University of Cyprus

DEPARTMENT OF BIOLOGICAL SCIENCES

SUSCEPTIBILITY OF *TUTA ABSOLUTA* TO INSECTICIDES, IDENTIFICATION OF RESISTANCE MUTATIONS AND BIOLOGICAL CONTROL WITH TWO GENERALIST PREDATORS

DOCTOR OF PHILOSOPHY DISSERTATION

GEORGIOS MICHAELIDES

2019

University of Cyprus

DEPARTMENT OF BIOLOGICAL SCIENCES

SUSCEPTIBILITY OF *TUTA ABSOLUTA* TO INSECTICIDES, IDENTIFICATION OF RESISTANCE MUTATIONS AND BIOLOGICAL CONTROL WITH TWO GENERALIST PREDATORS

GEORGIOS MICHAELIDES

A Dissertation Submitted to the University of Cyprus in Partial Fulfillment of the Requirements for the Degree of Philosophy

May 2019

VALIDATION PAGE

Doctoral Candidate: Georgios Michaelides

Doctoral Thesis Title: Susceptibility of *Tuta absoluta* to insecticides, identification of resistance mutations and biological control with two generalist predators

The present Doctoral Dissertation was submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy at the ***Department of Biological Sciences*** *and was approved on the 7th May 2019 by the members of the* ***Examination Committee****.*

Examination Committee:

Research Supervisor: _____

Spyros Sfenthourakis, Professor

Committee Member: _____

Anna Papadopoulou, Assistant Professor

Committee Member: _____

Dionysios Perdikis, Associate Professor

Committee Member: _____

Chrysoula Pitsouli, Assistant Professor

Committee Member: _____

Menelaos Stavrinides, Assistant Professor

DECLARATION OF DOCTORAL CANDIDATE

The present doctoral dissertation was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy of the University of Cyprus. It is a product of original work of my own, unless otherwise mentioned through references, notes or any other statements.

Georgios Michaelides

_____

ΠΕΡΙΛΗΨΗ

Ο υπονομευτής της τομάτας, *Tuta absoluta*, είναι ένας από τους πιο σοβαρούς εχθρούς της τομάτας και κατάγεται από τη Ν. Αμερική. Το έντομο εισέβαλε και εγκαταστάθηκε στην Ισπανία το 2006, που ήταν και η πρώτη αναφορά εκτός Ν. Αμερικής. Από την Ισπανία εισέβαλε σε γειτονικές χώρες και το 2010 αναφέρθηκε για πρώτη φορά στην Κύπρο. Σήμερα έχει φτάσει μέχρι τη Ρωσία, τη Νότιο Αφρική, την Ινδία και το Νεπάλ. Λόγω της ικανότητας των ανήλικων σταδίων του να τρέφονται με όλα τα υπέργεια μέρη των φυτών τομάτας, οι απώλειες που προκαλεί μπορεί να φτάσουν μέχρι και την πλήρη καταστροφή της καλλιέργειας αν δεν παρθούν οποιαδήποτε μέτρα εναντίον του. Εκτός από την τομάτα μπορεί και προσβάλλει όλα τα καλλιεργούμενα και αυτοφυή φυτά της οικογένειας Solanaceae.

Υπάρχουν διάφορα προληπτικά και κατασταλτικά μέτρα που μπορούν να εφαρμοστούν για την ελάττωση ή ακόμα και αποφυγή της προσβολής των καλλιεργειών τομάτας από το *T. absoluta*. Το κύριο μέτρο όμως της καταπολέμησής του υπονομευτή της τομάτας είναι η χημική καταπολέμηση με διάφορα εντομοκτόνα σκευάσματα.

Ωστόσο, η αλόγιστη χρήση εντομοκτόνων σκευασμάτων από τους παραγωγούς τομάτας μπορεί να προκαλέσει ανθεκτικότητα του *T. absoluta* στα εντομοκτόνα που χρησιμοποιούνται εναντίον του. Έχουν αναφερθεί πάρα πολλά παραδείγματα ανάπτυξης ανθεκτικότητας σε χώρες όπου υπάρχει ο εχθρός αυτός και αρκετά φυτοπροστατευτικά προϊόντα δεν είναι πλέον αποτελεσματικά για την αντιμετώπιση του.

Το πρώτο μέρος της παρούσας διδακτορικής διατριβής μελέτησε την ευαισθησία των Κυπριακών πληθυσμών του υπονομευτή της τομάτας στις τέσσερις πιο ευρέως χρησιμοποιούμενες δραστικές ουσίες για την καταπολέμησή του: Μελέτες στο εργαστήριο έδειξαν ότι ο υπονομευτής της τομάτας ανέπτυξε ανθεκτικότητα στα chlonathraniliprole και indoxacarb 188 και 28 φορές περισσότερο σε σχέση με τον ευαίσθητο πληθυσμό αντίστοιχα, ενώ δεν εντοπίστηκε ανθεκτικότητα στα ememactin benzoate και spinosad.

Στο δεύτερο μέρος της διατριβής διερευνήσαμε περαιτέρω την ανθεκτικότητα στα εντομοκτόνα chlorantraniliprole και indoxacarb. Πραγματοποιήθηκαν μοριακές αναλύσεις για να ανιχνευθούν τυχόν μεταλλαγές στις δύο διαμεμβρανικές πρωτεΐνες όπου δρουν τα εντομοκτόνα αυτά. Εντοπίστηκαν οι μεταλλαγές F1845Y και V1848I στους διαύλους

ιόντων Na+/K- στο νευρικό σύστημα των εντόμων όπου δρα το indoxacarb. Επιπλέον, βρέθηκαν οι μεταλλαγές G4903V και I4746M στους υποδοχείς της ρυανοδίνης στο μυϊκό σύστημα του *T. absoluta* όπου δρα το chlorantraniliprole. Οι μεταλλαγές αυτές βρέθηκαν προηγουμένως σε ανθεκτικούς ελληνικούς, ιταλικούς και ισπανικούς πληθυσμούς του *T. abosluta*.

Λόγω της τάσης που έχουν οι εχθροί των καλλιεργούμενων φυτών να αναπτύσσουν ανθεκτικότητα στα εντομοκτόνα και της σημασίας που δίνεται στην προστασία και διατήρηση του φυσικού περιβάλλοντος, τα τελευταία χρόνια άρχισε να εφαρμόζεται και η βιολογική καταπολέμησή τους με διάφορους ωφέλιμους οργανισμούς. Ως εκ τούτου, το τρίτο και τελευταίο μέρος της εργασίας αυτής αφορά δύο πολυφάγα έντομα της οικογένειας Miridae, τα *Macrolophus pygmaeus* και *Nesidiocoris tenuis* τα οποία χρησιμοποιούνται κυρίως σε θερμοκηπιακές καλλιέργειες για καταπολέμηση διάφορων εχθρών όπως αλευρώδεις, λεπιδόπτερα, ακάρεα κ.ά. Έχουν γίνει κάποιες μελέτες για την ικανότητα των δύο αυτών θηρευτών να τρέφονται με το *T. absoluta* όμως χρειάζεται περαιτέρω έρευνα για την αποτελεσματικότητά τους.

Η δική μας μελέτη εστίασε στα δύο αρπακτικά αυτά όταν τρέφονταν με τα αυγά του *T. absoluta*. Συγκεκριμένα, βρέθηκε Τύπος ΙΙΙ λειτουργικής απόκρισης και για τους δύο θηρευτές. Επιπλέον εντοπίστηκε ότι το *N. tenuis* είναι πιο αποτελεσματικός θηρευτής των αυγών του *T. absoluta* από το *M. pygmaeus* καθώς καταναλώνει μεγαλύτερη ποσότητα αυγών σε υψηλές πυκνότητές τους. Όσον αφορά τις διαειδικές αλληλεπιδράσεις μεταξύ των δύο θηρευτών, παρατηρήθηκε ανταγωνισμός σε μικρές πυκνότητες λείας και συνεργιστική κατανάλωση στη μεγαλύτερη πυκνότητα λείας. Στην περίπτωση των ενδοειδικών αλληλεπιδράσεων, άτομα του *N. tenuis* θα δώσουν καλύτερη καταστολή της λείας στις μικρές πυκνότητές της σε σχέση με άτομα του *M. pygmaeus*.

ABSTRACT

Tuta absoluta, known as the South American tomato pinworm, is one of the most disastrous pests of tomato cultivation worldwide. In 2006, *T. absoluta* invaded Spain from South America. Since then, it has spread rapidly to most European, African and Asian countries. Although tomato is the main host, the pest infests other cultivated and wild Solanaceae species. The S. American tomato pinworm is controlled mostly by chemical insecticides, and failure to control in an increasingly common phenomenon. The increase in pesticide use to control the pest increases production costs for farmers and impacts negatively the environment and non-target organisms.

Several examples of insecticide resistance for *T. absoluta* have been reported from different countries in the last 20 years. In order to develop a successful Insecticide Resistance Management (IRM) strategy for any major pest, one needs to identify the baseline toxicity to insecticides and then monitor susceptibility levels**.** In Cyprus, the current status of susceptibility levels to the main insecticides that are used to control *T. absoluta* has never been studied before.

Our first aim in the current work was to investigate the susceptibility levels of Cypriot *T. absoluta* populations against the four most commonly used insecticides (chlorantraniliprole, indoxacarb, emamectin benzoate, spinosad). More precisely, we found that the insecticides chlorantraniliprole and indoxacarb were not effective in controlling *T. absoluta* populations, where their resistance ratios found to fluctuate between 28 – 188 and 3 – 23, respectively. However, the insecticides emamectin benzoate and spinosad are still very effective against the pest.

To identify the mechanisms driving the development of resistance to indoxacarb and chlorantraniliprole we conducted molecular analyses to find out if there were any mutations at the transmembrane protein coding genes where the two insecticides act. Two point mutations for each gene were detected: The F1845Y and V1848I target-site mutations for indoxacarb; and the G4903V and I4746M mutations for chlorantraniliprole.

A sustainable method for pest control in modern agriculture is the use of natural enemies in Integrated Pest Management (IPM) programs. In the last part of this thesis we conducted a research with two generalist predators, *Nesidiocoris tenuis* and *Macrolophus pygmaeus* (Hemiptera: Miridae) to find out if they are effective predators of *T. absoluta* eggs. Specifically, their functional response and the intraspecific and interspecific

interactions when preyed on *T. absoluta* eggs were tested. We concluded that these two predators could not only control this pest, but also they suppressed high densities of *T. absoluta* eggs. Egg consumption increased with increasing egg density and the two predators exhibited a Type III functional response. Predation rates were strongly affected by prey density. Using the MRM, we found risk reduction at intraspecific treatments at high prey density. Applying the substitutive model, we detect risk enhancement at interspecific treatments at the highest egg density.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the encouragement and support of many people to which I am indebted and really grateful.

First of all, I would like to express my appreciation and sincere gratitude to my advisor, Professor Spyros Sfenthourakis, for his supervision. Without his knowledge and guidance this work would not have been possible. Special thanks to my co-advisor Nicos Seraphides for being motivating and supportive. Both have been constantly available to discuss whenever I got stuck with my research, providing the right direction to help me.

I would also like to thank the other members of my Ph.D committee, Assistant Professor Anna Papadopoulou, Associate Professor Dionysios Perdikis, Assistant Professor Crysoula Pitsouli and Assistant Professor Menelaos Stavrinides for their time and useful comments improving this work. Furthermore, special thanks to Assistant Professor Dr. Alexander Kirschel for being in my committee all these years.

Moreover, I would like to acknowledge my lab friend Andreas Dimitriou for sharing his knowledge with me, his valuable comments and his encouragement. He is a true friend.

Thanks are also due to the staff of the Insectary of Agricultural Research Institute. Many have contributed, in ways large and small, to this achievement. Thanks to everyone for their assistance, the good advice and encouragement. Thanks to Maria Panagiotou, Nicos Loizias and Andreas Kinezos.

Through my PhD I was lucky to meet Dr. Emmanuel Roditakis and all the members of the Institute of Olive Tree, Subtropical Plants and Viticulture, of the Department of Viticulture, Vegetable crops, Floriculture and Plant protection in Heraklion, Crete. I was also very lucky to get experienced in Dr. John Vontas lab and work with Dr. Aris Ilias at the Institute of Molecular Biology and Biotechnology in Heraklion, Crete.

In addition, I would like to thank my parents, my brothers and sister for their unconditional love and constant support at all times. Special thanks to my parents in law. I am thankful for their constant support and help.

Finally, I owe my biggest thank to my wife, Maria. The love of my wife has been my constant source of inspiration. Thank you for your support, patience and your endless help not only with the statistical analysis, but also with every daily negligible and serious problem. Thank you for standing by my side. I am also very grateful to you for giving birth to our children Fay and Nicolas.

DEDICATION

To Fay, Nicolas and Maria

“***Insecticide resistance is probably the best proof of the effectiveness of natural selection yet obtained***”

Theodosius Dobzhansky

Table of Contents

List of Figures

List of Tables

CHAPTER 1

Introduction

1.1 *Tuta absoluta* (Lepidoptera: Gelechiidae) taxonomy

The South American tomato pinworm *Tuta absoluta* (Lepidoptera: Gelechiidae) was first characterized by Meyrick in 1917 as *Phthorimaea absoluta*, relying on insects gathered from Huancayo (Peru).

The Gelechiidae micro-moth family consists of species that are forestry and agricultural pests, as well as species of ecological and biological interest (Lee et al., 2009). It includes 4700 described species from about 500 genera (Nieukerken et al., 2011). The Gelechiidae family is one of the most diverse Lepidoptera fauna in terms of species number, with distribution all around the world (Karsholt et al., 2013). Adults from this family are small to medium in size moths with a grey to brown color. Their larvae present various feeding behaviors. Some of them feed as leaf-miners, stem or fruit borers, into the flowers or they feed in rolled or tied leaves, hide in silken tubes in the earth or into other larvae case. Their diet includes a wide variety of plants including grasses, herbs, deciduous and coniferous trees, mosses, ferns, and lichens (Powell, 1980). Larvae of 258 species of this family, belonging in 85 genera, are pests of crops or stored food products, causing serious economic damage. Some of them are well-known species such as; *Tuta absoluta* (Meyrick) (South American tomato pinworm), *Phthorimaea operculella* (Zeller) (potato tuber moth), *Pectinophora gossypiella* (Saunders) (pink bollworm), *Anarsia lineatella* (Zeller) (peach twig borer) and *Sitotroga cerealella* (Olivier) (angoumois grain moth) (Zhang 1994).

Even though gelechiids include numerous harmful species, there are also many beneficial ones. Beneficial species are used as biological control agents to control invasive weeds worldwide, due to the fact that gelechiid larvae are broadly monophagous and they infest only a plant species or genus (Karsholt et al., 2013; Boggs et al., 1991; Klinken et al., 2003). Zhang (1994) listed the gelechiid pests, where the most harmful ones

are the borers and the miners, even though these feeding behaviors are rare throughout the subfamilies of Gelechiidae.

1.2 Morphology and biology of *T. absoluta*

The South American tomato pinworm is a holometabolous micro-lepidopteran insect. Adult individuals are 6-7 mm long and silver to grey in color, with filiform antennae. Females are larger than males and both have black spots on the anterior wings. Eggs are creamy yellow and cylindrical in shape, 0.35 mm long. First instar larvae are 0.5 mm long and light green or yellow in color. In addition, larger larvae become darker green and two black bands appear to the posterior head capsule. *T. absoluta* has four larval instars. Newly-formed pupae are greenish and turn into darker green when reaching adult emergence. Pupae are 5-6 mm in length (Coehlio and França, 1987; IRAC, 2011) (Figure 1).

Figure 1 demonstrates the *T. absoluta* life-cycle, which consists of four different stages: egg, larvae, pupae, and adult. Females lay their eggs on leaves, stems and fruits, but are generally located alongside the rachis (Coehlio and França, 1987; IRAC, 2011). Adult females live between 10-15 days, while male's lifespan is from 6 to 7 days (Estay, 2000). This insect mostly exhibits sexual reproduction, but two recent studies found that it can also reproduce with deuterotokous parthenogenesis under laboratory conditions (Caparros-Megido et al., 2012; Abbes and Chermiti, 2014), even though parthenogenesis is relatively scarce in the Lepidoptera (Suomalainen 1962; Lynch 1984).

Fitness of mated females is better than unmated ones because the former lay a greater number of eggs than the latter and the mortality of fertilized eggs is smaller compared to the unfertilized eggs. Nonetheless, asexual reproduction in field conditions has not been reported yet (Caparros-Megido et al., 2012). Females mate once per day, with coupling lasting 4-5h, with a maximum of six couplings during their lifespan. The most fertile period for oviposition is 7 days after mating, where females lay 76% of their eggs. A female can lay up to 260 eggs during its lifespan (Uchôa-Fernandes et al., 1995). *T. absoluta* adults can be found throughout the year under Mediterranean conditions (Vercher et al., 2010).

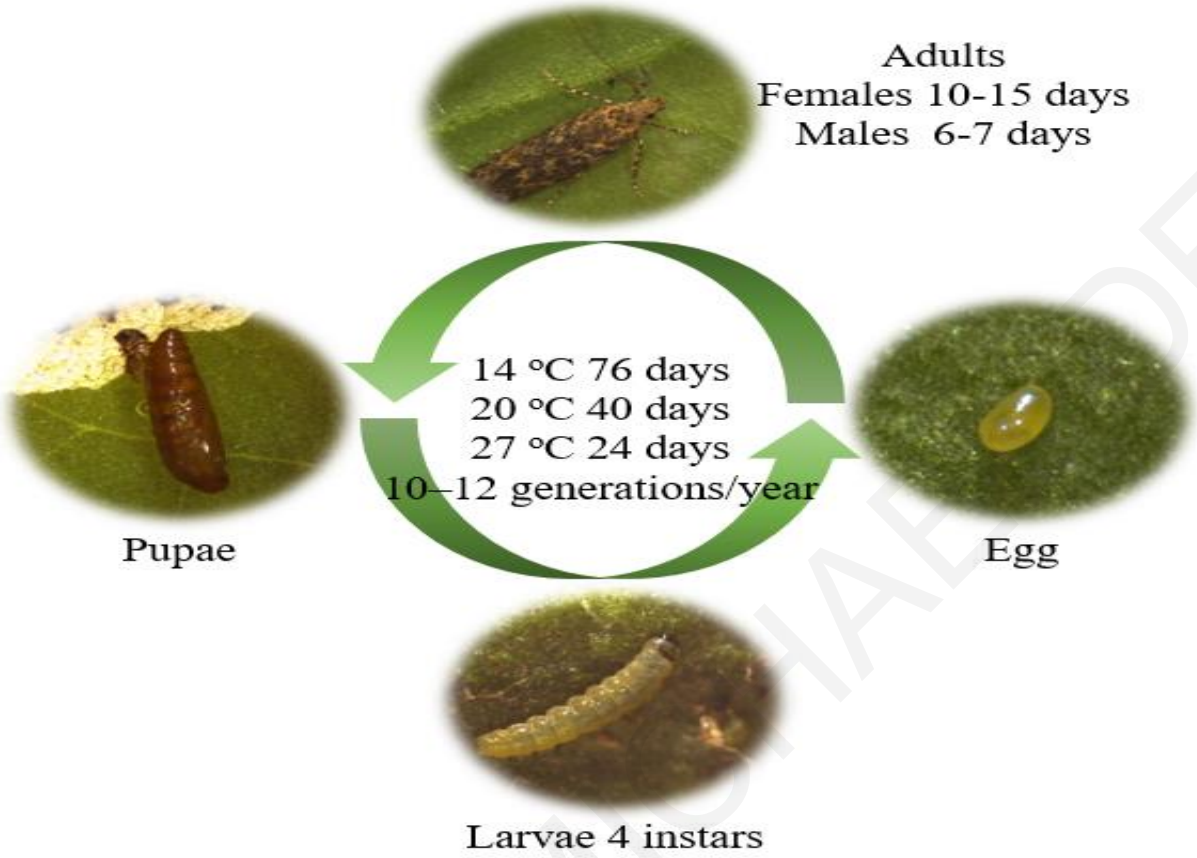

Figure 1: *T. absoluta* life cycle. Photos: https://www.cabi.org/isc/datasheet/49260.

Hatched larvae penetrate and enter plant tissues, where they feed and grow in order to complete the four larval stages. When larval development is completed the larvae fall on the ground to pupate. Pupating may also take place on plant tissues (Coehlio and França, 1987; IRAC, 2011). As Pereyra and Sánchez (2006) highlighted, *T. absoluta* is an r-selected species. Its life cycle duration depends on environmental conditions and lasts 76.3 days at 14˚ C, 39.8 days at 19.7˚ C and 23.8 days at 27.1˚ C (Barrientos et al., 1998). Below 4˚ C, insects suspend their biological activity. The tomato pinworm can complete 10-12 generations each year (Figure 1). If food is available throughout the year, larvae avoid getting into diapause. If diapause does not happen, depending on the environmental conditions the moth can overwinter as eggs, pupae or adults (Coehlio and França, 1987; Vercher et al., 2010; IRAC, 2011).

1.3 Hosts, damages, and economic importance

The tomato, *Lycopersicon esculentum* Mill., is among the 10 major agricultural products produced each year and it is the second most consumed vegetable in the world. The worldwide production reached 182.301.395 tonnes in 2017, produced on 4.7 million ha (Food and Agricultural Organization Data, FAOSTAT). Biondi et al. (2018) mentioned that USA, China, New Zealand, and Australia are still free of this pest, whereas their production was found to be 10.910.990, 59.626.900, 46.040, and 371.578 tonnes respectively, in 2017. USA and China yield 33% of the total worldwide tomato production. Having these numbers in mind, in 2010 the tomato pinworm infested 27.2% of the tomato world production. Before 2006 this percentage was only 5%, however in 2017 the infection percentage of world tomato production skyrocketed at 66% (Food and Agricultural Organization Data, FAOSTAT). A significant amount of money is needed each year for the control of *T. absoluta*, since its control costs around €100 - 150 per ha, per cropping season (Desneux et al., 2011). All these chemicals needed for the control of *T. absoluta* and the packing materials are eventually accumulated in agricultural ecosystems with all the negative consequences for biodiversity and environment. Thus, IPM strategies and biological control methods to control such pests are necessary, in order to eliminate the influx of pesticides in ecosystems.

Except from the tomato, *T. absoluta* can also complete its lifecycle on other cultivated Solanaceae plants such as: eggplant (*Solanum melongena* L.), potato (*S. tuberosum* L.), sweet pepper (*S. muricatum* L.), and tobacco (*Nicotiana tabacum* L.) (Vargas 1970; Campos 1976). Additionally, *T. absoluta* can feed, develop, and reproduce on wild Solanaceae. some of which are; *S. bonariense* L., *S eleagnifolium* L., *S. nigrum* L., *S. puberulum* Ph., *S. saponaceum* and *S. sisymbriifolium* Lam. Other native, non-cultivated hosts of the insect are *Datura ferox* L., *D. stramonium* L., and *N. glauca* Graham (Garcia and Espul 1982; Larraín 1986). The tomato pinworm has also been found on gooseberry (*Physalis peruviana*) (Tropea Garzia 2009), bean (*Phaseolus vulgaris*) (EPPO 2009), *Lycium* sp. and *Malva* sp. (Caponero 2009).

Damages on tomato plants are caused by the feeding behavior of all pinworm larva stages at buds, leaves, stems, and crops, where they mine galleries. If no control measures are taken against this pest, yield loss could reach 100% (IRAC, 2011). *T.*

absoluta can infest tomato plants at any developmental stage. Females prefer to lay their eggs on leaves (73%) and, secondary, on leaf vines and stem margins (21%), sepals (5%), and green fruits (1%) (Estay 2000). When hatched, first instar larvae infest the buds of young plants by penetration. When the plants grow and the foliage increases, larvae get into the mesophyll, where they mine galleries, thus, reducing photosynthetic ability and yield production. In addition, larvae permeate into the tomato fruits, causing significant yield losses (Figure 2) (Desneux et al., 2010; Guedes and Picanço 2012). Insecticide sprays are difficult to reach their target because of the plants anatomy and the larvae feeding behavior (Biondi et al., 2018).

Figure 2: Damages to tomato cultivations from the feeding behavior of *T. absoluta* larvae. Photos by G. Michaelides.

1.4 Geographic distribution of *T. absoluta*

Nowadays, globalization, international trade, and human travel increase the possibilities of the fast dispersion of invasive and alien species (Meyerson and Mooney, 2007; Hulme, 2009; Essl et al., 2011; Lowry et al., 2013). The spread of an invasive pest could be accelerated due to missing registrations of efficient insecticides at the place of its establishment and due to the existence of resistance alleles (Guedes et al., 2019). Colonization and establishment of invasive species could cause significant environmental and economic impacts (Hill et a., 2016). Great attention is paid on invasive arthropod pests (Keller et al., 2011). *T. absoluta* is such an invasive pest, the most disastrous pest for global tomato production and one of the most destructive enemies of solanaceous plants (EPPO, 2005; Desneux et al., 2010; 2011; Guedes and Picanço, 2012),

T. absoluta spread to Latin America countries in the 1960s, from the Peruvian central highlands from where it originated (Desneux et al., 2011; Campos et al., 2017). In early 1980s, *T. absoluta* reached Brazil (the main tomato producer country in S. America), and it is identified as the main pest for tomato cultivations, being extremely difficult to control (Guedes and Picanço 2012; Biondi et al., 2018). The tomato pinworm was restricted to South America until 2006, when it arrived to Spain, probably with tomato imports from Central Chile (Guillemaud et al., 2015). The establishment of the tomato pinworm in Mediterranean countries was followed by the increasing number of insecticide applications and increase in control cost (Potting et al., 2013).

Shortly after being detected in Spain, the tomato pinworm then quickly spread southwards and eastwards covering 800 km each year (Guillemaud et al., 2015), reaching and infesting tomato cultivations in Mediterranean countries (Desneux et al., 2010; 2011; Campos et al., 2017). In Cyprus, *T. absoluta* was first reported in Limassol, (November 2009), whereas until the end of that year the pinworm caused significant damages all around the island (ARI, 2010; EPPO, 2010b).

The tomato pinworm continued its spread eastwards and southwards, where it spread in the Middle East and in the eastern and western sub-Saharan areas, becoming a very serious worldwide threat for greenhouse and open field tomato crops. Colonizing Africa, *T. absoluta* reached South Africa in 2016 (Pfeiffer et al., 2013; Tonnang et al., 2015; Visser et al., 2017; Sylla et al., 2017; Campos et al., 2017; Biondi et al., 2018; Mansour et al., 2018; Santana et al., 2019). Spreading eastwards, *T. absoluta* reached India, the Himalayan mountain range, Tajikistan, and Pakistan in 2017 (Sankarganesh et al., 2017; Sharma and Gavkare 2017; Campos et al., 2017; Han et al., 2019; Santane et al., 2019). Fortunately, the USA, China, Australia, and New Zealand, which are some of the biggest tomato producing countries, are still free of this destructive pest (Biondi et al., 2018). Below is the distribution map of *T. absoluta*, up until the end of February 2019 (Figure 3).

Figure 3: Distribution map of *T. absoluta* as last updated on February 26th, 2019. (https://www.cabi.org/isc/datasheet/49260)

1.5 Control methods

The basic practices to diminish the possibilities of the arrival of an invasive pest species are quarantine measures and inspection. However, when an alien species colonizes a new area, its control depends mostly on pesticides (Lockwood et al., 2013; Liebhold et al., 2016). If control measures are not taken, cultivations are totally destroyed (Chermiti et al., 2009). The efficiency of the main chemical classes of insecticides used to control the tomato pinworm may be reduced when control methods rely only on insecticides, because the selection pressure increases (Guedes et al., 2019). When *T. absoluta* reached Cyprus, farmers tried to control the insect by spraying 10-12 times per cultivation cycle. Nowadays, they spray more than 20 times per cropping cycle, without having the expected results. The same happened in Brazil, where farmers applied insecticides more than 30 times per cultivation cycle (Guedes and Siqueira 2012).

Even though pest control principally depends on insecticides (Roditakis et al., 2009; Wang and Wu, 2012), the management of their populations should also include preventive and other corrective measures (Naranjo and Ellsworth, 2009). Such measures should combine pheromone traps, predators, parasitoids, insect pathogens, and plant extracts

(Urbaneja et al., 2009; Mollá et al., 2011; Biondi et al., 2013; Alili et al., 2014; Messelink and Janssen, 2014; Abd et al., 2016; Michaelides et al., 2018). Traps and mating disruption with sex pheromones were found to be important control strategies for the tomato pinworm (Desneux et al., 2010), since reproduction in *T. absoluta* is amphimictic and adult males emerge earlier from their cocoon than females (Garzia et al., 2012). However, two recent studies found that this pest can also reproduce by defterotokous parthenogenesis in laboratory conditions (Caparros-Megido et al., 2012; Abbes and Chermiti, 2014). This phenomenon could negatively affect the effectiveness of IPM programs, including pheromone-based control measures.

An alternatively method to control *T. absoluta* is biological control. Such an approach includes the release of natural enemies of this pest, including predators and parasitoids. A recent study summarized the predators and parasitoids of *T. absoluta* in South American, European, and Mediterranean basin countries (Ferracini et al., 2019). The predators found, belong to twenty-six different families, including eight orders of arthropods. However, parasitoids found to attack *T. absoluta* come from eleven different families corresponding to two orders (Diptera, Hymenoptera). The vast majority of them are Hymenoptera. The entomopathogenic nematode *Steinernema feltiae* was also found to cause significant larvae mortality of *T. absoluta* ranging between 78-100% (Garcia-del-Pino et al., 2013).

In Mediterranean countries, nine predators were found to feed on *T. absoluta* eggs and larvae including *Macrolophus pygmaeus*, *Nesidiocoris tenuis*, *Dicyphus errans* Wolff, *D. marrocannus* Wagner and *D. tamaninii* Wagner (Hemiptera: Miridae). The most commonly used are the predators *N. tenuis* and *M. pygmaeus*, which were also found in Cyprus (Zappalà et al., 2013). Both are commercially produced and used against *T. absoluta* in European and N. African countries (https://www.cabi.org/isc/datasheet/49260), since both of them are the most cost-effective predators and could be introduced into biological control programs alone or in combination with parasitoids (Chailleux et al., 2013a; b; De Backer et al., 2015; Mollá et al., 2014; Naselli et al., 2017) and selective pesticides (Zappalà et al., 2012; Martinou and Stavrinides, 2015). Releasement of predators could be combined by sprayings with the bacterium based (*Bacillus thuringiensis* (Bt) insecticide, since it has no side effects on

beneficial arthropods, and it is also very effective against the first instar larva of the tomato pinworm (Mollá et al., 2011).

1.6 Objectives of the current study

1.6.1 Aim 1: Susceptibility of Cypriot *T. absoluta* populations to four targeted insecticides and control failure likelihood

The susceptibility of *T. absoluta* to insecticides has been studied in many countries by different entomologists. *T. absoluta* has the ability to acquire multiple resistance in insecticides of different modes of action, and subsequently to increase its resistance levels (Roditakis et al., 2018). Thus, monitoring of the resistance levels in different countries and regions using the leaf-dip bioassay method is necessary (Roditakis et al; 2013a; b; 2015; 2018; Konuş et al., 2014; Ugurlu Karaağaç 2015; Cherif et al. 2018; Zibaee et al. 2018). Such studies raised our interest to explore the current status of insecticide susceptibility of Cypriot *T. absoluta* populations. Given the fact that Cypriot farmers reported control failure to many insecticides applied to control this pest, we decided to find out the current status of insecticide susceptibility of *T. absoluta* in Cyprus. Furthermore, knowing to which classes of insecticides the tomato pinworm is susceptible to, we are able to suggest a rotation scheme of insecticides of different modes of action to control it. It should be mentioned that such monitoring programs should take place every crop season because the status on resistance can change through time.

Considering the above, the level of resistance to the insecticides chlorantraniliprole, indoxacarb, emamectin benzoate, and spinosad was estimated for Cypriot populations of the tomato pinworm using the IRAC bioassay method No. 022 (presented in Chapter 2). This specific protocol is designed to evaluate the toxicity of insecticides which are used against the S. American tomato pinworm.

1.6.2 Aim 2: Molecular identification of mutations to indoxacarb and chlorantraniliprole of Cypriot *T. absoluta* populations

The intensive and continuous applications of insecticides to control *T. absoluta* lead to the existence of mutant alleles in invading populations of the insect. Hence, the early

detection of resistant genotypes using molecular diagnostic tests is very important to plan an appropriate management program. This program would encompass the use of insecticides that are still effective to control the populations examined (Guedes and Siqueira 2012; Haddi et al., 2012).

Expanding our research to the insecticides found not to be effective (Chapter 2), we contacted molecular assays to identify the mechanisms of resistance. Two target-site mutations were found for each insecticide that failed to control Cypriot *T. absoluta* populations. Low sensitivity to the insecticides chlorantraniliprole and indoxacarb in Cypriot populations of the pest is attributed to mutations G4903V and I4746M, F1845Y and V1848I identified herein.

1.6.3 Aim 3: Functional response and multiple predator effects of two generalist predators preying on *T. absoluta* eggs

The most common method to control the tomato pinworm is by spraying insecticides. An outcome of the frequent insecticide applications is the insensitivity of the target pest to insecticides. Some other negative effects include; the environmental pollution, side effects on non-target organisms, and impacts on human health (Ferracini et al., 2019). Consequently, the introduction of non-chemical and environmental friendly measures in *T. absoluta* control programs is a way to reduce the use of chemical insecticides.

Many studies using natural enemies of this pest to control its populations showed promising results. From the natural enemies, the generalist predators could give the best results. Literature review indicates that the predators *N. tenuis* and *M. pygmaeus* could control a wide range of agricultural pests, including *T. absoluta* (Zappala et al., 20130), and are native in Mediterranean countries. Therefore, we were able to design an experiment to explore if they can successfully control *T. absoluta* populations, In order to be used in biological control and IPM control programs.

To fulfill our third goal (presented in Chapter 4), we studied the combined effects of conspecific and heterospecific interactions of the predators *M. pygmaeus* and *N. tenuis* when preying on various densities of the South American tomato pinworm, *T. absoluta*, eggs.

CHAPTER 2

Susceptibility of Cypriot Tuta absoluta populations to four targeted insecticides and control failure likelihood*

Abstract

Tuta absoluta, known as the South American tomato pinworm, is one of the most disastrous pests of tomato cultivations, presently menacing tomato cultivations worldwide. In 2006, *T. absoluta* invaded Spain from South America. Since then, it was rapidly spread to most European, African and Asian countries. Such alien invasive species can minimize crop production, whereas the increasing use of insecticides raises various environmental concerns as well as on control costs, control failure and the toxicity to non-target organisms. The S. American tomato pinworm is mostly controlled by chemical insecticides, and failure to control it is not a rare phenomenon. Resistance to numerous insecticides has been reported and is mainly due to the fact that farmers do not follow a sustainable resistance management scheme. Several examples have been reported from several countries where the tomato pinworm is present. In order to develop a successful insecticide resistance management (IRM) strategy for any major pest, one needs to identify the baseline toxicity to insecticides and then monitor susceptibility levels**.** In Cyprus, the current status of susceptibility levels to the main insecticides that are used to control *T. absoluta* has never been studied before. Herein, nine Cypriot populations of the pest were subjected to laboratory bioassays between 2016 and 2018 using the main insecticides applied against it. We found that the insecticides chlorantraniliprole and indoxacarb could not control the Cypriot *T. absoluta* populations anymore, with a resistance ratio (RR) >28 and 3–23, respectively. Furthermore, mortality achieved by those two insecticides was 20.6%–72% for chlorantraniliprole and 27.5%–78% for indoxacarb. However, the insecticides emamectin benzoate and spinosad are very effective, since mortality to both of them ranged between 99.5% and 100%.

* In press: Michaelides G, Seraphides N, Pitsillou M, Sfenthourakis S. Susceptibility of Cypriot *Tuta absoluta* populations to four targeted insecticides and control failure likelihood. *J Appl Entomol*. 2019; 00:1–10. https://doi.org/10.1111/jen.12627

2.1 Introduction

In order to control the populations of *T. absoluta*, several insecticides with different modes of action [according to the classification by the Insecticide Resistance Action Committee (IRAC) Mode of Action Classification (IRAC, 2016)] are usually applied. When the generations of the insect overlap and the different groups of insecticides are not applied in rotation, the risk of insecticide resistance development is high (Siqueira et al., 2000b; 2001; Lietti et al., 2005; Silva et al., 2011).

In addition, excessive use of insecticides often leads to a reduction of their effectiveness due to survival of resistant genotypes (Perry et al., 1997). A fundamental problem that farmers worldwide have to deal with, is control failure of several pests due to such an acquisition of resistance to insecticides. There are numerous examples of arthropod pests that developed some level of resistance to many active ingredients of insecticides, while for a decreasing efficacy in pest control has been reported for a huge number of insecticides (Spark and Nauen, 2015).

The S. American tomato pinworm is such a case of a pest that developed resistance to several insecticides with different modes of action in many countries. According to the S. American countries, organophosphates and pyrethroids were the first classes of insecticides that used to control *T. absoluta* from the 1960s and 1980s, respectively (Siqueira et al., 2000a; 2000b; 2001; Lietti et al., 2005; Silva et al., 2011; Haddi et al., 2012; Gontijo et al., 2013; Campos et al., 2015a). When pyrethroids came to the foreground, they were used in combination with the nereistoxin analogue cartap and the avermectin abamectin, while the use of organophosphates eliminated. Nevertheless, resistance to organophosphate and pyrethroids insecticides detected initially after their use in Chile, Brazil and Agrentina (Salazar and Araya 2001; Siqueira et al. 2000b; Lietti et al. 2005). Resistance to abamectin and cartap were then found to be from low to moderate, but soon increased (Siqueira et al., 2000a; 2001; Silva et al., 2016). Extensive resistance found to pyrethroids was possibly introduced into European countries with the spread of *T. absoluta* from Latin America (Silva et al., 2011; Haddi et al., 2012). The, a few years afterwards (1990s, 2000s) farmers had the opportunity to control the tomato pinworm by applying the oxadiazine indoxacarb and chitin biosynthesis inhibitors (Silva et al., 2011). In the mid-2000s control failure detected to chitin biosynthesis inhibitors, as

well as medium levels of resistance to indoxacarb in S. American countries (Silva et al., 2011; 2016b). Later on new classes of insecticides established to control *T. absoluta* such as, pyrroles, spinosyns and diamides (Silva et al., 2011; 2016a; 2016b). However, the extensive use of spinosad and diamides resulted in their reduced efficiency in controlling *T. absoluta* populations from Brazil and Europe (Campos et al., 2015a; Roditakis et al., 2015; Silva et al., 2019).

When *T. absoluta* invaded tomato cultivations in Spain and other Mediterranean countries growers tried to control it by applying broad spectrum insecticides, such as pyrethroids (Balzan and Moonen 2012). Nonetheless, with such control approaches populations of tomato pinworm remained above the economic threshold. Therefore, the need of insecticides with novel active ingredients targeting lepidopteran pests and monitoring of their susceptibility required (Roditakis et al., 2013a, b). For this reason, insecticides from thirteen different modes of action registered for use against *T. absoluta* in Mediterranean countries until 2011 (Desneux et al., 2011).

At the website of the Ministry of Agriculture, Rural Development and Environment of Cyprus there are several chemical insecticides registered for the control of *T. absoluta*, such as chlorantraniliprole, chlorantraniliprole + l-cyhalothrin, chlorantraniliprole + abamectin, emamectin benzoate, spinosad, indoxacarb and metaflumizone, whereas *Bacillus thuringiensis* is the one and only microbial insecticide for use against Lepidoptera pests (www.moa.gov.cy/moa/da/da.nsf). However, four of them (chlorantraniliprole, indoxacarb, emamectin benzoate and spinosad) are the most extensively used against *T. absoluta* larvae in Europe (Sparks and Nauen, 2015). Chlorantraniliprole and spinosad have been registered for use in Cyprus to control *T. absoluta* on September, 2011 as Altacor 35WG and Steward 30WG, respectively. Two months later emamectin benzoate introduced for use against *T. absoluta* as Affirm 095SG. Spinosad enrolled as Tracer 48SC on January 2012 (http://www.kysyf.eu/about-us/law) (in greek). As we know from personal contact with farmers they control *T. absoluta* by spraying mostly the insecticides chlorantraniliprole and indoxacarb, alone or with mixtures with pyrethroids. Emamectin benzoate and spinosad are used less frequently by the farmers. However, none of them follow a rotation scheme or any IPM program. A few years after the registration of those insecticides they complaint for control failure while spraying with chlorantraniliprole and indoxacarb, where insecticide resistance found in this study.

Regarding the insecticides evaluated in our study, the susceptibility levels of *T. absoluta* populations were steady for some years (Roditakis et al., 2013b). However, resistance has been identified for diamides and Spinosyns in Brazilian populations of *T. absoluta*. A low resistance level to diamides has been reported from Greece but a high one from Italy, while spinosad resistance has been documented in the UK and Brazil (Campos et al., 2015a; AHDB, 2015; Roditakis et al., 2015; Silva et al., 2016b). In a recent study Roditakis et al., (2018) found a high likelihood of control failure for the diamide insecticide chlorantraniliprole in Italian, Greek and Israeli populations of the tomato pinworm. Resistance levels observed to be moderate to high in those countries, whereas no insecticide resistance were detected for this specific insecticide in populations from the UK and Spain. In the same study control failure was observed for oxadiazines, specifically for the insecticide indoxacarb, in four cases from Greece, one from Italy and one from Israel. The same authors, even though they found low to high resistance levels for emamectin benzoate and spinosad in Italian, Greek, Israeli, Spanish and UK populations of *T. absoluta*, could not identify any cases of control failure to those two insecticides.

Chlorantraniliprole is a diamide insecticide which belongs to the ryanodine receptor (RyR) modulators No. 28 Group mode of action (MoA) as classified by IRAC (IRAC, 2016). Ryanodine receptors are homotetrameric non-voltage-gated calcium channels and their function is to regulate the releasement of intracellular calcium stores needed for muscle contraction. Insecticides in this group activate the ryanodine receptors in the sarco- and endoplasmic reticulum of insect neuromuscular tissues, causing uncontrolled releasement of calcium stores which leads to irregular muscle function, paralysis and finally death (Lahm et al., 2005; 2009; Cordova et al., 2006). Resistance to diamides has been related to mutations to the RyR of the insects (Roditakis et al., 2017a).

Indoxacarb is classified in oxadiazines (IRAC MoA Group 22A) which are 'voltage-dependent sodium channel blockers' and when bioactivated by esterases in the target insects, they block action potential initiation in neuronal voltage-gated sodium channels (VGSC) at different binding sites than pyrethroids and DDT (Perry et al., 1997; Ahmad and Hollingworth, 2004; Silver et al., 2010). Resistance to indoxacarb is due on two mutations that took place on the sodium channel of *T. absoluta* and especially on segment 6 of domain IV (Roditakis et al., 2017b).

Emamectin benzoate belongs to avermectins (IRAC MoA Group 6). It acts as an antagonist for gamma-aminobutyric acid-gated chloride channels (Kass et al., 1980) and therefore activates the glutamate-gated chloride channels (GluCls) causing functional disorders in neuronal and muscular systems of the insects (Lasota and Dybas, 1991; Fischer, 1992; IRAC, 2016).

Spinosad is a spinosyn and falls in Group 5 of the IRAC MoA classification. Spinosyns are produced by fermentation of the soil actinomycete *Saccharopolyspora spinosa* (Mertz and Yao), and are mixtures of the Spinosyns A and D. Spinosyns have registered for use in organically produced tomatoes (Salgado and Sparks 2005; Racke, 2006; Puinean et al., 2013). Spinosad is a nicotinic acetylcholine receptor (nAChR) allosteric modulator that excites the neurons in the central nervous system of the insects (Salgado, 1998). In spinosad-resistant laboratory and field *T. absoluta* strains, the mutation G275E, has been reported (Silva et al., 2016).

In this Chapter, the level of resistance to the insecticides chlorantraniliprole, indoxacarb, emamectin benzoate and spinosad was estimated for Cypriot populations of the American tomato pinworm using the IRAC bioassay method No. 022.

2.2 Materials and Methods

The bioassay method which is widely used to identify the susceptibility of *T. absoluta* to insecticides is based on the Insecticide Resistance Action Committee (IRAC) method number 022. In this bioassay method tomato leaves are immersed into the insecticides aqueous dispersions and L2 instar larvae (4-5mm) are placed on the leaves for 72h.

2.2.1 Collection of *T. absoluta* populations

Infested tomato leaves with *T. absoluta* larvae were collected from seven distinct locations on Cyprus (Orounta: Nicosia, Koutrafas: Nicosia, Sotira: Ammochostos, Maroni: Larnaca, Parekklisia: Limassol, Timi: Pafos and Mantria: Pafos) from nine greenhouses during 2016-2018 (Figure 4). In all cases, growers had declared control failure for some insecticides. A singular recognition code was given to each population (Table 1) and is used for reference hereafter.

Figure 4: Collection areas of Cypriot *T. absoluta* populations.

About 300-400 infested tomato leaves with *T. absoluta* larvae were collected from each sampling area. The leaves were placed immediately in plastic bags and in a cool box, and were transferred to the insectary rooms of the Agricultural Research Institute (ARI), Nicosia (Cyprus), under controlled conditions (25 ± 1 ˚C, 65 ± 10% RH and 16L:8D photoperiod) within 1-2 hours after collection to avoid stressing the insects. The bags containing samples were transferred and opened into plastic rearing cages (50x50x50 cm) with four holes (30 cm diameter) on each side, covered with white organza fabric (mesh 0.3 x 0.3 mm) to reduce humidity. In each cage, two insect-free tomato potted plants were placed in order to allow the larvae to continue their lifecycle. All Cypriot *T. absoluta* populations tested were compared to the CY-Lab reference strain. The reference strain was collected for the first time in 2010 when *T. absoluta* invaded tomato cultivations in Cyprus.

Table 1: General information for collected *T. absoluta* populations.

	Location	Coordinates GPS	Sampling	Crop	Generation tested
Lab strain			2010	GHt	
CY-MAR1	Larnaca, Maroni	34°43'57.99"N 33°21'42.03"E	11/2016	GHt	F1
CY-TIM	Pafos, Timi	34°43'42.60"N 32°30'56.81"E	03/2017	GHt	F1-F2
CY-PAR1	Limassol, Parekklisia	34°45'37.50"N 33° 8'26.49"E	05/2017	GHt	F1
CY-ORU	Nicosia, Orounta	35° 5'50.08"N 33° 4'29.13"E	06/2017	GHt	F1-F2
CY-SOT	Ammochostos, Sotira	34°59'36.68"N 33°56'16.65"E	10/2017	GHt	F1
CY-KOU	Nicosia, Koutrafas	35° 5'45.29"N 32°59'23.18"E	10/2017	GHt	F1-F2
CY-MAR2	Larnaca, Maroni	34°44'11.59"N 33°22'9.71"E	11/2017	GHt	F1-F2-F3
CY-MAN	Pafos, Mantria	34°42'19.97"N 32°31'58.45"E	11/2017	GHt	F1-F2
CY-PAR2	Limassol, Parekklisia	34°45'49.89"N 33° 9'30.01"E	02/2018	GHt	F1

2.2.2 Rearing of tomato plants

Tomato plants were previously seeded in the nursery room of the insectary under controlled conditions (25±1 ◦C, 65±10% RH and 16L:8D photoperiod). Five to ten tomato seeds seeded in plastic pots where previously 2cm of sterilized mold where placed. When the seeds placed on the soil 2cm of soil placed, compressed lightly and watered. When the plants reached the stage of 2-3 leaves they were transplanted to bigger pots. Matured plants were positioned in the cages with the insects. If any infestation with pests or diseases were detected on any tomato plants, these were removed and destroyed. No pesticides were used.

2.2.3 Rearing with synchronous oviposition

T. absoluta adult individuals emerged about 15 days after collection. 40-60 adults were collected with a backpack aspirator (BioQuip, USA) and were released into a cage with two potted tomato plants. The insects were provided with water and honey and were allowed to oviposit for 48h. The infested plants with *T. absoluta* eggs (150-200 eggs) were then removed and placed into a clear cage to allow larvae reach the second instar. Immediately after removing the infested plants, new clear plants were placed in the oviposition area. In all bioassay experiments F1 generation, second-instar larvae (4-5mm) were used. Larvae larger than the second instar (>5 cm) were removed and placed in another cage to continue their development till F2 generation.

2.2.4 Insecticides

Commercial formulations of the insecticides chlorantraniliprole (Altacor® 35WG, DuPont, France), indoxacarb (Steward® 30WG, DuPont, France), emamectin benzoate (Affirm 095SG, Syngenta, UK) and spinosad (Tracer 48SC, Dow, USA) were used.

2.2.5 Bioassay method

The IRAC leaf dip bioassay protocol No 22 (www.irac-online.org) was used modified as described by Roditakis et al. 2013. This assay is a leaf-dip bioassay method using aqueous dispersions of commercial insecticide formulations. The concentrations tested

ranged from 0.66 - 672 mg L^{-1}, 0.59 - 600 mg L^{-1}, 0.01 – 228 mg L^{-1} and 0.06 - 480 mg L^{-1} for chlorantraniliprole, indoxacarb, emamectin benzoate and spinosad respectively, resulted in 0 - 100% mortality. Cut compound leaves were immersed for 5 s in sequential insecticide concentrations containing Tween 20 (0.05% v/v) as non-ionic wetting agent. The leaves were then left to dry for 2 h in moistened paper with distilled water. Subsequently, the leaves were placed in a transparent box (10.5 x 8.5 x 2.5 cm), with the dorsal surface up, containing filter paper moistened with 400μL of distilled water (Figure 5).

Figure 5: Left: Transparent boxes without leaves, Middle: Placement of the leaves in the boxes, Right: A leaf with five 2nd instar larvae. Photos: G. Michaelides.

A moistened cotton plug was attached around the peduncle of each leaf to provide them with water all along the bioassay. Before transferring the second-instar larvae on the leaves, they were carefully removed from inside the infested tomato leaves under a stereoscope. Five second-instar larvae were placed on the leaf in each box. Six boxes were used for each treatment corresponding to 30 larvae for each concentration. The box lids were then immediately fitted to prevent larvae from escaping. Before fitting the lids, sixteen vents (1 mm) were opened at each one with a pin to provide for adequate ventilation (Figure 5). In each box lid the collection location, the dose and the commercial name of the insecticide were written. The boxes with the larvae were then placed in an incubator at 25±1 ˚C, 65±10% RH and 16L:8D photoperiod. Larval mortality was evaluated 72 h after treatment under a stereoscope. A larva was counted dead if no movement was observed. A larva was considered moribund if no coordinated movement or inadequate response after mild touch with a fine paintbrush was observed. The total number of dead and moribund larvae was used to estimate the mortality rates. In the case that larvae were

in the gallery of the tomato leaves and vitality could not be observed, they were removed gently with tongs and a fine paintbrush to observe their response.

2.2.6 Statistical analysis

Mortality data acquired from dose-response bioassays were analyzed using the Finney's probit analysis (1964) carried out in R (R Core Team) with the 'drc' package (Ritz et al. 2015). This package tests the linearity of dose-mortality response and determines the slope, the lethal concentrations (LC) and the 95% fiducial limits (FL) of the lethal concentration for each mortality line. The relative potency ratio among responses was estimated using the same package. Responses were considered significantly different when no overlapping of the 95% FL of the LC_{50} was observed. Abbott's formula was used to correct mortality for control mortality (Abbot 1925).

Results were evaluated against the reference strain to estimate the resistance ratio. Moreover, the estimated LC_{80} and LC_{95} were compared to the maximum recommended label rate (RLR_{max}) to estimate to possibility of insecticide control failure based on the relevant studies of Silva et al. (2011) and Roditakis et al (2013a). The likelihood of an insecticide control failure could be estimated by comparing the predicted % mortality at the label rate to an 80% threshold (Guedes 2017). The RLRmax for Cyprus were: chlorantraniliprole: 42 mg L^{-1}, indoxacarb: 37.5 mg L^{-1}, emamectin benzoate: 14.2 mg L^{-1} and spinosad: 120 mg L^{-1}. The mortality expressed at the maximum recommended label rate was estimated based on the model. The 80% mortality was used as a threshold between control success and failure. If the 95% FL of the LC_{80} was higher than the recommended rate, then mortality reached.

Lastly, pair-wise comparisons were applied to examine the correlation among responses of the populations to the insecticides.

2.3 Results

Results from probit analysis are displayed in Table 2. *T. absoluta* populations showed a negligible trend to acquire insecticide resistance to the neurotoxic insecticides emamectin benzoate (2.00 – 5.00) and spinosad (2.00 – 5.00). However, high and medium levels of insecticide resistance were detected at the diamide chlorantraniliprole (RR: 28.00 – 188.00) and the oxadiazine indoxacarb (RR: 3.00 – 23.00), respectively. The effectiveness of the insecticides was evaluated on the basis of probit analysis. *T. absoluta* larvae mortality was 100% for emamectin benzoate and spinosad at the recommended field label rate. In contrast, control failure was detected for chlorantraniliprole and indoxacarb, for which mortality at the recommended label rate was between 20.6% – 72% and 27.5% - 78% respectively.

Table 2: Log-dose probit mortality data for *T. absoluta* populations with the insecticides chlorantraniliprole, indoxacarb, emamectin benzoate and spinosad. n: number of larvae tested; LC_{50}, LC_{80}, LC_{95}: in mg L-1; FL: fiducial limits 95%; RR: resistance ratio.

Population	n	LC_{50}	FL95%	RR	LC_{80}	FL95%	LC_{95}	FL95%	Slope	s.e.	X^2	df	p-value
						Chlorantraniliprole							
Lab	270	0.56	0.35-0.76		2.36	1.35–3.38	12.0	2.66 – 21.3	1.10	0.11	6.44	6	0.38
CY-MAR1	300	53.9	36.5-71.3	96	215	107- 324	1023	183 - 1862	1.49	0.05	3.02	7	0.88
CY-TIM	240	59.6	44.4-74.8	107	154	102- 206	448	200 - 696	2.08	0.06	4.32	5	0.50
CY-PAR1	210	67.4	46.2-88.6	121	204	94.2- 314	709	79.8 - 1338	1.79	0.06	3.06	4	0.55
CY-ORU	240	105	77.5-132	188	282	178- 387	860	342 - 1379	2.05	0.06	2.44	5	0.79
CY-SOT	240	15.9	11.2-20.4	28	50.6	30.2- 70.9	187	58.9 - 315	1.67	0.05	7.52	5	0.18
CY-KOU	270	24.6	18.7-30.5	44	59.0	40.5- 77.4	158	79.9 - 236	2.18	0.06	1.01	6	0.98

CY-MAR2	240	63.7	42.6-84.7	114	259	133- 385	125	173 - 2335	1.51	0.10	7.01	5	0.22
CY-MAN	240	42.6	31.0-54.3	76	119	77.4- 161	378	154 - 602	2.00	0.06	3.34	5	0.65
CY-PAR2	210	33.7	24.5-42.8	60	89.2	57.9- 121	267	1045 - 429	1.92	0.06	2.88	4	0.58
Indoxacarb													
Lab	240	4.69	3.74–5.63		8.80	6.51– 11.0	17.9	10.7–25.0	2.45	0.04	14.2	5	0.01
CY-MAR1	240	108	62.9-154	23	539	115- 962	3267	-8601	1.24	0.13	1.58	5	0.90
CY-TIM	240	46.8	32.7-60.9	10	156	92.7- 220	606	179-1033	1.56	0.05	5.27	5	0.38
CY-PAR1	240	36.6	27.7-45.5	8	88.5	59.6- 117	239	113-364	2.13	0.06	5.59	5	0.35
CY-ORU	240	29.8	21.3-38.3	6	92.9	56.0- 130	334	110-557	1.72	0.05	3.84	5	0.57
CY-SOT	210	14.6	11.0-18.2	3	34.0	23.3- 44.8	88.4	41.6-135	2.18	0.06	1.66	4	0.80

CY-KOU	210	17.7	11.4-23.9	4	55.4	34.3- 76.5	201	57.0-344	1.53	0.06	1.83	4	0.77
CY-MAR2	240	33.2	24.7-41.6	7	86.3	56.5- 116	253	110-395	1.90	0.06	3.62	5	0.60
CY-MAN	210	46.4	33.1-59.7	10	140	81.4- 198	483	134-833	1.68	0.05	2.52	4	0.64
CY-PAR2	210	39.5	27.9-51.0	8	121	71.0- 171	426	117-736	1.87	0.06	3.75	4	0.44
						Emamectin benzoate							
Lab	210	0.07	0.05-0.08		0.180	0.11- 0.24	0.52	0.20-0.83	2.15	0.06	3.12	4	0.54
CY-SOT	270	0.26	0.19-0.34	4	0.79	0.51- 1.07	2.70	1.10-4.37	1.48	0.05	7.98	6	0.24
CY-KOU	210	0.16	0.11-0.21	2	0.53	0.30- 0.75	2.04	0.47-3.60	1.59	0.06	5.06	4	0.28
CY-MAR2	180	0.33	0.24-0.42	5	0.86	0.53- 1.20	2.57	0.80-4.33	2.07	0.06	0.48	3	0.92
CY-MAN	210	0.21	0.15-0.26	3	0.52	0.34- 0.70	1.46	0.62-2.29	1.99	0.06	2.44	4	0.66

CY-PAR2	270	0.19	0.15-0.24	3	0.43	0.30-	0.56	1.06	0.56-1.56	2.20	0.06	2.02	6	0.92
						Spinosad								
Lab	210	0.11	0.07-0.14		0.32	0.20-	0.44	1.12	0.34- 1.90	1.51	0.06	3.50	4	0.48
CY-SOT	240	0.49	0.38-0.60	5	1.05	0.75-	1.35	2.47	1.34-3.60	1.91	0.06	8.45	5	0.13
CY-KOU	210	0.18	0.14-0.23	2	0.47	0.31-	0.64	1.37	0.56-2.18	2.01	0.06	2.47	4	0.65
CY-MAR2	210	0.27	0.20-0.34	3	0.69	0.45-	0.94	1.98	0.80-3.16	2.10	0.06	1.20	4	0.88
CY-MAN	210	0.24	0.17-0.31	2	0.72	0.43-	1.02	2.48	0.73-4.24	1.99	0.06	3.20	4	0.52
CY-PAR2	240	0.29	0.22-0.36	3	0.71	0.48-	0.94	1.94	0.92-2.96	1.91	0.06	3.79	5	0.58

2.3.1 Chlorantraniliprole

The response of nine populations was tested in order to identify their susceptibility levels against the insecticide chlorantraniliprole. Populations slopes ranged from 1.49 (CY-MAR1) to 2.18 (CY-KOU), indicating a homogeneous response to this insecticide. The LC_{50} ranged from 15.81 mg L^{-1} (CY-SOT) to 104.89 mg L^{-1} (CY-ORU), while the LC_{50} for control was 0.56 mg L^{-1} as shown in Figure 6. Tomato pinworm population from Sotira (CY-SOT) had the lowest LC_{50}, while the population from Orounta (CY-ORU) had the highest LC_{50}.

Statistical differences were detected between the responses (Table 3). In all cases tested, the LC_{80} values were higher than the recommended label rate (42mg L^{-1}), indicating control failure using this insecticide (see estimated mortality in Table 3). Moreover, the LC_{95} values ranged between 157.9 mg L^{-1} and 1254 mg L^{-1}. Statistical differences were detected within the responses.

The resistance ratio for CY-ORU population was 188 times that of the laboratory population, and it was the highest among populations and insecticides tested. For the case of CY-SOT RR, it was found to be 28 times more than of the laboratory population (Table 3). More specifically, the estimated mortality ranged between 20.6% and 72%, showing insecticide control failure at all cases. Control failures reported to fields sampled were probably due to resistance to diamide insecticides.

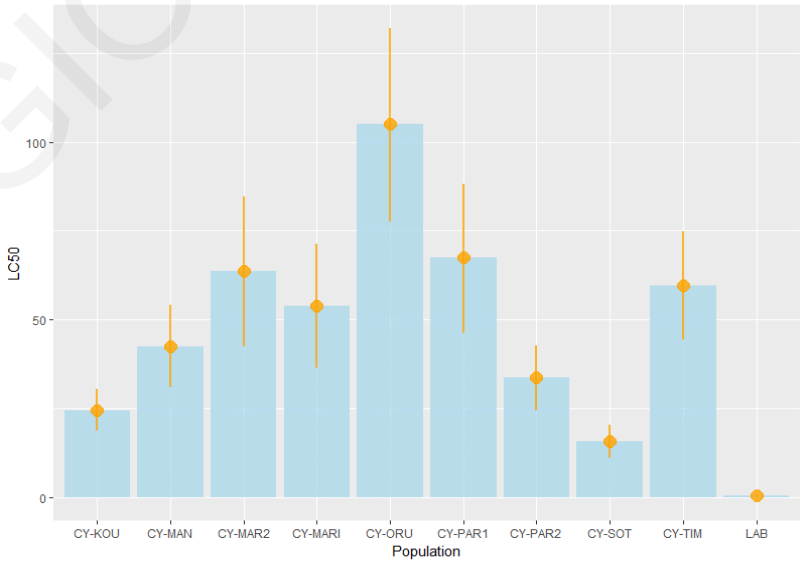

Figure 6: Chlorantraniliprole LC_{50} values for ten populations of Cypriot *T. absoluta* populations studied. Lower and upper 95%FL are presented.

2.3.2 Indoxacarb

The response of nine populations was estimated against the insecticide indoxacarb. Medium slopes of the response line to indoxacarb were observed, with values ranging from 1.24 to 2.18. The LC_{50} estimates for indoxacarb ranged from 17.65 mg L^{-1} (CY-KOU) to 108.33 mg L^{-1} (CY-MAR1) indicating statistical differences between the populations (Figure 7). Resistance to indoxacarb was detected in all populations tested from Cyprus, except for CY-SOT (LC_{80}: 34.081).

CY-SOT population is still susceptible to indoxacarb, while the RLR_{max} is within the fiducial limits of LC_{80}. The highest resistance ratio was detected in Maroni (CY-MAR1) and was higher than 23-fold, whereas the lower was detected in Sotira (CY-SOT) (threefold). The LC_{80} values ranged from 34.081 mg L^{-1} (CY-SOT) to 538.6 mg L^{-1} (CY-MAR1), and the RLR_{max} was 37.5 mg L^{-1} for the Cypriot populations (Table 3). Likelihood of control failure was detected in all cases tested, where the calculated mortality at the recommended label rate ranged between 27.5% and 78% (Table 3).

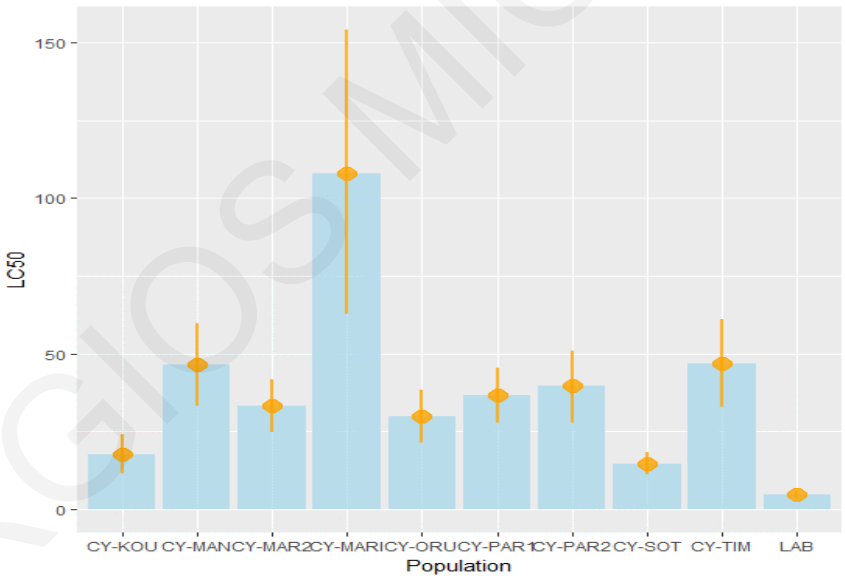

Figure 7: Indoxacarb LC_{50} values for ten populations of Cypriot *T. absoluta* populations studied. Lower and upper 95%FL are presented.

2.3.3 Emamectin benzoate

The response of five populations was evaluated versus the insecticide emamectin benzoate. The slopes varied from 1.48 (CY-SOT) to 2.20 (CY-PAR2), showing homogeneous responses to this neurotoxic insecticide. The highest LC_{50} was detected in CY-MAR2 population and was 0.327 mg L^{-1}, whereas the lowest

was observed in CY-KOU population and was 0.159 mg L^{-1}. LC_{50} for the reference strain was just 0.07 mg L^{-1} (Figure 8). LC_{80} in all populations tested were lower than the RLR_{max} (14 mg L-1).

In addition, the resistance ratio was always below fourfold, showing that all *T. absoluta* populations collected from Cyprus cannot be assumed to be resistant to emamectin benzoate (Table 2). In effect, we didn't observe any possibility of control failure in any case (Table 3).

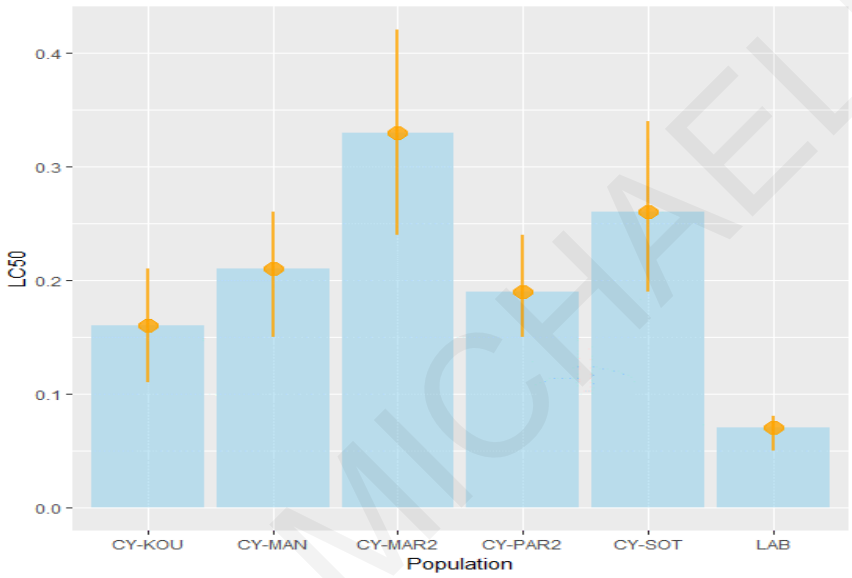

Figure 8: Emamectin benzoate LC_{50} values for six populations of Cypriot *T. absoluta* populations studied. Lower and upper 95%FL are presented.

2.3.4 Spinosad

The response of five populations was tested against the insecticide spinosad. The slopes ranged from 1.909 (CY-SOT) to 2.103 (CY-MAR2). The LC_{50} ranged from 0.185 mg L^{-1} (CY-KOU) to 0.493 mg L^{-1} (CY-SOT) as shown in Figure 9, indicating similar responses between all Cypriot populations. The highest RR was fivefold and the lowest was twofold. LC_{80} values ranged from 0.475 mg L^{-1} (CY-KOU) to 1.053 mg L^{-1} (CY-SOT), when the RLR_{max} for spinosad is 120 mg L^{-1} (Table 2). Estimated mortality for spinosad reached 100% for all tomato pinworm populations, except from CY-SOT where estimated mortality was 99.5% (Table 3).

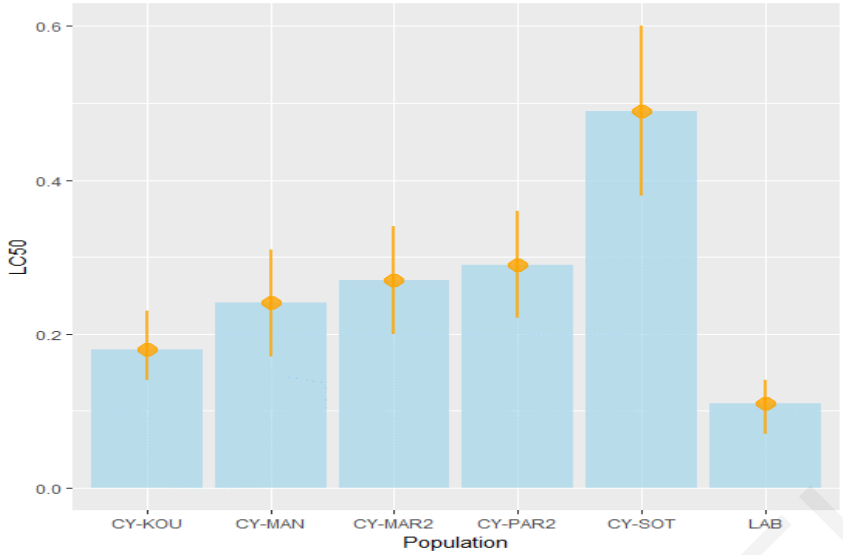

Figure 9: Spinosad LC_{50} values for six populations of Cypriot *T. absoluta* populations studied. Lower and upper 95%FL are presented.

Table 3: Estimated insecticide mortality (%) of Cypriot *T. absoluta* populations using European recommended label rates.

Insecticide/population	CY-MAR1	CY-TIM	CY-PAR1	CY-ORU	CY-SOT	CY-KOU	CY-MAR2	CY-MAN	CY-PAR2
chlorantraniliprole	42	37.7	35.5	20.6	72	65	34	46	52.8
indoxacarb	27.5	40.6	45	56	78	66	50.5	41	42.5
emamectin benzoate	N/A	N/A	N/A	N/A	99.5	100	100	100	100
spinosad	N/A	N/A	N/A	N/A	99.5	100	100	100	100

2.4 Discussion

Susceptibility levels of the four main insecticides registered for control of *T. absoluta* in Cyprus and the possibility of control failure to these insecticides were evaluated in this study. According to our knowledge, Cypriot populations of the American tomato pinworm have never been subjected to dose-mortality bioassays in any insecticide. The highest variability in the LC_{50} values were observed for the insecticides chlorantraniliprole and indoxacarb, resulting in significant differences among populations. For the insecticides emamectin benzoate and spinosad the

variability in the LC_{50} values observed was narrow and in most of the cases no marginal differences detected among populations. In order to follow an IRM strategy to keep under control a population of any economically important pest, its baseline and the subsequent susceptibility to insecticides should be identified (Ishtiaq et al. 2011; Gao et al. 2013). By following such monitoring programs, possible cases of insecticide resistance development can be detected early on and relevant authorities, interested parties and farmers have the opportunity to prevent or decelerate it (Zimmer et al. 2017). An excellent example of a pest that has the ability to develop resistance to insecticides is the tomato pinworm. Numerous researchers studied the baseline toxicity of many insecticides to *T. absoluta* and in most of them resistance development was found. All this information was transferred to local farmers in order to follow a more sustainable program of controlling the tomato pinworm (Siqueira et al. 20001, b; Lietti et al. 2005; Roditakis et al. 2013a, b; Campos et al. 2015b; Silva et al. 2015, 2016a, b).

Even though diamides are very effective to control lepidopteran pests, including *T. absoluta*, (Sparks and Nauen 2015) and are friendly to the environment and to non-target arthropods, their extensive use has resulted to high resistance levels in Italian, Brazilian, Greek and Israeli populations of the tomato pinworm (Roditakis et al. 2015; 2018; Silva et al. 2016). Such results were also found in our study, where control failure was detected for the diamide insecticide chlorantraniliprole. Control failure to indoxacarb has been reported only in European/Near East populations for which Roditakis et al. (2018) found resistance to indoxacarb in three populations from Greece and one from Israel whereas populations from S. America were susceptible to the insecticide (Silva et al. 2016b). Our findings showed that Cypriot *T. absoluta* populations are also resistant to indoxacarb, and control failure was detected in all cases tested. Only three cases of low resistance levels to the insecticide emamectin benzoate had been detected before, in Greece and Italy in 2016 (Roditakis et al. 2018). However, the only case of *T. absoluta* resistance to avermectins was detected for abamectin in Brazil in 2000 (Siqueira et al. 2000b). Our conclusions for emamectin benzoate are in agreement with Roditakis et al. (2018) in that this insecticide can effectively control *T. absoluta* populations. Concerning spinosyns our findings suggest that spinosad remains a highly effective insecticide against *T. absoluta* populations in Cyprus. This specific insecticide is also effective in controlling populations of the insect in other

European and Asian countries (Roditakis et al. 2018). Control failure for spinosad was reported in populations from S. America a few years ago (Reyes et al. 2012, Campos et al. 2015b). Resistance to spinosad by *T. absoluta* populations was also recently mentioned in the UK (AHDB 2015) and Portugal (Berger et al. 2016).

Despite the insecticide control failures in tomato cultivations reported from many countries colonized by the tomato pinworm, when IRM strategies are designed and applied consistently, *T. absoluta* populations in many cases do not develop resistance to insecticides. For example, even though resistance to diamides is widespread in S. American and European populations, in some regions of Spain diamides are still very effective against *T. absoluta* (Roditakis et al. 2018). This is due to the fact that, when the pest invaded tomato cultivations in these regions, all interested parties designed IRM strategies dependent on rotation of insecticides that have different modes of action, and adopted measures based on non-chemical control methods, such as insect-proof nets, pheromone traps and biological control (Bielza et al. 2016). Furthermore, in order to achieve their goals, IRAC and researchers in Spain organized meetings and conferences to inform and advice growers and agriculturists. Spanish growers adopted measures based on Integrated Pest Management (IPM). IPM measures included the release of the predator *Nesidiocoris tenuis* (Reuter) in nurseries where it established and successfully controlled the S. American tomato pinworm (Calvo et al. 2012). *N. tenuis* could also be used in combination with the predator *Macrolophus pygmaeus* (Rambur) in biological control programs of the tomato pinworm (Michaelides et al. 2018).

Nowadays, the development of a new insecticide is very costly and took many years from discovery to applying. Therefore, agricultural practices should include measures to delay resistance development to existing and recently developed insecticides (Sparks and Nauen 2015). Insecticide resistance evolvement could be restricted by following IPM and IRM programs. Moreover, farmers should use registered insecticides to control the pest and adhere to the instructions on the product's labels. Our findings could be used as a baseline data for further monitoring of the status of the insecticide resistance as well as a guideline in IRM strategies.

CHAPTER 3

Indoxacarb and Chlorantraniliprole target-site mutations of Cypriot *T. absoluta* populations

Abstract

A part of segment 6 of domain IV of the sodium channel of *Tuta absoluta* was amplified using a PCR assay and then digested with two restriction enzymes to examine the presence of two target-site resistance mutations. The existence of the F1845Y and V1848I resistance mutations was found in field collected Cypriot *T. absoluta* populations. Surprisingly, the F1845Y resistance mutation was present at the laboratory reference strain population, but in much lower frequency. The substitutions of these amino acids correspond to recently identified indoxacarb *T. absoluta* resistance mutations detected in Greek and Italian populations. Moreover, parts of S2 and S4 transmembrane-spanning domains of the Ryanodine Receptors of *T. absoluta* were amplified by applying two PCR diagnostic assays to investigate the presence of the I4746M and G4903V target-site mutations. All field collected Cypriot *T. absoluta* populations carried these mutations with one exception. G4903V mutation was missing from a field population. The substitutions of these amino acids were attributed to target-site missense mutations existing at the RyR of *T. absoluta*.

3.1 Introduction

Known mechanisms of resistance to insecticides include: (1) detoxification by metabolic enzymes (e.g. esterases, glutathione S-transferases, cytochrome P450s), (2) target-site missense mutations leading to low insecticide sensitivity, (3) insects' behavioral changes in response to the presence of the insecticide, and (4) reduced ability of chemical penetration through the exoskeleton (Li et al., 2007; Feyereisen et al., 2015). The resistance of *T. absoluta* to a wide variety of insecticides exhibiting different modes of action is most commonly attributed to the first two mechanisms (Guedes et al., 2019).

The Latin American origin of European populations is indicated by the genetic similarity between S. American and Mediterranean populations. In more detail, Cifuentes et al. (2011) work focusing on 33 different populations representing the two regions, revealed that the populations from the two sites of the Atlantic Ocean share a single COI and ITS haplotype. Given the genetic similarity and origin of European populations, the same pattern of insecticide resistance found in L. America is expected to be identified in Europe before any exposure of the insects to insecticides (Siqueira et al., 2000b; Lietti et al., 2005; Guedes & Siqueira 2012).

The frequency of alleles that confer resistance to insecticides increases with the use of insecticides. More specifically, it was shown that specific point mutations, such as M918T, T929I and L1014F lead to molecular changes on the para-type sodium channel IIS4-IIS6 that is not recognized anymore as a target by pyrethroids (Haddi et al., 2012; Silva et al., 2015).

Resistance to pyrethroids may also occur by overproduction of detoxification enzymes, such as esterases and CYP450s, which decompose the insecticides. However, such mechanisms do not seem to be common in *T. absoluta*.

Apart from pyrethroids, populations from Europe and S. America were shown to be resistant to organophosphate insecticides because of the A201S mutation of the acetylcholinesterase (AChE) gene. (Haddi et al., 2017; Zibaee et al., 2018). The first case of resistance to spinosad was reported in tomato pinworm populations from Chile, with CYP450 and esterases suggested as the principal mechanisms involved (Reyes et al., 2012). A few years later, autosomal, recessive, and monogenic resistance to spinosad was identified in a Brazilian population. The

insensitivity mechanism of this population was correlated with a target-site mutation (G275E) in the a6 subunit of the nAChr (Silva et al., 2016c; Campos et al., 2015b).

The molecular basis and target-site resistance to diamides in *T. absoluta* was first described by Roditakis et al., (2017a) from European and South American populations. The general mechanism of resistance of diamides was first described in the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae) from diamide resistant populations from Philippines and Thailand, by Troczka et al., (2012). These authors detected the G4946E mutation in the C-terminal transmembrane domain of the ryanodine receptor. Similarly to G4946E mutation in *Plutella xylostella*, the G4903V mutation on RyR receptor was identified for the first time as conferring resistance to diamides in the Italian *T. absoluta* strain (Figure 10). Resistance ratio for *T. absoluta* Italian strain was at least 300-fold higher than the reference strain (Roditakis et al., 2017a). In *T. absoluta* populations, esterases, glutathione S-transferases and cytochrome P450-dependent monooxygenases are not associated with resistance to chlorantraniliprole (Silva et al., 2019).

Furthermore, the mutation I4746M was found to be common among Italian *T. absoluta* populations. RyR target-site mutations are strongly associated with high levels of diamide resistance in *T. absoluta* compared to the recommended label rates (Roditakis et al., 2017a). The I4746M mutation identified in *T. absoluta* by Roditakis et al., (2017a) was first described as I4790M in Chinese

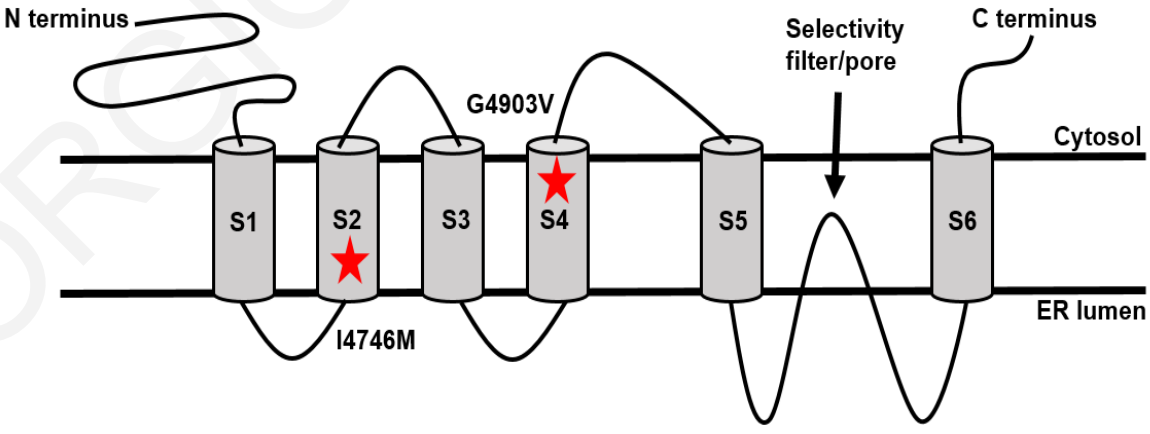

Figure 10: Representation of G4903V and I4746M mutations at the RyR of *T. absoluta.*

populations of diamondback moth by Guo et al., (2014a) (Figure 10). Moreover, two recently identified mutations, G4903V and I4746T, (Roditakis et al., 2017a) were

shown to be involved in the resistance mechanism of *T. absoluta* individuals. In other species of lepidoptera, RyR resistance to diamides is frequently related to amino acid substitution G4903E/V (Troczka et al., 2015; Nauen & Steinbach 2016).

Another group of insecticides which target the sodium channels of insects is oxadiazines. They act on voltage-gated sodium channels (VGSCs) α-subunits, which are composed of four homologous domains (I, II, III, IV). Every domain has six membranes spanning helical segments (S1 – S6) (Figure 11) (Catrerall, 2017). The target-site mutations F1845Y and V1848I on segment 6 of domain IV were diagnosed on the sodium channel of *T. absoluta* individuals (Figure 11), previously found to be resistant to indoxacarb (Roditakis et al., 2017b). The same mutations were earlier identified in *P. xylostella* by Wang et al., (2016). These mutations alter regionally the three dimensional structure of the protein which is no more recognized by the insecticides.

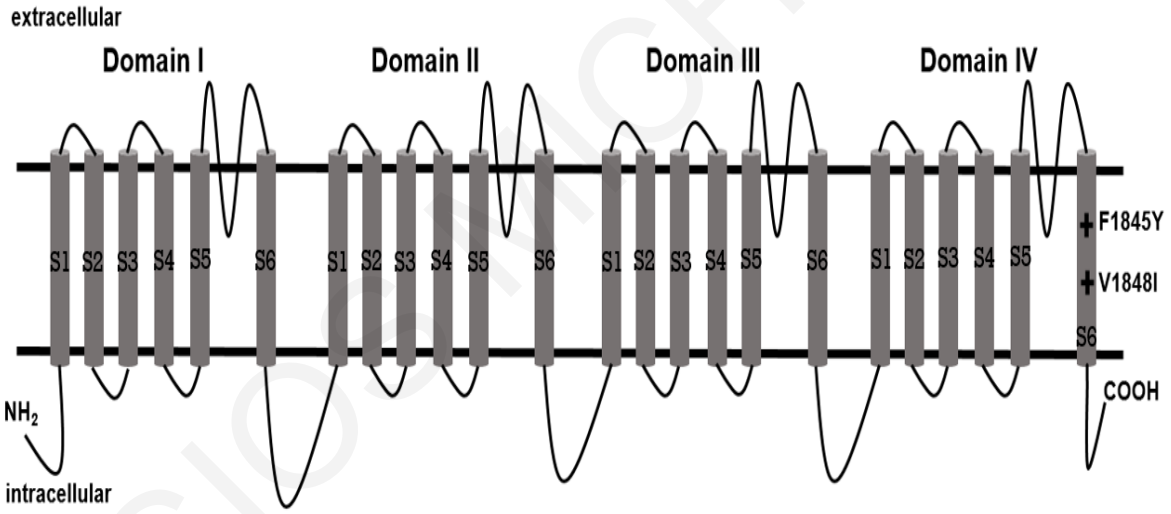

Figure 11: Representation of F1845Y and V1848I mutations at segment 6 of domain IV of *T. absoluta* sodium channel.

The increasing resistance of tomato pinworm to insecticides with novel modes of action reinforce concerns that the management of this pest will not be achievable with available insecticides within a few years (Biondi et al., 2018; Roditakis et al., 2018). For this reason, monitoring the status and understanding the genetic mechanisms of resistance to insecticides are key practices to control *T. absoluta* populations and avoid their spread in other non-infested regions.

3.2 Materials and methods

3.2.1 Collection of *T. absoluta* populations

Tomato leaves infested with *T. absoluta* larvae were collected from four different locations in Cyprus. Collection sites were Mantria (Pafos), Maroni (Larnaca), Sotira (Ammochostos), and Orounta (Nicosia) (Table 1, section 2.2.1). Samplings were carried out between September and November 2018. About 200 tomato leaves infested with *T. absoluta* larvae were collected from each sampling area. The leaves were transferred to the insectary and maintained as described before in section 2.1.1.

3.2.2 Identification of Indoxacarb resistance related mutations

Thirty individuals (adults and 4th instar larvae) from each sampling location were collected for this purpose. Furthermore, the same number of individuals from the laboratory population were included in the analyses to serve as control.

3.2.2.1 Genomic DNA extraction

Total genomic DNA was extracted using the Macherey-Nagel kit following the standard proposed protocol for DNA isolation. In more detail, tissues were placed in 1.5ml tubes with Proteinase K and incubated at 56 ˚C overnight. Then clean up steps including centrifugation were carried out before the final isolation of gDNA of high concentration and purity. The quality and quantity of extracted DNA was verified using NanoDrop 2000/200c (Thermo Fisher Scientific Inc., USA). DNA concentration was above 30ng/μl for all samples and the purity rate was over 1.6 (A260/A280 absorption rate). gDNA was stored at -20 ˚C until use.

3.2.2.2 Molecular diagnostic assays for indoxacarb resistance mutations

Modifying the diagnostics for detection of the resistance mutations to indoxacarb by Roditakis et al., 2017 a PCR diagnostic assay was prepared. PCR was performed using 0.025 U of Kapa Taq DNA polymerase, 5μl 1X Taq buffer A, 0.3mM dNTP's, 1.3mM $Mgcl_2$ (Kapa Biosystems), 1.3mM primer diagF, 1.3mM primer diarR (Eurofins Genomics), DMSO at a final concentration of 6% v/v, 3.0 μl of DNA extracted and DEPC Treated Water (Ambion®) at a final concentration of

50 µl. Primers pair diagF: 5′-GTGCTGGACGGCATCATCAA-3′ / diagR: 5′-CTCGAGAATGACGGCGATGT-3′, were used to amplify a 165bp part of segment 6 of sodium channel IV domain where F1845Y and V1848I mutations are found if present (Figure 11).

PCR conditions were 94 ˚C for 5 min, followed by 10 cycles of 94 ˚C for 30 s, 65 ˚C for 30 s and 72 ˚C for 30 s. Then 10 cycles of 94 ˚C for 30 s, 62 ˚C for 30 s and 72 ˚C for 30 s were pursued. Subsequently, 20 cycles of 94 ˚C for 30 s, 60 ˚C for 30 s and 72 ˚C for 30 s were heeled. The last step was 72 ˚C for 5 min. Using this touch down approach we manage to increase the final yield eliminating aspecific products (Korbie and Mattick, 2008).

PRC products, were loaded to 1% w/v agarose gels for electrophoresis. The size of PCR products was estimated using a ladder (3000 – 100bp) (Nippongenetics). DNA fragments were visualized under UV light. A band of 165 bp was confirmed for each reaction originating from diagF and diagR primers.

Identification of the F1845Y mutation was carried out with the enzymatic digestion using 2 U of HpyAV restriction enzyme, 1.5 µl of HpyAV CutSmart Buffer 1X (New England BioLabs®), 1.5 µl of BSA 10X, 7.5 µl of PCR product and DEPC Treated Water (Ambion®) in a total volume of 15 µl. The reaction was carried out at 37 ˚C for 3 hours and subsequently the enzyme was inactivated at 65 ˚C for 20 min. The enzymatic digestion for V1848I detection was achieved using 5 U of BclI restriction enzyme, 1.5 µl of BclI CutSmart Buffer 1X (New England BioLabs®), 1.5 µl of BSA 10X, 7.5 µl of PCR product and DEPC Treated Water (Ambion®) in a total volume of 15 µl. Incubation at 50˚C followed for 3 h. All digestion reaction products were electrophoresed in 3.5% w/v agarose gel. Expected genotypes for the HpyAV enzymatic digestions were 131bp, 165bp and 165-131bp for homozygous susceptible, homozygous mutant and heterozygous *T. absoluta* individuals, respectively. By contrast, predictable genotypes after the BCII enzymatic digestions were 165bp, 128bp and 165-128bp for homozygous susceptible, homozygous mutant and heterozygous *T. absoluta* samples, correspondingly (Roditakis et al., 2017b).

3.2.3 Identification of chlorantraniliprole resistance related mutations

In order to verify the presence and frequency of mutations related with chlorantraniliprole resistance, 20 individuals (adults and 4th instar larvae) of each sampling area plus 20 larvae of the laboratory population tested were used.

3.2.3.1 RNA extraction and cDNA synthesis

In order to eliminate non coding DNA regions nested within protein coding exons, we targeted mRNA. Total genomic RNA was isolated from 20 individuals from each study area following a Trizol based protocol (Invitrogen). Twenty individuals from each study area were used. Extracted RNA quality and quantity was tested using NanoDrop® 2000/200c (Thermo Fisher Scientific Inc., USA). RNA concentration was above 10ng/μl for all samples and the purity rate was over 1.6 (A260/A280 absorption rate).

Extracted RNA was treated with Turbo DNAse (Invitrogen) to avoid any possible contamination. DNAse was inactivated with the addition of 1.2 μl of EDTA 50mM. Subsequently, tubes were mix gently and incubated at 65 ˚C for 10 min. This step was followed by a centrifugation at 12000 rpm for 1.5 min and the upper phase was transferred to a new 1.5 ml tube.

SuperScript III RT (Invitrogen) was used to synthesize cDNA. Specifically, up to 4 μg of DNAse treated RNA were mixed with 0.5 μl 50 μM OlogodT (Invitrogen), 0.5 μl 10 μM dNTPs and DEPC Treated Water (Ambion®) at a final volume of 6.5 μl. Samples were incubated at 65 ˚C for 5 min and immediately placed on ice for >1 min. After this step a mixture of the following reagents were prepared and added to the samples: 2μl 5x FsB, 0.5 μl 0.1M DTT, 0.5 μl Superscript III RT 200 u/μl (Invitrogen) and 0.5 μl Anti-Rnase 40 u/μl (Ambion®). The final reaction was 6.5 μl. This was followed by an incubation of 50 ˚C for 50 min and then 70 ˚C for 15. cDNAs stored in -20 until their use in diagnostic assays.

3.2.3.2 Molecular diagnostic assays

In order to identify the mutation G4903V two forward and two reverse primers were used. These primers were the ‘Tabs_RyR_F5’ (5' CAAATCGTTCCCCGTGAACTAC 3') and ‘Tabs_RyR_R3’ (5' CGTGAGCATATCGTGACAGTTC 3') the universal forward and the universal

reverse primers, respectively. The susceptible forward and resistance reverse primers used were the 'Tabs_RyR_F3' (5' GCATCTGTTAGATGTCGCTGTTGG 3') and 'Tabs_RyR_R4' (5' AGGATCGTTCTCAACGTCTTAAACA 3'), correspondingly. For PCR reactions a master mix with the following reagents prepared: 2 μl of 1x Taq buffer A, 1.2 μl of 1.5 mM $Mgcl_2$, 0.6 μl of 0.3 mM dNTP's, 0.2 μl 0.025 u/μl Taq polymerase (Kapa Biosystems), 0.6 μl 0.3 mM of each primer (Tabs_RyR_F5, Tabs_RyR_R3, Tabs_RyR_F3, Tabs_RyR_R4) and DEPC Treated Water (Ambion®) in a total volume of 20 μl. PCR conditions were 94 ˚C for 5 min followed by 5 cycles of 94 ˚C for 30 sec, 65 ˚C for 30 sec and 72 ˚C for 30 sec. Then 5 cycles of 94 ˚C for 30 sec, 62 ˚C for 30 sec and 72 ˚C for 30 sec were attended. The conditions for the next 5 cycles were 94 ˚C for 30 sec, 60 ˚C for 30 sec and 72 ˚C for 30 sec. Another 10 cycles of 94 ˚C for 30 sec, 58 ˚C for 30 sec and 72 ˚C for 30 sec were performed. Subsequently, 20 cycles of 94 ˚C for 30 sec, 54 ˚C for 30 sec and 72 ˚C for 30 sec followed, accompanied with a last step at 72 ˚C for 5 min. Selected PCR conditions and concentrations were identified after performing gradient PCR reactions with different annealing temperatures and $MgCl_2$ concentrations. 3 μl of PCR products were electrophoresed in 1.5% agarose gel to confirm the presence of the bands. Expected bands were 502bp and 208bp for homozygous susceptible individuals, 502bp and 342bp for homozygous resistant insects and 502bp, 342bp and 204pb for heterozygous individuals.

Figure 12 illustrates how the DNA fragments are cut with the four primers. The primer Tabs_RyR_R4 is only bound (asterix) at the mutant position of the DNA sequence (Figure 12, Figure 13).

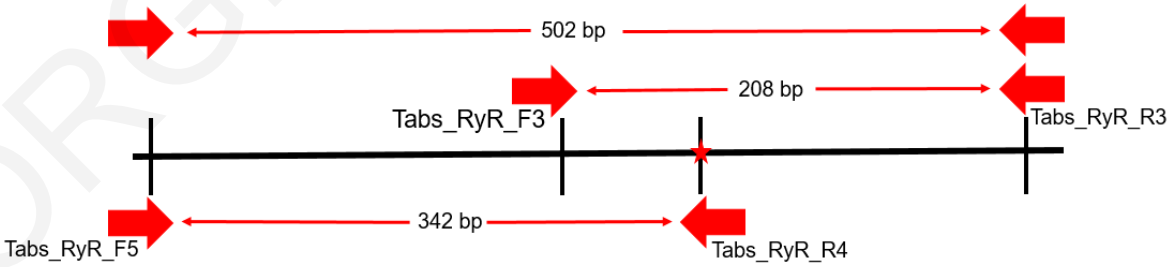

Figure 12: Diagrammatic explanation of G4903V mutation and positions that primers are binded. The asterisk shows the point of the mutation where the Tabs_RyR_R4 primer binded.

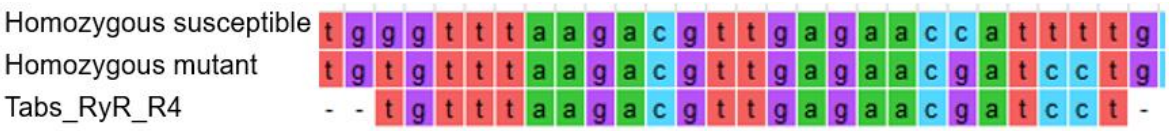

Figure 13: Alignment of the Tabs_RyR_R4 primer with a homozygous susceptible and a homozygous mutant individual.

The mutation I4746M was found if present within the frame enriched by 'Tabs_RyR_F1' (5' GCGAATCGGGTGAAGAAGAC 3') and 'Tabs_RyR_R2' (5' CGCTGAACGAGAAGTACCAC 3') primers. The PCR master mix consisted of 2 µl 1x Taq buffer A, 1.2 µl 1.5 mM $Mgcl_2$, 0.6 µl 0.3mM dNTP's, 0.2 µl 0.025 u/µl Taq polymerase (Kapa Biosystems), 0.6 µl 0.3mM of each primer (Tabs_RyR_F1 and Tabs_RyR_R2) and DEPC Treated Water (Ambion®) in a final volume of 20 µl. The PCR conditions were the same as previously described. Successful amplification of targeted fragment was ensured with gel electrophoresis where the presence of a band (531bp) was verified. PCR products were then digested with the enzyme N1aIII in order to identify the genotypes. N1aIII enzyme (CATG) digests the PCR products (531 bp) from homozygous susceptible and homozygous mutant individuals at two and three locations, respectively, as shown in Figure 14. At the mutation point (asterisk) the N1AIII enzyme recognizes only the mutant alleles and the 296 bp fragment found in susceptible individuals separated into two DNA fragments of 247 and 47 bp.

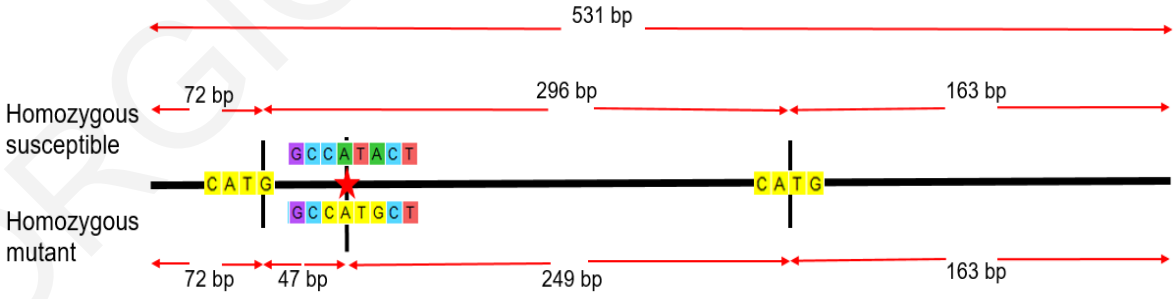

Figure 14: Diagrammatic explanation of I4746M mutation showing the places where the N1aIII enzyme digests the 531 bp amplicon. Asterisk shows the point of the mutation.

Expected genotypes were 296-163-72bp for homozygous susceptible *T. absoluta* insects, 249-163-72-47bp for homozygous resistant *T. absoluta* individuals

and 296-249-163-72-47bp for heterozygous individuals (Roditakis et al., 2017a). For this reason, 15 μl of PCR products were mixed with 2 μl of 1x N1aIII CutSmart Buffer, 0.2 μl of 10U N1aIII restriction enzyme (New England BioLabs®) and DEPC Treated Water (Ambion®) in a total volume of 20 μl. Samples were then incubated at 37 ˚C for 2 hours and afterwards electrophoresed in 2% agarose gel to determine the bands.

3.2.4 Statistical analysis

Initially, the frequencies of mutant alleles were computed. The differences between genotype or allele frequencies across the various populations of the study were examined via the non-parametric Fisher's exact statistical test. Lastly, a relevant correlation analysis was conducted in order to investigate to what extent the LC_{50} values (computed in section 2.2.6 of Chapter 2) were correlated to the frequencies of mutant alleles. All statistical analyses were carried out using R statistical software.

The propose of the correlation analysis was to examine possible relationships between the LC_{50} values found for the insecticide indoxacarb (Chapter 2) and the frequency of F1845Y and V1848 mutation alleles. Also, it was used to investigate whether the LC_{50} values calculated for the insecticide chlorantraniliprole (Chapter 2) are statistically correlated with the frequency of G4903V and I4746M mutation alleles. Comparisons of LC_{50} values and mutation allele frequencies were made on populations of the same origin. However, collecting of individuals for the purposes of these two analyses took place at different times. Specifically, LC_{50} values were calculated at least one year earlier than mutations' alleles frequencies.

3.2.5 Sanger sequencing

A number of samples exhibiting a different electrophoresis band pattern after digestion (i.e. homozygous susceptible, homozygous mutant, heterozygous) were sent for sequencing in order to cross check that the desired regions were amplified and digested as expected by the restriction enzymes. Prior to sequencing PCR products were purified using NucleoSpin® Gel and PCR clean up kit (Macherey-Nagel). Both DNA strands sequencing was performed at Macrogen Europe facilities.

The same procedure was followed for all mutations examined. Sequencing results were sent to us as chromatographs and inspected by eye using FinchTV 1.4.0. Low quality ends were trimmed before further analyses. Retrieved sequences similarity with already deposited data to NCBI GeneBank from previous studies was identified applying the BLAST algorithm.

3.3 Results

3.3.1 Indoxacarb molecular diagnostics

A PCR assay was performed to detect the indoxacarb resistance mutations F1845Y and V1848I. The presence of the 1845Y and 1848I alleles was accurately detected by PCR amplification of the flanking region and consequential two digestions with HpyAV and BclI restriction enzymes, respectively. After electrophoresis of the PCR product that confirmed the presence of the band (165 bp) (Figure 15), we moved on with HpyAV and BclI digestions and another gel electrophoresis.

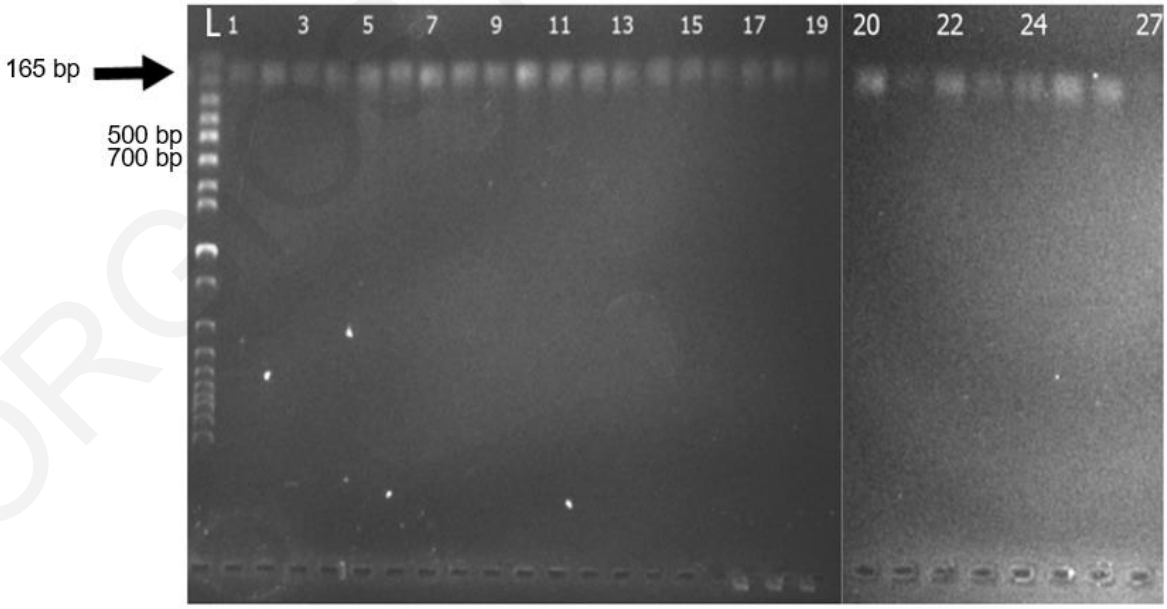

Figure 15: PCR for the 165 bp amplicon isolated with the primer set diagF and diagR L: Ladder.

Three different patterns in *T. absoluta* were demonstrated by each digestion. For the HpyAV restriction enzyme the three patterns were: the digested 131 bp fragment in the wild-type (FF) individuals, the undigested 165 bp band in the mutant (YY) individuals and two well-defined bands, one with 165 bp and one with 131 bp, for heterozygous (FY) individuals. Regarding the BcII restriction enzyme the three patterns were: the undigested 165 bp band in the wild-type (VV) individuals, the 128 bp digested fragment in the mutant (II) individuals and for heterozygotes (VI) the two distinct bands, 165 and 128 bp. Specifically, for the HpyAV restriction enzyme the homozygous wild-type samples are presented in wells (lanes) 1, 2, 4, 15 (Figure 16) where the 131 bp bands are visible. These lanes are products of the HpyAV enzymatic digestion. In lane 13 (165 bp) of the same figure an undigested homozygous mutant individual is represented. Heterozygous individuals are shown in the other lanes of Figure 16 where two bands (131 and 165 bp) appeared.

In contrast with HpyAV, homozygous wild-type samples for BcII enzymatic digestion are presented in lanes 1, 2, 5, 7, 10, 15 and 20 (Figure 17) where the undigested 165 bp products are shown. Homozygous mutant individuals are appeared in lanes 3, 6, 8, 9, 16 and 18 (Figure 17) where 128 bp bands are clearly visible. Heterozygotes band pattern revealed in lanes 4, 11, 12, 13, 14, 17 and 19 where both products (128 and 165 bp) are verified.

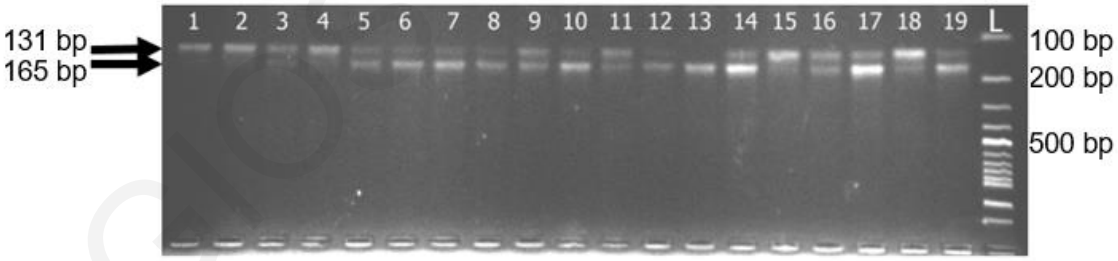

Figure 16: Samples after enzymatic digestion with HpyAV enzyme. L: Ladder.

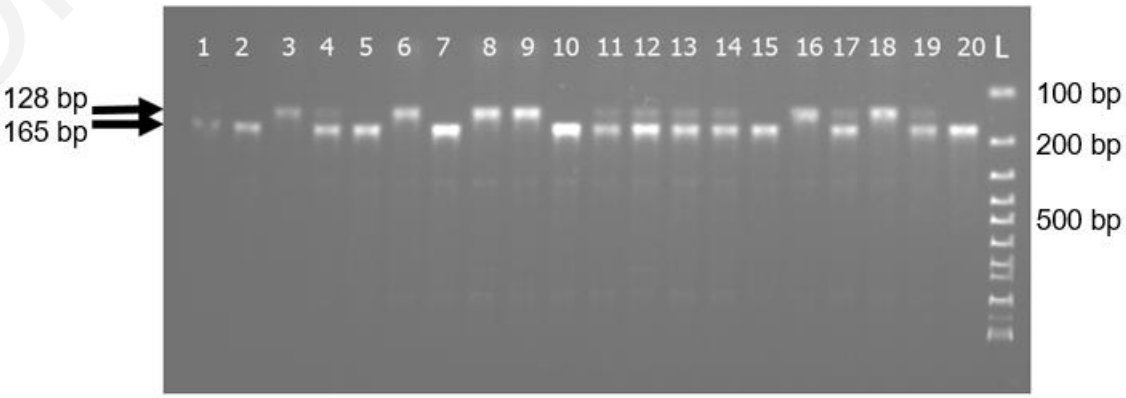

Figure 17: Samples after enzymatic digestion with BCII enzyme. L: Ladder.

A restriction site for HpyAV (5'-CCTTC-3') was decimated by the 1845Y mutation (Figure 18A) which is absent fromthe wild-type sequence F1845 (Figure 18B). A restriction site for the BclI enzyme (5'-TGATCA-3') is created by the mutation 1848I (Figure 18A), which is missing in the wild-type sequence V1848 (Figure 18B). At the point of F1845Y mutation (Figure 18B) two picks are visualized, representing a heterozygous individual.

In Figure 19 the amino acid sequence of the segment 6 of domain IV of the sodium channel of *T. absoluta* is shown. A mutant individual is represented at the position 1845, where the F1845Y mutation can be recognized at the 1845 amino acid position where the amino acid Phenylalanine was substituted by Tyrosine (Figure 19A). A mutant individual at the position 1848 can be recognized by the substitution of the amino acid Valine with Isoleucine (Figure 19B).

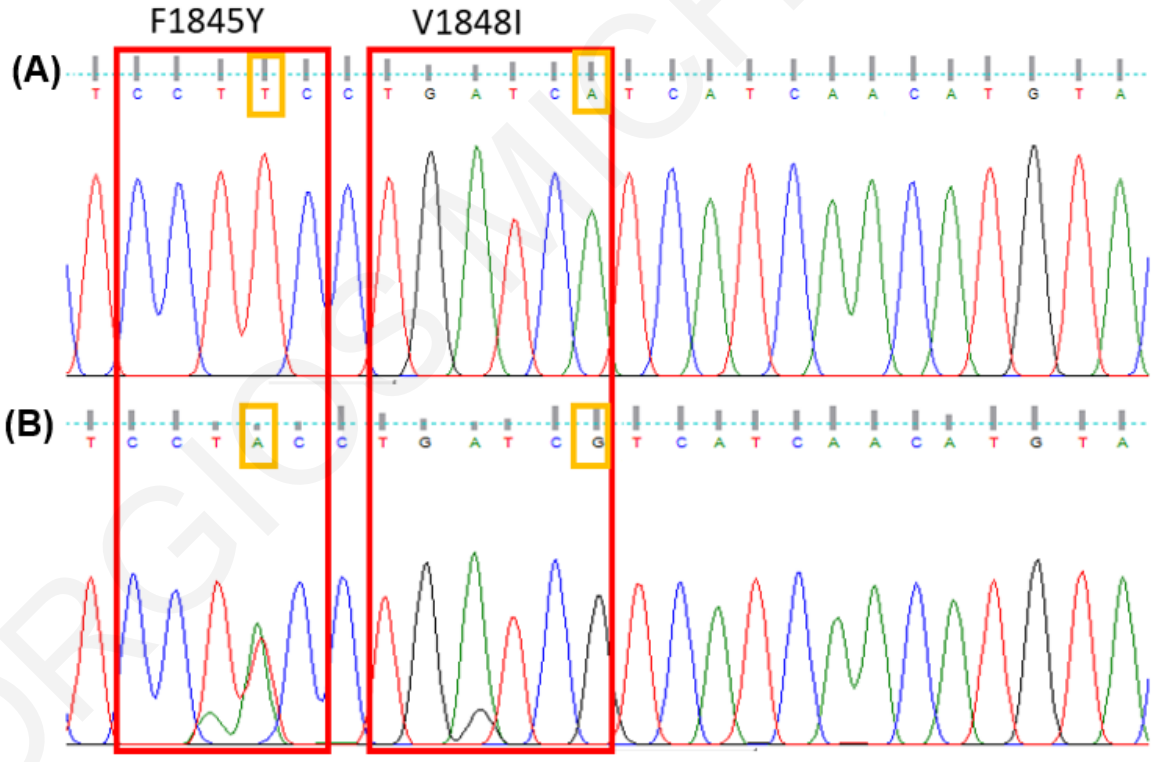

Figure 18: Amino acid sequence of the segment 6 of domain IV of the sodium channel of *T. absoluta*. A: Mutant individual at position 1845. B: Mutant individual at position 1848.

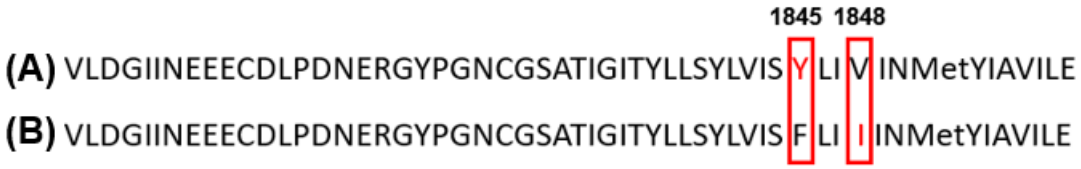

Figure 19:Amino acid sequence of the segment 6 of domain IV of the sodium channel of *T. absoluta*.

3.3.1.1 Frequencies of F1845Y and V1848I genotypes in the populations

The genotype frequencies of F1845Y and V1848I mutations of all resistance and susceptible populations were examined in order to identify their role in indoxacarb resistance. Genotype frequencies of F1845Y and V1848I mutations are shown in Tables 4 and 5 respectively. The percentage of 1845Y mutant alleles in the susceptible population (Lab) was 11.67% (Table 4), whereas no 1848I alleles were detected (Table 5).

Frequencies for 1845Y in the susceptible population ranged from 15% to 53.33% for Mantria (MAN) and Sotira (SOT), respectively (Table 4). The frequency of the 1845Y mutant alleles was significantly lower in the Lab population than in field collected populations, with the exception of MAN (Table 6).

The frequencies of the V1848I mutation ranged from 0% in the Lab to 50% in the MAN population (Table 5). Frequencies of V1848I were significantly higher in field collected than in the Lab population (Table 7).

The presence of all possible genotyping combinations of the two mutations in all populations were tested (Table 8). Double mutant homozygotes (YY/II) were not detected at any population. Likewise, heterozygosity was not paired with homozygosity for any of the resistant individuals (neither FY/II, nor YY/VI individuals were observed). Double heterozygotes were observed in all resistant populations tested, with the proportion ranging from 16.6% (ORU) to 50% (MAZ). All wild-type (VV) populations carried the mutant F1845Y allele in the heterozygous condition including the reference (Lab) population, whereas only three populations (MAZ, ORU, SOT) carried the mutant 1845Y allele in the homozygous condition. Concerning all wild-type (FF) populations, mutant V1848I alleles were observed as heterozygotes in all resistant populations tested. Individuals homozygous for mutant 1848I alleles were detected only in two resistant populations (MAN, SOT).

The computed LC_{50} values for indoxacarb (presented in Chapter 2) were negatively correlated with the frequency of the F1845Y mutation, but the relationship was not statistically significant (r = -0.02, *P* = 0.97). However, the same LC_{50} values had a statistically significant positive correlation with the frequency of the V1848I mutation (r = 0.89, *P* = 0.04). Moreover, LC_{50} values for indoxacarb were positively correlated with the combined frequencies of the two mutations (F1845Y and V1848I), but to a no statistical correlation (r = 0.57, P = 0.32).

Table 4: Frequencies of different genotypes of F1845Y mutation in *T. absoluta* populations.

		F1845Y						
		F/F		F/Y		Y/Y		
Population	N		Frequency (%)		Frequency (%)		Frequency (%)	Frequency of A (%)
Lab	30	23	76.67%	7	23.33%	0	0.00%	11.67% a
MAN	30	21	70.00%	9	30.00%	0	0.00%	15.00% a
MAZ	30	3	10.00%	26	86.67%	1	3.33%	46.67% b
ORU	30	9	30.00%	19	63.33%	2	6.67%	38.33% b
SOT	30	7	23.33%	14	46.67%	9	30.00%	53.33% b

Values followed by the same lower case letters were not statistically different among the populations.
A: Frequency of resistance alleles.

Table 5: Frequencies of different genotypes of V1848I mutation in *T. absoluta* populations.

		V1848I						
		V/V		V/I		I/I		
Population	N		Frequency (%)		Frequency (%)		Frequency (%)	Frequency of A (%)
Lab	30	30	100.00%	0	0.00%	0	0.00%	0.00% a
MAN	30	6	20.00%	18	60.00%	6	20.00%	50.00% b
MAZ	30	12	40.00%	18	60.00%	0	0.00%	30.00% b
ORU	30	17	56.67%	13	43.33%	0	0.00%	21.67% b
SOT	30	16	53.33%	12	40.00%	2	6.67%	26.67% b

Values followed by the same lower case letters were not statistically different among the populations.

A: Frequency of resistance alleles.

Table 6: The resulting *P values* from the Fisher's exact statistical tests performed between the frequencies of F1845Y mutation across the various populations of the study.

Populations tested	*P*
LAB-MAN	0.7891
LAB-MAZ	<0.0001
LAB-ORU	0.0013
LAB-SOT	<0.0001
MAN-MAZ	0.0003
MAN-ORU	0.0067
MAN-SOT	<0.0001
MAZ-ORU	0.4603
MAZ-SOT	0.5841
ORU-SOT	0.1424

Table 7: The resulting *P values* from the Fisher's exact statistical tests performed between the frequencies of V1848I mutation across the various populations of the study.

Populations tested	*P*
LAB-MAN	<0.0001
LAB-MAZ	<0.0001
LAB-ORU	<0.0001
LAB-SOT	<0.0001
MAN-MAZ	0.7230
MAN-ORU	0.1420
MAN-SOT	0.4700
MAZ-ORU	0.4041
MAZ-SOT	0.8402
ORU-SOT	0.6702

Table 8: Combined genotyping for F1845Y and V1848I mutations in the populations studied.

	Population (N; %)				
Genotype					
(F1845Y/V1848I)	LAB	MAN	MAZ	ORU	SOT
FF/VV	23; 76.6	4; 13.3	0; 0	1; 3.3	0; 0
FF/VI	0; 0	11; 36.6	3; 10	8; 26.6	5; 16.6
FF/II	0; 0	6; 20	0; 0	0; 0	2; 6.6
FY/VV	7; 23.3	2; 6.6	11; 36.6	14; 46.6	7; 23.3
FY/VI	0; 0	7; 23.3	15; 50	5; 16.6	7; 23.3
YY/VV	0; 0	0; 0	1; 3.3	2; 6.6	9; 30
FY/II	0; 0	0; 0	0; 0	0; 0	0; 0
YY/VI	0; 0	0; 0	0; 0	0; 0	0; 0
YY/II	0; 0	0; 0	0; 0	0; 0	0; 0

3.3.2 Chlorantraniliprole molecular diagnostics

Two chlorantraniliprole resistance mutations, I4746M and G4903V located at the S2 and S4 transmembrane-spanning domains, respectively, of the RyR of *T. absoluta* were detected by two PCR-RFLP assays.

To identify the G4903V mutation the four primers Tabs_RyR_F3, Tabs_RyR_F5, Tabs_RyR_R3 and Tabs_RyR_R4 were used. When the PCR products were electrophoresed we were able to recognize three different patterns of DNA fragments (Figure 20).

Wild-type individuals are shown in lanes 2, 3 and 4 where two bands are visible (502 and 208 bp). Homozygous mutant individuals are displayed in lane 1 where two bands (502 and 342 bp) appeared. In heterozygotes (lane 5) three clearly distinguished bands (502, 342, 208 bp) appeared.

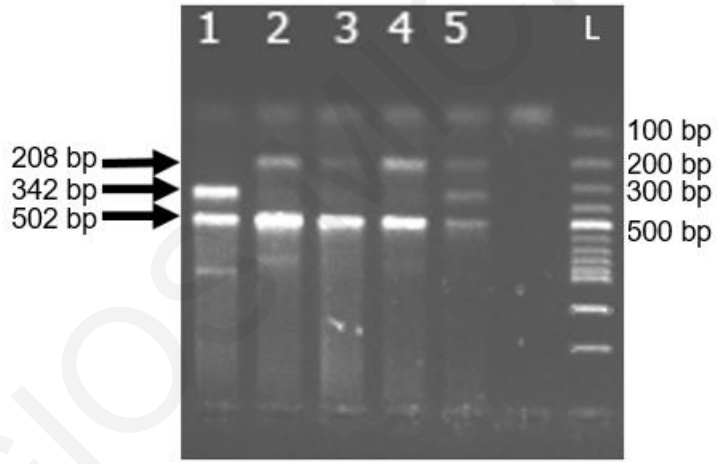

Figure 20: PCR-RFLP diagnostic assay of the G4903V chlorantraniliprole resistance mutation in T. absoluta. L: Ladder.

G4903V mutation is represented by the chromatogram (Figure 21B) where the mutation is boxed. The mutation is missing at the susceptible individual (Figure 21A).

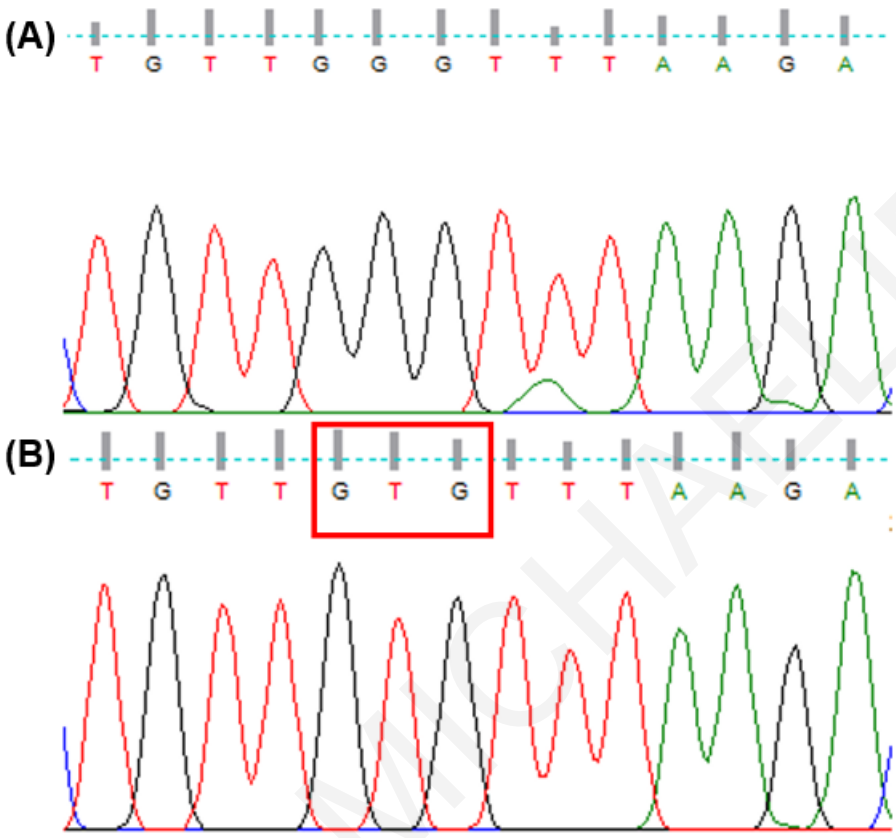

Figure 21: Representative chromatogram of the G4903V mutation in the RyR gene of *T. absoluta*. (A) Nucleotide sequence of a diamide susceptible individual. (B) Nucleotide sequence with the mutation (boxed).

In the diagram (Figure 22) a part of S4 transmembrane-spanning domain is shown, where Glycine substituted Valine at position 4903.

Figure 22: Amino acid substitution G4903V corresponding to the mutation.

To find the I4746M mutation a PRC-RFLP analysis was carried out using the primers Tabs_RyR_F1 and Tabs_RyR_R2. These two primers seclude a DNA

fragment of 531 bp of S2 transmembrane-spanning domain in *T. absoluta* RyR. This fragment could be visualized after PCR product electrophoresis (Figure 23).

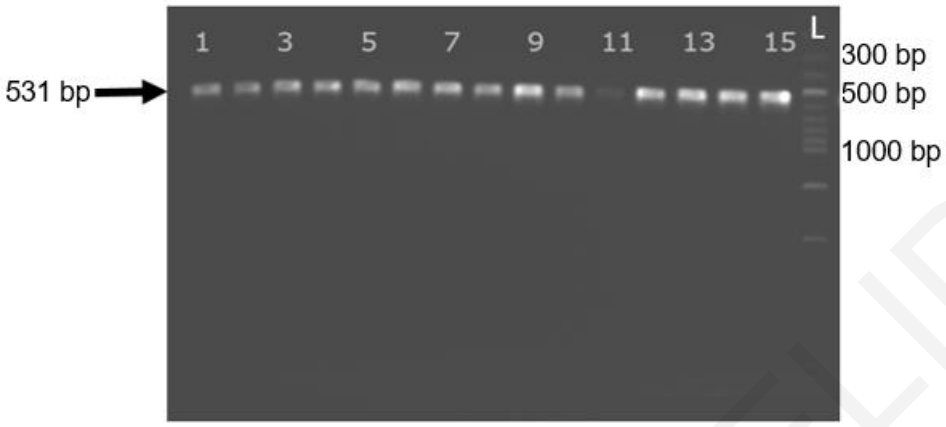

Figure 23: PCR-RFLP diagnostic assay of the I4746M chlorantraniliprole resistance mutation in *T. absoluta*. L: Ladder.

After the digestion with the restriction enzyme, two different band patterns represented the wild-type, homozygous mutant and heterozygous individuals (Figure 24). Specifically, for homozygous susceptible individuals three bands (296-163-72 bp) appeared. Specific individuals are shown in lanes 10, 12, 13 and 14. However, for homozygous resistant individuals (lanes 1, 6, 15) four bands (249-163-72-47 bp) were present. Heterozygotes are shown in lanes 2, 3, 4, 5, 7, 8, and 9. In these cases five bands (296-249-163-72-47 bp) were present (Figure 24).

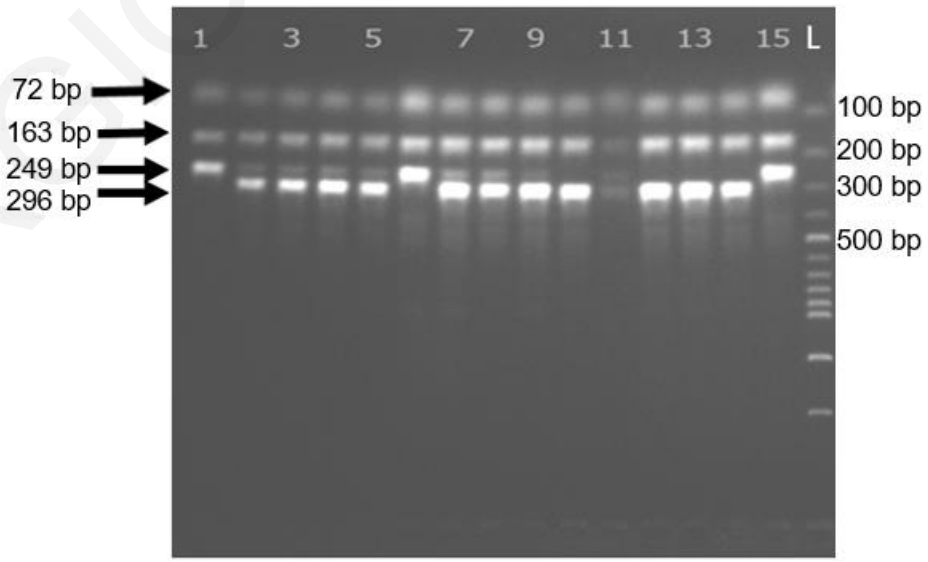

Figure 24: Samples after enzymatic digestion with N1aIII enzyme. L: Ladder.

Furthermore, representative sequencing chromatographs of the wild-type, homozygous mutant and heterozygous individuals are illustrated in Figure 25. A heterozygote is shown in the Figure 25C where at the point of the mutation allele two peaks are visible.

A part of amino acid sequence of the *T. absoluta* RyR C-terminal transmembrane domain is demonstrated in Figure 26. Particularly, the replacement of Methionine with Isoleucine in mutant individuals happened at 4746 position.

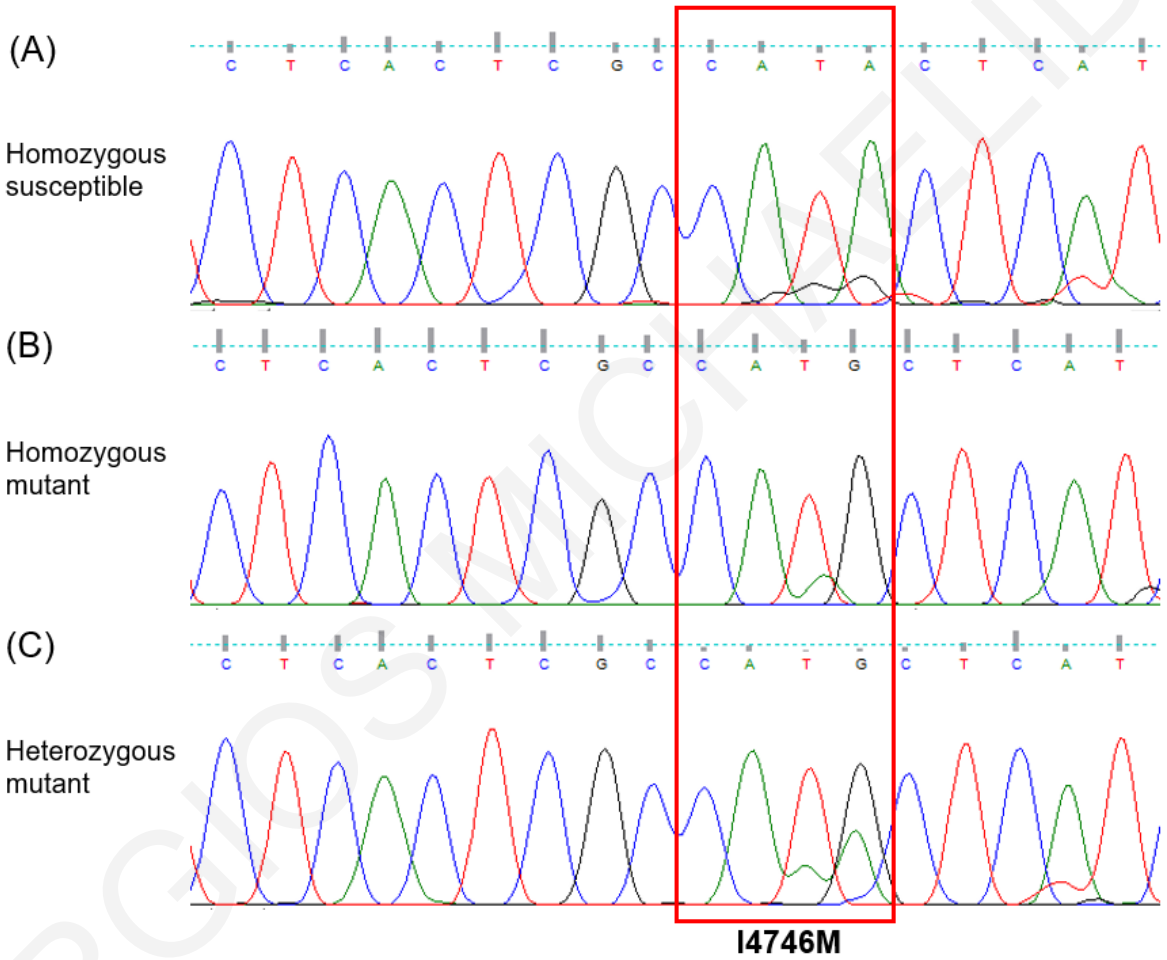

Figure 25: Representative chromatogram of the I4746M mutation in the RyR gene of *T. absoluta*. A: Homozygous susceptible individual, B: Homozygous mutant individual, C: Heterozygous mutant individual.

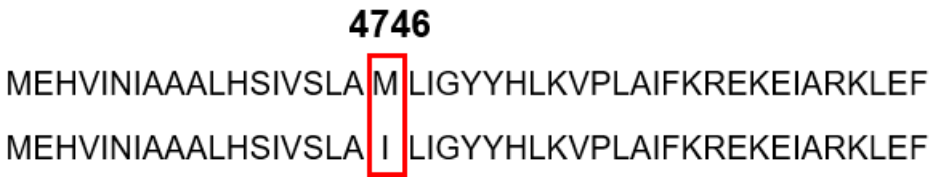

Figure 26: Amino acid substitution I4746M corresponding to the mutation.

3.3.2.2 Frequencies of G4903V and I4746M genotypes among the populations

The genotype frequencies of G4903V and I4746M mutations of all wild-type and mutant populations were investigated to identify their role in chlorantraniliprole resistance. Genotype frequencies of G4903V and I4746M mutations are presented in Tables 9 and 10 respectively. Considering the mutant alleles found in the resistant population, for the I4746M mutation the frequency of mutant alleles ranged from 47.50% (MAN) to 90.00% (SOT). However, for the G4903 mutation the frequencies of the resistant alleles were much lower, since they fluctuated from 10.00% (SOT) to 25.00% (MAN). This mutation was not detected in the *T. absoluta* population from MAZ population.

Fisher's exact tests were conducted in order to identify significant differences between the 4903V and 4746M allele frequencies across the various populations; the resulting *P-Values* are presented in Tables 11 and 12 respectively.

The presence of all possible genotyping combinations of the two mutations in all populations were also tested (Table 13). Double mutant homozygous individuals (VV/MM) were not detected in any population tested. Similarly, heterozygosity was not paired with homozygosity for GV/MM individuals. By contrast, a frequency of 5% was observed for VV/IM, homozygosity / heterozygosity. Double heterozygotes were observed only in MAN, ORU and SOT populations at a frequency of 20% in all cases. However, no double heterozygotes were detected at the wild-type (Lab) and MAZ *T. absoluta* populations. All wild-type (GG) populations, except from LAP, carried the mutant I4746M allele in the heterozygote and homozygote condition, apart from the SOT population where the combination GG/IM did not exist. Regarding the all wild-type (II) populations, mutant G4903V alleles were observed as heterozygotes and homozygotes in two (MAN, ORU) of the four resistant populations tested.

The frequencies of G4903V and I4746M mutant alleles were not statistically correlated to the LC_{50} values of chlorantraniliprole ($r = 0.49$, $P = 0.40$; $r = 0.34$, $P = 0.58$, respectively). Likewise, the combined alleles frequencies of the G4903V and I4746M mutations were not statistically correlated with the LC_{50} values found for the insecticide chlorantraniliprole ($r = 0.46$, $P = 0.43$)

Table 9: Frequencies of different genotypes of G4903V mutation in *T. absoluta* populations. Different small letters indicate significant statistical differences among populations.

		G4903V						
		G/G		G/V		V/V		
Population	N		Frequency (%)		Frequency (%)		Frequency (%)	Frequency of A (%)
Lab	20	20	100.00%	0	0.00%	0	0.00%	0.00% a
MAN	20	11	55.00%	8	40.00%	1	5.00%	25.00% b
MAZ	20	20	100.00%	0	0.00%	0	0.00%	0.00% a
ORU	20	13	65.00%	5	25.00%	2	10.00%	22.50% b
SOT	20	16	80.00%	4	20.00%	0	0.00%	10.00% a, b

Values followed by the same lower case letters were not statistically different among the populations.
A: Frequency of resistance alleles.

Table 10: Frequencies of different genotypes of I4746M mutation in *T. absoluta* populations. Different small letters indicate significant statistical differences among populations.

		I4746M						
		S/S		S/R		R/R		
Population	N		Frequency (%)		Frequency (%)		Frequency (%)	Frequency of A (%)
Lab	20	20	100.00%	0	0.00%	0	0.00%	0.00% a
MAN	20	6	30.00%	9	45.00%	5	25.00%	47.50% b
MAZ	20	1	5.00%	4	20.00%	15	75.00%	85.00% c
ORU	20	3	15.00%	12	60.00%	5	25.00%	55.00% b
SOT	20	0	0.00%	4	20.00%	16	80.00%	90.00% c

Values followed by the same lower case letters were not statistically different among the populations.
A: Frequency of resistance alleles.

Table 11: The resulting *P* values from the Fisher's exact statistical tests performed between the frequencies of G4903V mutation across the various populations of the study.

Populations tested	*P-Values*
LAB-MAN	0.0010
LAB-MAZ	1
LAB-ORU	0.0024
LAB-SOT	0.1156
MAN-MAZ	0.0010
MAN-ORU	1
MAN-SOT	0.1395
MAZ-ORU	0.0024
MAZ-SOT	0.1156
ORU-SOT	0.2247

Table 12: The resulting P values from the Fisher's exact statistical tests performed between the frequencies of I4746M mutation across the various populations of the study.

Populations tested	*P-Values*
LAB-MAN	<0.0001
LAB-MAZ	<0.0001
LAB-ORU	<0.0001
LAB-SOT	<0.0001
MAN-MAZ	<0.0001
MAN-ORU	0.6550
MAN-SOT	<0.0001
MAZ-ORU	0.0066
MAZ-SOT	0.7371
ORU-SOT	0.0009

Table 13: Combined genotyping for G4903V and I4746M mutations in the populations studied

Genotype	Population (N; %)				
(G4903V/I4746M)	LAB	MAN	MAZ	ORU	SOT
GG/II	20; 100	1; 5	1; 5	1; 5	0; 0
GG/IM	0; 0	5; 25	4; 20	7; 35	0; 0
GG/MM	0; 0	5; 25	15; 75	5; 25	16; 80
GV/II	0; 0	4; 20	0; 0	1; 5	0; 0
GV/IM	0; 0	4; 20	0; 0	4; 20	4; 20
VV/II	0; 0	1; 5	0; 0	1; 5	0; 0
GV/MM	0; 0	0; 0	0; 0	0; 0	0; 0
VV/IM	0; 0	0; 0	0; 0	1; 5	0; 0
VV/MM	0; 0	0; 0	0; 0	0; 0	0; 0

3.4 Discussion

A very important mechanism of insecticide resistance involves the changes in the structure of insecticide target transmembrane proteins, due to point mutations (Wang et al., 2008). Mutations F1845Y and V1848I of voltage-gated sodium channels conferring resistance to indoxacarb, and G4903V and I4746M mutations of Ryanodine receptors related with chlorantraniliprole resistance, were identified in Cypriot populations of *T. absoluta*.

Identified mutations conferring resistance to indoxacarb in the present study were the same as those found in Italian and Greek populations of *T. absoluta* (Roditakis et al., 2017b). The mutation F1845Y found in the laboratory population shows that this mutation probably was introduced during the invasion of the tomato pinworm in Cyprus. The 1845Y mutant alleles frequencies found in field-collected populations were statistically different from those of the reference strain, with one exception. However, the V1848I mutation was not found in the lab population.

Mutant alleles frequencies from all field-collected populations were not significantly different among one-another, but statistically different from the frequencies of lab population mutant alleles. These results probably show that for both indoxacarb related mutations the mutant alleles frequencies have increased through time.

The RyR mutation G4903E/V has been reported in *T. absoluta* (Roditakis et al., 2017a;), in *P. xylostella* as G4946V (Troczka et al., 2012) and in *Chilo suppressalis* (Lepidoptera: Crambidae) as G4910E (Yao et al., 2017). Troczka et al., (2015) and Nauen & Steinbach (2016) supported that resistance to diamides found at lepidopteran RyR caused mostly by the amino acid substitution G4903E/V. In contrast, according to our results resistance to chlorantraniliprole in Cypriot populations of *T. absoluta* is more frequently related to I4746M than G4903V mutations. The previous statement is strongly supported by the fact that I4746M mutation was found in much higher frequency than the mutation G4903V, in all populations tested. In addition, in the case of one of the studied populations, resistance to chlorantraniliprole was solely attributed to I4746M mutation since G4903V was absent. LC_{50} values found for chlorantraniliprole were not statistically correlated with both mutation alleles frequencies. We suppose that this is due to the fact that individuals for the two analyses were collected in different years, belonging to different generations, which is a limitation of this study.

Regarding resistance to indoxacarb it is not clear which mutation more commonly confers resistance. Even though the frequency of F1845Y mutation was higher than the frequency of the mutation V1848I in most populations tested, the frequency of the latter mutation was higher than the frequency of the first mutation in *T. absoluta* population from Mazotos. It is worth mentioning that the mutation F1845Y introduced with the invasion of the pest in Cyprus identified at a lower frequency (lab population: 11.67%) than from field-collected populations (MAN: 15.00%; MAZ: 46.67%; ORU: 38.33%; SOT: 53.33%) (see Table 4). In contrast, at the time of the pest's introduction in the island the mutation V1848I was probably not present. Therefore, the combination of the two mutations may play an important role in indoxacarb resistance.

LC_{50} values found in indoxacarb resistant populations and F1845Y mutation frequency were not statistically correlated. Similarly, both G4903V and I4746M mutation frequencies, were not significantly correlated to the LC_{50} values previously found in chlorantraniliprole resistant populations. However, the V1848I mutation

was found to be statistically correlated to the LC_{50} values found for indoxacarb. When mutant alleles frequencies for the F1845Y and V1848I mutations were summed up and compared with the LC_{50} values for indoxacarb, no significant correlation among them was observed.

Previous studies where Fisher's tests were conducted, found significant correlations between mutation frequencies and resistant levels (Guo et al., 2014b; Steinbach et al., 2015; Troczka et al., 2015; Roditakis et al., 2017b; Yao et al., 2017). Thus, our results could be attributed to the fact that individuals included in Fisher's exact statistical test were collected in different years. Moreover, we assume that LC_{50} values should was higher in late 2018 than in 2017.

In conclusion, the four mutations described in this Chapter are possibly related to indoxacarb and chlorantraniliprole resistance found in Chapter 2. Most of the mutant alleles frequencies are not statistically correlated with the LC_{50} values found in Chapter 2. However, it is assumed that the insecticide resistance detected is due to those mutations because LC_{50} values and mutant alleles frequencies identified, referred to different generations of the same populations. Such molecular analyses should be included in resistance monitoring programs for the early detection of mutant alleles.

CHAPTER 4

Functional response and multiple predator effects of two generalist predators preying on *Tuta absoluta* eggs*

Abstract

Interactions among invertebrate predators could affect a pest suppression. The hemipteran species *Macrolophus pygmaeus* (Rambur) and *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) are natural enemies of several pests in agroecosystems and coexist in tomato crops in Mediterranean countries. By using the multiplicative risk model (MRM) and the substitutive model, the multiple predator effects (MPEs) on prey suppression were calculated when two individuals of the predators foraged at the same densities on South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), eggs. Egg consumption increased with increasing egg density and the two predators exhibited a Type III functional response. Predation rates were strongly affected by prey density. Using the MRM, we found risk reduction at intraspecific treatments at high prey density. Applying the substitutive model, we detect risk enhancement at interspecific treatments at high egg density. At low prey densities, most of the interactions were independent, whereas at high densities most interactions were not independent and resulted in prey risk reduction, indicating antagonism between the individuals involved. We also showed that *N. tenuis* is a more competitive predator species for *T. absoluta* eggs than *M. pygmaeus*; however, combination of the two predator species will lead to better pest suppression at high *T. absoluta* population densities.

*Published: Michaelides, G., Sfenthourakis, S., Pitsillou, M. & Seraphides, N. 2018. Functional response and multiple predator effects of two generalist predators preying on *Tuta absoluta* eggs. *Pest Manag Sci*, 74, 332-339.

4.1 Introduction

Among the fundamental ecosystem services provided by biodiversity is the control of pests by beneficial organisms. A high diversity of natural enemies increases the possibilities of controlling a pest in a variety of environmental conditions (Wilby and Thomas, 2002; Tscharntke et al., 2005; Messelink et al., 2014). However, if more than one species of natural enemies is added to a system, they may show antagonistic interactions that result in a decrease in prey consumption rates (Rosenheim et al., 1995; Finke and Denno, 2004, 2005; Van Son and Thiel, 2006; Naselli et al., 2017). By contrast, higher consumption rates of a pest might occur when more than one single beneficial species co-occur (Losey and Denno, 1998; Cardinale et al., 2003; Straub and Snyder, 2006; Messelink and Janssen, 2014).

Multiple predator effects (MPEs) could emerge by interactions (collaboration, competition or intraguild predation) among multiple predators, or by prey anti-predator responses. Predation effects by multiple predators cannot always be predicted from evaluation of the independent effects of each predator on prey survival (Sih et al., 1998; Griffen, 2006; Schmitz, 2009). Interactions between multiple predators could affect prey survival separately, synergistically, causing prey risk enhancement (Sih et al., 1998; Soluk and Collins, 1988), or antagonistically, causing prey risk reduction (Sih et al., 1998). MPEs are important in applied ecology in the design of an effective plan of biological pest control through the introduction of multiple predators into cultivations (Hochberg, 1996).

Because *T. absoluta* is considered one of the most disastrous pests of tomato cultivations around the world (Miranda et al., 1998; Desneux et al., 2010; 2011), controlling the population is an essential goal. This could be effectively fulfilled taking advantage of natural enemies found at the place of origin of the targeted pest (Desneux et al., 2010). Among these, *N. tenuis* and *M. pygmaeus*, which are already present in the Mediterranean, could be used to serve this purpose (Arnó et al., 2009; Urbaneja et al., 2009; Mollá et al., 2014; Calvo et al., 2012; Urbaneja et al., 2012; Ingegno et al., 2013; Biondi et al., 2013).

These two predators co-occur in tomato cultivations and are efficient in reducing the population density of serious pests of vegetable crops (Albajes and Alomar, 1999; Castañé et al., 2011; Moreno-Ripoll et al., 2012a). Intraguild

interactions and cannibalism have been found between the two predators (Moreno-Ripoll et al., 2012a; Perdikis et al., 2014). Their multiple combined effect on prey suppression was studied by Lampropoulos *et al.* (2013) who showed prey risk enhancement at high prey densities and prey risk reduction at intermediate prey densities [*Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae)].

Preventive application of *M. pygmaeus* and *N. tenuis* has been increasingly used since *T. absoluta* has been established in new regions (Calvo et al., 2012; Urbaneja et al., 2012; Castañé et al., 2011), because both predators consume *T. absoluta* eggs (Arnó et al., 2009; Urbaneja et al., 2009; Mollá et al., 2011; Sanchez et al., 2014). Adult individuals of both predators exhibited a type II functional response when preying on *T. absoluta* and *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) eggs; however, *N. tenuis* was more effective than *M. pygmaeus* in consuming *T. absoluta* eggs (Sharifian et al., 2015). *M. pygmaeus* fifth instar nymphs also presented a type II functional response when fed on all juvenile instars of *Myzus persicae* (Sulzer) (Homoptera: Aphididae) (Fantinou et al., 2008) and on the second and third instar nymphs of *T. vaporariorum* (Lampropoulos et al., 2013). The previous results were also confirmed for *N. tenuis* (Lampropoulos et al., 2013). However, a predator species can reveal different types of functional response when presented different types of prey (van Lenteren et al., 2016). *M. pygmaeus* exhibited a type III functional response when adult females consumed first instar nymphs of *T. vaporariorum* (Engekaard et al., 2001).

In a greenhouse experiment, Messelink and Janssen (2014) found that the predators *M. pygmaeus* and *Orius laevigatus* (Fieber) (Heteroptera: Anthocoridae) controlled populations of western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) and green peach aphid, *M. persicae*, better when applied together than when applied separately. They also found that intraguild predation did not have any negative effects on prey control. Antagonistic interactions between predators do not necessarily lead to an increase in herbivorous insect populations (Lang, 2003), because the outcome of multiple predator interactions may vary depending on their mobility, predator–prey relative sizes, population structure, environmental heterogeneity, population density and the availability of intermediate prey (Polis et al., 1989; Lucas et al., 1998; Finke and Denno, 2002; Tommasini et al., 2002; Chacón and Heimpel, 2010; Lucas and Rosenheim 2011). Successful

biological control of pests could be closely related to interactions between generalist predators (Symondson et al., 2002).

Predators strongly affect prey communities, populations, habits and morphological traits (Lima and Dill, 1990). Research on effects of predators' interspecific interactions on prey populations has paid special attention to predator traits (Bruno and Cardinale, 2008) and prey behavior when confronted by predators (Soluk and Collins, 1988). A disadvantage of some studies exploring MPEs is that tests were carried out at one prey density (Soluk, 1993), whereas the determination of functional response curves is crucial to accurately define MPEs (Van Son and Thiel, 2006). Vance-Chalcraft and Soluk (2005a) found that interspecific interactions among predators are not associated with prey density; however, the functional response curves of the predators were not examined.

The effect of multispecies interactions may depend on prey density. This hypothesis was tested by McCoy et al. (2012) and Lampropoulos et al. (2013) using the Multiplicative Risk Model (MRM). This model estimates the possibility, from the additive model, of a prey individual being consumed twice by a predator (Griffen, 2006). McCoy et al. (2012) found that the additive model overestimates prey risk. Predation risk may also be reduced because predators become satiated. Lampropoulos et al. (2013) found conspecific and heterospecific interactions between the predators *M. pygmaeus* and *N. tenuis* when preying on various densities of *T. vaporariorum* nymphs. However, at densities close to their satiation level, the interactions were more intense, resulting in a reduction in prey risk. At higher prey densities predator interactions resulted in an increase in prey risk.

In this Chapter, the combined effects of conspecific and heterospecific interactions of the predators *N. tenuis* and *M. pygmaeus* when preying on various densities of *T. absoluta* eggs were studied.

4.2. Materials and methods

4.2.1 Rearing and preparation for bioassays

For the coordination of the bioassays tomato plants were previously seeded and were used as an oviposition substrate for *T. absoluta* females. The eggs were the prey for the predators. *M. pygmaeus* and *N. tenuis* colonies were reared at

tobacco plants infested with the whitefly *Bemisia tabaci*. Predators' juveniles fed on *B. tabaci* larvae. *T. absoluta* colony maintained at cages with tomato plants. The cages were kept at the insectary rooms of the Agricultural Research Institute (ARI) in Lefkosia, Cyprus under controlled conditions (T: 25°C, 65±5% RH, 16:8 Light: Dark).

4.2.2 Seeding of tomato and tobacco plants

Tobacco and tomato plants were previously seeded in the nursery room and maintained in the insectary rooms of the insectary under controlled conditions (25±1 ◦C, 65±10% RH and 16: 8 h light/dark) as previously described in 2.1.2 section. No pesticides were used.

4.2.3 Insects rearing

4.2.3.1 Rearing of the predators

Predator colonies were cultivated at the insectary of Agricultural Research Institute (ARI), Nicosia, Cyprus. The two species were collected from open field tomato cultivations using a backpack aspirator (BioQuip, USA), at Agios Theodoros, Larnaca, Cyprus, during May 2015. The insects were transferred to plastic rearing cages (50 × 50 × 50 cm) with four holes (30 cm diameter) on each side, covered with white organza fabric (mesh 0.3 × 0.3 mm) to reduce humidity (Figure 27), in the insectary rooms of ARI under controlled conditions at 25±1 ◦C, 65±10% RH and 16 L:8D photoperiod. In each cage two tobacco plants with 2-3 real leaves were placed. Subsequently, tobacco plants were infested with the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae). The two predator species were maintained at different rooms of the insectary in order to not cross contaminated. Both species completed their lifecycle on tobacco plants infested with *B. tabaci*. Fifth instar nymphs of the two predators were used in all experiments.

Twenty-five adults of the two predators were collected with the aspirator (BioQuip, USA) and placed at different cages with two tobacco plants infested with *B. tabaci* to oviposit for 48 hours and then removed. Around 10-13 days after oviposition 1st instar larvae of the predators *N. tenuis* and *M. pygmaeus* were

hatched, respectively. Subsequently, 5th instar larvae of *N. tenuis* and *M. pygmaeus* collected and used at the bioassays 15 and 20 days after oviposition, respectively (Figure 28). The developmental time for the juveniles of *N. tenuis* was approximately 3 days shorter than for *M. pygmaeus*, at 25 ◦C (Mollá et al., 2014; Ebrahimi et al., 2018).

Figure 27: *M. pygmaeus* and *N. tenuis* rearing cages. Photos: G. Michaelides.

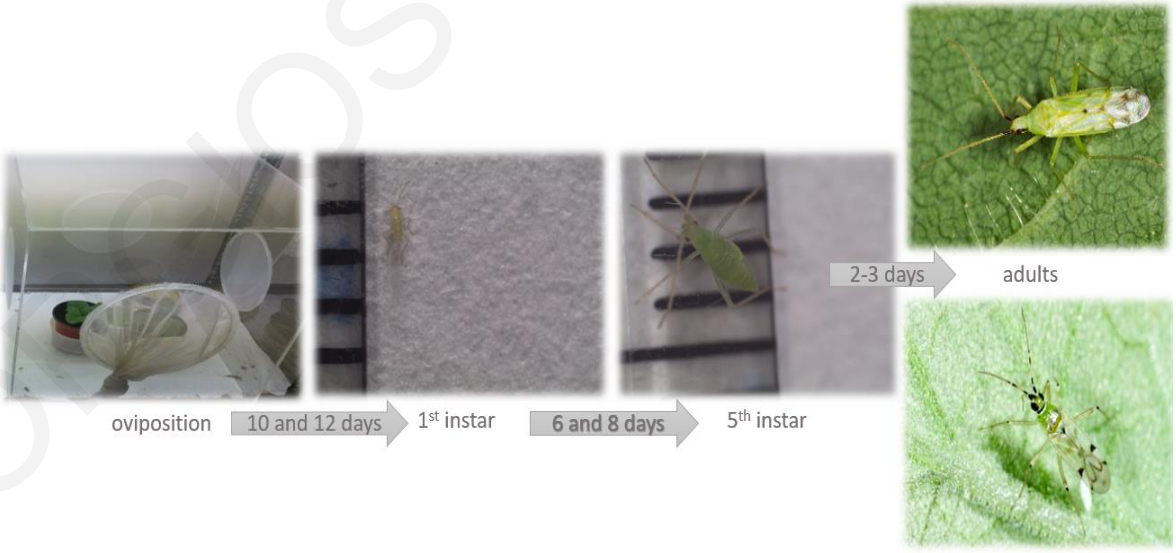

Figure 28: Days from oviposition to adulthood for *N. tenuis* and *M. pygmaeus* under controlled conditions. Photos: G. Michaelides.

4.2.3.2 Rearing of prey (*T. absoluta*)

The *T. absoluta* colony was established at the insectary in 2010 when the tomato pinworm first invaded cultivations in Parekklisia, Lemesos, Cyprus. This population of the insect is kept in a different room of the insectary (Figure 29). In every cage well developed tomato plants were placed in order the *T. absoluta* could complete its lifecycle. Numerous adults were collected with the aspirator and placed at a new cage with a single tomato plant to oviposit. Before placing the plant in the cage, it was checked of any pests or diseases infections.

Figure 29: *T. absoluta* rearing cages. Photos: G. Michaelides.

Newly laid eggs (< 24 h) were gently collected with a fine paintbrush under a stereoscope from the infested tomato plant (Figure 30). These were transferred on a filter paper to plastic Petri dishes (9 cm diameter) with a hole on the top (6 cm diameter) covered with white organza fabric (mesh 0.3 × 0.3 mm) (Figure 31). Petri dishes with the eggs were well sealed with parafilm and were then stored in an incubator (10±1 ◦C, 65±10% RH and 16: 8 h light/dark) to avoid hatching until use in the bioassays.

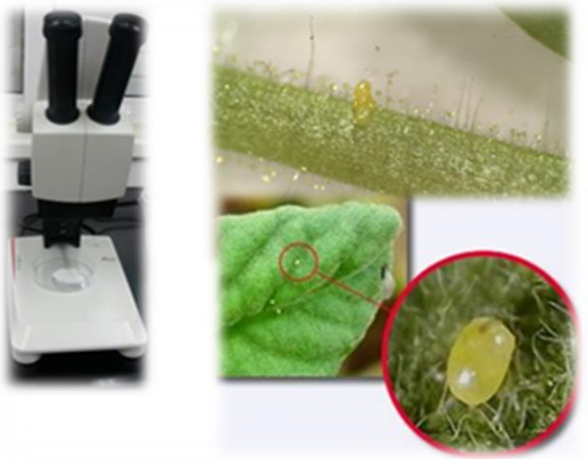

Figure 30: *T. absoluta* egg under a stereoscope. Photos: https://gd.eppo.int/taxon/GNORAB/photos

4.2.4 Bioassays

Experiments took place in plastic Petri dishes, under controlled conditions as previously described (Figure 31). Fifth instar nymphs of the two predators were transferred into plastic Petri dishes without any prey for 24 h to avoid effects of variable hunger levels. In each Petri dish a moistened cotton plug was fitted to preserve humidity.

We used predators of the same age and body size because size might affect the outcome (Jennings et al., 2001). To study predator responses to prey density, a single nymph of each predator was introduced into a plastic Petri dish with *T. absoluta* eggs (Figure 31). Petri dishes were immediately sealed with parafilm to avoid insects from escaping and placed at the insectary rooms under controlled conditions. The predation rate was recorded after 24 h, by counting the eggs sucked. Egg densities used were 10, 30, 50, 70 and 90 eggs per dish. These densities were used after preliminary tests that identified minimum and maximum egg consumption by the predators. Ten replicates for each prey density were conducted for each predator species.

Intraspecific interactions among *M. pygmaeus* nymphs when preyed on *T. absoluta* eggs were studied. Two 5th instar nymphs introduced into different Petri dishes without any prey for 14 hours as previously described. The two individuals were then placed into a Petri dish together with *T. absoluta* eggs at the densities as already defined. Egg consumption was recorded after 24 h. Intraspecific interactions among *N. tenuis* nymphs were also tested with the same way as for *M. pygmaeus*.

Interspecific interactions between *M. pygmaeus* and *N. tenuis* nymphs were finally tested by introducing an individual of each species simultaneously into a dish with *T. absoluta* eggs with the same eggs densities as previously mentioned. Egg consumption was recorded after 24 h.

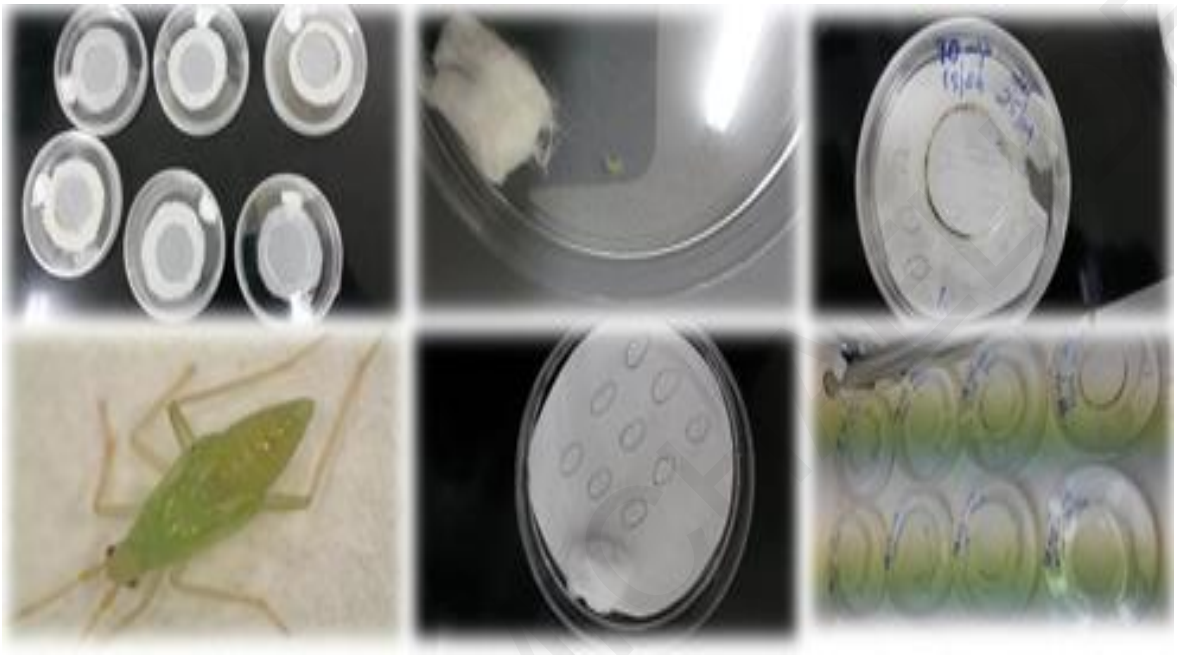

Figure 31: Up left and center: Petri dishes with predators, Down left: 5th instar individuals of the predator *M. pygmaeus*, Down center: *T. absoluta* eggs, Right up and down: *T. absoluta* eggs with predators' individuals. Photos: G. Michaelides.

4.2.5 Statistical analysis

4.2.5.1 Functional response

Estimation of predation rate for single fifth instar predator juveniles was carried out using a two-way analysis of variance (ANOVA), with the first factor being the predator species and the second prey density (five levels). Data related to prey density were first log-transformed.

The satiation level of each predator (*M. pygmaeus* or *N. tenuis*) and the relationship between the predation rate of each predator and the initial prey density were investigated by fitting functional response curves. The shape of the curve was determined by logistic regression of the proportion of *T. absoluta* eggs consumed as a function of the available eggs density. Logistic regression is appropriate because the dependent variable is dichotomous (egg consumed or not), and the

errors associated with such a variable are likely to be distributed binomially rather than normally. The polynomial function from Juliano (1993) was used:

$$\frac{N_e}{N0} = \frac{\exp(P0+P1N0+P2N0^2+P3N0^3)}{1+\exp(P0+P1N0+P2N0^2+P3N0^3)} \quad (1)$$

where *Ne* is the number of prey eggs consumed, *N*0 is the initial prey egg number, and *P*0, *P*1, *P*2 and *P*3 are the intercept, linear, quadratic and cubic coefficients, respectively, estimated through maximum likelihood. Estimates of the parameters *P*0 to *P*3 were obtained by applying logistic regression. A type I functional response is indicated when the linear term (*P*1) is 0. A type II response is indicated when *P*1 is negative, and a type III response is indicated when *P*1 is positive. Each term was considered different from zero if its 95% confidence interval did not include zero. Once the functional response type was determined, iterative non-linear least squares regression was used to fit the disc equation (Holling, 1959) after the transformation described by Livdahl and Stiven (1983). This transformation removes the statistical problems related to the transformation of Royama (1971) and Rogers (1972) and increases the explanatory power of the independent variable in the regression. This equation is:

$$\frac{1}{N\mathrm{a}} = \frac{1}{aTN0} + \frac{Th}{T} \quad (2)$$

where *Na* is the number of prey attacked, *N*0 is the initial prey density and *T* is the time (24 h) that the predatorwas free to forage. The parameter a is the attack rate and the parameter *T*h is the time required to handle a prey individual.

For type III functional response, a is no longer supposed to be constant, but increases as prey density increases, with a =(*d* +*bN*0)/(1+*cN*0), and *b*, *c* and d are fitted constants (Juliano, 1993; Hassell et al., 1977). The equation for type III functional response with prey reduction is:

$$\mathrm{Ne} = \mathrm{N0}\ (1 - \exp[(\mathrm{d} + \mathrm{b}\ \mathrm{N0})(\mathrm{T_h Ne} - \mathrm{T})/(1 + \mathrm{cN0})]) \quad (3)$$

Parameter estimates for equation 3 were made by iterative application on Newton's method (Juliano, 1993), carried out in R (R Development Core Team, 2016). The linearization of the random-predator equation (Carter et al., 1984) can generate biased estimates (Williams and Juliano, 1996), so Newton's method is more suitable. The parameters in equation 3 were estimated beginning with the full model

and then gradually moving on to the depletion model when the parameters c and/or d were not significantly different from 0. The parameters *b* and *T*h were always significantly different from 0.

4.2.5.2 Multiple predator effects

To investigate multiple predator interactions, two well-known models were used: the additive and the substitutive. The additive design correlates observed rate of predation by individuals of each species when foraging alone with the combined rate of predation when individuals of each species forage together. The substitutive model keeps the entire number of interacting predators stable while predator species richness is changed. The substitutive model only investigates the outcome of interactions among conspecifics in comparison with interactions among species. The additive design does not sufficiently explain the nature of interactions because it confuses alterations in whole predator density with alterations in the number of predator species involved (Sih et al., 1998; Griffen, 2006). Considering that the additive design explores whether non-additive effects occur because of interspecific interactions, and that the substitutive design explores whether non-additive effects due to interspecific interactions have the same intensity as non-additive effects due to intraspecific interactions (Jolliffe, 2000), the combination of these two designs should give us the most informative estimation of competition (Snaydon, 1991).

We further examined whether positive (facilitative prey consumption) or negative (interference, prey risk reduction) interactions between predators in the interspecific and intraspecific treatments occurred by applying the MRM (Soluk, 1993). With this model a misinterpretation by the additive design is solved, where a prey individual consumed by one predator cannot be eaten again from another predator. If two predators have independent effects, the observed proportion of prey eaten by the two predators should be:

$$P_{AB} = P_A + P_B - P_A P_B \quad (4)$$

Where *P*A and *P*B are the probabilities of an egg being consumed by the predator A or B in isolation, respectively, and *P*A*P*B is the probability of prey being eaten by one predator and is no longer available to the other. The observed predation when both predators were present was compared with the expected predation by:

$$C_{fs} = N0\ (P_A + P_B - P_A P_B) \quad (5)$$

where Cfs is the expected combined consumption for a specific initial prey density *N*0, and *P*A and *P*B are the probabilities of eggs being eaten over 24 h of exposure.

The response variable was the observed predation rate. To test for emergent MPEs, observed predation rates were compared with expected predation rates acquired from the model. A three-way ANOVA was used to carry out the analysis, with the following factors: (1) predator treatment with three levels (2 × *M. pygmaeus*; 2 × *N. tenuis*; *M. pygmaeus* × *N. tenuis*); (2) type of data: observed versus expected egg consumption rates; and (3) egg density, with five levels. Data used in the analysis were log transformed. The intensity of intra- versus interspecific interactions was further examined with the substitutive model, which predicts prey consumption of the interspecific treatment as:

$$E(1,2) = \sqrt{M(1,1) \cdot N(1,1)} \quad (6)$$

Where *M* (1,1) and *N* (1,1) are the prey consumption rates when the predators *M. pygmaeus* or *N. tenuis* forage on conspecific pairs, respectively (Griffen, 2006). The results of this model and the observed predation rates in the interspecific treatment were compared using a two-way ANOVA with the first factor having two levels: observed or predicted egg consumption by the model, and egg density being the second factor. Comparisons between means were performed using contrasts with the Tukey–Kramer HSD test.

4.2.6 Results

4.2.6.1 Functional response

Assays performed with one individual of each species in the dish with different densities of *T. absoluta* eggs showed an increase in the number of eggs eaten, at a decreasing rate with increasing egg density, reaching a plateau (Table 14). Predation rates were strongly affected by prey density ($F_{4,90} = 11.321$, $P < 0.001$). The effect of the predator species and the interaction of prey density and predator species was not significant ($F_{1,90} = 0.276$, $P = 0.600$ and $F_{4,90} = 0.268$, $P = 0.898$,

respectively). Predation rate of *N. tenuis* was significantly different from that of *M. pygmaeus* at all egg density treatments.

Logistic regression showed that the two predatory species exhibited a type III functional response. This is because the linear parameters of equation 1were positive, whereas the quadratic parameters were negative for both predators, and significantly different from zero (Table 15). The proportion of eggs consumed by *N. tenuis* and *M. pygmaeus* increased at the beginning and then decreased as egg density raised (see Figure 32, where a type III functional response is shown). *N. tenuis* and *M. pygmaeus* maximum predation rate reached a top at ~50 and 35 eggs, respectively (Table 14).

The handling times (*T*h) for *M. pygmaeus* and *N. tenuis* were ~0.5 and 0.3 h, respectively (Table 16), as calculated from equation 3. The attack rate (α) is a linear function of *N*0 for a type III functional response. The slope (*b*) for *M. pygmaeus* was 0.0013 (asymptotic SE=0.0005) and 0.0038 for *N. tenuis* (asymptotic SE=0.0013) (Table 16). *N. tenuis* showed shorter handling time than *M. pygmaeus* and this is line with the higher number of eggs consumed by *N. tenuis* and with *M. pygmaeus* shorter saturation level.

Table 14: Number (mean ± SE) of consumed prey by an individual of *N. tenuis* or *M. pygmaeus* at various densities of *T. absoluta* eggs.

Prey density	Predators			
	N. tenuis		*M. pygmaeus*	
10	5.80 ± 0.4774	Aa	3.80 ± 0.4557	Ab
30	18.10 ± 0.8314	Ba	11.30 ± 0.6132	Bb
50	33.90 ± 0.9961	Ca	27 ± 0.6409	Cb
70	50.70 ± 0.9509	Da	35.60 ± 0.7426	Db
90	50.60 ± 0.9020	Da	35.70 ± 0.5489	Db

Values followed by the same lower case letters were not significantly different among predator species within each prey density. Values followed by the same upper case letters were not significantly different among prey densities for each predator species separately.

Table 15: Maximum likelihood estimates from logistic regression of the proportion of *T. absoluta* eggs eaten by *M. pygmaeus and N. tenuis*, separately as a function of prey density.

Predator	Parameter	Estimate	SE	P
N. tenuis	Linear (P1)	0.04891	0.0089	<0.001
	Quadratic (P2)	-0.0004484	0.000075	<0.001
M. pygmaeus	Linear (P1)	0.04919	0.00909	<0.001
	Quadratic (P2)	-0.0004345	0.000076	<0.001

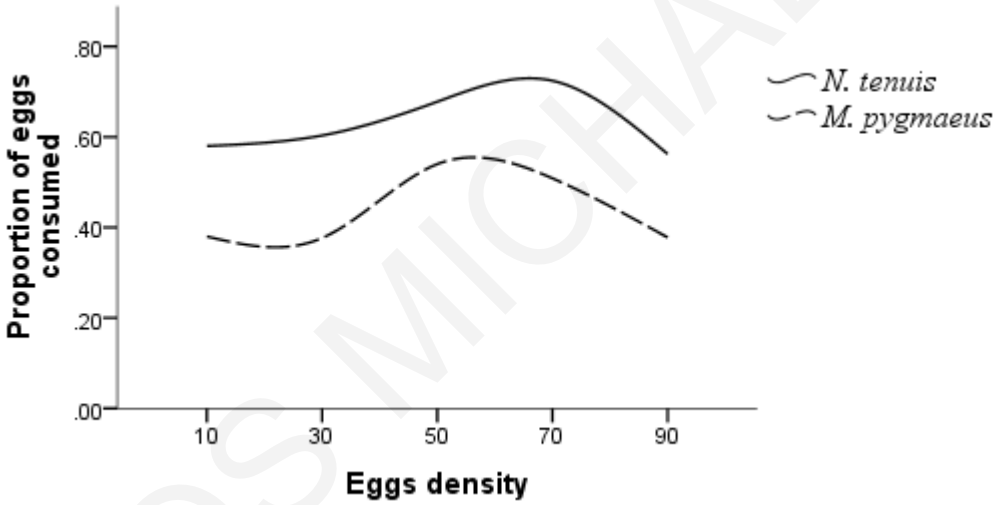

Figure 32: Proportion of *T. absoluta* eggs consumed by the predators *N. tenuis* and *M. pygmaeus* at various egg densities.

Table 16: Functional response of predators attacking *T. absoluta* eggs.

Parameter	Estimate ± SE	95% confidence intervals
M. pygmaeus		
b	0.0013 ± 0.0005	0.0003 – 0.0023
Th	0.5063 ± 0.1479	0.2164 – 0.7962
N. tenuis		
b	0.0038 ± 0.0013	0.0013 - 0.0063
Th	0.2631 ± 0.0603	0.1449 - 0.3813

4.2.6.2 Multiple predators' effects

The total numbers of eggs consumed when a conspecific (*M. pygmaeus–M. pygmaeus*, *N. tenuis–N. tenuis*) or heterospecific (*M. pygmaeus–N. tenuis*) pair was in the disc are presented in Figure 33. ANOVA indicated significant interactions between predator species and prey density. Prey density and observed/predicted mortality had also a highly significant effect on predation rates (Table 17). Observed values at higher egg densities (70, 90) were significantly lower than those predicted by the MRM at all conspecific treatments (*M. pygmaeus–M. pygmaeus*, *N. tenuis–N. tenuis*). Significantly lower observed values than predicted by the MRM were also found for prey densities of 10, 30 and 70 eggs in heterospecific treatments (*M. pygmaeus–N. tenuis*) and for conspecific treatments between individuals of *M. pygmaeus* at the intermediate prey density (50 eggs).

At lower prey densities (10, 30 eggs) observed predation rates under intraspecific interactions were similar to those predicted by the MRM. Similarly, at 50 eggs density between the individuals of *N. tenuis*, and at the 50 and 90 eggs densities between predators at heterospecific treatments, observed predation rates by the MRM did not differ from observed prey consumption (Figure 33).

The results from the substitutive model showed that predation rates were strongly affected by prey density. The interaction between observed and predicted prey consumption with egg density and the dissimilarity among predicted and observed predation rates were not significant (Table 18).

Using the substitutive model, we found risk enhancement, indicating synergistic consumption of prey at the highest prey density level when a heterospecific pair was present in the dish. However, using the MRM neither synergistic nor antagonistic interactions were observed between the two predator species at the highest prey density. Risk reduction was found at 30 and 70 eggs densities, results similar to those predicted by the MRM. At densities of 10 and 50 eggs, the results from the substitutive model did not statistically differ from observed consumption rates (Figure 34).

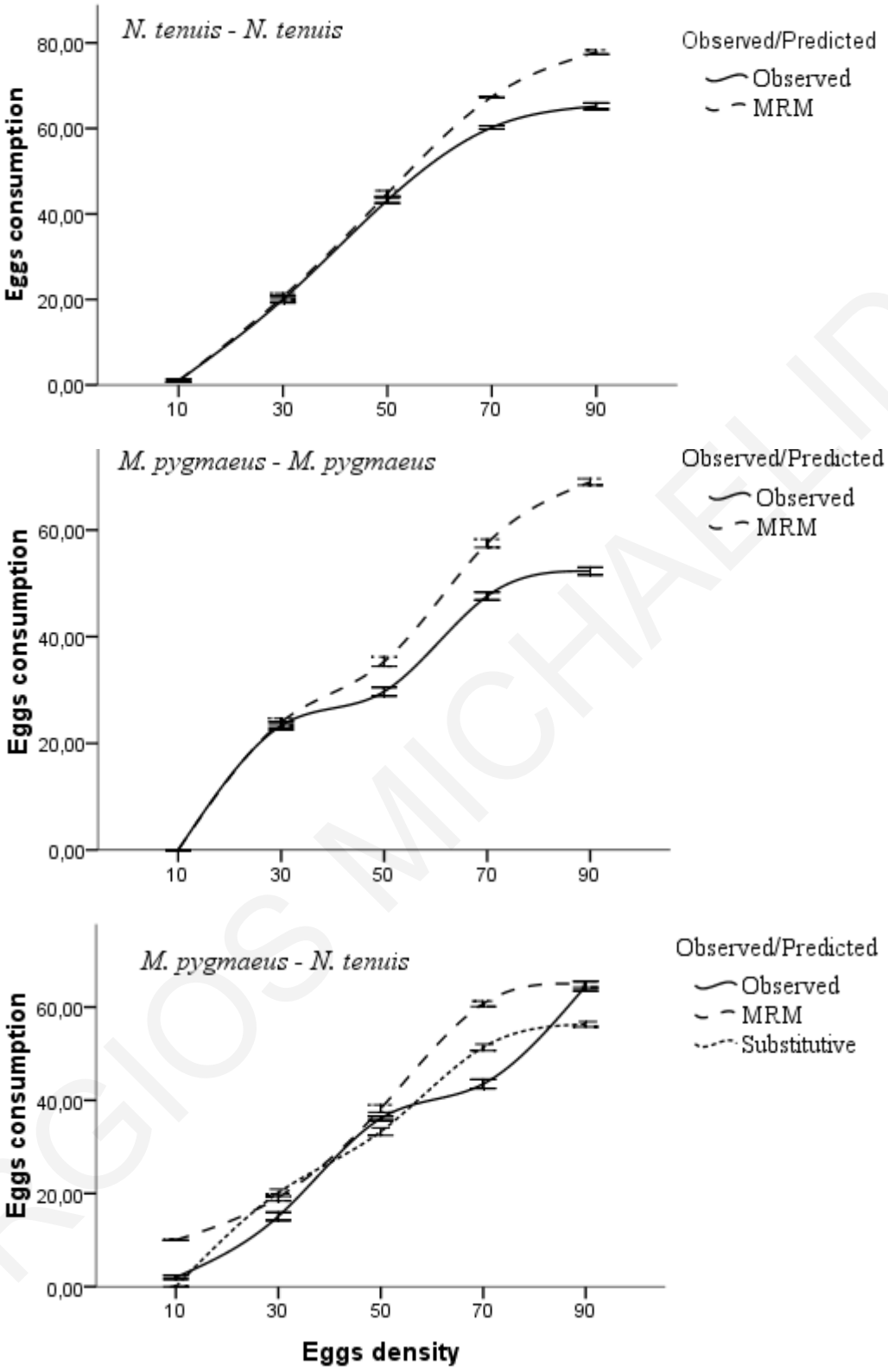

Figure 33: Observed and predicted by the MRM *T. absoluta* egg consumption by conspecific and heterospecific pairs of the predators *M. pygmaeus* and *N. tenuis*.

Table 17: Analysis of observed and predicted prey consumption (OP) by the MRM when various combinations of predators (Pr) were used at various levels of *T. absoluta* egg density (PD).

Source	df	SS	F	P
Pr	2	0.775	1.732	0.179
PD	4	92.521	106.146	<0.0001
OP	1	1.225	5.624	0.018
Pr x PD	8	6.078	3.487	0.001
Pr x OP	2	0.965	2.215	0.111
PD x OP	4	0.666	0.764	0.550
Pr x PD x OP	8	1.989	1.141	0.336
Error	270	58.835		

Table 18: Analysis of observed (OP) and predicted predation rates by the substitutive model when the predators *M. pygmaeus* and *N. tenuis* were paired at various levels of *T. absoluta* egg density (PD).

Source	df	SS	F	P
OP	1	1.767	1.480	0.227
PD	4	205.88	43.107	< 0.0001
OP x PD	4	0.983	0.206	0.935
Error	90			

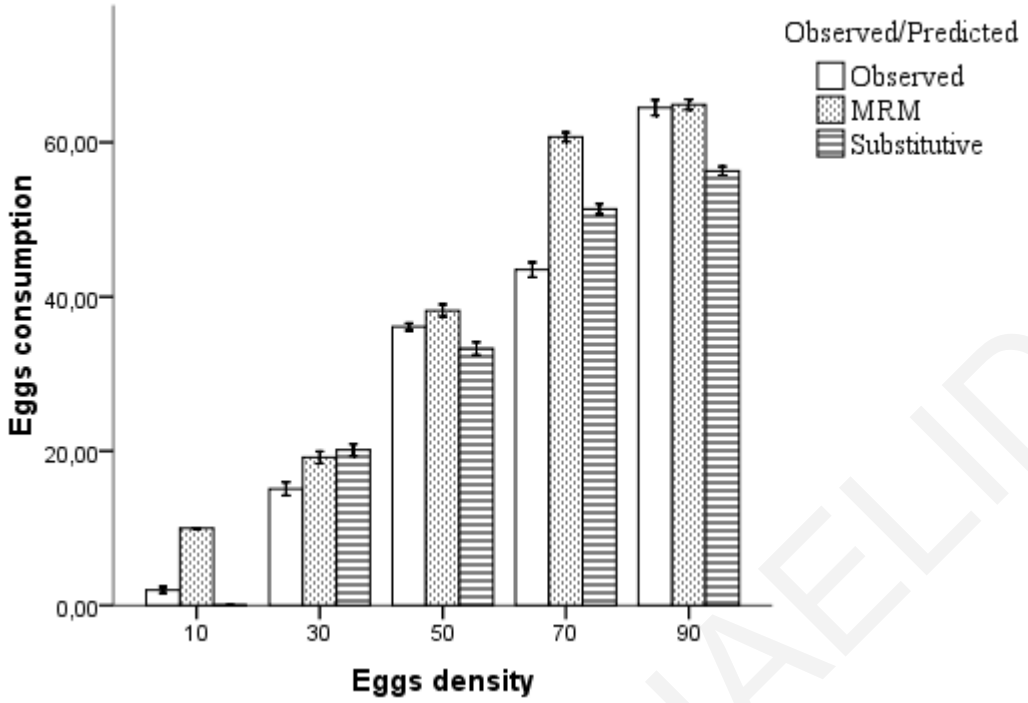

Figure 34: Observed and predicted *T. absoluta* egg predation rates when a heterospecific pair of predators was present, based on the MRM and the substitutive models at various densities of *T. absoluta* eggs.

4.2.7 Discussion

A predator species may show different types of functional response when preying on different types of prey (Enkegaard et al., 2001; Foglar et al., 1990). Queiroz *et al.* (2015) found that the predators *Amphiareus constrictus* (Stal) and *Blaptostethus pallescens* (Poppius) (Hemiptera: Anthocoridae) exhibited a type III functional response when preying on various densities of *T. absoluta* eggs. Enkegaard *et al.* (2001) found a type III functional response for the predator *M. pygmaeus* when preying on various densities of *T. vaporariorum* first instar nymphs. To the best of our knowledge, this is the first time that the predator *N. tenuis* has been found to exhibit a type III functional response. Type II and III functional responses are similar in that predators become saturated at high prey densities. These two types differ in that consumption of prey at low prey densities is lower in type III than in type II (Sharifian et al., 2015). This difference may be because the predators need some time to learn the environment and search for prey (Holling, 1959). Another factor may be that the prey was not their preferred 'meal' (Hassell et al., 1977). Predators with a longer handling time reach their saturation level faster than those with a shorter handling time (Kisdi and Liu, 2006). *N. tenuis* found to be

a more competitive predator than *M. pygmaeus* for *T. absoluta* eggs because it has a shorter handling time and reaches saturation levels at higher prey densities. However, both predators can be significant natural enemies of *T. absoluta*.

The predators *M. pygmaeus* and *N. tenuis* demonstrated similar predation characteristics in intraspecific interactions according to the MRM. However, *N. tenuis* is a more effective predator of *T. absoluta* than *M. pygmaeus* as it can consume more eggs at a higher density. In other words, intraspecific interactions among individuals of *N. tenuis* are weaker than among individuals of *M. pygmaeus*. The stronger interactions between *M. pygmaeus* individuals could be explained by the fact that cannibalism has been observed in this species (Hamdi et al., 2013). According to the MRM, prey density is a coefficient influencing outcomes that show prey risk reduction at high egg density at intraspecific interactions, as well as at low egg densities at interspecific interactions. Interspecific interactions occur at low prey densities, in contrast to intraspecific ones. Nevertheless, analysis with the substitutive model showed that the two predator species acted separately as would be expected for similar predators at low prey densities. Griffen (2006) also found that results differ by increasing prey density using the two models.

Analyses with the MRM also showed that interaction between predator species and prey density is a factor affecting the outcome. Indeed, intraspecific interactions followed a similar trend, whereas the combination of the two species followed a different pattern. At the level of 90 prey items, only treatments including interspecific interactions indicated the same observed consumption rate as predicted by the MRM. This results in neither negative nor positive prey risk.

The general trend at inter- and intraspecific treatments was an increase in the number of eggs consumed while prey density increased. Using the MRM, observed and expected predation rates differed significantly at high prey densities, showing that the predator effects were not independent and, in our case, translated to risk reduction. The strength of risk reduction did not differ between predator combinations and among the levels of prey density because no significant interactions were observed between the predator combination (Pr) and observed versus predicted predation rates (OP), as well as between prey density (PD) and OP respectively (Vance-Chalcraft and Soluk, 2005b).

Functionally similar predators may present stronger competition when prey is limited (Casula et al., 2006). This finding agrees only with the interspecific

treatments in our study, where competition at low prey densities between *N. tenuis* and *M. pygmaeus* individuals occurs. At prey densities where observed versus predicted predation rates were significantly different, we found risk reduction. This is possibly generated by intraguild predation between individuals of the two predator species used. Intraguild predation between the predators *N. tenuis* and *M. pygmaeus* has also been found in other studies (Moreno-Ripoll et al., 2012a; 2012b; Perdikis et al., 2014). Although intraguild predation occurs at both con- and heterospecific treatments, these two predator species could control population size of prey because the per capita predation rate is increasing with increasing prey density (see also Vance-Chalcraft and Soluk, (2005b) and Elliott and Sawrey (2003)).

When competition is decreased, prey depletion is greater and multiple species can coexist (Crowder et al., 2010). We found a result similar to that of Crowder *et al.*, (2010) because at 90 prey items competition between predators was reduced and the number of *T. absoluta* eggs consumed was greater in the interspecific treatments than that in intraspecific ones. Therefore, we can assume that the combination of the two predator species will lead to better pest suppression at high *T. absoluta* population densities, whereas at low prey densities better pest regulation can be achieved by introducing individuals of *N. tenuis* only. However, this contrasts to the finding that 'multiple predator species may only provide greater biological control than single species in systems where prey is scarce' (Werling et al., 2012).

Griffen (2006) compared seven studies that searched for MPEs and six of them found risk reduction when using the additive design, while risk enhancement when using the substitutive design. The latter study found risk reduction with both designs. However, Lampropoulos *et al.* (2013) found risk enhancement with both the MRM and the substitutive design. Biological control strategies could be more effective by knowing when multiple predator species provide better regulation of a pest than single species alone (Straub et al., 2008).

Because our experiment took place under highly simplified experimental conditions, and given the increased complexity of the natural environment, it is possible that interactions within and between individuals of the two predators may be different under field conditions. In any case, based on previous studies (Messelink and Janssen, 2014; Moreno-Ripoll et al., 2012a; Lampropoulos et al.,

2013; Schmitz, 2007), we assume that the proposed pattern of interactions will not be significantly affected by such unpredicted factors. More likely the competition between individuals of the predators in real field conditions would be milder, as positive MPEs may occur at more complex habitats (Tylianakis et al., 2008; 2010).

Moreover, the feeding behavior of *N. tenuis* on tomato plants can cause economic damage if prey is limited or absent (Castañé et al., 2011; Arnó et al., 2010). However, a recent study (Biondi et al., 2016) found that damage from this predator to tomatoes was minimized if an additional host plant [*Sesamum indicum* (L.) (Pedaliaceae)] of the predator is present in cultivations. This technique did not affect the preying efficiency of the predator on *T. absoluta eggs*, but damage to tomato plants was avoided and the predator could continue its lifecycle without prey and without making any damage to tomato plants (Biondi et al., 2016). However, when this predator foraged on whitefly nymphs, when prey was limited it caused serious damage to tomato crops. However, other plant sources were not provided (Sanchez, 2009).

CHAPTER 5

Conclusions

The general conclusions obtained from this thesis are listed below.

- Regarding Chapter 2, control failure was found for the insecticides chlorantraniliprole and indoxacarb with highest resistance ratios found to be 188 and 23 times more than that of the reference strain, which is maintained in controlled conditions without any applications since 2010.
- In contrast, spinosad and emamectin benzoate found to be highly effective to control this pest. The LC_{50} values for these two insecticides in all sampling areas tested were above the susceptible reference strain. Nevertheless, the LC_{80} values for spinosad and emamectin benzoate were well below the recommended label rates.
- In Chapter 3 molecular assays were conducted to identify the mechanisms conferring resistance to indoxacarb and chlorantraniliprole. The mutations F1845Y and V1848I from indoxacarb resistance populations and the mutations G4903V and I4746M from chlorantraniliprole resistance individuals were distinguished.
- Mutant allele frequencies for the F1845Y mutation ranged from 11.67% to 53.33%. Surprisingly, the lowest frequency was identified in the reference strain (collected in 2010 and no insecticides applied). For the V1848I mutant alleles, frequencies fluctuated between 0 and 50%. This mutation was not observed in the reference strain.
- No correlation between the LC_{50} values for indoxacarb and mutant allele frequencies for F1845Y mutation were found. In contrast, mutant allele frequencies for V1848I mutations were statistically correlated with LC_{50} values. However, different generations of the pest were analyzed in Chapter 2 and Chapter 3.
- Regarding G4903V mutant allele frequencies, these were found to be low and ranged from 0% to 25% in field-collected populations. In contrast, mutant allele frequencies for I4746M mutation were higher and vary from 47.50% to 90% in field-collected populations. The two mutations were missing from the reference strain.

- LC_{50} values found for chlorantraniliprole were not statistically significant with mutation alleles frequencies. However, we assumed that resistance phenotype is mainly attributed to I4746M mutation because mutant allele frequencies were much higher than the frequencies of G4903V mutation. This conclusion is supported also by the fact that G4903V mutation was missing from a resistant to chlorantraniliprole field-collected population.
- Regarding Chapter 4, we identified a Type III functional response in both predator species. Nonetheless, this is the first time that the predator *N. tenuis* has been found to exhibit a Type III functional response.
- Moreover, *N. tenuis* found to be a more effective predator than *M. pygmaeus* for *T. absoluta* eggs because it has a shorter handling time and reaches saturation levels at higher pray densities. In addition, predator rates were strongly related to prey density.
- Concerning multiple predator effects, interspecific interactions occur at low prey densities, in contrast to intraspecific ones. Furthermore, the combination of the two predators will lead to better pest suppression at high egg densities.
- To the contrary, at low egg densities better pest regulation can be achieved by introducing individuals of *N. tenuis* only.

From an applied ecology point of view, the findings of this work raises serious concerns about the management of this pest. Farmers should be informed immediately to avoid inadequate practices for the control of *T. absoluta*. Knowledge of which insecticides are not effective anymore and which predators could practically be introduced into Insecticide Resistance Management (IRM) programs can be a very useful tool in the fight against the tomato pinworm.

For the implementation of the results obtained from this PhD thesis in tomato cultivations all around Cyprus, we suggest the following IRM modified schedule (Figure 35):

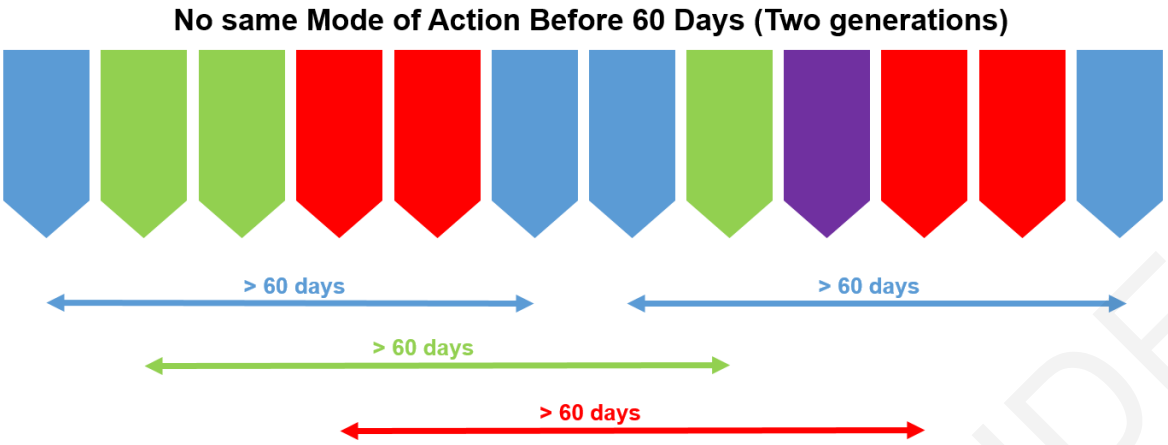

Figure 35: Rotation scheme of insecticide applications based on different modes of action. Each color represents insecticides from different modes of action. A single generation of *T. absoluta* lasts 30 days. Blue colour: *N. tenuis*, Green colour: Emamectin benzoate, Red colour: Spinosad, Purple colour: *N. tenuis* + *M. pygmaeus.*

- Application of insecticides chlorantraniliprole and indoxacarb must be avoided. These insecticides are not effective against *T. absoluta* anymore.
- Introduce (blue colour) *N. tenuis* individuals early in tomato cultivations because they can handle small egg densities of *T. absoluta*. *N. tenuis* can be further used (green and red arrows) if it suppresses effectively egg densities.
- Spray with the insecticides emamectin benzoate (green arrows) and spinosad (red arrows), but do no overdose or overuse.
- When the populations of the pest decrease, apply again individuals of *N. tenuis* only.
- Then, apply emamectin benzoate.
- If in any case the density of the eggs increases, introduce *M. pygmaeus* and *N. tenuis* individuals together.
- If egg densities do not decrease spay with spinosad.
- At the end, use *N. tenuis* individuals to avoid residuals on tomato fruits.

Knowing that *T. absoluta* is a pest species that rapidly develops resistance to insecticides, the need of an appropriate insecticide resistance management program with rotation of natural enemies is necessary. Furthermore, IRM programs will preserve the efficacy of insecticides (Yao et al., 2017). Elimination of resistant homozygous and heterozygous individuals can be achieved by rotation of the insecticides used to control *T. absoluta*. Such insecticides could be spinosyns avermectins, pyrazoles, oxadiazines and diamides (Campos et al., 2015b; Silva et

al., 2016b). However, according to our findings, oxadiazines and diamides are ineffective and should be avoided in Cyprus, at least until the reduction of resistant alleles.

An alternative method to control *T. absoluta* populations is the introduction of predators in management programs. Our findings in Chapter 4 can be part of an IRM program to control *T. absoluta*. According to these, the release of the predators *Nesidiocoris tenuis* and *Macrolophus pygmaeus* could provide satisfying pest suppression, depending on *T. absoluta* egg densities. By estimating *T. absoluta* egg densities on a cultivation, a farmer can release in a greenhouse either only *N. tenuis* or both predator species together. In cases of low egg densities, the release of *N. tenuis* is recommended. When egg densities are high, the release of both predators would lead to better control of the pest. These predators can be introduced in a tomato cultivation also before its infection with *T. absoluta* (Calvo et al., 2012). Nevertheless, when prey is limited, *N. tenuis* can cause damage to tomato fruits (Arnó et al., 2010; Castañé et al., 2011).

Possible extensions of this work could be a long-term monitoring of susceptibility to the insecticides over several crop seasons. Moreover, for the early detection of mutant alleles in spinosad and emamectin benzoate molecular techniques could be designed. Furthermore, monitoring of resistance alleles to indoxacarb and chlorantraniliprole should continue in order to identify their trends.

In addition, *in situ* applications of the predators *N. tenuis* and *M. pygmaeus* in tomato cultivations and the study of their conspecific and heterospecific interactions in field conditions would be a real challenge. Finally, further research should take into account that the presence of different beneficial insects and different pests at the same time in cultivations can affect the effectiveness of predators.

References

Abbot, W.S. 1925. A method of computing the effectiveness of an insecticide. *J Econ Entomol*, 18, 265–267.

Abd El-Ghany, N.M., Abdel-Razek, A.S., Ebadah, I.M.A. & Mahmoud, Y.A. 2016. Evaluation of some microbial agents, natural and chemical compounds for controlling tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *J Plant Prot Res*, 56, 372–379.

AHDB. 2015. PE 028—Tuta absoluta: investigating resistance to key insecticides and seeking alternative IPM compatible products. Jacobson R, Bass C (eds) Agriculture and Horticulture Development Board, https://horticulture.ahdb.org.uk/project/tuta-absoluta-investigating-resistance-key-insecticides-and-seeking-alternative-ipm. Accessed 17 Feb 2017.

Ahmad, M. & Hollingworth, R.M. 2004. Synergism of insecticides provides evidence of metabolic mechanisms of resistance in the obliquebanded leafroller *Choristoneura rosaceana* (Lepidoptera: Tortricidae). *Pest Management Science*, 60, 465-473.

Albajes, R. & Alomar, Ò. 1999. Current and potential use of polyphagous predators, in *Integrated Pest Disease Management in Greenhouse Crops*, ed. by R Albajes ML, Gullino JC, van Lenteren and Elad Y. Kluwer, Dordrecht, pp. 265–275.

Alili, D., Doumandji, A., Benrima, A., Doumandji, S., Doumandji, B. 2014. Pheromone trapping and control by insect-proof nets of *Tuta absoluta* (Meyrick, 1917) in greenhouses and in the field at Fouka Marine. *Bull Soc Zool Fr*, 139, 71–81.

ARI. Agricultural Research Institute 'Εντοπισμός ενός νέου εντομολογικού εχθρού στην Κύπρο', *Ηλεκτρονικό Ενημερωτικό Δελτίο* (online), Ιανουάριος 2010, 2: 3 <http://www.moa.gov.cy/moa/ari/ari.nsf/all/2DFDDB1B9C5BCD46C2257BA400396988/$file/january2010.pdf>.

Arnó, J., Castañé, C., Riudavets, J. & Gabarra, R. 2010. Risk of damage to tomato crops by the generalist zoophytophagous predator *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae). *Bull Entomol Res*, 100, 105–115.

Arnó, J., Gabarra, R., Estopà, M., Gorman, K., Peterschmitt, M., Bonato, O., *et al.* 2009. *Implementation of IPM Programs on European Greenhouse Tomato Production Areas: Tools and Constraints*. Edicions I publicacions de la UdL, Lleida, Spain.

Balzan, M.N. & Moonen, A.C. 2012. Management strategies for the control of *Tuta absoluta* (Lepidoptera: Gelechiidae) damage in open-field cultivations of processing tomato in Tuscany (Italy). *EPPO Bull*, 42, 217-225.

Barrientos, Z.R., Apablaza, H.J., Norero, S.A. & Estay, P.P. 1998. Temperatura base y constante térmica de desarrollo de la polilla del tomate, *Tuta absoluta* (Lepidoptera: Gelechiidae). *Ciencia e Investigación Agraria* 25, 133–137.

Berger. M., Puinean, A.M., Randall, E., Zimmer, C.T., Silva, W.M., Bielza, P., Field ,L.M., Hughes, D., Mellor, I., Hassani-Pak, K., Siqueira, H.A.A., Williamson, M.S. & Bass, C. 2016. Insecticide resistance mediated by an exon skipping event. *Mol Ecol*, 25, 5692–5704.

Bielza, P., García-Vidal, L. & Martínez-Aguirre, M.R. 2016. *Tuta absoluta*—insecticide resistance management of this invasive species. In: XXV international congress of entomology, 25–30 September, Orlando, FL, USA.

Biondi, A., Chailleux, A., Lambion, J., Han, P., Zappalà, L. & Desneux, N. 2013. Indigenous natural enemies attacking *Tuta absoluta* (Lepidoptera: Gelechiidae) in Southern France. *Egypt J Biol Pest Control*,23, 117–121.

Biondi, A., Desneux, N., Amiens-Desneux, E., Siscaro, G. & Zappalà, L. 2013. Biology and developmental strategies of the palaearctic parasitoid *Bracon nigricans* (Hymenoptera: Braconidae) on the neotropical moth *Tuta absoluta* (Lepidoptera: Gelechiidae). *J Econ Entomol*, 106, 1638–1647.

Biondi, A., Guedes, R.N.C., Wan, F.H. & Desneux, N. 2018. Ecology, worldwide spread, and management of the invasive South American tomato pinworm, *Tuta absoluta*: past, present, and future. *Annu Rev Entomol*, 63, 239–258.

Biondi, A., Zappalà, L., Di Mauro, A., Tropea Garzia, G., Russo, A., Desneux, N., *et al.* 2016. Can alternative host plant and prey affect phytophagy and biological

control by the zoophytophagous mirid *Nesidiocoris tenuis*? *BioControl*, 61, 79–90.

Boggs, K.W., Good, W.R., Harris, P. & Nowierski, R.M. 1991. *Metzneria paucipunctella* Zeller (Lepidoptera, Gelechiidae), a moth introduced against spotted knapweed: its feeding strategy and impact on two introduced *Urophora* spp. (Diptera; Tephrididae). *The Canadian Entomologist*, 123, 1001–1007.

Bruno, J.F. & Cardinale, B.J. 2008. Cascading effects of predator richness. *Front Ecol Environ*, 6, 539–546.

Calvo, F.J., Lorente, M.J., Stansly, P.A. & Belda, J.E. 2012. Preplant release of *Nesidiocoris tenuis* and supplementary tactics for control of *Tuta absoluta* and *Bemisia tabaci* in greenhouse tomato. *Entomol Exp Appl*, 143, 111–119.

Campos, M.R., Biondi, A., Adiga, A. & Guedes, R.N.C. 2017. From the Western Palaearctic region to beyond: *Tuta absoluta* 10 years after invading Europe. *J Pest Sci*, 90, 787–796.

Campos, M.R., Silva, T.B., Silva, W.M., Silva, J.E. & Siqueira, H.A.A. 2015a. Susceptibility of *Tuta absoluta* (Lepidoptera: Gelechiidae) Brazilian populations to ryanodine receptor modulators. *Pest Manag Sci*, 71, 537–544.

Campos, M., Silva, T.M., Silva, W., Silva, J. & Siqueira, H.A. 2015b. Spinosyn resistance in the tomato borer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *J Pest Sci*, 88. 405–412.

Campos, R.G. 1976. Control químico del ‘‘minador de hojas y tallos de la papa’’ (Scrobipalpula absoluta Meyrick) en el valle del Cañete. *Rev Per Entomol*, 19, 102–106.

Caponero, A. 2009. Solanacee, rischio in serre. Resta alta l’attenzione alla tignola del pomodoro nelle colture protette. *Colture Protette*,10, 96–97.

Cardinale, B.J., Harvey, C.T., Gross, K. & Ives, A.R. 2003. Biodiversity and biocontrol: emergent impacts of amulti-enemy assemblage on pest suppression and crop yield in an agroecosystem. *Ecol Lett*, 6, 857–865.

Carter, M.C., Sutherland, D. & Dixon, A.F.G. 1984. Plant structure and the searching efficiency of coccinellid larvae. *Oecologia*, 157, 394–397.

Castañé, C., Arnó, J., Gabarra, R. & Alomar, O. 2011. Plant damage to vegetable crops by zoophytophagous mirid predators. *Biol Control*, 59, 22–29.

Casula, P., Wilby, A. & Thomas, M.B. 2006. Understanding biodiversity effects on prey in multi-enemy systems. *Ecol Lett*, 9, 995–1004.

Catterall, W.A., 2017. Forty Years of Sodium Channels: Structure, Function, Pharmacology, and Epilepsy. *Neurochem. Res.*, 42, 2495–2504.

Chacón, J.M. & Heimpel, G.E. 2010. Density-dependent intraguild predation of an aphid parasitoid. *Oecologia*, 164, 213–220.

Chailleux, A., Bearez, P., Pizzol, J., Desneux, E., Ramirez, R. & Desneux., N. 2013a. Potential for combined use of parasitoids and generalist predators for biological control of the key invasive tomato pest *Tuta absoluta*. *J Pest Sci*, 86, 533–541.

Chailleux, A., Biondi, A., Han, P., Tabone, E. & Desneux, N. 2013b. Suitability of the pest—plant system *Tuta absoluta* (Lepidoptera: Gelechiidae)—tomato for Trichogramma (Hymenoptera: Trichogrammatidae) parasitoids and insights for biological control. *J Econ Entomol*, 106, 2310–2321.

Cherif, A., Harbaoui, K., Zappalà, L, & Grissa-Lebdi, K. 2018. Efficacy of mass trapping and insecticides to control *T. absoluta* in Tunisia. *J Plant Dis Prot*, 125, 51–61.

Chermiti, B., Abbes, K., Aoun, M., Ben Othmane, S., Ouhibi, M., Gamoon, W. & Kacem, S. 2009. First estimate of damage of *Tuta absoluta* (Povolny) (Lepidoptera: Gelechiidae) and evalutation of the efficiency of sec pheromone traps in greenhouses of tomato crops in the Bekalta region, Tunisia. *African Journal of Plant Science and Biotechnology*, 3, 49-52.

Cifuentes, D., Chynoweth, R. & Bielza P. 2011. Genetic study of Mediterranean and South American populations of tomato leafminer *Tuta absoluta* (Povolny, 1994) (Lepidoptera: Gelechiidae) using ribosomal and mitochondrial markers. *Pest Manag Sci*, 67, 1155-1162.

Clarke, J.F. 1962. New species of microlepidoptera from Japan. *Entomol News*, 73, 102.

Coelho, M.C.F. & França, F.H. 1987. Biologia e quemotaxia da larva e descriçãço da pupa e adulto da traça-do-tomateiro. *Pesqui Agropecu Bras*, 22, 129–135.

Cordova, D., Benner, E.A., Sacher, M.D., Rauh, J.J., Sopa, J.S., Lahm, G.P., Selby, T.P., Stevenson, T.M., Flexner, L., Gutteridge, S., Rhoades, D.F., Wu, L., Smith, R.M. & Tao, Y. 2006. Anthranilic diamides: a new class of insecticides with a novel mode of action, ryanodine receptor activation. *Pestic Biochem Physiol*, 84, 196–214.

Crowder, D.W., Northfield, T.D., Strand, M.R. & Snyder, W.E. 2010. Organic agriculture promotes evenness and natural pest control. *Nature*, 466, 109–112.

De Backer, L., Megido, R.C., Fauconnier., M.L., Brostaux, Y., Francis, F. & Verheggen, F. 2015. *Tuta absoluta*-induced plant volatiles: attractiveness towards the generalist predator *Macrolophus pygmaeus*. *Arthropod-Plant Interact*, 9, 465–476.

Desneux, N., Luna, M.G., Guillemaud, T. & Urbaneja, A. 2011. The invasive South American tomato pinworm, *Tuta absoluta*, continues to spread in Afro-Eurasia and beyond: the new threat to tomato world production. *J Pest Sci*, 83, 403–408.

Desneux, N., Wajnberg, E., Wyckhuys, K.A.G., Burgio, G., Arpaia, S., Narváez-Vasquez, C.A., *et al.* 2010. Biological invasion of European tomato crops by *Tuta absoluta*: ecology, geographic expansion and prospects for biological control. *J Pest Sci*, 83, 197–215.

Ebrahimi, M., Mahdian, K. & De Clercq, P. 2018. Life-history parameters and predation capacity of *Macrolophus pygmaeus* and *Nesidiocoris tenuis* (Hemiptera: Miridae) on eggs of *Plutella xylostella* (Lepidoptera: Plutellidae). *Agricultural and Forest Entomology*, https :// DOI: 10.1111/afe.12302.

Elliott, J.M. & Sawrey, F. 2003. A comparative study of the functional response of four species of carnivorous stoneflies. *Freshwater Biol*, 48, 191–202.

Enkegaard, A., Brødsgaard, H.F. & Hansen, D.L. 2001. *Macrolophus caliginosus*: functional response to whiteflies and preference and switching capacity between whiteflies and spider mites. *Entomol Exp Appl*, 101, 81–88.

EPPO. 2005. *Tuta absoluta*, data sheets on quarantine pests. *Bulletin OEPP/EPPO Bull*, 35, 454-435.

EPPO. 2009. EPPO Reporting service—Pest & Diseases. No 8, Paris, 2009-08-01.

EPPO. 2010b. First record of *Tuta absoluta* in Cyprus. EPPO Reporting Service 1, 2.

Essl, F., Dullinger, S., Rabitsch, W. et. al. 2011. Socioeconomic legacy yields an invasion debt. *Proc Natl Acad Sci USA*, 108, 203–207.

Estay, P. 2000. Polilla del Tomate *Tuta absoluta* (Meyrick) [WWW document]. URL http://alerce.inia.cl/docs/Informativos/Informativo09.pdf. Accesed 2 Feb 2010.

Fantinou, A.A., Perdikis, D.C., Maselou, D.A. & Lambropoulos, P.D. 2008. Prey killing without consumption: does *Macrolophus pygmaeus* show adaptive foraging behaviour? *Biol Control*, 47, 187–193.

Ferracini, C., Bueno, V.H.P., Dindo, M.L., Ingegno, B.L., Luna, M.G., Gervassio, N.G.S., Sánchez, N.E., Siscaro, G., van Lenteren, J.C., Zappalà, L., & Tavella, L. 2019. Natural enemies of *Tuta absoluta* in the Mediterranean basin, Europe and South America. *Biocontrol Science and Technology*, https :// DOI: 10.1080/09583157.2019.1572711.

Feyereisen, R., Dermauw, W. & van Leeuwen, T. 2015. Genotype to phenotype, the molecular and physiological dimensions of resistance in arthropods. *Pest Biochem Physiol*, 121, 61–67.

Finke, D.L. & Denno, R.F. 2002. Intraguild predation diminish in complex structured vegetation: implications for prey supression. *Ecology*, 83, 643–652.

Finke, D.L. & Denno, R.F. 2005. Predation diversity and the functioning of ecosystems: the role of intraguild predation in dampening trophic cascades. *Ecol Lett*, 8, 1299–1306.

Finke, D.L. & Denno, R.F. 2004. Predation diversity dampens trophic cascades. *Nature*, 429, 407–410.

Finney, D.J. 1964. Probit analysis. Cambridge University Press, Cambridge.

Fisher, M.H. & Mrozik, H. 1992. The chemistry and pharmacology of avermectins. *Annu Rev Pharmacol Toxicol*, 32, 537–553.

Foglar, H., Malausa, J.C. & Wajnberg, E. 1990. The functional response and preference of *Macrolophus caliginosus* (Heteroptera: Miridae) for two of its prey: *Myzus persicae* and *Tetranychus urticae*. *Entomophaga*, 35, 465–474.

Gao, C., Yao, R., Zhang, Z., Wu, M., Zhang, S. & Su, J. 2013. Susceptibility baseline and chlorantraniliprole resistance monitoring in *Chilo suppressalis* (Lepidoptera: Pyralidae). *J Econ Entomol*, 106, 2190–2194.

Garcia-del-Pino, F., Alabern, X. & Morton, A. 2013. Efficacy of soil treatments of entomopathogenic nematodes against the larvae, pupae and adults of *Tuta absoluta* and their interaction with the insecticides used against this insect. *BioControl*, 58(6), 723-731.

Garzia, G.T., Siscaro, G., Biondi, A. & Zappalà, L. 2012. Tuta absoluta, a South American pest of tomato now in the EPPO region: biology, distribution and damage. Bulletin OEPP/EPPO Bulletin [EPPO/FAO/IOBC/NEPPO Joint International Symposium on management of Tuta absoluta (tomato borer), Agadir, Morocco, 16-18 November 2011.], 42(2):205-210. http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1365-2338

Garcia, M.F. & Espul, J.C. 1982. Bioecología de la polilla del tomate (*Scrobipalpula absoluta*) en Mendoza, República Argentina. *Rev Invest Agropecuarias INTA (Argentina)*, 18, 135–146.

Gontijo, P.C., Picanço, M.C., Pereira, E.J.G., Martins, J.C., Chediak, M. & Guedes, R.N.C. 2013. Spatial and temporal variation in the control failure likelihood of the tomato leaf miner, *Tuta absoluta*. *Ann Appl Biol*, 162, 50–59.

Griffen, B.D. 2006, Detecting emergent effects of multiple predator species. *Oecologia*, 148, 702–709.

Guedes, R.N.C, Roditakis, E., Campos, M.R., Haddi, K., Bielza, P., Siqueira, H.A.A., Tsagkarakou, A., Vontas, J. & Nauen, R. 2019. Insecticide resistance in the tomato pinworm *Tuta absoluta*: patterns, spread, mechanisms, management and outlook. *Journal of Pest Sciences*, https://doi.org/10.1007/s10340-019-01086-9.

Guedes, R.N.C. & Picanço, M.C. 2012. The tomato borer *Tuta absoluta* in South America: pest status, management and insecticide resistance. *EPPO Bull*, 42, 211–216.

Guedes, R.N.C. 2017. Insecticide resistance, control failure likelihood and the First Law of Geography. *Pest Manag Sci*, 73, 479–484.

Guedes, R.N.C. & Siqueira, H.A.A. 2012. The tomato borer *Tuta absoluta*: insecticide resistance and control failure. *CAB Rev Perspect Agric Vet Sci Nutr Nat Resour*, 7, 1–7.

Guo, L., Liang, P., Zhou, X. & Gao, X. 2014a. Novel mutations and mutation combinations of ryanodine receptor in a chlorantraniliprole resistant population of *Plutella xylostella* (L.). *Sci Rep*, 4, 6924.

Guo, L., Wang, Y., Zhou, X., Li, Z., Liu, S., Pei, L. & Gao, X. 2014b. Functional analysis of a point mutation in the ryanodine receptor of *Plutella xylostella* (L.) associated with resistance to chlorantraniliprole. *Pest Manag Sci*, 70, 1083-1089.

Guillemaud, T., Blin, A., Le Goff, I., Desneux, N., Reyes, M., Tabone, E., Tsagkarakou, A., Ninõ, L. & Lombaert, E. 2015. The tomato borer, *Tuta absoluta*, invading the Mediterranean Basin, originates from a single introduction from Central Chile. *Sci Rep*, 5, 8371.

Haddi, K., Berger, M., Bielza, P., Cifuentes, D., Field, L.M., Gorman, K., Rapisarda, C., Williamson, M.S. & Bass, C. 2012. Identification of mutations associated with pyrethroid resistance in the voltage gated sodium channel of the tomato leaf miner (*Tuta absoluta*). *Insect Biochem Mol Biol*, 42, 506–513.

Hamdi, F., Chadoeuf, J., Chermiti, B. & Bonato, O. 2013. Evidence of cannibalism in *Macrolophus pygmaeus*, a natural enemy of whiteflies. *J Insect Behav*, 26, 614–621.

Han, P., Zhang, Y., Lu, Z., Wang, S., Ma, D., Biondi, A. & Desneux, N. 2018. Are we ready for the invasion of *Tuta absoluta*? Unanswered key questions for elaborating an Integrated Pest Management package in Xinjiang, China. *Entomol Gen*, 38, 113-125.

Han, P., Bayram, Y., Shaltiel-Harpaz, L., Sohrabi, F., Saji, A., Esenali, U.T., Jalilov, A., Ali, A., Shashank, P.R., Ismoilov, K., Lu, Z., Wang, S., Zhang, G., Wan, F., Biondi, A. & Desneux, N. 2019. *Tuta absoluta* continues to disperse in Asia: damage, ongoing management and future challenges. *J Pest Sci*, https :// doi.org/10.1007/s1034 0-018-1062.

Hassell, M.P., Lawton, J.H. & Beddington, J.R. 1977. Sigmoid functional response by invertebrate predators and parasitoids. *J Anim Ecol*, 46, 249–262.

Hill, M.P., Clusella-trullas, S., Terblanche, J.S. & Richardson, D.M. 2016. Drivers, impacts, mechanisms and adaptation in insect invasions. *Biol Invasions*, 18, 883–891.

Hochberg, M.E. 1996. Consequences for host population levels of increasing natural enemy species richness in classical biological control. *Am Nat*, 147, 307–318.

Holling, C.S. 1959. Some characteristics of simple types of predation and parasitism. *Can Entomol*, 91, 385–398.

Hulme, P.E. 2009. Trade, transport and trouble: managing invasive species pathways in an era of globalization. *J Appl Entomol*, 46, 10–18.

Ingegno, B.L., Ferracini, C., Gallinotti, D., Alma, A. & Tavella, L. 2013. Evaluation of the effectiveness of *Dicyphus errans* (Wolff) as predator of *Tuta absoluta* (Meyrick). *Biol Control*, 67, 246–252.

IRAC. 2011. *Tuta absoluta* – The Tomato Leafminer or Tomato Borer Recommendations for Sustainable and Effective Resistance Management. http://www.irac-online.org

IRAC. 2016. IRAC MoA classification scheme (version 8.1) http://www.irac-online.org. Accessed Jan 2017.

Ishtiaq, M., Saleem, M.A. & Razaq, M. 2011. Monitoring of resistance in *Spodoptera exigua* (Lepidoptera: Noctuidae) from four districts of the Southern Punjab, Pakistan to four conventional and six new chemistry insecticides. *Crop Protect*, 33, 13–20.

Jennings, S., Pinnegar, J.K., Polunin, N.V.C. & Boon, T.W. 2001. Weak cross-species relationships between body size and trophic level belie powerful size-based trophic structuring in fish communities. *J Anim Ecol*, 70, 934–944.

Jolliffe, P.A. 2000. The replacement series. *J Ecol*, 88, 371–385.

Juliano, S.A. 1993. Nonlinear curve fitting: predation and functinal response curves, in *Design and Analysis of Ecological Experiments*, ed.by Scheiner SM and Gurevitch J. Chapman and Hall, New York, pp. 158–183.

Karsholt, O., Mutanen, M., Lee, S. & Kaila, L. 2013. A molecular analysis of the Gelechiidae (Lepidoptera, Gelechioidea) with an interpretative grouping of its taxa. *Systematic Entomology*, 38, 334-348.

Kass, I. S., Wang, C.C., Walrond, J.P. & Stretton, A.O.W. 1980. Avermectin B(1a), a paralysing anthelmintic that affects interneurons and inhibitory motoneurons in Ascaris. *Proc. Natl. Acad. Sci. USA*, 77, 6211–6215.

Keller, R.P., Geist, J., Jeschke, J.M. & Kühn, I. 2011. Invasive species in Europe: ecology, status, and policy. *Environ Sci Eur*, 23, 23.

Kisdi, É. & Liu, S. 2006. Evolution of handling time can destroy the coexistence of cycling predators. *J Evol Biol*, 19, 49–58.

Klinken, R.D., van, Fichera, G. & Gordo, H. 2003. Targeting biological control across diverse landscapes: the release, establishment, and early success of two insects on mesquite (*Prosopis* spp.) insects in Australian rangelands. *Biological Control*, 26, 8–20.

Konuş, M. 2014. Analysing resistance of different *T. absoluta (*Meyrick) (Lepidoptera: Gelechiidae) strains to abamectin insecticide. *Turkish J Biochem*, 39, 291–297.

Korbie, D.J. & Mattick, J.S. 2008. Touchdown PCR for increased specificity and sensitivity in PCR amplification. Nature Protocols 3: 1452–1456. https://doi.org/10.1038/nprot.2008.133Lahm, G.P., Cordova, D. & Barry, J.D. 2009 New and selective ryanodine receptor activators for insect control. *Bioorg Med Chem*, 17, 4127–4133.

Lahm, G.P., Selby, T.P., Freudenberger, J.H., Stevenson, T.M., Myers, B.J., Seburyamo, G., Smith, B.K., Flexner, L., Clark, C.E. & Cordova, D. 2005. Insecticidal anthranilic diamides: a new class of potent ryanodine receptor activators. *Bioorg Med Chem Lett*, 15, 4898–4906.

Lampropoulos, P., Perdikis, D. & Fantinou, A. 2013. Are multiple predator effects directed by prey availability? *Basic Appl Ecol*, 14, 605–613.

Larraín, P.S. 1986. Plagas del tomate. *IPA, La Platina*, 39, 30–35.

Lang, A. 2003. Intraguild interference and biocontrol effects of generalist predators in a winter wheat field. *Oecologia*, 134, 144–153.

Lasota, J.A. & Dybas, R.A. 1991. Avermectins, a novel class of compounds: implications for use in arthropod pest control. *Annu Rev Entomol*, 36, 91–117.

Lee, S., Hodges, R.W. & Brown RL 2009. Checklist of Gelechiidae (Lepidoptera) in America North of Mexico. *Zootaxa*, 2231, 1-39.

Liebhold, A.M., Berec, L., Brockerhoff, E.G. et al. 2016. Eradication of invading insect populations: from concepts to applications. *Annu Rev Entomol*, 61, 335–352.

Lietti, M.M.M., Botto, E. & Alzogaray, R.A. 2005. Insecticide resistance in Argentine populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Neotrop Entomol*, 34, 113–119.

Lima, S.L. & Dill, L.M. 1990. Behavioral decisions made under the risk of predation: a review and prospectus. *Can J Zool*, 68, 619–640.

Livdahl, P. & Stiven, A.E. 1983. Statistical difficulties in the analysis of predator functional response data. *Can Entomol*, 115, 1365–1370.

Li, X., Schuler, M.A. & Berenbaum, M.R. 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Ann Rev Entomol*, 52, 231–253.

Lockwood, J.L., Hoopes, M.F. & Marchetti, M.P. 2013. Invasion ecology, 2nd edn. Wiley, New York.

Losey, J.E. & Denno, R.F. 1998. Positive predator–predator interactions: enhanced predation rates and synergistic suppression of aphid populations. *Ecology*, 79, 2143–2152.

Lowry, E., Rollinson, E.J., Laybourn, A.J. et, al. 2013. Biological invasions: a field synopsis, systematic review, and database of the literature. *Ecol Evol*, 3, 182–196.

Lucas, É. & Rosenheim, J.A. 2011. Influence of extraguild prey density on intraguild predation by heteropteran predators: a review of the evidence and a case study. *Biol Control*, 59, 61–67.

Lucas, É., Coderre, D. & Brodeur, J. 1998. Intraguild predation among aphid predators: characterization and influence of extraguild prey density. *Ecology*, 79, 1084–1092.

Lynch, M. 1984. Destabilizing hybridization, general-purpose genotypes and geographic parthenogenesis. *Q Rev Biol*, 59, 3, 257–290.

Mansour, R., Brévault, T., Chailleux, A., Cherif, A., Grissa-Lebdi, K., Haddi, K., Mohamed, S.A., Nofemela, R,S., Oke, A., Sylla, S., Tonnang, H.E.Z., Zappalà, L., Kenis, M., Desneux, N. & Biondi, A. 2018**.** Occurrence, biology, natural enemies and management of *Tuta absoluta* in Africa. *Entomol Gen*, 38, 83–112.

Martinou, A. & Stavrinides, M. 2015. Effects of Sublethal Concentrations of Insecticides on the Functional Response of Two Mirid Generalist Predators. PlosOne, 10(12): e0144413. doi:10.1371/journal.pone.0144413.

McCoy, M.W., Stier, A.C. & Osenberg, C.W. 2012. Emergent effects of multiple predators on prey survival: the importance of depletion and the functional response. *Ecol Lett*, 15, 1449–1456.

Messelink, G.J. & Janssen, A. 2014. Increased control of thrips and aphids in greenhouses with two species of generalist predatory bugs involved in intraguild predation. *Biol Control*, 79, 1–7.

Messelink, G.J., Bennison, J., Alomar, O., Ingegno, B.L., Tavella, L., Shipp, L. et al. 2014. Approaches to conserving natural enemy populations in greenhouse crops: current methods and future prospects. *BioControl*, 59, 377–393.

Meyerson, L.A. & Mooney, H.A. 2007. Invasive alien species in an era of globalization. *Front Ecol Environ*, 5, 199–208.

Michaelides, G., Seraphides, N., Pitsillou, M. & Sfenthourakis, S. 2019. Susceptibility of Cypriot *Tuta absoluta* populations to four targeted insecticides and control failure likelihood. *J Appl Entomol*, 2019;00:1–10. https://doi. org/10.1111/jen.12627.

Michaelides, G., Sfenthourakis, S., Pitsillou, M. & Seraphides, N. 2018. Functional response and multiple predator effects of two generalist predators preying on *Tuta absoluta* eggs. *Pest Manag Sci*, 74, 332-339.

Miranda, M.M.M., Picanço, M., Zanuncio, J.C. & Guedes, R.N.C. 1998. Ecological life table of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Biocontrol Sci Technol*, 8, 597–606.

Mollá, O., Biondi, A., Alonso-Valiente, M. & Urbaneja, A. 2014. A comparative life history study of two mirid bugs preying on *Tuta absoluta* and *Ephestia*

kuehniella eggs on tomato crops: implications for biological control. *BioControl*, 59, 175–183.

Mollá, O., González-Cabrera, J. & Urbaneja, A. 2011. The combined use of *Bacillus thuringiensis* and *Nesidiocoris tenuis* against the tomato borer *Tuta absoluta*. *BioControl*, 56, 883–891.

Moreno-Ripoll, R., Agustí, N., Berruezo, R. & Gabarra, R. 2012a. Conspecific and heterospecific interactions between two omnivorous predators on tomato. *Biol Control*, 62, 189–196.

Moreno-Ripoll, R., Gabarra, R., Symondson, W.O.C., King, R.A. & Agustí, N. 2012b. Trophic relationships between predators, whiteflies and their parasitoids in tomato greenhouses: a molecular approach. *Bull Entomol Res*, 102, 415–423.

Naranjo, S.E. & Ellsworth, P.C. 2009. Fifty years of the integrated control concept: moving the model and implementation forward in Arizona. *Pest Manag Sci*, 65, 1267–1286.

Naselli, M., Biondi, A., Garzia, G.T., Desneux, N., Russo, A., Siscaro, G. & Zappalà, L. 2017. Insights into food webs associated with the South American tomato pinworm. *Pest Manag Sci*, 73, 1352–1357.

Nauen, R. & Steinbach, D. 2016. Resistance to diamide insecticides in lepidopteran pests. In: Horowitz AR, Ishaaya I (eds) *Advances in insect control and in resistance management*. Springer, Dordrecht, pp. 219–240.

Nieukerken, E.J., van, Kaila, L., Kitching, I.J. *et al.* 2011. Order Lepidoptera Linnaeus, 1758. (ed. by Z.-Q. Zhang) Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness. *Zootaxa*, 3148, 212–221.

Perdikis, D., Lucas, E., Garantonakis, N., Giatropoulos, A., Kitsis, P., Maselou, D., *et al.* 2014. Intraguild predation and sublethal interactions between two zoophytophagous mirids, *Macrolophus pygmaeus* and *Nesidiocoris tenuis*. *Biol Control*, 70, 35–41.

Pereyra, P.C. & Sánchez, N.E. 2006. Effect of two solanaceous plants on developmental and population parameters of the tomato leaf miner, *Tuta*

absoluta (Meyrick) (Lepidoptera: Gelechiidae). *Neotrop Entomol*, 35, 671–676.

Perry, A.S., Yamamoto, I., Ishaaya, I. & Perry, R.Y. 1997. Insect resistance. In: Insecticides in Agriculture and Environment: Retrospects and Prospects. Springer-Veglar, Berlin, Germany, pp 208-220.

Pfeiffer, D.G., Muniappan, R., Sall, D., Diatta, P., Diongue, A. & Dieng E.O. 2013. First record of *Tuta absoluta* (Lepidoptera: Gelechiidae) in Senegal. *Fla Entomol*, 96, 661–662.

Polis, G.A.,,Myers, C.A. & Holt, R.D. 1989. The ecology and evolution of intraguild predation: potential competitors that eat each other. *Annu Rev Ecol and System*, 20, 297–330.

Potting, R.P., Van Der Gaag, D.J., Loomans, A., Van Der Straten, M., Anderson, H., MacLeod, A., Castrillón, J.M.G. & Cambra, V. 2013. Pest risk analysis – *Tuta absoluta*, tomato leaf miner moth. Ministry of Agriculture, Nature and Food Quality. Plant Protection Service of the Netherlands, Utrecht.

Powell, J.A. 1980. Evolution of larval food preferences in microlepidoptera. *Annual Review of Entomology*, 25, 133–159.

Puinean, A.M., Lansdell, S.J., Collins, T., Bielza, P. & Millar, N.S. 2013. A nicotinic acetylcholine receptor transmembrane point mutation (G275E) associated with resistance to spinosad in *Frankliniella occidentalis*. *J Neurochem*, 124, 590–601.

Queiroz, O.S., Ramos, R.S., Gontijo, L.M. & Picanço, M.C. 2015. Functional response of three species of predatory pirate bugs attacking eggs of *Tuta absoluta* (Lepidoptera: Gelechiidae). *Environ Entomol*, 44, 246–251.

Racke, K.D. 2006. A reduced risk insecticide for organic agriculture: spinosad case study. In: Felsot, A.S., Racke, K.D., editors. Crop protection products for organic agriculture: environmental, health and efficacy assessment, vol. 947. Washington: ACS. 92–108.

R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria; 2015. Available from: http://www.R-project.org/

R Development Core Team. 2016. *R: A Language for Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. http://www.R-project.org.

Reyes, M., Rocha, K., Alarcón, L., Siegwart, M. & Sauphanor, B. 2012. Metabolic mechanisms involved in the resistance of field populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) to spinosad. *Pestic Biochem Physiol*, 102, 45-50.

Ritz, C., Baty, F., Streibig, J.C. & Gerhard, D. 2015. Dose-Response Analysis Using R. PLOS ONE, 10:12. URL: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0146021.

Roditakis, E., Grispou, M., Morou, E., Kristoffersen, J.B., Roditakis, N.E., Nauen, R., Vontas, J. & Tsagkarakou, A. 2009, Current status of insecticide resistance in Q biotype Bemisia tabaci populations from Crete. *Pest Manag Sci*, 65, 313–322.

Roditakis, E., Mavridis, K., Riga, M., Vasakis, E., Morou, E., Rison, J. L., & Vontas, J. 2017b, Identification and detection of indoxacarb resistance mutations in the *para* sodium channel of the tomato leafminer, *Tuta absoluta. Pest Management Science*, 73, 1679-1688.

Roditakis, E., Skarmoutsou, C. & Staurakaki, M. 2013a. Toxicity of insecticides to populations of tomato borer *Tuta absoluta* (Meyrick) from Greece. *Pest Manag Sci*, 69, 834–840.

Roditakis, E., Skarmoutsou, C., Staurakaki, M., del Rosario Martínez-Aguirre, M., García-Vidal, L., Bielza, P., Haddi, K., Rapisarda, C., Rison, J-L., Bassi, A. & Teixeira, L.A. 2013b. Determination of baseline susceptibility of European populations of *Tuta absoluta* (Meyrick) to indoxacarb and chlorantraniliprole using a novel dip bioassay method. *Pest Manag Sci*, 69, 217–227.

Roditakis, E., Steinbach, D., Moritz, G., Vasakis, E., Stavrakaki, M., Ilias, A., García-Vidal, L., del Rosario Martínez-Aguirre, M., Bielza, P., Morou, E., Silva, J. E., Silva, W. M., Siqueira, H. A. A., Iqbal, S., Troczka, B. J., Williamson, M. S., Bass, C., Tsagkarakou, A., Vontas, J. & Nauen, R. 2017a. Ryanodine receptor point mutations confer diamide insecticide resistance in tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae). *Insect Biochemistry and Molecular Biology,* 80, 11-20.

Roditakis, E., Vasakis, E., García-Vidal, L., Martínez-Aguirre, M., Luc Rison, J., Haxaire-Lutun, M.O., Nauen, R., Tsagkarakou, A. & Bielza, P. 2018. A four-year survey on insecticide resistance and likelihood of chemical control failure for tomato leaf miner *Tuta absoluta* in the European/ Asian region. *J Pest Sci*, 91, 421-435.

Roditakis, E., Vasakis, E., Grispou, M., Stavrakaki, M., Nauen, R., Gravouil, M. & Bassi, A. 2015. First report of *Tuta absoluta* resistance to diamide insecticides. *J Pest Sci*, 88, 9–16.

Rogers, D. 1972. Random search and insect population models. *J Anim Ecol*, 41, 369–383.

Rosenheim, J.A., Kaya, H.K., Ehler, L.E., Marois, J.J. & Jaffee, B.A. 1995. Intraguild predation among biological control agents: theory and evidence. *Biol Control*, 5, 303–335.

Royama, T. 1971. A comparative study of models for predation and parasitism. *Res Popul Ecol Suppl*, 1, 1–91.

Salazar, E.R. & Araya, J.E. 2001. Tomato moth, *Tuta absoluta* (Meyrick) response to insecticides in Arica, Chile. *Agric Técnica*, 61,1–7.

Salgado, V.L. 1998. Studies on the mode of action of spinosad: insect symptoms and physiological correlates. *Pestic Biochem Phys*, 60, 91–102.

Salgado, V. L. & Sparks, T. C. 2005. 6.5 The spinosyns: chemistry, biochemistry, mode of action, and resistance. in: L.I. Gilbert (Ed.). *Comprehensive Molecular Insect Science*, (pp. 137-173). Elsevier: Amsterdam.

Sanchez, J.A. 2009. Density thresholds for *Nesidiocoris tenuis* (Heteroptera: Miridae) in tomato crops. *Biol Control*, 51, 493–498.

Sanchez, J.A., La-Spina, M. & Lacasa, A. 2014. Numerical response of *Nesidiocoris tenuis* (Hemiptera:Miridae) preying on *Tuta absoluta* (Lepidoptera: Gelechiidae) in tomato crops. *Eur J Entomol*, 111, 387–395.

Sankarganesh, E., Firake, D.M., Sharma, B., Verma, V.K. & Behere, G.T. 2017. Invasion of the South American Tomato Pinworm, *Tuta absoluta*, in north-eastern India: a new challenge and biosecurity concerns. *Entomol Gen*, 1, 335–345.

Santana, P.A. Jr., Kumar, L., Da Silva, R.S. & Picanço, M.C. 2019. Global geographic distribution of *Tuta absoluta* as affected by climate change. *J Pest Sci*. https ://doi.org/10.1007/s1034 0-018-1057-y.

Schmitz, O.J. 2007. Predator diversity and trophic interactions. *Ecology*, 88, 2415–2426.

Schmitz, O.J. 2009. Effects of predator functional diversity on grassland ecosystem function. *Ecology*, 90, 2339–2345.

Sharifian, I., Sabahi, Q. & Khoshabi, J. 2015. Functional response of *Macrolophus pygmaeus* (Rambur) and *Nesidiocoris tenuis* (Reuter) feeding on two different prey species. *Arch Phytopathol Plant Prot*, 48, 910–920.

Sharma, P.L. & Gavkare, O. 2017. New distributional record of invasive pest *Tuta absoluta* (Meyrick) in North-Western Himalayan Region of India. *Natl Acad Sci Lett*, 40, 217–220.

Sih, A., Englund, G. & Wooster, D. 1998. Emergent impacts of multiple predators on prey. *Trends Ecol Evol*, 13, 350–355.

Silva, J.E., Assis, C.P., Ribeiro, L.M. & Siqueira, H.A. 2016a. Field-evolved resistance and cross-resistance of Brazilian *Tuta absoluta* (Lepidoptera: Gelechiidae) populations to diamide insecticides. *J Econ Entomol*, 109, 2190–2195.

Silva, J.E., Ribeiro, L.M.S., Vinasco, N., Guedes, R.N.C. & Siqueira H.A.A. 2019. Field-evolved resistance to chlorantraniliprole in the tomato pinworm *Tuta absoluta:* inheritance, cross-resistance profile, and metabolism. *J Pest Sci*, https ://doi.org/10.1007/s1034 0-018-1064-z.

Silva, W.M., Berger, M., Bass, C., Balbino, V.Q., Amaral, M.H.P., Campos, M.R. & Siqueira, H.A.A. 2015. Status of pyrethroid resistance and mechanisms in Brazilian populations of *Tuta absoluta*. *Pestic Biochem Physiol*, 122, 8–14.

Silva, W. M., Berger, M., Bass, C., Williamson, M., Moura, D. N. M., Ribeiro, L. M. S. & Siqueira, H. A. A. 2016c. Mutation (G275E) of the nicotinic acetylcholine receptor α6 subunit is associated with high levels of resistance to spinosyns in *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Pesticide Biochemistry and Physiology,* 131, 1-8.

Silva, T.B.M., Silva, W.M., Campos, M.R., Silva, J.E., Ribeiro, L.M.S. & Siqueira, H.A.A. 2016b. Susceptibility levels of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) to minor classes of insecticides in Brazil. *Crop Protect*, 79, 80–86.

Silva, G.A., Picanço, M.C., Bacci, L., Crespo, A.L.B., Rosado, J.F. & Guedes, R.N.C. 2011. Control failure likelihood and spatial dependence of insecticide resistance in the tomato pinworm, *Tuta absoluta. Pest Manag Sci*, 67, 913–920.

Silver, K. S., Song, W., Nomura, Y., Salgado, V. L., & Dong, K. 2010. Mechanism of action of sodium channel blocker insecticides (SCBIs) on insect sodium channels. *Pesticide Biochemistry and Physiology,* 97, 87–92.

Siqueira, H.A.A., Guedes, R.N.C. & Picanco, M.C. 2000a. Cartap resistance and synergism in populations of *Tuta absoluta* (Lep., Gelechiidae). *J Appl Entomol*, 124, 233–238.

Siqueira, H.A.A., Guedes, R.N.C. & Picanco, M.C. 2000b. Insecticide resistance in populations of *Tula absoluta* (Lepidoptera: Gelechiidae). *Agric For Entomol*, 2, 147–153.

Siqueira, H.A.A., Guedes, R.N.C., Fragoso, D.B. & Magalhaes, L.C. 2001. Abamectin resistance and synergism in Brazilian populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Int J Pest Manag*, 47, 247–251.

Snaydon, R.W. 1991. Replacement or additive designs for competition studies. *J Appl Ecol*, 28, 930–946.

Soluk, D.A. & Collins, N.C. 1988. Synergistic interactions between fish and stoneflies: Facilitation and interference among stream predators. *Oikos*, 52, 94–100.

Soluk, D.A. 1993. Multiple predators effects: predicting combined functional response of steam fish and invertebrate predators. *Ecology*, 74, 219–225.

Sparks, T.C. & Nauen, R. 2015. IRAC: Mode of action classification and insecticide resistance management. *Pestic Biochem Physiol*, 121, 122–128.

Straub, C.S., Finke, D.L. & Snyder, W.E. 2008. Are the conservation of natural enemy biodiversity and biological control compatible goals? *Biol Control*, 45, 225–237.

Straub, C.S. & Snyder, W.E. 2006. Species identity dominates the relationship between predator biodiversity and herbivore suppression. *Ecology*, 87, 277–282.

Steinbach, D., Gutbrod, O., Lümmen, P., Matthiesen, S., Schorn, C. & Nauen, R. 2015. Geographic spread, genetics and functional characteristics of ryanodine receptor based target-site resistance to diamide insecticides in diamondback moth, *Plutella xylostella*. *Insect Biochem Mol Biol*, 63, 14–22.

Suomalainen, E. 1962. Significance of parthenogenesis in the evolution of insects. *Ann Entomol*, 7, 349–366.

Sylla, S., Brevault, T., Bal, A.B., Chailleux, A., Diatte, M., Desneux, N. & Diarra, K. 2017. Rapid spread of the tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae), an invasive pest in Sub-Saharan Africa. *Entomol Gen*, 36, 269–283.

Symondson, W.O.C., Sunderland, K.D. & Greenstone, M.H. 2002. Can generalist predators be effective biological control agents? *Annu Rev Entomol*, 47, 561–594.

Tommasini, M.G., Burgio, G., Mazzoni, F. & Maini, S. 2002. On intra-guild predation and cannibalism in *Orius insidiosus* and *Orius laevigatus* (Rhynchota Anthocoridae): laboratory experiments. *Bull Insectol*, 55, 49–54.

Tonnang, H.E.Z., Mohamed, S.F., Khamis, F. & Ekesi, S. 2015. Identification and risk assessment for worldwide invasion and spread of Tuta absoluta with a focus on Sub-Saharan Africa: implications for phytosanitary measures and management. *PLoS ONE*, 10:e0135283.

Troczka, B.J., Williams, A.J., Williamson, M.S., Field, L.M., Lüemmen, P & Emyr Davies, T.G. 2015. Stable expression and functional characterisation of the diamondback moth ryanodine receptor G4946E variant conferring resistance to diamide insecticides. *Sci Rep*, 5, 14680.

Troczka, B., Zimmer, C.T., Elias, J., Schorn, C., Bass, C., Emyr Davies, T.G., Field, L.M., Williamson, M.S., Slater, R. & Nauen, R. 2012. Resistance to diamide

insecticides in diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae) is associated with a mutation in the membrane spanning domain of the ryanodine receptor. *Insect Biochem Mol Biol*, 42, 873–880.

Tropea Garzia, G. 2009. *Physalis peruviana* L. (Solanaceae), a host plant of *Tuta absoluta* in Italy. *IOBC/WPRS Bull*, 49, 231–232.

Tscharntke, T., Klein, A.M., Kruess, A., Steffan-Dewenter, I. & Thies, C. 2005. Landscape perspectives on agricultural intensification and biodiversity–ecosystem service management. *Ecol Lett*, 8, 857–874.

Tylianakis, J.M. & Romo, C.M. 2010. Natural enemy diversity and biological control: making sense of the context-dependency. *Basic Appl Ecol*, 11, 657–668.

Tylianakis, J.M., Rand, T.A., Kahmen, A., Klein, A.M., Buchmann, N., Perner, J., *et al.* 2008. Resource heterogeneity moderates the biodiversity–function relationship in real world ecosystems. *PLoS Biol*, 6, 0947–0956.

Uchôa-Fernandes, M.A., Della Lucia, T.M.C. & Vilela, E.F. 1995. Mating, oviposition and pupation of Scrobipalpula absoluta (Meyrick) (Lepidoptera: Gelechiidae). *Anais da Sociedade Entomologica do Brasil*, 24, 159–164.

Urbaneja, A., González-Cabrera, J., Arnó, J. & Gabarra, R. 2012. Prospects for the biological control of *Tuta absoluta* in tomatoes of the Mediterranean basin. *Pest Manag Sci*, 68, 1215–1222.

Urbaneja, A., Montón, H. & Mollá, O. 2009. Suitability of the tomato borer *Tuta absoluta* as prey for *Macrolophus pygmaeus* and *Nesidiocoris tenuis*. *J Appl Entomol*, 133, 292–296.

Urbaneja, A., Vercher, R., Navarro, V., García Marí, F. & Porcuna, J.L. 2007. La polilla del tomate, *Tuta absoluta*. *Phytoma Esp*, 194, 16-23.

Ugurlu Karaağaç, S. 2015. Enzyme activities and analysis of susceptibility levels in Turkish *T. absoluta* populations to chlorantraniliprole and metaflumizone insecticides. *Phytoparasitica*, 43, 693–700.

Vance-Chalcraft, H.D. & Soluk, D.A. 2005a. Estimating the prevalence and strength of non-independent predator effects. *Oecologia*, 146, 452–460.

Vance-Chalcraft, H.D. & Soluk, D.A. 2005b. Multiple predator effects result in risk reduction for prey across multiple prey densities. *Oecologia*, 144, 472–480.

van Lenteren, J.C., Hemerik, L., Lins, J.C. & Bueno, V.H.P. 2016. Functional responses of three neotropical mirid predators to eggs of *Tuta absoluta* on tomato. *Insects*, 7, 1–10.

Van Son, T.C. & Thiel, M. 2006. Multiple predator effects in an intertidal food web. *J Anim Ecol*, 75, 25–32.

Vargas, H.C. 1970. Observaciones sobre la biología y enemigos naturales de la polilla del tomate, *Gnorimoschema absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Idesia*, 1, 75–110.

Vercher, R., Calabuig, A. & Felipe, C. 2010. Ecología, muestreos y umbrales de *Tuta absoluta* (Meyrick). *Phytoma España*, 217, 23–26.

Visser, D., Uys, V.M., Nieuwenhuis, R.J. & Pieterse, W. 2017. First records of the tomato leaf miner *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) in South Africa. *Bio Invasions Rec*, 6, 301–305.

Wang, J. J., Dong, H. G., & Yuan, L. Z. 2008. Selection for indoxacarb resistance in *Spodoptera litura* and investigation on cross-resistance in the selected population. *Acta Phytophylactica Sinica*, 35, 525–529.

Wang, X-L., Su, W., Zhang, J-H, Yang, T-H., Dong, K. & Wu, Y-D. 2016. Two novel sodium channel mutations associated with resistance to indoxacarb and metaflumizone in the diamondback moth, *Plutella xylostella*. *Insect Sci*, 23, 50–58.

Wang, X. & Wu, Y. 2012. High levels of resistance to chlorantraniliprole evolved in field populations of *Plutella xylostella*. *J Econ Entomol*, 105, 1019–1023.

Werling, B.P., Lowenstein, D.M., Straub, C.S. & Gratton, C. 2012. Multi-predator effects produced by functionally distinct species vary with prey density. *J Insect Sci*, 12, 1–7.

Wilby, A. & Thomas, M.B. 2002. Natural enemy diversity and pest control: patterns of pest emergence with agricultural intensification. *Ecol Lett*, 5, 353–360.

Williams, F.M. & Juliano, S.A. 1996. Functional responses revisited. *Environ Entomol*, 25, 549–550.

Yao, R., Zhao, D.D., Zhang, S., Zhou, L.Q., Wang, X., Gao, C.F. & Wu, S.F. 2017. Monitoring and mechanisms of insecticide resistance in *Chilo suppressalis*

(Lepidoptera: Crambidae), with special reference to diamides. *Pest Manag Sci*, 73, 1169-1178.

Zappalà, L., Biondi, A., Alma, A., Al-Jboory, I. J., Arnò, J., Bayram, A., Chailleux, A., El-Arnaouty, A., Gerling, D., Guenaoui, Y., Shaltiel-Harpaz, L., Siscaro, G., Stavrinides, M., Tavella, L., Vercher Aznar, R., Urbaneja, A. & Desneux, N. 2013. 'Natural enemies of the South American moth, *Tuta absoluta*, in Europe, North Africa and Middle East, and their potential use in pest control strategies', *Journal of Pest Science*, 86, 635-647.

Zappalà, L., Siscaro, G., Biondi, A., Mollá, O., González-Cabrera, J. & Urbaneja, A. 2012. Efficacy of sulphur on *Tuta absoluta* and its side effects on the predator *Nesidiocoris tenuis*. *J Appl Entomol*, 136, 401–409.

Zhang, B.C. 1994. *Index of Economically Important Lepidoptera.* CAB International, Wallingford.

Zibaee, I., Mahmood, K., Esmaeily, M., Bandani, A.R. & Kristensen, M. 2018. Organophosphate and pyrethroid resistances in the tomato leaf miner *T. absoluta* (Lepidoptera: Gelechiidae) from Iran. *J Appl Entomol*, 142, 181–191.

Zimmer, C.T., Panini, M., Singh, K.S., Randall, E.L., Field, L.M., Roditakis, E., Mazzoni, E. & Bass, C. 2017. Use of the synergist piperonyl butoxide can slow the development of alpha-cypermethrin resistance in the whitefly *Bemisia tabaci*. *Insect Mol Biol*, 26, 152–163.