**GENETICS AND MECHANISMS OF INSECTICIDE
RESISTANCE IN *Ceratitis capitata* AND ITS IMPLICATIONS
FOR RESISTANCE MANAGEMENT**

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Licenciada en Biotecnología

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ALIMENTARIA Y DE BIOSISTEMAS

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Reconocimientos

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Glossary of acronyms and abbreviations

ABC: ATP-Binding Cassette

ACh: Acetylcholine

AChE: Acetylcholinesterase

BC: Backcross

BSA: Bulk Segregant Analysis

cDNA: Complementary DNA

CNV: Copy Number Variation

DDT: Dichlorodiphenyltrichloroethane

df: Degrees of Freedom

D_{LC}: Dominance value

EAG: Electroantennography

FL: Fiducial Limits

GABA: Gamma-Aminobutyric Acid

GBS: Genotyping-By-Sequencing

gDNA: Genomic DNA

GLM: Generalized Linear Model

GluCl: Glutamate-gated Chloride Channel

GPDH: Glycerol-3-Phosphate Dehydrogenase

GSTs: Glutathione S-Transferases

IF: Initial Frequency

IRAC: Insecticide Resistance Action Committee

IRM: Insecticide Resistance Management

Kdr: Knockdown resistance

LC₅₀: Lethal Concentration 50

LD₅₀: Lethal Dose 50

nAChR: Nicotinic Acetylcholine Receptor

nACh α 6: α 6 subunit of the nicotinic Acetylcholine Receptor

OPs: Organophosphate compounds

P450s: Cytochrome P450s

PBO: Piperonyl Butoxide

PCR-RFLP: Polymerase Chain Reaction – Restriction Fragment Length Polymorphism

qPCR: Quantitative PCR

QTL: Quantitative Trait Loci

RR: Resistance Ratio

SIT: Sterile Insect Technique

SNPs: Single Nucleotide Polymorphisms

TM: Transmembrane

VGSC: Voltage Gated Sodium Channel

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ABSTRACT

The Mediterranean fruit fly (medfly), *Ceratitis capitata*, is one of the main insect pests of fruits. Current control practices in citrus crops in the Comunitat Valenciana mainly rely on the use of insecticides (spinosad and lambda-cyhalothrin as bait sprays, and deltamethrin as lure and kill traps) and the release of sterile males. However, the sustainability of medfly management programs is threatened by the reports of resistance to lambda-cyhalothrin in field populations and the potential for spinosad resistance development, which has been obtained under laboratory selection. The aim of this work was to study the mechanisms and genetics of spinosad and lambda-cyhalothrin resistance and their implications for resistance monitoring and management.

Spinosad resistance has been associated with different mutant alleles of the $\alpha 6$ subunit of the nicotinic acetylcholine receptor (*Ccα6*) that lead to loss-of-function phenotypes in the laboratory selected JW-100s strain. Using the GAL4>UAS system in a resistant *Drosophila melanogaster* strain, lacking the endogenous $\alpha 6$ subunit, we demonstrated that the ectopic expression of *Ccα6* wild-type isoforms rescue susceptibility, while the expression of the truncated allele *Ccα6*^{3aQ68*} (carrying the mutation 3aQ68*) does not. These results confirmed that truncated transcripts do not produce a response to spinosad. We also generated *C. capitata* isolines homozygous for the alleles: 1) *Ccα6*^{3aQ68*Δ3b-4}, which contains the mutation 3aQ68* and a deletion of exons 3b and 4; and 2) *Ccα6*^{3aQ68*-K352*}, which contains the mutations 3aQ68* and K352*. Both were present in JW-100s and neither of them produced complete *Ccα6* transcripts. We confirmed that both alleles contributed equally to the resistant phenotype. However, their frequency declined when in competition with individuals carrying the wild-type allele, suggesting a fitness cost associated with them. Through extensive testing of both biological and behavioral fitness traits, we identified a reduced ability of *Ccα6*^{3aQ68*Δ3b-4} males to detect the parapheromone trimedlure and to mate with females carrying the *Ccα6*^{3aQ68*-K352*} allele in competition experiments.

Lambda-cyhalothrin resistance in the laboratory selected W-1Kλ strain had previously been associated with P450-metabolic detoxification mediated by over-expression of the *CcCYP6A51* gene. However, our results indicated that other traits must also be

involved. Thus, we showed that the expression of several P450s, including *CcCYP6A51*, was highly variable and induced in some individuals in response to lambda-cyhalothrin treatments in both W-1Kλ and C strains. Besides, we proved that the inheritance pattern is polygenic, dominant and autosomic. A genotyping-by-sequencing analysis confirmed the polygenic character of resistance and identified seven candidate regions associated with the resistant phenotype. However, the subsequent analysis showed that they have a minor contribution with a marked cumulative effect. Moreover, none of the seven candidate regions were at or near P450 gene loci. As a result, we have not found a proper diagnostic marker to confidently monitor lambda-cyhalothrin resistance in field populations of *C. capitata*.

The susceptibility to available insecticides was monitored in Spanish medfly field populations in the period 2015-2017. We have found that the level of resistance to lambda-cyhalothrin (3-8 fold) is similar to that reported in previous years. Resistance to deltamethrin has been detected for the first time in field populations exposed to MagnetMEDTM traps, which are coated with this insecticide. All populations analyzed are susceptible to spinosad, though resistant alleles have been detected at low frequency by the combined use of an F1-screen assay and the search of mutations in the *Ccα6* gene. Modelling of spinosad resistance evolution was carried out considering the frequency of resistant alleles in the field, type of inheritance, fitness costs, level of exposition and the use of other insecticides. In addition, laboratory simulation studies were performed to evaluate different insecticide treatment scenarios in a multiresistant laboratory strain, generated by crossing JW-100s and W-1Kλ. The results indicated that the best option for resistance management is the rotation of insecticides with different modes of action and no cross-resistance.

In conclusion, medfly control is compromised by the development of resistance to available insecticides. Hence, the implementation of resistance management strategies is required. It is advisable to perform rotations of spinosad and lambda-cyhalothrin, or to use spinosad in those orchards where lure-and-kill traps of deltamethrin are deployed. On the contrary, the simultaneous use of lambda-cyhalothrin and deltamethrin is discouraged, as there is a risk of cross-resistance between them. These strategies must be harmonized with other control methods, such as cultural practices and the sterile insect technique.

RESUMEN

La mosca mediterránea de la fruta, *Ceratitis capitata*, es uno de los principales insectos plaga de los frutales. Las prácticas actuales de control en los campos de cítricos de la Comunitat Valenciana se basan principalmente en el uso de insecticidas (spinosad y lambda-cihalotrina mediante pulverización-cebo, y deltametrina en trampas de atracción y muerte) y la suelta de machos estériles. Sin embargo, la sostenibilidad de los programas de manejo está amenazada por la detección de resistencia a lambda-cihalotrina en poblaciones de campo y por la capacidad de esta especie para desarrollar resistencia a spinosad bajo presión de selección en laboratorio. El objetivo de este trabajo fue estudiar los mecanismos y la genética de la resistencia a spinosad y lambda-cihalotrina y su implicación en el monitoreo y manejo de la resistencia.

La resistencia a spinosad ha sido asociada en la línea seleccionada de laboratorio JW-100s con diferentes alelos mutantes de la subunidad $\alpha 6$ del receptor nicotínico de acetilcolina (*Ccα6*), que da lugar a fenotipos de pérdida de función. Con la implementación del sistema GAL4>UAS en una línea resistente de *Drosophila melanogaster*, que carecía de la subunidad $\alpha 6$ endógena, demostramos que la expresión ectópica de isoformas silvestres de *Ccα6* restablecen la susceptibilidad, mientras que la expresión del alelo truncado *Ccα6*^{3aQ68*} (que porta la mutación 3aQ68*) no lo hace. Estos resultados confirmaron que los transcritos truncados no producen una respuesta a spinosad. También generamos isolíneas de *C. capitata* homocigotas para los alelos: 1) *Ccα6*^{3aQ68*Δ3b-4}, que contiene la mutación 3aQ68* y una delección de los exones 3b y 4; y 2) *Ccα6*^{3aQ68*-K352*}, que contiene las mutaciones 3aQ68* y K352*. Ambos estaban presentes en JW-100s y ninguno de ellos producía transcritos completos de *Ccα6*. Confirmamos que ambos alelos contribuyen de igual manera al fenotipo resistente. Sin embargo, su frecuencia descendía cuando se encontraban en competencia con individuos que portaban el alelo silvestre, sugiriendo la existencia de un coste biológico. Diferentes caracteres biológicos y comportamentales fueron analizados, identificándose una reducción en la habilidad de los machos *Ccα6*^{3aQ68*Δ3b-4} para detectar la paraferomona trimedlure y para aparearse en experimentos de competencia con hembras que portan el alelo *Ccα6*^{3aQ68*-K352*}.

La resistencia a lambda-cihalotrina en la línea seleccionada de laboratorio W-1Kλ ha sido previamente asociada con detoxificación metabólica mediada por sobreexpresión del gen *CcCYP6A51*. Sin embargo, nuestros resultados indicaron que otros caracteres debían también estar implicados. Así, mostramos que la expresión de varios P450, incluyendo *CcCYP6A51*, era altamente variable e inducida en algunos individuos en respuesta al tratamiento con lambda-cihalotrina, tanto en W-1Kλ como en la línea control C. Además, demostramos que el patrón de herencia es poligénico, dominante y autosómico. Un análisis de genotipado por secuenciación confirmó el carácter poligénico de la resistencia, identificándose siete regiones candidatas asociadas con el fenotipo resistente. Sin embargo, el análisis posterior mostró que tenían una contribución menor con un marcado efecto aditivo. Además, ninguna de las regiones candidatas se encontraba dentro ni cerca de los genes P450. Como resultado, no hemos encontrado un marcador de diagnóstico robusto para monitorizar la resistencia a lambda-cihalotrina en poblaciones de campo de *C. capitata*.

La susceptibilidad a los insecticidas utilizados actualmente se monitorizó en poblaciones españolas de *C. capitata* durante el periodo 2015-2017. Hemos encontrado que el nivel de resistencia a lambda-cihalotrina (3-8 veces) es similar a la encontrada en años anteriores. La resistencia a deltametrina se detectó por primera vez en poblaciones de campo expuestas a trampas MagnetMED™, recubiertas con este insecticida. Todas las poblaciones analizadas permanecen susceptibles a spinosad, aunque se han detectados alelos de resistencia a baja frecuencia mediante el uso combinado de ensayos “F1-screen” y la búsqueda de mutaciones en el gen *Ccα6*. La evolución de resistencia a spinosad fue modelizada considerando la frecuencia de los alelos en el campo, tipo de herencia, coste biológico, nivel de exposición y el uso de otros insecticidas. Además, se realizó un estudio de simulación en laboratorio para evaluar diferentes escenarios de tratamiento con insecticidas con una línea de laboratorio multirresistente, generada mediante el cruce entre JW-100s y W-1Kλ. Los resultados indicaron que la rotación de insecticidas con diferentes mecanismos de acción y sin resistencia-cruzada es la mejor opción para el manejo de la resistencia.

En conclusión, el control de *C. capitata* está comprometido por el desarrollo de resistencia a los insecticidas disponibles, siendo necesaria la implementación de estrategias de manejo de la resistencia. Es recomendable realizar rotaciones entre

spinosad y lambda-cihalotrina, o utilizar spinosad en aquellos cultivos en los que se emplean trampas de atracción y muerte con deltametrina. Por el contrario, el uso simultáneo de lambda-cihalotrina y deltametrina está desaconsejado, ya que existe riesgo de resistencia cruzada entre ambos. Estas estrategias deben de ser armonizadas con otros métodos de control, tales como las prácticas culturales y la técnica del insecto estéril.

CHAPTER 1. INTRODUCTION

CHAPTER 1. INTRODUCTION

1.1. The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann, 1824)

1.1.1. Taxonomical classification

Ceratitis capitata (Wiedemann, 1824) (Diptera: Tephritidae) is commonly named as “the Mediterranean fruit fly” or “medfly”. According to Richards and Davies (1984) it can be classified as follows:

Class: Insecta

Order: Diptera

Suborder: Brachycera

Infraorder: Cyclorrhapha

Family: Tephritidae

Genus: *Ceratitis*

Species: *Ceratitis capitata*

1.1.2. Description

C. capitata is a holometabolous insect, with four life stages (egg, larva, pupa and imago or adult) in its biological cycle. The adult fly is about 3 to 5 mm long. The color of the body is yellowish with brown tinge on the abdomen, legs and some markings on the wings. The male has a pair of bristles with enlarged endings (Figure 1.1.a and b), while the female can be distinguished by its long ovipositor at the apex of the abdomen (Figure 1.1.c). The eggs are white, elongated and slightly curved, with a length between 0.2 and 1 mm (Figure 1.1.d). The larvae are elongated and yellowish white colored, and they reach their full development in the third instar measuring about 7 to 9 mm long (Figure 1.1.e). The pupae are cylindrical, 4 mm in length, dark reddish brown (Figure 1.1.f) (White and Elson-Harris, 1992).

C. capitata is a multivoltine species with short and overlapping generations (Bateman, 1972). The duration of the life cycle in optimal conditions is less than a month, but can be extended up to 2-3 months depending on temperature and other factors as food and water availability (Thomas *et al.*, 2010). During courtship, males aggregate in groups known as leks and emit a pheromone to attract females for mating (Field *et al.*, 2002). The fertilized females lay 1 to 10 eggs under the skin of fruit that is just

beginning to ripen, and may lay up to 800 eggs during its lifetime (Christenson and Foote, 1960; McDonald and McInnis, 1985). The eggs hatch and develop into larvae that feed inside the fruit pulp. Larvae leave the fruit when they reach the third instar, dropping to the ground and pupating in the soil. Adults emerge from the pupae and feed on plant glandular secretions, nectar and fruit juice (Christenson and Foote, 1960). Both males and females reach sexual maturity (ability to perform and to receive the proper courtship stimuli leading to copulation) 2 days after emergence, although females need 4 days to reach reproductive maturity (presence of mature eggs) (Arita, 1982).

Figure 1.1. *Ceratitis capitata*: a) adult male, b) male's bristles with enlarged endings, c) adult female, d) eggs, e) larvae and f) pupa.

In favorable areas, such as the Mediterranean basin of Spain, medfly adults can be found during the whole year. It generally completes 6 generations per year (Generalitat Valenciana, 2001), but it can reach up to 7 or 8 generations in the south coast (Muñiz and Gil, 1984). The population levels increase at the beginning of the summer, descending again in the warmest months and reaching the highest peak in autumn, declining rapidly in the cooler season (Generalitat Valenciana, 2001; Martínez-Ferrer *et al.*, 2007).

1.1.3. Importance as a pest

C. capitata is native from East Africa but has spread to many tropical and subtropical areas (Figure 1.2) (Fischer-Colbrie and Busch-Petersen, 1989; Fletcher, 1989; EPPO, 2019). It is reported to attack more than 300 species of fruits and vegetables (USDA, 2019), and it is considered one of the most destructive and economically damaging pests of citrus and other fruit crops. In Spain, it is widely distributed and especially abundant in the Mediterranean basin (Fimiani, 1989), and it is considered a pest of special incidence in agriculture since 1955. Some of the main fruit hosts in Spain are apricots (*Prunus armeniaca*), peaches (*Prunus spp.*) and figs (*Ficus carica*) in spring and summer, and persimmons (*Diospyros kaki*) and citrus (*Citrus spp.*) in fall and winter (Generalitat Valenciana, 2001; Martínez-Ferrer *et al.*, 2007).

Figure 1.2. Geographical distribution of *Ceratitidis capitata* (EPPO, 2019). Different dots correspond to areas where the pest is present (yellow) or transient -under eradication- (purple).

The relevance of this species in Spain is related to the importance of the citrus sector. Spain is the main producer of citrus in the European Union with more than 6-7 million Tm of production (MAPA, 2019a) in about 300000 ha (MAPA, 2018), and the first exporting country of fresh citrus in the world. The most important damage is produced on extra early varieties of citrus (satsumas and clementines) that reach maturity between September and November, when adult populations reach maximum levels (Martínez-Ferrer *et al.*, 2007).

Direct damage caused by *C. capitata* results from larval feeding that destroys the pulp. In addition, the holes made on the surface of the fruit during the oviposition provide

the entry of microorganisms, as fungi or bacteria, and induces a necrotic reaction around, it all causing the premature drop of the fruits and preventing its use for human consumption. There are a number of indirect losses that also have significant economic implications. They are derived from the imposition of quarantine protocols by the importing countries on fresh products from medfly infested areas to prevent the entry of the pest, and include costs related with pre- and post-harvesting surveillance and control actions (Castañera, 2003; Delrio and Cocco, 2012).

1.1.4. Control

The European legislation promotes the use of control methods that are environmentally friendly and respectful with human health, and aims to keep the use of pesticides only to levels that are economically and ecologically justified (European Comission, 2019). Sustainable biological, physical and cultural practices and other methods such as the sterile insect technique (SIT) must be preferred to chemical controls if they provide satisfactory pest control. In addition, the monitoring of field populations is a useful tool for a sustainable use of control strategies. In Spain, cultural practices and SIT are implemented at present for medfly control. However, for an efficient control in citrus crops, insecticide treatments are also required. Several biological control approaches have also been studied, for instance entomopathogenic agents as *Bacillus thuringiensis* (Vidal-Quist *et al.*, 2010) and natural predators (Monzó *et al.*, 2010, 2011) and parasitoids (Falcó *et al.*, 2006; De Pedro, 2017).

Cultural practices

Cultural control is considered a preventive practice that modifies the growing environment to reduce the prevalence of the pest in the field. The most effective cultural practices against medfly are the removal of fallen fruits, the elimination of pupae by ploughing and digging in orchards and the harvesting of non-commercial fruits in residential areas to remove suitable places for females to lay eggs (Chueca, 2007). In addition, the removal of abandoned and feral host plants nearby commercial orchards is useful to eliminate potential reservoirs for the flies (Castañera, 2003).

Sterile insect technique (SIT)

This methodology is based on the mass rearing and release of sufficient number of competitive sterile males into infested areas in order to compete with wild males. Thus, sterile males transfer their sterile sperm to females during mating, so they produce infertile eggs and natural pest populations are reduced (Reddy and Rashmi, 2016). In Spain, a SIT pilot program was initiated in the Comunitat Valenciana in 2002, with sterile males coming from a biofactory located in Mendoza (Argentina) (Argilés and Tejedo, 2007). In 2007, a biofactory was built in Caudete de las Fuentes (Comunitat Valenciana) with a production capacity of 500 million of pupae weekly. At present, 250-300 million of sterile males are liberated every week to cover a total surface of 140000 ha (Generalitat Valenciana, 2016). This technique is especially effective in spring, when population levels are low, but medfly outbreaks when temperature increases would require the combination with other control methods (Argilés and Tejedo, 2007; Navarro-Llopis *et al.*, 2011) for a satisfactory management (Juan-Blasco *et al.*, 2014). Nevertheless, special attention should be given to the coordination and timing when used in combination with insecticide treatments (Juan-Blasco *et al.*, 2013).

Chemical control

C. capitata control has traditionally relied on the use of insecticide bait sprays, which utilize hydrolyzed protein as food-based attractant, for both aerial applications and ground treatments. In Spain, medfly control in citrus crops from the 1990's was mainly based on malathion treatments (Primo-Millo *et al.*, 2003). However, the decision of non-inclusion of malathion in Annex 1 of the Directive 91/414/EEC, that regulates the process of revision of phytosanitary products, resulted in the withdrawal of this insecticide from the European Union in 2009. Since then, lambda-cyhalothrin and spinosad as bait sprays have been the most widely used insecticides for medfly control in citrus crops. They are mainly used for ground treatments, although aerial treatments with spinosad are also applied punctually. At present, malathion has been authorized again with restrictions in its use, and etofenprox, phosmet and chlorpyrifos-methyl are also registered for *C. capitata* control in citrus crops (MAPA, 2019b), although their use is very limited.

A more environmentally acceptable alternative for insecticides is their use in bait stations designed for mass-trapping (catch the flies) or lure and kill (flies are not retained) strategies. These devices attract as many flies as possible by combining visual (as the color of the trap) and olfactory (as food and/or sexual) attractants, and are impregnated with insecticides that kill the flies once they come into contact with the bait (Navarro-Llopis *et al.*, 2013, 2015). Mass-trapping

Figure 1.3. Mass-trapping device for *Ceratitidis capitata* control. Source of picture: Navarro-Llopis and Vacas, 2013.

in the Comunitat Valenciana started in 2000. First, conventional mass-trapping systems (Figure 1.3) with dichlorvos were used until 2010, when it was prohibited and traps' insecticide changed to deltamethrin. Lure and kill disposals (Figure 1.4) combining olfactory attraction and insecticide were commercialized with lambda-cyhalothrin in a first attempt, and changed in 2012 to deltamethrin, which has been used to date. First, M3[®] bait traps (Biagro, 0.004 g deltamethrin/trap) were used in small agricultural areas (Figure 1.4.a). Since then, the use of different bait stations with this insecticide, especially MagnetMED[™] traps (Suterra, 0.010 g deltamethrin/trap), has increased year after year (Figure 1.4.b). The "Conselleria de Agricultura" distributes half of the recommended number of traps (50 traps/ha) to be collocated in crops of citrus, figs, cherries, grapes, kakis and pomegranates. This organism distributed 265000 traps to cover a surface of 10600 ha in 2016 (Generalitat Valenciana, 2016), and 312000 traps for 12500 ha in 2018 (Ignacio Pla, TRAGSA, personal communication). This technique provides a feasible management option when used on low-density and isolated pest populations, though for larger populations it might be necessary to reinforce the control using chemical spray treatments.

Figure 1.4. Attract and kill devices for *Ceratitis capitata* control, a) M3® (Biagro), and b) MagnetMED™ (Suterra). Source of pictures: Navarro-Llopis and Vacas, 2013.

Apart from mass-trapping with insecticides, a pilot program with traps coated with the chemosterilant lufenuron started in the Comunitat Valenciana in 2001 with 200 ha (Navarro-Llopis *et al.*, 2007), and was extended between 2002 and 2009, when 3600 ha of citrus, prunus and kakis orchards were exposed to this substance in a rate of 24 traps/ha (Navarro-Llopis *et al.*, 2010). However, its commercialization stopped in 2013. The insecticides mentioned above can be classified in four groups according to their mode of action (IRAC, 2019a). Spinosad is a mixture of two macrocyclic lactones, spinosyns A and D, two natural compounds with insecticidal properties produced through fermentation by the actinomycete *Saccharopolyspora spinosa*. This insecticide is primarily an allosteric agonist of insect nicotinic acetylcholine receptor (nAChR) (Salgado and Sparks, 2005). Lambda-cyhalothrin, deltamethrin and etofenprox are pyrethroids, which modify the gating kinetics of voltage-gated sodium channels (VGSC) by slowing both the activation and inactivation of the channel (Silver *et al.*, 2014). This group of insecticides is characterized by a rapid knock-down effect due to its neurotoxic action. Malathion, phosmet and chlorpyrifos-methyl are organophosphate compounds (OPs). They belong to the group of inhibitors of esterases and its target site is the acetylcholinesterase (AChE), which plays a key role in the nervous impulse transmission by hydrolyzing the neurotransmitter acetylcholine (ACh). The inhibition of AChE by OPs allows the accumulation of ACh in the synaptic cleft, resulting in a continuous impulse and the death of the insect (Eto, 1974). Lufenuron is a

phenylbenzoylurea that inhibits chitin synthesis. When female medflies ingest bait containing lufenuron or when they mate with lufenuron treated males, they subsequently lay non-viable eggs (Navarro-Llopis *et al.*, 2004)

1.2. Insecticide resistance

1.2.1. General aspects

According to the Insecticide Resistance Action Committee (IRAC), insecticide resistance is defined as “a heritable change in the sensitivity of a pest population that is reflected in a repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species” (IRAC, 2019b).

Development of resistance is a problem concerning all groups of insecticides, as well as an example of natural selection and adaptation (Feyereisen *et al.*, 2015; Hawkins *et al.*, 2019). The development of resistance occurs when natural selection by an insecticide allows some insects with pre-existing or *de novo* traits to survive and pass the resistance trait on to their offspring. The continued application of insecticides with the same action mechanism provokes that the proportion of resistant insects increases in the population because of their selection against their susceptible counterparts. As a consequence, the insecticide becomes no longer effective. The eventual evolution of resistance in the field is a complex phenomenon influenced by biological (generation time, number of offspring per generation, migration, etc.), genetic (initial frequency, inheritance of resistant alleles, etc.) and operational (timing, dose and formulation of insecticides used, method of application, etc.) factors (Devine and Denholm, 2009). Knowledge of these factors and of the mechanisms by which resistance is acquired is essential for devising proactive resistance management strategies that can prevent or retard resistance development. Besides, it is important to detect resistance when it is at incipient levels and to monitor its evolution and geographical spread so that appropriate measures can be taken.

When an insect population is resistant to more than one insecticide, it is necessary to distinguish between multiple and cross-resistance. Multiple resistance occurs when insects develop resistance to several compounds by expressing multiple resistance mechanisms as a result of selection with different insecticides. Otherwise, cross-resistance happens when resistance to one insecticide confers resistance to another

insecticide because the resistance mechanism is shared, even when the insect has not been exposed to the latter product (Devine and Denholm, 2009).

Development of insecticide resistance is an increasing problem for the effective control of several agricultural pest species, resulting in economic losses, increased pesticide usage and potential associated risks for the environment and human health (Gould *et al.*, 2018). The first case of insecticide resistance was reported in 1947 to dichlorodiphenyltrichloroethane (DDT) in *Musca domestica* (Sacca, 1947). Since then, the incidence of resistance has increased annually at an alarming rate. Nowadays, more than 600 species of insects have been recounted showing resistance to one or more pesticide active ingredients, and the 32% of these species belong to the order Diptera (Gould *et al.*, 2018; IRAC, 2019c).

1.2.2. Mechanisms of resistance

1.2.2.1. Types

There are a number of ways insects can become resistant to insecticidal products (Feyereisen *et al.*, 2015). Five main categories of resistance mechanisms can be distinguished.

Behavioral resistance

Behavioral resistance is defined as the development of behaviors that reduce an insect's exposure to toxic compounds, or that allow an insect to survive in an environment that is harmful to the majority of insects of the same species (Sparks *et al.*, 1989). Resistant insects may detect or recognize a danger and avoid the exposure to toxic compounds as a result of stimulus-dependent or –independent mechanisms. In the first case, insects are stimulated by the repellency and/or irritancy of some insecticides to avoid them; for example, they stop feeding if they come across certain insecticides. In the second case, the population changes its habits to minimize contact with treated areas; for instance, they may move to the underside of a sprayed leaf or fly away from the target area (Zalucki and Furlong, 2017). This mechanism of resistance has been reported for several classes of insecticides, including OPs and pyrethroids (IRAC, 2019b).

Penetration resistance

Penetration resistance occurs when absorption of the insecticide into the insect decreases due to changes in its cuticle, involving a reduction in the dose of insecticide that reaches the site of action (Liu *et al.*, 2006). This mechanism does not usually confer high levels of resistance; however, it frequently appears along with other forms of resistance acting as an enhancer (Scott and Georgiou, 1986; Wu *et al.*, 1998; Valles *et al.*, 2000).

Metabolic resistance

Metabolic resistance consists on the metabolization of insecticides to non-toxic or less toxic forms that are finally excreted through a process called “detoxification”, reducing the amount of molecules that reach its target site. As a consequence of this, resistant insects detoxify the noxious products faster than susceptible insects, quickly ridding their bodies of toxic molecules (Li *et al.*, 2007a). The detoxification of insecticides involves complex multi-gene detoxification enzyme families: esterases (Montella *et al.*, 2012), glutathione S-transferases (GSTs) (Ketterman *et al.*, 2011) and cytochrome P450s (P450s) (Feyereisen, 2012). Esterases hydrolase and/or sequester the insecticide molecules by binding permanently to them. P450 introduce hydrophilic functional groups into the insecticide molecules to transform them to less harmful metabolites and to enhance their water solubility. The GSTs catalyzes the transfer of glutathione to electrophilic compounds to detoxify and favor the excretion of endogenous and exogenous compounds.

Metabolic resistance can be achieved by mutations that increase the affinity of the enzyme for the insecticide or by transcriptional up-regulation or gene amplification of detoxification enzymes. It is dependent on the chemical structure of the insecticides, and it can range from compound-specific to broad cross-resistance to several insecticides with different modes of action (Li *et al.*, 2007a; Liu, 2015).

Increased excretion

The ATP-binding cassette (ABC) transporters are a family of transmembrane proteins that transport a large diversity of substances (endogenous metabolites, cellular synthesis products and xenobiotics as insecticides) across lipid membranes in

eukaryotic cells with consumption of ATP (Higgins, 1992; Gott *et al.*, 2017). In insects, there are eight subfamilies of ABC proteins (ABCA-ABCH), and three of them (ABCB FTs/P-gps, ABCCs and ABCGs) have been reported to be implicated in insecticide transport and resistance (Dermauw and Van Leeuwen, 2014). They are abundant in the Malpighian tubules, which are responsible for the majority of the excretion of toxins, and increased expression of some of these proteins has been observed in these tissues (Robinson *et al.*, 2013).

Target-site resistance

Target-site resistance occurs when the target site of insecticides suffers structural modifications that interfere with their binding affinity, reducing or even eliminating their toxic action (Casida and Durkin, 2013). Studies about target-site resistance have made clear that many insecticides bind to specific biophysical forms of their receptors. For instance, pyrethroids have a higher affinity for the open state of the sodium channel (O'Reilly *et al.*, 2006). Consequently, mutations associated with insecticide resistance can be located directly within the insecticide-binding site or, otherwise, they can modify the stability of the receptor conformation for which the drug has more affinity (Ffrench-Constant *et al.*, 2016).

Target-site resistance has been associated with alterations in important molecules as the enzyme AChE, target of OPs (Oakeshott *et al.*, 2005; Hotelier *et al.*, 2010); and in different ion channels, as gamma-aminobutyric acid (GABA) receptor for cyclodienes (Buckingham *et al.*, 2017), glutamate-gated chloride channel (GluCl) for avermectins (Wolstenholme, 2012), VGSC for pyrethroids (Dong *et al.*, 2014), and nAChRs for neonicotinoids (Crossthwaite *et al.*, 2014) and spinosyns (Salgado and Sparks, 2005; Watson *et al.*, 2010).

1.2.2.2. Resistance to spinosad

High levels of spinosad resistance have been recorded in field populations of several pest species (Sparks *et al.*, 2012; Geng *et al.*, 2013), including the tephritid flies *Bactrocera zonata* (Nadeem *et al.*, 2012), *Bactrocera oleae* (Kakani *et al.*, 2010) and *Bactrocera cucurbitae* (Hsu *et al.*, 2012a). Moreover, selection in the laboratory has resulted in several highly resistant insect strains including *Drosophila melanogaster*

(Watson *et al.*, 2010), *Bactrocera dorsalis* (Hsu *et al.*, 2012) and *C. capitata* (Ureña *et al.*, 2019).

Spinosad acts in insects as an allosteric agonist of ACh by binding to nAChRs (Salgado and Sparks, 2005; Ihara *et al.*, 2017). nAChRs belong to the family of the Cys-loop ligand-gated ion channel and mediate fast action potentials at cholinergic synapses when activated by ACh (Lester *et al.*, 2004; Grutter *et al.*, 2005). These receptors are pentameric proteins composed by five ligand-binding nAChR subunits surrounding a central cation permeable pore that opens when activated by agonists, allowing a net influx of cations into the cell. Each one of their five subunits contain an extracellular N-terminal domain including six loops (A-F, where the ACh binding site is), four transmembrane segments (TM1–4, of which TM2 is part of the receptor channel pore), and a large intracellular domain (between TM3 and TM4) (Jones and Sattelle, 2010). It can be distinguished between homomeric or heteromeric nAChR subtypes depending on if they are formed by a single type of α subunit or if they are a mix of both α and β subunits. Insect species code in their genome for 10-16 subunit genes, being 10 in *D. melanogaster* (α 1-7 and β 1-3) (Sattelle *et al.*, 2005) and 11 in *C. capitata* (α 1-8 and β 1-3) (Papanicolaou *et al.*, 2016).

The specific binding of spinosad at an allosteric site of α 6 subunits is believed to mediate the insect death (Perry *et al.*, 2007; Somers *et al.*, 2015). nAChR α 6 subunits share a high degree of conservation between insect species and also with the α 7 subunits in vertebrates (Grauso *et al.*, 2002). The insect α 6 gene contains 12 different exons, with two mutually exclusive variants of exon 3 (exon 3a and exon 3b) and generally three mutually exclusive variants of exon 8 (exon 8a, 8b and 8c). These possibilities for alternative splicing, together with frequent A-to-I pre-mRNA editing, confer to this gene an extraordinary capacity to generate a huge diversity of mRNA products that will have different properties when assembled into pentameric complexes (Grauso *et al.*, 2002; Jin *et al.*, 2007). Interestingly, exon 3 contains the ACh binding loop D, exon 8 includes part of the TM2 domain involved in the formation of the pore, and some of the editing sites described in α 6 are located in the proximity of the ACh binding pocket (Sattelle *et al.*, 2005; Jin *et al.*, 2007).

The first mutation conferring spinosad resistance described in the nAChR α 6 subunit gene was a null mutation in *D. melanogaster* that resulted in >1000-fold resistance

(Perry *et al.*, 2007). Since then, several studies have shown that *D. melanogaster* nAChR $\alpha 6$ subunit gene knock out and point mutations generating premature stop codons or amino acid changes next to the Cys-loop motif result in resistance to spinosad (Watson *et al.*, 2010; Somers *et al.*, 2015; Zimmer *et al.*, 2016). Moreover, the generation of $\alpha 6$ truncated transcripts or amino acid changes due to mis-splicing, insertions, deletions and point mutations has also been associated with spinosad resistance in *Plutella xylostella* (Baxter *et al.*, 2010; Rinkevich *et al.*, 2010; Wang *et al.*, 2016), *Tuta absoluta* (Berger *et al.*, 2016; Silva *et al.*, 2016), *Frankliniella occidentalis* (Puinean *et al.*, 2013), *Thrips palmi* (Bao *et al.*, 2014) and *B. dorsalis* (Hsu *et al.*, 2012a). It seems that any mutation causing a loss of $\alpha 6$ function will confer resistance to spinosad by avoiding its binding to the nAChR. This big variety of possibilities to develop spinosad resistance makes its evolution easier than if a specific mutation was required (Somers *et al.*, 2015).

Although target-site resistance is the main cause of spinosad resistance, there are evidences of metabolic resistance occasioned by P450 and esterases (Geng *et al.*, 2013; Campos *et al.*, 2015). However, with the exception of the over-expression of *CYP6A1*, *CYP6D1*, *CYP6D3* and *CYP6G4* in spinosad resistant strains of *M. domestica* (Markussen and Kristensen, 2012; Højland *et al.*, 2014), the genes involved are unknown.

1.2.2.3. Resistance to pyrethroids

Field resistance to pyrethroids has been reported for many insects, including resistance to lambda-cyhalothrin in tephritids as *B. zonata* (Nadeem *et al.*, 2012) and *C. capitata* (Arouri *et al.*, 2015), and to deltamethrin in *B. dorsalis* (Wang *et al.*, 2013). Resistance to pyrethroids is mainly caused by two mechanisms: target site insensitivity (knockdown resistance or *kdr*) and metabolic detoxification mediated by P450 enzymes, esterases and GSTs (Li *et al.*, 2007a; Davies *et al.*, 2008).

Knockdown resistance (kdr)

The VGSC are the physiological target of pyrethroid insecticides (Elliott *et al.*, 1978; Dong *et al.*, 2014). They are integral transmembrane proteins mediating voltage-dependent changes in sodium transport in the generation and propagation of action

potentials in the neurons of insects (Dong, 2007). Their structure is highly similar to their equivalent proteins in mammals, with a pore-forming α -subunit composed of four homologous repeats (I-IV) with six transmembrane segments (S1-S6) each one (Catterall, 2012). The majority of insects species have a single copy of the VGSC gene that encodes the α -subunit (Dong *et al.*, 2014; Scott, 2019), such as the *DmNaV* (formerly *para*) in *D. melanogaster*. Nevertheless, alternative splicing and RNA editing of the transcripts of this gene help to generate a collection of sodium channel variants (Olson *et al.*, 2008; Lin *et al.*, 2009; Dong *et al.*, 2014). There is also a small auxiliary transmembrane protein, TipE, and four TipE-homologous genes (Teh1-4), which have been found to be critical for sodium channel expression and neuronal activities in *D. melanogaster* (Feng *et al.*, 1995; Warmke *et al.*, 1997) and other insects (Bourdin *et al.*, 2013; Du *et al.*, 2013).

Pyrethroids interaction with VGSC slows both activation and inactivation of the channel in a way that it remains open and interferes with Na^+ flux, leading to nerve exhaustion and death (Narahashi, 1992; Scott, 2019). Resistance to pyrethroids and DDT results from the modification in the sodium channel gene that affects the binding of these insecticides (Shono, 1985). Mutations in the VGSC gene have been linked to resistance to the knock-down effect caused by pyrethroids in many insect species. The most recent review lists 61 mutations or combination of mutations associated to insecticide resistance (Dong *et al.*, 2014), and there is evidence that different *kdr* alleles can confer different levels of resistance (Scott, 2019). The replacement L1014F, first described in a DDT resistant strain of *M. domestica* (Ingles *et al.*, 1996), has been identified in more than 20 different arthropods species (Dong *et al.*, 2014), including the fly *Haematobia irritans* (Jamroz *et al.*, 1998) and many species of mosquitoes (Silva *et al.*, 2014), and confers 10-20 fold resistance to pyrethroids. Alternative substitutions in the same homologous site have also been identified, such as L1014S in *Anopheles gambiae* (Pinto *et al.*, 2006) and *Culex pipiens* (Chen *et al.*, 2010), L1014C in *C. pipiens* (Taskin *et al.*, 2016) and *Anopheles sinensis* (Tan *et al.*, 2012), and L1014H in *M. domestica* (Rinkevich *et al.*, 2006). Other mutations associated with pyrethroids resistance in dipterans, concretely in mosquitoes, are N1575Y in *A. gambiae* (Jones *et al.*, 2012) and I1011M/V, G923V, L982W, V1016G/I, D1794Y, F1534C in *Aedes aegypti* (Vontas *et al.*, 2012). Mutations conferring up to 500-fold resistance (super-*kdr*) have

been identified in pyrethroid resistant arthropods either singly or in combination with *kdr* (Dong *et al.*, 2014). One of the most frequent of these mutations is M918T, identified in combination with L1014F in the flies *M. domestica* (Williamson *et al.*, 1996; Shono *et al.*, 2002) and *H. irritans* (Guerrero *et al.*, 1997). Other mutation described conferring super-*kdr*-like levels of resistance in combination with L1014F in *M. domestica* is T929I (Sun *et al.*, 2017).

Metabolic resistance mediated by P450s

P450s are a big family of proteins present in all living organisms (Werck-Reichhart and Feyereisen, 2000). Most of them have monooxygenase activity, but they can also act as oxidases, reductases, desaturases, isomerases, etc. (Guengerich, 2001), what gives them the potential to metabolize xenobiotics. In insects, their role in metabolizing toxic compounds has conferred resistance to a wide range of insecticides. However, they are also involved in the biosynthesis, activation, and/or catabolism of endogenous substrates, such as the juvenile and molting hormones (Feyereisen, 2012). The total amount of P450 genes in insects is very variable, ranging from 36 CYP genes in *Pediculus humanus* (Lee *et al.*, 2010) to 170 in *Culex quinquefasciatus* (Arensburger *et al.*, 2010). In *C. capitata*, 103 CYP genes and 9 pseudogenes have been described, with a characteristic cluster of 18 consecutive CYP genes (13 of them belonging to the CYP6A subfamily) (Papanicolaou *et al.*, 2016). Those genes linked to insecticide resistance mostly belong to CYP4, CYP6, CYP9 and CYP12 families (Feyereisen, 2012). There is a high evolutionary plasticity in this gene family that provokes that a single species, even under similar conditions, can evolve resistance using different P450s (Scott, 2017).

In resistant strains, elevated levels of P450 enzymatic activity can be observed, and synergist piperonyl butoxide (PBO) has proved to reverse resistance. Some of these facts have already been reported for *C. capitata* (Arouri *et al.*, 2015) among other tephritids (Hsu *et al.*, 2004; Margaritopoulos *et al.*, 2008), and studies with other dipterans have also shown a positive correlation between pyrethroids resistance and constitutive or induced over-expression of one or more CYP genes (David *et al.*, 2013). Functional studies have also proved that transgenic expression of some of these genes conferred resistance to pyrethroids to susceptible *D. melanogaster* strains (Zhu *et al.*,

2010; Riveron *et al.*, 2013; Tsakireli *et al.*, 2019), whereas suppression of their expression by RNAi increased the susceptibility in resistant strains (Bautista *et al.*, 2009; Zhu *et al.*, 2010).

The increased production of P450s in resistant insects had been shown to occur mostly through upregulation via changes in cis- or trans-acting regulatory loci (Hemingway *et al.*, 2004; Li *et al.*, 2007a). However, gene duplication or amplification of P450s has also been related with resistance of some insect species (Bass and Field, 2011).

1.2.3. Inheritance and genetics of resistance

The type of inheritance of insecticide resistance determines in great manner how resistance would be transmitted to the offspring and hence, how fast its spread would be. Important aspects to be considered are to find out if the resistant traits are recessive or dominant, if they are autosomal or sex-linked, and if they are under monogenic or polygenic control (Gassmann *et al.*, 2009).

In some cases it has been possible to establish an association between dominance and molecular mechanism (Bourguet and Raymond, 1998). For example, spinosad resistance mediated by null or truncated transcripts of the $\alpha 6$ subunit of the nAChR is usually inherited as an autosomal recessive trait (Perry *et al.*, 2007; Baxter *et al.*, 2010; Ureña *et al.*, 2019). The inheritance of pyrethroids resistance as a consequence of VGSC mutations has been found to be incompletely recessive (Farnham, 1977; Scott, 2019). However, resistance to pyrethroids mediated by P450s varies from dominant, as observed in *M. domestica* for deltamethrin (Khan *et al.*, 2015) and for lambda-cyhalothrin (Abbas *et al.*, 2014a) resistance, to recessive, as for permethrin resistance in *C. quinquefasciatus* (Li and Liu, 2010).

The general paradigm of resistance development is that field populations usually favor the appearance of a monogenic response involving one gene, or very few genes of major effect (Ffrench-Constant, 2013). Genetic data support that target-site resistance is usually monogenic involving mutations at a single gene locus (for instance, mutations at ligand-gated ion channels, voltage-gated ion channels and AChE). However, transcriptomic studies suggest that a more complex set of changes in multiple gene/enzyme systems may occur. For instance, a large number of closely related genes can be upregulated in resistant strains, mutations can arise in an

upstream regulatory gene, or even a group of genes can have different contributions to resistance in different strains according to insect physiology or other parameters (Oakeshott *et al.*, 2013). In the case of metabolic resistance, multigene enzyme systems may be involved. When this happens, finding the specific role of single genes or enzymes can turn out to be more difficult due to different family members have similar or overlapping substrate specificity. Thus, pyrethroid resistance mediated by P450s can be controlled by a major gene, as in alphamethrin resistance in *Anopheles stephensi* (Prasad and Shetty, 2013) or by more than one gene (Abbas *et al.*, 2014a; Khan *et al.*, 2015). The P450s enzymes are an example of multigene system in which, if a specific P450 gene is altered to cope with the metabolism of an insecticide, other related enzymes might be able to replace its natural metabolic function (Feyereisen, 2012).

1.2.4. Cost of resistance

Insecticide resistance mechanisms selected in insects can provoke the appearance of fitness cost when individuals carrying resistant alleles present significant disadvantages that diminish their fitness in comparison to their susceptible counterparts. The fitness cost linked to insecticide resistance has an effect on the development and spread of resistant populations. The higher the fitness cost, the longer the time for resistant individuals to spread in the population, thus adding an important factor to take into consideration when developing resistance management programs (Kliot and Ghanim, 2012).

For cases of target site resistance, mutations located in essential genes (for instance, coding for necessary enzymes or fundamental proteins) can provoke severe functional constraints as a consequence of an alteration in the natural (and hence, the optimal) physiological state of the insect (Ffrench-Constant and Bass, 2017). An example of a mutation in an essential enzyme is observed in the *ace1* gene (encoding the AChE targeted by organophosphates) of *P. xylostella* that confer resistance to chlorpyrifos. Authors showed that resistant allele was costly in the absence of insecticide affecting longevity and fecundity, and that costs were greater at higher temperatures (Zhang *et al.*, 2015). As an example of alterations in a protein constituting important channels, a study reported that the knocked-out of the $\alpha 1$ subunit of the nAChR in *D.*

melanogaster resulted in changes in courtship, longevity, sleep and insecticide resistance, revealing potential fitness cost associated with changes in this subunit of the receptor (Somers *et al.*, 2017a,b). However, not all mutations in subunits of this receptor necessarily confer constraints. Actually, it has been seen in *D. melanogaster* that the complete removal of the $\alpha 6$ subunit of the nAChR is not lethal and does not carry obvious fitness costs under laboratory conditions for the insect (Perry *et al.*, 2007).

For metabolic resistance, if the over-expression of an enzyme associated with resistance is energetically costly, resistant individuals over-producing such an enzyme in the absence of the insecticide would be at an energetic disadvantage (Ffrench-Constant and Bass, 2017). For instance, it has been reported that individuals of *C. pipiens* with permethrin resistance mediated by P450 detoxification had lower energetic reserves (glycogen and lipids) than their susceptible counterparts (Hardstone *et al.*, 2010).

1.2.5. Resistance management

Insecticide Resistance Management (IRM) attempts to preserve the utility of insecticides through strategies aimed at either overcoming resistance to currently used compounds or preventing the development of resistance to extant or new insecticides (Devine and Denholm, 2009; Sparks and Nauen, 2015). Effective IRM is essential for insect control, as the availability of replacement insecticides results really challenging given the time, increasing expense and strict regulatory requirements (Sparks, 2013). To accomplish these goals, an early detection of resistance events is required, as well as the reduction of selection pressure directed toward a particular insecticide, which favors the increase of resistant alleles in the population.

Related to the monitoring of resistance, bioassays are conceived as a primarily warning signal, although they have also been used for long periods to quantify the level of resistance by recording mortality and survival across a series of insecticide concentrations. However, bioassays require large numbers of insects, and they are affected by environmental and physiological conditions, which imply a handicap for widespread monitoring (Weetman and Donnelly, 2015). For these reasons, biochemical or molecular approaches have been developed targeting transcriptional

variation, enzyme activity or DNA (Devine and Denholm, 2009). In particular, DNA diagnostic markers allow to identify polymorphisms linked to insecticide resistance. The development of these markers requires knowing which genes are involved in resistance mechanism so as to associate DNA variations with a resistant phenotype. Nonetheless, “hypothesis-free” or agnostic genome-wide studies can be used when no candidate information is available, scanning the genome or transcriptome for phenotype-associated variants. Both approaches require a subsequent validation by *in vivo* or *in vitro* experiments to functionally explain the association with resistance (Donnelly *et al.*, 2016). DNA markers can early detect the onset of resistance and track its increase in populations, they are applicable to all field-collected samples, and have no specific requirements of insects’ storage. However, it is essential to keep in mind that resistance mechanisms are in continuous evolution and new polymorphisms will appear, so the markers also need to be updated (Weetman and Donnelly, 2015).

With the aim of managing resistance, different insecticide use strategies have been proposed: sequences, which consists on the use of a chemical until the emergence of resistance and then change its use to a second chemical, and so on; mosaics, which establish spatial patterns of application for at least two chemicals in close fields; mixtures, simultaneous use of two or more chemicals over time and space; and rotations, which implies temporal cycles that alternate several products (Bourguet *et al.*, 2013). In particular, the rotation is one of the simplest and more used approaches and it is based on the alternative use of compounds with different mechanisms of action to which significant levels of resistance have not been selected yet (Hemingway, 2018), establishing “windows” or treatments stages according to the biology of the pest and the crop. One or more applications of a given insecticide may be acceptable within a window, depending on local conditions. However, it is critical that the next window or generation is not treated with a compound with the same action mechanism as the previous window/generation. So, the IRM programs require studies of cross-resistance to find out how useful the mixtures or alternation of insecticides can be. Furthermore, the combination of chemical products with other control practices or techniques would contribute to the management of resistance.

Other useful tool, which is progressively gaining importance in the management of resistance, is the use of predictive models. The models encompass relevant aspects

that influence the evolution of resistance, such as frequencies and inheritance of resistant alleles, type of insecticides used and number of treatments, and predict how different scenarios could influence it. The information obtained is of fundamental importance because it could contribute to the decision making in IRM. This type of models are especially implemented in mosquitos in the fight against malaria, where they are used to predict insecticide resistance evolution and the best strategies to prevent it (South and Hastings, 2018; Wairimu *et al.*, 2018).

1.3. Insecticide resistance in *C. capitata*

1.3.1. Appearance and evolution of insecticide resistance

At a general level, few cases of insecticide resistance were reported in fruit flies before the 1990's (Keiser, 1989), and it was suggested that their mobility and broad host range would have caused incomplete selection pressure (Orphanidis *et al.*, 1980). Nevertheless, resistance to commonly used insecticides have spread quickly in the last two decades in tephritid flies, being currently recognized as a major problem for some fruit pests of great economic importance (Vontas *et al.*, 2011). In Spain, no cases of field resistance were reported for *C. capitata* during the 1980s and 1990s (Viñuela, 1998). Particularly, laboratory analyses of field populations collected from citrus and peach orchards found no sign of resistance to OPs (diazinon, malathion and trichlorphon) which were being used to control this pest since treatments became mandatory in citrus crops in 1955 (Viñuela and Arroyo, 1982).

Resistance to insecticides in Spanish field populations of *C. capitata* was first reported to malathion in 2004, and it was suggested to be caused by extensive application of this insecticide by ground and aerial treatments to control this pest in citrus crops (Magaña *et al.*, 2007). Populations from different geographical areas showed resistance to malathion (6- to 201-fold compared to laboratory susceptible strain), and the differences in susceptibility were related to the frequency of field treatments. Interestingly, low levels of resistance were even detected in field populations collected from fields that had never been treated, suggesting that resistant flies may have dispersed from areas with high selection pressure to untreated areas. Indeed, the high level of gene flow existing among Spanish medfly population favors the spread of resistance (Beroiz *et al.*, 2012). Since 2009, malathion was retired from the European

Union and other insecticides gained importance in medfly control, such as spinosad and the pyrethroid lambda-cyhalothrin. However, it was not long until resistance to lambda-cyhalothrin was detected in 2009-2010 in Spanish populations from the Mediterranean area (Arouri *et al.*, 2015). On the contrary, field populations remain susceptible to spinosad (Ureña *et al.*, 2019). The use of the pyrethroid deltamethrin in lure and kill devices has increased in the last years, but there are no records of the susceptibility of Spanish field populations to this insecticide. In Greece, an analysis of spinosad and deltamethrin susceptibility of field populations reported no significant levels of resistance to either of them (Voudouris *et al.*, 2018).

Apart from resistance events found in the field, *C. capitata* has also demonstrated its capacity to develop resistance under laboratory selection pressure with different insecticides: DDT (15-fold) (Orphanidis *et al.*, 1980), dieldrin (68-fold) (Busch-Petersen and Wood, 1986), permethrin (up to 20-fold) (Busch-Petersen and Wood, 1983), lambda-cyhalothrin (205-fold) (Arouri *et al.*, 2015) and spinosad (about 500-fold) (Ureña *et al.*, 2019). The potential to develop spinosad resistance under selection pressure is especially relevant, since it is at present the main insecticide used for the control of this pest in Spanish citrus crops.

1.3.2. Mechanisms of resistance to insecticides in *C. capitata*

To date, mechanisms of insecticide resistance in *C. capitata* have been elucidated for malathion, lambda-cyhalothrin and spinosad.

Related to malathion resistance, the G328A mutation in the coding sequence of the acetylcholinesterase *Ccace2* gene was first reported in individuals from the field-derived malathion resistant strain W of *C. capitata* (Magaña *et al.*, 2008). This mutation is adjacent to the glutamate of the catalytic site of AChE, and it was demonstrated that homozygous adult flies for this mutation showed reduced AChE activity and less sensitivity to inhibition by malaoxon, the active form of malathion. In order to detect the G328A mutation, a Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) method was designed (Couso-Ferrer, 2012). This mutation resulted widely distributed throughout Spain, including the Balearic and the Canary Islands (Couso-Ferrer, 2012) and it was also found later in field populations of Brazil (Elfekih *et al.*, 2014), although it still has not been found in other

continents where it has been analyzed (Couso-Ferrer, 2012). To deeply study the mechanism of resistance, the malathion resistant strain W was further selected under laboratory conditions to obtain the W-4Km and W-10Km strains. Studies with W-4Km showed that this malathion resistant strain presented moderate (3 to 16-fold) cross-resistance to other OPs (trichlorphon, diazinon, phosmet and methyl-chlorpyrifos), the carbamate carbaryl, the pyrethroid lambda-cyhalothrin, and the benzoylphenylurea derivative lufenuron (Couso-Ferrer *et al.*, 2011). Couso-Ferrer (2012) demonstrated that some individuals in the resistant strains carried a duplication of the *Ccace2* gene and that only one of the copies carried the G328A mutation. Individuals carrying the duplication of the *Ccace2* gene were also detected in field populations of *C. capitata* collected in Spain in the period 2004-2007. This mechanism of resistance had already been reported in other insects as *C. pipiens* (Alout *et al.*, 2009) and *A. gambiae* (Djogbénou *et al.*, 2008). Biological consequences of this mutation and duplication of *ace* gene have been analyzed (Lee *et al.*, 2015), suggesting that a mutation in such an essential gene carries a severe fitness cost that favors the fixation of a new and functional copy of *ace*. Actually, a higher fitness cost was observed in resistant individuals carrying the G328A mutation, which showed a delay in the developmental time from egg to pupa and a reduction of pupal weight when compared to individuals carrying the duplication of the *Ccace2* gene (Arouri, 2013). In addition to target site insensitivity, metabolic resistance to malathion mediated by esterases has also been described in *C. capitata* (Magaña *et al.*, 2008), as reported for other tephritids (Wang *et al.*, 2015).

Lambda-cyhalothrin resistance in medfly has been associated to metabolic detoxification mediated by P450 enzymes (Arouri *et al.*, 2015). Almost complete reversion of resistance was observed when the laboratory-selected resistant strain W-1Kλ was treated with the P450 inhibitor PBO. In addition, study of expression of P450 genes belonging to CYP4, CYP6, CYP9 and CYP12 families showed that *CcCYP6A51*, located at one end of the CYP cluster previously mentioned, was over-expressed in the resistant strain (16 fold). A recent study has functionally validated this gene by *in vitro* metabolism assays, which revealed that it was capable of metabolizing lambda-cyhalothrin and deltamethrin, and by *in vivo* expression via GAL4>UAS system in *D. melanogaster*, that allows driving its expression with detoxification tissue-specific

drivers. In this case, bioassays with the transgenic flies and lambda-cyhalothrin and deltamethrin indicated that *CcCYP6A51* confers knock-down resistance (Tsakireli *et al.*, 2019). However, the inheritance and fitness cost of this resistance remains unknown, and the expression of P450 genes is not a reliable method for resistance monitoring. The resistant *C. capitata* strain W-1kλ also showed high levels of cross-resistance to etofenprox (240-fold) and deltamethrin (150-fold) (Arouri *et al.*, 2015), which seriously compromises the effectiveness of pyrethroids to control medfly.

Spinosad resistance has been associated to a target-site resistance process based on the appearance of truncated transcripts of the α6 subunit of the nAChR (Ureña *et al.*, 2019). Authors investigated the resistance mechanism in the laboratory-selected strain JW-100s, originated from a field population, and found different resistant alleles that had varied during the selection process with spinosad. First, *Ccα6*^{3aAG>AT} allele, with the AG>AT mutation in the 5'splicing site of exon 3a, and *Ccα6*^{3aQ68*} allele, with mutation Q68* in exon 3a, were dominant at generation F29. However, *Ccα6*^{3aAG>AT} allele was not detected again since generation F45, when allele *Ccα6*^{3aQ68*-K352*}, which have not been detected previously, became dominant. This allele carried mutations Q68* in exon 3a and K352* in exon 10, both of them provoking the appearance of stop codons. Finally, since generation F85 the population was mainly constituted by individuals homozygous for *Ccα6*^{3aQ68*-K352*} (92%), resulting in the generation of transcripts truncated in exon 3a and transcripts with exon 3b truncated in exon 10. Authors also demonstrated that inheritance of spinosad resistance in medfly was completely recessive, but left unsolved the fitness costs associated with these resistant alleles. Knowledge of mutations associated to resistance in *C. capitata* allowed the design of a Multiplex PCR and a PCR-RFLP methods to monitor the presence and evolution of resistant alleles (Ureña *et al.*, 2019).

1.4 Aims of the Doctoral Thesis

With the aim of improving the management of insecticide resistance in *C. capitata*, this thesis was focused on the study of the mechanisms and genetics of spinosad and lambda-cyhalothrin resistance and in the development of molecular tools that facilitate the monitoring of resistant alleles in field populations.

The specific objectives of this work were:

- 1) Validating by functional genomics and the generation of isolines the contribution to spinosad resistance of the mutated *Ccα6* alleles found in the laboratory strain JW-100s, as well as revealing the fitness costs associated to them, by analyzing biological and behavioral parameters of the insect.
- 2) Revealing the inheritance of lambda-cyhalothrin resistance in the resistant strain W-1Kλ, as well as improving our knowledge in the resistance mechanism and searching for molecular markers associated to the resistant phenotype.
- 3) Informing about the current situation of resistance in field populations of *C. capitata* by performing toxicity bioassays and molecular monitoring, and predicting by the implementation of mathematical models the evolution of resistance in different insecticide treatments scenarios.

**CHAPTER 2. FUNCTIONAL CHARACTERIZATION AND FITNESS COST OF
SPINOSAD RESISTANT ALLELES IN *Ceratitis capitata***

CHAPTER 2: FUNCTIONAL CHARACTERIZATION AND FITNESS COST OF SPINOSAD**RESISTANT ALLELES IN *Ceratitis capitata*****2.1. RATIONALE FOR THE STUDY**

Spinosad in bait sprays is one of the most used insecticides for *Ceratitis capitata* (medfly) control in citrus crops in Spain (MAPA, 2019b). There is still no evidence of resistance to spinosad in the field, but a highly resistant strain to this insecticide, JW-100s, has been obtained after laboratory selection (Ureña *et al.*, 2019). The mechanism of resistance has been studied in this strain, and it has been reported to be associated to three different mutant alleles of the $\alpha 6$ subunit of the nicotinic acetylcholine receptor (nAChR), the target site of spinosad: 1) $Cc\alpha 6^{3aAG>AT}$ allele, 2) $Cc\alpha 6^{3aQ68*}$ allele, and 3) $Cc\alpha 6^{3aQ68*-K352*}$ allele (Ureña *et al.*, 2019). The frequency of these resistant alleles varied along the selection process and, since generation F85, JW-100s is constituted by individuals homozygous for $Cc\alpha 6^{3aQ68*-K352*}$ (92%) and individuals heterozygous for $Cc\alpha 6^{3aQ68*}/Cc\alpha 6^{3aQ68*-K352*}$ (8%). None of the $Cc\alpha 6$ transcripts from the $Cc\alpha 6^{3aQ68*-K352*}$ allele can generate complete $Cc\alpha 6$ protein products, which is expected to confer resistance to spinosad (Perry *et al.*, 2007). However, it still remains unknown why individuals with $Cc\alpha 6^{3aQ68*}$ or $Cc\alpha 6^{3aAG>AT}$ alleles, which only affect isoforms containing exon 3a while generating wild-type full-length $Cc\alpha 6$ isoforms with exon 3b, show a resistant phenotype (Ureña *et al.*, 2019).

The eventual evolution of spinosad resistance in the field will depend, among other biological, genetic and operational factors, on the inheritance and cost of resistance (Devine and Denholm, 2009). Resistance to spinosad in the JW-100s strain is recessive (Ureña *et al.*, 2019), consistent with other reported examples of $\alpha 6$ based resistance (Bielza *et al.*, 2007a; Perry *et al.*, 2007; Baxter *et al.*, 2010; Wang *et al.*, 2016). There is evidence that the knock-out of $\alpha 6$ in *Drosophila melanogaster* does not cause lethality (Perry *et al.*, 2007). However, when different trade-offs related to growth and developmental traits were analyzed in other species, fitness cost ranged from absent or minor (Bielza *et al.*, 2008; Abbas *et al.*, 2014b) to substantial (Wang *et al.*, 2010; Okuma *et al.*, 2018), and reversion of resistance in the absence of spinosad selection has been reported in other dipterans (Ferguson, 2004; Khan *et al.*, 2014). Only a few studies have considered how spinosad resistance affects behavior and the impact that

loss of function or truncated $\alpha 6$ transcripts could have, despite nAChRs may have many roles in behavior (Perry and Batterham, 2018). The known roles for the different subunits identified in *D. melanogaster* currently include sleep, D α 1, D α 3 and D α 4 (Shi *et al.*, 2014; Wu *et al.*, 2014; Somers *et al.*, 2017a), courtship, D α 1 (Somers *et al.*, 2017a) and escape response, D α 7 (Fayyazuddin *et al.*, 2006). Changes to feeding behavior in *Frankliniella occidentalis* (Li *et al.*, 2017) and reduced competitive mating in *Heliothis virescens* (Wyss *et al.*, 2003) have been reported, but the molecular basis for spinosad resistance was unknown in both cases.

The aim of this work was: 1) to determine whether or not the relationship between spinosad resistance and $Cc\alpha 6^{3aQ68*}$ and $Cc\alpha 6^{3aQ68*-K352*}$ alleles is casual; 2), to assess the stability of spinosad resistance in *C. capitata* linked to these two alleles; and 3) to examine whether there are fitness costs associated with these resistant alleles, referred to both biological and behavioral traits.

2.2. MATERIALS AND METHODS

2.2.1. *C. capitata* strains and generation of isolines

The susceptible laboratory strain of *C. capitata*, C, was established from medfly individuals from non-treated experimental fields of the Instituto Valenciano de Investigaciones Agrarias (IVIA, València, Spain) in 2001. Since then, it has been reared in the laboratory (Magaña *et al.*, 2007) without any exposure to insecticides.

The *C. capitata* isolines described here were derived from the spinosad resistant laboratory strain JW-100s (Ureña *et al.*, 2019). To obtain isolines homozygous for the mutant alleles a process of crossing and genotyping was performed. All of the crosses were initiated with 50 virgin females (isolated as pupae) and 10 males under standard rearing conditions (16 : 8 hours light : dark photoperiod and a temperature of 26 ± 2 °C (light) and 22 ± 2 °C (dark) with water and food *ad libitum*). After 7-10 days, individual females were placed in tubes containing around 20 g of larval rearing medium (standard rearing conditions) for oviposition. When the offspring of these females had developed into larvae and pupae, their female parents were genotyped and the offspring of females carrying the allele of interest were selected for the next cross. The genotyping was done according to two PCR protocols described in Ureña *et al.* (2019), a PCR-RFLP for the detection of the K352* mutation in exon 10, identified via the

presence of an *MseI* restriction site in the mutant allele, and a multiplex (“multiplex PCR-2” in Ureña *et al.* (2019)) to detect the Q68* mutation in exon 3a. Both protocols allow mutant and wild type homozygotes and heterozygotes to be distinguished. Homozygous lines were sequenced to ensure that all individuals carried the expected genotype. The sequencing was performed by Secugen (Madrid, Spain) on a 3730 XL DNA Analyzer (Applied Biosystems) using BigDye[®] Terminator v3.1 reagents. The DNA sequences produced were analysed using Geneious 11.0.5 (<https://www.geneious.com>).

In order to obtain the Q68*-K352* isoline, a cross between JW-100s females and C males, homozygous for the wild allele ($Cc\alpha 6^+/Cc\alpha 6^+$), was performed. F1 offspring which female parent carried the $Cc\alpha 6^{3aQ68^*-K352^*}/Cc\alpha 6^{3aQ68^*-K352^*}$ genotype were selected and inter-crossed. The offspring F2 embryos were placed on larval rearing medium (stock prepared by mixing 300 g of sugar, 11.2 g of N-propyl-p-hydroxibenzoate (Sigma-Aldrich, Japan), 11.2 g of methyl-4-hydroxibenzoate (Sigma-Aldrich, India), 10 g of benzoic acid (Merck, China), 145.2 g of dry beer yeast (Difco Laboratories, Erembodegem, Belgium), 1000 g of wheat bran and 2400 ml of distilled water) containing 5 ppm of spinosad to eliminate the $Cc\alpha 6^+/Cc\alpha 6^+$ and $Cc\alpha 6^+/Cc\alpha 6^{3aQ68^*-K352^*}$ genotypes. F2 survivors were used to establish the homozygous Q68*-K352* isoline ($Cc\alpha 6^{3aQ68^*-K352^*}/Cc\alpha 6^{3aQ68^*-K352^*}$) (Figure 2.1.a).

To obtain Q68* isoline, an initial cross was done between JW-100s females and males of the Q68*-K352* isoline. F1 females carrying the $Cc\alpha 6^{3aQ68^*}$ allele were selected for crossing to C ($Cc\alpha 6^+/Cc\alpha 6^+$) males, and the same process was repeated with F2 females. F3 individuals carrying the $Cc\alpha 6^+/Cc\alpha 6^{3aQ68^*}$ genotype were inter-crossed and the resulting F4 embryos were placed on larval rearing medium containing 5 ppm spinosad to eliminate the $Cc\alpha 6^+/Cc\alpha 6^+$ and $Cc\alpha 6^+/Cc\alpha 6^{3aQ68^*}$ genotypes. F4 survivors were used to establish the Q68* isoline (Figure 2.1.b), homozygous for a new allele $Cc\alpha 6^{3aQ68^*\Delta 3b-4}/Cc\alpha 6^{3aQ68^*\Delta 3b-4}$ (see section 2.3.2).

Figure 2.1. Generation of Q68*-K352* (a) and Q68* (b) isolines from JW-100s strain.

a)

b)

2.2.2. Genomic DNA extraction, PCR of intronic/exonic boundaries and sequencing of *Ccα6*

Genomic DNA (gDNA) extraction from single flies from Q68* and Q68*-K352* isolines, and JW-100s and C strains of *C. capitata* was carried out with tissue homogenization and DNA isolation using SpeedTools Tissue DNA Extraction (Biotools, Madrid, Spain), according to the manufacturer's instructions. gDNA was quantified using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

The boundaries between intronic regions and exons 2, 3a, 3b, 4 and 5 of *Ccα6* were PCR amplified. The reactions were performed in a volume of 10 µl using 0.4 µM of each oligonucleotide (Table 2.1), 0.5 U of AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific, Austin, USA), 10x PCR Buffer II, 1.5 mM MgCl₂, 0.2 mM dNTPs (Thermo Fisher Scientific, Austin, USA) and 100-200 ng of the corresponding template. PCR conditions were as follows: an initial denaturation step at 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; and a final step of 72 °C for 7 min for full extension. PCR products were analyzed by electrophoresis on 1.5% agarose gel (Agarose D2, Conda Pronadisa, Madrid, Spain) and directly purified (QIAquick PCR Purification Kit, QIAGEN, Germany). As required, bands of interest were purified from the gel with Ultrafree®-DA Centrifugal Filter Units (Millipore, Ireland) following the manufacturer's instructions. The *Ccα6* regions were then sequenced with the same primers used for the amplification (Secugen, Madrid, Spain).

2.2.3. RNA extraction, RT-PCR, qPCR of *Ccα6* exons, PCR and sequencing of codifying regions

Three replicates of Q68* and Q68*-K352* isolines, and C strain were used to analyze isoform expression. Each replicate consisted of a pool of 3 males and 3 females. Total RNA extraction was performed with TRIzol® Reagent (Life Technologies, Van Allen Way, Carlsbad, California, USA) according to the manufacturer's instructions, and RNA was dissolved in RNasefree Resuspension Solution (Life Technologies) followed by incubation at 60 °C during 10 min and quantification with Nanodrop ND-1000 spectrophotometer. Reverse transcription of complementary DNA (cDNA) was performed using 2 µg of RNA as a template and a commercial kit including oligo(dT) for

the reaction (Thermo Scientific, Massachusetts, USA), with modifications of the protocol as described in Ureña *et al.* (2019).

Table 2.1. Oligonucleotides used for PCR and qPCR analysis.

Technique	Exon and gene	Primer forward	Primer reverse
PCR	1-3aQ68* (<i>Cca6</i>)	FnACh6ex1 (CAACGGAAGCTGAAATCTAAGGAC)	RnACh6Q68 (AACCACGCATTTGTGGTCAGAATC)
PCR	2-5 (<i>Cca6</i>)	FnACh6ex2-76 (CGTCTACTTAACCACCTATTATCC)	RnACh6-535 (CCAAATCCAAGTATTTCCATCGT)
PCR	5-7 (<i>Cca6</i>)	FnACh567ex5 (GCGGAYGAGGGWTCGATGGAAC)	RnACh6-774 (ATCGGGAGGTAGTGTAATCCTAA)
PCR	10-12 (<i>Cca6</i>)	FnACh6-996 (TAAATCCGTTTTCTGCAATGGCT)	RnACh6-ex12nest (TCGCTGTTTGCCGTGTTGATCTTT)
PCR	2-3a (<i>Cca6</i>)	FnACh6ex2-76 (CGTCTACTTAACCACCTATTATCC)	Ex2-3a-R (ATTCCAAATTTAACCACGCATT)
PCR	2-4 (<i>Cca6</i>)	FnACh6ex2-76 (CGTCTACTTAACCACCTATTATCC)	Ex3a-R(2) (CGCCTCCATATTCTGATTC)
PCR	3a-5 (<i>Cca6</i>)	Ex3a-F (AGATTCTGACCACAAATGC)	RnACh6-ex5 (CGTGCCATCGAATCCCTCATCC)
PCR	After 2 (<i>Cca6</i>)	PEx2-F (GGCTTAACGCTGCAACAAAT)	PEx2-R (TTTCATGTTTCGCGTGTGTT)
PCR	After 3a (<i>Cca6</i>)	PEx3a-F (ACCACAAATGCGTGGTTAAA)	PEx3a-R (TAGCTGCGTGAAAACCATG)
PCR	Before 3b (<i>Cca6</i>)	AEx3b-F (TTTCATTCTTCATGCCTCTTTTT)	AEx3b-R (CCAACGAAAGCCAAAGATTG)
PCR	Before 4 (<i>Cca6</i>)	AEx4-F (CTGGAAAAATAATAATGCAATAC)	AEx4-R (CATCCGGTTTCCAGAGCTTA)
PCR	After 4 (<i>Cca6</i>)	PEx4-F (ATGGAGGCGTGAAAGATTTG)	PEx4-R (GCCTGCGTGTTCCTTTTGT)
PCR	Before 5 (<i>Cca6</i>)	AEx5-F (CGTAGCAAATGCGCTATCAA)	AEx5-R (TTGGTGTGATACGTGCCATC)
qPCR	2-3b (<i>Cca6</i>)	Ex2-3b-F (AACGCTGCAACAAATAATTGATG)	Ex2-3b-R (CCATTCCAACGAAAGCCAAA)
qPCR	3b-4 (<i>Cca6</i>)	Ex3b-F (AATCAACTTCTTATAACCAATCTT)	Ex3b-R (TTCACGCCTCCATATTCT)
qPCR	2-3a (<i>Cca6</i>)	Ex2-3b-F (AACGCTGCAACAAATAATTGATG)	Ex2-3a-R (ATTCCAAATTTAACCACGCATT)
qPCR	3a-4 (<i>Cca6</i>)	Ex3a-F (AGATTCTGACCACAAATGC)	Ex3a-R(2) (CGCCTCCATATTCTGATTC)
qPCR	7 (<i>Cca6</i>)	ComúnF (TAGTTGGACTTACGATGG)	ComúnR (GACGATGGTATTCTTCTTG)
qPCR	<i>actine</i>	FqActinCc (CGAGGAACACCCTGTTTTGT)	RqActinCc (CGACCAGAAGCGTACAGTGA)

Technique	Exon and gene	Primer forward	Primer reverse
qPCR	α -tubuline	FqATCc (TGCCAATAACTACGCTCGTG)	RqATCc (CCACCGAATGAGTGGAAGAT)
qPCR	GPDH	FqGPDHCc (GGCTGCCGCGACATAAAGTA)	RqGPDHCc (GCGCACCACAACTTCAACG)

Quantitative PCR (qPCR) for the analysis of the expression of exons 3a, 3b and 7 was carried out in a Corbett Rotor-Gene 6000 (QIAGEN) in a volume of 15 μ l using 0.3 μ M of each oligonucleotide (Table 2.1), Brilliant III Ultra Fast SYBR Green QPCR 2X (Agilent Technologies, Texas, USA) and 5 μ l of cDNA template diluted 50-fold. qPCR conditions were as follows: an initial step at 95 °C for 3 min; 45 cycles of 95 °C for 10 s and 60 °C for 30 s; 50 °C for 3 min and a melting ramp of 50-99 °C raising by 1 °C and waiting for 5 s in each step. Actin-5C, glycerol-3-phosphate dehydrogenase (GPDH) and α -tubulin were used as reference genes to normalize the data.

PCR for the coding regions was performed in a volume of 25 μ l using 0.8 μ M of each oligonucleotide (Table 2.1), 0.5 U of AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific, Austin, USA), 10x PCR Buffer II, 1.5 mM MgCl₂, 0.2 mM dNTPs (Thermo Fisher Scientific, Austin, USA) and 5 μ l of the corresponding cDNA as template. PCR conditions were as described in section 2.2.2, and then PCR products were purified and sequenced.

2.2.4. GAL4>UAS targeted expression of Ccα6 isoforms in *D. melanogaster*

D. melanogaster transgenic flies with an insertion of wild-type Ccα6 isoforms 3a8b (MK251469) (strain UAS-Ccα6^{3a8b}) and 3b8a (MK251470) (strain UAS-Ccα6^{3b8a}) and the mRNA isoform carrying 3aQ68* mutation (strain UAS-Ccα6^{3aQ68*}) of the Ccα6 gene were created in the *dα6^{nx}* background (as per Perry *et al.*, 2015) to specifically express Ccα6 isoforms using the GAL4>UAS method (Brand and Perrimon, 1993).

2.2.4.1. Cloning of wild-type and mutant mRNA isoforms of Ccα6

Mutated and wild-type sequences of Ccα6 were obtained by conventional PCR from JW-100s resistant and C wild-type individuals, respectively. Samples were acquired by RNA extraction of single flies and cDNA synthesis was performed as described above. The oligonucleotides used (Sigma-Aldrich, Spain) are listed in Table 2.2.

The mutant allele was amplified through PCR in a volume of 25 µl using 0.4 µM of forward FnAch6ex1 and reverse RnAch6Q68 oligonucleotides, with reagents and conditions as mentioned above. Wild-type isoforms were amplified in a volume of 50 µl using 0.5 µM of forward FnAch6ex1nest and reverse RnAch6ex12 oligonucleotides, 2 U of iTaq™ DNA Polymerase (Biorad, California, USA), 5x HF Buffer, 0.2 mM dNTPs, and 8 µl of the corresponding cDNA diluted 100-fold as template. PCR conditions were as follows: an initial denaturation step at 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 1.5 min; and a final step of 72 °C for 7 min for full extension.

PCR products were cloned into the pGEM-T-easy vector (Promega Corporation, Madison, USA) following manufacturer's instructions. Transformation was performed by electroporation with a 2.5 V electric pulse for 4.6 ms using a MicroPulser™ electroporator (Bio-Rad, USA). Minipreps were performed using the alkaline lysis method (Engebrecht *et al.*, 1991). The amount of plasmid DNA was quantified using a NanoDrop (Thermo Fisher Scientific, Massachusetts, USA) and sequenced (Secugen, Madrid, Spain) to identify the isoform present and to detect any mutations introduced in the cloning process.

Table 2.2. Oligonucleotides used in the cloning process of *Ccα6* mRNA isoforms.

Primer	Sequence 5'→3'	Use
FnAch6ex1	CAACGGAAGCTGAAATCTAAGGAC	Mutant genotype
RnAch6Q68	AACCACGCATTTGTGGTCAGAATC	Mutant genotype
FnAch6ex1nest	TCGCTGTTTGCCGTGTTGATCTTT	Wild-type genotype
RnAch6ex12	GTCTATCCTTAAACCTCAATTTCC	Wild-type genotype
M13F	GTAAAACGACGGCCAG	Confirm transformation pGEM-T Easy
M13R	AACAGCTATGACCATG	Confirm transformation pGEM-T Easy
T7	TAATACGACTCACTATAGGG	Confirm transformation pUAS
T3	ATTAACCCTCACTAAAGGGA	Confirm transformation pUAS

2.2.4.2. Construction of vectors for transformation of *D. melanogaster*

Clones were digested with *NotI* (New England Biolabs, NEB) and ligated into digested pUASattB (Bischof *et al.*, 2007) using 1U DNA T4 ligase (Promega Corporation, Madison, USA) and 2X buffer. Transformation was performed using a standard heat

shock protocol. Clones with proper orientation were selected and cultures established. Plasmids were purified (Wizard® Plus SV Minipreps DNA Purification System, Promega, USA) and sequenced (Macrogen, South Korea) with T7 and T3 oligonucleotides (Table 2.2).

2.2.4.3. Microinjection of *Cca6* constructs to generate transgenic lines

Transgenic UAS-*Cca6* lines of *D. melanogaster* were produced by embryonic microinjection into 786G strain, which lacked expression of the *nACh α 6* gene (*d α 6^{nx}*) (Perry *et al.*, 2015). Plasmid DNA (100-250 mg/ μ l) was injected into the posterior pole of dechorionated 0-30 mins old embryos using a Femtojet microinjector (Eppendorf, USA) with a Femtoll needle (Eppendorf, Hamburg, Germany). Single-pair crosses were established with survivors that developed to the adult stage and then F1 individuals carrying the transgene (distinguishable by their eye color) were crossed to obtain homozygous lines.

To drive the expression of UAS constructs, homozygous transgenic males were crossed to females homozygous for the X-linked *D α 6>GAL4* which drives expression in cells that normally express *D α 6* (Perry *et al.*, 2015). This was conducted in the *d α 6^{nx}* background.

2.2.5. Susceptibility bioassays with spinosad

2.2.5.1. Susceptibility of *C. capitata*

The susceptibility of *C. capitata* Q68* and Q68*-K352* isolines to spinosad was tested with a topical application assay with increasing doses of this insecticide (Dow AgroSciences 88% p/p, Indianapolis, USA). Dilutions were prepared using acetone as a solvent for spinosad and for the non-treated control. A volume of 0.5 μ l of each solution was applied to each fly with an automated microapplicator 900X (Burkard Manufacturing Co., Hertfordshire, United Kingdom). Four replicates of each dose were performed with 15 flies per replicate. After the insecticide treatment, flies were confined in ventilated plastic dishes (89 mm in diameter and 23 mm in height) with water and the rearing diet (4 : 1 sugar : yeast) *ad libitum*, in an environmentally controlled chamber (Sanyo MLR-350-H, Sanyo, Japan), at 25 $\pm$ 1 °C and 16 h light and 8

h dark photoperiod (standard conditions). Mortality was recorded after 48 h. Flies were considered dead if they were ataxic.

2.2.5.2. Susceptibility of *D. melanogaster*

The *D. melanogaster* transgenic strains were also tested for susceptibility to spinosad. The resistant 786G strain (carrying $d\alpha 6^{nX}$) and $d\alpha 6^{nX};UAS-D\alpha 6-3a3b$ flies (that carries a susceptible nACh $\alpha 6$ transcript from *D. melanogaster*, isoform 3a3b) were used as positive and negative controls, respectively. Bioassays were performed by placing F1 first instar larvae in vials containing food with increasing doses of spinosad (0, 0.05, 0.5 and 5 ppm). Food was prepared using Instant Drosophila Medium (Carolina™, Burlington, North Carolina, USA). The experiment included five replicates of each spinosad concentration and five vials with non-treated food, with 50 larvae per vial. Vials were kept in the environmentally controlled chamber in constant darkness at 25 ± 1 °C. The number of flies that had emerged after 16-19 days was recorded.

2.2.6. Stability of $Cc\alpha 6^{3aQ68*\Delta 3b-4}$ and $Cc\alpha 6^{3aQ68*-K352*}$ alleles in *C. capitata*

Pupae from the Q68* ($Cc\alpha 6^{3aQ68*\Delta 3b-4}/Cc\alpha 6^{3aQ68*\Delta 3b-4}$) and Q68*-K352* ($Cc\alpha 6^{3aQ68*-K352*}/Cc\alpha 6^{3aQ68*-K352*}$) isolines and C strain ($Cc\alpha 6^+/Cc\alpha 6^+$) were isolated and the adults sexed immediately after emergence to guarantee virginity. Males and females from each strain were placed separately into ventilated plastic dishes and maintained with water and rearing diet for two days. The following reciprocal crosses were performed: 100 ♂Q68* x 100 ♀C and 100 ♀Q68* x 100 ♂C to obtain QC-G0 (genotype $Cc\alpha 6^{3aQ68*\Delta 3b-4}/Cc\alpha 6^+$); 100 ♂Q68*-K352* x 100 ♀C and 100 ♀Q68*-K352* x 100 ♂C to obtain KC-G0 (genotype $Cc\alpha 6^{3aQ68*-K352*}/Cc\alpha 6^+$); and 100 ♂Q68* x 100 ♀Q68*-K352* and 100 ♀Q68* x 100 ♂Q68*-K352* to obtain QK-G0 (genotype $Cc\alpha 6^{3aQ68*\Delta 3b-4}/Cc\alpha 6^{3aQ68*-K352*}$). The offspring of reciprocal crosses were pooled at G0. Three replicates of QC and KC crosses were established and maintained for 9 generations without insecticide selection pressure. Three replicates of the QK cross were established and maintained without selection until G1. At generation G2, each replica of QK was split into two, one continued without exposure to insecticide (QKnt) whereas the other one was exposed to 100 ppm spinosad for 72 hours (QKs) every

generation for 9 generations. Between 20 and 40 flies of each cross and generation were frozen at -20 °C for genotyping (see section 2.2.1).

2.2.7. Fitness of resistant isolines and the susceptible strain of *C. capitata*

Several assays were performed to analyze biological and behavioral traits in Q68* and Q68-K352* isolines and the C strain.

2.2.7.1. Developmental time to pupation and adults' weight

Eggs (corresponding to a volume of 50 µl) were collected and spread on larval rearing medium (160 g approx.) in containers (130 x 90 x 25 mm) covered with an aluminum foil to avoid desiccation. Containers were placed in 2 l ventilated plastic boxes allowing third instar larvae that jump from the food container and pupate to be collected. Eggs were kept in the environmentally controlled chamber in constant darkness at 25 ± 1 °C. Three replicates of each strain were prepared. The number of pupae obtained was recorded every day and the pupae removed from the box. 50 females and 50 males (2-3 days old adult flies) of each strain were collected and weighed with a precision balance (AM100, Mettler-Toledo, Switzerland).

2.2.7.2. Attraction to a parapheromone in a wind tunnel

The attraction of males for trimedlure (a parapheromone) was evaluated in a wind tunnel (Jang *et al.*, 1994). A piece of trimedlure dispenser weighing 0.52 g (Aralure, Agrisense, Pontypridd, United Kingdom) was suspended from the top of the wind tunnel (63 x 63 x 215 cm, Plexiglas flight tunnel, Analytical Research Systems, Gainesville, FL, USA) at a height of 20 cm, next to the air inlet, while 50 male adult flies were released downwind at the other end of the tunnel. A yellow sticky trap (Koppert, Berkel en Rodenrijs, The Netherlands) was located next to the trimedlure so that flies approaching the lure would be stuck. The rate of air flow was 0.2 m/s, the temperature 25 °C and the humidity 40%. Flies were left inside the tunnel for 30 minutes. The time taken for the first fly of each strain to reach the trap was recorded, as well as the number of caught flies in each 5 minutes interval. Each isolate/strain was evaluated 4 times. A control assay (same conditions, flies and yellow trap without trimedlure) was

performed to measure any effect of the yellow traps on flies. The number of flies stuck in the presence of trimedlure was corrected by the number of flies stuck without it.

2.2.7.3. Electrophysiological Antennal Activity

Electroantennography (EAG) was performed to determine if males were able to produce a response when stimulated with trimedlure (Cossé *et al.*, 1995). Heads of 3-7 days old adult flies were removed from the rest of the body and connected to two electrodes of an EAG probe type PRG-2 (Syntech) coated with a conductive Spectra 360 electrode gel (Parker Laboratories Inc., Fairfield, NJ, USA); one electrode was inserted into the tip of one antenna and the other into the back of the head. A Syntech IDAC 2 acquisition controller and a Syntech CS-55 stimulus controller (Ockenfels Syntech GmbH, Kirchzarten, Germany) were used to evaluate the electroantennographic response. GC-EAD 32 (v 4.3) software was employed to record the data. Serial dilutions of the tested compound were made in re-distilled HPLC-grade hexane. The solution (5 µl) was applied to a filter-paper strip located inside a 15 cm long Pasteur pipette. Control puffs of air (pipet containing a filter-paper with no impregnated solution) and a blank (filter-paper plus hexane) were performed with each head before the exposure to trimedlure. The response to air was used as a negative control as it showed low EAG activity, very close to 0 mV. The response to hexane was used as a positive control as *C. capitata* individuals show EAG activity when exposed to this compound. Between 5 and 15 replicates of each strain/isoline were performed. Thus EAG amplitudes recorded when exposed to trimedlure were corrected by the value observed with hexane exposure.

2.2.7.4. Male competition in mating

Mating assays were performed to study the mating success of males of the three strains. Virgin 3-5 days old males and females were used to make crosses with 10 females of the same strain and 12 males of each of the three strains. Five replicates of each cross were established. Flies were located in ventilated methacrylate boxes (12 cm diameter, 5 cm in height) provided with water and food *ad libitum*. Crosses were kept during 6 hours in a climatic room, at 26 ± 2 °C during the light hours. Then, individual females were placed in separate tubes containing rearing medium for the

laying of the eggs, and under standard conditions until F1 pupae were obtained (approximately 10 days). Three pupae derived from each female were genotyped (see section 2.2.1). The presence of $Cc\alpha 6^{3aQ68^*-K352^*}$, $Cc\alpha 6^{3aQ68^*\Delta 3b-4}$ and $Cc\alpha 6^+$ alleles allowed the genotype of the male mating with each female to be determined.

2.2.8. Statistics

In all experiments, Levene and Shapiro-Wilk tests were used to statistically analyze the homogeneity and normality of the data, respectively. Developmental time to pupation, weight of adult flies, first fly to reach the source of stimuli in the wind tunnel, competitive mating assays with *C. capitata* and Arcsin transformed susceptibility to spinosad of *D. melanogaster* transgenic lines were analysed by ANOVA followed by the Student Newman Keuls *post hoc* test. Expression levels of *Ccα6* exons were performed on Cq' values (log2 transformation of NRQs) using Student's t-Test (exonic regions 3a-4 and 2-3b) or ANOVA followed by Student Newman Keuls *post hoc* test (exonic regions 2-3a and 7). The attraction to trimedlure of a group of flies in wind tunnel was evaluated by a Repeated Measures ANOVA, also followed by Student Newman Keuls *post hoc* test. A Kruskal-Wallis test was performed for the analysis of EAG. χ^2 test was completed to test the fit of the data to expected genotypic frequencies in the stability assay. For the analysis of susceptibility to spinosad of *C. capitata* isolines/strains, mortality data were used to estimate LD₅₀ values (dose needed to cause 50% mortality) and Probit analysis was performed using the program POLO-PC (LeOra Software14), which corrects exposed samples' mortality with the control (non-treated) mortality using Abbott's transformation (Abbott, 1925). Resistance ratios (RR = LD₅₀ (lab strain/isoline) / LD₅₀ (C strain)) were considered significant if their 95% fiducial limits did not include 1 (Robertson and Preisler, 1992).

2.3. RESULTS

2.3.1. Expression of $Cc\alpha 6^{3aQ68^*}$, $Cc\alpha 6^{3a8b}$ and $Cc\alpha 6^{3b8a}$ mRNA isoforms in *D. melanogaster*

In order to study the contribution that the different *Ccα6* alleles make to spinosad resistance/susceptibility, it is important that they be compared in an identical genetic background. Such experiments are readily performed in *D. melanogaster*. Thus we

generated UAS transgenic *D. melanogaster* flies carrying wild-type mRNA isoforms *Cca6*^{3a8b} (UAS-*Cca6*^{3a8b}) and *Cca6*^{3b8a} (UAS-*Cca6*^{3b8a}), and mutant mRNA isoform *Cca6*^{3aQ68*} (UAS-*Cca6*^{3aQ68*}) (see section 2.2.4). We used the methods of Perry *et al.* (2015) to introduce the mRNA isoforms of *Cca6* into the resistant background of 786G strain, carrying the null *dα6*^{nx} allele.

Figure 2.2. Mortality percentages obtained from exposure to increasing spinosad concentrations of: a) F1 transgenic *Drosophila melanogaster* flies coming from the cross between the *Dα6*>GAL4 and the UAS-*Cca6* lines *Cca6*^{3a8b}, *Cca6*^{3b8a}, *Cca6*^{3aQ68*}, 786G and UAS-*Dα6*-3a3b (with a susceptible nACh6 transcript); and b) parental UAS-*Cca6* lines of *D. melanogaster* flies. All flies used in the crosses were homozygous for the *dα6*^{nx} allele (no *Dα6* expression). Different letters within each concentration indicate significant differences (ANOVA, Student Newman Keuls *post hoc* test, *p*≤0.05. Data were previously Arcsin transformed).

Bioassays of susceptibility to spinosad were performed with F1 flies from the cross between transgenic UAS-*Cca6* strains and the *Dα6*>GAL4;*dα6*^{nx} driver strain (Figure 2.2.a). Flies expressing UAS-*Cca6*^{3a8b} and UAS-*Cca6*^{3b8a} had close to 100% mortality when exposed to 0.5 ppm of spinosad in the food (red and green histogram bars,

respectively, in Figure 2.2.a). Those flies expressing UAS-Ccα6^{3aQ68*} only had approximately 20% mortality, even at the highest concentration tested (5 ppm) (purple histogram bar in Figure 2.2.a). As expected, the offspring from the control cross (*Dα6>GAL4;dα6^{nx}* x 786G) did not exhibit significant mortality at any of the tested concentrations of spinosad, while mortality of the F1 produced by crossing *Dα6>GAL4;dα6^{nx}* with *dα6^{nx};UAS-Dα6-3a3b* (the susceptible *nACh6* transcript) was greater than 60% at all concentrations tested (blue and orange histogram bars, respectively, in Figure 2.2.a). We found that UAS-Ccα6^{3a8b} and UAS-Ccα6^{3b8a} strains also showed spinosad susceptibility when we tested them without driving the expression of the transgene (Figure 2.2.b). This could be due to basal expression of the transgenes, and suggests that minimal expression levels of Ccα6 are enough for spinosad to be toxic. Thus, rescue experiments showed that *nACh6* wild-type mRNA isoforms 3a8b and 3b8a are capable of rescuing the response to spinosad, while the mRNA isoform carrying the 3aQ68* mutation do not.

2.3.2. Characterization of Q68* and Q68*-K352* isolines of *C. capitata*

To study the contribution of resistant alleles *Ccα6*^{3aQ68*} and *Ccα6*^{3aQ68*-K352*} to the resistant phenotype of the laboratory strain JW-100s, we generated two isolines homozygous for each allele and we named them Q68* (*Ccα6*^{3aQ68*}/*Ccα6*^{3aQ68*}) and Q68*-K352* (*Ccα6*^{3aQ68*-K352*}/*Ccα6*^{3aQ68*-K352*}) (see section 2.2.1). When obtained, we characterized them molecularly to check if they carried the expected genotypes.

We first sequenced the cDNA of *Ccα6* and found that Q68* isline had transcripts where exon 3a was directly followed by exon 5, and transcripts with exon 2 followed by exon 5, indicating that exons 3b and 4 were not transcribed. In addition, we observed that when exons 3b and 4 were absent, a change in the reading frame of *Ccα6* occurred, producing the appearance of a premature stop codon at the beginning of exon 5. Next, we observed that exons 3b and 4 were not present in gDNA of Q68* isline, while intronic region downstream of exon 3a and upstream of exon 5 were present, indicating that a deletion must have occurred in between these exons. Conversely, when we checked the sequence of both the gDNA and the cDNA of isline Q68*-K352*, we found that all exons were present and transcribed. To understand if the deletion of exons had occurred during the generation of Q68* isline or if it was

already present in JW-100s, we checked the gDNA and cDNA of JW-100s individuals carrying the allele $Cca6^{3aQ68*}$ of generations F29 (30% of individuals homozygous $Cca6^{3aQ68*}/Cca6^{3aQ68*}$) and F85 (92% of individuals homozygous $Cca6^{3aQ68*-K352*}/Cca6^{3aQ68*-K352*}$ and just 8% of individuals heterozygous $Cca6^{3aQ68*}/Cca6^{3aQ68*-K352*}$). We found that $Cca6^{3aQ68*}$ allele carried all exons in F29, while exons 3b and 4 were absent in F85, suggesting that the deletion happened at an unknown generation during the selection of JW-100s strain in the laboratory. Furthermore, qPCR analysis to test the expression of exons 3a and 3b in Q68* and Q68*-K352* isolines confirmed that exons 3b and 4 were not expressed in Q68* (Figure 2.3). The results also indicated that expression of both 3a and 3b exons was significantly lower in Q68*-K352* isolate than in C strain (Figure 2.3).

Figure 2.3. Expression of *Cca6* gene in C strain, and Q68* and Q68*-K352* isolines of *Ceratitis capitata*. Oligonucleotides were designed for the exonic regions: 2-3a, 3a-4, 3b and 7. Data are mean $\pm$ standard error of 3 pools of 6 individuals normalized to the reference genes *actine*, *glycerol-3-phosphate dehydrogenase (GPDH)* and α -*tubuline*, and relative to C strain. Different letters within each exonic region indicate significant differences (exonic regions 3a-4, 2-3b, 3b-4: Student's T-test; exonic regions 2-3a and 7: ANOVA, Student Newman Keuls *post hoc* test, $p \leq 0.05$).

The compendium of results of sequence and expression of *Cca6* allowed to elucidate that $Cca6^{3aQ68*}$ allele had lost exons 3b and 4 during the selection of JW-100s giving

rise to a new allele $Cca6^{3aQ68*\Delta3b-4}$, which gives rise to isoforms that truncate at exon 3a, and to isoforms lacking exons 3b and 4 that truncate at exon 5 (Figure 2.4). On the other hand, Q68*-K352* isolate kept the allele $Cca6^{3aQ68*-K352*}$ as originally was in JW-100s strain, though the expression of both 3a and 3b isoforms was reduced when compared to the C strain.

We corroborated that the multiplex protocol developed by Ureña *et al.* (2019) to identify the Q68* mutation in exon 3a in the $Cca6^{3aQ68*}$ allele (see section 2.2.1) can also be used to detect this mutation in the $Cca6^{3aQ68*\Delta3b-4}$ allele.

Figure 2.4. *Cca6* gene structure and its mRNA isoforms in: a) C strain; b) JW-100s strain at generation F29; c) Q68* isolate and d) Q68*-K352* isolate of *Ceratitis capitata*. Dark grey structures are truncated isoforms carrying stop codons, light grey means isoforms lacking some exons and white refers to non-truncated isoforms. Sections a) and b) have been extracted from Ureña *et al.* (2019).

We performed bioassays on the isolines in order to compare the levels of resistance in them with that reported for JW-100s. Feeding spinosad at the concentration used in field treatments (260 ppm in bait sprays) did not cause mortality in either isolate. Feeding bioassays could not be performed at higher concentrations because of the limited solubility of spinosad in aqueous solutions. Topical bioassays with a range of doses of spinosad (Table 2.3) indicated that both Q68* and Q68*-K352* isolines had similar levels of resistance, with resistance ratios (RR) of 3139 and 3602, respectively, when compared to the susceptible C strain. These data indicate that both alleles contribute equally to the resistant phenotype of JW-100s (RR = 1794, Ureña *et al.*, 2019).

Table 2.3. Resistance levels to spinosad of JW-100s and C strains and Q68* and Q68*-K352* isolines of *Ceratitis capitata*.

Isoline/strain	n ⁽¹⁾	slope ± SE	LD ₅₀ ⁽²⁾ (95%FL)	χ^2 ⁽³⁾	df	RR (95%FL) ⁽⁴⁾
JW-100s ⁽⁵⁾	419	4.74 ± 0.61	185 (142-224)	83.7	22	1794 (1156-2785)#
Q68*-K352*	390	2.26 ± 0.30	371 (284-483)	35.3	21	3602 (2253-5757) #
Q68*	390	3.59 ± 0.45	323 (266-378)	26.6*	21	3139 (2000-4927) #
C	306	2.34 ± 0.67	0.10 (0.08-0.18)	15.6*	18	1

⁽¹⁾ Number of flies considered in the Probit analysis (including non-treated).

⁽²⁾ Lethal dose (LD₅₀) in µg/g of insect (fresh weight assuming an average weight of 10 mg) for topical assays at 48 h performed with spinosad 88% technical grade insecticide (a 0.5 µL drop of insecticide solution in acetone or acetone alone was applied to the dorsal thorax of each fly by using an automatic microapplicator).

⁽³⁾ Good fit (*) of the data to the probit model (p>0.05).

⁽⁴⁾ Resistance ratio (RR) = [LD₅₀ (selected strain) / LD₅₀ (C strain)]. The fiducial limits for RR were calculated according to Robertson and Preisler (1992). # RR is significant (p≤0.05) if the 95% FL does not include 1.

⁽⁵⁾ Data from Ureña *et al.* (2019).

2.3.3. Stability of spinosad resistant alleles *Ccα6*^{3aQ68*-K352*} and *Ccα6*^{3aQ68*Δ3b-4} in

C. capitata

In order to study the stability of *Ccα6*^{3aQ68*-K352*} and *Ccα6*^{3aQ68*Δ3b-4} alleles in *C. capitata*, we performed crosses among Q68* and Q68*-K352* isolines and C strain to obtain individuals heterozygous for one of the resistant alleles and the wild-type *Ccα6*⁺

allele or heterozygous for both resistant alleles. We then followed the frequency of the different genotypes in the successive generations (Table 2.4).

The fate of the different *Ccα6* alleles depended on the crosses performed (Table 2.4). In QC and KC crosses that carried only one of the resistant alleles (*Ccα6*^{3aQ68*Δ3b-4} or *Ccα6*^{3aQ68*-K352*}) and the wild type allele (*Ccα6*⁺), the genotypic frequencies obtained in G1 corresponded to those expected under Mendelian inheritance (25% homozygous wild-type, 50% heterozygous and 25% homozygous resistant). After this we observed a rapid and continuous decline in the frequency of homozygous resistant genotypes, not observed after G7. Heterozygous individuals remained at low frequency after 9 generations. On the other hand, when both *Ccα6*^{3aQ68*-K352*} and *Ccα6*^{3aQ68*Δ3b-4} alleles coexisted in the QK cross, the typical frequencies of the Mendelian inheritance were maintained in Hardy-Weinberg equilibrium until the end of the experiment (G9), in both untreated (“nt” in Table 2.4) and spinosad selected conditions (“s” in Table 2.4). This result indicates that *Ccα6*^{3aQ68*Δ3b-4} and *Ccα6*^{3aQ68*-K352*} resistant alleles have a fitness cost compared to the wild type allele, and that it is higher in homozygosis than in heterozygosis. However, no difference in fitness cost was observed between both strains.

Table 2.4. Genotype frequencies (%) in the offspring of the crosses between Q68* and Q68*-K352* isolines and C strain of *Ceratitis capitata*.

Crosses ⁽¹⁾	Generation	Genotypes ($\bar{X} \pm SE$) ⁽²⁾			Fit the model (χ^2 test) ⁽³⁾	
Q68*x Q68*-K352* (QK)	nt	$Cca6^{3aQ68*\Delta3b-4}$ / $Cca6^{3aQ68*\Delta3b-4}$	$Cca6^{3aQ68*\Delta3b-4}$ / $Cca6^{3aQ68*-K352*}$	$Cca6^{3aQ68*-K352*}$ / $Cca6^{3aQ68*-K352*}$		
		G0	0 ± 0	100 ± 0	0 ± 0	*
		G1	40.8 ± 3.8	50.9 ± 4.7	8.3 ± 8.3	
		G3	16.7 ± 4.2	66.7 ± 4.2	16.7 ± 4.2	*
		G5	46.7 ± 3.3	33.3 ± 8.8	20 ± 11.6	
		G7	20 ± 5.8	46.7 ± 3.3	33.3 ± 3.3	*
	G9	30 ± 5.8	56.7 ± 12	13.3 ± 6.7	*	
	s	G3	25 ± 12.5	58.3 ± 18.2	16.7 ± 8.3	*
		G5	23.3 ± 3.3	36.7 ± 8.8	40 ± 5.8	*
		G7	40 ± 10	36.7 ± 6.7	23.3 ± 14.5	*
G9		40 ± 10	46.7 ± 8.8	13.3 ± 3.3	*	
Q68* x C (QC)		$Cca6^+ / Cca6^+$	$Cca6^{3aQ68*} / Cca6^+$	$Cca6^{3aQ68*\Delta3b-4}$ / $Cca6^{3aQ68*\Delta3b-4}$		
		G0	0 ± 0	100 ± 0	0 ± 0	*
		G1	34.4 ± 3.6	52.6 ± 4.6	13 ± 5	*
		G3	54.2 ± 10.5	45.8 ± 10.5	0 ± 0	
		G5	55.6 ± 13.6	39.7 ± 8.8	4.8 ± 4.8	
		G7	75.4 ± 17	24.6 ± 17	0 ± 0	
		G9	70.6 ± 7.6	29.4 ± 7.6	0 ± 0	

Crosses ⁽¹⁾	Generation	Genotypes ($\bar{X} \pm SE$) ⁽²⁾			Fit the model (χ^2 test) ⁽³⁾
		$Cca6^+/Cca6^+$	$Cca6^{3a \text{ Q68*}-K352*}/Cca6^+$	$Cca6^{3a \text{ Q68*}-K352*}/Cca6^{3a \text{ Q68*}-K352*}$	
Q68*-K352* x C (KC)	G0	0 $\pm$ 0	100 $\pm$ 0	0 $\pm$ 0	*
	G1	23.3 $\pm$ 18.6	60 $\pm$ 17.3	16.7 $\pm$ 12	*
	G3	58.3 $\pm$ 15	33.3 $\pm$ 18.2	8.3 $\pm$ 4.2	
	G5	52.2 $\pm$ 7.8	42.2 $\pm$ 2.2	5.6 $\pm$ 5.6	
	G7	75 $\pm$ 25	25 $\pm$ 25	0	
	G9	80 $\pm$ 10	20 $\pm$ 10	0	

⁽¹⁾ Individuals of the Q68* and Q68*-K352* isolines were crossed among them (QK) and with individuals of the C strain (QC and KC, respectively). Both QC and KC were maintained without selection pressure for 9 generations. QK was split at G2 in two different populations: one maintained without selection pressure (QKnt) and the other one selected with spinosad (QKs).

⁽²⁾ There were three replicates of each cross. Between 20 and 40 flies of each cross and generation were genotyped.

⁽³⁾ Good fit (*) of data to the expected genotypic frequencies (0, 100 and 0 % in G0, and 25, 50 and 25 % for all the other generations for first homozygous, heterozygous and second homozygous, respectively). χ^2 test was performed for data analysis ($p > 0.05$).

2.3.4. Fitness of Q68* and Q68*-K352* isolines of *C. capitata*

We analyzed biological and behavioral traits in the Q68* and Q68*-K352* isolines of *C. capitata* and compared them to C strain in order to associate the existence of any fitness cost with the presence of a mutant allele conferring resistance.

2.3.4.1. Biological traits

We compared the embryo to pupal viability of the isolines and the C strain by analyzing the number of pupae obtained from a volume of 50 µl of eggs. No significant differences were observed (Table 2.5).

The analysis of embryo to pupa development time showed that Q68* isoline developed significantly slower than C strain, with the Q68*-K352* isoline developing even slower (Table 2.5). We also checked if there were differences in adult weight between these genotypes. It was equivalent for females of all strains (Table 2.5), but males from the resistant Q68* and Q68*-K352* isolines were significantly heavier than those from the susceptible C strain.

Table 2.5. Biological parameters of adult flies from C strain and Q68* and Q68*-K352* isolines of *Ceratitis capitata*.

	C	Q68*	Q68*-K352*
Number of pupae⁽¹⁾	878 ± 102 (a)	755 ± 52 (a)	909 ± 45 (a)
Days from egg to pupae⁽²⁾	8.77 ± 0.01 (a)	9.3 ± 0.01 (b)	9.46 ± 0.01 (c)
Females' weight (g)⁽³⁾	8.27 ± 0.14 (a)	8.6 ± 0.15 (a)	8.5 ± 0.15 (a)
Males' weight (g)⁽³⁾	7.34 ± 0.11 (a)	8.29 ± 0.15 (b)	7.95 ± 0.13 (b)

⁽¹⁾ Number of pupae obtained from a volume of eggs of 50 µl. Three replicates were performed with each strain/isoline.

⁽²⁾ n = number of pupae obtained for each strain/isoline in the table.

⁽³⁾ n = 50 adults 2-3 days old.

Data are mean ± standard error. Different letters within each row indicate significant differences (ANOVA, Student Newman Keuls *post hoc* test, $p \leq 0.05$).

2.3.4.2. Behavioral traits

We performed a wind tunnel assay to study the ability of male flies of *C. capitata* to get attracted by the synthetic parapheromone trimedlure. We saw that males from

Q68* isoline had a lower response to trimedlure than males from C strain and Q68*-K352* isoline, when considered the percentage of males responding to trimedlure during an exposition of 30 minutes (Figure 2.5.a) and the time needed by the first fly to reach the parapheromone (Figure 2.5.b). On the contrary, the ability of males of Q68*-K352* isoline to respond to the parapheromone resulted similar to that of the wild type.

We also carried out an EAG assay to analyze if males' antennal receptors were able to detect the parapheromone trimedlure. Among all the concentrations tested, we observed significant differences only with a dose of 5 ng of trimedlure, where the response in Q68*-K352* was higher than in the C males (Figure 2.6). We did not observe any significant difference between Q68* and C strain at any of the concentrations tested.

We conducted competitive mating assays to find out if there were differences in fecundation success when males of the different strain/isolines were put together with a group of females of a specific strain/isoline. We did not observe significant differences in the number of fecundated females when females belonged to C strain or Q68* isoline. However, Q68*-K352* males were more successful in fecundating Q68*-K352* females than Q68* males (Figure 2.7).

The results of the wind tunnel, the EAG and the mating assays indicate that males carrying the $Cc\alpha 6^{3aQ68*\Delta 3b-4}$ allele have impaired ability to detect the parapheromone and to mate females carrying the $Cc\alpha 6^{3aQ68*-K352*}$ allele.

Figure 2.5. Wind tunnel assay with males of C strain and Q68* and Q68*-K352* isolines of *Ceratitis capitata*; a) percentage of males that respond to trimedlure during 30 minutes; b) time needed by the first fly of each strain/isoline to reach the trimedlure source. Data are mean $\pm$ standard error of 4 replicates with 50 adult males in each one. Different letters within each row indicate significant differences (Repeated Measures ANOVA in male response during 30 minutes and ANOVA in first fly attracted, in both cases followed by Student Newman Keuls *post hoc* test, $p \leq 0.05$).

Figure 2.6. Electroantennography response (mV) of males of C strain and Q68* and Q68*-K352* isolines of *Ceratitis capitata* when exposed to increasing doses of trimedlure. Data are mean $\pm$ standard error of 5-15 replicates. Different letters within each amount of trimedlure indicate significant differences (ANOVA, Student Newman Keuls *post hoc* test, $p \leq 0.05$).

Figure 2.7. Number of males of C strain and Q68* and Q68*-K352* isolines of *Ceratitis capitata* that, under competitive conditions, mated with females of the three strain/isolines. Data are mean $\pm$ standard error of 5 replicates with 10 females of a specific strain/isoline and 12 males of all strain/isolines. Different letters within each group indicate significant differences (ANOVA, Student Newman Keuls *post hoc* test, $p \leq 0.05$).

2.4. DISCUSSION

In a previous study it was shown that medflies homozygous for $Cc\alpha 6^{3aQ68*}$ allele were resistant to spinosad, despite generating wild-type full-length $Cc\alpha 6$ isoforms with exon 3b (Ureña *et al.*, 2019). Here, we have used the GAL4>UAS expression system to introduce in *D. melanogaster* $\alpha 6$ loss of function mutants three different $Cc\alpha 6$ isoforms: 1) the isoform carrying 3aQ68* mutation, encoded by $Cc\alpha 6^{3aQ68*}$ allele; 2) the wild-type mRNA isoform 3b8a, encoded by both $Cc\alpha 6^{3aQ68*}$ and $Cc\alpha 6^{+}$ alleles; and 3) the wild-type mRNA isoform 3a8b, encoded by $Cc\alpha 6^{+}$ allele. As expected, the expression of the isoform carrying mutation 3aQ68* does not rescue susceptibility to spinosad, confirming that the truncated transcripts do not produce a response to spinosad (Baxter *et al.*, 2010; Rinkevich *et al.*, 2010; Hsu *et al.*, 2012b). On the contrary, we found that the expression of both wild-type $Cc\alpha 6^{3a8b}$ and $Cc\alpha 6^{3b8a}$ isoforms rescued spinosad susceptibility in the $\alpha 6$ -deficient strain, as already reported for $\Delta\alpha 6$ isoforms from susceptible *D. melanogaster* strains and of orthologues from susceptible strains of a variety of species (Perry *et al.*, 2011, 2015; Anstead *et al.*, 2015). Ureña *et al.* (2019) hypothesized that the absence of wild type isoforms containing exon 3a in individuals homozygous for $Cc\alpha 6^{3aQ68*}$ allele could be enough to confer resistance to spinosad in medfly, since it has been suggested that some isoforms could be more responsive to spinosad or better able to form functional receptors than others (Perry *et al.*, 2015). However, our results indicate that medfly 3b8a isoform is enough to mediate spinosad toxicity. We must keep in mind the fact that the GAL4>UAS system usually confers higher expression levels than that observed under native conditions, which could mean that the susceptible phenotype observed could be due to 3b8a isoform being expressed at abnormally high, non-physiological levels. Nonetheless, we have found that UAS- $Cc\alpha 6^{3b8a}$ construct in the absence of GAL4 driver is sufficient to partially rescue the susceptibility to spinosad, as also observed with UAS- $Cc\alpha 6^{3a8b}$, which suggests that basal expression of both isoforms is enough for spinosad to be toxic, as observed previously (Perry *et al.*, 2015). Alternative explanations for resistance conferred by $Cc\alpha 6^{3aQ68*}$ allele, despite the expression of susceptible 3b isoforms, have been proposed: i) that truncated 3a isoforms may interfere with the correct assembly of nAChR in medfly, and ii) the presence of undetected mutations in non-coding regions that could affect the transcript stability or

the translation of full-length wild-type 3b isoforms (Ureña *et al.*, 2019). Further studies are required to prove if the 3b8a isoform encoded by the $Cc\alpha 6^{3aQ68*}$ allele has affected its functionality in medfly.

Remarkably, when we tried to obtain an isoline homozygous for the $Cc\alpha 6^{3aQ68*}$ allele, we realized that the resulting Q68* iseline was homozygous for a new $Cc\alpha 6^{3aQ68*\Delta 3b-4}$ allele composed by isoforms that truncate at exon 3a and isoforms lacking exons 3b and 4 as a consequence of a deletion in the gDNA that truncate in exon 5 (Figure 2.4). We confirmed that the allele $Cc\alpha 6^{3aQ68*}$ expressing full-length wild-type 3b isoforms was present in JW-100s individuals from generation F29, as already reported (Ureña *et al.*, 2019). However, we found that heterozygous $Cc\alpha 6^{3aQ68*\Delta 3b-4}/Cc\alpha 6^{3aQ68*-K352*}$ individuals of JW-100s strain from generation F85, about 10 generations previous to the parents that were used to generate the Q68* iseline, already lacked exons 3b and 4. Thus, $Cc\alpha 6^{3aQ68*\Delta 3b-4}$ allele might have appeared at an undetermined point during the selection of JW-100s with spinosad. It is probable that this new allele had been generated from $Cc\alpha 6^{3aQ68*}$. Nevertheless, we cannot discard that $Cc\alpha 6^{3aQ68*\Delta 3b-4}$ was already present at very low frequency in JW-100s. Regardless of its origin, it could have been selected instead of $Cc\alpha 6^{3aQ68*}$ because of better stability or lower fitness cost. Insect *nAChR* subunit genes have a complex process of splicing and different isoforms are usually expressed in natural conditions (Sattelle *et al.*, 2005). Indeed, the existence of *nAChR* transcripts lacking some exons appear to be common in insects (Jones *et al.*, 2006). This includes $\alpha 6$ transcripts missing both forms of exon 3 that have been identified at low proportion in wild-type populations of *Bombyx mori*, *Tribolium castaneum*, *Tuta absoluta* and *Plutella xylostella* (Shao *et al.*, 2007; Rinkevich and Scott, 2009; Berger *et al.*, 2016; Wang *et al.*, 2016). Indeed, Berger *et al.* (2016) reported that spinosad resistance in *T. absoluta* was associated with altered *nAChR* $\alpha 6$ transcripts that lacked exon 3a/3b, though in this case both exons were present and unaltered at the genomic level, indicating that their absence in mRNA results from exon skipping rather than a deletion in gDNA. It is not known if *nAChR* transcripts with stop codons and/or exclusion of exons are translated, and if so, the functional role of the resulting proteins, though some regulatory and modulatory roles have been proposed (Sattelle *et al.*, 2005). In any case, our results and those from other studies

highlight the plasticity of nAChR $\alpha 6$ to generate a tremendous diversity of isoforms, including truncated isoforms potentially unaffected by spinosad.

An important factor that will determine the progress of spinosad resistance in field populations is the fitness cost of resistance. We found that both $Cca\alpha 6^{3aQ68*\Delta 3b-4}$ and $Cca\alpha 6^{3aQ68*-K352*}$ alleles were not stable in the absence of spinosad when individuals from isolines homozygous for these two alleles were crossed with individuals carrying the wild-type allele $Cca\alpha 6^+$. Our results indicate that both resistant alleles may impart a competitive disadvantage with respect to the wild-type allele. However, no differences among the stability of $Cca\alpha 6^{3aQ68*\Delta 3b-4}$ and $Cca\alpha 6^{3aQ68*-K352*}$ alleles was perceived when they competed between them, both in the presence or absence of insecticide. Besides, both resistant alleles conferred similar levels of spinosad resistance, which indicated that differences in susceptibility did not play a relevant role in the competition between resistant alleles in the presence of the insecticide. Fitness cost associated with spinosad resistance has been reported to range from minor (Wyss *et al.*, 2003) or absent (Bielza *et al.*, 2008; Abbas *et al.*, 2014b) to sustancial (Li *et al.*, 2007b, 2017; Sayyed *et al.*, 2008; Wang *et al.*, 2010; Rehan and Freed, 2015; Okuma *et al.*, 2018). However, the genetic mechanism underlying spinosad resistance was only known in two of these studies, and in both cases it was associated with overexpression of detoxifying enzymes (Sayyed *et al.*, 2008; Rehan and Freed, 2014). In order to identify trade-offs associated with spinosad resistance in *C. capitata*, extensive testing of both biological and behavioral traits was undertaken. We found that the effect on the biological traits tested was moderate, with Q68* ($Cca\alpha 6^{3aQ68*\Delta 3b-4}/Cca\alpha 6^{3aQ68*\Delta 3b-4}$) and Q68*-K352* ($Cca\alpha 6^{3aQ68*-K352*}/Cca\alpha 6^{3aQ68*-K352*}$) isolines showing longer developmental time but higher male weight than C strain, whereas females of both resistant isolines and susceptible C strain did not differ in weight. However, mutations that would lead to a loss-of-function phenotype may affect behavioral patterns, as it has been reported for a D α 1 knock-out mutant with a significant fitness cost in terms of severe mating behavior defects and an altered sleep phenotype (Somers *et al.*, 2017a). Thus, we explored the possibility that behavioral fitness traits (parapheromone detection by wind tunnel and electrophysiological antennal assays, and mating success) could be affected in males of the Q68* and Q68*-K352* isolines. We found that males from Q68* isolate, homozygous for $Cca\alpha 6^{3aQ68*\Delta 3b-4}$ allele, had impaired ability to find the

parapheromone source in a wind tunnel and to mate females, though EAG assays demonstrated that this impediment was not due to a failure in the response of receptors in the antennae. This could point to a failure in the flight tools to properly move inside the tunnel, or to a difficulty to orient themselves when they receive a stimulus, though further studies would be needed to confirm these hypotheses. A study focused on the competitive mating of a spinosad resistant strain of *H. virescens* concluded that resistant adults must have a reproductive disadvantage, but they were not able to discern if it was due to differences in mating success or in fecundity (Wyss *et al.*, 2003). On the contrary, a homozygous $Cc\alpha 6^{3aQ68^*-K352^*}$ allele in the Q68*-K352* isoline does not imply a handicap for the male's ability to detect the parapheromone and to mate with females when compared to males of the C strain. Therefore, other fitness traits may be affected by the $Cc\alpha 6^{3aQ68^*-K352^*}$ allele that make it unstable when in competition with the wild-type allele, as it is possible that different isoforms generated by each resistant allele may interfere differently with the normal function of the receptor and with other nAChR subunits that $\alpha 6$ may also co-assemble with.

Our results highlight the plasticity of *C. capitata* to generate a wide range of mutations in nAChR $\alpha 6$ that can lead to loss-of-function phenotypes potentially conferring spinosad resistance. However, these resistant alleles have a fitness cost that makes them unstable when in competition with wild-type alleles, which could be the reason for the absence of resistance events in the field (Ureña *et al.*, 2019). In addition, the rate of resistance evolution in field populations is expected to be slow, due to the completely recessive nature of spinosad resistance in this species (Ureña *et al.*, 2019). Nonetheless, once resistance evolved it would be expected to increase to fixation if the selection pressure eliminated all susceptible insects. Moreover, the selection of compensatory mutations that ameliorate the fitness cost of existing resistant genotypes, or the selection of less costly new resistant alleles could accelerate the evolution of resistance (Raymond *et al.*, 2001; Ffrench-Constant and Bass, 2017). Thus, insecticide resistance management strategies should be implemented to ensure the sustainability of spinosad.

**CHAPTER 3. INHERITANCE OF RESISTANCE TO LAMBDA-CYHALOTHRIN
AND SEARCH OF MOLECULAR MARKERS ASSOCIATED WITH RESISTANCE**

CHAPTER 3: INHERITANCE OF RESISTANCE TO LAMBDA-CYHALOTHRIN AND SEARCH OF MOLECULAR MARKERS ASSOCIATED WITH RESISTANCE

3.1. RATIONALE FOR THE STUDY

Lambda-cyhalothrin is one of the most widely used insecticides for *Ceratitis capitata* (medfly) control in Spain. However, evidences of field resistance to this insecticide have already been reported (Arouri *et al.*, 2015). The resistance mechanism was studied in the laboratory selected lambda-cyhalothrin resistant strain W-1Kλ, in which reversion of resistance by the synergist piperonyl butoxide (PBO) and over-expression of *CcCYP6A51* gene detected by qPCR pointed to a P450 mediated metabolic type of resistance (Arouri *et al.*, 2015). In addition, functional validation by expression of this gene in *Escherichia coli* and in *Drosophila melanogaster* confirmed, respectively, its ability to metabolize pyrethroids and that its induced expression confer a resistant phenotype (Tsakireli *et al.*, 2019). Nonetheless, the role of *CcCYP6A51* in resistance to lambda-cyhalothrin in field populations still has to be demonstrated.

Knowledge of the inheritance and genetics of resistance is needed for devising effective insecticide resistance management strategies. Previous studies in dipterans have found that resistance to lambda-cyhalothrin (Abbas *et al.*, 2014a) and other pyrethroids (Li and Liu, 2010; Khan *et al.*, 2015) varies from dominant to recessive when mediated by P450s. Besides, a single gene or polygenic traits can be involved in metabolic resistance to pyrethroids, including different CYP genes (Scott, 2017) and other genes associated with pesticide metabolism (Pedra *et al.*, 2004; Qiu *et al.*, 2013). However, the inheritance of resistance to lambda-cyhalothrin in *C. capitata* has not been determined.

A previous study about lambda-cyhalothrin resistance mechanism in *C. capitata* highlighted the difficulty to develop a diagnostic marker based on the expression of P450 genes because of the expected variability in field populations (Arouri *et al.*, 2015). Indeed, it has been reported that the expression of *CcCYP6A51* presents a very heterogeneous pattern with high variability both between and within field populations susceptible to pyrethroids from Greece (Voudouris *et al.*, 2018). Besides, we cannot discard that more than one CYP gene was involved in the resistance mechanism, so resistance arose as a combination of several genes with different pattern expression.

Thus, a more detailed description of what exactly is happening with these P450 genes at the molecular level in resistant strains is required for the potential development of a diagnostic tool based on the resistance mechanism.

DNA diagnostic markers are more simple and reliable methods than those based on transcripts (Devine and Denholm, 2009). A source of DNA polymorphism that has not been checked is the possibility of copy number variation (CNV) of genes involved in resistance (Feyereisen *et al.*, 2015; Weetman *et al.*, 2018), since P450 gene amplification has been associated with insecticide resistance in a number of species (Wondji *et al.*, 2009; Bariami *et al.*, 2012; Zimmer *et al.*, 2018). In addition, the availability of medfly genome sequence (Papanicolaou *et al.*, 2016) makes possible genome-wide studies scanning the genome or transcriptome to identify polymorphisms linked to insecticide resistance (Devine and Denholm, 2009). Variant calling analysis by Genotyping-By-Sequencing (GBS) approach analyzes all the genome by using methylation-sensitive restriction enzymes and has been considered the simplest and cost effective approach due to its simple library preparation and high level multiplexing capacity (Elshire *et al.*, 2011). In insects, GBS has been used to identify a quantitative trait loci (QTL) controlling resistance to the Bt toxin Cry1Fa in *Ostrinia nubilalis* (Coates and Siegfried, 2015).

The aims of our work were: 1) to study the inheritance of resistance to lambda-cyhalothrin in the resistant strain W-1Kλ; and 2) to analyze the expression and CNV of *CcCYP6A51* and other P450 genes aimed to improve our knowledge in the resistance mechanism; and 3) to search for molecular markers associated to the resistant phenotype.

3.2. MATERIALS AND METHODS

3.2.1. C. capitata strains

The laboratory strain W-1Kλ is resistant to lambda-cyhalothrin and was obtained from W-4Km (a malathion resistant strain derived from a field population collected in 2004 in Castelló) by laboratory selection with this insecticide (Arouri *et al.*, 2015). C, the susceptible strain, was originated from individuals belonging to non-treated experimental fields of the Instituto Valenciano de Investigaciones Agrarias (IVIA, València, Spain). It was set up in 2001 and it has been reared in the laboratory without

any exposition to insecticides since then. For the standard rearing conditions, insects were kept in an environmentally controlled chamber (Sanyo MLR-350-H, Sanyo, Japan), at 25 ± 1 °C and 16 h light and 8 h dark photoperiod, with water and rearing diet (4 : 1 sugar : yeast) *ab libitum*.

3.2.2. Study of the inheritance of lambda-cyhalothrin resistance

Adults from W-1Kλ and C strains were collected and their sex determined immediately after adult emergence. Males and females from each strain were placed separately into ventilated plastic dishes and maintained in standard conditions for two days. Reciprocal crosses (120 ♂W-1Kλ x 120 ♀C and 120 ♀W-1Kλ x 120 ♂C) were performed to obtain the F1 generation. The offspring of both reciprocal crosses was pooled and kept in the absence of selection pressure to produce the F2 generation. F1 was also backcrossed to the susceptible parent strain, C ((30 ♂F1.A, 30 ♂F1.B) x 60 ♀C and (30 ♀F1.A, 30 ♀F1.B) x 60 ♂C), to obtain the backcross (BC henceforth) (Figure 3.1).

Figure 3.1. Crosses performed for the study of the inheritance of lambda-cyhalothrin resistance in *Ceratitis capitata*. Parental strains used were W-1Kλ, selected in laboratory for lambda-cyhalothrin resistance, and susceptible laboratory strain C.

Susceptibility of the crosses F1, F2 and BC was determined by feeding bioassays with increasing concentrations of lambda-cyhalothrin (KarateZeon 10% p/v (100 g/l), Syngenta Limited, Surrey, United Kingdom) in the food (0.9 rearing diet : 0.1 insecticide (w/w)). Water was used to prepare dilutions, and also to mix with food for the non-treated control. For the assay, 4 replicates were set up, each one consisting on 15 flies 3 to 5 days old that were kept into ventilated plastic dishes (89 mm in diameter and 23 mm in height) at standard conditions. Flies were starved for 24 h before the exposition to insecticide and mortality was recorded after 48 h. Flies were considered dead if they were ataxic.

The dominance value (D_{LC}) of W-1K λ resistance was calculated using the formula $D_{LC} = \{[(2 \log LC_{50}F1 - \log LC_{50}P1 - \log LC_{50}P2) / (\log LC_{50}P1 - \log LC_{50}P2)] + 1\} / 2$; where P1 and P2 corresponded to parental strains W-1K λ and C, respectively (Bourguet *et al.*, 2000). Values ranged between 0 for completely recessive and 1 for completely dominant.

3.2.3. Bioassays with the synergist PBO

The synergist piperonyl butoxide (PBO; 90% technical, Aldrich, Milwaukee, WI) was diluted in acetone and applied topically to adult flies. Flies (3–5 days old) were anesthetized with CO₂ and treated with a 0.5 μ l drop of PBO solution in acetone or only acetone (used as control) on the dorsal thorax by using an automatic microapplicator 900 $\times$ (Burkard Manufacturing Co., Hertfordshire, United Kingdom). The dose applied (0.5 μ g PBO per insect) showed no mortality on adults. Three to four replicates of 10–15 adults were performed. After PBO treatment, insects were placed in ventilated plastic dishes containing water and rearing diet with lambda-cyhalothrin as previously described. The mortality was recorded after 48 h.

3.2.4. Expression and CNV analysis of P450 genes from *C. capitata*

The P450 genes selected for this study are listed in Table 3.1. They were *CcCYP6A51*, which was overexpressed in a previous work of resistance to lambda-cyhalothrin (Arouri *et al.*, 2015), and a group of genes that although did not show a significant over-expression in that work, presented low p-values. Nine are located with *CcCYP6A51* in the same cluster of 18 consecutive CYP genes (Papanicolaou *et al.*, 2016), while *CcCYP6D14* is located out of the cluster.

The expression of P450 genes was studied in pools and single individuals of the strains W-1K λ and C that had previously been exposed or not to lambda-cyhalothrin (as in section 3.2.2). Three pools of three males and three females were analyzed at each treatment tested, whereas 6 or 3 males of each treatment were evaluated at the individual level, depending on the experiment. Total RNA extraction was performed over the abdomens from single or pooled individuals with TRIzol[®] Reagent (Life Technologies, Van Allen Way, Carlsbad, California, USA) according to manufacturer's indications, and RNA was quantified using Nanodrop ND-1000 spectrophotometer

(Thermo Fisher Scientific, Waltham, MA, USA). It was followed by retrotranscription to complementary DNA (cDNA) using 2 µg RNA as a template and a commercial kit (Thermo Scientific, Massachusetts, USA), as described in Ureña *et al.* (2019). qPCR for the analysis of the P450 genes expression was carried out in a Corbett Rotor-Gene 6000 (QIAGEN) in a volume of 15 µl using 0.3 µM of each oligonucleotide (Table 3.1), Brilliant III Ultra Fast SYBR Green QPCR 2X (Agilent Technologies) and 5 µl of cDNA template diluted 5-fold. qPCR conditions were as follows: an initial step at 95 °C for 3 min; 45 cycles of 95 °C for 10 s and 60 °C for 30 s; 50 °C for 3 min and a melting ramp of 50-99 °C raising by 1 °C and waiting for 5 s in each step. To normalize the data, actin, glycerol-3-phosphate dehydrogenase (GPDH) and α -tubulin were used as reference genes.

Table 3.1. CYP genes studied and oligonucleotides used for qPCR analysis.

Gene ⁽¹⁾	Primer forward	Primer reverse
<i>CcCYP6A10</i>	ATCCACTCACCGGTCAACTC	CCACACAGACACAGATGGG CCACACAGACACAGATGGG
<i>CcCYP6A11</i>	CTGCGCGTTCGGTATAGATT	GGCGCTGATCTACAAACACA
<i>CcCYP6D14</i>	AGCCGGTAGTGAGACCACAT	AAGAGGATCACTGGGCTTCA
<i>CcCYP6A48</i>	GACGGAAGGCATTAATCGAA	TACGCACTGCACACACATTC
<i>CcCYP6A49</i>	CAGTCATTGCCATCGCTCTA	TGTTTTCGTCACGCCATTTA
<i>CcCYP6A50</i>	TGATGAGCATGTGTGGGATT	AATCCGACGTCCCATTACAC
<i>CcCYP6A51</i>	CGGAATACTCCCTGATCCA	TATGTGAGACCGACCAACGA
<i>CcCYP6A66</i>	ACTACCGCATTTGGTTCGTC	TGGCAATAACGGGTATGGTT
<i>CcCYP6A67</i>	TCGTCACCGCTCTAACACTG	AAAGTGCGCGTCTTCAGAAT
<i>CcCYP6A70</i>	ACGTCGCCACTTCAGCTACT	GTACCGATGCCGCTCATATT
<i>CcCYP6A71</i>	CACTCACCGGTCACCTCTTT	CAAGCATGCACCAACTCACT
<i>actine</i>	CGAGGAACACCCTGTTTTGT	CGACCAGAAGCGTACAGTGA
<i>α-tubuline</i>	TGCCAATAACTACGCTCGTG	CCACCGAATGAGTGGAAGAT
<i>GPDH</i>	GGCTGCCGCGACATAAAGTA	GCGCACCACAACTTCAACG
<i>Ccp0</i>	TCCAGGCTCTCTCCATACCA	CGACTTTGTACCAGGCTTC

⁽¹⁾ All P450 genes are named according to Papanicolau *et al.*, 2016.

The number of gene copies was predicted in the same group of P450 genes. Genomic DNA extraction of 7 males from C strain and 7 males from W-1K λ (neither of them had been exposed to insecticide) was performed with the kit SpeedTools Tissue DNA

Extraction (Biotools), following the manufacturer's indications, and it was quantified using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The predicted copy number of P450 genes was analyzed by performing qPCR (same conditions as previously mentioned) using 5 ng of DNA as template, and oligonucleotides listed in Table 3.1. As a reference, a ribosomal gene (*Ccp0*) was used.

3.2.5. Search of markers involved in lambda-cyhalothrin resistance by variant calling analysis

Parental and F2 samples from the study of inheritance (section 3.2.2) were analyzed and compared to develop a variant calling analysis. For parental sample, a pool (males and females randomly selected) of 20 individuals of each strain, W-1Kλ and C, was studied. F2 sample consisted on 150 single individuals randomly selected among survivors and dead to 300 ppm of lambda-cyhalothrin. DNA of all of them was extracted with the kit Puregene Core Kit A (QIAGEN), following the manufacturer's indications, and quantified by a fluometric method (Qubit™, Invitrogen, Thermo Fischer Scientific, Eugene, Oregon, 2014).

GBS was performed for the analysis of F2 samples while whole-genome sequencing was implemented with the parental pools (Centre Nacional de Recerca Genòmica, CNAG, Barcelona, Spain). GBS approach required 400 ng of gDNA from each sample, which was digested using the endonuclease *ApeKI* (recognition site: G/CWCG). The digested product was ligated using T4 DNA ligase enzyme to uniquely barcoded adaptors. GBS libraries were constructed by mixing in equal proportion the products ligated to adaptors. They were then amplified, purified to remove the excess of adaptors and used for sequencing on HiSeq 2500 Illumina platform.

Bioinformatics analysis was performed at the Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV-UPV; València, Spain). Raw sequencing reads were checked using FastQC (Andrews, 2014), cleaned and aligned to *C. capitata* reference genome (assembly Ccap_2.1) using BWA-MEM (Li, 2013). Variant calling analysis was performed with FreeBayes (Garrison and Marth, 2012) to detect short sequence variations in read alignments, as single nucleotide polymorphisms (SNPs) and indels. Then, SnpEff (Cingolani *et al.*, 2013) was used to annotate and predict the effect of these genetic variants. Due to high variability in parental strains, an *in silico* Bulk

Segregant Analysis (BSA) was chosen in order to find SNPs with different allele frequency between the resistant and susceptible populations.

The polymorphic regions that resulted candidates for association to resistance were analyzed. Data were adjusted to a generalized linear model (GLM) assuming a binomial distribution using R (R Core Team, 2013). In addition, putative resistance haplotypes were manually estimated, and the cumulative effect on resistance of the combination of some of them was calculated by a punctuation system in which a score was given to each genotype according to the total amount of resistance haplotypes that carried.

3.2.6. Statistics

For the study of susceptibility to lambda-cyhalothrin of *C. capitata* crosses, LC₅₀ values (concentration needed to cause 50% mortality) were estimated with mortality data and Probit analysis, using the program POLO-PC (LeOra Software14). Abbott's transformation was used to correct samples' mortality by control (non-treated) mortality (Abbott, 1925). Resistance ratios (RR = LC₅₀ (W-1Kλ or crosses)/LC₅₀ (C strain)) were considered significant if their 95% fiducial limits did not include 1 (Robertson and Preisler, 1992). In the inheritance study, a χ^2 test was performed to prove the fit of the data to a monogenic model. In the analysis of the expression and the copy number of P450 genes, Levene and Shapiro-Wilk tests were performed to check homogeneity and normality of the data, respectively. A t-test was used to compare gene copy number between C and W-1Kλ strains. In the case of expression analysis, an ANOVA and Student Newman Keuls *post hoc* tests were completed on Cq' values (log2 transformation of NRQs) to compare between groups. In the sequencing analysis, genotypic frequencies obtained in the candidate regions were analyzed by a χ^2 test to check the fit of the data to expected genotypic frequencies in a random segregation.

3.3. RESULTS

3.3.1. Study of the inheritance of lambda-cyhalothrin resistance

Susceptibility bioassays with lambda-cyhalothrin were performed with the laboratory strains W-1Kλ, resistant to lambda-cyhalothrin, and C, susceptible, as well as with the F1, F2 and BC crosses (Figure 3.1). A LC₅₀ value of 17 ppm was estimated for the C

strain (Table 3.2.b), with mortality values higher than 95% at concentrations over 100 ppm of lambda-cyhalothrin (Table 3.2.a). However, mortality values below 15% were obtained with the W-1K λ strain for concentrations up to 1000 ppm (Table 3.2.a). W-1K λ strain could not be tested at concentrations over 1000 ppm because of the repellent effect of lambda-cyhalothrin on *C. capitata* at higher concentrations (personal observations). As we could not estimate the exact LC₅₀ value for W-1K λ , we assumed that it was over 1000 ppm, and thus the resistance ratio (RR) of W-1K λ with respect to C was estimated as >59 (Table 3.2.b). The mortality values of F1 reciprocal crosses turned out to be equivalent to the resistant parent's and equal in both reciprocal crosses (Table 3.2.a), indicating that lambda-cyhalothrin resistance was completely dominant and autosomic. As before, we assume that LC₅₀ values for F1 crosses were over 1000 ppm, which allows us to estimate the RR values as >59 and the dominance values (D_{LC}) as close to 1 (Table 3.2.b). The RR for F2 and BC crosses were 12.0 and 3.7, respectively (Table 3.2.a and 3.2.b). In both cases, the observed mortality at the discriminating concentration of 300 ppm (96% mortality for susceptible parental C and 3% mortality for resistant parental W-1K λ) did not fit the mortality expected for a dominant character under a monogenic model (Table 3.2.c), meaning that the inheritance of lambda-cyhalothrin resistance is not controlled by a single gene with Mendelian genetics.

3.3.2. Effect of PBO treatment on lambda-cyhalothrin resistance

We applied a topical pre-treatment with the synergist PBO (inhibitor of P450s) to flies from the W-1K λ strain that were next exposed to lambda-cyhalothrin. We observed that resistance to lambda-cyhalothrin was reverted, meaning that PBO pre-treatment dramatically increased the susceptibility of flies to lambda-cyhalothrin (60, 67 and 81% mortality at 30, 100 and 300 ppm of lambda-cyhalothrin, respectively), compared to that of control flies topically pre-treated with acetone (10% mortality at 300 ppm of lambda-cyhalothrin).

Table 3.2. Study of the inheritance of *Ceratitis capitata* resistance to lambda-cyhalothrin by analyzing in the parents W-1Kλ and C, F1, F2 and BC: a) the susceptibility to different concentrations of the insecticide, b) estimation of LC₅₀, resistance ratio (RR) and dominance value (D_{LC}), and c) fit of the inheritance segregation to a monogenic model.

a)	Mortality (%) ± SE (n ⁽¹⁾) to lambda-cyhalothrin (ppm)						
	3	10	30	100	300	1000	Non-treated
Parents							
C	2 ± 2 (60)	35 ± 12 (58)	84 ± 4 (57)	98 ± 2 (59)	96 ± 2 (55)		16 ± 8 (55)
W-1Kλ					3 ± 2 (60)	14 ± 5 (59)	0 (60)
F1 crosses							
F1A (♂W1Kλ x ♀C)		0 (30)	0 (30)	0 (30)	0 (30)	30 ± 12 (30)	0 (30)
F1B (♀W1Kλ x ♂C)		0 (30)	0 (30)	0 (30)	3 ± 3 (40)	27 ± 3 (30)	3 ± 3 (30)
F2 crosses							
F2 (♂♀F1A x ♂♀F1B)		10 ± 6 (60)	22 ± 11 (60)	42 ± 5 (60)	65 ± 10 (60)	77 ± 4 (60)	8 ± 6 (60)
Back crosses							
BC (♂♀F1AB x ♂♀C)		2 ± 2 (59)	42 ± 6 (60)	73 ± 3 (60)	84 ± 3 (120)	92 ± 4 (60)	7 ± 5 (59)
b)	n ⁽²⁾	Slope ± SE	LC ₅₀ ⁽³⁾ (95% FL)	χ ²	df	RR ⁽⁴⁾ (95% FL)	D _{LC} ⁽⁵⁾
Parents							
C	344	2.34 ± 0.24	17 (7-35)	116.4*	18	1	
W-1Kλ	239		>1000			>59	
F1 crosses							
F1A (♂W1Kλ x ♀C)	180		>1000			>59	~1
F1B (♀W1Kλ x ♂C)	190		>1000			>59	~1
F2 crosses							
F2 (♂♀F1A x ♂♀F1B)	360	1.41 ± 0.18	205 (115-348)	35.0*	18	12.0 (7.4-19.4) [#]	
Back crosses							
BC (♂♀F1AB x ♂♀C)	418	1.57 ± 0.18	63 (38-98)	38.0*	18	3.7 (2.4-5.7) [#]	
c)	Observed ⁽⁶⁾		Expected ⁽⁷⁾	χ ²	df		
F2	39/60 (65%)		25%	51.2 ⁺	1		
BC	101/120 (84%)		50%	56.0 ⁺	1		

- (1) Number of flies tested (3-8 replicates of 10-15 flies each).
- (2) Number of flies considered in the Probit analysis (including non-treated).
- (3) Lethal concentration (LC_{50}) expressed in ppm of lambda-cyhalothrin in the diet.
- (4) Resistance ratio (RR) = LC_{50} (selected lab strain or cross) / LC_{50} (C strain). The fiducial limits for RR were calculated according to Robertson and Preisler (1992).
- (5) Dominance value (D_{LC}) calculated following the formula $D_{LC} = \{[(2 \log LC_{50} F1 - \log LC_{50} P1 - \log LC_{50} P2) / (\log LC_{50} P1 - \log LC_{50} P2)] + 1\} / 2$; where P1 and P2 corresponded to parental strains W-1Kλ and C, respectively (Bourguet *et al.*, 2000).
- (6) Number and percentage of observed dead individuals in the inheritance experiment when exposed to 300 ppm of lambda-cyhalothrin in the diet.
- (7) Percentage of expected dead individuals in a monogenic model.
- * Do not fit to the probit model ($p \leq 0.05$).
- + Do not fit to the monogenic model ($p \leq 0.05$).
- # RR is significant ($p \leq 0.05$) if the 95% FL does not include 1.

3.3.3. Analysis of P450 expression and CNV

We first analyzed the expression of *CcCYP6A51* in abdomens from pooled (three males and three females) or single (males) individuals from the W-1K λ and C strains (Figure 3.2). We found a consistent over-expression of this gene when pooled individuals of W-1K λ exposed to lambda-cyhalothrin were analyzed. However, the gene was overexpressed in untreated individuals of W-1K λ in one of the two experiments with pooled individuals (Figure 3.2.a), but not in a repetition of the same experiment with other pooled individuals (Figure 3.2.b). A similar trend was observed when single individuals were analyzed (Figure 3.2.c), but differences were not significant because of the high variability in the relative expression of *CcCYP6A51* found in this experiment (with respect to the average expression in adult flies from C strain): 0.006-3.33 for the untreated C strain, 0.12-1.06 for untreated W-1K λ and 0.54-21.89 for W-1K λ treated with the insecticide.

We then checked the expression of *CcCYP6D14*, *CcCYP6A48*, *CcCYP6A51* and *CcCYP6A70* in single (males) individuals of both strains when exposed to concentrations of lambda-cyhalothrin close to the LC₅₀ values for W-1K λ (600 ppm) and C (10 ppm) (Figure 3.3). Once again, high variability for *CcCYP6A51* expression was found among individuals, with not significant differences despite the higher average values in W-1K λ exposed to 600 ppm and C exposed to 10 ppm. Similar results were found for the other CYP genes analyzed, this induction resulting significant in *CcCYP6A70* and *CcCYP6D14* for W-1K λ exposed to 600 ppm, and in *CcCYP6A70* for C exposed to 10 ppm of the insecticide. However, not all genes were induced in all individuals (Figure 3.3.e), adding a new source of variability. In addition, it was remarkable that none of these genes (Figure 3.3) nor the rest of the genes analyzed (Table 3.3) were induced when W-1K λ individuals were exposed to 10 ppm of lambda-cyhalothrin (Table 3.3).

Figure 3.2. Relative expression of *CcCYP6A51* in adult flies from C and W-1Kλ strains of *Ceratitis capitata*. The assays were performed with a) and b) abdomens of pooled (3 males and 3 females) individuals; and c) 6 individual males. Induction of expression as a consequence of exposure to lambda-cyhalothrin can be seen. The numbers in parenthesis refer to the concentration of insecticide present in the food. Different letters refer to significantly different groups (ANOVA, Student Newman Keuls *post hoc* test, $p \leq 0.05$).

Figure 3.3. Relative expression of four CYP genes in adult flies from C and W-1Kλ strains of *Ceratitidis capitata*. Induction of expression as a consequence of exposure to lambda-cyhalothrin can be seen. The numbers in parentheses refer to the concentration of insecticide present in the food. The values in e) correspond to the relative expression of each single individual encompassed in the graphics a)-d), and the values in blue and red refer to moderate and high induction of expression, respectively. Different letters refer to significantly different groups (ANOVA, Student Newman Keuls *post hoc* test, $p \leq 0.05$).

Table 3.3. Relative expression of CYP genes in adult flies from C and W-1Kλ strains of *Ceratitis capitata* using qPCR. No significant differences were found (ANOVA, Student Newman Keuls *post hoc* test, $p \leq 0.05$).

CYP	C, 0 ppm	W-1Kλ, 0 ppm	W-1Kλ, 10 ppm
CcCYP6A10	1 ± 0.31	1.35 ± 0.35	1.29 ± 0.31
CcCYP6A11	1 ± 0.12	1.18 ± 0.21	1.31 ± 0.17
CcCYP6A49	1 ± 0.58	0.51 ± 0.28	0.55 ± 0.3
CcCYP6A50	1 ± 0.22	0.65 ± 0.12	0.95 ± 0.27
CcCYP6A66	1 ± 0.09	1.01 ± 0.22	0.94 ± 0.11
CcCYP6A67	1 ± 0.35	0.41 ± 0.18	0.63 ± 0.22
CcCYP6A71	1 ± 0.4	0.86 ± 0.25	1.14 ± 0.45

We also studied the CNV of CYP genes to check if gene amplification could be associated to lambda-cyhalothrin resistance. The results showed no statistically significant differences among strains except for *CcCYP6D14* that was lower (0.87) in W-1Kλ (Figure 3.4), so there is no more than one copy of the analyzed CYP genes in W-1Kλ with respect to C.

Figure 3.4. Copy number of CYP genes in C and W-1Kλ strains of *Ceratitis capitata*. An asterisk refers to significant differences between both strains for a given gene (t-test $p \leq 0.05$).

3.3.4. Search of markers involved in lambda-cyhalothrin resistance by variant calling analysis

We performed a Genotyping By Sequencing (GBS) analysis with a F2 cross between W-1Kλ and C strains to look for genetic variants that could be used as a marker for lambda-cyhalothrin resistance. We found a total of 41278 variants (variant rate of 1 out of 9831 bases), with special presence of SNPs (35580). They were distributed all along the genome, with the highest frequency located in the transcripts (33.51%). However, in view of the total amount of variants, the 70.78% was silent and only the 0.017% was detected as “high impact” (considered by the software to produce a relevant change in the amino acidic sequence).

We obtained 7 candidate regions that may be associated to lambda-cyhalothrin resistance, 3 of them with unknown products and the other 4 coding for different biological function genes (Table 3.4). Each region included one or several significant variations that constituted specific haplotypes. QTL1 had the highest contribution to the resistant phenotype, according to the “odds” parameter, which results from the GLM adjustment assuming a binomial distribution and indicates the relation between the probabilities of surviving/dying when a resistant allele appears. We confirmed that all the regions except the one named QTL3 had no random segregation of the genotypes among resistant and susceptible individuals, suggesting a significant correlation between them and the resistant phenotype. In addition, the GLM adjustment also indicated that QTL3 had the lowest contribution to the resistant phenotype, and that its effect on resistance was not significant.

QTLs 1, 6 and 7 included two possible haplotypes, while QTLs 2 to 5 concerned several. To allow the treatment of the data in these cases, we selected the haplotype that seemed to be contributing to a greater extent to the resistant phenotype, and group the rest of them as they were a second haplotype. Particularly, region “QTL3” had a vast amount of haplotypes that needed to be simplified, and probably the difficulties in this simplification provoked that the statistic test interpreted it as a random segregation character.

Table 3.4. Candidate regions for resistance association in *Ceratitis capitata* genome obtained in the sequencing analysis, and the distribution of their genotypes in resistant and susceptible individuals.

Code	Scaffold	Position	Annotation	Genotypic frequencies in resistant individuals (%)			Genotypic frequencies in susceptible individuals (%) ⁽¹⁾			χ^2	Odds ⁽²⁾	p-value
				A A	A B	B B	A A	A B	B B			
QTL1	AOHK02000269.1	630036- 630037	Unknown	7.55	30.19	62.26	0	11.46	88.54	16.83	4.43	0.0002
QTL2	AOHK02003133.1	197356- 19173 and 470785- 470786	Leucine-rich repeat neuronal protein 3 (LRRN3)	22.64	16.98	60.38	2.08	15.63	82.29	17.60	2.76	0.0003
QTL3	KZ344538.1	927205- 956841	Endocuticle structural glycoprotein SgAbd-2 (CUD2) Ankyrin repeat and BTB/POZ domain- containing protein	24.53	24.53	50.94	15.63	17.71	66.66	3.61*	1.47	0.0700
QTL4	KZ344569.1	249110- 474611	Gustatory receptor for sugar taste 64f (Gr64f) Gustatory receptor 5a for trehalose (Gr5a) Neuropeptide capsa receptor (CapaR)	18.87	50.94	30.19	4.17	33.33	62.50	17.52	3.10	7.3602e-05
QTL5	KZ344569.1	1132022- 1159288	Transcription factor Mef2A Glycine-tRNA ligase CTP synthase	26.42	49.06	24.53	7.29	37.50	55.21	17.21	2.87	7.8597e-05
QTL6	KZ344684.1	475491- 475492	Unknown	56.60	35.85	7.55	23.96	48.96	27.08	18.03	3.01	6.6047e-05
QTL7	KZ344967.1	859348- 859349	Unknown	77.36	22.64	0	52.08	40.63	7.29	10.66	3.10	0.0017

⁽¹⁾ Frequencies of genotypes of individuals resistant and susceptible to 300 ppm of lambda-cyhalothrin, from the F2 cross performed for the analysis of lambda-cyhalothrin resistance inheritance. In all cases, allele “A” corresponds to the most frequent haplotype observed among the resistant individuals, while “B” refers to the combination of all other haplotypes found for the alternative allele.

⁽²⁾ Odds ratio indicates the relation between the probabilities of surviving/dying when a resistant allele appears.

* Good fit of the data ($p > 0.05$) to a random segregation of the genotypes, non-depending on the phenotype.

A punctuation approach was performed to check if the addition of traits was associated with the resistant phenotype. As QTLs 4 and 5 were located in the same scaffold and presented high genotypic differences among resistant and susceptible individuals, the analysis of cumulative effect started with the association of these two traits and the successive addition of further QTLs. Results showed a trend in which the accumulation of candidate QTLs provoked that individuals with a resistant phenotype increased in the highest scores, while susceptible individuals presented lower ones. Actually, the most traits were added, the most perceptible was the difference between resistant and susceptible individuals' scores (Figure 3.5).

Figure 3.5. Distribution of the quantity of candidate resistance haplotypes (score) in resistant (R) and susceptible (S) individuals of an F2 cross of *Ceratitis capitata* to 300 ppm of lambda-cyhalothrin. The cumulative effect of candidate QTLs is represented by the combination of a) QTL4 and QTL5; b) QTL4, QTL5 and QTL6; c) QTL4, QTL5, QTL6 and QTL7; d) QTL1, QTL4, QTL5, QTL6 and QTL7; e) QTL1, QTL2, QTL4, QTL5, QTL6 and QTL7; and f) QTL1, QTL2, QTL3, QTL4, QTL5, QTL6 and QTL7.

Unexpectedly, no CYP gene was located in the candidate regions associated to lambda-cyhalothrin resistance, nor even close to them. Thus, a marker focused on this detoxification pathway could not be developed.

3.4. DISCUSSION

This work demonstrated that resistance of W-1Kλ strain of *C. capitata* to lambda-cyhalothrin was completely dominant and autosomic and does not fit a monogenic model. The level of dominance of insecticide resistance varies according to the resistance mechanism, although environmental conditions and insecticide exposure can also mark a difference (Bourguet *et al.*, 2000). When resistance to pyrethroids is caused by alteration of the voltage-gated sodium channel (VGSC) target-site, it usually has an incompletely recessive inheritance pattern (Farnham, 1977; Scott, 2019). On the contrary, the inheritance of metabolic resistance is variable, going from recessive, as observed in *Culex quinquefasciatus* for permethrin resistance (Li and Liu, 2010), to dominant, as lambda-cyhalothrin resistance in *Musca domestica* (Abbas *et al.*, 2014a). The number of genes involved in resistance is also in concordance with the resistance mechanism. Pyrethroids resistance occasioned by an alteration in the VGSC gene is usually monogenic (Dong *et al.*, 2014), while metabolic resistance has been reported in the literature as controlled by one (Prasad and Shetty, 2013) or several genes (Abbas *et al.*, 2014a; Khan *et al.*, 2015). The inheritance of lambda-cyhalothrin resistance in W-1Kλ fits with the proposed metabolic resistance mechanism mediated by P450s (Arouri *et al.*, 2015). However, our results indicate that, in addition to the reported *CcCYP6A51* gene (Arouri *et al.*, 2015; Tsakireli *et al.*, 2019), other polygenic traits must be involved. Our results showed that the expression of several P450s, including *CcCYP6A51*, was induced in W-1Kλ in response to a high, but sublethal for this strain, concentration (600 ppm) of lambda-cyhalothrin. However, we found that their expression was highly variable in W-1Kλ strain, that resistant individuals did not show a basal over-expression in most experiments, and that they were not always equally induced in all individuals. These results suggest that different P450 genes must be contributing to confer resistance when exposed to high concentrations of the insecticide. Studies with other insect species have shown how insects display a vast plasticity in their adaptation to insecticide selection, and how resistance mediated by P450 can evolve by over-

expression of different CYP genes (Ffrench-Constant *et al.*, 2004; Scott and Kasai, 2004). Thus, different populations selected with the same insecticide can evolve resistance by using different P450s, as reported for lambda-cyhalothrin resistance in *Anopheles funestus* (Riveron *et al.*, 2013; Samb *et al.*, 2016) and permethrin resistance in *M. domestica* (Scott and Kasai, 2004). Besides, several P450s have been reported to contribute to pyrethroid resistance in a permethrin resistant strain of *C. quinquefasciatus* (Komagata *et al.*, 2010). Nonetheless, the over-expression of a particular CYP gene does not automatically need to be linked to insecticide resistance, as they participate in different metabolic processes. Indeed, some of the P450 genes analyzed in this study were also induced when the C strain was exposed to 10 ppm of lambda-cyhalothrin, which is toxic for this susceptible strain, whereas their expression was not induced in resistant W-1Kλ individuals exposed to the same concentration. These results indicated that: i) the induction of the P450 genes analyzed is not enough to confer resistance to the C strain; and ii) other P450 genes or other resistance mechanism must be responsible for the survival of W-1Kλ flies at 10 ppm of the insecticide, otherwise they would die as observed in flies from the C strain. Thus, more complex machinery must be involved in resistance to lambda-cyhalothrin in the W-1Kλ strain, with other resistant traits acting, at least, at low insecticide concentrations.

The results obtained in the variant calling analysis also reinforce the idea that resistance is polygenic, as they outlined seven candidate regions that could be associated to resistance. The analysis of the data indicated that they had a cumulative effect, as it was observed that the consecutive addition of these traits explained a higher percentage of the resistant phenotype. The genes located in the candidate regions were not major insecticide target sites, nor associated with pesticide metabolism. However, their biological function indicated that they could be implicated in other process of resistance, as the detection of the insecticide (gustatory receptor for sugar and trehalose, located in the QTL4), signaling routes (leucine-rich repeat neuronal transmembrane proteins, located in QTL2, that modulate trafficking and signaling) and excretion regulation (the neuropeptide capa receptors located also in QTL4 participate in the transduction pathway that gives rise to Malpighian tubule fluid secretion). Nonetheless, none of these genes were over-expressed in the transcriptome analysis of W-1Kλ (unpublished results), and none of the mutations had

a high impact in the resulting protein, as they do not produce relevant alterations in the amino acid sequence nor in the reading frame. Thus, they might be just markers associated with the resistant phenotype. Further studies are required to determine if the detection of lambda-cyhalothrin, repellent for *C. capitata* at high concentrations, is playing a role in resistance.

In this work, we have found some differences with those previously reported (Arouri *et al.*, 2015). Specifically, Arouri *et al.* (2015) showed that among the 53 CYP genes analyzed, only *CcCYP6A51* was constitutively overexpressed in the resistant W-1Kλ strain, and induced when exposed to lambda-cyhalothrin. However, we found here that the expression of 12 of these CYP genes, including *CcCYP6A51*, was induced in W-1Kλ, but none of them was constitutively overexpressed. We cannot discard the possibility that some of the differences found between these and the previous study are due to changes in the expression profile of P450 genes and other genes of minor effect, which would have occurred during the time that W-1Kλ has been reared in the laboratory. CYP genes still appear to have an important contribution for resistance, since they are induced at sublethal concentrations in the resistant strain. Furthermore, we corroborated that resistance was reverted by PBO, as previously reported (Arouri *et al.*, 2015), and no mutations in the VGSC target-site were found in individuals from the W-1Kλ strain (see Chapter 4). However, other genes may have been selected during laboratory selection, adding small levels of resistance to the basal resistance mechanism. Indeed, it has been reported that the selection of polygenic traits with smaller effect is likely to happen in laboratory strains (Ffrench-Constant, 2013).

The complex mechanism of lambda-cyhalothrin resistance in medfly, with implication of several genes, has not made possible to find a robust diagnostic marker of resistance. The high variability found for the expression of *CcCYP6A51* and other CYP genes in resistant and susceptible strains makes them inappropriate for monitoring purposes. Likewise, Voudouris *et al.* (2018) reported that the expression of *CcCYP6A51* presented a high variability in field populations. At present, several studies have associated CNV of P450 genes with insecticide resistance in a number of insect species (Wondji *et al.*, 2009; Bariami *et al.*, 2012; Zimmer *et al.*, 2018), which can then potentially be used as DNA diagnostic markers. However, we did not find variations in the number of copies for any of the P450 genes analyzed between the resistant W-1Kλ

and the susceptible C strains. Using a variant calling analysis we have found that seven candidate regions were associated to resistance. However, none of them discriminate between resistant and susceptible insects, showing a marked cumulative effect. Further analysis should be performed to find an appropriate marker to monitor lambda-cyhalothrin resistance in field populations.

**CHAPTER 4. DETECTION AND MANAGEMENT OF INSECTICIDE
RESISTANCE IN *Ceratitis capitata***

CHAPTER 4: DETECTION AND MANAGEMENT OF INSECTICIDE RESISTANCE IN

Ceratitis capitata

4.1. RATIONALE FOR THE STUDY

Current control practices against *Ceratitis capitata* (medfly) in the Comunitat Valenciana rely on the release of sterile males and the use of insecticides. At present, spinosad and lambda-cyhalothrin as bait sprays, and deltamethrin as lure and kill traps are mostly used in citrus crops (MAPA, 2019b). Monitoring of Spanish field populations susceptibility to insecticides performed in 2007-2011 found that some populations had developed resistance to lambda-cyhalothrin (Arouri *et al.*, 2015), whereas they were highly susceptible to spinosad (Ureña *et al.*, 2019). Nonetheless, resistance to spinosad has been obtained under laboratory selection (Ureña *et al.*, 2019), highlighting the potential for resistance development. The use of the pyrethroid deltamethrin in citrus orchards is increasing due to the implementation of lure-and-kill traps as MagnetMEDTM, although cross-resistance between lambda-cyhalothrin and deltamethrin has been previously noticed in medfly (Arouri *et al.*, 2015). Malathion has been recently re-authorized with restrictions in its use (MAPA, 2019b), but resistance to this insecticide was widespread in Spain by the time of its withdrawal in 2009 (Magaña *et al.*, 2007). In this context, the implementation of Insecticide Resistance Management (IRM) strategies is required for both the improvement of medfly control programs and the sustainability of available insecticides.

Key components of IRM strategies are the early detection of resistance and understanding the mechanisms and genetics of resistance, so that appropriate measures can be taken. Resistance to spinosad is recessive and provoked by the appearance of truncated transcripts of the $\alpha 6$ subunit of the nicotinic acetylcholine receptor (*Ccα6*) (Ureña *et al.*, 2019), which imposes a fitness cost to resistant individuals compared to susceptible ones (Chapter 2). Lambda-cyhalothrin resistance is completely dominant, caused by detoxification mediated by P450 enzymes (Arouri *et al.*, 2015) with active implication of more than one gene (Chapter 3), although fitness costs have not been studied yet. In addition, knowledge about the mechanisms conferring resistance have led to the design of molecular markers for spinosad resistance in *C. capitata* (Ureña *et al.*, 2019), useful for the early detection of resistant

alleles in field populations, and studies are under way for lambda-cyhalothrin (Chapter 3).

The use of laboratory simulation studies and theoretical evolutionary models are also relevant tools for resistance management. Laboratory simulation studies allow to test different treatment strategies (Zhao *et al.*, 2010). Current practices include the repeated use of spinosad and lambda-cyhalothrin alone or in rotations, and the simultaneous use of one of these two chemicals with lure-and-kill traps of deltamethrin. Evolutionary models are based on empirical data (Griffiths *et al.*, 2008) and help to understand how resistant alleles can evolve in a population depending on biological, genetic and operational factors (South and Hastings, 2018; Wairimu *et al.*, 2018).

The aim of this work was: 1) to determine the current levels of resistance to approved insecticides for *C. capitata* control in Spanish citrus crops; 2) to study with laboratory strains how multiple resistance could develop under different insecticide treatment strategies; and 3) to predict how spinosad resistance would evolve in the field based on evolutionary models.

4.2. MATERIALS AND METHODS

4.2.1. Laboratory strains, field populations and generation of a multiresistant strain of *C. capitata*

The susceptible laboratory strain of *C. capitata*, C, was originally set up in 2001 from non-treated experimental fields at the Instituto Valenciano de Investigaciones Agrarias (IVIA, València, Spain). It has been reared in the laboratory without any insecticide exposure since then. The lambda-cyhalothrin resistant strain W-1Kλ was obtained from W-4Km, the malathion laboratory resistant strain, by laboratory selection with this insecticide (Arouri *et al.*, 2015). The strain JW-100s derived from field individuals collected in Xàbia in 2007 and it has been selected with spinosad in the laboratory since then (Ureña *et al.*, 2019). At the time the experiments were performed (F88), the strain was constituted by individuals homozygous for $Cc\alpha 6^{3aQ68*-K352*}$ allele and heterozygous for $Cc\alpha 6^{3aQ68*-K352*}$ and $Cc\alpha 6^{3aQ68*\Delta 3b-4}$ alleles (Ureña *et al.*, 2019; Chapter 2). The isolate Q68*-K352* is homozygous for the allele $Cc\alpha 6^{3aQ68*-K352*}$ and was obtained from JW-100s strain (Chapter 2).

Table 4.1. Field populations of *Ceratitis capitata*.

Population	Year	Host	Field Treatments ⁽¹⁾
Sagunt (València)	2015	Citrus	Spinosad since 2008
	2016	Citrus	Spinosad and deltamethrin in 2016
Albal (València)	2015	Citrus	Spinosad in 2015
Algarrobo Costa (Málaga)	2015	Cherimoya	Non-treated in the last years (experimental field)
	2016	Cherimoya	Non-treated in the last years (experimental field)
	2017	Cherimoya	Non-treated in the last years (experimental field)
Blanca (Murcia)	2016	Citrus	Non-treated in the last year
Oliva (València)	2016	Citrus	Spinosad in 2016
Sanet i Negrals (Alacant)	2017	Citrus	Spinosad in 2017 (5X)
Onda (Castelló)	2017	Citrus	Spinosad in 2017 (3X)
Alcalà de Xivert (Castelló)	2017	Citrus	Non-treated / Spinosad in 2017 (3X) ⁽²⁾
Betxí (Castelló)	2017	Citrus	Deltamethrin and spinosad in 2017 (2X)
Vinaròs (Castelló)	2017	Citrus	Deltamethrin and lambda-cyhalothrin in 2017 (2X)
Vila-real (Castelló)	2017	Citrus	Deltamethrin and lambda-cyhalothrin in 2017 (6X)
Puçol (València)	2018	Citrus	Spinosad in 2018 (3X)

⁽¹⁾ The number between brackets refers to the amount of field applications of bait formulations with lambda-cyhalothrin (by ground treatment) or spinosad (by ground or aerial treatments) per year. Deltamethrin treatments were applied by the use of MagnetMED™ attract and kill traps.

⁽²⁾ This population came from two different fields, one that had no insecticide treatment and another treated with spinosad.

Field populations were collected from orchards sited in different localities of East Spain, in the Mediterranean area (Table 4.1). Fruit punctured by *C. capitata* was taken from the field to the laboratory, placed in ventilated plastic boxes (15 x 21 x 28 cm) and kept at controlled conditions (photoperiod of 16 : 8 hours light : dark and a temperature of 26 ± 2 °C (light) and 22 ± 2 °C (dark)). New pupae were harvested every two-three days and were kept in ventilated boxes (12 cm in diameter and 5 cm height). Emerged-adults were provided with water and rearing diet (4 : 1 sugar : yeast) *ad*

libitum, in an environmentally controlled chamber (Sanyo MLR-350-H, Sanyo, Japan), at 25 ± 1 °C and 16 h light and 8 h dark photoperiod (standard conditions).

The laboratory strains W-1Kλ and JW-100s were crossed to generate the multiresistant MR strain. Adults from both strains were collected and their sex determined immediately after adult emergence to assure virginity. Males and females from each strain were then placed separately into ventilated plastic dishes and maintained with water and rearing diet for two days. Reciprocal crosses (100 ♂ W-1Kλ x 100 ♀ JW-100s and 100 ♀ W-1Kλ x 100 ♂ JW-100s) were performed to obtain the F1 generation. The offspring of both reciprocal crosses was kept at standard conditions without insecticides exposure to produce the F2 generation. This was considered the initial MR strain for the experiment (G1), where all the possible allelic combinations were represented. At this point, the MR strain was divided into four strains to emulate four different treatment scenarios that could occur in the field: T1 was exposed to 100 ppm of spinosad in each generation; T2 alternated three consecutive generations with a treatment of 100 ppm of spinosad with a generation with 125 ppm of lambda-cyhalothrin; T3 alternated a treatment of 100 ppm of spinosad in two consecutive generations with 125 ppm of lambda-cyhalothrin during the next two generations; and T4 remained unexposed to insecticides. Treatments were applied during 10 consecutive generations (Figure 4.1).

Generation	T1	T2	T3	T4
G1	S	S	S	-
G2	S	S	S	-
G3	S	S	λ	-
G4	S	λ	λ	-
G5	S	S	S	-
G6	S	S	S	-
G7	S	S	λ	-
G8	S	λ	λ	-
G9	S	S	S	-
G10	S	S	S	-

Figure 4.1. Treatment scenarios T1-T4 for the MR laboratory strain of *Ceratitis capitata*. “S” in yellow means a treatment with 100 ppm of spinosad, “λ” in green means a treatment with 125 ppm of lambda-cyhalothrin and “-” in blue means absence of insecticide treatment.

4.2.2. Bioassays

Feeding bioassays were performed with lambda-cyhalothrin (KarateZeon 10% p/v (100 g/l), Syngenta Limited, Surrey, United Kingdom), spinosad (Dow AgroSciences 88% p/p, Indianapolis, USA) and malathion (Fyfanon 440 g/l, Cheminova, Lemvig, Denmark). Adult flies 3-5 days old were tested. Several concentrations of each insecticide were added to the rearing diet (9 rearing diet : 1 insecticide w/w) and tested in the concentration-response assays. In addition, a discriminant-concentration assay with 125 ppm of lambda-cyhalothrin was performed. Dilutions of insecticides were prepared with water in the case of lambda-cyhalothrin and malathion, and with a buffer composed of acetic acid : sodium acetate (1 : 3, pH 4.7) in the case of spinosad. These solvents (water or buffer) were mixed with the rearing diet for the non-treated controls. Two-4 replicates of 10-15 flies per replica were performed. During the assays, flies were kept into ventilated plastic dishes (89 mm in diameter and 23 mm in height) at standard conditions. Laboratory strains were starved for 24 hours before the exposition to insecticide. Mortality was recorded after 48 h and flies were considered dead if they were ataxic.

Deltamethrin susceptibility was evaluated with assays of exposition to MagnetMED™ traps (Suterra Europe Biocontrol SL, València, Spain). They are laminated devices of 16 x 18 cm coated with deltamethrin in their entire surface (10 mg per dispositive) and baited with a BioLure® Unipack dispenser placed inside. These assays were performed with 3-5 days old flies in ventilated aluminum boxes (50 x 65 x 60 cm) located in a green-house where the environmental conditions were $T = 25 \pm 2$ °C and natural photoperiod of October in Madrid, Spain (approximately, 11 : 13 hours light : dark). During

Figure 4.2. Ventilated aluminium box with a MagnetMED™ trap, fly diet and water for susceptibility assays.

the entire assay, a trap was hung from the upper side of the cage (Figure 4.2). Three replicates, containing 30 flies each, were performed. Flies were provided with water and food *ad libitum*. Mortality was recorded after 1, 4, 24 and 48 hours of exposition. Flies were considered dead if they were ataxic. This methodology was used to determine the susceptibility of field populations to new traps and to test the efficacy of traps that had been exposed to field conditions against the laboratory C strain. Traps exposed to field conditions were located in single trees in Bétera (València) for 5 weeks (between 18th August and 22nd September 2017, with 34.2 mm of rain and average minimum-maximum temperature of 16.4-28.8 °C) and for 3 months (between 18th August and 17th November 2017, with 43.2 mm of rain and 13.0-26.1 °C), and to intensive rain in a citrus grove in Carcaixent (València) for 7 weeks (between 1st September and 18th October 2018, with 110.1 mm of rain and 16.9-29.0 °C). Climate conditions were provided by the nearby climate station (SIAR, 2018).

4.2.3. F1-screen assays

Males from field populations were crossed with virgin females from the laboratory Q68*-K352* isoline or JW-100s strain, both resistant to spinosad (Ureña *et al.*, 2019). Crosses were performed in methacrylate cages (20 x 20 x 20 cm) with a net in one of their sides for the laying of the eggs, containing water and rearing diet *ad libitum*. All

the resultant F1 eggs were spread over larvae rearing diet (stock prepared by mixing 300 g of sugar, 11.2 g of N-propyl-p-hydroxibenzoate (Sigma-Aldrich, Japan), 11.2 g of methyl-4-hydroxibenzoate (Sigma-Aldrich, India), 10 g of benzoic acid (Merck, China), 145.2 g of dry beer yeast (Difco Laboratories, Erembodegem, Belgium), 1000 g of wheat bran and 2400 ml of distilled water) with 5 ppm of spinosad, covered with an aluminum foil and kept at standard conditions. The development was checked frequently to isolate and provide with water and rearing diet those individuals that reached the adult stage.

4.2.4. Detection of mutations in laboratory strains and field populations of *C. capitata*

Genomic DNA (gDNA) extraction was performed with the SpeedTools Tissue DNA Extraction kit (Biotools, Madrid, Spain) and total RNA extraction was performed with TRIzol® Reagent (Life Technologies, Van Allen Way, Carlsbad, California, USA), in both cases according to manufacturer's indications. RNA was incubated with RQ1 DNase (Promega, Madison, WI, USA) at 37 °C for 35 min to remove gDNA, and the reaction was stopped by incubation with RQ1 DNase Stop solution (Promega) at 70 °C for 10 min. Quantification of nucleic acids was performed using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription to complementary DNA (cDNA) used 2 µg RNA as a template and a commercial kit with oligo(dT) (Thermo Scientific, Massachusetts, USA). PCR products were analysed by electrophoresis on 1.5% agarose gel (Agarose D2, Conda Pronadisa) and purified (QIAquick PCR Purification Kit, QIAGEN, Germany). When needed, bands were cut from the gel and eluted with centrifugal filter units (Merck Millipore, Darmstadt, Germany). PCR products were sequenced (Secugen, Madrid, Spain) and analysed using Genious 11.0.5 (<https://www.geneious.com>).

The domains II and III of the voltage-gated sodium channel (VGSC) gene (XM_020861574) were partially sequenced to cover most of the codons associated to *kdr* resistance mutations in more than one insect species and functionally confirmed in *Xenopus* oocytes (Dong *et al.*, 2014) (Figure 4.3.a). We analyzed 20 flies from the populations of Vinaròs and Betxí (2017) that survived their exposition to MagnetMED™ traps, and 20 from Blanca (2016) that survived in a feeding bioassays

with lambda-cyhalothrin. Whole body DNA was extracted and PCR was performed in a volume of 50 µl using 0.6 µM of oligonucleotides NaCh899_F (5'-TCGAGTTTTTAACTTGCCAAA) and NaCh932_R (5'-TTTCCGAACAGTTGCATTCC) for region "A", Kdr_F (5'-TCGTTTTTCGTGTGCTATGC) and Kdr_R (5'-CCAGGCTTTAAAACGCGATA) for region "B", NaCh1528_F (5'-AAGCAACCAATCCGTGAAAC) and NaCh1575_R (5'-TCGGTCTAGGAATGGCTTTT) for region "C" (Figure 4.3.b); 0.04 U of AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific, Austin, USA), 10x PCR Buffer II, 1.5 mM MgCl₂, 0.2 mM dNTPs (Thermo Fisher Scientific, Austin, USA) and 100-200 ng of the corresponding template. PCR conditions were as follows: an initial denaturation step at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s; and a final step of 72 °C for 7 min for fully extension.

The gene of the α6 subunit of the nicotinic acetylcholine receptor (*Ccα6*) was studied in surviving F1-screen assay flies. RNA extraction and cDNA synthesis was performed on heads of adult flies when they were in good state and on whole body when flies were amorphous or the head was not in good conditions. PCR was performed in a volume of 10 µl using 0.4 µM of oligonucleotides FnACh6ex1 (5'-CAACGGAAGCTGAAATCTAAGGAC) and RnACh6ex12nest (5'-TCGCTGTTTGCCGTGTTGATCTTT), 2 U of iTaq™ DNA Polymerase (Biorad, California, USA), 5x HF Buffer, 0.2 mM dNTPs (Thermo Fisher Scientific, Austin, USA) and 5 µl of the corresponding cDNA diluted 2-fold as template. PCR conditions were as follows: an initial denaturation step at 98 °C for 30 s; 40 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 1.5 min; and a final step of 72 °C for 7 min for fully extension. Nested-PCR was performed following the same procedure, using 5 µl of the first PCR product as a template and oligonucleotides FnACh6ex2-76 (5'-CGTCTACTTAACCACCTATTATCC), RnACh6ex5 (5'-CGTGCCATCGAATCCCTCATCC), RnACh6ex11-1382 (5'-CTATCCACAACCATGCGCAAAC) and RnACh6ex12-nest. PCR products were sequenced and analyzed.

Ccα6 alleles associated to spinosad resistance were analyzed in the MR strain by implementing the genotyping tools previously developed for *C. capitata* (Ureña *et al.*, 2019). gDNA was extracted from the thorax of 20 individuals alternating generations. For the detection of K352* mutation, it was performed a PCR-RFLP to recognize a *MseI*

restriction site that is generated in the presence of the mutation, while a Multiplex PCR was performed for the detection of Q68* mutation.

Figure 4.3. a) Location of *kdr* resistance mutations in the domains I-IV of the voltage-gated sodium channel (VGSC) observed in more than one insect species (Dong *et al.*, 2014) and functionally confirmed in *Xenopus* oocytes (adapted from Dong *et al.*, 2014). b) Partial sequence of the domains II-III of the VGSC in *Ceratitis capitata*, and location of the primers for the search of potential mutations. Numbers over the yellow regions refer to the codon affected. Mutations are massed in the three sequenced areas: “A”, amplified with oligonucleotides NaCh899_F and NaCh932_R (in red); “B”, with Kdr_F and Kdr_R (in green); and “C”, with NaCh1528_F and NaCh1575_R (in blue).

4.2.5. Evolutionary model of spinosad resistance

A model to predict the evolution of spinosad resistance was established. It was assumed a panmictic population with discrete generations, and it was considered that

the effective population size is large enough to rule out genetic drift effects. Several parameters with different values were tested in the simulation (Table 4.2).

Table 4.2. Parameters used in the evolutionary model for spinosad resistance in *Ceratitis capitata*.

Parameter	Definition	
w_{XY}	Fitness cost of genotype XY = {(S/S), (R/R), (S/R)}; $0 \leq w_{XY} \leq 1$	
$s(s)_{XY}$	Sensitivity to spinosad (s) of genotype XY; $0 \leq s(i)_{XY} \leq 1$	
$s(i)_{XY}$	Sensitivity to insecticide (i) with no cross-resistance to spinosad of genotype XY; $0 \leq s(i)_{XY} \leq 1$	
$e(i)$	Exposition to insecticide (i), understood as the percentage of insects in the population contacting the insecticide, $e(i) = \{(0.2), (0.5), (0.8), (0.95)\}$	
IF_{XY}	Initial frequency of genotype XY = {(S/S), (R/R), (S/R)} used to calculate the predicted rate of resistance evolution	
Fitness cost of genotype XY (w_{xy}) (see Supplementary Figure SF1)		
w (S/S)	w (R/R)	w (S/R)
0	0.4	0.2
Expected mortality of the genotypes to field spinosad concentration (260 ppm)		
$s(s)$ (S/S)	$s(s)$ (R/R)	$s(s)$ (S/R)
1	0	1
Expected mortality of the genotypes with an insecticide with no-cross resistance to spinosad		
$s(i)$ (S/S)	$s(i)$ (R/R)	$s(i)$ (S/R)
1	1	1
Initial frequencies IF1; F(R) = 0.00227272 (2 resistant alleles/880)		
F(S/S)	F(R/R)	F(S/R)
0.9954597107	0.00000516529	0.004535123967
Initial frequencies IF2; F(R) = 0.00454545 (4 resistant alleles/880)		
F(S/S)	F(R/R)	F(S/R)
0.9909297521	0.00002066115702	0.009049586777

The simulation was developed to detect the evolution of three different genotypes that could compose a field population: S/S, R/R and S/R, being S a susceptible allele and R a resistant allele, carrying any mutation or indel. The initial frequency (IF) of each genotype was calculated considering the frequency of appearance of resistant

alleles, taking this information from the F1-screen performed with populations collected during the years 2016-2018 (see section 4.3.2). Two situations were simulated: considering that both resistant individuals found in the field (from 440 tested) were heterozygous for the resistant allele ($IF1 = 0.00227272$) or that they were homozygous ($IF2 = 0.00454545$). The case corresponding to one heterozygous individual and one homozygous was not considered.

To calculate the fitness of each genotype (equivalent to $1-w_{XY}$, being w_{XY} the fitness cost or selection coefficient of genotype XY), it was considered that, by definition, the wild-type (S/S) had no fitness cost ($w_{SS} = 0$). To simplify the understanding of the evolutionary model, from here hence we will always talk about fitness cost instead of selection coefficient. For the remaining genotypes, trial and error modelling was performed to find out the combination of fitness cost values that better adjusted the expected evolution of allelic frequencies to the experimental frequency of alleles $Cca6^{3aQ68*\Delta3b-4}$ and $Cca6^{3aQ68*-K352*}$ during a study of stability (Chapter 2), and resulted 0.4 for RR and 0.2 for RS in both cases (see section 4.3.4).

We predicted the evolution of resistance under six different scenarios of insecticide treatments strategies that combined none, one or two insecticide treatments during three consecutive generations of *C. capitata*, using spinosad only or in combination with a second insecticide without cross resistance to spinosad. The simulation covered 60 generations (10 years, since *C. capitata* usually has six generations per year in the area of study).

The concentration of spinosad was selected according to the established for field treatments (260 ppm in bait sprays). Considering that spinosad resistance is inherited as a recessive trait (Ureña *et al.*, 2019), the sensitivity to spinosad was defined as $s(i)_{SS} = s(i)_{SR} = 1$, as both heterozygous and wild-type homozygous would die when exposed to a field dose of spinosad. As demonstrated in Chapter 2, this concentration has no effect on resistant homozygous individuals $s(i)_{RR} = 0$. The expected mortality for all genotypes when exposed to an insecticide with no cross-resistance was considered as $s(i)_{XY} = 1$. As the insecticide treatment is considered to reach only a part of the population, four levels of insecticide exposure ($e(i)$) were considered, 20, 50, 80 and 95%. So, the population was divided into two sub-populations depending on whether or not the insect contacted with the insecticide. Note that the putative migration is

implicit in the parameter $e(i)$. The general equation for calculating the relative genotype frequency through generations was:

$$F_{n+1}(XY) = \frac{\left\{ \left[\sum [F_n(XY) \times F_n(XY) \times F(XY)] \right] \times (1 - w_{XY}) \times (1 - s(s)_{XY}) \times (1 - s(i)_{XY}) \times e(i) \right\} + \left\{ \left[\sum [F_n(XY) \times F_n(XY) \times F(XY)] \right] \times (1 - w_{XY}) \times (1 - e(i)) \right\}}{\left[\sum F_{n+1}(\text{all XY genotypes}) \right]}$$

Genotype (XY) = {(S/S), (R/R), (S/R)}

$F_{n+1}(XY)$ = frequency of genotype XY at generation ($n + 1$)

$F_n(XY)$ = frequency of genotype XY at generation (n)

$[F_n(XY) \times F_n(XY)]$ = frequency of any cross between genotypes XY and XY

$F(XY)$ = frequency of genotype XY produced by a given cross

4.2.6. Statistics

Data were statistically analyzed with Levene and Shapiro-Wilk tests to check homogeneity and normality, respectively. Susceptibility to a discriminant concentration of lambda-cyhalothrin (percentage mortality data were previously Arcsin transformed) were compared by ANOVA followed by Student Newman Keuls *post hoc* test. Susceptibility to MagnetMED™ traps and evaluation of traps' efficacy along time (percentage data were Arcsin transformed) were evaluated by a Repeated Measures ANOVA followed by Student Newman Keuls *post hoc* test. χ^2 test was completed to test the fit of the data to expected genotypic frequencies in the evolution of spinosad resistant alleles in the MR strain. Susceptibility to different insecticides of *C. capitata* field populations, laboratory strains and MR crosses was analyzed using mortality data to estimate LC₅₀ values (concentration needed to cause 50% mortality). Probit analysis was performed using the program POLO-PC (LeOra Software14), which corrects samples' mortality by control (non-treated) mortality using Abbott's transformation (Abbott, 1925). Resistance ratios (RR =LC₅₀ (field or lab strain) / LC₅₀ (C strain)) were considered significant if their 95% fiducial limits did not include 1 (Robertson and Preisler, 1992).

4.3. RESULTS

4.3.1. Susceptibility to insecticides in field populations of *C. capitata*

We tested spinosad susceptibility of several field populations from the Mediterranean area by concentration-response assays. We found that all of them presented resistance ratios slightly but significantly higher than the susceptible C strain (Table 4.3). However, the LC_{50} values were far below the recommended field dose for this insecticide (260 ppm), proving that field populations of *C. capitata* are still susceptible to spinosad.

Susceptibility to lambda-cyhalothrin was tested by concentration-response assays (Table 4.3) and discriminatory concentration assays at the concentration recommended for field treatments (125 ppm) (Table 4.4). Results showed variability in the susceptibility, with populations highly resistant (Algarrobo Costa 2017 and Vila-real 2017, with less than 50% of mortality at 125 ppm, and Blanca 2016 with $LC_{50} > 125$ ppm), moderate but significantly different to the susceptible C strain (Algarrobo Costa 2016 with $LC_{50} < 125$ ppm, and Alcalà de Xivert 2017 and Vinaròs 2017 with more than 50% of mortality to 125 ppm) or susceptible (Sagunt 2016, with LC_{50} not significantly different from the susceptible C strain).

Table 4.3. Susceptibility to spinosad and lambda-cyhalothrin of field populations and the susceptible laboratory strain (C) of *Ceratitis capitata*.

Insecticide	Population	Year	n ⁽¹⁾	slope ± SE	LC ₅₀ ⁽²⁾ (95% FL)	χ^2	df	RR (95%FL) ⁽³⁾
Spinosad	Sagunt (València)	2015	199	2.56 ± 0.69	1.46 (1.12-1.75)	7.59*	10	2.50 (1.84-3.40) #
	Albal (València)	2015	172	2.21 ± 0.56	1.01 (0.57-1.33)	13.45*	12	1.73 (1.12-2.65) #
	Algarrobo Costa (Málaga)	2015	133	6.91 ± 1.22	0.96 (0.77-1.21)	18.30	8	1.65 (1.25-2.17) #
	Laboratory (C)	-	305	4.44 ± 1.01	0.58 (0.50-0.73)	12.20*	18	1.00
Lambda-cyhalothrin	Algarrobo Costa (Málaga)	2016	233	1.09 ± 0.18	46.13 (27.55-74.06)	14.71*	14	2.70 (1.56-4.67) #
	Blanca (Murcia)	2016	201	1.19 ± 0.24	135.70 (70.41-430.82)	16.39*	10	7.93 (3.87-16.26) #
	Sagunt (València)	2016	311	0.54 ± 0.18	18.89 (1.37-43.72)	14.47*	17	1.11 (0.16-7.58)
	Laboratory (C)	-	344	2.33 ± 0.24	17.10 (7.25-35.07)	116.38*	18	1.00

⁽¹⁾ Number of flies considered in the Probit analysis (including non-treated).

⁽²⁾ Lethal concentration (LC₅₀) in ppm of insecticide in the diet for feeding bioassays at 48h.

⁽³⁾ Resistance ratio (RR) = LC₅₀ (selected field strain) / LC₅₀ (C strain). The fiducial limits for RR were calculated according to Robertson and Preisler (1992).

* Good fit of the data to the probit model ($p > 0.05$).

RR is significant ($p \leq 0.05$) if the 95% FL does not include 1.

Table 4.4. Susceptibility to a discriminating concentration of lambda-cyhalothrin of field populations and the susceptible (C) and the resistant (W-1Kλ) laboratory strains of *Ceratitis capitata*.

Population	Year	Mortality % ± SE (n) ⁽¹⁾	
		Non-treated	Lambda-cyhalothrin (125ppm)
Alcalà de Xivert (Castelló)	2017	0 (30)	60 ± 6 (60) ^c
Vinaròs (Castelló)	2017	0 (30)	53 ± 5 (60) ^c
Algarrobo Costa (Málaga)	2017	0 (30)	23 ± 2 (60) ^d
Vila-real (Castelló)	2017	0 (30)	45 ± 9 (60) ^c
Laboratory (C)	-	0 (60)	98 ± 2 (60) ^a
W-1kλ	-	0 (60)	3 ± 2 (60) ^b

⁽¹⁾ The total number of flies tested is indicated between brackets (3-4 replicates of 10-15 flies each).

Different letters within each treatment indicate significant differences (ANOVA, Student Newman Keuls *post hoc* test, $p \leq 0.05$).

We analyzed susceptibility to deltamethrin by performing assays with MagnetMED™ traps, which are coated with this insecticide. Previously, we checked efficacy and durability of these traps in field conditions. We tested MagnetMED™ traps with the susceptible C strain and detected high efficacy (mortality 80-100%) at 48 hours with both new traps and traps that had remained in the field for up to 3 months in regular climatic conditions. However, their efficacy decreased (mortality of 48.9%) when they were exposed in the field to extreme weather conditions for 7 weeks in 2018 (Figure 4.4.a). These extreme conditions were characterized by high precipitations, which points that the reduction in the efficacy of the trap could be due to a wash of the insecticide. It explains why its efficacy was lower in 2018 than that of the traps used in 2017 (when precipitations were considerably lower, see section 4.2.2.), even when they stayed in the field for longer than the year before. We then exposed field populations to MagnetMED™ traps in the laboratory and found differences in susceptibility (Figure 4.4.b). Vila-real, Alcalà de Xivert and Algarrobo Costa populations showed no significantly different susceptibility from that of the susceptible C strain. On the contrary, individuals from Vinaròs and Betxí showed certain resistance to deltamethrin, with mortality below 40%. Remarkably, MagnetMED™ traps were totally inefficient when used with the lambda-cyhalothrin selected W-1Kλ strain, suggesting the existence of cross-resistance between lambda-cyhalothrin and deltamethrin (Figure 4.4.b).

Figure 4.4. Susceptibility of: a) laboratory C strain of *Ceratitis capitata* to MagnetMEDTM traps when they are new and after exposure of the devices to field conditions; and b) laboratory C and W-1Kλ strains and field populations of *C. capitata* collected in 2017 to new MagnetMEDTM traps. Different letters within each figure indicate significant differences (Repeated Measures ANOVA, Student Newman Keuls *post hoc* test, $p \leq 0.05$).

4.3.2. Search of resistant alleles in field populations of *C. capitata*

We developed a F1-screen assay to look for resistant alleles of *Ccα6* in the field. This assay is based on crosses between field collected (of unknown genotype) and laboratory individuals of the resistant isolate Q68*-K352*, homozygous for the resistant allele *Ccα6*^{3aQ68*-K352*}, or from the resistant strain JW-100s, with all individuals carrying *Ccα6*^{3aQ68*Δ3b-4} or *Ccα6*^{3aQ68*-K352*} alleles. Since spinosad resistance is completely recessive (Ureña *et al.*, 2019), only the F1 offspring with a resistant allele from the field collected parental and a resistant allele from the resistant isolate/strain will survive when exposed to a discriminatory concentration of spinosad.

When we crossed males from field populations with females from JW-100s strain, F1 survivors (pupae, pharates or adults) to spinosad were found in the crosses with Oliva, Algarrobo Costa and Puçol populations (Table 4.5). The *Ccα6* gene was sequenced in all F1 survivors (see section 4.2.4). In all cases, mutations Q68* and K352* corresponding to the parental JW-100s strain or Q68*-K352* isolate were found in one of the copies of the *Ccα6* gene in the resultant F1 individuals. However, we only found alterations in the second copy of *Ccα6* in two F1 survivors: one coming from the cross with males of Algarrobo Costa (K352*) and the other from the cross with males of Puçol (deletion of exons 5-11). So, in total, we found two mutations for a total of 440 males screened.

Table 4.5. F1-screen performed by crossing males from *Ceratitis capitata* field populations and females from the laboratory JW-100s strain or Q68*-K352* isolate, and detection of mutations in *Ccα6* in the F1-survivors.

Population/Strain	Year	Tested males (n)	F1 survivors (n) ⁽¹⁾	Mutations at <i>Ccα6</i> ⁽²⁾ in the F1 survivors
Oliva (València)	2016	70	1 pharate + 2 pupae	None
Algarrobo Costa (Málaga)	2016	140	3 adults + 1 pupa	K352* in <i>Ccα6</i> in 1 adult
Sanet i negrals (Alacant)	2017	25	0	None
Alcalà de Xivert (Castelló)	2017	25	0	None
Onda (Castelló)	2017	40	0	None
Puçol (València)	2018	140	4 adults + 2 pharates	Deletion exons 5-11 in 1 adult

⁽¹⁾ Number of F1 survivors to a discriminatory dose of 5 ppm in larvae diet.

⁽²⁾ Mutations refer to the copy of the *Ccα6* gene corresponding to the “tested” parental. In all cases, mutations Q68* and K352* corresponding to the parental strains JW-100s or Q68*-K352* were found in the other copy of the *Ccα6* gene.

Besides, we could confirm the hypothesis that alterations in both copies of the *Ccα6* lead to a phenotype of spinosad resistance due to a loss of gene function, and that the mutations in the “tested” parental can be different to those in the resistant laboratory isolate/strain. We did not find mutations or indels in the rest of F1 survivors. This could mean they were false positives that would have ingested a sublethal dose of the insecticide in the diet. However, we cannot discard that mutations in a gene different to *Ccα6* or alterations in non-coding parts of the genome could be causing the resistance.

The three regions of the *VGSC* gene that concentrate most of the *kdr* mutations associated to pyrethroids resistance described in the literature (Dong *et al.*, 2014) were sequenced in flies from Vinaròs, Betxí and Blanca that survived to MagnetMED™ traps or lambda-cyhalothrin. We did not find mutations in any of the 20 individuals analyzed from each population.

4.3.3. Impact of different treatment scenarios in the progression of resistance in the multiresistant laboratory strain MR of *C. capitata*

We generated the multiresistant MR strain by reciprocal crosses of JW-100s and W-1Kλ strains and keeping the offspring for two generations without exposure to insecticides. At this point (G1), we established four scenarios of insecticide treatments (Figure 4.1) and we observed how insecticide resistance evolved depending on the treatments received.

Referred to spinosad resistance (Table 4.6), MR strain presented in generation G1 a low increase in mortality rate in response to increasing concentrations of the insecticide, which is indicative of a heterogeneous group of individuals. We found that 18% of the individuals survived at 100 ppm, which probably corresponded to the expected ¼ of spinosad resistant homozygous individuals in this generation. Accordingly, 71% died when exposed to 10 ppm, probably due to expected ¼ homozygous and ½ heterozygous susceptible to this concentration. At 1 ppm, 56% mortality was obtained, which is also expected since the LC_{50} of susceptible individuals is 0.58 ppm (Ureña *et al.*, 2019), and ¼ of the individuals are expected to be homozygous for a resistant allele. However, when we tested susceptibility to spinosad in G5, we found no mortality to 100 ppm in the scenarios T1, T2 and T3. In MR-T1

resistance was fixed because individuals were exposed to a constant treatment of spinosad in each generation. MR-T2 and MR-T3, which combined 3 treatments with spinosad with a treatment with lambda-cyhalothrin, and 2 treatments with each insecticide, respectively, also fixed spinosad resistance, due to the first treatment they received was done with this insecticide. In MR-T4, susceptibility increased along the experiment due to the absence of spinosad treatments.

Table 4.6. Susceptibility to spinosad of the multiresistant strain MR of *Ceratitis capitata* under four different scenarios of insecticide treatments, T1: spinosad treatment in each generation; T2: alternation of spinosad for three generations and lambda-cyhalothrin for one generation; T3: alternation of spinosad for two generations and lambda-cyhalothrin for two generations; and T4: non-treated. See Figure 4.1 for more details.

Scenario	Generation	Mortality % $\pm$ SE (n) ⁽¹⁾				
		Non-treated	0.5 ppm	1 ppm	10 ppm	100 ppm
Initial MR	G1	2 $\pm$ 2 (45)	-	56 $\pm$ 11 (45)	71 $\pm$ 6 (45)	82 $\pm$ 4 (44)
T1	G5	0 $\pm$ 0 (36)	-	0 (12)	3 $\pm$ 3 (36)	0 $\pm$ 0 (36)
	G7	0 $\pm$ 0 (32)	-	-	-	7 $\pm$ 7 (32)
	G9	0 $\pm$ 0 (30)	-	-	-	0 $\pm$ 0 (30)
	G11	2 $\pm$ 2 (45)	-	-	-	13 $\pm$ 10 (45)
T2	G5	0 $\pm$ 0 (36)	0 $\pm$ 0 (24)	0 $\pm$ 0 (36)	0 (12)	0 $\pm$ 0 (36)
	G7	0 $\pm$ 0 (36)	-	-	-	0 $\pm$ 0 (36)
	G9	0 $\pm$ 0 (30)	-	-	-	3 $\pm$ 3 (30)
	G11	2 $\pm$ 2 (45)	-	-	-	0 $\pm$ 0 (45)
T3	G5	0 $\pm$ 0 (29)	0 $\pm$ 0 (20)	0 $\pm$ 0 (30)	0 (10)	0 $\pm$ 0 (30)
	G7	0 $\pm$ 0 (36)	-	-	-	3 $\pm$ 3 (36)
	G9	0 $\pm$ 0 (30)	-	-	-	3 $\pm$ 3 (30)
	G11	2 $\pm$ 2 (45)	-	-	-	0 $\pm$ 0 (45)
T4	G5	3 $\pm$ 3 (36)	8 $\pm$ 8 (24)	67 $\pm$ 25 (36)	100 (12)	97 $\pm$ 3 (36)
	G7	6 $\pm$ 3 (36)	77 $\pm$ 3 (35)	89 $\pm$ 3 (36)	97 $\pm$ 3 (36)	94 $\pm$ 6 (36)
	G9	3 $\pm$ 3 (30)	53 $\pm$ 3 (30)	84 $\pm$ 3 (31)	97 $\pm$ 3 (30)	93 $\pm$ 3 (30)
	G11	0 $\pm$ 0 (45)	69 $\pm$ 4 (45)	98 $\pm$ 2 (45)	98 $\pm$ 2 (45)	100 $\pm$ 0 (45)

⁽¹⁾ Number of flies tested (1–4 replicates of 10–15 flies each).

The evolution of *Ccα6* alleles associated with spinosad resistance in the four scenarios was analyzed by genotyping alternating generations with the markers for mutations

Q68* and K352* (see section 4.2.4). In G1, all the possible allelic combinations were represented and the observed genotypic frequencies corresponded to the expected for a Mendelian segregation (Figure 4.5.a), based on the genotypic frequencies of $Cc\alpha 6^{3aQ68*\Delta 3b-4}/Cc\alpha 6^{3aQ68*-K352*}$ (8%) and $Cc\alpha 6^{3aQ68*-K352*}/Cc\alpha 6^{3aQ68*-K352*}$ (92%) observed in the parental JW-100s strain, and $Cc\alpha 6^{+}/Cc\alpha 6^{+}$ (100%) in W-1Kλ. The genotypic frequencies of strains MR-T1, MR-T2 and MR-T3 evolved equally due to spinosad resistance was fixed since the very first treatment performed with this insecticide. Drastically, after one treatment with spinosad all the individuals analyzed were homozygous for the resistant $Cc\alpha 6^{3aQ68*-K352*}$ allele, thus ousting $Cc\alpha 6^{3aQ68*\Delta 3b-4}$ and $Cc\alpha 6^{+}$ from the MR strain (Figure 4.5.b). Although, we cannot discard that $Cc\alpha 6^{3aQ68*\Delta 3b-4}$ still remained in strains MR-T1, MR-T2 and MR-T3 in such a low frequency that did not allow its identification. On the contrary, in MR-T4 the absence of selection with spinosad implied a progressive loss of resistant alleles accompanied by an increase of $Cc\alpha 6^{+}$. After G5, all individuals carrying two resistant alleles were lost, and just heterozygous $Cc\alpha 6^{3aQ68*\Delta 3b-4}/Cc\alpha 6^{+}$ and $Cc\alpha 6^{3aQ68*-K352*}/Cc\alpha 6^{+}$ and homozygous $Cc\alpha 6^{+}/Cc\alpha 6^{+}$ individuals were found (Figure 4.5.c).

With regard to lambda-cyhalothrin resistance (Table 4.7), the initial situation (G1) showed a very heterogeneous response in the susceptibility of the MR strain to this insecticide. The variability in individuals' response, observed by a low slope and high fiducial limits ($LC_{50} = 50.63$ (3.89-129.87) ppm), was expected considering that in G1 (corresponding to a typical F2 cross) all genotypes were represented, including homozygous resistant, heterozygous and homozygous susceptible individuals. Furthermore, as resistance to lambda-cyhalothrin is controlled by several genes (Chapter 3), the percentage of each genotype would not coincide with 25, 50 and 25%, respectively, expected in a monogenic model. MR-T1 (exposed to a constant treatment of spinosad) did not display significant changes in the susceptibility to lambda-cyhalothrin with respect to G1. In MR-T2, LC_{50} values increased after the lambda-cyhalothrin treatment (G5 and G9), while they diminished again after two generations with spinosad treatment (G7 and G11). Thus, although we proved that lambda-cyhalothrin resistance evolved fast in the presence of selection pressure, a generation of relaxation of this pressure was enough to go backwards in the evolution of resistance. In MR-T3, we found the highest LC_{50} values in G5 and G9 (although they

were not significantly different in this generation, probably due to the high fiducial limits), after two generations of lambda-cyhalothrin treatments. Interestingly, these values were higher than those found in MR-T2 (G5 and G7), where only a generation with lambda-cyhalothrin exposure was introduced between the spinosad treatments, suggesting that several consecutive treatments with lambda-cyhalothrin have a powerful effect in selection of resistance. The relaxation of selection pressure was noticeable in G7 and G11, after two generations with spinosad treatments. MR-T4 (non-treated) showed random changes in susceptibility accompanied by big fiducial limits, which could mean that some individuals from this strain still kept the resistance mechanisms from the parental W-1Kλ and were able to escape the insecticide action. When testing resistance to malathion (Table 4.8), MR strain showed a low variability in the response to the insecticide at G1, as indicated by a high slope and reduced fiducial limits ($LC_{50} = 58.89$ (52.53-67.16) ppm). Later, susceptibility to this insecticide was not altered under the different scenarios, with similar LC_{50} values along the generations and RR that did not significantly differ from the RR of the initial MR strain (G1). This was predictable, as this insecticide was not used for selection during the experiment.

Figure 4.5. Evolution of genotypic frequencies associated to spinosad susceptibility of the multiresistant strain MR of *Ceratitidis capitata* from an initial generation G1 (a) under four different scenarios of insecticide treatments, b) T1: spinosad treatment in each generation; T2: alternation of spinosad for three generations and lambda-cyhalothrin for one generation; T3: alternation of spinosad for two generations and lambda-cyhalothrin for two generations; and c) T4: non-treated. See Figure 4.1 for more details. In each generation, 20 individuals from each group were genotyped. * indicates that observed genotypic frequencies correspond to the expected in a Mendelian inheritance segregation (χ^2 test, $p > 0.05$).

Table 4.7. Susceptibility to lambda-cyhalothrin in the multiresistant strain MR of *Ceratitis capitata* under four different scenarios of insecticide treatments, T1: spinosad treatment in each generation; T2: alternation of spinosad for three generations and lambda-cyhalothrin for one generation; T3: alternation of spinosad for two generations and lambda-cyhalothrin for two generations; and T4: non-treated. See Figure 4.1 for more details.

Scenario	Generation	n ⁽¹⁾	Slope $\pm$ SE	LC ₅₀ ⁽²⁾	95% FL	χ^2	df	Significancy	RR ⁽³⁾	95% FL	Significancy
Initial MR	G1	285	0.80 $\pm$ 0.15	50.63	3.89-129.87	36.63	13		1		
T1	G5	179	2.48 $\pm$ 0.34	84.29	61.75-115.21	10.37	10	*	1.66	0.59-4.71	
	G7	229	2.16 $\pm$ 0.33	30.52	22.47-39.46	15.24	14	*	0.60	0.22-1.69	
	G9	150	1.90 $\pm$ 0.30	101.21	72.36-148.29	6.38	10	*	2.00	0.69-5.82	
	G11	300	1.29 $\pm$ 0.20	145.40	101.14-219.26	6.65	14	*	2.87	0.98-8.39	
T2	G5	179	1.71 $\pm$ 0.25	175.87	124.96-245.44	7.38	10	*	3.47	1.20-10.05	#
	G7	180	1.60 $\pm$ 0.38	27.59	11.52-41.30	7.17	10	*	0.54	0.16-1.82	
	G9	199	2.06 $\pm$ 0.35	233.76	168.85-345.03	15.23	13	*	4.62	1.63-13.10	#
	G11	225	1.06 $\pm$ 0.34	21.78	0.30-62.90	7.55	10	*	0.43	0.02-8.66	
T3	G5	149	1.87 $\pm$ 0.29	301.06	173.08-579.44	18.68	10		5.95	2.02-17.54	#
	G7	262	2.07 $\pm$ 0.31	130.70	91.10-209.64	35.05	15		2.58	0.93-7.19	
	G9	250	1.69 $\pm$ 0.23	272.79	159.31-460.66	48.07	18		5.39	0.01-1947.02	
	G11	224	1.03 $\pm$ 0.34	21.97	0.19-65.11	8.59	10	*	0.43	0.02-11.11	
T4	G5	181	1.81 $\pm$ 0.32	172.74	110.12-334.75	13.22	10	*	3.41	1.14-10.22	#
	G7	212	1.52 $\pm$ 0.26	23.05	8.08-39.78	27.21	13		0.46	0.14-1.41	
	G9	120	3.38 $\pm$ 0.61	32.38	22.55-47.18	10.44	7	*	0.64	0.23-1.82	
	G11	300	1.25 $\pm$ 0.20	246.94	128.7-913.9	37.89	14		4.88	1.55-15.32	#

⁽¹⁾ Number of flies considered in the Probit analysis (including non-treated).

⁽²⁾ Lethal concentration (LC₅₀) in ppm of lambda-cyhalothrin in the diet for feeding bioassays at 48h.

⁽³⁾ Resistance ratio (RR) = LC₅₀ (selected strain) / LC₅₀ (MR-G1 strain). The fiducial limits for RR were calculated according to Robertson and Preisler (1992).

* Good fit of the data to the probit model ($p > 0.05$).

RR is significant ($p \leq 0.05$) if the 95% FL does not include 1.

Table 4.8. Susceptibility to malathion of the multiresistant strain MR of *Ceratitis capitata* under four different scenarios of insecticide treatments, T1: spinosad treatment in each generation; T2: alternation of spinosad for three generations and lambda-cyhalothrin for one generation; T3: alternation of spinosad for two generations and lambda-cyhalothrin for two generations; and T4: non-treated. See Figure 4.1 for more details.

Scenario	Generation	n ⁽¹⁾	Slope $\pm$ SE	LC ₅₀ ⁽²⁾	95% FL	χ^2	df	Significancy	RR ⁽³⁾	95% FL	Significancy
Initial MR	G1	222	4.95 $\pm$ 0.65	58.89	52.53-67.16	4.70	10	*	1.00		
T1	G5	180	3.40 $\pm$ 0.53	44.30	34.61-58.61	17.12	10	*	0.75	0.60-0.95	
	G7	230	2.37 $\pm$ 0.43	36.34	29.12-44.20	4.59	14	*	0.62	0.47-0.80	
	G9	150	4.23 $\pm$ 0.64	42.50	34.21-53.42	14.90	10	*	0.72	0.58-0.90	
	G11	225	4.65 $\pm$ 0.61	46.83	37.95-57.87	19.05	10		0.80	0.66-0.97	
T2	G5	180	3.04 $\pm$ 0.51	55.97	43.05-83.43	17.25	10	*	0.95	0.73-1.24	
	G7	178	2.70 $\pm$ 0.49	42.74	35.11-52.80	7.80	10	*	0.73	0.56-0.94	
	G9	150	4.33 $\pm$ 0.65	43.20	36.41-51.73	10.15	10	*	0.73	0.60-0.90	
	G11	223	3.19 $\pm$ 0.50	51.75	42.57-64.41	10.21	10	*	0.88	0.70-1.11	
T3	G5	149	3.87 $\pm$ 0.65	61.73	47.14-96.84	20.62	10		1.05	0.80-1.37	
	G7	175	2.71 $\pm$ 0.50	47.28	37.17-63.04	11.37	10	*	0.80	0.62-1.05	
	G9	250	3.03 $\pm$ 0.43	51.51	40.75-71.60	39.34	18		0.87	0.70-1.10	
	G11	225	3.20 $\pm$ 0.47	42.45	32.86-54.89	16.99	10	*	0.72	0.58-0.91	
T4	G5	180	3.71 $\pm$ 0.63	49.43	38.26-63.90	14.15	10	*	0.84	0.66-1.07	
	G7	297	2.60 $\pm$ 0.40	31.50	23.51-40.09	27.74	18	*	0.53	0.42-0.68	
	G9	150	4.36 $\pm$ 0.74	37.59	31.37-44.29	5.94	10	*	0.64	0.51-0.80	
	G11	299	3.71 $\pm$ 0.47	58.88	51.06-69.81	15.11	14	*	1.00	0.82-1.21	

⁽¹⁾ Number of flies considered in the Probit analysis (including non-treated).

⁽²⁾ Lethal concentration (LC₅₀) in ppm of malathion in the diet for feeding bioassays at 48h.

⁽³⁾ Resistance ratio (RR) = LC₅₀ (selected strain) / LC₅₀ (MR-G1 strain). The fiducial limits for RR were calculated according to Robertson and Preisler (1992).

* Good fit of the data to the probit model ($p > 0.05$).

RR is significant ($p \leq 0.05$) if the 95% FL does not include 1.

4.3.4. Modelling of spinosad resistance evolution

Simulations for the evolution of the three possible genotypes (S/S, S/R and R/R) under different scenarios were performed. The analysis consisted in calculating the frequencies of these genotypes over generations. Knowing the genotypic frequencies, the calculation of gene frequencies was straightforward. To facilitate the interpretation of the figures, only the evolution of the susceptible S allele is shown (note that knowing the frequency of allele S, that of allele R is complementary as both frequencies equal 1).

The fitness cost for resistant homozygous (R/R) and heterozygous (S/R) genotypes was estimated by assigning different combinations of fitness cost values to these genotypes and comparing the expected evolution of the R allele in each one of these situations with the frequency obtained experimentally in laboratory crosses during the study of the stability of spinosad resistant alleles *Ccα6*^{3aQ68*-K352*} and *Ccα6*^{3aQ68*Δ3b-4} (Chapter 2). The combination of fitness cost values that better adjusted the expected evolution of allelic frequencies to the experimental data was the same for both resistant alleles, and resulted 0.4 for R/R and 0.2 for S/R (Figure 4.6).

When the values for fitness cost estimated under laboratory conditions were considered, the model predicted that the S allele will rapidly get fixed in the population, and the R allele will be eliminated (Figure 4.7). We observed this result for both initial frequencies of the resistant alleles considered (IF1 or IF2), non-depending on the insecticide treatments applied and even at extreme conditions of insecticide exposure, with 6 treatments per year and when spinosad is supposed to reach the 80 and 95% of the individuals. The evolution at lower levels of insecticide exposure (20 and 50%) displayed the same trend (Figure 4.7). So, considering that spinosad resistance had a fitness cost in the wild equivalent to the one we found in the laboratory, resistant alleles would disappear from the field populations at their natural frequency of appearance in a few generations.

Figure 4.6. Estimation of the fitness cost associated to spinosad resistance under laboratory conditions. Experimental frequency (F) evolution of alleles $Cc\alpha 6^{3aQ68^*-K352^*}$ (a) and $Cc\alpha 6^{3aQ68^*\Delta 3b-4}$ (b) obtained in a study of their stability when in competition with individuals carrying the wild-type allele $Cc\alpha 6^+$ (Chapter 2, experimental data from Table 2.4). Different tests represent scenarios with different fitness cost values assigned to each genotype. The test with more intersections with the allelic frequency obtained from experimental data is considered the one that best adjusts to the evolution of resistance in the laboratory.

Treatment	G1	G2	G3	G4	G5	G6
TC	-	-	-	-	-	-
T1	-	-	-	-	s	-
T2	-	-	-	s	i	s
T3	-	-	-	s	s	s
T4	-	-	-	s,i	s,i	s,i
T5	-	-	-	s,s	s,s	s,s

Figure 4.7. Predicted rate of the evolution of the spinosad susceptible allele (S) frequency in field populations (24 generations, six generations/year) when using spinosad (s) and a second insecticide without cross resistance (i), under different resistance management strategies (T1-T5 and TC). Test conditions correspond to the parameters in the model that best adjust to the evolution of resistance in the field, with fitness cost values $w_{RR} = 0.4$ and $w_{RS} = 0.2$ (see Table 2). Four levels of insecticide exposure $e(i)$, 20, 50, 80 and 95%; and two initial frequencies corresponding to the presence of two (IF1; $F(S) = 0.9977273$) or four (IF2; $F(S) = 0.9954545$) resistant alleles (R) out of 880 analyzed were considered.

Treatment	G1	G2	G3	G4	G5	G6
TC	-	-	-	-	-	-
T1	-	-	-	-	s	-
T2	-	-	-	s	i	s
T3	-	-	-	s	s	s
T4	-	-	-	s,i	s,i	s,i
T5	-	-	-	s,s	s,s	s,s

Fitness cost of genotype XY (w_{XY})			
Code	w (S/S)	w (R/R)	w (S/R)
SC1	0	0.13	0.07
SC2	0	0.04	0.02
SC3	0	0.01	0.007
SC4	0	0.005	0.002

Figure 4.8. Predicted rate of the evolution of the spinosad susceptible allele (S) frequency in field populations (60 generations, six generations/year) when using spinosad (s) and a second insecticide without cross resistance (i), under different resistance management strategies (T1-T5 and TC) and different values of fitness cost: a) SC1, b) SC2, c) SC3 and d) SC4. Test conditions correspond to the parameters in the model that best adjust to the evolution of resistance in the field (see Table 4.2). A level of insecticide exposure $e(i) = 80\%$ and an initial frequency corresponding to the presence of four (IF2; $F(S) = 0.9954545$) resistant alleles (R) out of 880 analyzed were considered.

However, there is a chance that the fitness cost of spinosad resistance in the field is different to that observed in the laboratory. To explore this possibility, scenarios with different fitness costs were analyzed. It was observed that the lower the fitness cost was for the resistant allele, the faster increased its frequency in the population. This result was especially remarkable when considering an initial frequency IF_2 $F(R) = 0.0045455$ and high insecticide exposure (80%) (Figure 4.8). Under these conditions, resistant alleles are predicted to increase in the population if fitness cost is reduced 40 times (SC3, $w_{RR} = 0.01$ and $w_{SR} = 0.007$) and 6 treatments are applied per year (T5) (Figure 4.8.c). When fitness cost is reduced 80 times (SC4, $w_{RR} = 0.005$ and $w_{RS} = 0.002$), resistant alleles are expected to increase even with two treatments per year (T2), and with 6 treatments per year (T5) they would reach 5% before 10 years (60 generations) (Figure 4.8.d). It suggests that, if spinosad resistance evolved in the field associated to a low fitness cost, resistant individuals would rise in the population if the treatments with this insecticide were abundant, while the application of only one treatment of spinosad per generation, or the alternation between this insecticide and another without cross-resistance, would relax its selection pressure.

4.4. DISCUSSION

The current situation of insecticide resistance in Spanish field populations of *C. capitata* reveals certain concern. Resistance to lambda-cyhalothrin remains similar to previous years. In this work we found 2.70-7.93 fold resistance, slightly lower compared to a previous study in the period 2009-2010 in which authors detected 6-14 fold resistance (Arouri *et al.*, 2015). Nevertheless, mortality was below 50% in three of the seven populations tested at field concentration (125 ppm). Here, we have detected for the first time resistance to deltamethrin in field populations exposed to MagnetMED™ traps, which are coated with this insecticide. Field populations remain susceptible to spinosad, with LC_{50} values far below the recommended field concentration (260 ppm). However, their susceptibility is significantly lower (1.5-2.5 fold) compared to the susceptible laboratory strain C and, more importantly, resistant alleles of the *Ccα6* gene have been found at low frequency, so attention should be paid to avoid the development of resistance.

Medfly control with pyrethroids is compromised by the information obtained in this study about resistance to lambda-cyhalothrin and deltamethrin in field populations. Remarkably, deltamethrin resistance has been found in geographic areas where lambda-cyhalothrin resistance already existed. There is a chance that resistance to lambda-cyhalothrin is conferring cross-resistance to deltamethrin; however, they may have evolved independently. We have shown that W-1Kλ, a lambda-cyhalothrin resistant strain, is also resistant to MagnetMED™. Since this strain has never been selected with deltamethrin, we can conclude that in this case resistance to lambda-cyhalothrin confers cross-resistance to deltamethrin. Likewise, Arouri *et al.* (2015) showed that W-1Kλ had cross-resistance to deltamethrin and etofenprox by ingestion. However, the situation in the field may be different. We analyzed if individuals from two field populations resistant to deltamethrin and one also resistant to lambda-cyhalothrin had resistance-associated mutations in the VGSC gene, which cause insensitivity to the insecticide in many species (Dong *et al.*, 2014). Mutations were not found in those regions of the VGSC gene that concentrate most of the point mutations previously associated with *kdr* and super *kdr* resistance (Dong *et al.*, 2014), suggesting that target-site resistance was not present in field populations. Nevertheless, though the presence of mutations in other regions of the VGSC gene is unlikely, it cannot be discarded. It has been demonstrated that lambda-cyhalothrin resistance in the W-1Kλ strain is dominant and polygenic, with implication of P450 detoxification enzymes (Chapter 3, Arouri *et al.*, 2015). Further studies are needed to test if field resistance to the pyrethroids lambda-cyhalothrin and deltamethrin is mediated by P450s and if they have the same molecular mechanism, which will indicate cross-resistance. In any case, resistance management strategies must be implemented when using these two pyrethroids to prevent possible control failures. We simulate the evolution of lambda-cyhalothrin resistance in the multiresistant MR strain and found that a relaxation of resistance occurred when lambda-cyhalothrin treatments were introduced as a part of a rotation strategy with an insecticide with no cross-resistance as spinosad. However, resistance increased again after each treatment with lambda-cyhalothrin, probably due to the fact that inheritance of resistance in the parental W-1Kλ strain is dominant (Chapter 3). Our results indicate that the use of lambda-cyhalothrin bait sprays should be alternated with other insecticides with no cross-resistance and that the repeated

use of lambda-cyhalothrin bait sprays in the same fields should be avoided. Thus, farmers should be aware that lambda-cyhalothrin must not be used in those orchards where lure-and-kill traps of deltamethrin are deployed, as there is a risk of cross-resistance between them.

Though spinosad is still very effective in controlling *C. capitata*, resistant alleles exist in the field at low frequency. These resistant alleles would give rise to truncated isoforms of *Ccα6*, as those already reported in laboratory selected strains (Ureña *et al.*, 2019). Mathematical modeling predicted that, if the fitness cost of field resistant individuals is equivalent to that estimated for resistant laboratory isolines ($w_{RR} = 0.4$ and $w_{SR} = 0.2$), the resistant alleles will decline over time resulting in the disappearance of resistant individuals from the population. The model assumes that spinosad resistance is recessive, as determined for spinosad resistant strain JW-100s (Ureña *et al.*, 2019), and entails a high fitness cost, as observed for Q68* and Q68*-K352* isolines (Chapter 2). Actually, resistant isoline Q68* resulted to be defective to detect the parapheromone trimedlure and to mate females (Chapter 2). However, it is relevant to outline that fitness cost observed in resistant laboratory strains is not necessarily the same that in field resistant populations. Indeed, care should be taken when extrapolating laboratory results to field populations because their environmental conditions and genetic backgrounds are not comparable, and field genotypes are not always well-known (Ffrench-Constant and Bass, 2017; Lenormand *et al.*, 2018). Our simulations showed that a considerable reduction in fitness cost would be required to result in an increase of resistant alleles in the population. Nonetheless, there are several examples of absence of trade-offs in field that developed resistance as a result of selection of alleles with no fitness cost, selection of compensatory mutations that ameliorate the fitness of resistant genotypes or replacement of resistant alleles by less costly ones (Raymond *et al.*, 2001; Ffrench-Constant and Bass, 2017). Indeed, it has been suggested that genetic variability in field populations can compensate at some extent the fitness cost associated to some resistant genotypes (Ffrench-Constant and Bass, 2017). Since field resistance to spinosad in *C. capitata* has not evolved yet, experimental data are not available.

Treatments with spinosad for medfly control in citrus crops are normally performed during generations G4-G6 (*C. capitata* usually has six generations per year in the area

of study) in a period of two-three months from September to October/November, due to the phenology of the crop. During this period, 1 to 6 bait sprays treatments are applied, mainly ground treatments although aerial treatments can also be used punctually, depending on the level of the attack. They can be performed with spinosad as the only insecticide used, rotated with lambda-cyhalothrin or in combination with lure-and-kill traps of deltamethrin. It is noteworthy that medfly populations can also be treated with insecticides early in the year, coinciding with generations G2-G3, when it is attacking other crops. Our simulation model showed that even at the worst-case scenario with high exposition to spinosad (80%) and very low fitness cost ($w_{RR} = 0.005$ and $w_{SR} = 0.002$), the selection of resistant alleles can be delayed to avoid control failures by: i) reducing the number of spinosad treatments to a maximum of once per generation; and/or ii) applying spinosad on rotation with a second insecticide without cross-resistance. However, care should be taken in those fields where only spinosad is used year after year. Our study with the multiresistant strain MR showed that a single treatment with spinosad would be enough to kill susceptible and heterozygous individuals and fixate resistant alleles in a population if an exposure of 100% of the individuals was reached. Hence, although the recessive inheritance pattern and fitness cost of spinosad resistance together with the influence of migration will help to slow down the development of resistance in field populations, spinosad overuse would favor its quick fixation in small or isolated populations, as reported in other species (Bielza *et al.*, 2007b).

Malathion has been recently re-authorized for medfly control, with a restriction in its use of no more than one application per year (MAPA, 2019b). In the simulations performed here, we observed that treatments with spinosad or combinations of spinosad and lambda-cyhalothrin had no effect on selecting malathion resistance. Previous studies showed no cross-resistance between spinosad and malathion (Ureña *et al.*, 2019). Some cross-resistance was detected between malathion and lambda-cyhalothrin (Couso-Ferrer *et al.*, 2011), but the selection of the W-1Kλ strain with lambda-cyhalothrin was accompanied with a decline in the resistance to malathion (Arouri *et al.*, 2015), suggesting different resistance mechanisms. Thus, in principle, malathion may be considered as an additional alternative to spinosad and pyrethroids. Nevertheless, resistant alleles were already spread in Spain (Magaña *et al.*, 2007)

before its withdrawal occurred in 2009. Field resistance was mainly caused by alterations in the acetylcholinesterase: i) the G328A mutation in the *Ccace2* gene; and ii) a duplication of the *Ccace2* gene with one of the copies carrying the G328A mutation (Magaña *et al.*, 2008; Couso-Ferrer, 2012). Remarkably, studies performed by our group have determined that both resistant alleles are currently present in field populations from the Comunitat Valenciana (unpublished results), thus they may be rapidly selected. At present, its use is restricted to one application per year, which must be verified to avoid the selection of resistant alleles before its use as a viable alternative is considered.

Considering it all, it would be advisable for medfly control to combine insecticides of different chemical group, for instance spinosad and a pyrethroid (lambda-cyhalothrin or deltamethrin), and avoid the repeated use of a single insecticide in the same field. This can be performed by the rotation of spinosad and lambda-cyhalothrin as bait sprays, and by the use of spinosad bait sprays in those orchards where lure-and-kill traps of deltamethrin are deployed. However, the simultaneous use of lambda-cyhalothrin and deltamethrin is discouraged. The use of pyrethroids as part of a rotation strategy is expected to restrain the further evolution and spread of resistance. Actually, even for spinosad, for which resistance has not been developed yet, rotation should be implemented as the preferred control strategy to delay the appearance of resistance. Nevertheless, these resistance management strategies must be reinforced and harmonized with other control strategies implemented at present, such as cultural practices and the sterile insect technique.

CHAPTER 5. GENERAL DISCUSSION

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Field resistance to insecticides is recognized as a major problem for pest control of several tephritid species of economic importance (Vontas *et al.*, 2011). Only a few cases of insecticide resistance in fruit flies were reported before the 1990s (Keiser, 1989). However, the number of cases of resistance that result in a reduction of insecticide efficacy have risen in the last years, including organophosphates, pyrethroids and/or spinosad resistance in *Bactrocera cucurbitae* (Hsu and Feng, 2002; Jin *et al.*, 2016), *Bactrocera dorsalis* (Wei *et al.*, 2019), *Bactrocera oleae* (Margaritopoulos *et al.*, 2008; Pavlidi *et al.*, 2018) and *Bactrocera zonata* (Nadeem *et al.*, 2012). Insecticide resistance to malathion and lambda-cyhalothrin has also been reported for Spanish field populations of *Ceratitis capitata* (Magaña *et al.*, 2007; Arouri *et al.*, 2015). In this work (Chapter 4), we have corroborated that the current situation has become even more challenging. The levels of resistance to lambda-cyhalothrin remain similar to previous years, which jeopardizes the efficacy of this insecticide. More importantly, we have reported for the first time resistance to deltamethrin, an insecticide whose use has increased in the last years. This novel case of resistance could be due to the existence of cross-resistance between lambda-cyhalothrin and deltamethrin or as an independent mechanism. Field populations remain susceptible to spinosad, though resistant alleles to this insecticide have already been detected at a very low frequency. Likewise, resistant alleles to malathion are still present in field populations (unpublished results), even though this insecticide has not been used since its withdrawal in 2009. So far, no cases of field resistance have been reported for *C. capitata* in other countries (Voudouris *et al.*, 2018). Nonetheless, the G328A mutation in AChE that causes target site insensitivity to malathion in Spanish field populations of *C. capitata* (Magaña *et al.*, 2008) has been detected in field populations from Brazil (Elfekih *et al.*, 2014) and Tunisia (unpublished results). Besides, resistant flies may colonize new areas because of geographical dispersion (Gasperi *et al.*, 2002; Beroiz *et al.*, 2012) or trading activities, favoring the spread of resistance.

Effective monitoring systems are required for the correct diagnosis of insecticide resistance in field populations (Devine and Denholm, 2009; Hemingway *et al.*, 2013). Routine monitoring by bioassays remain the basis of most programs, but much

attention is being paid to developing techniques that offer greater precision (Devine and Denholm, 2009; Donnelly *et al.*, 2016). Concretely, the knowledge of the molecular mechanism of spinosad resistance (Ureña *et al.*, 2019) has allowed the detection of resistant alleles in field populations before a reduction of insecticide efficacy could be detected with bioassays (Chapter 4). Different mutations in the *Cca6* spinosad target site, giving rise to truncated transcripts, can lead to resistance (Ureña *et al.*, 2019; Chapter 2). We have proved that an F1-screen assay, which is based on the cross between field collected (of unknown genotype) and laboratory individuals of a resistant strain, could be used to detect mutations in the *Cca6* gene associated to spinosad resistance in field populations (Chapter 4). Interestingly, we were able to identify both a mutation previously identified in the resistant strain (K352*), and a new resistant allele (deletion of exons 5-11 in *Cca6*). In addition, the F1-screen assay has allowed a first estimation of the allelic frequency of spinosad resistant alleles in Spanish field populations, which has proved essential for modelling the potential evolution of resistance under different treatment strategies. However, the complexity of the resistance mechanism or the polygenic nature of some types of resistance makes it difficult to obtain a proper marker to confidently monitor resistance in field populations. This is the case of lambda-cyhalothrin resistance in *C. capitata*, in which several P450 genes and some QTLs of minor effect with a marked cumulative effect seem to contribute to the resistance phenotype (Chapter 3). Other species in which it has been demonstrated that different P450s contribute to pyrethroids resistance is *Anopheles funestus* (Wondji *et al.*, 2009). In this case, the expression of key P450 genes involved in resistance varied between regions in Africa (Riveron *et al.*, 2013; Samb *et al.*, 2016), exemplifying the difficulties of diagnostic tools based on transcripts. Only recently, a cis-regulatory polymorphism driving the overexpression of the major resistance gene *CYP6P9a* allowed to design a DNA-based assay that tracked the spread of pyrethroid resistance in Africa (Weedall *et al.*, 2019). Consequently, further studies are required to obtain robust molecular markers for lambda-cyhalothrin resistance in *C. capitata*, being bioassays still essential for field monitoring.

Understanding the mechanisms of field-developed resistance is essential for improving both insecticide resistance monitoring and management programs. However, most studies to discern these elements are performed with laboratory selected strains,

which may not be representative of what is occurring in the field (Ffrench-Constant, 2013). Essentially, the author reasoned that selection in the field acts on large population sizes and on a potentially limitless source of mutations, favoring a monogenic response involving a rare variant. Whereas, selection of a few inbred individuals in the laboratory favors an accumulation of a number of traits of minor effect. Even in those cases in which a laboratory resistant strain derives from a field-selected population, rearing conditions, selection pressure with insecticide, and environment would have marked a difference in the evolution of resistance. In the case of *C. capitata*, malathion resistance mechanisms were determined in a laboratory strain derived from a field resistant population (Magaña *et al.*, 2007, 2008) and later confirmed their presence in field populations (Couso-Ferrer, 2012), pointing to a robust determination of the mechanism. However, the mechanisms of spinosad (Ureña *et al.*, 2019, Chapter 2) and lambda-cyhalothrin (Arouri *et al.*, 2015; Chapter 3) resistance were elucidated in laboratory selected strains derived from field populations susceptible to these insecticides. For spinosad resistance, some of the mutations in the $\alpha 6$ subunit of the nAChR found in the laboratory resistant strain, but also new mutations, were detected in field populations (Chapter 4), when using the F1-screen method previously mentioned. Nevertheless, we cannot discard that other resistant alleles could be present in field populations that escape the detection control. In the case of lambda-cyhalothrin, the role of *CcCYP6A51* and other P450s, as well as other potential resistant genes of minor effect associated with the markers obtained by variant calling analysis (Chapter 3) has yet to be demonstrated in resistant field populations. Nonetheless, we have recently demonstrated that resistance to lambda-cyhalothrin in field populations was reverted by PBO (unpublished results), as reported for the laboratory selected strain (Arouri *et al.*, 2015; Chapter 3). Apart from differences in the mechanism, the inheritance and fitness costs of resistance can also vary between laboratory and field environments (Ffrench-Constant and Bass, 2017; Lenormand *et al.*, 2018). Ideally, resistant strains should be field derived and the costs and inheritance of resistance should be studied in the field. Thus, data obtained in the laboratory needs to be carefully used and corroborated across environments, whenever possible, to determine the rate of resistance evolution in the field.

An important outcome of this work is the potential of medfly populations to develop resistance to different classes of insecticides by multiple resistance mechanisms. Currently, the repertoire of effective insecticides against this pest in Spain is becoming very limited, farmers being constrained to use only one or a few effective insecticides. Thus, we are in a situation in which medfly control may be seriously compromised if resistance management strategies are not reinforced. Our results, based on evolutionary models and laboratory simulation studies (Chapter 4), indicate that the best option is the rotation of insecticides with different modes of action and no cross-resistance (spinosad and a pyrethroid), since this favours susceptible alleles in *C. capitata* populations. On the contrary, the repeated use of a single insecticide in the same field or the simultaneous use of lambda-cyhalothrin and deltamethrin is discouraged. A more efficient medfly control will result in a reduction of costs, and would also contribute to a reduction in the use of insecticides. For a sustainable use of chemical products and a better management of resistance, the simultaneous implementation of other control strategies results essential. For instance, the area-wide sterile insect technique program implemented in the Comunitat Valenciana since 2007 provides an effective medfly control in spring (Juan-Blasco *et al.*, 2014), contributing to reduce the population levels along the year. Thus, the combination of different control strategies should be harmonized to favor an efficient control management.

CONCLUSIONS

CONCLUSIONS

- 1) Resistance to the pyrethroids lambda-cyhalothrin and deltamethrin has been detected in Spanish field populations of *Ceratitis capitata*. At present, field populations remain susceptible to spinosad, although resistant alleles for this insecticide have been found at very low frequency.
- 2) Our results highlight the plasticity of the $\alpha 6$ subunit gene of the nicotinic acetylcholine receptor (*Cc $\alpha 6$*) of *C. capitata* to generate a wide range of mutations, which can lead to loss-of-function phenotypes potentially conferring spinosad resistance.
- 3) It has been confirmed that both wild-type isoforms of *Cc $\alpha 6$* , carrying exon 3a or 3b, are targeted by spinosad. The contribution to resistance of some of the mutated *Cc $\alpha 6$* alleles found in the laboratory resistant strain JW-100s have been validated by functional genomics and the generation of isolines.
- 4) The spinosad resistant alleles are unstable when in competition with wild-type alleles, thus suggesting that there must be a fitness cost associated to resistance. We have reported for the first time that a mutation in the *Cc $\alpha 6$* that leads to truncated transcripts has an effect on behavioral fitness traits.
- 5) Resistance to lambda-cyhalothrin in the laboratory resistant strain W-1K λ is inherited as a dominant, polygenic and not sex-linked trait.
- 6) The results obtained showed considerable variability in the expression of several P450 genes, including *CYP6A51*, in the resistant W-1K λ strain, indicating that several genes may be involved in metabolic detoxification mediated by P450 enzymes.

- 7) We have found by variant calling analysis that seven QTLs of minor effect with a marked cumulative effect are associated with resistance to lambda-cyhalothrin in the laboratory strain W-1Kλ.
- 8) We have not found a robust diagnostic marker to confidently monitor lambda-cyhalothrin resistance in field populations of *C. capitata*.
- 9) Resistance management strategies should be implemented to guarantee the sustainability of available insecticides. Our results indicate that it would be advisable to combine insecticides of different chemical groups, as spinosad and a pyrethroid lambda-cyhalothrin or deltamethrin. On the contrary, the repeated use in the same field of a single insecticide, or insecticides from the same group as these two pyrethroids, is discouraged.

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