005).

UGTs catalyse the conjugation of a modified sugar molecule to a hydrophobic molecule reducing toxicity and increasing hydrophilicity expediting excretion. In Humans, UGT's have been extensively investigate with regards their contribution to drug metabolism. They catalyse the conjugation of glucuronic acid, derived from UDP-glucuronic acid (UDPGA), to typically lipophilic substrates bearing nucleophilic acceptor functional groups - producing a more water-soluble glucuronide (Tukey and Strassburg 2000). Transfer of glucuronic acid to available substrates can be formed through hydroxyl, carboxyl, sulfuryl, carbonyl, and amino (primary, secondary, or tertiary) linkages. This flexibility in substrate specificity allows for targeted glucuronidation of thousands of agents (Tukey and Strassburg 2000). This process is analogous in insects except for the use of UDP-glucose as the main sugar donor (Ahn, Vogel, and Heckel 2012). UGTs have become increasingly associated with insecticide resistance in a large range of insects for a broad range of pesticides with varying modes of action (Li et al. 2018).

1.5.5 Target-site resistance

Pesticides most frequently target pathways in the neuro-muscular system. The binding of pesticides perturbs function of these targets resulting in insect mortality. If the target-site can tolerate structural alterations, resulting from mutations, that mitigate the effects of the pesticide without major costs to fitness, then they will be selected for and proliferation of resistant phenotypes within the pest population will occur. Amino acid substitutions can occur agonistically, directly altering the biochemical properties of the pesticide binding site (Tmimi et al. 2018), or antagonistically, indirectly altering target-site binding affinity through conformational change of the molecule. Furthermore, mutations may occur that allow the binding of the pesticide to persist but alter the sensitivity of the target to its toxic effects, allowing it to function in the presence of the pesticide. Due to the conserved structure of functional regions of many proteins within the neuro-muscular system, the number and location of non-lethal mutations are often highly constrained. Therefore convergent evolution of resistance phenotypes across different insect species through alteration of the same amino acid is common.

One of the earliest examples of this convergent evolutionary response to pesticide application is knock down resistance (kdr), the reduction in sensitivity of the insect nervous system to pyrethroids, a voltage-gated sodium ion channel. Comparisons of sequences in the housefly gene revealed a leucine to phenylalanine substitution at position 1014 (Williamson et al. 1996). This specific alteration (L1014F) was functionally validated as conferring resistance though its expression in *Xenopus laevis* oocytes resulting in a 17-

fold reduction in sensitivity to pyrethroids (Soderlund and Knipple 2003).This knockdown resistance (kdr) mutation has since been found in insects from the orders Blattodea (Miyazaki et al. 1996), Diptera (Guerrero et al. 1997), Lepidoptera (Schuler et al. 1998), Coleoptera (Lee et al. 1999) and Hemiptera (Martinez-Torres et al. 1999). Some species including the housefly *Musca domestica* and the horn fly *Hematobia* irritans can tolerate a second mutation (*M918T)* resulting in a super kdr (skdr) phenotype (Williamson et al. 1996; Guerrero et al. 1997). In the absence of pyrethroids however, these mutations result in a fitness cost and are unstable over time (Hanai, Hardstone Yoshimizu, and Scott 2018).

Target site resistance in now present for a wide range of pesticide chemistries used. Since *T. absoluta's* pest status was recognised in South America in the 1960's, it has been controlled with the use of; methamidophos (organophosphate), cartap (carbamate), avermectins, the pyrethroids deltamethrin and permethrin, spinosyns and diamides (Hostetler and Brenner 1994; Nansen et al. 2016). With up to 36 applications of pesticides in one growing season selection was strong and resistance quick to evolve (Siqueira, Guedes, and Picanço 2000).

1.5.6 Organophosphate and carbamate resistance

Organophosphates and carbamates differentially target the acetylcholinesterase enzyme which catalyses the hydrolysis of the neurotransmitter acetylcholine (ACh) (Fukuto 1990). Inhibition of ACh comes about through a chemical reaction in which the serine hydroxyl moiety at the

enzymes' active site is either phosphorylated by organophosphates or formulates an enzyme-inhibitor complex with carbamides leading to carbamylation of the serine hydroxyl. Both reactions are analogous to regular acetylation of AChE except that the phosphorylated and carbamylated enzymes have greater stability preventing regeneration of enzymatic activity (Fukuto 1990). The phosphorylated or carbamated enzyme no longer enables hydrolysis resulting in build-up of the neurotransmitter at nerve synapses or neuromuscular junctions (Fukuto 1990). This build-up of neurotransmitter is the causal factor of the symptomatology of organophosphate poisoning. Resistance to these pesticides however has been reported in more than 25 arthropod species (Fournier 2005).

A variety of structural alterations of AChE (amino acid substitutions) result in reduced affinity of the pesticide-enzyme complex and is a convergent evolutionary response in many insect species for resistance to organophosphates and carbamides (Fournier 2005). In *Drosophila melanogaster* five mutations at different sites confer resistance through alteration of the insecticide AChE binding site (Mutero et al. 1994). Resistant strains of *Culex pipiens* display a single missence mutation (G119S) resulting in an altered active site reducing insecticide toxicity (Weill et al. 2004). In *P. xylostella* general enhanced enzyme activity contributes to some organophosphate resistance however, three mutations of the *ace-1* gene, G119S, F331W and S331F were the major contributing factors (Baek et al. 2005; Lee et al. 2007). In *Aphis gossypii* mutations in the *ace1* gene (S431F and A302S) were associated with insensitivity of acetylcholinesterase to

carbamate and organophosphate (Andrews et al. 2004). Reports of organophosphate and carbamate resistance in *T. absoluta* go back to 1983 in Bolivia (Moore 1983). Work on cartap, showed the addition of a synergist (piperonyl butoxide), an enzymatic inhibitor of cytochrome P450 monooxygenases, implicating xenobiotic metabolism as the resistance mechanism in Brazilian populations, generating resistance ratios of 2.3 - 21.9-fold (Siqueira, Guedes, and Picanço 2000b). A report of resistance in Spanish strains showed that all resistant phenotypes were homozygous for the A201S alteration. This substitution is the structural equivalent of A302S in *Aphis gossypii* having the same functional consequences, resulting in an LC90 higher than the recommended field application rate for chlorpyrifos (560 mg/l) (Haddi et al. 2017) .

1.5.7 Avermectin resistance

Avermectins are a complex of macrocyclic lactones produced through fermentation by the actinomycete *Streptomyces avermitilis* (Burg et al. 1979). Avermectin alters the permeability of glutamate-gated chloride ion channels (GluCl) and specifically the γ-aminobutyric acid (GABA) receptor. GABA is a major inhibitory neurotransmitter preventing nerve impulses through an increased flux of chloride ions across insect neuronal membranes (Tanaka and Matsumura 1985; Clark et al. 1995). This ion flux into neurons hyperpolarises the resting potential of the neuron and reduces the chance that an action potential can be elicited. Inhibiting this process results in ataxia and paralysis of the insect (Shoop, Mrozik, and Fisher 1995). Pesticide interaction with the glutamate-gated chloride ion channel is through allosteric modulation.

Evidence for this was provided by expression of the channel from *C. elegans* in *Xenopus laevis* oocytes which resulted in avermectin-sensitive chloride currents (Arena et al. 1991). This current could be directly activated by glutamate with avermectin potentiating its activity (Arena et al. 1992). The GluCl was shown to be composed of α and β subunits in *C. elegans,* the co-expression of which produced an ivermectin (a derivative of avermectin) sensitive GluCl (Cully et al. 1994). In *D. melanogaster* however only GluCl-α is required to enable glutamate gating (Cully et al. 1996). Ivermectin activates GluCl-α, while simultaneously rendering the receptor susceptible to further activation by glutamate, therefore acting as a partial allosteric agonist. Ivermectin binds at subunit interfaces on the periphery of the transmembrane domains. It inserts deeply into the subunit interface and makes important contacts with the pore lining. The ivermectin binding-site in GluCl is shared, at least in part, by many important modulators of the receptor. The interaction of ivermectin with the GluCl pore-lining increases both its affinity for the receptor and its ability to stabilize the open state (Hibbs and Gouaux 2011).

The effect of synergists has been used to suggest metabolic resistance to abamectin in many arthropod species. Metabolic resistance was observed in Colorado potato beetle where resistant strains had significantly elevated levels of cytochrome P450's resulting in resistance ratios of up to 38 (Argentine, Clark, and Lin 1992). In field populations of *P. xylostella* 5000-fold resistance to abamectin was observed and when selected, reached 23,670-fold. The oxidase inhibitor PBO showed 7.5-fold and 3.2-fold synergism to abamectin in the selected and unselected strain respectively. This indicated

that metabolic resistance was partly involved in abamectin resistance. However 3,140-fold resistance remained after PBO co-application, suggesting that enhanced oxidative metabolism is not the major mechanism responsible for abamectin resistance (Pu et al., 2010). A 36-bp deletion in the GluCl of *P. xylostella* was identified in the linker between the m3 and m4 transmembrane domains and was found to be present in 61% of resistant individuals and only 16% and 20% of individuals in susceptible strains. The much lower frequency of susceptible individuals carrying the deletion in the field compared with the resistant strain suggests that the 36-bp deletion may play a role in abamectin resistance (Liu et al. 2014). A mutation (A309V) in the m3 transmembrane domain was found in 94.7% of another *P. xylostella* population and associated with 10-fold resistance to abamectin (X. Wang et al. 2016). In the two-spotted spider mite, *Tetranychus urticae* a point mutation resulting in a G323D conferred a 17.9-fold resistance when homozygous in an individual (Kwon et al. 2010) None of these alterations to the GluCl structure occur in regions associated with abamectin or glutamate binding sites suggesting they act as allosteric modulators.

Abamectin resistant populations of *T. absoluta* were reported in Chile in 2000 and had resistance ratios up to 9.4 (Siqueira, Guedes, and Picanço 2000). Brazilian populations in 2001 had similar levels of resistance reaching 9.4-fold. The main mechanism for this resistance appeared to be metabolic detoxification as application of the synergist, piperonyl butoxide, returned sensitivity to the pesticide. The occurrence of resistance in different geographic populations of this pest was proposed to be a result of widespread

pesticide use (Siqueira et al. 2001). Resistance ratios, however, were relatively low, attributed to the low dose of abamectin used in the field along with its fast degradation in the environment and limited bioaccumulation (Clark et al. 1995). The detoxification enzymes glutathione S-transferases, cytochrome P450-dependent monooxygenases, and esterases were inhibited with the use of the synergists diethyl maleate, piperonyl butoxide and triphenyl phosphate respectively. Piperonyl butoxide completely suppressed abamectin resistance in one population, with suppression being close to complete in the rest. Partial suppression of abamectin resistance due to inhibition of gluthatione-S-transferases by diethyl maleate indicated this enzyme group might be of only minor importance. Triphenylphosphate completely suppressed abamectin resistance in four *T. absoluta* populations and provided partial suppression in two populations. The results suggest a major involvement of esterases as an abamectin resistance mechanism in Brazilian populations, with cytochrome P450-dependent monooxygenases playing a secondary role. The coexistence of different resistance mechanisms in the same insect populations suggests a polygenic basis for abamectin resistance (Siqueira et al. 2001).

1.5.8 Pyrethroid resistance

Pyrethroids were originally derived from the plant *Chrysanthemum cinerariaefolium*, and their primary mode of action is the inhibition of voltage-gated sodium channels (VGSCs) of the insect nervous system (Zlotkin 1999). Sodium channels generate the Na+ currents which initiate and propagate action potentials in nerve and muscle cells, this includes transmission of

sensory information through the nervous system to the brain and initiating contractions in muscle (de Lera Ruiz and Kraus 2015). Pyrethroids interfere with the normal function of the voltage-sensitive sodium channel by altering the gating kinetics of the channel (Soderlund 2010). Type I pyrethrins produce repetitive discharges or short sensory bursts whereas type II compounds produce stimulus dependant nerve depolarisation which results in long sensory bursts (Soderlund and Bloomquist 1989). Although type I and II pyrethroids produce different symptoms they interfere with sodium channel function via the same mechanism - the prolonged activation of the sodium current, resulting in hyperexcitation (Soderlund and Bloomquist 1989; Narahashi et al. 1992). Pyrethroids prolong the time sodium channels are open, thereby prolonging Na+ discharge across the membrane. Only a small population of sodium channels needs to be modified by pyrethroids to cause pathology (Narahashi et al. 1992).

Insect VGSCs are encoded by a single gene, *para*, first cloned from the fruit fly *Drosophila melanogaster* (Loughney, Kreber, and Ganetzky 1989). VGSCs are capable of expressing many splice variants, which while they share the same binding site for pyrethroids display variation in their sensitivity to these insecticides (Gilbert and Gill 2010). The VGSC is a large protein with four internally homologous domains (Noda et al. 1984) positioned around a central pore (Sato et al. 2001). Each domain contains 6 alpha-helical transmembrane segments (S1-S6) (Noda et al. 1984). The different α subunits contain the receptor sites for drugs and toxins that act on Na+ channels (de Lera Ruiz and Kraus 2015). Four of the six helical segments are in a transmembrane

orientation and function as voltage sensing domains, whilst two helical segments line the pore (Payandeh et al. 2011). The voltage dependant activation of the sodium channel depends on membrane electric field activation. This results in conformational change and selective outward movement of 'gating charges' in the form of Na+ ions. These move through the pore across the membrane electric field. Whilst the pore is undergoing depolarisation, permeability for Na+ rises rapidly and then decays as the channel converts to an inactivated state (Hodgkin and Huxley 1952).

Pyrethroids prolong the activation and deactivation of sodium channels shown by slowly-decaying sodium tail currents that flow following a depolarization–repolarization cycle (Toshio 1992; Toshio Narahashi 1996). A diverse array of pyrethroids stabilize multiple channel states, slowing the transitions between them (Soderlund et al. 2002). Many amino acid sequence polymorphisms across many species are associated with kdr. Most polymorphisms occur in distinct molecular environments of the sodium channel protein. This includes mutations in the S5 and S6 transmembrane segments of domain two surrounding the pore channel, mutations in the pore-forming region lying between the S5 and S6 segments, mutations in the short intracellular loops connecting the S4 and S5 transmembrane helices, and mutations occurring near the beginning or the end of the intracellular linkers that separate the homology domains (Soderlund and Knipple 2003). The most common mutation however in a range of insects is L1014F in the S6 segment of domain 2 (Davies et al. 2007). Resistance of *T. absoluta* to pyrethroids was observed in Brazil in 2000 (Siqueira, Guedes, and Picanço 2000). Since then

kdr-type mutations have been observed in populations across 12 countries from Argentina to Israel, and were shown to be fixed in populations from Europe and Brazil. A second mutation concurrent to kdr L1014F was also observed in most individuals, M918T or T929I (Haddi et al. 2012). M918T confers a skdr phenotype in *M. domestica* (Williamson et al. 1996), and when functionally expressed in *D. melanogaster* results in 100-fold reduction in sensitivity to deltamethrin (Vais et al. 2000). The T929I substitution is a likely binding residue for pyrethroids (Davies and Williamson 2009) The effect of T929I in combination with L1014F it was found to make the sodium channels expressed in *Xenopus laevis* oocytes ~300,000 times less sensitive to deltamethrin than the wild-type channel (Vais et al. 2003).

1.5.9 Spinosad resistance

Spinosad is an economically important biopesticide utilised for the control of *T. absoluta*. It is derived from a soil dwelling bacteria, *saccharopolyspora spinosa,* that was first isolated from sugarcane bagasse from the Virgin Islands in 1990 (Mertz and Yao 1990). In 1992 structurally unique, fermentation-derived tetracyclic macrolides were isolated (Kirst et al. 1992) from the bacteria. These metabolites were shown to have agricultural importance through their insecticidal properties (Salgado 1998) and were made commercially available as a pesticide in 1997. The insecticidal activity comes from a natural mixture of two metabolites, spinosyn A and spinosyn D which differ only by a methyl group (Kirst 2010). When spinosad was first introduced to the market it had a novel mode of action giving negative assay results to over 60 target-site assays and its effects were shown to cause

widespread excitation of neurons in the central nervous system of a cockroach. This resulted in an induced inward baseline current shift and prolonged nicotinic acetylcholine receptor response (Salgado, Watson, and Sheets 1997). Further more spinosyn A did not appear to interact with known imidacloprid nicotinic target sites or abamectin GABA target sites (Orr et al. 2009).

Spinosad targets nicotinic acetylcholine receptors (nAChR) involved in rapid neurotransmission (Tomizawa and Casida 2001). They have long been recognised as a target for insecticidal action (Gepner, Hall, and Sattelle 1978). The nAChR is made up from 5 subunits (Figure 1.2) (Arias 1997) that are arranged in a pentameric transmembrane complex, arranged around a central, cation-permeable channel (Tomizawa and Casida 2001). 10-12 subunit genes have been identified in a range of insect species (Rinkevich and Scott 2009) and further splicing of these genes increases subunit diversity with 18 unique transcripts from 1 subunit gene being identified in *Tribolium castaneum* (Rinkevich and Scott 2009). Subunits possess an N-terminal extracellular domain, four transmembrane regions (one of which lines the ion channel) and a large intracellular loop. The N-terminal domain consists of two cysteines separated by thirteen residues. The ACh-binding site is also located in the N-terminal extracellular domain and is formed by several distinct regions. Subunits that have the two adjacent cysteine residues in loop C are essential for ACh binding, and are referred to as alpha subunits (Sattelle et al., 2005).

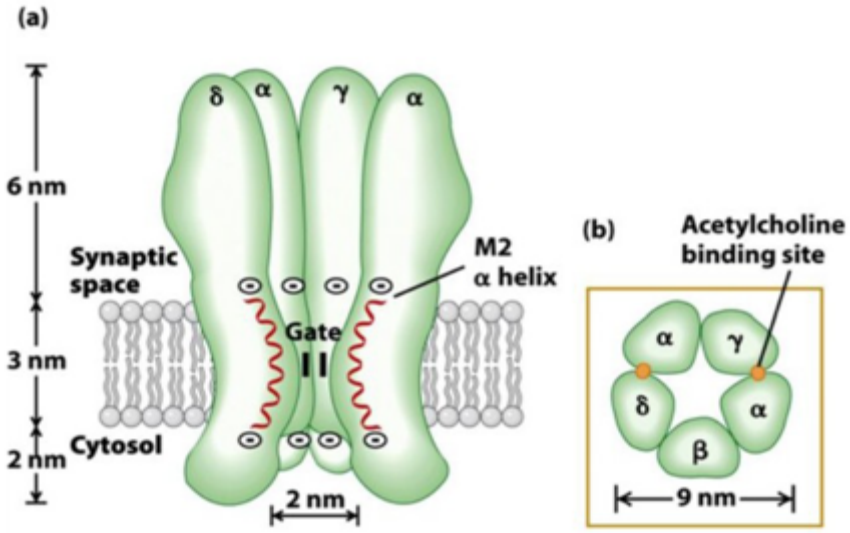


Figure 1.2. Structure of acetylcholine receptor showing (a) variation in subunit composition and (b) ACh binding site (Lebbe et al. 2014).

Each of the two alpha subunits have non-equivalent binding sites located at the extracellular region of the complex, these recognise the endogenous neurotransmitter ACh. Specific binding of agonists allows the movement of ions down their electrochemical gradient depolarising the membrane (Arias, 1997). This electrochemical transduction activates many responses in the cell; contraction of muscles, neurotransmitter release, glucose use and transference of frequency encoded information for memory and learning processes (Arias 1997). Competitive antagonists that compete with ACh for binding sites can bring about loss of function of the receptor. The binding site for spinosad is likely the α6 subunit. A loss of function mutation in an nAChR α6 subunit gene in *D melanogaster* resulted in 1181 fold resistance (Perry, McKenzie, and Batterham 2007). Mutations in *P. xylostella* α6 resulted in a stop codon and non-functional α6 subunit was found in strains with 18000-fold resistance to spinosad (Rinkevich and Scott, 2009), Alternative splicing was

also attributed to production of non-functional α6 subunits resulting in resistance (Baxter et al. 2010a).

Spinosad is highly effective against Lepidoptera as well as some Diptera, Coleopteran, Orthoptera, Thysanoptera and Hymenoptera (Sparks et al. 1995; Bret et al. 1997). Novel modes of action are invaluable in IPM as they replace long serving pesticides that through evolution of resistance have become redundant. They also relieve selection pressures on remaining efficacious pesticides through alternating between different modes of action. Spinosyns appeared to have no crossover target-site or metabolic resistance from use of other compounds (Sparks et al. 1995) and a review of cross-resistance to a broad range of pesticides in a variety of insect species show this to still be the case with few exceptions (Sparks et al. 2012). Additional advantages to spinosad are that it has bio pesticide status enabling its use in organic farming (*Commission Regulation (EC) No 889/2008* 2008). Spinosad also has a minimal environmental impact when compared to synthetic compounds. Half-life in water due to photolysis is 1-2 days, which is the primary cause of degradation. Spinosad also undergoes biotransformation and will partition its self from water to organic matter (Cleveland et al. 2002). In the field spinosad degrades quickly with little residual toxicity at 3-7 days post-application (Williams, Valle, and Viñuela 2003). Although spinosad can be classed as highly toxic to some beneficial insects such as bees (Rabea, Nasr, and Badawy 2010) once the application to the plant has dried the effects were negligible (Mayer et al. 2001). Effects on insect natural enemies showed that in 71% of laboratory studies and 79% of field studies spinosad

was non harmful and had insignificant sub-lethal effects (Williams, Valle, and Viñuela 2003) . Hymenopteran parasitoids were significantly more susceptible to spinosad than predatory insects with 78% of laboratory studies and 86% of field-type studies returning a moderately harmful or harmful result and showed sub-lethal effects including loss of reproductive capacity and reduced longevity (Williams, Valle, and Viñuela 2003).

Resistance studies of spinosad on different insect species showed increasing levels of tolerance and range of mechanisms involved. The house fly, *Musca domestica,* showed that the P450 inhibitor piperonyl butoxide increased toxicity of spinosad (Scott 1998) and there was variation in susceptibility between populations demonstrating potential evolution of metabolic resistance. In North Carolina, Laboratory strains of the tobacco budworm, *Heliothis virescens,* were selected with spinosad over many generations producing 245-fold increase in resistance (Young et al. 2000). Field populations of the beet armyworm, *S. exigua,* from southern U.S.A. and South East Asia showed a 3.0 to 70-fold increase in tolerance to spinosad (Moulton, Pepper, and Dennehy 1999). After laboratory selection of a Chinese population of beet armyworm, resistance increased 345 times. Addition of synergist PBO to spinosad application supressed resistance and had a synergistic ratio of 9.8, implicating monooxygenases in it's detoxification (Wang et al. 2006).

Resistance evolved in Hawaiian populations of the diamondback moth, *Plutella xylostella,* after ~2.5 years of field application. Populations collected

from Pearl City showed 991-fold increase in tolerance to spinosad compared to a susceptible strain. Further laboratory selection increased the tolerance to 13,100-fold. Synergists had no significant effect on toxicity suggesting no contribution of metabolism to resistance and genetic studies showed the resistance mechanism to be autosomally inherited and incompletely recessive (Zhao et al. 2002). Characterisation of the mechanism of resistance from selected Pearl City strain was characterised, a point mutation in the nAChR *Pxa6* gene. This was predicted to produce a truncated subunit through the introduction of a premature stop codon was discovered in spinosad resistant individuals (Baxter et al. 2010).

By 2010 low levels of resistance (<5-fold) were being found in South American populations of *T. absoluta* (Silva et al. 2011). In a study of Chilean populations of *T. absoluta*, four out of five field populations displayed significantly lower susceptibility to spinosad with all populations showing a significant increased expression of mixed function oxidases suggesting metabolic component to the resistance (Reyes et al. 2012). In 2014 resistance in Brazilian populations showed variation in tolerance with resistance ratios ranging from 8.9 to 93.8 times that of susceptible strains (Campos et al. 2015). Resistance was on the rise, driven by excessive pesticide use. Although some mechanisms of resistance to spinosad are unstable (Campos et al. 2014) excessive use of pesticides in South America (4 - 6 applications per week) (Guedes and Picanço 2012) had the potential to drive any resistant genotypes to fixation. Characterisation of target-site resistance to spinosad was identified in South American populations that were

cloned for their nAChR α6. A point mutation resulting in G275E was present in a field population showing a resistance ratio of 284. This population was further selected showing RR of 48900 (Silva et al. 2016). This mutation was functionally characterised by the induction of a missence mutation resulting in G275E alteration of the nAChR α6 subunit of *D. melanogaster* using CRISPR/Cas9. The resulting altered subunit induced a 66-fold reduction in sensitivity to spinosad (Zimmer et al. 2016).

Concern for UK growers increased as resistance started to appear across Greece Portugal and Spain. Portuguese populations were analysed for mode of resistance. Populations with a ~8-fold resistance were selected resulting in 277-fold resistance. Analysis a6 nAChR transcripts showed a 45 bp deletion corresponding to the exon 3 position in the nAChR (Berger et al. 2016). This process of exon skipping excises an important ACh binding region from the protein (Grutter and Changeux 2001). The functional result of exon 3 skipping was examined by excising exon 3 from human nAChR α7 and expressing the modified RNA in *Xenopus laevis* oocytes. The modified receptors were unable to discharge current across the membrane following ACh application. Furthermore expression of the homozygous genotype significantly correlated with the resistant phenotype (Berger et al. 2016).

1.5.10 Diamide resistance

In the continuous race to stay ahead of resistance and maintain control on pest species diamides were introduced to the market, these were a new class of chemicals with a new mode of action. Flubendiamide, a substituted phthalic

acid diamide, was introduced in 1997, having an excellent profile against Lepidoptera. Further development of this new chemistry led to the anthranilic diamide, chlorantraniliprole (Jeanguenat 2013). By 2013 global turnover of this pesticide class was $1.2 billion (Sparks and Nauen 2015).

Diamides have a novel mode of action, targeting the ryanodine receptor (RyR) involved in Ca^{2+} signalling. Ca^{2+} regulates many processes in eukaryotic cells including gene regulation, membrane transport processes, hormone biosynthesis, muscle contraction, neurotransmitter release and apoptosis (Lümmen 2013). There are two main families of calcium release channels, the inositol triphosphate receptor and the RyR, each having different isoforms. RyRs are homotetrameric proteins that surround a large release channel that regulates Ca^{2+} from intra cellular stores of the endo/sarcoplasmic reticulum. They respond to surface membrane action potentials and/or change in concentration of secondary messengers and are linked to muscle excitation-contraction coupling (E-C coupling) (Fill and Copello 2002). E-C coupling requires the presence of extracellular Ca^{2+}. In mammalian cardiac muscle the dihydropyridine receptor (DHPR), an L-type Ca^{2+} channel, senses a membrane potential from transverse tubule membranes (Feher 2017) and induces a small Ca^{2+} influx that activates the RyR channel (Sham, Cleemann, and Morad 1995). A similar process governs the RyR channels in some invertebrate skeletal muscles (Fill and Copello 2002). Mammal express three isoforms of RyR which are approximately 5000 amino acids long sharing about 66% sequence identity (Otsu et al. 1990). Insects express one RyR gene and sequencing of the *D. melanogaster* RyR revealed a gene with 26

exons and a predicted protein of 5216 amino acids that has 45%-47% identity to mammalian RyRs (Takeshima et al. 1994). The hydropathicity profile of the insect RyR was also similar to the mammalian RyR, consisting of four transmembrane segments at the carboxyl terminal channel region and a largely hydrophilic cytoplasmic region (Takeshima et al. 1994). *D. melanogaster* RyRs are expressed in embryonic development as well as adult muscle and nervous tissue (Hasan and Rosbash 1992).

The RyR is named after a natural alkaloid ryanodine (Ry), which is a natural pesticide and was important in RyRs characterisation (Cannell, Vaughan-Jones, and Lederer 1985). Ry inhibits both mammalian and insect RyR by locking channels in a sub-conductance state (Fill and Copello 2002). The binding of pesticides Ry, flubendiamide and chlorantraniliprole all occur at distinct sites on the receptor (Isaacs et al. 2012) and most likely occur between residue R4475 and the carboxyl terminus (Figure 1.3) (Callaway et al. 1994). Binding site studies in *Bombyx mori* agreed with this, the flubendiamide binding site was suggested to be at the C terminal end between residues 4111-5084 (Kato et al. 2009). Phthalic diamides bind with high affinity to RyR, increasing their sensitivity to Ca^{2+} at low calcium concentrations. This response is not observed in mammalian RyR indicating that the phthalic diamides binding site was specific to insect RyRs (Ebbinghaus-Kintscher et al. 2006). Both mechanisms have effectively the same result, depleting internal Ca^{2+} stores interfering with muscle contraction and ultimately resulting in paralysis (Cordova et al. 2006). Development of anthranilic diamides from phthalic diamides resulted in compounds with

excellent lepidopteran specificity (Lahm et al. 2005). This included chlorantraniliprole which showed differential selectivity for insect ryanodine receptors over mammalian as well as greater affinity within insects for lepidopteran, coleopteran, and some dipteran pests (Bentley, Fletcher, and Woodward 2010). This has the added benefit of making it a viable pesticide in IPM strategies that included Hemipteran natural enemies. Mortality of *M. pygmaeus* (a predator of *T. absoluta*) caused by chlorantraniliprole did not differ significantly from control mortality and it had no significant effect on predation rate (Martinou, Seraphides, and Stavrinides 2014). Similar results were observed for other predatory bugs with chlorantraniliprole causing low mortalities in *M. basicornis* (Passos et al. 2018).

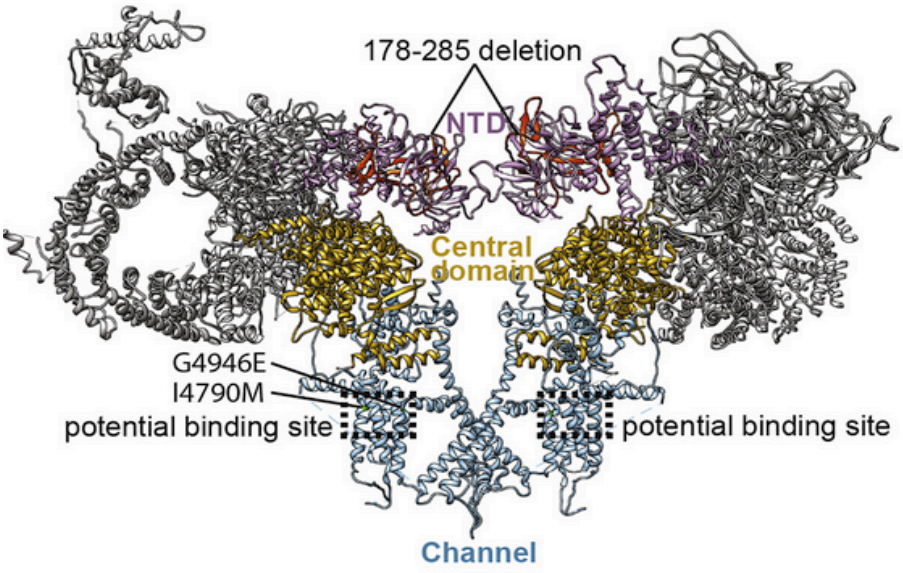


Figure 1.3. A) Ryanodine receptor showing calcium gating channel, potential diamide binding sites, and previously described resistance associated mutations (Lin et al. 2018).

In spite of chlorantraniliproles' effective lepidopteran profile resistance has recently evolved in the field. 2008-2009 Chinese populations of *P. xylostella* displayed a 1.8 - 8.9-fold increase to chlorantraniliprole with synergism assays suggesting metabolic resistance (Xingliang Wang et al. 2010). In 2010-2011 South Chinese populations were suggested to have developed target-site resistance resulting in 2000-fold resistance (Xingliang Wang and Wu 2012). High resistance has also seen in Brazilian population showing 27793-fold resistance (Ribeiro et al. 2014). Cloning of the *P. xylostella* RyR reviled a 15495 bp open reading frame coding a protein of 5164 amino acid residues. A mutation resulting in the amino acid substitution G4946E was presumed to confer resistance in Thailand, Philippine and Chinese populations (Troczka et al. 2012; Guo, Liang, et al. 2014a). Further mutations in the ryanodine receptor gene have since been identified resulting in E1338D, Q4594L and I4790M substitutions. These correlated with resistance under diamide selection. Mutation frequencies increased from 34.3% to 90.9% for E1338D, and, 34.3% to 87.9% for Q4594 and I4790. Furthermore these mutations were present in 100% of the population with 2128-fold resistance and none were present in a population with a resistance ratio of 1 (Guo, Liang, et al. 2014a). Resistance to diamides in *T. absoluta* has also been reported in Italian and Brazilian populations (Roditakis et al. 2015; J. E. Silva et al. 2016). Identification of target-site mutations at sites G4903 and I4746 were implicated in reduced target-site binding of chlorantraniliprole (Roditakis, Steinbach, et al. 2017). CRISPR/Cas9 editing in *S. exigua* gave functional validation to these resistance associated mutations confirming their causal effect by increasing resistance 223-fold (Zuo et al. 2017). These results are

worrying for UK growers. Although no resistance to diamides has been reported in UK populations', importation of resistance alleles is a real threat to the efficacy of chlorantraniliprole and its role in IPM.

1.6 Current IPM

Pesticides are an invaluable part of any IPM strategy and the loss of compounds due to resistance can increase the selection for resistance to any remaining pesticides that are approved for pest control. Due to the loss of efficacy of many pesticide classes on *T. absoluta* initial control measures were ineffective and economic damage was devastating with growers losing up to £50K per hectare. At the start of this research (2017) two pesticides with complimentary modes of action were being introduced into *T. absoluta* IPM, spinosyns (discussed in chapter 1) and diamides (discussed in chapter 2). Both these compounds effectively supressed *T. absoluta* populations and have excellent environmental profiles. The IPM strategy incorporating these compounds involved monitoring of pest levels early in the growing season, primarily by the use of delta traps, in which a pheromone attractant lures males to sticky paper. By distributing these throughout the crop, they can provide information on the prevalence and distribution of the pest throughout the crop. Light traps are also useful for monitoring *T. absoluta*; working in a similar manner to pheromone traps except the attractant UV light lures both males and females. The next step in the IPM is the introduction of the biocontrol agent, *Macrolophus pygmaeus. M. pygmaeus* feeds on eggs and larvae through the leaf but is slow to establish and may take up to 4 months to

reach an effective population size. During this time the growth of *T. absoluta* populations must be slowed down. This is done by systemic application of spinosad via the irrigation, just after mines are visible, about 4-5 weeks into crop development. Other less effective ways to slow down the pest are the use of light/sticky traps for adult moths and sticky floor treatments to capture late instar larvae that typically repel to the floor to pupate. If the biocontrol was ineffective at eradicating *T. absoluta* post spinosad, a second round of pesticide application was required. This time chlorantraniliprole was applied through a foliar spray and having a different mode of action to spinosad it would reduce target-site selection pressures. At the end of the growing season any remaining reservoirs of the pest were eradicated using the insecticide indoxacarb to delay early establishment of the pest in the next growing season. Indoxacarb was selected is it was shown to be effective against *T. absoluta* (Roditakis et al. 2013; Berxolli and Shahini 2018) and has a different mode of action to spinosad and chlorantraniliprole - aiding resistance management. However, in contrast to spinosad and chlorantraniliprole it has negative effects on both pollinators and biocontrol agents, so can only be used at the end of the season (Jacobson and Howlett 2014). This IPM approach has had much success in limiting the pest status of *T. absoluta,* however in spite of the resistance management strategies included in the IPM protocols, resistance to spinosad and chlorantraniliprole has been confirmed in Europe and control failure has been reported in Glasshouses in the UK (R. Jacobson Personal communication).

1.7 Objectives

At the start of this thesis there were no confirmed cases of resistance in *T. absoluta* in the UK to control measures used in the current IPM. However, tomato growers have reported to horticultural consultants (Rob Jacobson Consultancy) that these measures were having reduced impact on the control of *T. absoluta.* The aim of this thesis is to investigate the following key questions:

1. Is resistance to spinosad and chlorantraniliprole present in UK populations of *T. absoluta*? Levels of spinosad resistance will be tested in chapter 3 and chlorantraniliprole resistance in chapter 4.
2. What are the underlying mechanisms of resistance in these strains? In chapter 2 I will investigate the target site of spinosad, the nicotinic acetylcholine receptor, for structural alterations and associate them with resistance.
3. Can *T. absoluta* rapidly evolve resistance under selection, and does this result from *de novo* mutation or the selection of standing genetic variation? In chapter 3 I will select populations of *T. absoluta* with chlorantraniliprole. I will analyse the target site of chlorantraniliprole, the Ryanodine receptor, to characterise the molecular basis of resistance in this compound.
4. Does the genetic background of *T. absoluta* possess the capacity to detoxify pesticides through pre-existing metabolic detoxifications genes, or, are these modified/differentially expressed to provide

protection from pesticides? In chapter 5 I will analyse gene expression in strains of *T. absoluta* that can tolerate high concentrations of chlorantraniliprole to assess their contribution to resistance. I will also look at the diversity and phylogeny of genes in these families to generate a resource to facilitate future resistance analysis.

5. Finally, can *T. absoluta* adapt in the face of mating disruption and evolve strategies to mitigate the effects of this novel control strategy? Chapter 5 will assess the capacity for parthenogenesis in *T. absoluta* and look for evidence that this can be selected for to alleviate population suppression by this control measure.

2. General methods

2.1 Tuta absoluta

T. absoluta were collected from glasshouses across the UK as well as acquired from Syngenta (Switzerland) and Rothamsted research centre (Harpenden) (Table 2.1)

Table 2.1 Insect strains and locations

Strain	Supplier	Location	Resistance status
TA1	Rothamsted	Spain	Suceptable
EVH	R & L Holt	Evesham, UK	Spinosad resistance
EVH2016	R & L Holt	Evesham, UK	Spinosad resistant
EVH2019sel	R & L Holt	Evesham, UK	Diamide resistant
EVH2019	R & L Holt	Evesham, UK	Suspected parthenogenesis
NY2016	Jan Bezemer & Sons	Stokesley, UK	Spinosad reistant
LAN2016	Flavour Fresh Salads Ltd	Lancashire, UK	Spinosad resistant
IOW2016	Wight Salads ltd.	Isle of Wight, UK	Suceptable
Sus	Pablo Bielza	Spain	Diamide resistant
Mur	Pablo Bielza	Spain	Diamide resistant
Ssus	Syngenta	Spain	Diamide resistant
Sres	Syngenta	Spain	Diamide resistant

2.2 Insect cultures

All insect stages were housed in bug dorm cages (MegaView science co.) in CE rooms at 24°C 16L:8D and 65% humidity. Cultures were supplied with tomato plants (var. Money Maker) *ad libitum*. Once adults had eclosed >50 were collected with an electric pooter and transferred to a new cage with a fresh tomato plant.

2.3 Leaf dip bioassays.

Leaf dip assays were conducted according to test method 22 guidelines produced by the Insecticide Resistance Action Committee (Roditakis et al. 2013) which subjects pooled replicates of larvae 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 LC50 of the susceptible strain.

2.4 RNA extraction

T. absoluta larvae (L2-L4) were collected from culture as soon as populations had established (1-2 generations), snap frozen in liquid nitrogen and stored at -80°C prior to RNA extractions. RNA extractions were carried out on pools of 10 individuals using ISOLATE II RNA Mini Kit (Bioline, London, UK). Micro pestles were used to homogenise samples in 1.5ml Eppendorf tubes while partially submerged in liquid nitrogen. 350 µl of lysis buffer was added and vortexed. The lysate was filtered. 350 µl of ethanol was added to the filtrate

and homogenized. The filtrate was the added to an RNA binding column and centrifuged at 11000 g. The bound RNA was then desalted using 350 of MEM buffer. 95 µl of 1:10 DNase I was added to the RNA bound membrane and incubated at room temperature for 15 min. three wash steps were performed before RNA was eluted in 60 µl of RNase-free water. Concentration and purity of RNA was assessed using a spectrophotometer (NanoDrop®, USA) Integrity of the RNA was assessed mixing RNA with 1x loading buffer, heating for 5 minutes at 65 °C to denature running on 1.5% agarose gel. Any RNA not directly used for RNA synthesis was stored at -80°C.

2.5 cDNA synthesis

All cDNA for PCR reactions was synthesized using Maxima H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, US) according to the manufacturer's protocol. 4µg of total RNA was added to 1 µl of 100 pmol oligo (dT) primers, 1 µl of 100 pmol random hexamer primers, 1 µl of 10mM dNTP mix. Nuclease free water was added to bring the volume to 15 µl. 4 µl of 5x RT buffer and 1 µl of Maxima H Minus Enzyme Mix was added to bring the total reaction volume up to 20 µl. The solution was incubated at 25°C for 10 min then 50°C for 30 min. The reaction was terminated by heating to 85 °C for 5 minutes. Complimentary DNA generated for RT-qPCR used the same protocol except with 0.25 µl of random hexamer primers and 0.25 µl of oligo (dT) primers. The cDNA was stored at - 20°C

2.6 Primer design

All primers were designed in Geneious v10.2.6 (Biomatters Ltd.) and synthesized by Sigma-Aldrich. Primers were 18-25 nucleotides long with Tm of close to 58°C and GC content between 40-60%. All primers were diluted in nuclease free water to a concentration of 10μM prior to inclusion in PCR.

2.7 Standard PCR protocol

1μl of cDNA was added 12.5 μl of DreamTaq Green PCR master mix (Thermo Fisher Scientific), 1 μl of forward primer, 1 μl of reverse primer and made to a total reaction volume of 25 μl with nuclease free water. Temperature cycling conditions comprised 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. Negative controls were run to check for contamination. For amplification of gDNA temperature cycling was carried out for 40 cycles.

2.8 PCR purification

PCR products were purified using the GeneJET PCR purification kit (Thermo Scientific). A 1:1 ratio of Binding buffer was added to the PCR product. Where products were <500 bp a 1:2 volume of 100% isopropanol was added. The solution was then added to a purification column and centrifuged for 30s. 700 μl of wash buffer was the added to the column and centrifuged for 30s. The purification column was then centrifuged for 1 min to completely dry it. The DNA was then eluted in 50 μl of elution buffer. DNA was quantified by spectrophotometry (NanoDrop, Thermo-Fisher).

2.9 Sequencing

Sequencing was conducted by Eurofins Genomics. 2 µl of either forward or reverse primer was added 15 µl of purified PCR product diluted to 5 ng/µl.

2.10 qPCR

QPCR was used to test expression of *T. absoluta* UGT in transgenic *D. melanogaster.* Primers were designed to be 18-23 bp long, have a Tm of 60 °C, and a GC content of 40-60%. 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, 1.5 µl of diluted cDNA (10 ng μl^{-1}), and 9 µl of nuclease free water. 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. A final melt-curve step was included increasing temperature from 72 °C to 95 °C by 1 °C every 5 s to confirm the absence of non-specific amplification. Primer efficiency was assessed using a serial dilution from 100 ng to 0.01 ng of cDNA. Four biological replicates were used in each qPCR each with two technical replicates. Positive control reactions used primers that target housekeeping genes and negative controls were run to assess for contamination. Analysis was conducted according to the ΔΔCT method (Pfaffl 2001) and normalised to previously published housekeeping genes (Vandesompele et al. 2002) (Table 2.2).

Table 2.2 Primers for *D. melanogaster* housekeeping genes

Housekeeping primer	5'-3' sequence
SDHAF	CACGACCCTCCATGATCTCG
SDHAR	CGGATGTCTCATCACCGAGG
RPL32F	GCGCTTGTTCGATCCGTAAC
RPL32R	GCCCAAGGGTATCGACAACA

2.11 E.Z.N.A insect DNA extraction (Omega Bio-tek)

Individual larvae were homogenized in liquid nitrogen using a micro-pestle. 350 µL CTL Buffer and 25 µl Proteinase K Solution was added and then vortexed. This was incubated for ~ 30 min at 60°C until the sample was solubilized. 350 µl chloroform:isoamyl alcohol (24:1) was added and vortexed. The solution was the vortexed at 10000g. The clear supernatant was the transferred to a clean 1.5 ml microcentrifuge tube thoroughly. Repeat Steps 5-6 above. BL Buffer was then added at the same volume as the extracted supernatant along with 2 µl RNase A. This was vortexed at maximum speed for 15 seconds and then incubated at 70°C for 10 minutes. An equal volume of 100% ethanol was added and vortexed at maximum speed for 15 seconds. The solution was then transferred to a HiBind® DNA Mini Column and was centrifuged for 1 min at maximum speed. This was repeated until all the solution was passed through the column. The column was transferred to a new collection tube and had 500 µl of HBC added. This was then centrifuged for 30 s and the filtrate discarded. The column was then washed with 700 µl of DNA wash buffer centrifuged for 1 min at max speed. This wash step was repeated the collection tube emptied and centrifuged for a further 2 min to fully dry the column. Elution buffer was heated to 70°C and 50 µl was added

to the column and left to sit for 5 min. the column was centrifuged at max speed for 1 min and DNA was stored at -20 °C

2.12 DNAzol extraction (Thermo Fisher)

Individual Larvae were added to 350 µl of DNAzol reagent and homogenized with a micro-pestle and left for 10 min. Samples were then centrifuged for 10 min at 10000g and the supernatant extracted. 350 µL of 100% ethanol was added to precipitate the DNA. Tube was inverted several times until all the liquid became cloudy. Collect the DNA using a micro pipette tip and position it on the side of the tube then pour out the ethanol and rest for 1 minute. Wash the DNA twice with 75% ethanol. Each time add 0.8 ml of ethanol, invert the tube, allow the DNA to settle at the bottom then pipette off the ethanol. After the second wash step air-dry the DNA for about 15 s and the dissolve in 200 µl of 0.8 mM µL NaOH.

2.13 QIAGEN Genomic-tip DNA extraction

Pooled samples of 10 individuals were fully homogenized on liquid nitrogen using a micro pestle and transferred to a screw cap tube. 4 µl of RNase was added to a 2 ml aliquot of G2 buffer. This was added to the screw cap tube with 0.1 ml of proteinase K stock solution and vortexed. This was then incubated at 50°C for 2 h. this was centrifuged at 5000g for 10 minutes at 4°C and the supernatant transferred to a new tube. The Genomic tip was equilibrated with 2 ml of buffer QBT and allowed to empty by gravity. The sample was vortexed for 10s at max speed and applied to the equilibrated genomic tip. The QC buffer was warmed to 50°C then used to wash the G-tip

(3 time with 1 ml of buffer). Buffer QF was warmed to 50°C. Two applications of 1 ml QK buffer were used to elute the DNA into a 2 ml tube. The DNA was then precipitated by adding 1.4 µl of room temperature isopropanol. This was mixed and centrifuged at 5000g for least 15 min at 4°C. The supernatant was then removed and the pellet was washed with 1ml of cold 70% ethanol then vortexed briefly and centrifuged at > 5000g for 10 min at 4°C. The supernatant was removed and the pellet was air dried for 10-15 minutes before being suspended in 1:1 TE:deionised water.

2.14 QIAprep Spin Miniprep Kit

5 ml of bacterial culture was pelleted by centrifugation at 7000g for 3 min at room temperature. The pellet was resuspended in 250 µl of P1 buffer and transferred to a microcentrifuge tube. 250 µl of P2 buffer was added and mixed thoroughly by inverting the tube 4–6 times until the solution became clear. 350 µl of N3 buffer was added and mixed by inverting the tube 4–6 times. The solution was then centrifuged for 10 min at 17900g. 800 µl of the supernatant was added to the spin column. This was centrifuged for 60 s and the flow-through was discarded. The spin column was washed with 0.5 ml of PB buffer. This was centrifuged for 60s and the flow-through was discarded. Wash the spin column again with 0.75 ml of PE buffer and centrifuge for 60 s discarding the flow-through. Centrifuge for 1 min to remove residual wash buffer and place the spin column to a new collection tube and add 50 µl of nuclease free water to elute.

2.15 Gel electrophoresis

0.5g of agar was dissolved in 50 ml of boiling TAE and allowed to cool to handling temperature. 2.5 µl of redsafe was added and mixed through. Suitable tray with a comb in was clamped and the solution was poured in. This was allowed to set for 30 min. The tray was then placed in an electrophoresis tank and a molecular ladder loaded into the first lane. Roughly 20ng of DNA was loaded in to relevant lanes and run at suitable voltage and for a suitable time depending on amplicon length.

3. Target-site resistance to spinosad in UK populations of *T. absoluta*

The results detailed in this chapter have been published in *Pest management science* (Appendix 1)

Grant, Charles, Rob Jacobson, Aris Ilias, Madeleine Berger, Emmanouil Vasakis, Pablo Bielza, Christoph T. Zimmer, Martin S. Williamson, Richard H. ffrench-Constant, and John Vontas. 2019. “The Evolution of Multiple-Insecticide Resistance in UK Populations of Tomato Leafminer, Tuta Absoluta.” *Pest Management Science* 75 (8): 2079–85.

3.1 Introduction

Spinosad is comprised of the secondary metabolites spinosyn A and D produced by the soil bacteria *Saccharopolyspora spinosa* (Salgado, 1998). Spinosad inhibits the function of the insect nervous system by targeting the α6 subunit of the nicotinic acetylcholine receptor (nAChR), a neurotransmitter-gated ion channel that plays a critical role in nerve signalling at the postsynaptic membrane. High levels of spinosad resistance have been reported in field populations of *T. absoluta* in Brazil (Campos et al. 2015). A G275E amino acid substitution in the α6 subunit was identified in resistant selected strains and resistant field populations (Silva et al., 2016). This mutation was confirmed as being the causal factor for the resistant phenotype through CRISPR/Cas9 gene editing (Zimmer et al. 2016). Another mechanism of α6 alteration in *T. absoluta* is exon skipping (Berger et al. 2016) with spinosad-selected lines derived from a field strain from Portugal expressing mRNA transcripts lacking the normally mutually exclusive exon 3a or 3b. This

is predicted to result in a truncated α6 subunit lacking a key ACh binding loop. The mutations leading to the aberrant splicing remain uncertain, however, methylation may have played a role (Berger et al. 2016). The methylation ratio of the selected strain at a CpG 21bp downstream of exon 3a were 30% compared to 0% and 0.5% in the unselected strains. This position matched sequences thought to be targets of splice binding proteins. Another potential co-factor, however, is the fact that transcripts encoding putative splice factor proteins were significantly down regulated (Berger et al., 2016).

Following these studies some UK growers reported a loss of efficacy to spinosad against *T. absoluta* however, this had not been experimentally confirmed (R. Jacobson personal communication). The resistance mechanisms described above have not yet been described in UK populations of *T. absoluta*. However, the rapid invasion of this species into the UK from Europe suggests that European populations carrying resistance alleles may be readily imported into the UK, especially as *T. absoluta* is no longer a notifiable (quarantine) pest in the UK. Alternatively, the use of spinosad in the UK for the control of *T. absoluta* might lead to the *de novo* emergence of resistance. In this chapter I explored these possibilities by carrying out phenotypic and molecular characterisation of resistance to spinosad in several UK populations of *T. absoluta.*

3.2 Methods

3.2.1 Insects

Following reports of control failure using spinosad, *T. absoluta* were collected in 2015 from four commercial tomato glasshouse sites across the UK. ~200 larvae were collected from; R & L Holts, Evesham (EVH), Bezemer and Sons, North Yorkshire (NY), Flavour Fresh Salads ltd. Lancashire (LAN) and Wight Salads ltd. Isle of Wight (IOW). A second strain was collected from Evesham in 2016 (EVH2016) after spinosad had not been used for a growing season and *T. absoluta* had become re-established in the crop. To compare levels of resistance a spinosad 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).

3.2.2 Leaf dip assays

Leaf dip assays were conducted according to test method 22 guidelines produced by the Insecticide Resistance Action Committee (see methods section 2.3). 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} from the target population by the LC_{50} of TA1.

3.2.3 Nicotinic acetylcholine receptor α6 subunit amplification (nAChR)

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) (see methods 2.4 and 2.5). From this cDNA a 1585 bp region of the nAChR *Taα6* sub unit was amplified using primers listed in table 3.1 designed in Geneious (v8.1.9, Biomatters Ltd) based on the previously published *Taα6* (Berger et al. 2016b). 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 (Figure1).

Table 3.1 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

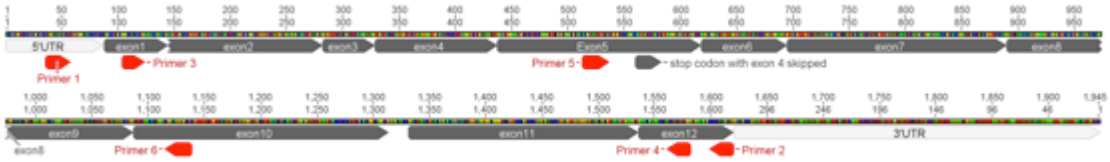


Figure 3.1 Position of primers (Table 1) on nAChR α6 sub unit

3.2.4 Genomic amplification of nAChR α6 subunit exon 4 region

Genomic DNA was extracted from EVH2016 survivors of the leaf dip bioassay using E.N.Z.A insect DNA extraction kit (Omega Bio-Tek Inc., Norcross, Georgia, USA) according to the manufacturer's protocol (see methods 2.11). Extractions were made on 10 individuals. Genomic DNA was amplified using standard PCR protocol (see methods 2.7) and primers listed in table 3.2. PCR products were purified using GeneJET PCR purification kit (Thermo Scientific) (general methods 2.8) according to the manufacturers protocol and submitted for Sanger sequencing in accordance with Eurofins Genomics guidelines (see methods 2.8 and 2.9)

Table 3.2. Primers for the amplification of genomic DNA region of *nAChR α6* subunit spanning exon 4.

Primer	Sequence 5'-3'
NAChR.E4.F	ATACGCCTATATCATTTCGGACG
NAChR.E4.R	AGTACGTCTGGCTTCCACAG

3.2.5 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 spinosad 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 spinosad. 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 (see methods 2.12). 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 3.3. 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 3.3. 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. 3 Results

3.3.1 Sensitivity of T. absoluta to spinosad

The sensitivity of UK strains of *T. absoluta* to spinosad was compared to a susceptible strain (TA1) from Spain by leaf dip bioassays (see appendix 2). Whilst Chi-sq. values were unable to be generated for TA1, EVH2016, NY2016 due to excessive 0's in the data, differences in the LC_{50} values obtained was observed between four of the UK strains (EVH, EVH2016, LAN2016 and NY2016) and TA1 (Table 3.4). IOW showed low level resistance with overlap in 95% confidence limits compared to TA1. The LAN strain showed moderate levels of resistance (28.7-fold) with no overlap in 95% confidence limits. The most potent resistance was observed in the EVH (165-fold) EVH2016 (>96-fold) and NY2016 (> 480.8-fold) strains. Confidence limits could not be generated to the EVH2016 and NY2016 due to their high levels of survival when exposed to spinosad.

Table 3.4. 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	Supplier	Origin	LC 50 (ppm)	Chi-square	95% CLs	RR
TA1	Rothamsted	Italy	5.2	N/A	3.1 - 7.3	1.0
EVH	R & L Holt	Evesham	860	0.03	484-2114	165.4
EVH2016	R & L Holt	Evesham	>500	N/A	N/A	>96
NY2016	Bezemer and sons	North Yorkshire	>2500	N/A	N/A	> 480.8
LAN2016	Flavour Fresh Salads Ltd.	Lancashire	149	0.13	31-5385	28.7
IOW2016	Wight Salads ltd.	Isle of Wight	8.6	0.14	5.3	1.7

3.3.2 Assessment of Taα6 transcripts 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 bp that precisely corresponds to the position and size of exon 4. This deletion results in a frameshift leading to the introduction of a premature termination codon (PTC) in exon 5 (Figure 3.2). Amplification and sequencing of the Taα6 gene from genomic DNA of the NY and EVH strains revealed that exon 4 was present and unaltered in both strains confirming that the loss of this exon in mRNA results from exon skipping rather than a deletion of the exon in genomic DNA. While variation was seen in the intronic genomic sequence up and downstream of exon 4 no variation was observed in conserved *cis*-elements at flanking exon-intron junctions such as the 5′ or 3′ splice sites which might explain the observed exon skipping.

The second alteration, observed only in LAN strain, was an in-frame triplet deletion resulting in the loss of a phenylalanine amino acid at position 238 (Figure 3.2). The F238del mutation occurs in the first α-helical transmembrane domain (TM1) of the *Taα6* subunit. A comparison of insect nAChR α6 sequences from a range of insect species (Figure 3.2) demonstrate that this residue is highly conserved across the Arthropoda. 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 spinosad resistance the LAN strain was treated with a discriminating dose of spinosad and alive, dead and affected larvae genotyped. Of the 200 individuals exposed to 150 mg/L of spinosad 108 died, 30 were affected (alive but were unable to respond in a coordinated manor to stimuli), 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 (Fig. 3.3). 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 spinosad exposure (χ^2 (4) = 78.50, P<0.001). Post-hoc analysis (Table 3.5) 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 3.4).

Table 3.5. Results of Chi-square post-hoc analysis of genotype frequency at F238 in response to spinosad exposure.

			Genotype			
			F238del/ F238del	F238/ F238del	F238/ F238	Total
Phenotype	Affected	Count	9	17	2	28
		Expected Count	9	14.1	5	28
		% within Phenotype	32.10%	60.70%	7.10%	100.00%
		Adjusted Residual	0.01768	1.20421	-1.59863	
		p value	0.98589	0.22851	0.1099	
	Dead	Count	8	61	28	97
		Expected Count	31	48.8	17.2	97
		% within Phenotype	8.20%	62.90%	28.90%	100.00%
		Adjusted Residual	-7.51171	3.71788	4.30898	
		p value	0	0.0002	0.00002	
	Survived	Count	39	10	1	50
		Expected Count	16	25.1	8.9	50
		% within Phenotype	78.00%	20.00%	2.00%	100.00%
		Adjusted Residual	8.25045	-5.06785	-3.44365	
		p value	0	0	0.00057	

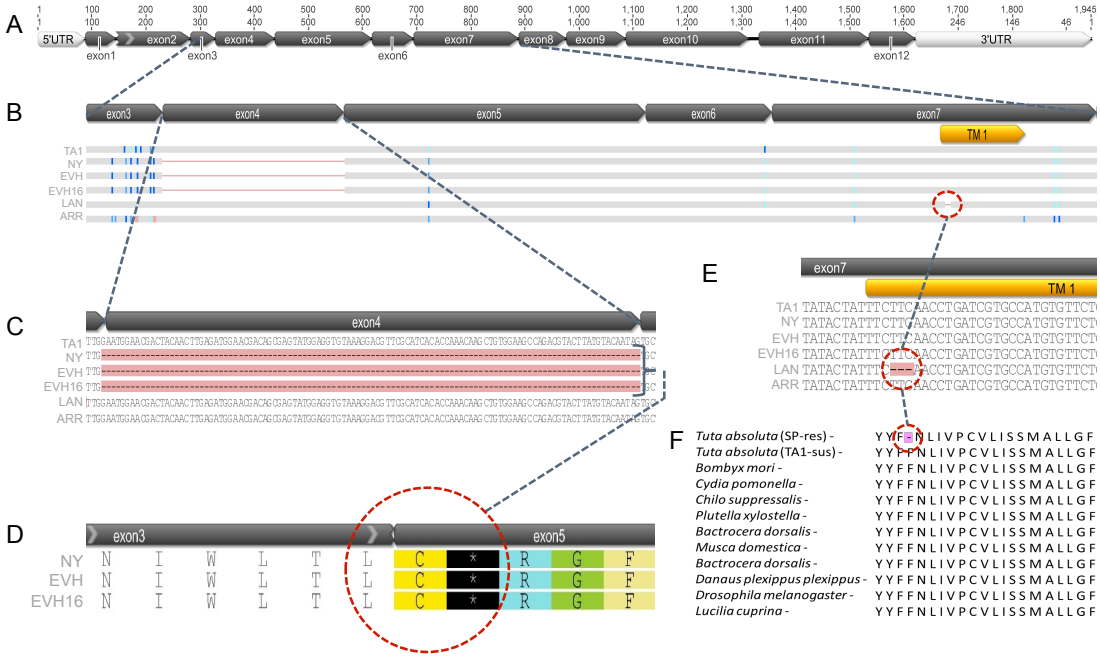
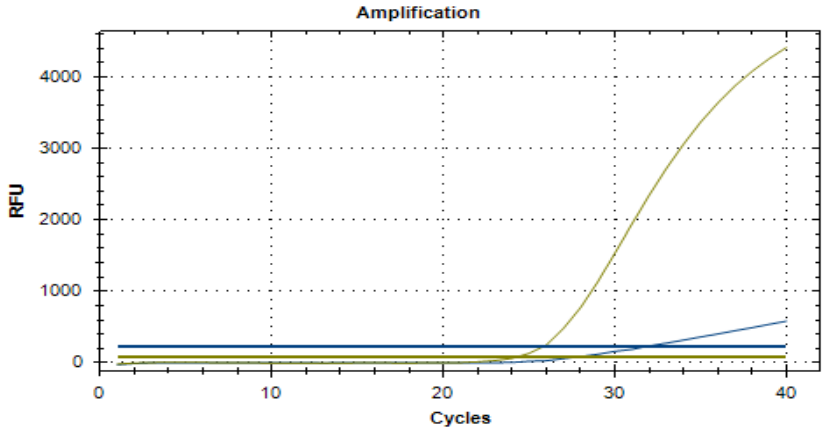
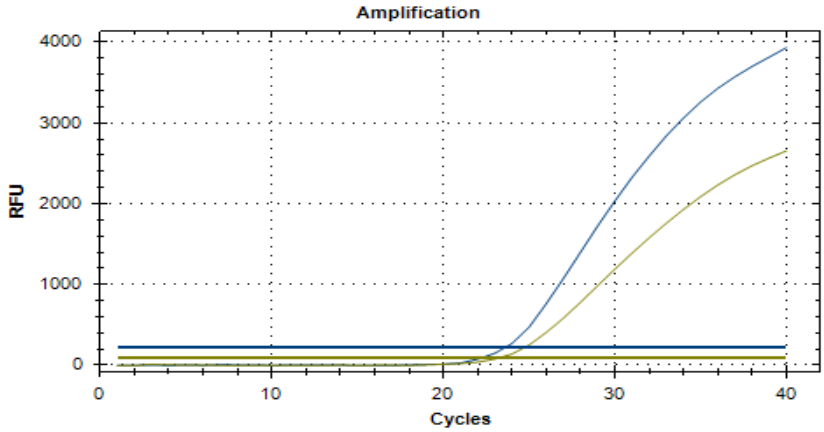


Figure 3.2. Diagram of mutations identified in *Taα6* transcript of UK strains of *T. absoluta.* (A) Exonic structure of the α6 sub-unit. B-D Highlight the skipping of exon 4 in three of the spinosad-resistant strains and show the introduction of a premature stop codon in exon 5. (E,F) Triplet deletion in exon 7 of the LAN2016 strain. This in-frame deletion occurs in the first transmembrane domain of the *Taα6,* a region that is highly conserved across Arthropoda.

A.



B.



C.

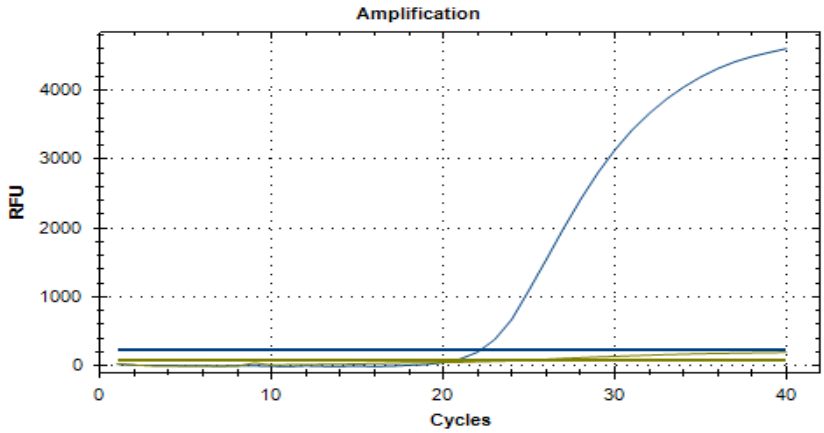


Figure 3.3. Fluorescence of MGB probes (VIC = brown, wild-type genotype 6FAM = blue F238del mutation) in TaqMan assay of different genotypes A) Homozygous wild type, B) heterozygote, C) F238del homozygote.

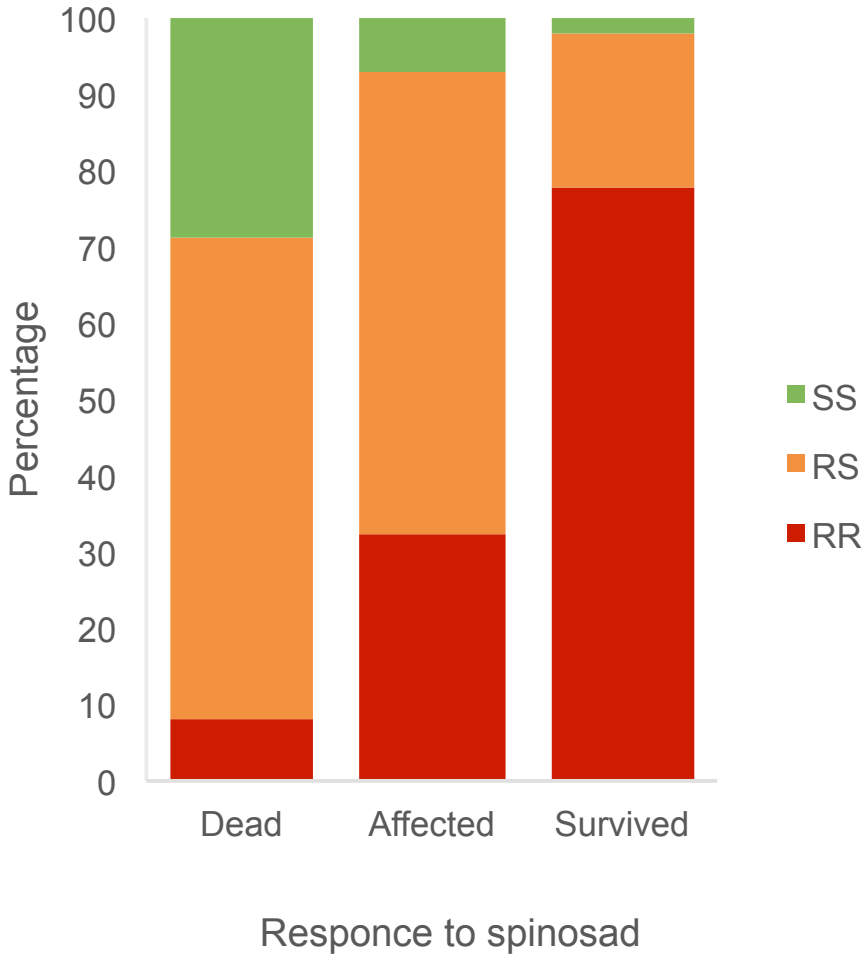


Figure 3.4. A graph showing the percentage of Dead, Affected and Survived individual for each genotype when exposed to 150ppm of spinosad. RR = Homozygous (F238del) resistant, RS = Heterozygotes (F238/F238del) and SS = Homozygous susceptible (F238/F238).

3.4 Discussion

This data reveals high levels of spinosad resistance in strains of *T. absoluta* collected from nurseries in the north and south of the UK. Only one of the UK strains sampled (from the Isle of Wight) exhibited full susceptibility to this compound. While the sample size is small this finding is consistent with reports by several British tomato growers of poor efficacy of spinosad against *T. absoluta* and suggests that resistance to this insecticide may be widespread in UK populations. In three of the strains, NY, EVH and EVH16, the levels of resistance observed were extremely high and would certainly be expected to compromise the use of spinosad at the recommended field rate (87-100 mg L^{-1}). Lower levels of resistance were observed in the LAN strain, however, the LC_{50} value determined for this strain indicates that a significant percentage of exposed individuals would be expected to survive exposure to the field rate application.

Sequence characterisation of transcripts encoding the nAChR a6 subunit revealed two different mutations in the spinosad resistant strains. The first of these, observed in the three most resistant strains, is skipping of exon 4. This alteration has profound consequences for the encoded protein which would lack the ~36 amino acids encoded by exon 4, and would also be significantly truncated as the loss of exon 4 leads to a frameshift and the introduction of a PTC in exon 5. Together this would result in a protein of just 160 amino acids (compared to 510 amino acids in the wild-type) that lacks all four transmembrane domains and would almost certainly be non-viable. More

likely, however, is that nonsense-mediated decay, a conserved mRNA surveillance pathway in all eukaryotes that degrades transcripts containing PTCs, would hydrolyze the truncated *Taα6* transcripts prior to them being translated. Work on *Drosophila melanogaster* first demonstrated that null mutations of α6 lead to spinosad resistance with a strain with a variant of *Daα6* lacking the TM3 and TM4 cytoplasmic loops, and the extracellular C-terminal tail domains exhibiting >1000-fold levels of resistance to spinosad (Perry, McKenzie, and Batterham 2007). Significantly, this study also demonstrated that loss-of-function mutations in insect α6 transcripts do not result in catastrophic loss of fitness as insects without a functional copy of the α6 gene are still viable (Perry, McKenzie, and Batterham 2007). Subsequent studies on several insect crop pests have described genetic alterations in nAChR α6 sequences that result in truncated non-functional proteins leading to resistance. For example, several mutations resulting in mis-splicing and the introduction of PTCs in nAChR α6 transcripts are associated with spinosad resistance in the diamondback moth, *Plutella xylostella*, and the oriental fruit fly *Bactrocera* dorsalis (Baxter et al. 2010; Hsu et al. 2012; Rinkevich et al. 2010). In a previous study of a spinosad resistant *T. absoluta* strain derived from a population collected in Portugal, resistance was conferred by skipping of exon 3 rather than exon 4, however, in this case the reading frame of the altered *Taα6* transcripts was unaffected by the exclusion of exon 3 (Berger et al. 2016). Sequencing of surrounding intronic regions was unable to identify genomic alterations in conserved *cis*-elements at exon-intron junctions that might be responsible for causing skipping of exon 4. If the causal mutation could be identified in future studies it would provide a marker for the

development of DNA-based diagnostics and would allow this mechanism to be monitored in *T. absoluta* populations more simply and affordably.

A more subtle alteration, F238del, was observed in transcripts of *Taα6* in the LAN strain, and this was correlated with lower levels of resistance to spinosad. This triplet deletion results in the loss of a phenylalanine amino acid in exon 7 and occurs in TM1 of the nAChR Ta*α6* subunit (Figure 3.2). This amino acid is highly conserved in nAChR *α6* subunit proteins across arthropods, suggesting that this residue is functionally constrained. The loss of the amino acid at this position is thus extremely unusual and consistent with it being a *bona fide* resistance mutation. I provide further evidence of a causal role of this mutation in resistance by demonstrating that it cosegregates with spinosad resistance in survival bioassays. Finally, the F238del mutation exhibits parallels with a previously reported amino acid substitution (G275E) shown to confer spinosad resistance in both thrips and *T. absoluta* (the latter in strains from Brazil) (Silva et al. 2016; Zimmer et al. 2016; Bao et al. 2014; Puinean et al. 2013). Like F238del this mutation also occurs in a α-helical transmembrane domain (TM3) of the α6 subunit (Figure 3.5) (Puinean et al. 2013). X-ray crystallisation studies of the glutamate-gated chloride channel (GluCl), a pentameric receptor with close structural similarity to nAChRs, bound to ivermectin, another macrocyclic lactone with structural similarity to spinosad, have shown that ivermectin makes direct associations (by hydrogen bonding and van der Waals interactions) with TM1, TM2 and TM3 (Hibbs and Gouaux 2011). This finding is consistent with spinosad acting via an allosteric transmembrane site distinct from the conventional agonist

binding site (Puinean et al. 2013), and it is thus likely that the F238del mutation confers resistance by modifying the spinosad binding site (Fig. 3.5).

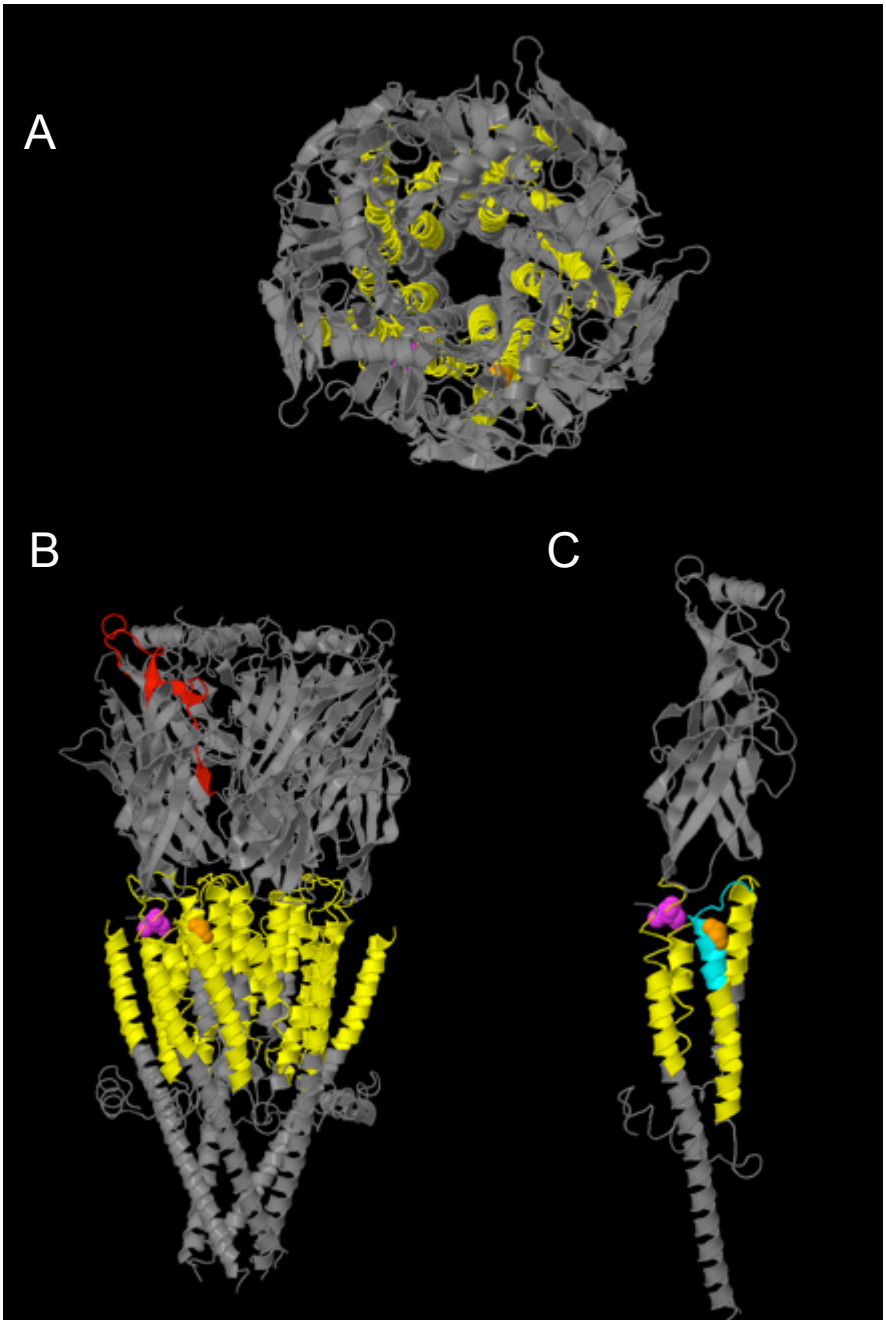


Figure 3.5. Human acetylcholine receptor showing important functional regions and sites of alterations from resistant strains of *T. absoluta* mapped to them based on sequence alignments. (A) The top view showing the ion pore.

(B) The side view showing homologous positions of *T. absoluta* exon 4 in red, transmembrane regions (Tm 1-3) highlighted in yellow, G275E in orange and LAN F238del in magenta. (C) The structure of a single subunit – highlighting the close proximity of G275E and F238del and the position of analogous cis-loop receptor in cyan. The cis-loop binding-site of the macrocyclic lactone ivermectin is based on sequence homology from GluCl (Hibbs and Gouaux 2011; Puinean et al. 2013) (Images produced in Geneious 10.2.6 Biomatters Ltd.). It is notable that neither the G275E mutation or exon 3 skipping were observed in UK strains of *T. absoluta* suggesting that the mutations identified in my study arose *de novo* in UK populations rather than being imported via transfer of insects on plant material or packaging from outside the UK. The application of spinosad via the irrigation system in the UK, together with the redundancy of the nicotinic acetylcholine receptor (nAChR) α6 subunit, in which any nonfunctionalizing mutation will confer resistance, likely facilitated the rapid evolution of resistance.

My results have significant applied implications in relation to the control of *T. absoluta* in the UK. Spinosad performs a key role in the IPM strategy introduced against this pest in 2013, and is applied at the start of the season to provide control while the natural enemy *M. pygmaeus* is becoming established (Jacobson and Howlett 2014). If necessary, a high volume spray of chlorantraniliprole is applied as a second line of defence later in the season to keep the pest and predator populations in balance (Jacobson and Howlett 2014). The fact that I identified three strains with two independent mechanisms of resistance to spinosad is concerning as it shows a clear

redundancy to the *Taα6* demonstrates that *T. absoluta* with resistance spinosad can readily evolve and are viable. It is likely that these mutations evolved independently in the UK under spinosad selection as (1) they have not been previously described in populations outside of the UK and (2) they are not likely to be the result of cross-selection from pesticides that also target the nAChR such as neonicotinoids (Mota-Sanchez et al. 2006). It is unlikely that the resistance to spinosad is mechanistically related to neonicotinoids resistance as spinosad has a novel site of action in the nAChR receptor that is clearly distinct from all other insecticidal sites of action (Orr et al. 2009). Lack of cross-resistance is expected when non-metabolic mechanisms are implicated and the majority of studies show spinosad does not confer resistance to other classes of nAChR-acting insecticides like neonicotinoids (Sparks et al. 2012)

It would be useful to screen UK populations of *T. absoluta* more widely for the resistance mechanisms identified in this study. If such monitoring indicates that, as suggested by my study, spinosad resistance is widespread then I suggest that use of this insecticide is suspended for a period to examine if susceptibility is restored to populations in the absence of selection. The likelihood of this is dependent on any fitness costs associated with the resistance mechanisms I have identified. In *D. melanogaster* no evidence of fitness costs were identified under laboratory conditions in a Dα6 null mutant, suggesting the costs of loss of function mutations in this subunit may be minimal (Perry, McKenzie, and Batterham 2007). However, work on a spinosad selected strain of *T. absoluta* found that resistance was unstable in

the absence of spinosad selection and dropped rapidly over relatively few (<10) generations (Campos et al. 2014). In this study I observed high levels of spinosad resistance in a strain (EVH16) collected from a nursery where this compound had not been used for a year suggesting the exon 4 skipping identified in this strain has a low fitness penalty. Additional work is required to clarify fitness costs associated with this mutation and the others identified in this study in both the α6 nAChR subunit and the RyR under field-realistic conditions.

4. Target-site resistance to diamide insecticides in UK populations of *T. absoluta*

The results detailed in this chapter have been published in *Pest management science* (Appendix 1)

4.1 Introduction

Diamides are the second class of neurological insecticides, along with spinosad, that were implemented in the initial IPM strategy developed to control UK populations of *T. absoluta* (Jacobson and Howlett 2014). In this framework diamides are regarded as a potential second line of defence following the utilisation of the pesticide spinosad and the biocontrol agent *Macrolophus pygmaeus.* Diamides, or tetramethylazodicarboxamides are a chemical family that have powerful and fast acting insecticidal activity through their interaction with ryanodine receptors (RyRs). They are especially effective against Lepidoptera (Temple et al. 2009) and show negligible effects on beneficial insects (Martinou, Seraphides, and Stavrinides 2014)

RyRs are named after a plant-derived alkaloid, ryanodine, extracted from the stem and roots of the plant *Ryania speciosa.* It was shown that ryanodine bound with high affinity to membrane bound receptors in the sarcoplasmic reticulum (Fill and Copello 2002). The sarcoplasmic reticulum is a calcium ion storage organelle that consists of a network of tubules that that run through the muscle cells. They surround myofibrils and regulate excitation-contraction

(E-C) coupling (Endo 1977) through the controlled release of Ca^{2+} which controls cycles of muscular contraction and relaxation. Inhibition of this coordinated control by diamides induces spasms, paralysis and death of the insect.

Despite the preferable insecticidal profile of diamides, resistance has evolved independently in at least 9 species of Lepidoptera (Richardson et al. 2020) including *T. absoluta.* The first accounts of resistance to diamides in Europe were in Sicilian and Greek populations that displayed LC_{50} values ranging between 47.6–435 mg L^{-1} and 0.14–2.45 mg L^{-1} for chlorantraniliprole (Roditakis et al. 2015). High levels of resistance were also recorded in Brazil with LC_{50} values reaching 1236 mg L^{-1} (Silva et al. 2016). The molecular basis for this resistance was characterised by comparisons of RyR sequences from resistant and susceptible strains. 100% of resistant individuals from Brazil were found to carry the G4903E mutation and a low proportion had the substitutions I4746M or I4746T, all of which are situated in the C-terminal transmembrane region of the RyR (Roditakis, Steinbach, et al. 2017). These mutations were absent from the susceptible genotype and have been previously functionally characterised as bona fide resistance causing mutations in *Plutella xylostella* (Guo, Liang, et al. 2014).

Diamide selection of an Italian strain resulted in strong resistance (LC_{50} value of 1700 mg L^{-1}), and when sequenced individuals were found to be homozygous for either G4903E or G4903V, and also carried the I4746M replacement at high frequency. The Greek strain displayed moderate

phenotypic resistance (55-fold), and this correlated with a low frequency of G4903E and I4746M alterations in this strain. Finally, in a Spanish strain displaying low levels of resistance, the only alterations observed were I4746M in about one third of the individuals tested. Taken together, these results suggest that G4903E/V contributes to strong resistance with I4746M giving mild protection. Finally, the relative contribution of these mutations to resistance were functionally validated by CRISPR/Cas9 genome modification in *Drosophila melanogaster, Plutella xylostella and Spodoptera exigua* (Douris et al. 2017; Wang et al. 2020; Zuo et al. 2017). G4946V modification in *D. melanogaster* showed high resistance to chlorantraniliprole (194.7-fold) and flubendiamide (91.3-fold) compared to cyantraniliprole (15.3-fold) (Douris et al. 2017). The I4790M edited strain of *P. xylostella* showed moderate resistance to flubendiamide (40.5-fold) and low resistance to chlorantraniliprole (40.5-fold) and cyantraniliprole (7.7-fold) (Wang et al. 2020). Finally the G4946E edited strain of *S. exigua* showed high resistance to all three compounds, chlorantraniliprole (223-fold), cyantraniliprole (336-fold) and flubendiamide (>1000-fold) (Zuo et al. 2017).

While high levels of resistance to diamides have been described in European and South American populations similar phenotypes had not been previously reported in *T. absoluta* populations in the UK. Recently, however, glasshouses in the UK have reported some control failure of *T. absoluta* using chlorantraniliprole (Rob Jacobson; personal communication). As chlorantraniliprole is an important second line of defence for the current IPM strategy it is vital to test whether; (1) diamide resistance is present in UK

populations of *T. absoluta* and to what level. (2) To identify the mechanism by which resistance is enabled with the intention to update and advance current IPM and (3) assess the future potential of chlorantraniliprole as an effective pesticide in IPM.

4.2 Methods

4.2.1 Insect strains

Four strains of *T. absoluta* were collected in 2015 from commercial glasshouses at four UK sites; IOW from Wight salads, Isle of Wight, LAN from Flavourfresh Salads Ltd, Lancashire, NY from Jan Bezemer and sons, North Yorkshire and EVH from Sandylands Nurseries, Worcester. In 2016 a second strain, EVH16, was collected from Sandylands Nurseries, Worcester after reports of control failure using chlorantraniliprole. An additional Spanish strain that is susceptible to spinosad and chlorantraniliprole was acquired from Rothamsted Research, Hertfordshire. 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).

4.2.2 Leaf dip assays

Leaf dip assays were conducted according to test method 22 guidelines produced by the Insecticide Resistance Action Committee (see methods 2.3). 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} from the target population by the LC_{50} of TA1.

4.2.3 Chlorantraniliprole selection

T. absoluta were selected with chlorantraniliprole 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 (fig. 4.1). Fresh tomato leaves were submerged in chlorantraniliprole 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 diamide concentrations of 5 mg L^{-1}, 10 mg $L^{-1}$and 50 mg L^{-1}. The population was then bioassayed to assess resistance.

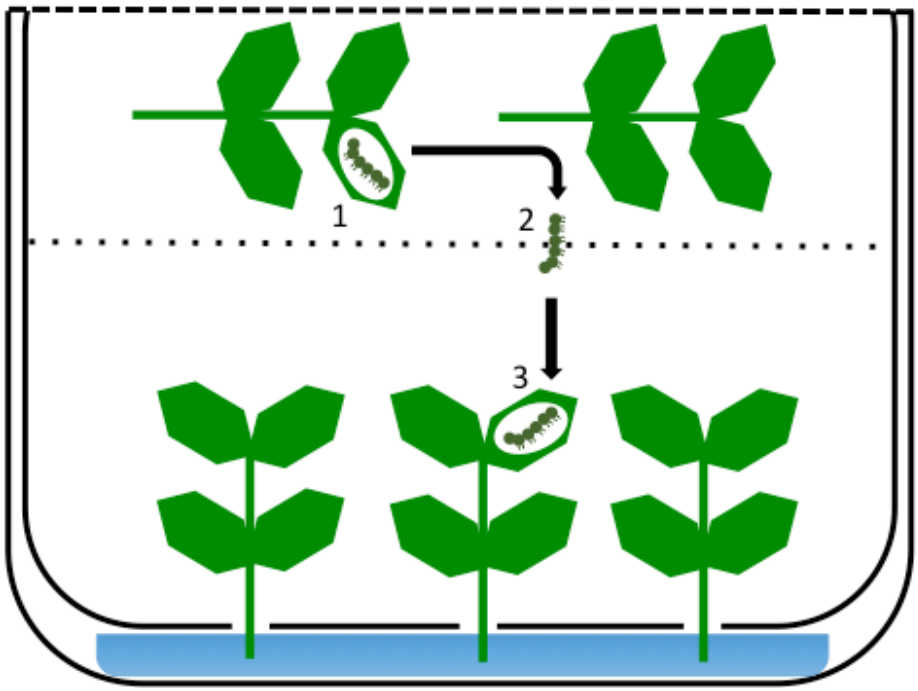


Figure 4.1. Selection chamber. (1) Infested tomato leaves placed over course mesh. (2) Larvae repel down through course mesh as leaves wilt. (3) Larvae mine leaves dipped in chlorantraniliprole.

4.2.4 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 (see methods 2.4 and 2.5).

4.2.5 Cloning and sequencing

To analyse the structure of the *T. absoluta* RyR gene a 1353 bp region (encompassing the C-terminal domain) containing sites of previously reported diamide resistance-conferring mutations (Kato et al. 2009; Troczka et al. 2012) was amplified by PCR. The standard PCR protocol was used (see methods 2.7) with the primers listed in table 4.1. PCR products were purified as detailed in 2.8 and sequenced as described in 2.9.

Table 4.1. 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

4.2.6 Analysis of G4903V proximity to chlorantraniliprole binding site.

To implicate target site alterations with the chlorantraniliprole 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 (see results fig. 4.3) and aligned in Geneious v10.2.6 (Biomatters Ltd.) to show consensus. G4903V and the chlorantraniliprole binding site were then mapped to the rabbit RyR to highlight physical association.

4.3 Results

4.3.1 Bioassays and selection

The sensitivity of UK strains to chlorantraniliprole was compared to a known susceptible strain (TA1) using full dose-response bioassays (see appendix 2). Out of these, only NY and EVH2016 showed tolerance to chlorantraniliprole with EVH, LAN and IOW showing no differences in sensitivity compared to TA1 (Table 2). 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 (27 mg L^{-}.) and its LC_{50} was 79-fold higher than the EVH population from the previous year. Selection of the NY strain with chlorantraniliprole failed to rapidly result in a strain with higher levels of resistance. However, progressive selection of the EVH2016 strain with chlorantraniliprole over just 12 generations resulted in a strain with potent resistance to this compound (LC_{50} of >5000 mg L^{-1}).

Table 4.2. Lead dip bioassay results for the LC_{50}s of chlorantraniliprole 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	Rothamsted	Italy	1.23	0.02	0.095-5	1.0
EVH2016	R & L Holt	Evesham	9.53	0.02	5.025 - 18.09	7.94
NY2016	Bezemer and sons	North Yorkshire	33	<0.001	11.2 - 97.9	27.5
EVH2016sel	Evesham	Evesham	>5000	N/A		>4166.67
LAN2016	Flavour Fresh Salads Ltd.	Lancashire	4.75	<0.001	1.7 - 12.8	3.96
IOW2016	Wight Salads ltd.	Isle of Wight	4.75	0.25	1.7 - 12.8	3.96

4.3.2 Sequencing

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 diamides in *T. absoluta* (Roditakis, Steinbach, et al. 2017) and *P. xylostella* (Troczka et al. 2012) was amplified from the chlorantraniliprole 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 (fig. 4.2). 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.

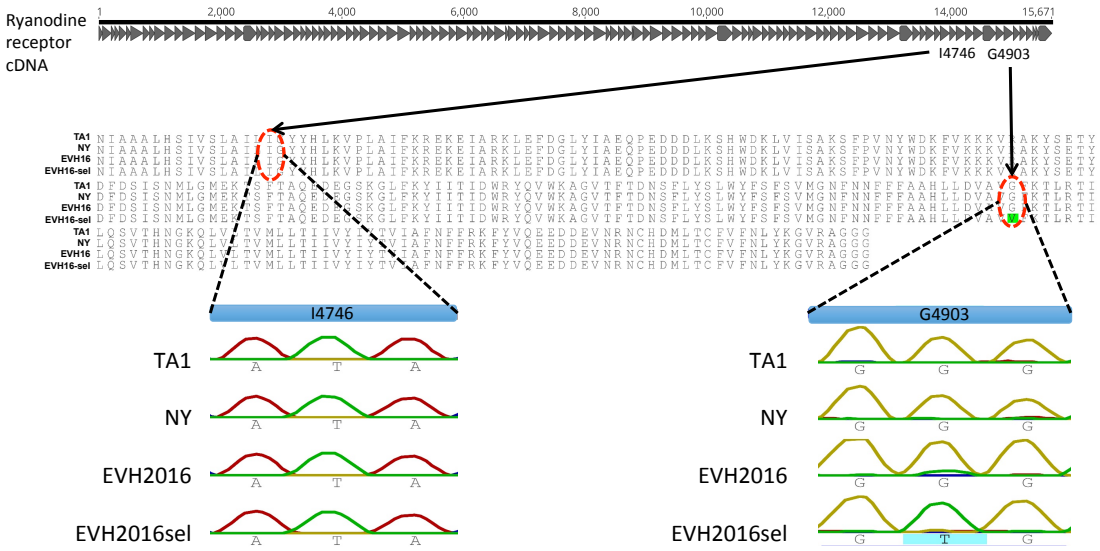


Figure 4.2. Alignment of RyR sequences (TA1, NY, EVH2016 and EVH2016sel) showing genotypes at diamide resistance mutation sites.

4.3.3 Physical association of G4903V with chlorantraniliprole binding

Alignment of rabbit (*Oryctolagus cuniculus*), human (*Homo sapien*), wild boar (*sus scrofa*), *C. suppressalis, H. armigera, M. persicae, P. xylostella, T. castaneum* and *T. absoluta* showed a high level of consensus in the transmembrane region believed to be the binding site of chlorantraniliprole (Lin et al. 2020) (fig 4.3). Mapping G4903V to the 3D structure of the rabbit RyR showed it was positioned in the transmembrane region in the proposed chlorantraniliprole binding site (fig. 4.4)

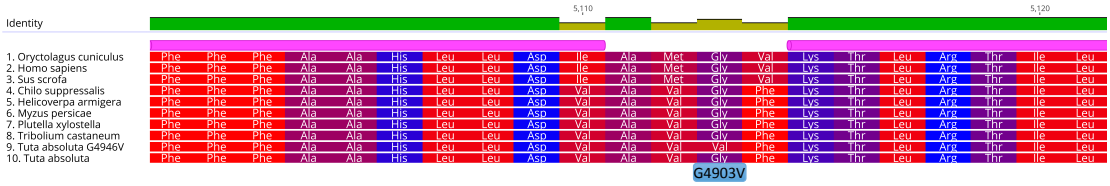


Figure 4.3. Alignment of region surrounding position G4903 with other species including mammals and insects. Green bar represents alignment consensus. Pinks bars represent alpha helices (of the tm region in fig. 4.4), the blue annotation shows the position of the G4903V mutation site and the colour of the amino acids denotes their hydrophobicity where red is the most hydrophobic and blue is the most hydrophilic (Gly = 0.501 and Val = 0.825).

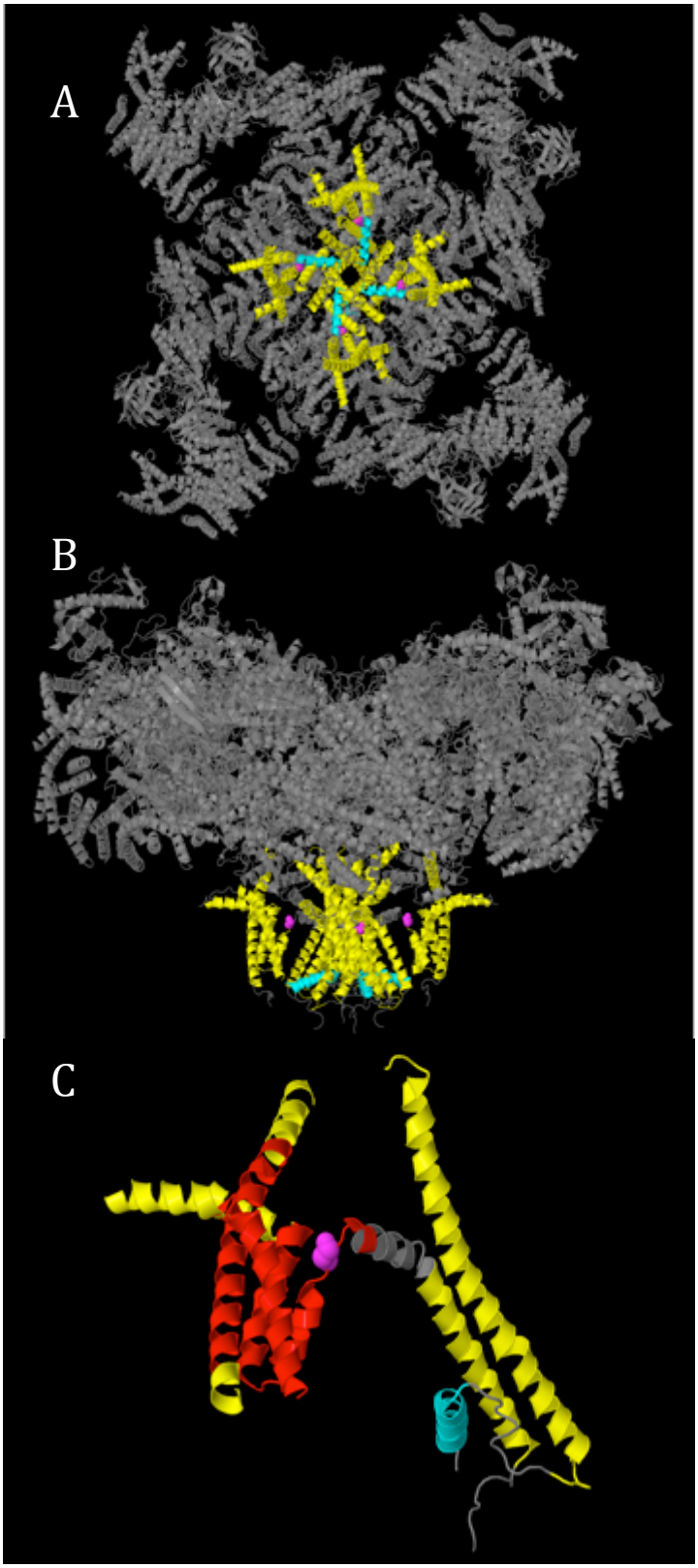


Figure 4.4. Image of a rabbit RyR (PDB ID:5J8V) with homologous *Plutella xylostella* transmembrane (TM) regions S1-S6 in yellow, pore helix in cyan

(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 diamide binding regions in red as described by Lin et al. (2020).

4.4 Discussion

Two of the field strains (EVH2016 and NY) tested in my study showed tolerance to chlorantraniliprole. While the levels of tolerance displayed were relatively modest (8-fold and 27.5-fold respectively), selection of EVH2016 rapidly resulted in a line with potent resistance to this compound (>4166-fold). The fact that the resistance ratio, prior to lab selection, rose from 0.1-fold in EVH to 9.53-fold in EVH2016 (strains that were collected from the same glasshouse before and after continued use of chlorantraniliprole) suggests that resistance alleles were already present in the population. It is likely they had already been exposed to selection in the field environment by chlorantraniliprole application. The level of resistance was then further elevated through laboratory selection resulting in a population with >4166-fold resistance. This suggests that the pre-existing resistance alleles present in the field populations are capable of rapidly inducing ‘complete’ resistance to chlorantraniliprole, whilst maintaining population viability, if subject to continued selection.

The NY strain showed levels of resistance that were higher than that of EVH2016, however, sequencing showed no suggestion of G4903V or I4746M

in the population and attempts to select for resistance to chlorantraniliprole in this population failed. This suggests that any alleles conferring resistance in this strain were of small effect, and thus selection for them did not result in large shifts in sensitivity - as seen following the selection of a mutation of large effect in the EVH2016 strain (see below). Alternatively, the further selection of these alleles in the NY strain could have been constrained by strong fitness costs.

Sequencing of a region of the RyR that encompassed the sites of known resistance mutations confirmed the presence of G4903V at low frequency in the EVH2016 strain. It also showed that in the selected line (EVH2016sel) this alteration had reached fixation correlating with the potent resistant phenotype of this strain detected by the bioassays. The causal role of this mutation has been previously demonstrated by CRISPR/Cas9 editing in *D. melanogaster showing high resistance to* chlorantraniliprole and flubendiamide (Douris et al. 2017). In *S. exigua* its presence at 100% frequency in the population results in high resistance to chlorantraniliprole, cyantraniliprole and flubendiamide compared to strains that lack the mutation (Zuo et al. 2017). Together, with my findings, this confirms the presence of this mutation as the mechanism of resistance in UK populations of *T. absoluta* - as well as demonstrating its potential to rapidly reach fixation under constant application of chlorantraniliprole.

G4903 is situated at the end of the S4 transmembrane helix which carries the gating charges in most voltage gated ion channels (Lin et al. 2020). The

mutation resulting in G4903V would alter the charge (fig. 4.3) of the predicted binding site of chlorantraniliprole (fig. 4.4 C), which is likely the reason for reduced binding affinity of chlorantraniliprole to the RyR (Lin et al. 2020). Our findings identified G4903V as the sole contributor of resistance to the anthranilic diamide chlorantraniliprole in this strain of *T. absoluta*. However, other studies on *T. absoluta* have demonstrated the contribution of this mutation in cross-resistance to phthalic diamides. Alterations at the G4903V in *T. absoluta* resulted in 742-fold resistance to chlorantraniliprole and >2500-fold resistance to flubendiamide (Roditakis, Steinbach, et al. 2017). These findings suggest that the use of commercially available flubendiamide as an alternative may not be suitable as a replacement for chlorantraniliprole; rather a pesticide with different mode of action should be considered.

These results have direct implications for the control of *T. absoluta* in the UK. Chlorantraniliprole is vital as a second line of defence to supress *T. absoluta* populations if the first line of defence, the pesticide spinosad and biocontrol agent *M. pygmaeus* are ineffective, or in the latter case, slow to become established (Jacobson, Ltd, and Howlett 2014). The fact that EVH2016 showed complete resistance to spinosad (chapter 3) and also the potential for complete resistance to chlorantraniliprole is concerning. It demonstrates that *T. absoluta* with resistance to both compounds can readily evolve and are viable. The data collected in this investigation is vital for informing future IPM with the purpose of directing the appropriate use of chlorantraniliprole in field environments, maintaining its efficacy and encouraging research into viable alternatives. Guidelines for the purpose of resistance prevention were

produced by the Insecticide Resistance Action Committee (IRAC) which state resistance episodes should be; (1) tracked (bioassays of NY, EVH and EVH2016), (2) resistance mechanisms identified (G4903V), and (3) future events predicted (selection of EVH2016 and bioassay of EVH2016sel) (Sparks and Nauen 2015). The fact that these mutations were only identified in the EVH2016 strain in our study suggests that with effective resistance management it may be possible to prevent the further development of resistance to this compound.

These results clearly demonstrate that the use of chlorantraniliprole in glasshouses should be minimised and applied in coordination with resistance monitoring - using population genotyping based on G4903V as described in Troczka et al. (2012) - to provide accurate assessment of resistance allele frequency in the populations. This dual process would prevent the proliferation of resistance alleles to a point of fixation, stopping the second important chemical defence in the IPM program from becoming redundant - as predicted by the selection of EVH2016 in this study. It would also be advisable for the rotation of this insecticide with insecticides of alternative modes of action and/or the use of alternative control methods. In regards to the latter, recent trials in the UK using a synthetic sex pheromone as a mating disruption strategy against *T. absoluta* have provided encouraging results (AHDB 2017) and may allow the frequency of insecticide applications to be significantly reduced. However, our study clearly illustrates the danger of complacency and we suggest that the control of *T. absoluta* in the UK (and worldwide) should continue to rely on the use of a range of integrated approaches in

order to reduce selection pressure on any single control in order to prevent or slow the emergence of resistance.

5. Metabolic resistance to chlorantraniliprole

5.1 Introduction

The toxicodynamic process of contemporary insecticides typically involves interactions with target receptors/signalling pathways in the insect nervous system. Their effects can be overcome through the evolution of one or both of two mechanisms, target-site resistance and metabolic resistance. *T. absoluta* has shown the ability to resist the impact of diamides through target-site resistance – mutations to the RyR gene (positions G4946 and I4790) that maintain the receptors function whilst altering its structure/electrochemical profile sufficiently to minimise toxicological effects of the insecticide. This has been demonstrated for *T. absoluta* populations in Europe (Roditakis, Steinbach, et al. 2017). Target-site resistance to diamides has also been identified at low frequencies in the UK (EVH2016) with populations showing moderate tolerance to diamides (9.53-fold). Furthermore, these target site mutations were rapidly selected to fixation resulting in a strain with potent (>5000-fold) resistance. A second strain from North Yorkshire (NY2016) also showed tolerance higher than that of a reference susceptible strain (TA1), however, selection failed to elevate resistance in this population. Furthermore analysis of the RyR in this strain before and after selection identified no alterations to the RyR. An absence of target-site resistance was also observed in European populations with tolerance to the compound (Grant et al. 2019).

The identification of diamide resistant *T. absoluta* strains that do not carry known target-site mutations suggests that other mechanisms may underpin their resistance, such as enhanced metabolic detoxification. Metabolic resistance is a toxicokinetic process that involves interception, modification and excretion of xenobiotics, reducing cellular concentrations thus minimising interactions with the target-site. In some insects, the detoxification processes is so efficient that pesticides are fully metabolised before reaching their target (Taylor and Feyereisen 1996). Metabolic detoxification enzymes fall into one of five major families: ATP-binding cassette transporters (ABC's), Cytochrome P450s (CYP), esterases (EST's), Uridine 5'-diphospho-glucuronosyltransferase (UGT's) and Glutathione S-transferases (GST's). These can function independently, but most often in a three phase, step-wise process.

ABC transporter genes are involved in transporting a wide range of molecules across lipid membranes for downstream processing as well as passing compounds directly to the excretory system (Merzendorfer 2014b). Cytochrome P450s and ESTs are involved in xenobiotic modification resulting in the formation of polar regions of the xenobiotic reducing its toxicity, increasing solubility and increasing its reactive profile for further modification (Xianchun Li, Schuler, and Berenbaum 2007). These processes are referred to as phase 1 metabolism. Phase 2 metabolism involves conjugation reactions and is typified by the addition of a modified sugar in the form of UDP-glucose in insects (Bock 2016). This molecule greatly increases the

hydrophobicity of xenobiotics, facilitating excretion. This process typically includes UGT's and GST's (Enayati, Ranson, and Hemingway 2005). Previous studies in *T. absoluta* demonstrate the action of metabolic detoxification in the resistance of pesticides with no evidence of target site resistance (Campos et al. 2015; Barati, Hejazi, and Mohammadi 2018). A key trait of enzyme-mediated detoxification is the increased expression of genes involved in specific or a broad spectrum of metabolic processes (Xianchun Li, Schuler, and Berenbaum 2007). Fluorescence and absorbance assays were used to show elevated P450 and EST activity in spinosad resistant populations from Chile (Reyes et al. 2012), elevated levels of CYPs and GSTs in pyrethroid resistant populations from Brazil (Silva et al. 2015), and enhanced activity of CYPS and ESTs in organophosphate resistant Iranian populations (Barati, Hejazi, and Mohammadi 2018).

Metabolic resistance has not been linked to diamide resistance in *T. absoluta,* however, in *Plutella xylostella,* transcriptome profiling of chlorantraniliprole resistant strains showed that the flavin monooxygenase gene *PxFMO2* was highly overexpressed in a strain that lacked any target-site resistance. Functional expression of this gene in transgenic *Drosophila melanogaster* demonstrated that PxFMO2 confers resistance to chlorantraniliprole in vivo (Mallott et al. 2019). Furthermore, synergist assays showed that PBO, DEM and DEF can partially suppress chlorantraniliprole resistance suggesting P450s, GSTs, and esterases role in resistance (Xingliang Wang et al. 2013; Roditakis, Steinbach, et al. 2017). Additionally, short-term exposure to chlorantraniliprole increased the detected levels of GST after exposure to a

sub-lethal concentration - demonstrating a strong correlation between GST activity and resistance (Hu et al. 2014).

In *Chilo suppressalis,* the over-expression of P450s was suggested as a resistance mechanism to chlorantraniliprole. Injection of dsRNAs that reduced expression of *CYP6CV5, CYP9A68, CYP321F3*, and *CYP324A12* resulted in significant larvae mortality in the presence of chlorantraniliprole (Xu et al. 2019). Another study on the same species showed that inhibition of ABC transporters with the synergist verapamil significantly increased toxicity of chlorantraniliprole and larval mortality (Meng et al. 2020). UGT's were also implicated in chlorantraniliprole resistance in another strain of resistant *C. suppressalis*, where they were found to be significantly overexpressed in the Malpighian tubules, fat body, and midgut. Suppression of their expression through RNAi injections resulted in significantly elevated levels of larval mortality, providing evidence of a role in chlorantraniliprole resistance (Zhao et al. 2019).

Chlorantraniliprole is still a highly effective and important pesticide in the control of *T. absoluta.* Losing the efficacy of this pesticide through the evolution of resistance would be a significant economic blow to the tomato industry as there are limited compounds that can replace it in the current IPM. It is therefore vital for resistance management strategies to monitor its efficacy and identify the mechanisms through which resistance evolves. This chapter aims to (1) assess the differential expression of genes between chlorantraniliprole resistant and susceptible strains of *T. absoluta.* (2)

Generate a short-list of potential candidate-resistance genes. (3) Functionally validate any genes of interest to implicate their causal effects as resistance mechanisms to chlorantraniliprole and to (4) describe the structure and diversity of any gene families that are associated with resistance to chlorantraniliprole to facilitate further future analysis of resistance in *T. absoluta*.

5.2 Methods

5.2.1 Transcriptome resources

All *T. absoluta* transcriptomes were sequenced from 4 replicate pools of 10 whole larvae (l2-l4). Two strains of *T. absoluta* and their transcriptomes were acquired from Syngenta (Switzerland) that were collected from commercial glasshouses in Spain (Ssus and Sres). Ssus was susceptible to chlorantraniliprole 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 chlorantraniliprole.

5.2.2 Sequencing and assembly of a reference T. absoluta genome

5.2.2.1 Generation of sequenced strains

To maximise homozygosity and facilitate genome assembly *T. absoluta* were first inbred. Pupae from a single pair mating were collected sexed and paired in isolated chambers containing a single tomato leaf (var. Money Maker). All pupae were collected from the most fecund pair and sexed. The sibling

offspring were then paired off again in isolated chambers to breed. This process was repeated over 6 generations until further mating's were inhibited by inbreeding depression.

5.2.2.2 DNA extraction

gDNA libraries for genome assembly were generated for two sequencing approaches, Illumina paired-end sequencing and PAC-BIO long single molecule sequencing at the Earlham Institute. For Illumina paired end (250-bp) libraries 2-5 µg of gDNA (min 35ng/µl in 60 µl) with absorbance ratios of (260/280) 1.6–2.0 nm and (260/230) 1.8 –2.4 nm was required. For the generation of PAC-BIO libraries 20 µg of gDNA was required with absorbance ratios of (260/280) 1.6–2.0 nm and (260/230) 1.8-2.4 nm and a total molecular weight of > 40kb.

T. absoluta were snap frozen in liquid nitrogen and stored at -80°C prior to DNA extraction. DNA was extracted using E.Z.N.A.® Insect DNA Kit (Omega Bio-Tek Inc., Norcross, Georgia, USA) in accordance with their protocol (see methods 2.11). This extracted gDNA at a concentration of 70 ng μl^{-1} with a 260/280 ratio of 1.89 and a 260/280 of 1.89. These metrics were sufficient for Illumina sequencing; however, they fell short of the requirements required for PAC-BIO sequencing. Thus, a second gDNA extraction was carried out using QIAGEN Genomic-tip (Hilden, Germany) according to the manufacturers protocol. This resulted in gDNA with a concentration of 170 ng μl^{-1} with both 260/280 and 260/230 ratios of 1.88 with an average molecular weight >60kb suitable for PAC-BIO library generation.

5.2.2.3 Genome assembly (completed by Kumar Singh)

PAC-BIO long-reads were assembled using Canu v2.0 (using default parameters) (Koren et al. 2017) and then polished (correction of minor errors using high fidelity reads) with both long- and short-reads. To deal with the two sets of data DBG2OLC v20180222 (Ye et al. 2016), a hybrid assembler, was also used to assemble both short- and long-reads together (using default parameters). The Canu and DBG2OLC assemblies were then merged using QuickMerge v0.3 (Chakraborty et al. 2016). Redundancy in the merged assembly was removed (de-duplicated) using the Redundans assembler. Quality checks were performed to check the completeness and contiguity of the assembly using KATv2.4.2 (Mapleson et al. 2017) and BUSCO tools v4.1.2 (Simão et al. 2015). For annotation, *Tuta absoluta* RNAseq data were used in the Braker2 v2.1.2 pipeline (Brůna et al. 2021) to obtain final gene-models. The predicted gene-models were then annotated using BLAST2GO (Götz et al. 2008).

5.2.3 Differential gene expression analysis

5.2.3.1 Transcriptome mapping and gene expression

Replicated RNAseq data was acquired from Syngenta (Switzerland) for all four strains Sus, Ssus, Mur and Sres. Before transcripts were mapped to the genome files adaptor sequences and low quality base calls were removed using trim-galore (Babraham Bioinformatics - Trim Galore!) in bioconda. Transcripts were mapped to the genome using HISAT2 and StringTie protocol (Pertea et al. 2016). First information about gene structure is required.

Genome annotation data is contained in GFF (General feature format) files generated from the genome. To be used in StringTie GFF files need to be converted to GTF (Gene transfer format). From these GTF files, exon and splice site data are extracted and combined to build a HISAT2 index for the genome. This HISAT2 index is then used to map both files of the paired end read libraries to the genome. This process outputs a SAM (sequence alignment map) file that needs to be converted into a BAM (binary alignment map) that reduces computational requirements. StringTie is then used to assemble and quantify the expressed genes and transcripts. The replicate data from all strains are then merged. Differential gene expression analysis was conducted in blast2GO pro (Conesa et al. 2005) using edgeR (Robinson, McCarthy, and Smyth 2010). Pairwise comparisons of expression profiles from all four strains were produced that identified genes with a corrected p-value threshold of $p < 0.05$ and a fold change > 2. Lists of differentially expressed genes were compared using Jvenn (Bardou et al. 2014).

5.2.3.2 Assessment of target-site modifications

Assessment of sequence reads mapped to the RyR, the chlorantraniliprole 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.

5.2.3.3 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 chlorantraniliprole 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.

5.2.4 Functional validation of candidate gene

5.2.4.1 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.

5.2.4.2 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).

5.2.4.3 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.

5.2.4.4 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.

5.2.4.5 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.

5.2.4.6 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.

5.2.4.7 Fly insecticide bioassays

Chlorantraniliprole 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.

5.2.4.8 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 5.1) 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 5.1. 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

5.2.6 UGT phylogenetics

To assess the diversity of *T. absoluta* UGTs, all genes were extracted from the *T. absoluta* genome based on annotations generated from BLAST hits. Transcripts were aligned to each candidate UGT on the genome in Geneious (Geneious v10.2.6, Biomatters Ltd.). Exons for each UGT were extracted and concatenated and coding sequences were translated into their protein sequence. Each UGT amino acid sequence (based on genome and

transcriptome data) was searched for in the protein BLAST database and named corresponding to the top blast hit. For the purposes of comparison UGTs from three other lepidopteran species were downloaded from the National centre for biotechnology information (N.B.C.I.) 21 UGTs were downloaded for *Plutella xylostella* (Li et al. 2018), 32 for *Spodoptera exigua* (Hu et al. 2019) and 24 for *Chilo suppressalis* (Zhao et al. 2019). All sequences were aligned in Geneious using M.U.S.C.L.E. Alignments were trimmed manually for homologous regions. A phylogenetic tree was generated using MEGAX maximum likelihood model with 1000 bootstrap replications.

5.3 Results

5.3.1 Bioassay results

Results from leaf dip bioassays 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 strains Ssus. High levels of resistance (44613.6-fold) were identified in Sres with an LC_{50} of 3926 (Table 5.2).

Table 5.2. 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

5.3.2 Genome statistics

Pac-Bio and Illumina sequencing of an inbred line of *T. absoluta* generated an 819 Mbp genome. K-mer analysis revealed two coverage peaks at around 18X and 36X (Fig. 5.1) with an estimated a heterozygosity rate of 1.45%. The assembled genome comprised 3111 scaffolds with an N50 of 1124.7 Kb. Gene completeness of the assembled genome was assessed using the Benchmarking Universal Single-Copy Orthologues (BUSCO). 1013 BUSCOs groups were searched in the arthropod data set. Analysis identified 93.3% as complete BUSCOs (Table 5.3).

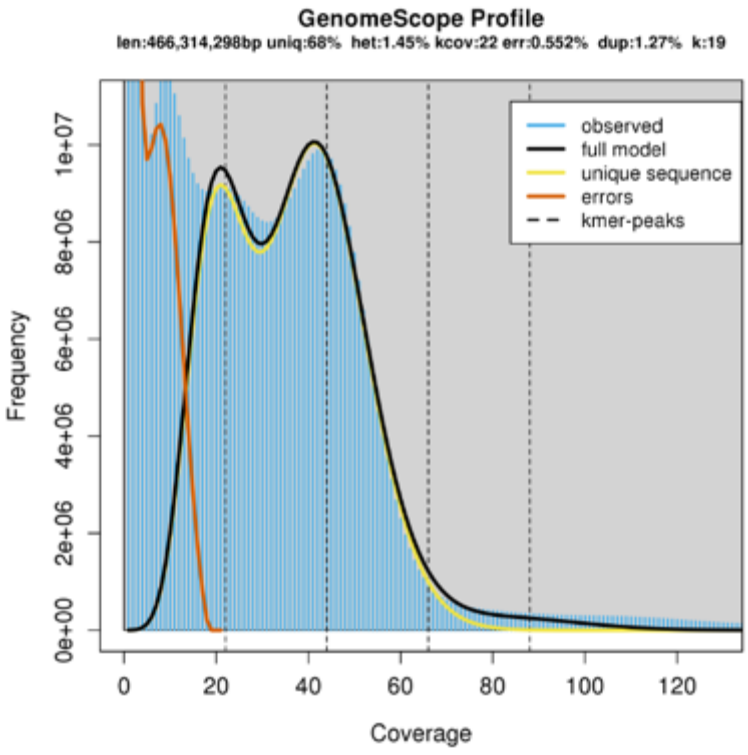


Figure 5.1. Genome scope profile showing homozygous k-mer peak at 18X and a heterozygous peak at 36X. Estimate of the heterozygous portion is 1.45%

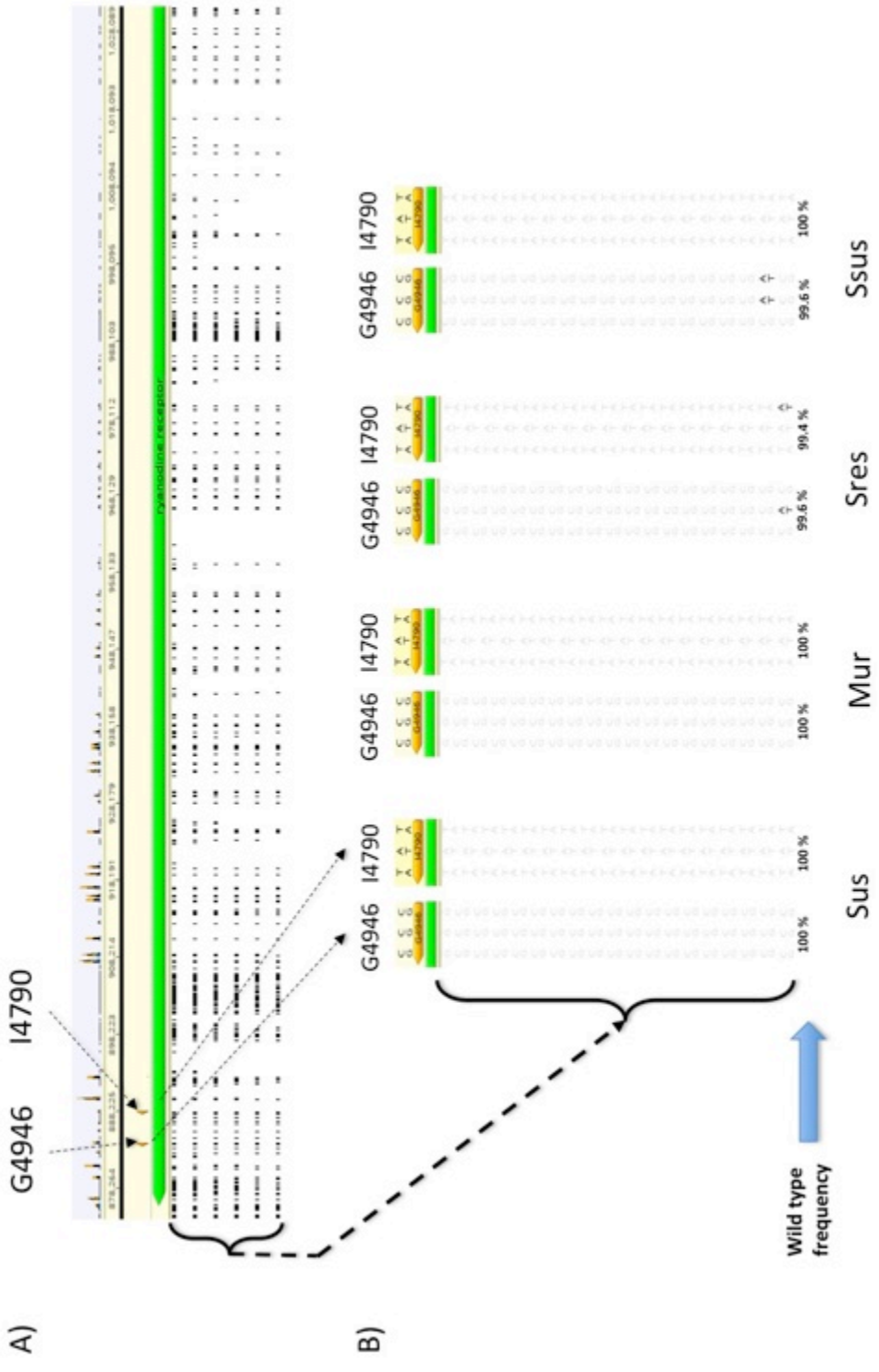
Table 5.3. Summary statistics for *T. absoluta* genome assembly including BUSCO score (1013 BUSCO groups searched in arthropod data set).

Statistic	Value
N50	1124664
L50	167
Shortest sequence	5306
Longest sequence	20421755
Mean length	263115
Median length	74935
Total sequence	3111
Total bases	818551225
GC content	35.453207
Complete Buscos	93.30%

5.3.3 Analysis of the RyR

Mapping of RNAseq data to the *T. absoluta RyR* gene (Fig. 5.2) 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 5.2. (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.



5.3.4 Differential gene expression

Analyses of differentially expressed genes revealed that 228 genes were upregulated (Fig 5.3) and 290 were downregulated (Fig 5.4) in the comparison of the Sus and Mur strains. Between Sus and Sres, 2632 genes were upregulated and 3868 were downregulated. A total of 4128 upregulated and 2747 downregulated genes were identified in the comparison of the Ssus and Mur strains. Lastly, between Ssus and Sres 1794 genes were upregulated and 1718 genes were downregulated. Cross referencing of genes upregulated in all the comparisons of resistant and susceptible strains (Fig. 5.3) and downregulated in all comparisons (Fig. 5.4) generated a short list of 20 upregulated genes (Table 5.4) and 24 downregulated genes (Table 5.5).

Figure 5.3. Venn diagram showing genes commonly upregulated in comparisons of chlorantraniliprole susceptible and resistant strains.

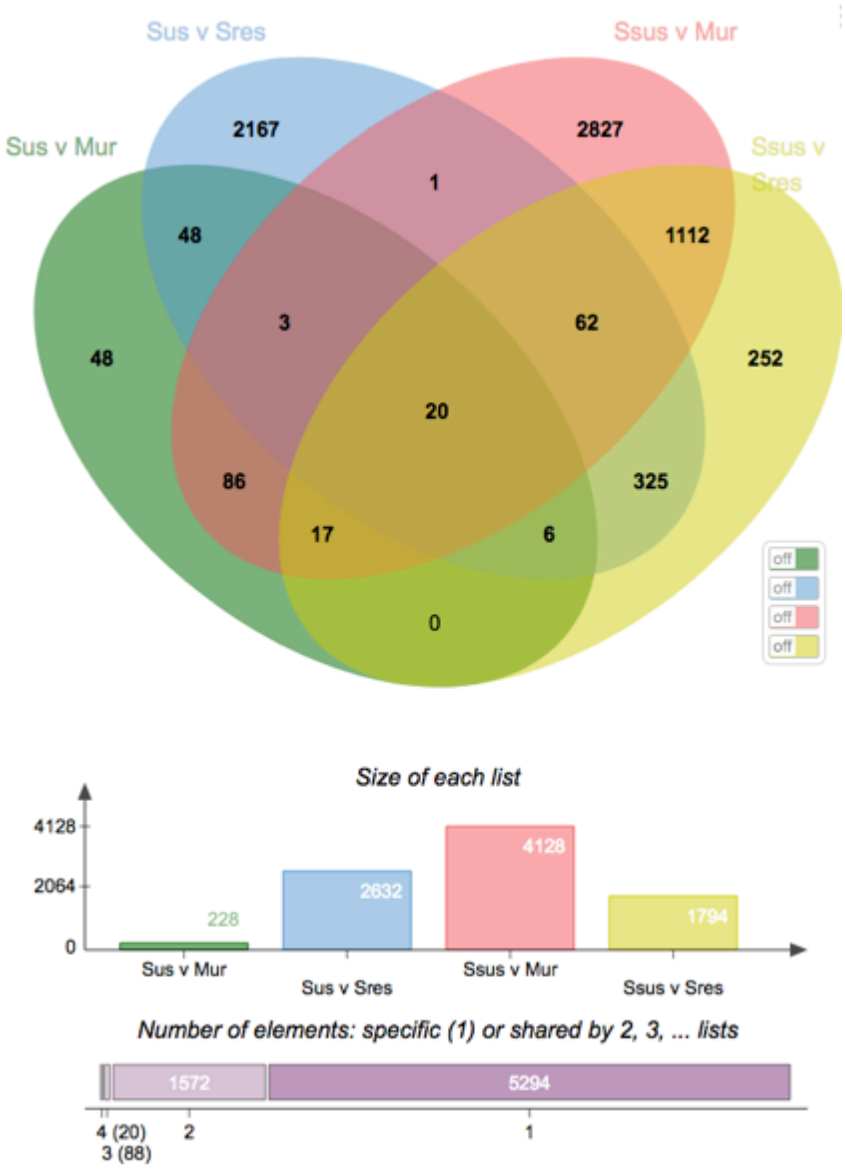


Table 5.4. Short list of upregulated genes across all comparisons of chlorantraniliprole susceptible and resistant strains, with gene number from genome annotation, sequence description from BLAST annotation and fold increase for each comparison. Colours represent level of expression with green being 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

Figure 5.4. Venn diagram showing genes commonly downregulated in comparisons of chlorantraniliprole susceptible and resistant strains.

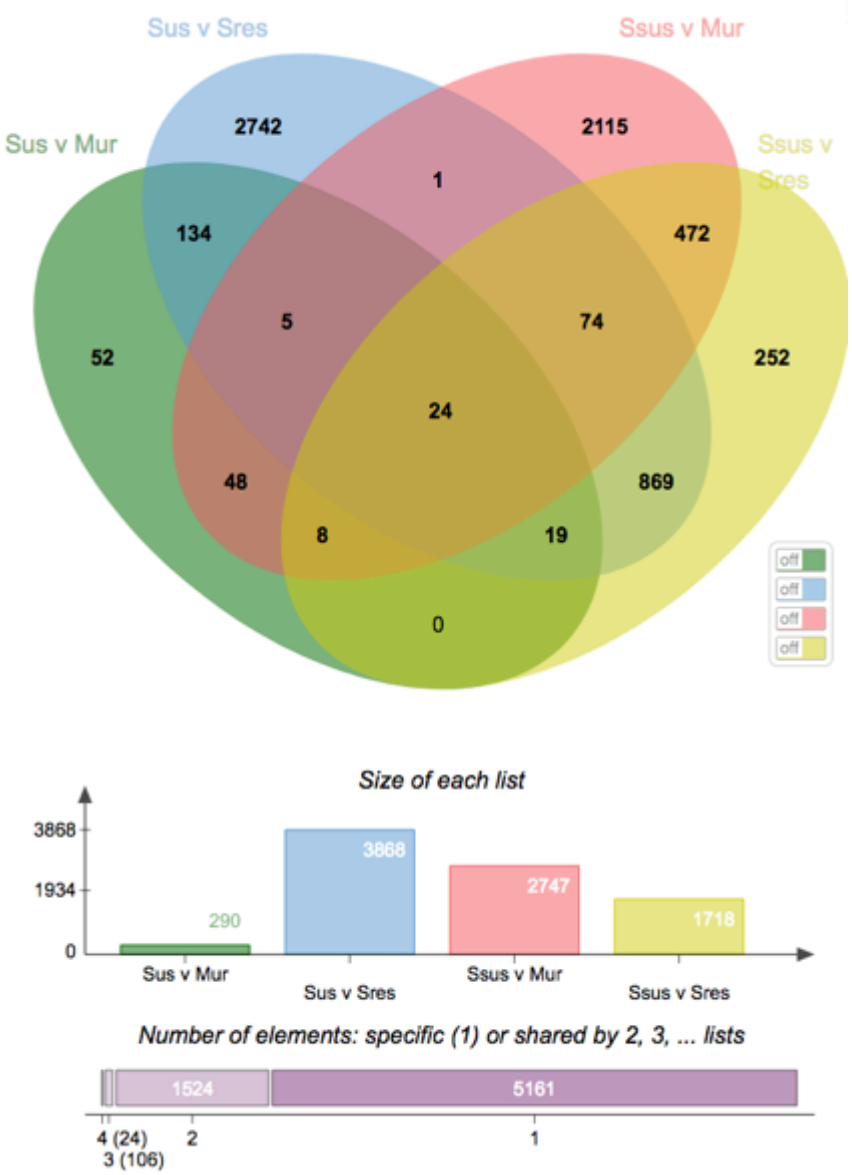


Table 5.5. Short list of downregulated genes across all comparisons of chlorantraniliprole susceptible and resistant strains, with gene number from genome annotation, sequence description from BLAST annotation and fold change for each comparison.

Gene	Sequence description	SpainSus v Mur	SpainSus v SpainRes	Sus v Mur	Sus v SpainRes
g25876	PREDICTED: glutaredoxin-1-like	-3	-227	-3	-182
g25872	RRM domain-containing protein ECU09_1470-like	-2	-222	-2	-239
g25875	uncharacterized transmembrane protein DDB_G0289901-like	-3	-156	-2	-140
g25880	uncharacterized transmembrane protein DDB_G0289901-like	-2	-145	-2	-135
g25877	PREDICTED: uncharacterized protein LOC106123703	-2	-129	-2	-113
g6351	Chymotrypsin-2	-9	-36	-2	-9
g1122	uncharacterized protein LOC110369752	-6	-9	-3	-4
g34070	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	-2	-8	-4	-14
g16403	piggyBac transposable element-derived protein 4-like	-8	-8	-7	-8
g25933	hypothetical protein B5V51_255	-6	-7	-3	-3
g6400	protein ORAOV1 homolog	-4	-6	-4	-7
g14844	---NA---	-31	-5	-22	-5
g11743	---NA---	-3	-5	-2	-4
g21426	vanin-like protein 2 isoform X2	-4	-4	-3	-3
g34057	zonadhesin-like isoform X5	-3	-4	-5	-8
g16718	hypothetical protein g.3773, partial	-5	-4	-3	-3
g3061	hypothetical protein RR48_04999	-2	-4	-5	-10
g16717	UDP-glucuronosyltransferase 2B19-like	-5	-4	-3	-2
g7814	---NA---	-2	-4	-3	-4
g27147	hypothetical protein g.4678	-8	-4	-6	-2
g15675	hypothetical protein g.8144	-2	-3	-3	-3
g17936	glutamate dehydrogenase, mitochondrial	-3	-3	-3	-2
g11540	hypothetical protein g.18910	-3	-3	-5	-4
g6401	hypothetical protein g.15803	-3	-2	-3	-2

5.3.5 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. Mapping of RNAseq data to this loci of the genome confirmed that a full length UGT was expressed identifying a 1566 bp open reading frame that translated into a protein with a 522 amino acid open reading frame. BLAST hits gave good

sequence homology to fully characterised UGTs from a range of lepidopterans (see below). Comparison of the consensus sequences derived from the four strains revealed 19 sites where amino acid residues varied within and between strains uncovering surprising heterozygosity as well as variation in frequency of genotypes present in the different phenotypes. Out of the four strains Sus was most similar to Ssus with different amino acids occurring at four sites. Sres was most similar to Mur with difference being identified at two sites (Fig. 5.5). The rest of the variation was between sus and Ssus compared to Mur and Sres. Comparison in amino acid frequency at each site revealed the majority of amino acids present in Sres at high frequency were present in Mur at moderate frequency and the two susceptible strains at low frequency (with the exception of T320, N332, A506 and E514 which were also present in one of the susceptible strains at high frequency) (Table 5.6).

Figure 5.5. Alignment of g995 consensus sequences showing variation in amino acid residues between four strains of *T. absoluta*.

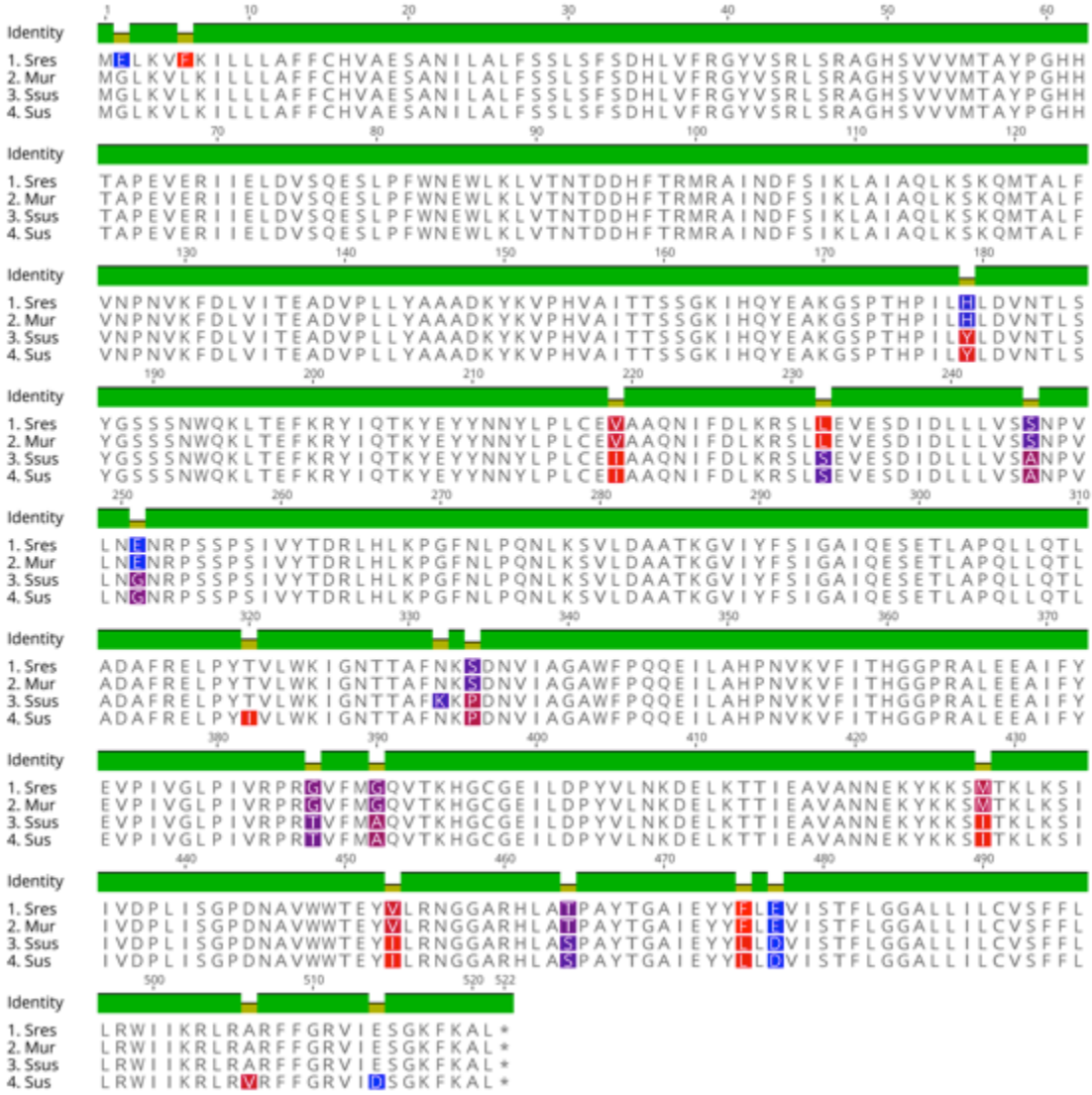


Table 5.6. Frequency of UGT g995 amino acid variation across all four strains of *T. absoluta*. LC_{50} for chlorantraniliprole and relative fold expression are included for comparison.

			Percent of transcriptswith Sres concensus residues			
Position	Sres concensus	alternative residue	Sres	Mur	Sus	Ssus
2	E	G	66	29	6	1
6	F	L	73	42	33	22
179	H	Y	81	69	20	3
219	V	I	85	68	22	7
232	L	S	83	64	13	9
245	S	A	79	57	9	5
251	E	G	82	56	9	5
320	T	I	93	95	37	99
332	N	K	98	95	90	47
334	S	P	90	78	16	15
386	G	T	82	70	10	2
390	G	A	82	53	8	2
428	M	I	89	52	9	2
453	V	I	90	69	12	6
464	T	S	86	69	13	2
475	F	L	84	61	10	2
477	E	D	84	61	9	3
506	A	V	87	72	15	98
514	E	D	92	82	34	96
	LC_{50}		3962	1.6	0.15	0.088
	Fold expression		149.1	22.6	2	1

5.3.6 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 5.7). Thus, the expression of UGT g995 in *D. melanogaster* (Fig. 5.6) resulted in a 10.8-fold increase in tolerance to chlorantraniliprole.

Table 5.7. 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

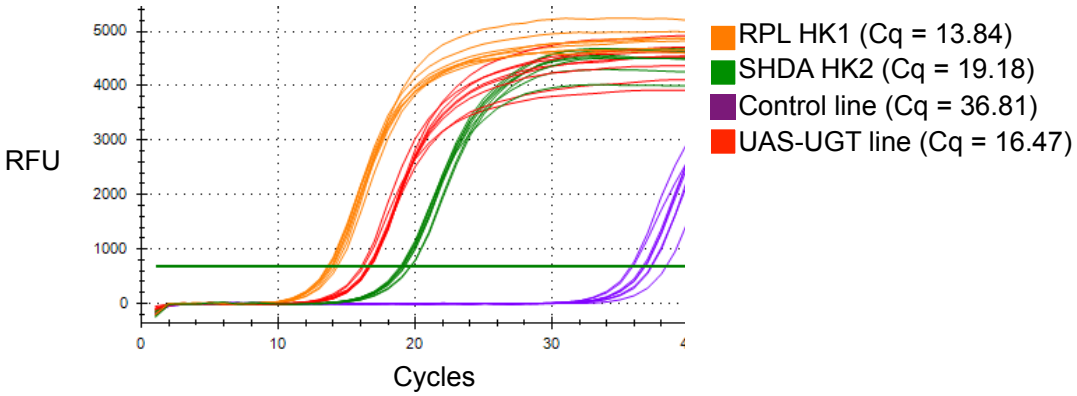


Figure 5.6. Expression of UGT g995 in UAS-UGT and Control lines compared to *D. melanogaster* housekeeping genes (HK1 and HK2).

Table 5.8 Expression of UGT in *D. melanogaster* normalised to housekeeping genes.

Treatment	Fold change in expression	Standard error
Control average	1.02	0.12
UGT average	1320351	145615

5.3.7 Phylogenetic analysis of all *T. absoluta* UGTs.

Forty UGTs were identified from the *T. absoluta genome* on the basis of sequence similarity to other insect UGT's (Table 5.9) (for sequences see appendix 3). Alignment of these sequences showed good sequence identity in conserved regions characteristic of UGTs (Fig. 5.8). Phylogenetic analysis distributed the *T. absoluta* genes throughout 11 gene families containing genes from all comparison species. Most genes (15) fell into family UGT33 (Fig. 5.9). UGT g995 fell in a small cluster of UGT34 genes.

Table 5.9. List of UGTs extracted from *T. absoluta* (sequences available in appendix 3).

Candidate *T. absoluta* UGT's	Sequence length	Top BLAST hit	% identity	Allocated Name
g995	522	UDP-glucuronosyltransferase 2C1-like [Vanessa tameamea]	64.44	UGT2C1-Like
g2599	512	UDP-glucuronosyltransferase 2B20-like [Ostrinia furnacalis]	59.45	UGT2B20-Like
g5824	323	UDP-glycosyltransferase UGT33J1 [Helicoverpa armigera]	60.75	UGT33J1-Like
g6741	506	UDP-glucuronosyltransferase 2B15-like [Hyposmocoma kahamanoa]	54.35	UGT2B15-Like
g6742	521	UDP-glycosyltransferase UGT5 [Plutella xylostella]	54.56	UGT5-Like
g7425	506	UDP-glucuronosyltransferase 2B20-like [Ostrinia furnacalis]	73.4	UGT2B20-Like
g9081	475	UDP-glucuronosyltransferase 2C1-like [Galleria mellonella]	53.46	UGT2C1-Like
g10479	356	UDP-glucuronosyltransferase 3A1-like [Papilio xuthus]	53.89	UGT3A1-Like
g12880	530	UDP-glucuronosyltransferase 2B30-like [Hyposmocoma kahamanoa]	59.69	UGT2B30-Like
g13473	521	UDP-glucuronosyltransferase 2C1-like [Vanessa tameamea]	65.2	UGT2C1-Like
g13521	555	UDP-glucuronosyltransferase 1-1-like [Hyposmocoma kahamanoa]	79.18	UGT1-1-Like
g13565	519	UDP-glucuronosyltransferase 2B20-like [Ostrinia furnacalis]	61.38	UGT2B20-Like
g13867	383	UDP-glucuronosyltransferase 2B10-like [Amyelois transitella]	59.65	UGT2B10-Like
g15009.1	529	UDP-glucuronosyltransferase 2B15-like [Ostrinia furnacalis]	53.03	UGT2B15-Like
g15009.2	533	UDP-glycosyltransferase UGT5 [Plutella xylostella]	51.8	UGT5-Like
g16225.1	518	UDP-glycosyltransferase UGT33J1 [Helicoverpa armigera]	59.33	UGT33J1-Like
g16225.2	519	UDP-glucuronosyltransferase 2B4 isoform X1 [Manduca sexta]	61.79	UGT2B4-Like
g16225.3	517	UDP-glucuronosyltransferase 2B20-like [Ostrinia furnacalis]	55.98	UGT2B20-Like
g16226	516	UDP-glucuronosyltransferase 2B20-like [Ostrinia furnacalis]	55.8	UGT2B20-Like
g16227	483	UDP-glucuronosyltransferase 2B20-like [Ostrinia furnacalis]	60.73	UGT2B20-Like
g16228	507	UDP-glucuronosyltransferase 2B20-like [Ostrinia furnacalis]	55.14	UGT2B20-Like
g16232	504	UDP-glycosyltransferase UGT33AF1 [Chilo suppressalis]	58.25	UGT33AF1-Like
g16717	518	UDP-glucuronosyltransferase 2B20-like [Ostrinia furnacalis]	55.04	UGT2B20-Like
g20872	516	UDP-glucuronosyltransferase 2B2-like [Papilio machaon]	60.74	UGT2B2-Like
g21063	147	UDP-glucuronosyltransferase 2B19-like [Spodoptera litura]	55.98	UGT2B19-Like
g22125	523	UDP-glucuronosyltransferase 2B20-like [Ostrinia furnacalis]	72.22	UGT2B20-Like
g22850	162	UDP-glycosyltransferase UGT50A4 [Zygaena filipendulae]	68.42	UGT50A2-Like
g22851	230	UDP-glycosyltransferase UGT50A2 [Danaus plexippus plexippus]	40.48	UGT50A4-Like
g22875	522	UDP-glucuronosyltransferase 2C1 [Hyposmocoma kahamanoa]	76.72	UGT2C1-Like
g27172	418	UDP-glucuronosyltransferase 2B20-like [Spodoptera litura]	58.91	UGT2B20-Like
g27899	497	2-hydroxyacylsphingosine 1-beta-galactosyltransferase-like [Hyposmocoma kahamanoa]	68.35	2-hydroxyacylsphingosine 1-beta-galactosyltransferase-
g28274	516	UDP-glucuronosyltransferase 2B20-like [Ostrinia furnacalis]	55.9	UGT2B20-Like
g29883	257	UDP-glucuronosyltransferase 2B1-like [Galleria mellonella]	63.53	UGT2B1-Like
g30114	391	UDP-glucuronosyltransferase 2C1 [Hyposmocoma kahamanoa]	72.04	UGT2C1-Like
g31445	389	2-hydroxyacylsphingosine 1-beta-galactosyltransferase-like [Hyposmocoma kahamanoa]	67.01	2-hydroxyacylsphingosine 1-beta-galactosyltransferase-
g31448	249	2-hydroxyacylsphingosine 1-beta-galactosyltransferase-like [Hyposmocoma kahamanoa]	69.42	2-hydroxyacylsphingosine 1-beta-galactosyltransferase-
g32146	495	UDP-glucuronosyltransferase 2B2-like [Papilio machaon]	63.51	UGT2B2-Like
g32147	525	UDP-glucuronosyltransferase 2B10-like [Hyposmocoma kahamanoa]	78.41	UGT2B10-Like
g33223	440	UDP-glucuronosyltransferase 2C1 [Hyposmocoma kahamanoa]	73.19	UGT2C1-Like
g182652	145	UDP-glucuronosyltransferase 2B20-like [Spodoptera litura]	51.74	UGT2B20-Like

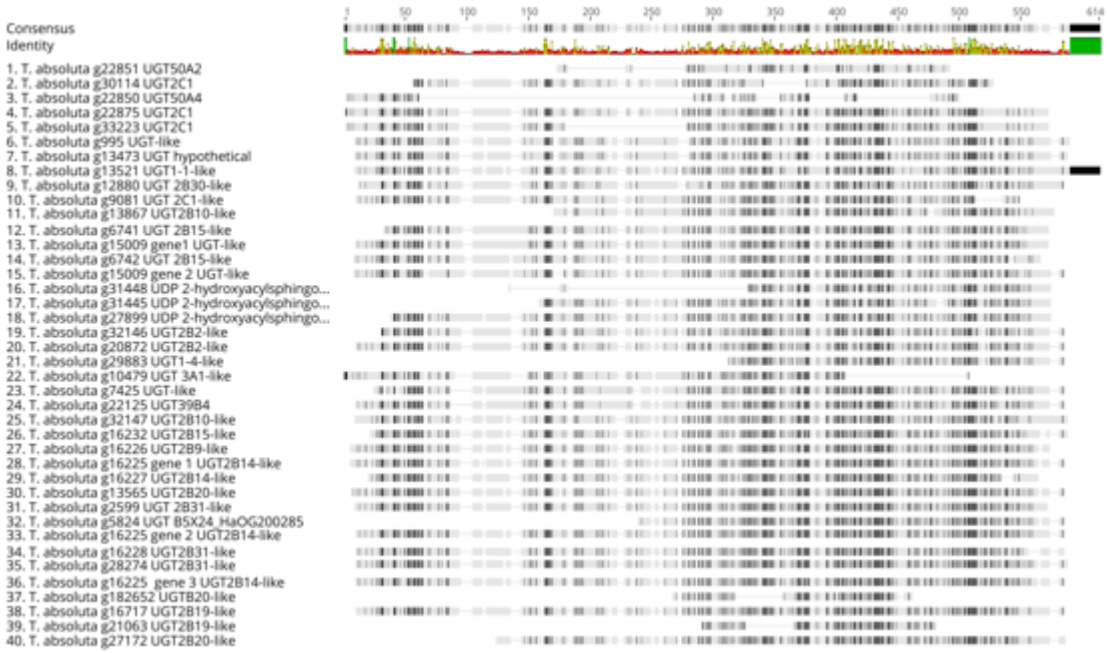


Figure 5.8. Alignment of all candidate *T. absoluta* UGTs. Dark black bars indicate sequence identity between alignments.

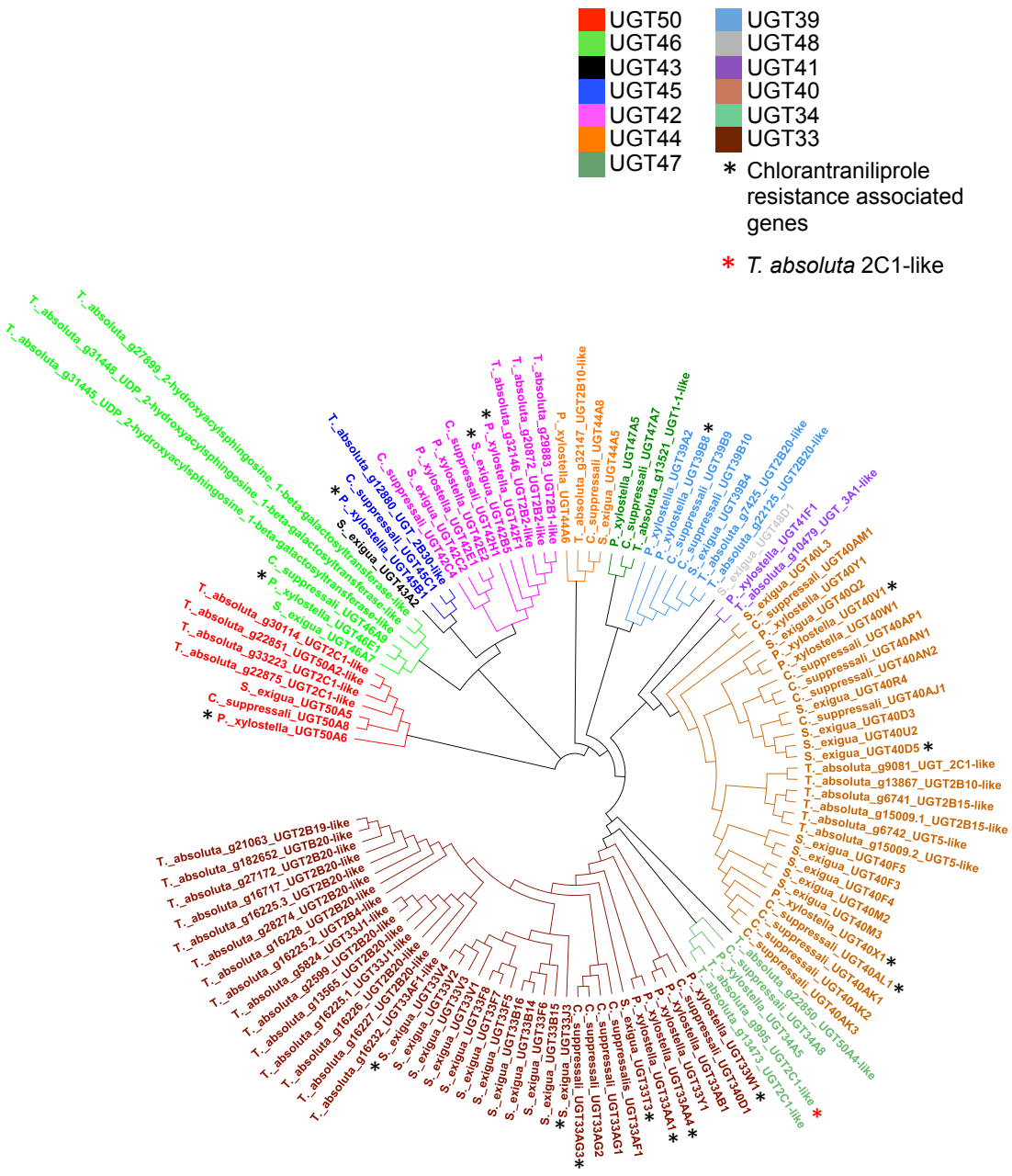


Figure 5.9. Maximum likelihood phylogenetic tree showing family-wise distribution of UGT genes in *T. absoluta, P. xylostella, S. exigua* and *C. suppressalis.*

5.4 Discussion

In this chapter I provide several lines of evidence that metabolic detoxification is a key determinant of the resistance of *T. absoluta* to the pesticide chlorantraniliprole, through the increased expression of UGT 2C1-like (g995). Differential gene expression analyses provided a short list of 20 genes that were consistently and significantly overexpressed in both chlorantraniliprole resistant strains compared to both susceptible strains and 24 genes that were consistently downregulated in these comparisons. All these genes had a fold change between resistant and susceptible strains of greater than two. Of these genes there was one clear candidate resistance gene. UGT 2C1-like was selected for further analysis based in its expression profile and its presumed function based on sequence homology to the UGT family of detoxification enzymes. Furthermore, the level of overexpression of this gene in the resistant strains correlated with their sensitivity to chlorantraniliprole, with UGT 2C1-like was >100-fold overexpressed in comparisons of the highly resistant Sres with both susceptible strains, and >20-fold overexpressed in the moderately resistant Mur strain.

The mapping of transcripts from all strains to the region of the genome that contained UGT 2C1-like revealed amino acid variation suggesting that all populations were heterozygous at 19 sites (Fig. 5.5). Furthermore, the expression of the two different isoforms seemed to correlate with resistant phenotypes. The consensus sequence alignment between the two susceptible strains and the resistant and tolerant strains suggests that the two isoforms

were differentially expressed in the two different phenotypes. The consensus of Sres and Mur differed at 2 sites G2E and L6F both of these were unique to Sres. Ssus and sus differed at four sites T320I, K332N, V506A and D514E. The rest of the consensus differences were between Ssus and sus compared to Sres and Mur. Selection therefore may have driven overexpression of UGT g995 in conjunction with selection for increased gene frequency of the isoform observed in Sres and Mur. These results suggest that while shift in expression of isoform frequency may contribute partially to the resistance profile, i.e. the differences in resistance between the two susceptible strains and Mur, the fact that the difference in resistance between Mur and Sres was so great (2476-fold) implies the major contributing factor to the highly resistance phenotype of Sres is the overall increase in its expression. For this reason only the isoform expressed in Sres was synthesised and expressed in *D. melanogaster* lines. The marked (~11-fold) tolerance of flies expressing UGT g995 compared to flies of the same genetic background but lacking the transgene demonstrates that overexpression of this gene is sufficient to confer resistance in vivo. Thus both expression and functional analyses implicate this gene as a *bona fide* chlorantraniliprole resistance mechanism in *T. absoluta*. Furthermore, these results are consistent with studies on *P. xylostella* which showed up regulation of UGT2B17 in response to chlorantraniliprole application (Li et al. 2017) and *C. suppressalis* (Li et al. 2017; Zhao et al. 2019) in which up regulation of UGT UGT40AL1 and UGT33AG3 were also correlated with tolerance to chlorantraniliprole. Together these results clearly show that differential regulation of UGTs is a convergent response to chlorantraniliprole application.

As this chlorantraniliprole resistant population of *T. absoluta* was detected in Span, there is a high risk that it could be imported to the UK. Spain exported 112 025 metric tonnes of tomatoes to the UK in 2018 (Blazquez 2021), and this, combined with the fact that *T. absoluta* is no longer a quarantinable pest, suggests a possible route for chlorantraniliprole resistance to enter the UK. My results are also important to pest management strategies as they demonstrate that resistance to chlorantraniliprole can develop in the absence of target-site resistance. Molecular-based resistance assays to identify mutations in insecticide target-sites have been proposed as alternatives to traditional bioassays to determine the resistance status of pest populations and inform pesticide application (Troczka et al. 2012). However, the presence of metabolic resistance could lead to false predictions from data derived from such assays. To detect the metabolic resistance described in this chapter attempts to assess field resistance through molecular assays would have to be complemented by phenotyping by bioassays. The latter could also include the use of inhibitors of UGTs and/or other enzyme systems (i.e. synergist assays) to provide evidence of potential metabolic resistance in the population. Such assays my also help facilitate selection of appropriate synergist assays for the inhibition of UGT activity. In this regard the findings presented in this chapter could inform the development of pesticide formulations through the incorporation of synergists. Specifically, the knowledge that UGTs confer diamide resistance should encourage the testing of compounds known to inhibit UGTs for use in such formulations. This practice has been used successfully in the past with PBO being added to pyrethroid based pesticides to maintain their efficacy in the face of metabolic

detoxification by P450s (Gleave et al. 2018). PBO has been shown to reduce the production of CHP-glucose conjugates, the products of UDP-glucosyltransferase detoxification reactions in *M. domestica* exposed to pyraclofos (S.-W. Lee et al. 2006). In an imidacloprid resistant population of *Diaphorina citri,* UGTs were highly over expressed. Application of two synegists, 5-nitrouracil and sulfinpyrazone resulted in a significant increase of pesticide toxicity (5.89- and 8.15-fold, respectively) (Tian et al. 2019). In human drug research a wide range of compounds have been identified that inhibit a broad range of UGT activity including atazanavir and erlotinib for 1A1, Hecogenin for 1A4, Niflumic acid for 1A9, Fluconazole for 2B7 and *S*-Nicotine for 2B10. The fact that UGT g995 is readily expressed in *D. melanogaster* as a functional enzyme means these lines could be used as a screening tool for the assessment of novel synergists aiding the enhancement of contemporary pesticide products and extending the effective lifespan of active ingredients. The importance of maintaining diamide pesticides through such processes is heightened due to the loss of efficacy of other pesticides used in current UK IPM strategies such as spinosad.

The full complement of insect UGTs have been identified for several lepidopteran species, but few have been linked with pesticide resistance. Genomic analysis of *P. xylostella,* a polyphagous pest, revealed a UGTome consisting of 23 UGTs belonging to 11 families (Li et al. 2018). The expression of these UGTs were then examined in the presence of 10 pesticides with 10 significantly up regulated in response to either one or several pesticides. All UGTs were also identified from the *S. exigua* genome

comprising 32 genes. Exposure of this species to pesticides was associated with the upregulation of 5 UGTs. Analysis of the *T. absoluta* genome identified 40 UGT-like genes and phylogenetic analysis revealed they were distributed among 11 families with most of the UGTs (15) falling into the lepidopteran specific family UGT33 (Ahn, Vogel, and Heckel 2012) as was also observed in *P. xylostella* (Li et al. 2018) and *S. exigua* (Hu et al. 2019). The phylogenetic analysis also showed that UGTs identified as being differentially expressed in response to pesticide resistance in *P. xylostella* and *S. exigua* had a closely related UGT in *T. absoluta* (Fig. 5.9). This finding suggests that *T. absoluta* UGTs may also have the direct potential, through upregulation, or the evolutionary potential, through adaptation of substrate specific sites, to facilitate detoxification to a broad range of pesticides. The genome will aid any future gene expression analysis by allowing differentially expressed genes to be identified more accurately. It will be a powerful resource for a wide range of future studies on *T. absoluta* including future work on resistance. It can provide sequence data on pesticide target sites as well as all metabolic resistance genes such as ABCs, P450s esterases and GSTs. This will increase the speed at which resistance mechanisms can be identified - informing IPM and aiding resistance management.

Prior to commencement of our *T. absoluta* genomic and transcriptomic sequencing project the *T. absoluta* transcriptome had been sequenced by Camargo et al. (2015) As no genome had been published they utilised a *de novo* assembly approach using transcriptomes from different *T. absoluta* life stages. This identified target genes for silencing through the development of

RNAi tools - inhibiting *T. absoluta* development. Subsequently, Tabuloc *et al.* (2019) released a draft genome to facilitate the successful creation of a diagnostic for accurately identifying *T. absoluta* species from closely related and morphologically similar species- assisting accurate monitoring of potential *T. absoluta* invasion to the USA. Our sequencing approach differed from Tabuloc et al. (2019) through the additional use of Pac Bio long read technology along with high fidelity Illumina short read sequencing, as used in their assembly, allowing reads to span the low complexity and repetitive regions creating accurate *de novo* assemblies (Pollard et al. 2018). Our approach gave a larger genome (819/677Mbp) with more contiguous sequencing N50 (1124.66/26.36Kb) and lower L50 (167/10813). Our assembly also had a higher BUSCO score with (93.3/92.4%) identified as complete (Tabuloc et al. 2019). Our genome assembly builds on these previous resources, all of which being valuable assets for future research into monitoring and control of *T. absoluta.*

Organisms that are polyphagous like *T. absoluta* are exposed to a broad range of plant secondary metabolites and are therefore likely to have a broad range of metabolic enzymes capable of detoxifying them such as UGTs. In *O furnacalis* UGT's are implicated in the detoxification of the secondary metabolite cyclic hydroxamic acid (Kojima et al. 2010). UGT's in *B. mori* showed substrate specificity for flavonoids, coumarins, terpenoids and simple phenols (Luque, Okano, and O'Reilly 2002). In *M. separate* UGTs were shown to be important in the detoxification of benzoxazinoids (Sasai et al. 2009). Having gene families evolved to detoxify a broad spectrum of natural

xenobiotics increases the likelihood of cross-resistance to other compounds including synthetic pesticides. Duplication of genes within these families also allows for greater capacity for adaptive mutations in specific genes giving greater potential to generate genes with novel substrate specificity. UGT detoxification has been linked to resistance to a wide range of pesticides including DDT in *D. melanogaster* (Pedra et al. 2004), pyrethroids in *A. gambiae* (Vontas et al. 2005), organophosphates in *M. domestica* (S.-W. Lee et al. 2006) and carbamates in *M. persicae* (Silva et al. 2012), neonicotinoids in *B. tabaci* (Yang et al. 2013) and chlorantraniliprole in *C. suppressalis* (Zhao et al. 2019). Deeper understanding of UGT family diversity, sequence information and functional specificity is especially important for pest species to greater understand their potential to evolve resistance and facilitate quick detection of resistance mechanisms. UGT phylogeny provided by this study is a valuable resource to facilitate detection of their contribution to metabolic resistance to any pesticide through the qPCR of these specific genes in resistant strains rather than whole transcriptome analysis, reducing cost and increasing speed of results.

In summary, these results show that the >100-fold increase in expression of UGT g995 contributes to the >45000-fold increase in resistance to chlorantraniliprole observed in the resistant Sres strain of *T. absoluta* compared to the susceptible strain. This is supported by expression of the gene in transgenic fly lines resulting in a 10.8-fold increase in resistance to the compound. Analysis of the *T. absoluta* genome revealed 40 UGTs and phylogenetic analysis of these showed UGTs were distributed in a broad

range of gene families. These gene families were also present in other pest Lepidoptera, which differentially regulated UGT expression in association with a broad range of pesticides. These findings are of value to pest management as they (1) Identify UGT mediated detoxification as a novel chlorantraniliprole resistance mechanism in *T. absoluta.* (2) They provide a potential screening tool to test the effects of potential synergistic compound that could facilitate development of effective pesticide formulations and (3) they provide resources to aid assessment of UGTs contribution to newly arising resistant phenotypes to all pesticides.

6. Parthenogenesis in UK populations of *T. absoluta*

The results detailed in this chapter have been published in *Pest management science* (appendix 4).

Grant C, Jacobson R, Bass C. Parthenogenesis in UK field populations of the tomato leaf miner, Tuta absoluta, exposed to the mating disruptor Isonet T. Pest Manag Sci. 2021

6.1 Introduction

Tuta absoluta is a highly destructive pest of tomato crops globally. First identified as serious agricultural pest of tomato crops in South America, *T. absoluta* reached the UK in 2009 and is now present in most of Europe, Africa, the Middle East and parts of Asia (Campos et al. 2017). The economic impact of *T. absoluta* on the tomato growing industry can be profound resulting in 100% yield loss if left untreated, with 60% of global tomato crops estimated to have been affected (Campos et al. 2017). In the UK, growers have reported losses of up to £ 50 000 per hectare (Rob Jacobson, personal communication). Control of *T. absoluta* has been achieved through the implementation of an integrated pest management (IPM) strategy, incorporating crop monitoring, biological control and application of pesticides. This strategy, once established, proved extremely effective, however, over time it became compromised due to the emergence of resistance to several of the insecticides used for control (Siqueira, Guedes, and Picanço 2000; Grant et al. 2019). To restore full control through IPM, a mating disruptor was developed by Shin Etsu Chemical Co. Ltd and introduced to the market under

the product name of Isonet T. This product works by inundating closed glasshouse environments with a synthetic version of the female sex pheromone of *T. absoluta* (3E,8Z,11Z)-3,8,11-Tetradecatrienyl acetate) (Attygalle et al. 1996). When deployed, the high levels of synthetic pheromone in the environment prevent male moths effectively detecting semiochemical concentration gradients emitted by females, inhibiting location of a mate and thus preventing reproduction. The incorporation of Isonet T into contemporary IPM proved a spectacular success and was found to eradicate outbreaks in as little as one generation (Jacobson 2018). This product not only eliminated yield losses, but also decreased reliance on chemical pesticides, thus providing additional environmental benefits. In spite of Isonet T's remarkable success in many UK commercial tomato glasshouses, one grower in Evesham subsequently reported loss of effective control of *T. absoluta* by the product (Rob Jacobson, personal communication). *T. absoluta* at this site were also highly resistant to spinosad (Grant et al. 2019), a pesticide used in previous IPM strategies, resulting in limited options for alternative chemical control.

The effectiveness of mating disruption in eliminating pest populations can be severely compromised if the target pest has the capacity to reproduce in the absence of sex. In this regard, research by Megido and Verheggen (2012) previously indicated that French populations of *T. absoluta* have the capacity to reproduce asexually through the process of deuterotokous parthenogenesis. The authors found that 4 out of 20 virgin females laid viable eggs, and 57 larvae reached adulthood with a sex ratio of 1/1.5 (male/female).

However, the fecundity of these adult moths was not reported. Following this, work by Abbes and Chermiti (2014) demonstrated that individual females from three virgin populations of Tunisian *T. absoluta* (30 individuals for each population) could lay eggs (30%, 13.33% and 50%), of which 11.36%, 16.67 and 17.02% hatched. However, out of a collective 10 F1 female virgins only one egg was laid and this was not viable. Both of these studies demonstrate parthenogenetic reproduction in *T. absoluta*. It is therefore possible that the loss of efficacy of Isonet T in the Evesham glasshouse was caused, at least in part, by parthenogenetic reproduction circumventing the effectiveness of mating disruption.

Asexual reproduction in *T. absoluta* is likely tychoparthenogenetic, a process by which a small proportion of unfertilized eggs hatch from females of typically sexually reproducing species (Ball 2001). Offspring survival of this reproductive mode is typically much lower than for sexual reproduction (Kramer, Templeton, and Miller 2002). However, tychoparthenogenesis can result in a positive feedback system in which males are lost through female-biased sex ratios and increasing mate limitation. As a result, the strength of selection for tychoparthenogenesis increases in concert with the proportion of tychoparthenogenetic offspring in the sexual population (Schwander et al. 2010).

In the current study we assessed (1) the level of parthenogenetic reproduction in UK populations of *T. absoluta*, and (2) looked for evidence of any potential shift in frequency or viability of parthenogenetic reproductive output as a result

of selection through the use of Isonet T. The data provided by this study is of direct relevance to the control of UK populations of *T. absoluta* and the robustness of current IPM strategies for this pest.

6.2 Methods

6.2.1 Insect cultures

Insects were collected from R & J Holts Sandylands Nurseries (Evesham) in 2016 and 2019, i.e. before and after the deployment of Isonet-T. All insect stages were housed in bug dorm cages (MegaView science co.) in CE rooms at 24°C 16L:8D and 65% humidity. Cultures were supplied with tomato plants (var. Money Maker) *ad libitum*. Insects were collected and sexed for experimentation at pupal stage.

6.2.2 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.

6.2.3 *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.

6.3 Results

6.3.1 F0 virgin reproductive traits

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 6.1 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 6.2). 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.

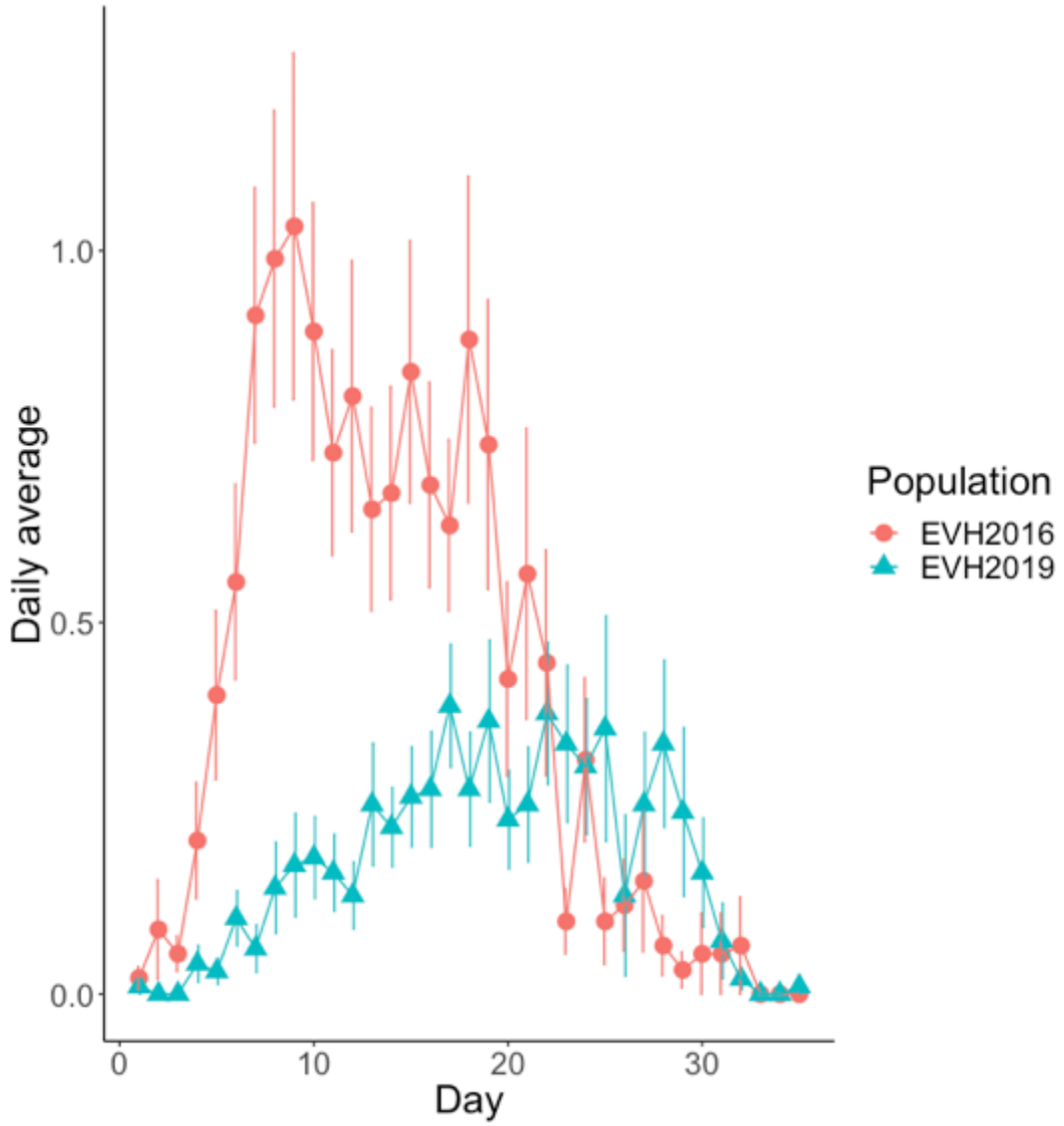


Figure 6.1 Differences in average daily egg production by virgin females of the EVH2016 and EVH209 strains over 35 days (W = 806, p-value = 0.02). Bars represent ± SEM.

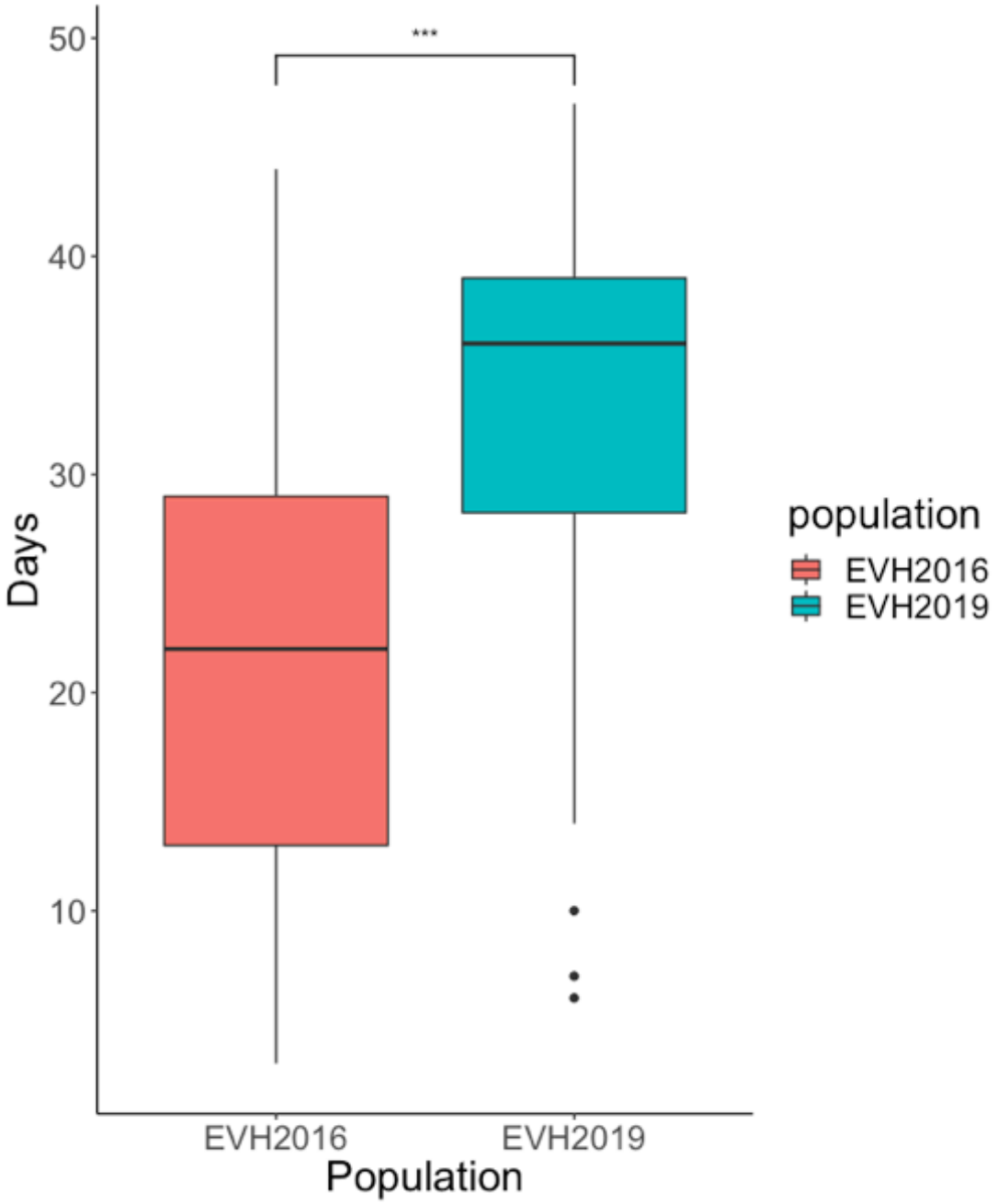


Figure 6.2 Box plots showing range of lifespan of virgin females from EVH2016 and EVH2019 strains (W = 1682, p-value < 0.001).

6.3.2 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 6.3).

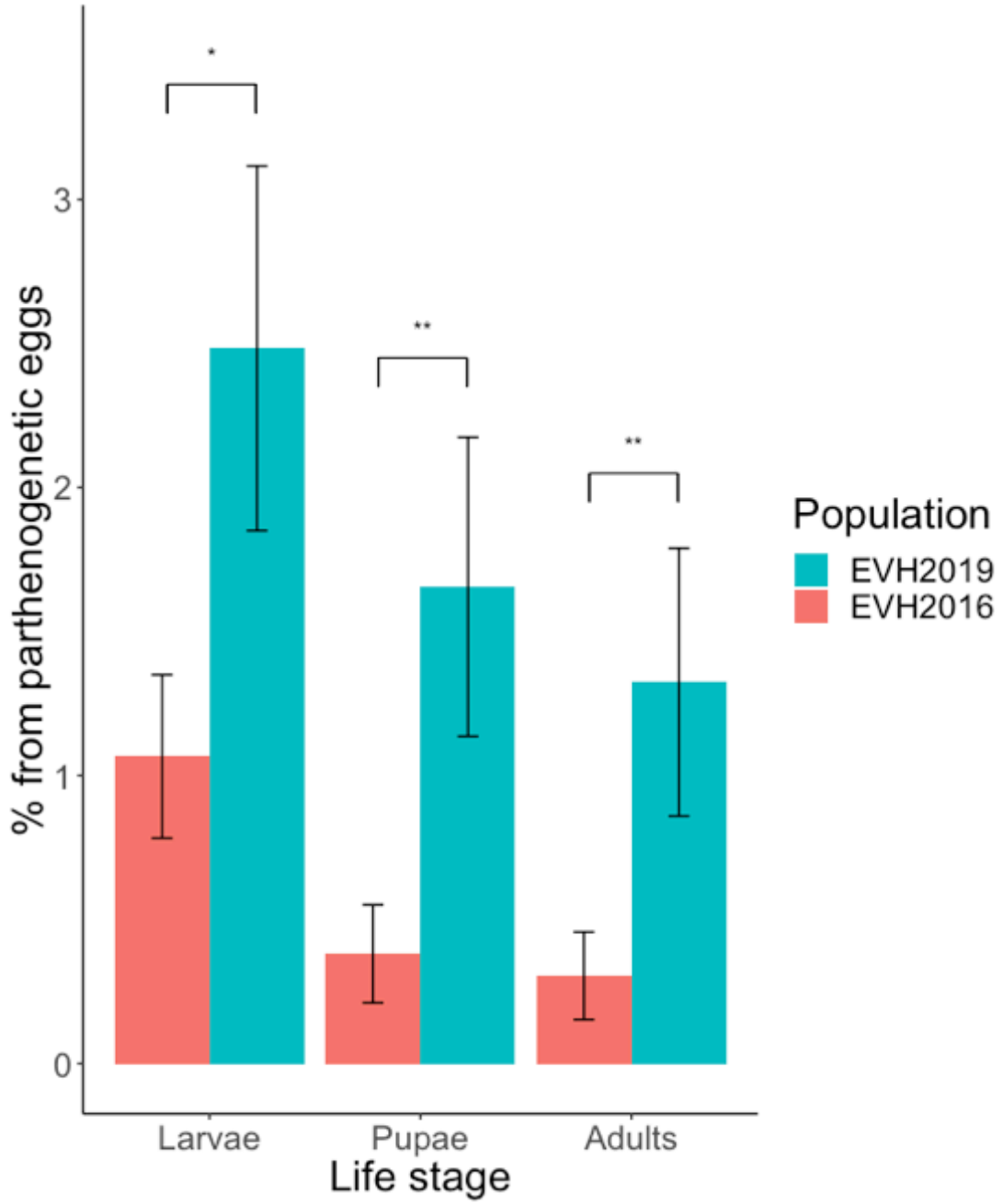


Figure 6.3 Percentage differences in larvae, pupae and adults from total number of eggs laid by EVH2016 and EVH2019 (* $p \leq 0.05$, ** $p \leq 0.01$).

6.4 Discussion

Our results reveal clear differences in life history traits associated with reproduction between two populations of virgin *T. absoluta* that were differentially exposed to mating disruption in the field. These included marked differences in the number of eggs laid (Figure 6.1), the start date of laying and lifespan (Figure 6.2). Both populations from Evesham had a low frequency of virgin females capable of laying viable eggs, and significant differences between these populations in the number of larvae, pupae and adults produced were not observed. However, a significant increase in their proportions as a function of eggs laid was observed (Figure 6.3).

What are the implications of these results for the resilience of control employing mating disruption against *T. absoluta* in the UK? Firstly, the very low levels of parthenogenesis observed in both the Isonet T exposed and unexposed populations suggests changes in the rate of parthenogenesis, in isolation, are unlikely to explain the reduced levels of *T. absoluta* control reported at the Evesham glasshouse in 2019. A small but significant increase in the proportion of eggs developing parthenogenetically was observed in the EVH2019 population compared to EVH2016. However, the reduced total egg production of the former strain in comparison to the latter resulted in no significant increase in total number of larvae, pupae and adults produced between the two strains. This mitigated any effect of the increased rate of parthenogenesis of the EVH2019 on population size. Furthermore, in both populations no first generation virgin females went on to produce viable eggs.

This suggests that the ability of *T. absoluta* to persist over more than one generation by parthenogenesis is limited, however, given the small sample size of the second generation in this study, more extensive testing is required to confirm this.

Secondly, the marked differences in life history traits between virgin females of the EVH2016 and EVH2019 strains may also have relevance for IPM incorporating mating disruption. Evolutionary theory shows that life history traits are controlled by energetic trade-offs between intrinsically controlled factors such as reproductive output and longevity (Roff 2002). It is plausible therefore that in a mate-limited environment, selection would result in the diversion of energetic resources away from egg production and reallocation to longevity. The reduction in egg production would therefore be an antagonistic pleiotropic result of increased longevity - a strategy that would increase the likelihood of encountering a mate. Furthermore, the delay in average egg laying date observed in the EVH2019 strain would postpone energetic expenditure in reproductive output, facilitating longevity (Wu et al. 2018), until a mating occurs, which in turn stimulates egg production.

Both parthenogenesis (Schwander et al. 2010) and longevity (Mori and Evenden 2013) have been shown to result from populations that are male limited or at low density, which is consistent with our data. Furthermore if parthenogenesis has a selective advantage in pheromone inundated environmental conditions, as suggested by the observed significant increase in its frequency, it could further proliferate as a result of a positive feedback

loop, with males lost through a skewed parthenogenetic sex ratio, as observed in EVH2019. The overall reduction in egg production of this population could further drive parthenogenetic evolution, as a reduction in overall offspring produced from a low frequency of sexual encounters within the population would increase the relative fitness of any offspring produced through parthenogenesis. Thus, the increased rate of deuterotokous parthenogenesis in combination with a shift in longevity and egg laying date may allow the persistence of populations within the crop - allowing populations to re-establish if there was any diminution of mating disruption. Furthermore, while sampling of additional field populations is required, our data supports the notion that there could be an inherited genetic component to asexual reproduction in *T. absoluta* that can be selected for under conditions that limit sexual reproduction.

Finally, the changes in *T. absolutas'* life history traits may also have synergistic effects on the efficacy of biocontrol agents used as part of IPM in the UK. The predatory bug, *Macrolophus pygmaeus*, requires time to build effective population sizes in the crop. This means that pesticide applications are often required to knock back *T. absoluta* populations until the natural enemy is established. Later egg laying and longer life span of *T. absoluta* would increase the time available for this process to occur. However, *M. pygmaeus* is polyphagous and can cause damage to the crop if densities are too high. Therefore a reduction in density of the applied biocontrol may be required to mirror any reduced reproductive output of *T. absoluta*.

In summary, the observed increase in rate of parthenogenesis in UK glasshouse populations of *T. absoluta* following the deployment of Isonet T is unlikely to explain partial control loss of mating disruption. This news is especially welcome in light of *T. absoluta's* capacity for resistance to chemical control (Grant et al. 2019). However, the marked differences in life history traits in UK populations that differ in exposure to mating disruption has implications for the current use of this control method, resulting in populations that may be more resilient at persisting within the crop at lower densities. Thus, further sampling of UK populations is warranted to examine the extent to which the modified life history traits identified in this study are observed in *T. absoluta* populations from glasshouses using Isonet T for control. Furthermore, the fact that deuterotokous parthenogenesis exists, the fact that populations are male restricted, and the fact that populations persist at low densities, does - according to evolutionary theory - provide the right circumstances for further changes in the rate of parthenogenesis to evolve.

7. General discussion

7.1 Importance of studying resistance in *T. absoluta*

Tomatoes are the second most important vegetable crop after potato (Wakil, Brust, and Perring 2017). *T. absolutas'* potential to reduce the yield of tomato production is considerable, destroying whole crops if left unchecked. Originating in South America, which accounts for 3.8% of global tomato production (FAOSTAT), *T. absoluta* has spread over a 15 year period to a near global distribution and has infested over 60% of global tomato crops (Campos et al. 2017). *T. absoluta* arrived in the UK in 2009 and immediately had a devastating effect on the tomato industry with 30% of fruits being damaged and losses of £50K per hectare. Lack of practical knowledge about a pest with a comprehensive pre-existing resistome meant control measures were difficult to implement. In spite of a limited number of available pesticides, in 2014, a robust IPM strategy was established. Field trials demonstrated that the incorporation of the insecticides spinosad and chlorantraniliprole with the biocontrol agent *M. pygmaeus* was successful at supressing populations to the point where the pest was no longer an immediate concern (Jacobson and Howlett 2014). However, the author of the IPM strategy warned of the potential of evolution of resistance and cautioned against complacency in the long-term effectiveness in the program without relief of selection pressure imposed by the pesticides from alternative control measures.

7.2 Spinosad resistance

Loss of control to spinosad was reported in glasshouses in the UK in 2015. The research presented in chapter 3 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 spinosad 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 spinosad 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 spinosad 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 from Evesham and North Yorkshire. The excision of exon 4 from mRNA resulted in a frame shift and PTC 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 spinosad resistance, however this

alteration didn't result in a frame shift or the introduction of a PTC. In spinosad resistant strains of *B. doralis,* α6 transcripts were non functional and expressed PTC in exon7 (Hsu et al. 2012). Loss-of-function mutations were also reported in spinosad resistant strains of *D. melanogaster* where α6 subunits lacked the TM3, cytoplasmic loop, TM4 and extracellular C-terminal tail domains. These individuals also showed no loss of fitness under laboratory conditions (Perry, McKenzie, and Batterham 2007). Spinosad resistance mechanisms appear to fall into one of two categories, either target-site alterations that impede the binding of spinosad (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 NMD, 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). Beyond insects, work on nAChR composition in mice showed that α6 knockouts showed no signs of defects and were viable (Champtiaux et al. 2002). This was associated with the upregulation of α4 subunits. nAChRs were then purified and their subunit content determined confirming replacement of the α6 subunit with α4 subunits (Champtiaux et al. 2003). Insects have a range of nAChR subunits including 12 subunits in *B. mori* (Shao, Dong, and Zhang 2007), 10 in *D. melanogaster* (Sattelle et al. 2005), and *A. gambiae* (Jones, Grauso, and Sattelle 2005) and 11 in *Apis mellifera* (Jones et al. 2006). In insects α5, α6 and α7 have been assigned as a single

group due to their high sequence homology to vertebrate subunits (Jones, Brown, and Sattelle 2007). Together these finding suggest the possibility α6 alterations result in the inhibition of α6 incorporation into nAChR pentameric structure and that an alternative subunit – one lacking the spinosad target site specific to α6 – is up regulated and included in its place. This would also explain the differences in resistance profiles observed between α6-knockout mutations (no target site – high resistance) and spinosad binding site mutations (moderate resistance). nAChR receptors are an integral part of the signal transition from nerve cells to muscle cells. It would be of interest then to assess the effects of the structural alterations of the α6 subunit on fitness. 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? Whilst no fitness studies were carried out on *T. absoluta* personal observations of populations containing resistance genes indicated no clear reduction in their consumption of tomato plants or population growth. While this is consistent with fitness studies in *D. melanogaster* (Perry, McKenzie, and Batterham 2007) and observations in mice (Champtiaux et al. 2002), fitness costs evident in the field or glasshouse may not be present under ideal conditions in the lab (Ribeiro et al. 2014). 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 spinosad resulted in withdrawal of its use in some glasshouses, however the pesticide was still effective in the Isle of Wight (IOW). These results and those of previous resistance mechanisms should be used to diagnose all outbreaks in the IOW regardless of the effect of spinosad 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 spinosad would be invaluable in the decision to weigh the short-term gain of crop protection in the immediate growing season from the application of spinosad to the long term gain of maintaining the efficacy of spinosad 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 spinosad resistance, selection for wild-type alleles in the absence of spinosad molecular diagnostics would allow for the monitoring of the return of susceptibility.

7.3 Chlorantraniliprole resistance

At the start of this PhD, chlorantraniliprole was still a highly effective compound in supressing *T. absoluta* pest populations. Chlorantraniliprole was used as a second line of defence if spinosad and the biocontrol failed to fully supress *T. absoluta.* Therefore selection pressures for resistance were lower than that of spinosad. With the increasing resistance to spinosad however the

reliance on diamides increased, and consequently so has selection for resistance. In 2016 two growers from Evesham and North Yorkshire reported reduced efficacy of chlorantraniliprole in some of their glasshouses (R. Jacobson, personal communication), glasshouses that had previously reported loss of control to spinosad. 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. Previous work on chlorantraniliprole resistance in pest species like *P. xylostella* had confirmed through assessment of the chlorantraniliprole target site (RyR) and functional validation that amino acid substitutions at G4946 had resulted in high levels of resistance (Guo, Liang, et al. 2014; Guo, Wang, et al. 2014). Alterations at this site have also evolved independently and contributed to resistance in 8 other species (Richardson et al. 2020) including *T. absoluta.*

The research undertaken in chapter 4 analysed the RyR and found evidence of G4903V (equivalent to G4946 in *P. xylostella*) 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 a 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 chlorantraniliprole resistance (Richardson et al. 2020). My findings are important for direction of the appropriate use of chlorantraniliprole in the control of *T. absoluta* in current IPM. The fact that spinosad resistance is present in the UK means chlorantraniliprole is the only remaining chemistry in

the current IPM compatible with biocontrol and pollinators. The fact that resistance is low, but alleles to chlorantraniliprole 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.

Molecular diagnostics have been developed that can asses resistant allele frequency of populations such as pyrosequencing of *P. xylostella* for G4946E frequency (Troczka et al. 2012). In *T absoluta* PCR-RFLP assays were highly successful at detecting both resistance mutations 1845Y and V1848I in field populations of indoxacarb resistant populations (Roditakis, Mavridis, et al. 2017). TaqMan assays described in chapter two for the F238del mutation add to this list of diagnostic tools. For G4903V identified in diamides resistant strains either TaqMan approaches or pyrosequencing could be easily utilised to assess frequencies in field populations in as little as a week of infestations being detected at glasshouses. This is compared to the months it can take to perform a traditional bioassay. These results would then inform the farmer on when to apply the pesticide (low resistant allele frequency) and when to hold off pesticide application (high resistant allele frequency). Once initial infestations are detected samples should be collected. This would give one month to carry out analyses before the next generation of *T. absoluta* were at the destructive larval stage - when the pesticide would usually be applied. The cost to crop production of not applying pesticides in any particular growing

season would be justified through the maintenance of the pesticide as a long-term control measure.

7.4 Metabolic resistance of chlorantraniliprole

Chapter 4 identified metabolic resistance as a second, independent mode of chlorantraniliprole resistance. Spanish populations of *T. absoluta* showing no target-site alterations in the RyR had 44614-fold resistance to chlorantraniliprole. With the importance of chlorantraniliprole increasing with the loss of efficacy of spinosad, 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 chlorantraniliprole in other pest species and was functionally validated for *T. absoluta* in this study through expression in transgenic lines of *D. melanogaster* resulting in chlorantraniliprole 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.

7.5 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 loose control due to spinosad resistance and the addition of this technology not only restored control but also relived selection pressure on the second line of defence – chlorantraniliprole. However, soon after the introduction of Isonet T Evesham 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 in chapter 6 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 at Evesham. 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 at the Evesham glasshouse 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 IMP strategy, *T. absoluta* has managed to evolve two independent mechanisms of resistance to spinosad, and, resistance genes have been detected in the field for chlorantraniliprole. 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.

7.6 Research summary

1. UK *T. absoluta* populations are resistant to spinosad 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 chlorantraniliprole 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 chlorantraniliprole 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.

7.7 IPM recommendations

1. Applications of spinosad should be stopped to prevent fixation of resistance alleles in the population.
2. Chlorantraniliprole 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.

7.8 Concluding thoughts

Almost as soon as *T. absoluta* was identified a crop pest in South America in 1960's chemicals have been used to supress the damage it has caused, and almost as quickly it has evolved a diverse array of molecular mechanisms to overcome them. The work in this thesis adds to the long list of resistance mechanisms evolved by *T. absoluta* to circumvent these measures. The findings in this thesis are vital for informing IPM with the aim of maintaining the number of pesticides that are available to control the pest. Reliance on individual compounds is the strongest driver for the evolution of resistance to that compound. Maintaining as many chemistries within the arsenal of compounds effective against *T. absoluta* – especially chemistries with different modes of actions – is the best way to ensure long term control of *T. absoluta* and pests in general. Historic responses to pesticide failures have included 'if it doesn't work add more' approaches and personal communications with agronomists and growers suggests that this ideology still goes on in parts of the world. Knowledge gained from this thesis of the mechanisms by which resistance arises means these mechanisms can be used to detect resistance genes early and at low frequencies in the field. IPM can then be proactive at preventing the evolution of resistance. Understanding these mechanisms can offer new lines of research into methods that circumvent target-site resistance through diagnostically informed pesticide rotation or pesticide breaks, and, mitigate metabolic resistance through development of synergists. To preserve pesticides from resistance, selection must be released from the mechanisms that drive it. While short term this puts

great strain on growers especially operating with tight profit margins, long term it offers a more sustainable approach to pest control.

Appendix 1

Research Article

Received: 20 December 2018 Revised: 15 February 2019 Accepted article published: 20 February 2019 Published online in Wiley Online Library: 25 March 2019

(wileyonlinelibrary.com) DOI 10.1002/ps.5381

The evolution of multiple-insecticide resistance in UK populations of tomato leafminer, *Tuta absoluta*

Charles Grant,[a] Rob Jacobson,[b] Aris Ilias,[c] Madeleine Berger,[d] Emmanouil Vasakis,[e] Pablo Bielza,[f] Christoph T Zimmer,[a†] Martin S Williamson,[d] Richard H ffrench-Constant,[a] John Vontas,[c,g] Emmanouil Roditakis[e] and Chris Bass[a*]

Abstract

BACKGROUND: The tomato leafminer, *Tuta absoluta*, is an economically important pest of tomatoes in Europe, Africa, Asia and South America. In the UK this species is controlled using an integrated pest management (IPM) programme which incorporates the insecticides spinosad and chlorantraniliprole. In response to UK grower concerns of loss of efficacy of these compounds at certain sites, insecticide bioassays were performed on five populations collected from four commercial glasshouses and potential mechanisms of resistance investigated.

RESULTS: We observed high levels of resistance to spinosad in four of the strains, and in two of these tolerance to chlorantraniliprole. Selection of one of these strains with chlorantraniliprole rapidly resulted in a line exhibiting potent resistance to this compound. Sequencing of messenger RNA encoding the nicotinic acetylcholine receptor (nAChR) α6 subunit, target of spinosad, revealed *Taα6* transcripts in the spinosad-resistant strains that lack exon 4 and encode a highly truncated protein, or contain a triplet deletion in the predicted first transmembrane domain resulting in the loss of a highly conserved amino acid. Sequencing of the ryanodine receptor gene, encoding the target of diamide insecticides, of the chlorantraniliprole-selected line revealed an amino acid substitution (G4903V) that has been previously linked to diamide resistance in populations of *T. absoluta* in the Mediterranean and South America.

CONCLUSION: Taken together our results reveal emerging resistance in UK populations of *T. absoluta* to two of the most important insecticides used as part of IPM, with significant implications for the control of this species in the UK.

Supporting information may be found in the online version of this article.

Keywords: spinosad; chlorantraniliprole; resistance; tomato leafminer

1 INTRODUCTION

The tomato leafminer, *Tuta absoluta,* is an economically important pest of tomatoes which spread from South America to Spain in 2006, subsequently invading a range of other European countries, Africa and the Middle East.[1] *T. absoluta* arrived in the UK in 2009[2] and caused profound damage to commercial tomato crops at sites across the country before an integrated pest management (IPM) strategy was developed that provided effective control.[3] This IPM strategy integrated biological control using the predatory bug *Macrolophus pygmaeus*, with some physical control measures and three insecticides: the spinosyn spinosad, the diamide chlorantraniliprole and the oxadiazine indoxacarb. These three insecticides were selected in part due to their different modes of action in order to prevent or delay the emergence of resistance. Recently, however, some UK growers have reported a loss of efficacy of both spinosad and chlorantraniliprole against *T. absoluta* (R. Jacobson personal communication). While, to date, resistance in UK populations of *T. absoluta* to these compounds has not

* *Correspondence to: C Bass, College of Life and Environmental Sciences, Biosciences, University of Exeter, Penryn Campus, Penryn, Cornwall, UK. E-mail: c.bass@exeter.ac.uk*

† *Present address: Syngenta Crop Protection, Werk Stein, Schaffhauserstrasse, Stein CH4332, Switzerland.*

a *College of Life and Environmental Sciences, Biosciences, University of Exeter, Penryn Campus, Penryn, UK*

b *RJC Ltd, Bramham, UK*

c *Institute of Molecular Biology & Biotechnology, Foundation for Research & Technology Hellas, Crete, Greece*

d *Department of Biointeractions and Crop Protection, Rothamsted Research, Harpenden, UK*

e *Hellenic Agricultural Organisation – 'Demeter', Institute of Olive Tree, Subtropical Crops and Viticulture, Heraklion, Greece*

f *Departamento de Producción Vegetal, Universidad Politécnica de Cartagena, Cartagena, Spain*

g *Department of Crop Science, Agricultural University of Athens, Athens, Greece*

been confirmed, both resistance and the underlying mechanisms involved have been characterised in populations of *T. absoluta* in other countries. In the case of spinosad, resistance has been described in field populations from Brazil with subsequent selection of one of these strains resulting in a line with extremely high levels of resistance to this compound.[4] Spinosad acts on the insect nervous system by targeting the α6 subunit of the nicotinic acetylcholine receptor (nAChR), a neurotransmitter-gated ion channel that plays a critical role in nerve signalling at the postsynaptic membrane. Sequencing of the gene encoding this subunit in the spinosad selected strain identified a point mutation in exon 9 of *Ta*α6 that results in a G275E amino acid substitution.[5] The causal role of this mutation in resistance was subsequently confirmed by CRISPR/Cas9 gene editing.[6] In a second study, characterisation of the *Ta*α6 gene in a resistant strain selected from a field population collected in Portugal revealed an alternative mechanism of resistance conferred by skipping of exon 3 in transcripts of *Ta*α6.[7]

Resistance to chlorantraniliprole has been recently described in populations of *T. absoluta* from Italy, Greece, Spain and Brazil.[8–10] Sequencing of the gene encoding the ryanodine receptor (RyR), a ligand-gated calcium-channel located in the sarco- and endoplasmic reticulum of neuromuscular tissues, identified several target-site mutations in the resistant strains.[8] Two of these, G4903E and I4746M, had been previously identified in diamide resistant diamondback moth,[11,12] while two others, G4903V and I4746T, were novel to *T. absoluta*. Radioligand binding studies and CRISPR/Cas9 gene editing provided unequivocal functional evidence that these mutations alter the affinity of the RyR to diamides and confer resistance.[8,13,14]

The resistance mechanisms described above have not yet been described in UK populations of *T. absoluta*. However, the rapid invasion of this species into the UK from Europe suggests that European populations carrying resistance alleles may be readily imported into the UK, especially as *T. absoluta* is no longer a notifiable (quarantine) pest in the UK. Alternatively, the use of spinosad and chlorantraniliprole in the UK for the control of *T. absoluta* might lead to the *de novo* emergence of resistance. In the current study we explored these possibilities by carrying out phenotypic and molecular characterisation of resistance in several UK populations of *T. absoluta*.

2 MATERIALS AND METHODS

2.1 Insect strains

After reports of control failure using spinosad, four strains of *T. absoluta* were collected in 2015 from commercial glasshouses at four UK sites: IOW from Wight salads, Isle of Wight, LAN from Flavourfresh Salads Ltd, Lancashire, NY from Jan Bezemer and Sons, North Yorkshire and EVH from R and L Holt, near Evesham, Worcestershire. In 2016 a second strain, EVH16, was collected from R and L Holt after reports of control failure using chlorantraniliprole. An additional Spanish strain that is susceptible to spinosad and chlorantraniliprole was acquired from Rothamsted Research, Hertfordshire. Insects were housed in controlled environment rooms at 25 °C, 60% RH, 16:8 light–dark cycle and fed *ad libitum* on tomato plants (var. Money Maker).

2.2 Leaf-dip assays

Leaf-dip bioassays were completed according to test method 22 guidelines produced by the Insecticide Resistance Action Committee.[15] Probit analysis and the calculation of LC_{50} values were performed with GenStat 15th Edition (VSN International, Hemel Hempstead, UK).

2.3 Chlorantraniliprole selection

Infested tomato leaves containing in excess of 500 larvae of the EVH16 strain were taken from cages and placed on a course net suspended over tomato branches that had been treated with chlorantraniliprole. This was repeated over the course of 12 months at concentrations of 1, 5, 10 and 50 ppm. After each round of selection the line was left unselected for two full generations to allow the population to recover. The resulting selected line was named EVH16sel.

2.4 RNA extraction and cDNA synthesis

T. absoluta L2–L4 larvae were snap frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. RNA extractions were carried out using the ISOLATE II RNA Mini Kit (Bioline, London, UK) on individual larvae or pools of ten larvae. cDNA was then synthesized from 1 µg of total RNA using the Maxima H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, US) according to the manufacturer's protocol.

2.5 Amplification and sequencing of the RyR and nAChR α6 subunit

A 1353 bp region of the *T. absoluta* RyR gene (encompassing the sites of previously reported diamide resistance-conferring mutations) was amplified by PCR using DreamTaq Green PCR master mix (Thermo Fisher Scientific, Waltham, US) and the primers detailed in Table S1. Primers were designed based on the previously published *T. absoluta* RyR mRNA sequence.[8] Ten nanograms (1 µL) of cDNA was used as template. Temperature cycling conditions comprised 3 min at 95 °C followed by 35 cycles of 95 °C for 30 s, 54.5 °C for 30 s and 72 °C for 60 s, and a final extension step of 72 °C for 6 min. Amplification of the nAChR α6 subunit gene was performed using the primers detailed in Table S1 designed on the previously published *Ta*α6 mRNA sequence.[7] A nested PCR protocol was used with 10 ng (1 µL) of cDNA used in the primary PCR, and 1 µL of a 1:10 dilution of the product used in a secondary reaction. Temperature cycling conditions were as above. PCR products were purified using the GeneJET PCR purification kit (Thermo Fisher Scientific) and purified DNA quantified by spectrophotometry (NanoDrop, Thermo Fisher Scientific, Waltham, US) prior to Sanger sequencing using the primers detailed in Table S1.

2.6 Association of F238del with spinosad resistance

To examine the association of the F238del mutation and resistance to spinosad we utilised the LAN strain as it is heterogeneous for this mutation (see Results). Twenty replicates of ten larvae of this strain were placed on tomato leaves treated with 150 ppm of spinosad. 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 S1. Each probe was labeled with a 5′ reporter dye specific to either

the wild-type or mutant allele. The VIC reporter dye was used to detect the wild-type allele and the 6FAM reporter dye to detect the mutant allele. Each probe also carried a 3′ non-fluorescent quencher and a minor groove binder at the 3′ end that provides more accurate allelic discrimination by increasing the melting temperature (TM) between matched and mis-matched probes. PCR reactions contained 2 µL genomic DNA, extracted from individual insects using DNAzol reagent, 7.5 µL of SensiFAST SYBR No-ROX kit (Bioline), 800 nM of each primer and 200 nM of each probe. Samples were run on a CFX96 real-time PCR machine (Bio-Rad Hercules, US) using the temperature cycling conditions of 10 min at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. The increase in fluorescence of 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. Pearson chi-square test was performed to examine the association between the F238del mutation and survival to spinosad exposure. To analyse 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).

3 RESULTS

3.1 Sensitivity of *T. absoluta* strains to spinosad and chlorantraniliprole

The sensitivity of the five UK strains of *T. absoluta* (EVH, EVH16, LAN, IOW and NY) to spinsoad and chlorantraniliprole was compared to a reference susceptible strain (TA1) from Spain by full dose–response bioassays. In the case of spinosad significant differences in the LC_{50} values obtained were observed between four of the UK strains (EVH, EVH16, LAN and NY) and TA1 with resistance ratios ranging from 29 to >480 (Table 1). The LAN strain exhibited moderate resistance to spinosad with an LC_{50} of 149 mg L^{-1} while the EVH, EVH16 and NY strains showed high levels of resistance to this compound with LC_{50} values of 860, >500 and >2500 mg L^{-1}, respectively (Table 1). Just two of the UK strains, NY and EVH16, exhibited tolerance to chlorantraniliprole. The LC_{50} of the two strains was 8–28-fold higher than that of the susceptible TA1 strain. Selection of the NY strain with chlorantraniliprole failed to rapidly result in a strain with higher levels of resistance. However, progressive selection of the EVH strain with chlorantraniliprole over just 12 generations resulted in a strain with potent resistance to this compound (LC_{50} of >5000 mg L^{-1}).

3.2 Identification of altered nAChR α6 transcripts in spinosad resistant strains of replace with *T. absoluta*

Amplification and sequencing of the nAChR α6 mRNA revealed the presence of two altered *Taα6* transcripts 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, EVH and EVH16 strains was a deletion of 109 bp that precisely corresponds to the position and size of exon 4. This deletion results in a frameshift leading to the introduction of a premature termination codon (PTC) in exon 5 (Fig. 1). Amplification and sequencing of the *Taα6* gene from genomic DNA of the NY and EVH strains revealed that exon 4 was present and unaltered in both strains, confirming that the loss of this exon in mRNA results from exon skipping rather than a deletion of the exon in genomic DNA.

The second alteration, observed only in the LAN strain, was an in-frame triplet deletion resulting in the predicted loss of a phenylalanine amino acid at position 238 (*T. absoluta* numbering) (Fig. 1). The F238del mutation occurs in the first α-helical transmembrane domain (TM1) of the Taα6 subunit and comparison of insect nAChR α6 sequences from a range of insect species (Fig. 1) demonstrates that this residue is highly conserved across the Arthropoda. Unlike exon 4 skipping this alteration was observed in both the *Taα6* gene and mRNA transcript.

To examine the association of the F238del mutation with spinosad resistance the LAN strain was treated with a discriminating dose of spinosad and alive, dead and affected (larvae that were alive but unable to crawl their own body length away from mechanical pressure) larvae genotyped. Of the 200 individuals exposed to 150 mg L^{-1} of spinosad 108 died, 30 were affected, 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 labelled probes when tested with sequence verified DNA samples (Fig. S1).

Table 1. Sensitivity of UK strains of T. absoluta to the insecticides spinosad and chlorantranilprole. The resistance ratio (RR) of these strains was compared to a susceptible strain from Spain (TA1)

Treatment	Strain	Location	LC_{50} (mg L^{-1})	95% CI	RR
Spinosad	TA1	Spain	5.2	3.1–7.3	1
	NY	North Yorkshire	>2500		>481
	EVH	Evesham	860	484–2114	165.38
	EVH16	Evesham	>500		>96
	LAN	Lancashire	149	31–5385	28.65
	IOW	Isle of Wight	8.6	5.3–13.9	1.65
Chlorantraniliprole	TA1	Spain	1.2	0.095–5.00	1
	NY	North Yorkshire	33	11.2–97.9	27.5
	EVH	Evesham	0.12	0.005–1.00	0.1
	EVH16	Evesham	9.53	5.025–18.09	7.94
	EVH16sel	Evesham	>5000		>4167
	LAN	Lancashire	4.75	1.7–12.8	3.96
	IOW	Isle of Wight	3.6	0.9–14	3

CI, confidence interval.

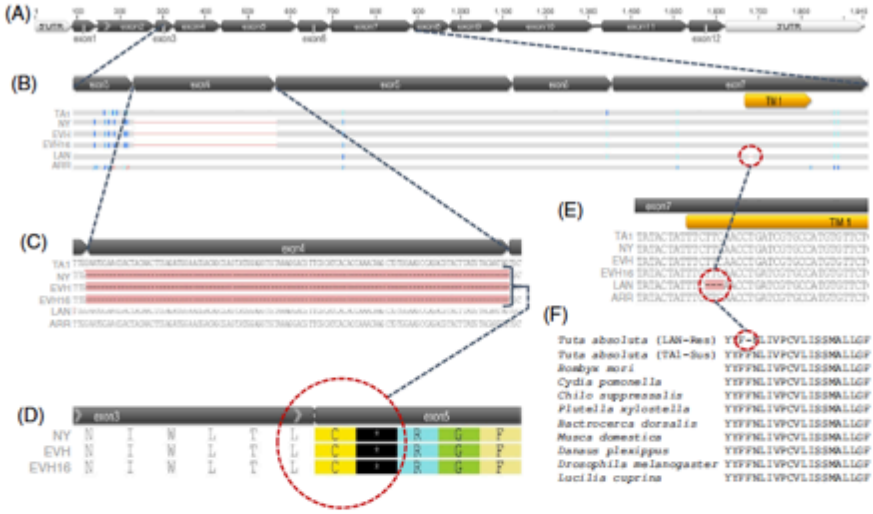


Figure 1. Schematic of mutations identified in *Tαα6* transcripts of spinosad-resistant *T. absoluta* strains. (A) Schematic of the exon structure of *Tαα6* (defined previously[7]). (B–D) Alignment of the sequences obtained from six *T. absoluta* strains illustrating the skipping of exon 4 observed in three of the spinosad-resistant strains that leads to the introduction of a premature termination codon in exon 5. (E, F) The LAN strain has a triplet deletion in the encoded first transmembrane domain of *Tαα6* that leads to an in-frame deletion of an amino acid that is highly conserved in nAChR *α6* subunit sequences across Arthropoda.

One hundred and seventy five individuals from the discriminating dose assay were successfully genotyped using this assay, with 31 individuals scored as homozygous for the wildtype allele, 56 as homozygous for the mutant allele and 88 heterozygous, indicating an overall mutation frequency of 0.57 in the LAN strain. A significant association was observed between the individual's genotype and its response to spinosad exposure (χ^2 (4) = 78.499, $P < 0.001$). Post-hoc analysis (Table S2) 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 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 (Fig. 2).

A 1353 bp region of the RyR encompassing the sites of mutations previously shown to confer resistance to diamides in *T. absoluta*[8] and *Plutella xylostella*[12] (G4903E/V and I4746M/T in *T. absoluta* corresponding to G4946E and I4790M in *P. xylostella*) was amplified from the chlorantraniliprole selected line (EVH16sel), the parent strain from which it was derived, (EVH16), NY and TA1 (Fig. 3). The sequence obtained from a pooled sample of the parental EVH16 strain encoded the wildtype amino acid at all the positions detailed above, however, a very small secondary peak was observed in the sequence chromatogram at position 4903, suggesting the G4903V substitution was present at very low frequency in this strain (Fig. 3). This was confirmed when the RyR sequence from the selected line was examined, which showed fixation of the mutation leading to this amino acid substitution.

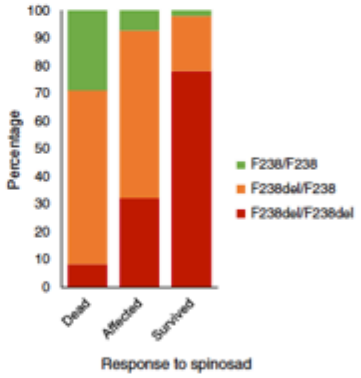


Figure 2. Genotype of *T. absoluta* larvae for the F238del mutation that died, survived or were affected following exposure to a discriminating dose of spinosad. *n* dead = 108, *n* affected = 30, *n* survived = 55.

No mutations were observed in RyR sequences of the NY and TA1 strains.

4 DISCUSSION

Our data reveal high levels of spinosad resistance in strains of *T. absoluta* collected from nurseries in the north and south of the

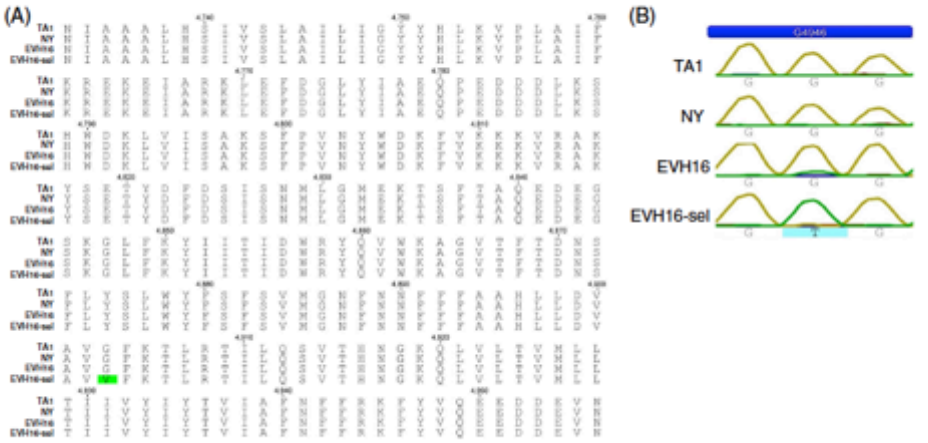


Figure 3. Identification of the G4903V amino acid substitution in a diamide-resistant strain of *T. absoluta*. (A) Alignment of RyR amino acid sequences obtained from three UK strains of *T. absoluta* and a strain from Spain. (B) Sequence chromatograms of the same strains at the G4903 codon illustrating a slight secondary 'T' peak in the second nucleotide of the codon EVH16 strain that has become fixed following chlorantraniliprole selection (EVH16sel).

UK, indeed, only one of the UK strains, sampled from the Isle of Wight, exhibited full susceptibility to this compound. While our sample size is small this finding is consistent with reports by several British tomato growers of poor efficacy of spinosad against *T. absoluta* and suggests that resistance to this insecticide may be widespread in UK populations. In three of the strains, NY, EVH and EVH16, the levels of resistance observed were extremely high and would certainly be expected to compromise the use of spinosad at the recommended field rate (87–100 mg L^{-1}). Lower levels of resistance were observed in the LAN strain, however, the LC_{50} value determined for this strain indicates that a significant percentage of individuals would be expected to survive exposure to the field rate application.

Sequence characterisation of transcripts encoding the nAChR α6 subunit revealed two novel mutations in the spinosad-resistant strains. The first of these, observed in the three most resistant strains, is skipping of exon 4. This alteration has profound consequences for the encoded protein, which would lack the ~36 amino acids encoded by exon 4, and would also be significantly truncated as the loss of exon 4 leads to a frameshift and the introduction of a PTC in exon 5. Together this would result in a protein of just 160 amino acids (compared to 510 amino acids in the wildtype) that lacks all four transmembrane domains and would almost certainly be non-viable. Work on *Drosophila melanogaster* first demonstrated that null mutations of α6 lead to spinosad resistance with a strain with a variant of *Dα6* lacking the TM3 and TM4 cytoplasmic loops, and the extracellular C-terminal tail domains exhibiting >1000-fold levels of resistance to spinosad.[16] Significantly, this study also demonstrated that loss-of-function mutations in insect α6 transcripts do not result in catastrophic loss of fitness as insects without a functional copy of the α6 gene are still viable.[16] Subsequent studies on several insect crop pests have described genetic alterations in nAChR α6 sequences that result in truncated non-functional proteins leading to resistance. For example, several mutations resulting in mis-splicing and the introduction of PTCs in nAChR α6 transcripts are associated with spinosad resistance in the diamondback moth, *Plutella xylostella*,[17,18] the oriental fruit fly *Bactrocera dorsalis*,[19] the western flower thrips, *Frankliniella occidentalis*[20] and the lesser grain borer, *Rhyzopertha dominica*.[21] In a previous study of a spinosad resistant *T. absoluta* strain, derived from a population collected in Portugal, resistance was conferred by skipping of exon 3 rather than exon 4, however, in this case the reading frame of the altered *Taα6* transcripts was unaffected by the exclusion of exon 3.[7] Finally, it is worth noting that while there is parity in the frequency of exon 4 skipping in the NY, EVH and EVH16 strains some variation was seen in their respective sensitivity to spinosad (Table 1), with the NY strain exhibiting the greatest resistance to this compound. This suggests that other additional mechanisms of resistance may be present in the NY strain, and it would be interesting to explore the potential role of metabolic mechanisms in the resistance of this strain to spinosad in future studies.

A more subtle alteration, F238del, was observed in transcripts of *Taα6* in the LAN strain and this was correlated with lower levels of resistance to spinosad. This triplet deletion results in the loss of a phenylalanine amino acid in exon 7 and occurs in TM1 of the nAChR Taα6 subunit. This amino acid is highly conserved in nAChR α6 subunit proteins across Arthropoda, suggesting that this residue is functionally constrained. The loss of the amino acid at this position is thus extremely unusual and consistent with it being a *bona fide* resistance mutation. We provide further evidence of a causal role of this mutation in resistance by demonstrating that it cosegregates with spinosad resistance in survival bioassays. The loss of one or more amino acids in the nAChR α6 subunit has been linked to spinosad resistance previously with a three amino acid deletion in the fourth transmembrane region of the receptor shown to confer resistance in *P. xylostella*.[22] Furthermore, the F238del mutation exhibits parallels with a previously reported amino acid substitution (G275E, *P. xylostella* numbering) shown to confer spinosad resistance in both thrips and *T. absoluta*

(the latter in strains from Brazil).[5,6,23–25] Like F238del, this mutation also occurs in a α-helical transmembrane domain (TM3) of the α6 subunit.[24] X-ray crystallisation studies of the glutamate-gated chloride channel (GluCl), a pentameric receptor with close structural similarity to nAChRs, bound to ivermectin, another macrocyclic lactone with structural similarity to spinosad, have shown that ivermectin makes direct associations (by hydrogen bonding and van der Waals interactions) with TM1, TM2 and TM3.[26] This finding is consistent with spinosad acting via an allosteric transmembrane site distinct from the conventional agonist binding site,[24] and it is thus likely that the F238del mutation confers resistance by directly modifying the spinosad binding site.

It is notable that neither the G275E mutation or exon 3 skipping, previously identified in strains of *T. absouta* from Brazil and Portugal, respectively, were observed in UK strains of *T. absoluta*, suggesting that the mutations identified in our study arose *de novo* in UK populations rather than being imported via transfer of insects on plant material or packaging from outside the UK. The application of spinosad via the irrigation system in the UK, together with the redundancy of the nicotinic acetylcholine receptor (nAChR) α6 subunit, in which any non-functionalizing mutation will confer resistance, likely facilitated the rapid evolution of resistance.

Two of the strains tested in our study also showed tolerance to chlorantraniliprole, while the levels of tolerance displayed were relatively modest selection of one of these strains rapidly resulted in a line with potent resistance to this compound. This finding suggests that resistance alleles were almost certainly present in the population prior to selection. Sequencing of a region of the RyR encompassing the sites of known resistance mutations confirmed this and revealed that a mutation leading to a G4903V substitution present in the parental strain at low frequency had reached fixation in the selected line. This mutation has recently been reported in *T. absoluta* strains from Greece and Italy[8], suggesting that *T. absoluta* with this resistance allele may have been imported into the UK. The functional significance of the G4903V substitution has been confirmed *in vitro* using radioligand binding studies and *in vivo* by CRISPR/CAS9 genome editing in Drosophila.[13] In the case of the latter genome modified flies bearing the mutation exhibited high levels of resistance to both the diamides flubendiamide (91-fold) and chlorantraniliprole (195-fold) when compared to flies of the same genetic background without the mutation.[13]

Our results have significant applied implications in relation to the control of *T. absoluta* in the UK. Spinosad performs a key role in the IPM strategy introduced against this pest in 2013, and is applied at the start of the season to provide control while the natural enemy *M. pygmaeus* is becoming established.[3] If necessary, a high-volume spray of chlorantraniliprole is applied as a second line of defence later in the season to keep the pest and predator populations in balance.[3] The fact that we identified one strain with resistance mechanisms to both spinosad and chlorantraniliprole is concerning as it demonstrates that *T. absoluta* with resistance to both compounds can readily evolve and are viable. It would be useful to screen UK populations of *T. absoluta* more widely for the resistance mechanisms identified in this study. If such monitoring indicates that, as suggested by our study, spinosad resistance is widespread, then we suggest that use of this insecticide is suspended for a period to examine if susceptibility is restored to populations in the absence of selection. The likelihood of this is dependent on any fitness costs associated with the resistance mechanisms we have identified. In *D. melanogaster* no evidence of fitness costs were identified under laboratory conditions in a Dα6 null mutant, suggesting the costs of loss of function mutations in this subunit may be minimal.[16] However, work on a spinosad-selected strain of *T. absoluta* found that resistance was unstable in the absence of spinosad selection and dropped rapidly over relatively few (less than ten) generations.[27] In our study we observed high levels of spinosad resistance in a strain (EVH16) collected from a nursery where this compound had not been used for a year, suggesting that the exon 4 skipping identified in this strain may have a low fitness penalty. However, additional work is required to clarify fitness costs associated with this mutation and the others identified in this study in both the α6 nAChR subunit and the RyR under field-realistic conditions.

Mutations conferring resistance to chlorantraniliprole were only identified in a single strain in our study, suggesting that with effective resistance management it may be possible to prevent the further development of resistance to this compound. This would require the rotation of this insecticide with insecticides of alternative modes of action and/or the use of alternative control methods. In regards to the latter recent trials in the UK using a synthetic sex pheromone as a mating disruption strategy against *T. absoluta* have provided encouraging results[28] and may allow the frequency of insecticide applications to be significantly reduced. However, our study clearly illustrates the danger of complacency and we suggest that the control of *T. absoluta* in the UK (and worldwide) should continue to rely on the use of a range of integrated approaches in order to reduce selection pressure on any single control to prevent or slow the emergence of resistance.

ACKNOWLEDGEMENTS

This work was funded by PhD studentship awards from the Agriculture and Horticulture Development Board (AHDB) (CP 162) and the Biotechnology and Biological Sciences Research Council (BBSRC) (grant number: 1096240). ER was supported by funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under the STomP project, grant agreement no. 219262 (ARIMNet).

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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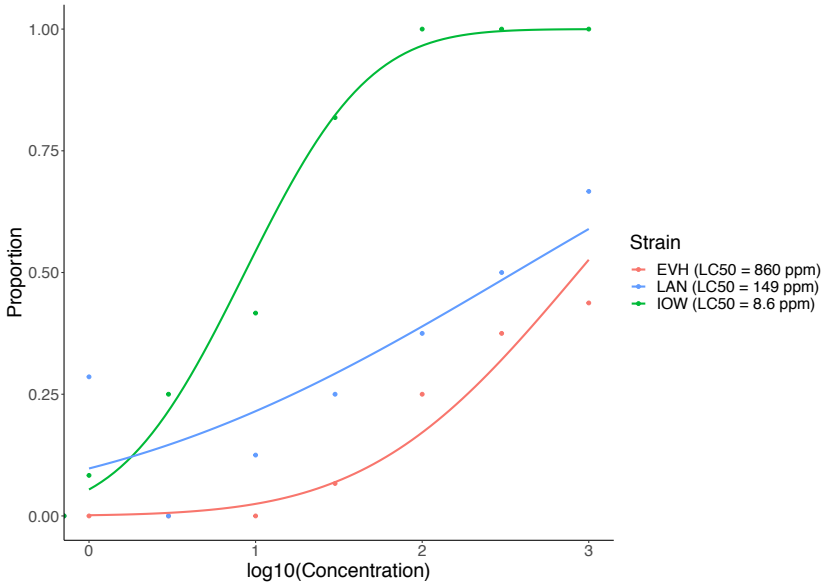
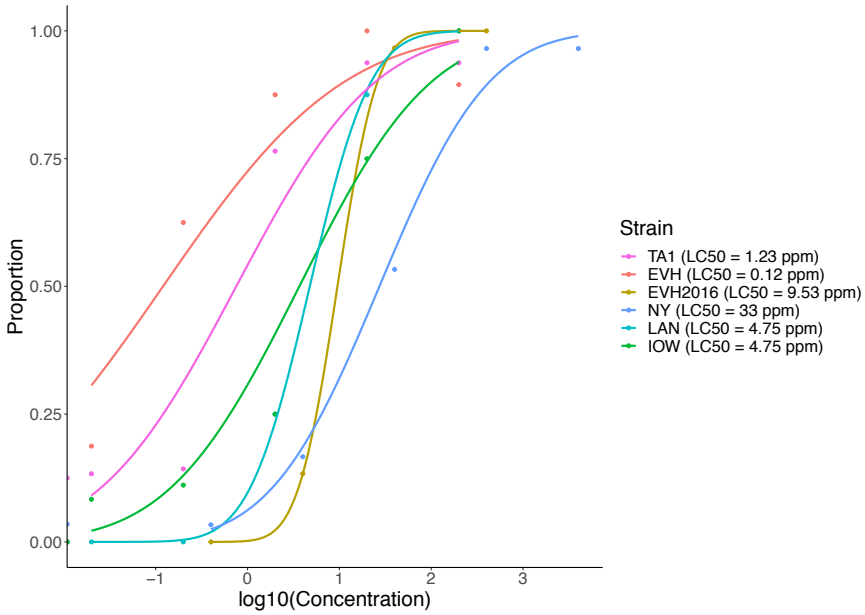
Appendix 2

Dose response curves for (A) spinosad and (B) chlorantraniliprole. For excluded strains, data was insufficient to generate dose response curves.





Appendix 3

UGT sequence list.

T. absoluta g995 UGT2C1-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MELKVFKILLLAFFCHVAESANILALFSSLSFSDHLVFRGYVSRLSRAGH
SVVVMTAYPGHHTAPEVERIIELDVSQESLPFWNEWLKLVTNTDDHFTRM
RAINDFSIKLAIAQLKSKQMTALFVNPNVKFDLVITEADVPLLYAAADKY
KVPHVAITTSSGKIHQYEAKGSPTHPILHLDVNTLSYGSSSNWQKLTEFK
RYIQTKYEYYNNYLPLCEVAAQNIFDLKRSLLEVESDIDLLLVSSNPVLN
ENRPSSPSIVYTDRLHLKPGFNLPQNLKSVLDAATKGVIYFSIGAIQESE
TLAPQLLQTLADAFRELPYTVLWKIGNTTAFNKSDNVIAGAWFPQQEILA
HPNVKVFITHGGPRALEEAIFYEVPIVGLPIVRPRGVFMGQVTKHGCGEI
LDPYVLNKDELKTTIEAVANNEKYKKSMTKLKSIIVDPLISGPDNAVWWT
EYVLRNGGARHLATPAYTGAIEYYFLEVISTFLGGALLILCVSFFLLRWI
IKRLRARFFGRVIESGKFKAL*
```

T. absoluta g2599 UGT2B20-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MSGQIYLLISLLCSVAISESARILAVFPTPSISHQVVFRPLTQELARRGH
EVVILTPDPVFEKGQAPENLTEIDVHDMSYDLWRKHFLVHATGKADDLYK
QVEVVFDLQLKIFHMQLNTSEFKKIIDEKQQFDLLLIEAWLKPVLLLTHF
LKAPVIQVSSFGAIWDNYESFGAPGHPMLYPTSLHKRLYNLTLWEKMGEL
YSHWRFVNLMNNVEKSQDDFIKKTYGSDIPSLHELSNNVDMLFLNIHPIF
EGNTPWPPGIISTWGIHQKPEKPLPKELQSYLDTSKHGVIYMSFGTNVDP
AYLPPEKIQMFIKVFSKLPYDVLWKWNQDVLPGKSNNINISRWFPQSDLL
RHPKVKLFITQGGLQSTDEAIVAGVPLIGIPMLGDQWYNTEKYVYHGIGK
KLDIETITEEELMKTINEVIDKERYRHNIKNLGALMRDEPMSGLQRAVWW
TEHVLRHGGARHLRAPAANISWSQYLELELVSVVVFALLALMVLVFVVFR
IFYRHIKIFNYC
```

T. absoluta g5824 UGT33J1-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MGELYSHWRIDNLMNNVEKNQDDFIKKTYGSDFPSLHELSNNVDMLFLNI
HPIFEGNTPWPPGIISTWGIHQKPEKPLPKELQSYLDTSKHGVIYMSFGT
NVDPAYLPPEKIQMFIKVFSKLPYDVLWKWNQDVLPGKSNNINISRWFPQ
SDLLRHPKVKLFITQGGLQSTDEAIVAGVPLIGIPMLGDQWYNTEKYVYH
GIGKKLDIETITEEELMKTINEVIDNGRYRHNIKNLGALMRDEPMSGLQR
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IVFRIFYRLISRFSITARRKKLN
```

T. absoluta g6741 UGT2B15-like

```
1       10       20       30       40       50
|       |        |        |        |        |
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FATEDMKKLLNDPKEKFDLVIIEWMFAEVYAGLATVFDCPYIWVSSNNPH
WMVLSLIDEIPNPAFNPEIMSSNIPPLGFSERVRELFMTATWGVLRMFLR
RNDEKTYNEVFTPIMVKKGRPLPSYKEMIHSASLMFGNTHESLSWSRSLP
QNYKSIGGYHIDPEVKPLPKDLQKIMDSAKDGVIYFSLGSNVKSKDLPDE
IKQSLLKMFQQLKYTVLWKFEEQLIGLPKNVHILKWAPQQSILAHSNCKL
FITHGGLLSTIETIHFGVPIIGIPVFADQFTNVELSKKKGFALRVDISYE
MADDLKVAIEEILGNPKYKQTIERLNLIFHDRPITPAQELVHWVNHVIKT
KGAPHLRSMALMTPWWQKLYLDLLALVLIVIFVIVKIIKAFCCRKKKTVS
SKKKNN
```

T. absoluta g6742 UGT5-like

```
1       10       20       30       40       50
|       |        |        |        |        |
MWKLLCAAVALFTLIVDMSEGSKILVVFPFPGKSHNILGEGFVRALLKAG
HEVTYVTPFPKKEVAQNLRYIDLSDIVADFQASMAADPALDLKKIADKTS
EPLVMTFAKGLISGNAKPITTMPNQTLHNANFQKFMNDPNEKFDLIIIEW
MFTDVFSGLSAVFDCPYIWVCSSNPHWMVLDLIDEAPNPAFTPEIMSSNI
PPLDFSQRARELVMTTVWGGLRMLMRRVSKQTYHNVFSPILAQKGKPLPP
FEEIIHNGSFMFSNSHESTGWARNLPQNFKNIAGYHIDPEVKPLPKDLKN
IMDNAKNGVIYFSLGSNVKSKDLPDEIKQSLLKMFGGLKYTIIWKFEEQL
TSLPKNVHILNWAPQPSILAHPNCKLFITHGGLLSTIETIHFGVPIIGIP
VFADQFTNVELSKAKGFALRVDISYQMADDLKAAIEEILGNPKYKETIEN
LNFIFHDRPVSPSQELVHWVNHVIKTKGAPHLRSMALMTPWWQKLYLDLL
ASNEDYITHKELLKQCCLASC
```

T. absoluta g7425 UGT2B20-like

```
1       10       20       30       40       50
|       |        |        |        |        |
MCKHSSISIPSHQKSTTSAEAIGLELARRGHNVTVITAFKETDHPPNYHQ
VKVDDKEIWETTGQKRPNVFTMVDISAEEFHDKILWGGGFGFTEVALNSS
DVQKFLKEDHKFDLVIAEQFFQEATFILAHKYQAPLVLITTYGNCMRHNI
VTRNPLQLATVVQEFLDVKDPSSFLGRLRNWYFTVYEYVWWKYWFLPKNE
ELVKKYVPNLPQPVPSLFEMQKNAAMILLNSHFSFDPPTAYLPNVIEVGG
LHLSKSNDKLPQDLQQILDEAKHGVVYVNFGSNVRSSELPLEKKNAFLKV
FSELKQTVLWKWEDDSLQNQPANVFVRKWLPQKAVLAHPNIKVFVSHGGL
IGTQEAVFHGVPIIGIPIYADQYNNLLRAQDIGIGRILLYHDITEDTLRN
TLNDVIKDNTYLLKAKETSARFKDRPMSALDTAMFWIEYVIRNKGADFIK
NPARDLSWFAYTMYDVYVFVLVSSLGFLYLVSKIVRILLSQSSSKQTVKQ
DKKKMY
```

T. absoluta g9081 UGT 2C1-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MKLLNFLCVALISLFKCEAYRILVVYPFPAKSHNILGNGVVKHLLKAGHE
VTYITPFPIESHDSNLRQVDVSAVTNLLKGMEDALNIRSYLSKGAEINDE
TYLFSVIFNVAAAAVKTENVQKFLNDPTQRFDVVVNEWMFNDLYAGFAAV
FNCPYIWTYPYEPYIHVLSLIDEVPNPAYTSNLMSSNVPPFNFAQRAEEL
FSQIKSSILRTYYQIPLEKAVYEETFGPVLKKQGRILPTYDELRHNVSMI
LGNSHVSFGLSTRLPESYKSIAGYYIDENVETLPEDLQKLMDDAKDGVIY
FSLGSNLKSKDFPESIKKDLIKVFAELKEVVLWKFEEQYEDLPKNLHVTK
WAPQQSILAHPNCKTFITHGGLLSTTEAAHFGVPTIGIPVFVDQFNNVDR
AVHRGYALRVDLSYSLANDLRKAIKEITTDYKYTTRAREISSIYHDRLVS
PATELVHWVEYVYFNIIGSYLLEQI
```

T. absoluta g10479 UGT 3A1-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MMVPNYVDSRMKLSLLLSVLTLSHQVVGYKILVISPTPSRSHATLARGIV
TPLLEAGHQVTWGVPFMDQKPHPNLRLIDLSATLNHVPKMDMTQIRNDHG
IVKEFATNISRTTALNKDIRDTLVNDNYDAVVTEWFFSDTDAGFAAVQQV
PWIMLSGMIMHPHVEGLVDEVRSISTVPMVFHDFTVPMSFYERVVNTFTF
IMMRIGAILDESTNAARYESYFSPLAAARGVPLPPYSEAIYNISVVLVQS
HPSIAPALSLPPNVVDIGGYHIDKPQPLPQDIQYILDSSPQGVVYFSMGS
VIKGYRLPLGLRKDLIAMFGELPYTVLWKFEEELQGLPKNVVVRKWWPQA
GVLGQF
```

T. absoluta g12880 UGT 2B30-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MKILLCLLFCAHHALALNILAIASLPLRSHYMAFHPFFRELATRGHHVTV
MNNYPDDNAPPLMRFVNMNADNNGMIYITPLNVYEKFNSDYLHLYNFYRH
LVLSTPSAKTDCENFFTNENVKAQLAKGIKYDVIFVEMFGVDCGLAFAGA
MFDAPIIGIASHVMQPLAYPRLGLPFDFGSDAFYYSNAGFNPSLYQKVEA
FIINIIDHIYVWYTHNFIVYEVFNRYLPNNSLDIEWVARERVKMYFSYQH
FSLTGARVSSPQVLEIGGIHIGKAKPLDQKLEKFLSNADQGAIFVSFGSN
LKANGMSPEKRQEFLNAFMKIPQKVIWKYENETLAEEYHDKVYFGNWLPQ
LDILCHPKVVGFVSHGGMLSLSESTHCGKPLVVTPFFGDQFSNAAAAEQA
GIGILLHFDQLDGDSLADAIQYITSSTMQQNAKTISKLWHDRPMPVMESA
IYWTEYVARNHDADAPPSLPSKRSTWFEKSLIDVALILLAIFLAPILFLA
AVVKLMKSLFFKSEETAETKKANKKKPKKS
```

T. absoluta g13473 UGT2C1-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MGLKVFKILLLAFFCYVAESANILALFSSLSFSDHLVFRGYVSRLSRAGH
SVVVMTAYPGHHTASEVERIIELDVSQESLPFWNEWLKLVTNTDDHFTRM
RAINDFSIKLAIAQLKSKQMTALFVNPNVKFDLVITEADVPLLYAAADKY
KVPHVAITTSSGKIHQYEAKGSPTHPILYLDVNTMSYGSSSNWQKLTEFK
RYIQTKYEYYNNYLPLCEIAAQNIFDLKRSLSEVESDIDLLLVSANPVLN
GNRPSSPSIVYTDRLHLKPGFNLPQNLKSVLDAATKGVIYFSIGAIQESE
TLAPQLLQTLADAFRELPYTVLWKIGNTTAFNKPDNVIAGAWFPQQEILA
HPNVKVFITHGGPRALEEAIFYEVPIVGLPIVRPRIVFMAQVTKHGCGEI
LDPYVLNKDELKTTIEAVANNEKYKKSITKLKSIIVDPLISGPDNAVWWT
EYILRNGGARHLASPAYTGAIEYYLLDVISTFLGEALLILCVSFFLLRWI
IKRLRARFFGRVIESGKFKAL
```

T. absoluta g13521 UGT1-1-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MQAVVWLVMCLAAAAPAADAARILAVLPTNTRSHYAMYGRLIEALARKDH
QMTIISHFPMKNPPPNVETISLAGTIPEITNNLTRQNQSLKPSFVVNLEN
IMKECLQACETVSRLPAVKALLNSTIAFDLVIVEVFGSECFLPLGERFQA
PVVGLLSSVPLPWVNEQLGNPEATSYVPAYMMGFGQRMNLWERFANTISV
LWAKMLYKYKSQIPSQVIADRLFGAGLKLEDLAKNYSLVLSNSHFSINEV
RPVVPAMVEVGGLHLDNSQKLSNEMKTLLDSSPEGVIYWSFGSMSRIETI
PSEKLAQIFEALSRMSQTVLIKMNRGMLARNLTVPDNVYTMDWIPQYATL
CHPNVKLFIGHGGLLGTQEAVACGVPMLMVPLYADQALNAHAMTDRGVAR
THMLKEGDANTWTEAMRDLLRNPTYKENALKLRDVFLDRPMPPLDTGVYW
IEYVIRHKGAKHLRSPALDLPLTQYLLLDVVALSLAATIMTIFILHTLFR
YLCTRCIKWWAKQKTRISREKLSGKNISLFLCLLMVRSLRLTSYLTHMLL
FGKSK
```

T. absoluta g13565 UGT2B20-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MSRQILVLISLLSSVVLSESARILAVFPTPSISHQVVFRPLTQELARRGH
EVVILTTDPVFPKGKSPANLTEIDVHDMSYDLWRKHFLAHATGKANDLYS
QMEVLFDIALKIFHMQLNTSEFKKIIDEKQHFDLLLIEAWMKPAFLLTHF
FKAPVIQVSSFGRVWSNYENMGAPVHPILYPISLRQRLCNLTLWEKMTEL
YSHWQFVNLYDQFERSEDEFNKKTYGSDTPSIHELSNNVDMLFLNIHPIF
EGNTPWPPGIISTWGIHHKPEKPLPKDLQSYLDTSKHGVIYMSFGTNVDP
ANLPPETIQMFIKVFSKLPYDVLWKWNQDVLPGKTEKINISKWFPQSDLL
RHPKVKLFITQGGLQSTDEAIVAGVPLIGIPMLGDQWYNVEKYLYLGIGL
KLDIETITEEGLIRAINEVIDNERYRQNIKKLSTLMRDEPMSGLQRAVWW
TEHVLRHGGARHLRAPAANISWAQYLELELVFVLLFAILVFVVIVFSVLR
VLYTLLSKFSLTVSKKKLN
```

T. absoluta g13867 UGT2B10-like

```
1       10       20       30       40       50
|       |        |        |        |        |
MFNDLYAGFAAVFNCPYIWTYPYEPYIHVLSLIDEVPNPAYTSNLMSSNV
PPFNFAQRAEELFSQIKSSILRTYYQIPLEKAVYEETFGPVLKKQGRILP
TYDELRHNVSMILGNSHVSFGLSTRLPESYKSIAGYYIDENVETLPEDLQ
KLMDDAKDGVIYFSLGSNLKSKDFPESIKKDLIKVFAELKEVVLWKFEEQ
YEDLPKNVHVTKWAPQQSILAHPNCKTFITHGGLLSTTEAAHFGVPTIGI
PVFVDQFNNVDRAVHRGYALRVDLSYSLANDLRKAIKESLLITTREISSI
YHDRLVSPATELVHWVEYVVRTRGARHLRSPALQLPWYQKLYLDLAAVLI
VLFLFVFSTLTLSVRIYLNKYKSNLPKKNKKAS
```

T. absoluta g15009.1 UGT2B15-like

```
1       10       20       30       40       50
|       |        |        |        |        |
MHFLYFALAIITLPLSEGARILVMFPFPSRSHNILGEGVVRILLKDGHEI
TYITPFPKAEVTPHLRYVNISGNAENFNNQNDSSIDLKKIADKTADSIIW
TFIKNMFANDGKPTGFASLPILTFATEDMKKLLNDPKEKFDLVIIEWMFA
EVYAGLATVFDCPYIWVSSNNPHWMVLSLIDEIPNPAFNPEIMSSNIPPL
GFSERVRELFMTATWGVLRMFLRRNDEKTYNEVFTPIMVKKGRPLPSYKE
MIHSASLMFGNTHESLSWSRSLPQNYKSIGGYHIDPEVKPLPKDLQKLMD
SAKDGVIYFSLGSNVKSKDLPDEIKQSLLKMFQQLKYTVLWKFEEQLIGL
PKNVHILEWAPQQSILAHSNCRLFITHGGLLSTIETIHFGVPIIGIPVFA
DQFTNVELSKKKGFALRVDISYEMADDLKVAIEEILGNPKYKQTIERLNL
IFHDRPITPAQELVHWVNHVIKTKGAPHLRSMALMTPWWQKLYLDLLALV
IIVILVIVKIIKAFCCRKKKTVSSKKKNN
```

T. absoluta g15009.2 UGT5-like

```
1       10       20       30       40       50
|       |        |        |        |        |
MWKLLCAAVALFTLSLDMSEGSKILVVFPFPGKSHNILGEGFVRALLKAG
HEVSDICYTVSEERSGSKIYGTFDLSDIVADFQASMAADPALDLKKIADK
TSEPLVMTFAKGLISGNAKPITTMPNQTLHNANFQKFMNDPNEKFDLIII
ELSAVFDCPYIWVCSSNPHWMVLDLIDEVPNPAFTPEIMSSNIPPLDFSQ
RARELVMTTVWGGLRMLMRRVSKQTYHNVFGPILAQKGKPLPPFEEIIHN
GSFMFSNSHESTGWARNLPQNFKNIAGYHIDPEGKPLPKDLKNIMDNAKN
GVIYFSLGSNVKSKDLPDEIKQSLLKMFGGLKYTIIWKFEEQLTNLPKNV
HILNWAPQPSILAHPNCKLFITHGGLLSTIETIHFGVPIIGIPVFADQFT
NVELSKAKGFALRVDISYQMADDLKAAIEEILGNPKYKETIENLNFIFHD
RPVSPSQELVHWVNHVIKTKGAPHLRSMALMTPWWQKLYLDLLALVAVVM
FVTFKALKFLCCSKKTLESSSSKKKKEKAKKIN
```

T. absoluta g16225.1 UGT33J1-like

```
1       10       20       30       40       50
|       |        |        |        |        |
MTSKYIVIFVSLLCTIVTNESARILAVYPTPSISHQVVFRPLTLELVKRG
HEVVVLTTDPVYTKGKAPANLTEIDIHDMSYDIWREKIIAHGTAKDDFSV
IKKMSKTLVELFYEQIQTSEFKKIIDEKQQFDLLLLEACSRPALGLTHIF
KAPVIQVSSLGSVFSNYQDLGAPYHPILYPSVINKRLYNLTLWEKASILY
LEWQYKNLLDDVDAELDVWMKKRFGHDTPSFKELSNNIDMLFLNIHPIWE
GNMPWPPSIIHTWGIHHKPEKPLPQDIQTYLDTSKYGVIYFSLGTNVDPA
NLPAEKLQMFIRVFSKLPYDVLWKWNHDVLPGKTENIRISKWLPQSDLLR
HPKVKVFITQGGLQSTDEAIVAGVPLIGIPMLADQWYNVEKYVHHGIGLK
LDIKTVTEEELTIAIKEVIDNDRYRKNIKKLSALMHDEPMSGLQRAVWWT
EHVLRHGGARHLRAPAANISWAEYLELELVSIVLMALLIGIVLVYSVLKR
CYKFVSRITTTIHKNKKN
```

T. absoluta g16225.2 UGT2B4-like

```
1       10       20       30       40       50
|       |        |        |        |        |
MSFLHLFITLLLNLVFAHAARILAVFPVPSISHQVVFRPLVQELAKRGHE
VVVITTDPAFPKGQAPTNLTEIDVHDMSYSTWTEFVAKSTGKSNDQYDQI
KTALDLFTIIVEKQIQNAEVQKILKDKSKTFDLLLLEACVRQALGFSYVY
KVPVIQVSSLGPVFDNYKSVGGPTHPLLYPNILNQRLYNLTIWEKLWVVY
YYYQVEYLYYDHENEENAMLKRNFGPDTPTVTELSENIDMLFLNVHPIWE
GNFPVPPSVIYMGGIHQKPSKDIPQDLKLFLDSSKHGVIYMSFGTNTNPS
TLPPERIQMFVKTFAQLPYDILWKWDKDSLPGQSKNIRIGKWFPQSDLLK
HPNIKLFITQGGLQSTDEAISAGVPLLGIPMLGDQWYNVEKYVYHKIGVK
LDLETITEEKFDNAIKTVINNESYRLNIKRLKELMHDEPMSGLQRAVWWT
EHVLRHGGARHLRAPAANISWSEYLELELVSIVLIALLIVIVLVYSVLKR
CYKIVSRILSNVDENKKNR
```

T. absoluta g16225.3 UGT2B20-like

```
1       10       20       30       40       50
|       |        |        |        |        |
MRLLSFVACVAFVAEIDCANILAYIPTPSISHQLPFRSFFKELAIQGHNL
TVITTDPAFSKGNAPSNITEIDVHDISYGPLLSSSLKEFTKGFKSDFVNQ
ASLAHSLFTPVIDNQLSDPEVHNAIFGNKYDLLFIEAIARPALAISYVQK
APVILLSSVGATFDVMEAMGAPTHPILYPLPWSRQRLYNLTLREKFSELY
THFTLLNVIATLEDHENRMLQKHFGSDIPALNKLYDNVDMLFLNTNPIFE
DNRPVPPSVIYMWGIHSKPEKQLPKDLKTYLDSSRHGVIYMSFGTNVDPS
LLSPEKIAIFVNVFCKLPYAVLLKWNQDELPGKCENIKIGKWFPQFDLLR
HPNIKAFVTQGGLQSTDESISAGVPLVGIPMLLDQYYNTEKYVHLNIGVQ
LDVETLTEEKFKNALTTVIDDESYRKNIKKLSALMRDEPMSGLQRAVWWT
EHVLRHGGARHLRAPAANISWSEYLELELVSLVLLAILVVILLIFTLLLT
CYRFHRNPLTVRKTKIN
```

T. absoluta g16226 UGT2B20-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MAYAAVYILFTLLFTSWVIKSESARILAVFPTPSYSHQIVFRPLTHELAR
RGHEVVVVTTDPVFLKENIPGNLTEIDVHDISYDIWRKKFLEIPTGKKRN
LQDNLKVIYNIVGEVFDEQLKNTEFKKIIDERQRFDLLILEAWIRPTLVL
SHIFKAPVIQISSLGRLWYNYEDLGAPVHPFLNPLPVRQKLYNLTIWEKI
NELFIEWKYNSVMNDVEMIYDEWIKSTFGPETPKLKELSNNIAMFFNNKH
PIWEGNNPIPPSIIHIWGINQKPEKQLPKELQTYLDSSKVGVIYISFGTN
VDTAKLPIETIQVFINVFSKLEFNVLWKWNQDVLPGKSDNIRISKWFPQS
DLLRHPNIKLFITQGGQQSTDEAIVAGVPLIGIPMLSDQWYNAEKYIHHG
IGKKLFIESLTEVELRATIEDVIGNESYRKNIKKLSALMHDEPMSGLQRA
VWWTEHVLRHGGARHLRAPAANISWAQYLELELVAILLFTIIVLIFLILG
ILRLLLVCFQNFHEKG
```

T. absoluta g16227 UGT2B20-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MCSVDINDSARILAVFPTASISHQVVFRPLTQELARRGHEVIVVTPDPAF
KKGQAPANLTEIDVHDMSYEVFREFLDHTTGKANDVHSQMEVAWNLAHKI
FHMQINTKEFKRMIDEKQQFDVIIIEAWLKPVLLVSHFFKAPVIQFSSLG
RIWSMYENLGAPVHPILYPDSLRQRLYNLTLWEKITELYSHWKFKMLTNN
IEEEQDNFIKKTYGADIPTLHELSNNVDMLFLNTHPMFEGNTPWPPGIIS
TWGIHHKPEMPLSKDLQTYLNSSKDGVIYMSFGTNVDPANLPPETIQMFI
RVFSKLPYDVLWKWNQDVLPGKTDNIRISKWFPQSDLLRHPKVKLFITQG
GLQSTDEAIVAGVPLIGIPMLADQWYNTEKYLHHGIGLKLNIETITEDIF
FTAIKKVIEDESYSKNIKRMGAIMRDQPMSGLQRAVWWTEHVLRHGGARH
LRAPAANISWAQQIENITFNYSARNSTHVCLKS
```

T. absoluta g16228 UGT2B20-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MKLLTAFICVAFITFSNGARILVYIPTPSISHQEPFRPLSQQLAKQGHEV
IVITADPAFPKGKTPPNLTEIDLHDMSYEFIKDTVGALKRGSKDEFIDQI
QVAVFDFLIDLLDLQYATKEVSDVIRDGKFDLLILEAFARPLLGITHLIK
APVILASSLGPLFFNSGAVGIPTHPILYPAATRQKLYNLTFWEKLRELYN
EYKIHRVIIGTEAKEHKMLQKHFGNDVPTVNELQNNIDMIFLNTHPVFEG
NYPVPPSVVYMRGVHKRPQKELPKDIKSYLDSSVHGIIYMSFGTNTDPTA
LSQDTMQMFVKVFCQLPYDVLWKWSQDELPGRCPNIRIGKWFPQSDLLRH
PNVKLFVTQGGMQSTDEAISAGVPLIGIPMLGDQWYNVERYKYLKIGMGL
DFETLTEEQFRDAVTTVIGDDSYRKNIRNLDTLMRDEPMSGLQRAVWWTE
YVLRHGGARHLRAPAANISWGQYFELEFVVLLVTGLLETMILFAISIFGN
FIAEYDI
```

T. absoluta g16232 UGT33AF1-like

```
1       10       20       30       40       50
|       |        |        |        |        |
MNFVSESARILAVFPTPSISHQVVFRPLTQELARRGHEVVVITTDPAYPK
GETPGNLTEVDVHDVSYTIWSKVMSMTSTGKRQDLHSQVEFSLRLFGEIF
EKQIQSDEVRAIIRDKKQKFDLILVEALIAPTLAFSHVFKAPVIQISSFT
SVRGDYEAMGAPTHPILYPEMTNQKLYNLSIWERLSVVWKWLLIRDLYSE
IEVLENEMLKRNFGPNMPELAELRNSVDMLFLNMHPIWEDNRPVPPGVVF
MGSIHQNPVKELPKELKSYLEESKNGVIYVNLGTNIETILPTDKVQTMLK
VFSKMPQNILMKWNAVELNTSSTNIKISKWFPQSDLLRHPKVKLFITQGA
LQSTNEAISAGVPLIGIPMFGDQWFNVEKYVKHNIGLRVDVDSITEEGFE
SAISTVLNDKSYRKSIIRLRSIMRDEPMSGLQRAVWWTEHVLRHGGARHL
RAPAANISWVQYLELELVVILLALLLSVIIALLGAAYFAWVFWEYTDDQK
VKTS
```

T. absoluta g16717 UGT2B20-like

```
1       10       20       30       40       50
|       |        |        |        |        |
MKLFFVFVFIILIVPIIDGAKILAYIPSPAISHQAPFHALFQELARRGHQ
VDVITTDPAFPKGSTPPNLTEIDVHDVSYGAVVEKFRNSTSTNHGDIFSQ
MWIGLNMFTEFMDDQLSVKEVHDAIHGKKYDLLFLEACARPALALTHVHK
VPVILLSSFGPMFNIMNVMGAPTHPLLYQSFTRQRIYNLTIWEKINELYV
HYRLHIMYANVEKRENKMLQKHFGTDLPSLSTMYDNIDMVFLNTHPILED
NRPVPPSVVYIWGSHKRPEKELSKDLKEFLDSSKHGVIYMSFGTNTDPSL
LPAKKIKNFVKVFCQLPYDVLWKWNKDELPGNCKNIKFGKWFPQSDLLRH
QNVKAFVTQGGLQSTEEAIRAGVPLIGIPMGRDQFANVEKYVHHNIGVQL
NLKSLTEDNFKDALRTVIEDESYRRNIKTLDALMQDEPMSGLERAIWWTE
HVLRHGGARHLRAPAANISLVQYLELELVAVVVLTLSAIFVIAFTVLIFC
CRLVRKRNAVYVKKTKIH
```

T. absoluta g20872 UGT2B2-like

```
1       10       20       30       40       50
|       |        |        |        |        |
MNLSFVSILVFLINSVASFNILAIYPYNGKSHSLVFRVLLRELATKGHNI
TVISHYPEEDPPENYHDISLAGSMRAVEGAVSIPFPTNHFKRYLNIAIAG
WYLVKSGERTCEVLLENKQVQDLINSKPKFDVIVLELFQSDCALGVAHIL
GAPVVGTASSIFLPFHYDRFGIPYNPSYVPFHFLEGGTKPNLIQRLERVV
FNFYMKSIFYWVSQRANQNTLAKYFDGIPPLEDLGREMKLVLAYQNFVLT
GSRIQPANVIDVAAYHVDKTKPLTGDLKEFVEQAKEGVIYINFGSMMKTS
SLPADKVEAILGAMNEFPHRFIWKWEDKTLKYDKNKLFINSWLPQVDILG
HPKTLAFYSHAGMGGTSEAIHFGVPMVAMPAFGDQPSNAAAIEEAGFGVQ
LHLRDLTKDSLVAALKKVLDPGFQAKAKEVSSAWHDRPQTALETAVFWIE
FVARHPNLTYRTAAADVPFYQYYCLDIAAVFLSLLGSFLFLISLCRGSKK
SVPEPRRQKTKKSKRE
```

T. absoluta g21063 UGT2B19-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MVFLNTHPIFEDNRPVPPSVVYIWGSHKRPEKELPKVFCQLPYDVLWKWN
KDKLPGNCKNIKFGKWFPQSDLLRHQNVKAFVTQGGLQSTEEAIRAGVPL
IGIPMGRDQFANVEKYVHHNIGVQLNLKSLTEDNFKDALRTVIEDER
```

T. absoluta g22125 UGT2B20-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MICIRILVIFVAINYCSCANILYSIPFTSKSHYIMLKAIGLELARRGHNV
TVITAFKETDHPPNYHQVKVDDKEIWETTGQKRPNVFTMVDISAEEFHDK
ILWGGGFGFTEVALNSPDVQKFLKEDHKFDLVIAEQFFQEATFILAHKYQ
APLVLITTYGNCMRHNIVTRNPLQLATVVQEFLDVKDPSSFLGRLRNWYF
TVYEYVWWKYWFLPKNEELVKKYVPNLPQPVPSLFEMQKNAVMILLNSHF
SFDPPTAYLPNVIEVGGLHLSKSNDKLPQDLQQILDEAKHGVVYVNFGSN
VRSSELPLEKKKAFLKVFSELKQTVLWKWEDDSLQNQPANVFVRKWLPQK
AVLAHPNIKVFVSHGGLIGTQEAVFHGVPIIGIPIYADQYNNLLRAQDIG
IGRILLYHDITEDTLRNTLNDVIKDNTYLLKAKETSARFKDRPMSALDTA
MFWIEYVIRNKGADFIKNPARDLSWFAYTMYDVYVFVLLSLLGFLYLVSK
IVRILLSQLSSKQTVKQDKKKMY
```

T. absoluta g22850 UGT50A4-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MTKNLSSKKGWLPWMLLPLLAGCAFGSSILMLTMGGTKSHKMPFWELARG
LNQKQVQIVTDEASILSRDKNHNITFISAFPPDFHLEGLEELAPEGLVSY
VRSYMVHDLVGARMRGQDPLPIQDIFRYGYEACDAFLSDYETRSFLRSGR
NFDLIVLDRRIP
```

T. absoluta g22851 UGT50A2-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MLCDDDLARSSEKTLWTQIPHVYDMAKNVSFVAARTDIIPCLIRDRICLM
LPRWPASIVKKQRGWILKLKNGSPAHGEAGFIYISMGSSVKTTKMPLAVH
RLIVNALGACHQRVVWKQDGDQNMTDIPSQREGCSDGCPQQGLTWSPKNQ
SIRNPRRSTQLCMKLSYHGVPIVVHPSLLRPRRQRSKSRGRRLCEKLDLQ
KSIQRKTIQSHQEVINDPTTEERSQKDSSC
```

T. absoluta g22875 UGT2C1-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MRIRSVSYLKGWLPWMLLPLLAGCAFGSSILMLTMGGTKSHKMPFWELAR
GLIRRNHNITFISAFPPDFHLEGLEELAPEGLVSYVRSYMVHDLVGARMR
GQDPLPIQDIFRYGYEACDAFLSDYETRSFLRSGRNFDLIILDGAYPECG
LGLVHRLKVPFMYINTVGFYAMPLSISGSPTPWSVTPFFGKAYTDNMGLI
DRAMNTAWYFGAYSMHAVMTTILQGVLRRHFGPQIPHVYDMAKNVSFVLQ
NGHYSVSYPRPYLPNVAEVACIHCKEAKRLDSEIEEWISGAGEAGFIYIS
MGSSVKTTKMPLAVHRLIVNALGRLPQRVVWKQDGDQNMTDIPSNVRLFR
WLPQQDLLGHPKIKAFVTHGGLLSMYETVYHGVPIVSIPVFCDHDANAAK
AEVDGYAKKLDLQNLSSERLYKAIKEVINDPTYRREVTKRQFLLRDQKET
PLERAVYWTEYVIRHKVASTFAIIAYVLRTGFNKLVDHVQNKRMDKFILK
SNTLLKRSKKLINHSTLAKKKL
```

T. absoluta g27172 UGT2B20-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MWIGLNMFTEFMDDQLSVKEVHDAIHGKKYDLLFLEACARPALALTHVHK
VPVILLSSFGPMFNIMNVMGAPTHPLLYQSFTRQRIYNLTIWEKINELYV
HYRLHIMYANVEKRENKMFQKHFGTDLPSLSTFYDNIDMVFLNTHPIFED
NRPVPPSVVYIWGSHKRPEKELPKDLKDFLDSSKYGVIYMSFGTNTDPSL
LPAKKIENFVKVFCQLPYDVLWKWNKDKLPGNCKNIKFGKWFPQSDLLRH
QNVKAFVTQGGLQSTEEAIRAGVPLIGIPMGRDQFANVEKYVHNNIGVQL
NLKSLTEDNFKDALRTVIEDESYRRNIKNLDALMQDEPMSGLERAIWWTE
HVLRHGGARHLRAPAANISLIQYLELELVAVVVLTLSAIFVIAFTVLIFC
CRLVRKRNAVHVKKTNIN
```

T. absoluta g27899 2-hydroxyacylsphingosine 1-beta-galactosyltransferase-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MGRSHQMVFEPLLQKLANRGHHVTTISFYPLKNPPANYTDVSLHGISDLG
LESVDLEMFEQSNKVLQWLGVERILMQLFAFWPLNEFALSTCKKLVDWPG
VAEAMKQPYDLVIMEYFNSYCMLGLLHVYRVNAPIVALSSTGLMPWTPSR
IGLDENPSYVPLLSSSFTTKMDFWQRLENSILQVYYKYWFKTEIQAKEQE
IIEKHFARKIPDLGELAKNVSLVIQNTHPSLHGVKPLLPGVVEAGGMHLD
HTRKPIPEYIERFINESDHGVILLSFGSLIKTASLPAYKEEMIVNACAKM
KQRVIWKYENSGDEGTLTGNILRVRWLPQMELLQHPKVLAFVAHGGLLGM
TEAVYAGKPMVVVPFFGDQPLNAAAAEARGMAKIVSYVQLSEKSLVEAME
KAVSAEMRLNARLVSQMWKDRPAQPLDTAVYWTERVLRWGHHDPLHSSAR
DLAFYEIALLDVAAAVILVILAALITLKILLSFIPKFLFGAKKQKLH
```

T. absoluta g28274 UGT2B20-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MKLCCVFVCVALIANIEAANILAYIPTPSISHQSPFRALFQELAKRGHDV
SVITTDPAFPKGQTPPNLTEYDIHDVSYEIMIREFHKMSKGVKSDLIDQM
RLASFMFVNIIDAQLNTTEVQTAIHGKKYDLLLIEAVCRPALALSYVHKA
PVIMLSSFGAFHIVMEAFGAPTHPLLYPLMMQQRIYNLTLWEKIDNLYRH
YQMNDLTYMTEELENKILKKYFGDGFPPVNELYDNVDMLFLNMHPIFEDN
RPIPPSVIYMWGVHSKPQKELPKDLKDYLDSSKNGVIYMSFGTNTDPTLL
PADVISKFVKVFCELPYDVLWKWNADELPGKCANIKTQKWFPQPDLLKHS
NIKAFVTQGGIQSTDEAITAGVPLVGIPMLGDQWYNVEKYVHLKIGVKLD
METLTEEKFKNAITTVINDESFRRNIKQLDALMRDEPMTGLERAVWWTEH
VLRHRGGRHLRAAAANISWAQYLELEVVSVIFLGLLFVVVSVLILLRLVY
KSVANIFSQKQKIKNH
```

T. absoluta g29883 UGT2B1-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MLPAYHVDKTKPLTGDLKEFVEQAKEGVIYINFGSMMKTSSLPADKVEAI
LGAMNEFPHRFIWKWEDKTLKYDKNKLFINSWLPQVDILGHPKTLAFYSH
AGMGGTSEAIHFGVPMVAMPAFGDQPSNAAAIEEAGFGVQLHLRDLTKDS
LVAALKKVLDPGFQAKAKEVSSAWHDRPQTALETAVFFWIEFVARHPNLT
YRTAAADVPFYQYYCLDIAAVFLSLLGSFLFLISLCRGSKKSVPEPRRQK
TKKSKRE
```

T. absoluta g30114 UGT2C1-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
XNHNITFISAFPPDFHLEGLEELAPEGLVSYVRSYMVHDLVGARMRGQDP
LPIQDIFRYGYEACDAFLSDYETRSFLRSGRNFDLIVLDGAYPECGLGLV
HRLKVPFMYINTVGFYAMPLSISGSPTPWSVTPFFGKAYTDNMGLIDRAM
NTAWYFGAYSMHAVMTTILQGVLRRHFGPQIPHVYDMAKNVSFVLQNGHY
SVSYPRPYLPNVAEVACIHCKEAKRLDSTYRERTRAPASEGWCGKQDGDQ
NMTDIPSNVEAVSDGCHKQDLLGHPKIKAFVTHGGLLSMYETVYHGVPIV
SIPVFCDHDANAAKAEVDGYAKKLDLQNLSSERLYKAIKEVINDPTYRRE
VTKRQFLLRDQKRTPLEQSCLWTEYVIRHKCAYHLQSLQKT
```

T. absoluta g31445 UDP 2-hydroxyacylsphingosine 1-beta-galactosyltransferase-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MKQPYDLVIMEYFNSYCMLGLLHVYRVNAPIVALSSTGLMPWTPSRIGLD
ENPSYVPLLSSSFTTKMDFWQRLENSILQVYYKYWFKTEIQAKEQEIIEK
HFARKIPDLGELAKNVSLVIQNTHPSLHGVKPLLPGVVEAGGMHLDHTRK
PIPEYIERFINESDHGVILLSFGSLIKTASLPAYKEEMIVTACAKMKQRV
IWKYENSGDEGTLTGNILRVRWLPQMELLQHPKVLAFVAHGGLLGMTEAV
YAGKPMVVVPFFGDQPLNAAAAEARGMAKIVSYVQLSEKSLVEAMEKADA
SERPSGLSIWKDRPAQPLDTAVYWTERVLRWGHHDPLHSSARDLAFYEIA
LLDVAAAVILVILAALITLKILLAFIPKFLFGAKKQKLH
```

T. absoluta g31448 UDP 2-hydroxyacylsphingosine 1-beta-galactosyltransferase-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MCCGCGIICLYIERFINESDHGVILLSFGSLIKTASLPAYKEEMIVNACA
KMKQRVIWKYENSGDEGTLTGNILRVRWLPQMELLQHPKILAFVAHGGLL
GMTEAVYAGKPMVVVPFFGDQPLNAAAAEARGMAKIVSYVQLSEKSLVEA
MEKAVSAEMRLNARLVSQMWKDRPAQPLDTAVYWTERVLRWGHHDPLHSS
ARDLAFYEIALLDVAAAVILVILAALITLKILLSFIPKFLFGAKKQKLH
```

T. absoluta g32146 UGT2B2-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MLAIYPYNGKSHWLVYKVLLRELAAKGHNVTVISHFPEKDPHKNYHHISL
AGSMHAVEGDVSIPFPTNQFNRYLNIAMVGWYLVDSGATTCEVLLGNKQV
QDLIKSKPKFDVIVLEVFNSDCALGIAHKLGAPVVGTTSSVFMPFHYNRF
GIPYNPSYVPFHFLEGGTKPNLIQRLERVIFHFYIKSIFYWVSQRANQNT
LAKYFDDIPPLEDLAREIKFVLAYQNFALTGSRIQPANVIDVAAYHVEKP
KPLTGDLNKFIEEAKDGVIYINFGSVMKTSSLPADKVEAILGAMDEFPHR
FIWKWEDKTLKYDKNKLYIDSWLPQVDILGHPKTLAFYSHAGMGGTSEAI
HYGVPMVAMPAFGDQPSNAAAIEESGFGVKLHFRDLTKDSLVAALKKVLD
PGFQARAKEVSSAWQDRPQTSLETAVFWTEFAARHPSLTYRAPSADVPCY
QYYCLDIAAVFLGFLSSFWLLISLCKGSKKSAPVSKRQKTKRKRE
```

T. absoluta g32147 UGT2B10-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MAKYGFLVFYFVATIFATDSYKILGIFPSLDRSNYLTYRGLFAELANRDH
EVTLISHFELPNAPASYKDILLSDKKVYKQLSYDSVIANEVSRVPFETLV
STKAGNDDCKTLMNNHQVLHLINSRAKFDVIIVESYNSDCGLALASNLSA
PYISFNPQPLQSWQYNRLGINFNSASVPQPGLPYGKEPWFLDRLRSYVIY
YVSNWVYYVGSQVTDHVYLYKYLGDDLPTLESIASNASLVFVNTHQSVFG
GVPRPDNVIDVGGIHIRQPKIIPTEIDRFISEAEYGVVYVNLGSTVTDST
LPKDKLDELVATFRKLPHRVLWKWEGVVDNLPKNVMTMRWFPQYDVLKHD
NVKVFISHAGILSTIEAIDAGVPVVAVPLFGDQYGNAAAMQDAGIATIVS
YQDLNKEYLLDAINDVLDPKSQQQAQLVSRIWHDRPTSPLETAIYWTEYV
ARYGGAPNLQATSVQKPLYQQYQLDVLAFVALVVYILTKVSCKILSACCC
TCCCTVDDTSVTVVEERKTKRVKFE
```

T. absoluta g33223 UGT2C1-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MTKNLSSKKGWLPWMLLPLLAGCAFGSSILMLTMGGTKSHKMPFWELARG
LIRRNHNITFISAFPPDFHLEGLEELAPEGLVSYVRSYMVHDLVGARMRG
QDPLPIQDIFRYGYEACDAFLSDYETRSFLRSGRNFDLIVLDGAYPECGL
GLIPHVYDMAKNVSFVLQNGHYSVSYPRPYLPNVAEVACIHCKEAKRLDS
EIEEWISGAGEAGFIYISMGSSVKTTKMPLAVHRLIVNALGRLPQRVVWK
QDGDQNMTDIPSNVRLFRWLPQQDLLGHPKIKAFVTHGGLLSMYETVYHG
VPIVSIPVFCDHDANAAKAEVDGYAKKLDLQNLSSERLYKAIKEVINDPT
YRREVTKRQFLLRDQKETPLERAVYWTEYVIRHKVASTFGIIAYVLRTGF
NKLVDHVQNKRMDKFILKSNTLLKRSKKLINHSTLAKKKL
```

T. absoluta g182652 UGTB20-like

```
 1       10       20       30       40       50
 |       |        |        |        |        |
MLQKHFGTDLPSLSTMYDNIDMVFLNTHPILEDNRPVPPSVVYIWGSHKD
QKRKLSKVFCQLPYDVLWKWNKDKLPGNCKNIKFGKWFQQSDLLRHQNVK
AFVTQGGLQSTEEAIRAGVPLIGIPMGRDQFANVKNMCTIILAYN
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Appendix 4

Research Article

Received: 9 February 2021 Revised: 30 March 2021 Accepted article published: 30 March 2021 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.6394

Parthenogenesis in UK field populations of the tomato leaf miner, *Tuta absoluta*, exposed to the mating disruptor Isonet T

Charles Grant,[a] Rob Jacobson[b] and Chris Bass[a*]

Abstract

BACKGROUND: The tomato leafminer, *Tuta absoluta* is a damaging pest of tomato crops worldwide. In the UK *T. absoluta* is controlled using an integrated pest management (IPM) strategy that includes pheromone-based mating disruption. However, some growers have reported a loss of efficacy of this technology, and there are concerns that *T. absoluta* may evolve resistance *via* changes in its capacity to reproduce asexually. In this study we investigated the reproductive capacity of virgin populations of *T. absoluta* collected from a UK glasshouse before (EVH2016) and after (EVH2019) the introduction of the mating disrupter Isonet T.

RESULTS: In line with earlier reports, we demonstrate that UK populations of *T. absoluta* can reproduce parthenogenetically, and observed 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 were also observed between the two virgin populations, with the EVH2019 strain producing fewer eggs, a delayed onset of egg laying and increased lifespan.

CONCLUSION: The low rate of parthenogenetic reproduction seen 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, and, together with the increased rate of parthenogenesis, may reflect selection from the use of Isonet T. Thus, regular monitoring of the reproductive capacity of UK populations should be conducted, and mating disruption used only as part of IPM to avoid the emergence of resistance.

Keywords: Tuta absoluta; resistance; Isonet T; mating disruption; parthenogenesis

1 INTRODUCTION

Tuta absoluta is a highly destructive pest of tomato crops globally. First identified in South America, *T. absoluta* reached the UK in 2009 and is now present in most of Europe, Africa, the Middle East and parts of Asia.[1] The economic impact of *T. absoluta* on the tomato growing industry can be profound, resulting in 100% yield loss if left untreated, with 60% of global tomato crops estimated to have been affected.[1] In the UK, growers have reported losses of up to £ 50 000 per hectare (Rob Jacobson pers. comm.). Control of *T. absoluta* has been achieved through the implementation of an integrated pest management (IPM) strategy, incorporating crop monitoring, biological control and application of pesticides. This strategy, once established, proved extremely effective, however, over time it became compromised due to the emergence of resistance to several of the insecticides used for control.[2,3] To restore full control through IPM, a mating disruptor was developed by Shin Etsu Chemical Co. Ltd and introduced to the market under the product name of Isonet T. This product works by inundating closed glasshouse environments with a synthetic version of the female sex pheromone of *T. absoluta* ((3E,8Z,11Z)-3,8,11-Tetradecatrienyl acetate).[4] When deployed, the high levels of synthetic pheromone in the environment prevent male moths effectively detecting semiochemical concentration gradients emitted by females, inhibiting location of a mate and thus preventing reproduction. The incorporation of Isonet T into contemporary IPM proved a spectacular success and was found to eradicate outbreaks in as little as one generation.[5] This product not only eliminated yield losses, but also decreased reliance on chemical pesticides, thus providing additional environmental benefits. In spite of the remarkable success of Isonet T in many UK commercial tomato glasshouses, one grower in Evesham subsequently reported loss of effective control of *T. absoluta* by the product (Rob Jacobson pers comm.). *T. absoluta* at this site were also highly resistant to spinosad,[3] a pesticide used in previous

* *Correspondence to: C Bass, College of Life and Environmental Sciences, Biosciences, University of Exeter, Penryn Campus, Penryn, Cornwall TR10 9FE, UK. E-mail: c.bass@exeter.ac.uk*

a *College of Life and Environmental Sciences, Biosciences, University of Exeter, Penryn Campus, Penryn, Cornwall, UK*

b *RJC Ltd, Bramham, West Yorkshire, UK*

IPM strategies, resulting in limited options for alternative chemical control.

The effectiveness of mating disruption in eliminating pest populations can be severely compromised if the target pest has the capacity to reproduce in the absence of sex. In this regard, research by Megido and Verheggen (2012) previously indicated that French populations of *T. absoluta* have the capacity to reproduce asexually through the process of deuterotokous parthenogenesis.[6] The authors found that 4 out of 20 virgin females laid viable eggs, and 57 larvae reached adulthood with a sex ratio of 1/1.5 (male/female). However, the fecundity of these adult moths was not reported. Following this, work by Abbes and Chermiti (2014) demonstrated that individual females from three virgin populations of Tunisian *T. absoluta* (30 individuals for each population) could lay eggs (30%, 13.33% and 50%), of which 11.36%, 16.67% and 17.02% hatched.[7] However, out of a collective 10 F1 female virgins only one egg was laid and this was not viable. Both of these studies demonstrate parthenogenetic reproduction in *T. absoluta*. It is therefore possible that the loss of efficacy of Isonet T in the Evesham glasshouse was caused, at least in part, by parthenogenetic reproduction circumventing the effectiveness of mating disruption.

Asexual reproduction in *T. absoluta* is likely tychoparthenogenetic, a process by which a small proportion of unfertilized eggs hatch spontaneously. Offspring survival of this reproductive mode is typically much lower than for sexual reproduction.[8] However, tychoparthenogenesis can result in a positive feedback loop in which males are lost through female-biased sex ratios and increasing mate limitation. As a result, the strength of selection for tychoparthenogenesis increases in concert with the proportion of tychoparthenogenetic offspring in the sexual population.[9]

In the current study we assessed the level of parthenogenetic reproduction in UK populations of *T. absoluta*, and looked for evidence of any potential shift in frequency or viability of parthenogenetic reproductive output as a result of selection through the use of Isonet T. The data provided by this study is of direct relevance to the control of UK populations of *T. absoluta* and the robustness of current IPM strategies for this pest.

2 MATERIALS AND METHODS

2.1 Insect cultures

Insects were collected from R & J Holts Sandylands Nurseries (Evesham) in 2016 and 2019, *i.e.* before and after the deployment of Isonet T. All insect stages were housed in bug dorm cages (MegaView science co.) in CE rooms at 24 °C 16L:8D and 65% humidity. Cultures were supplied with tomato plants (var. Money Maker) *ad libitum*. Insects were collected and sexed for experimentation at pupal stage.

2.2 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).[10] One hundred female pupae were placed in individual chambers. A plastic beaker with a small hole in the bottom was placed in another plastic beaker containing 100 mL of water and fertilizer. The petiole of a tomato leaf was inserted through the hole with any left-over space blocked with cotton wool. A 1.5 mL plastic tube 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 number of eggs produced was 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.

2.3 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 used to determine significance and reject the null hypothesis that no difference exists. Where outcomes were categorical, χ^2 tests were used to detect differences in the populations, and where assumptions of χ^2 were not met Fishers exact test was used.

3 RESULTS

3.1 F0 virgin reproductive traits

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 ($\chi^2 = 2.6316$, 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 ($\chi^2 = 1.8601$, df = 1, P-value = 0.17) with 84 females laying eggs in the EVH16 population compared to 82 females in EVH19.

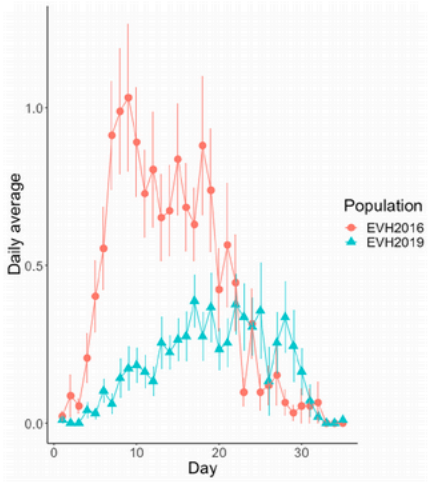

Figure 1. Differences in average daily egg production by virgin females of the EVH2016 and EVH2019 strains over 35 days (W = 806, P-value = 0.02). Error bars represent ± SEM.

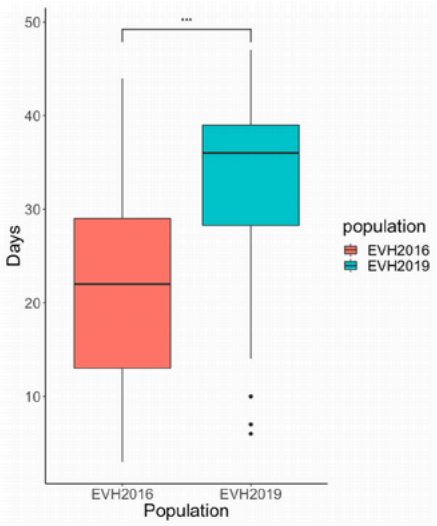

Figure 2. Box plots showing range of lifespan of virgin females of the EVH2016 and EVH2019 strains (W = 1682, *P*-value <0.001).

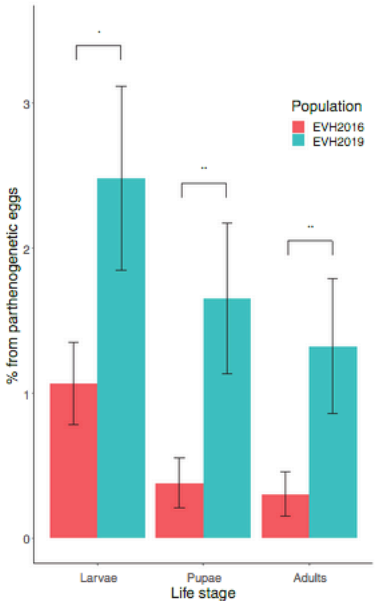

Figure 3. Percentage differences in larvae (χ^2 = 4.666, df = 1, *P*-value = 0.03), pupae (*P*-value <0.01) and adults (*P*-value = 0.01) from total number of eggs laid by virgin females of the EVH2016 and EVH2019 strains. Error bars display standard error for all proportions.

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 1 shows the differences between average daily increase in egg number across the two populations. Significant 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 (Fig. 2). Significant 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 in 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.

3.2 Parthenogenesis

Six 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, three females and one male eclosed. These females laid 27, 57 and eight 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 six females and two 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 (χ^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 (Fig. 3).

4 DISCUSSION

Our results reveal clear differences in life history traits associated with reproduction between two populations of virgin *T. absoluta* that were differentially exposed to mating disruption in the field. These included marked differences in the number of eggs laid, the start date of laying and lifespan. Both populations from Evesham had a low frequency of virgin females capable of laying viable eggs, and significant differences between these populations in the number of larvae, pupae and adults produced were not observed. However, a significant increase in their proportions as a function of eggs laid was observed.

What are the implications of these results for the resilience of control employing mating disruption against *T. absoluta* in the UK? Firstly, the very low levels of parthenogenesis observed in

both the Isonet T exposed and unexposed populations suggests changes in the rate of parthenogenesis, in isolation, are unlikely to explain the reduced levels of *T. absoluta* control reported at the Evesham glasshouse in 2019. A small but significant increase in the proportion of eggs developing parthenogenetically was observed in the EVH2019 population compared to EVH2016. However, the reduced total egg production of the former strain in comparison to the latter, resulted in no significant increase in total number of larvae, pupae and adults produced between the two strains. This mitigated any effect of the increased rate of parthenogenesis of the EVH2019 on population size. Furthermore, in both populations no first generation virgin females went on to produce viable eggs. This suggests that the ability of *T. absoluta* to persist over more than one generation by parthenogenesis is limited, however, given the small sample size of the second generation in this study, more extensive testing is required to confirm this.

Secondly, the marked differences in life history traits between virgin females of the EVH2016 and EVH2019 strains may also have relevance for IPM incorporating mating disruption. Evolutionary theory shows that life history traits are controlled by energetic trade-offs between intrinsically controlled factors such as reproductive output and longevity.[11] It is plausible therefore that in a mate-limited environment, selection would result in the diversion of energetic resources away from egg production and reallocation to longevity. The reduction in egg production would therefore be an antagonistic pleiotropic result of increased longevity - a strategy that would increase the likelihood of encountering a mate. Furthermore, the delay in average egg laying date observed in the EVH2019 strain would postpone energetic expenditure in reproductive output, facilitating longevity,[12] until a mating occurs, which in turn stimulates egg production.

Both parthenogenesis[9] and longevity[13] have been shown to result from populations that are male limited or at low density, which is consistent with our data. Furthermore, if parthenogenesis has a selective advantage in pheromone inundated environmental conditions, as suggested by the observed significant increase in its frequency, it could further proliferate as a result of a positive feedback loop. Specifically, males could be lost through a skewed parthenogenetic sex ratio, as observed in EVH2019. The overall reduction in egg production of this population could further drive parthenogenetic evolution, as a reduction in overall offspring produced from a low frequency of sexual encounters within the population would increase the relative fitness of any offspring produced through parthenogenesis. Thus, the increased rate of deuterotokous parthenogenesis in combination with a shift in longevity and egg laying date may allow the persistence of populations within the crop. This would allow populations to re-establish if there was any diminution of mating disruption. Furthermore, while sampling of additional field populations is required, our data supports the notion that there could be an inherited genetic component to asexual reproduction in *T. absoluta*, which can be selected for under conditions that limit sexual reproduction.

Finally, the changes in *T. absolutas*' life history traits may also have synergistic effects on the efficacy of biocontrol agents used as part of IPM in the UK. The predatory bug, *Macrolophus pygmaeus*, requires time to build effective population sizes in the crop. This means that pesticide applications are often required to knock back *T. absoluta* populations until the natural enemy is established. Later egg laying and longer life span of *T. absoluta* would increase the time available for this process to occur. However, *M. pygmaeus* is polyphagous and can cause damage to the crop if densities are too high. Therefore a reduction in density of the applied biocontrol may be required to mirror any reduced reproductive output of *T. absoluta*.

A limitation of our study is the fact that, a) only two strains were tested and, b) these differed in the duration spent in culture under controlled environment conditions in the lab. In particular we cannot discount the fact that differences observed between the strains in certain life-history traits, at least in part, result from differences in acclimation to laboratory conditions. Thus further testing of field-strains is required to provide additional evidence that the findings reported in this study are associated with the use of pheromone-based mating disruption.

5 CONCLUSION

In summary, the observed increase in rate of parthenogenesis in UK glasshouse populations of *T. absoluta* following the deployment of Isonet T is unlikely to explain partial control loss of mating disruption. This news is especially welcome in light of *T. absoluta*'s capacity for resistance to chemical control.[3] However, the marked differences in life history traits in UK populations that differ in exposure to mating disruption has implications for the current use of this control method, resulting in populations that may be more resilient at persisting within the crop at lower densities. Thus, further sampling of UK populations is warranted to examine the extent to which the modified life history traits identified in this study are observed in *T. absoluta* populations from glasshouses using Isonet T for control. Furthermore, the fact that deuterotokous parthenogenesis exists, the fact that populations are male restricted, and the fact that populations persist at low densities, does - according to evolutionary theory - provide the right circumstances for further changes in the rate of parthenogenesis to evolve.

ACKNOWLEDGEMENTS

This work was funded by a PhD studentship award from the Agriculture and Horticulture Development Board (AHDB) (CP 162). The authors also thank the members of the British Tomato Growers' Association for their support and co-operation throughout the study.

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Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

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