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Control methods of *Trioza erytreae* Del Guercio (Hemiptera: Psylloidea: Triozidae), vector of Huanglongbing, and their implications in citrus agroecosystems

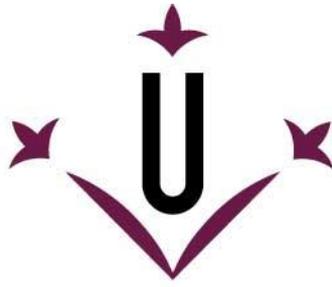
Paula Molina Melgares

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Universitat de Lleida

TESI DOCTORAL

Control methods of *Trioza erythrae* Del Guercio (Hemiptera: Psylloidea: Triozidae), vector of Huanglongbing, and their implications in citrus agroecosystems

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Table of contents

Abstract.....	1
Resum	3
Resumen	5
General introduction.....	7
Objectives	17
CHAPTER 1	21
Efficacy of selected pesticides for the chemical control of <i>Trioza erytreae</i>	
CHAPTER 2	33
Side effects of six systemic candidate pesticides to control <i>Trioza erytreae</i> on the parasitoid <i>Anagyrus vladimiri</i>	
CHAPTER 3	45
Development of a PCR-based method for the screening of potential predators of the African citrus psyllid <i>Trioza erytreae</i>	
CHAPTER 4	61
Potential natural enemies for a fortuitous biological control of <i>Trioza erytreae</i> in the Mediterranean basin	
General discussion.....	75
Conclusions	83
References	87

Abstract

The psyllid *Trioza erytreae* is one of the vectors of the citrus disease Huanglongbing (HLB), the main global citrus groves threat. Since its detection in the north-western Iberian Peninsula in 2014, its contention and eradication have been a priority to prevent its spread. The present Doctoral Thesis provides effective management approaches to control this vector, considering chemical and biological control. The efficacy of several pesticides with varying modes of action on the different developmental stages of *T. erytreae* was evaluated by applying them either by contact or by a systemic way in laboratory bioassays. Spinetoram caused the highest mortality on eggs (80-90%) and dimethoate, lambda cyhalothrin, spinetoram, cyantraniliprole and paraffin oil showed over 90% mortality on nymphs. Dimethoate, spinetoram and paraffin oil also demonstrated high efficacy on adults. The impact of six selected systemic pesticides (acetamiprid, dimethoate, cyantraniliprole, flupyradifurone, spirotetramat and sulfoxaflor) was assessed on the citrus mealybug parasitoid *Anagyrus vladimiri* when the parasitoid fed on pesticide solutions and on nectar of *Fagopyrum esculentum* plants irrigated with these pesticides. All of them, except spirotetramat, caused mortality on *A. vladimiri* females after feeding on pesticide solutions. They all reduced fertility, except flupyradifurone. When *F. esculentum* plants were watered with pesticides, a significant mortality was observed in females after 6 days feeding, except for flupyradifurone, ranging from 50% to 96% depending on the pesticide. Fertility was only reduced in those females that fed on spirotetramat and dimethoate watered plants. With the aim to determine which predators incorporated *T. erytreae* into their diet in real field conditions, a specific pair of primers was designed for its detection in predator gut content. Then, field-collected potential predators from *T. erytreae*-infested citrus trees in the Canary Islands and Galicia were analysed by PCR with the designed specific primers. A wide range of predatory taxa ingested *T. erytreae*, like some species of Coccinellidae, Anthocoridae, Chrysopidae, Hemerobiidae, Forficulidae, Miridae, Syrphidae, Formicidae, Erythraeidae and Araneae, with detection percentages ranging from 20 to 100% of the analysed individuals. Finally, seven ornamental trees located near the citrus growing areas infested with psyllids were sampled for natural enemies. The families Anthocoridae and Coccinellidae, together with the order Araneae were the most abundant generalist predators. Those psylla-tree associations with more diversity of predators were *Acizzia jamatonica* on *Albizia julibrissin*, *Cacopsylla alaterni* on *Rhamnus alaternus*, and *Lauritrioza alacris* on *Laurus nobilis*. The results of this Doctoral Thesis provide relevant tools to manage *T. erytreae* to be included in future Integrated Pest Management programs.

Resum

El psíl·lid *Trioza erythrae* és un dels vectors de la malaltia del cítrics Huanglongbing (HLB), la principal amenaça d'aquest cultiu. Des de la seva detecció al nord-oest de la península Ibèrica al 2014, la seva contenció i erradicació ha estat una prioritat per tal d'evitar la seva propagació. Aquesta Tesis Doctoral pretén aportar nous coneixements pel control químic i biològic d'aquest vector. D'una banda, s'ha avaluat l'eficàcia de diversos insecticides amb diferents modes d'acció sobre els diferents estadis de desenvolupament de *T. erythrae*, aplicats per contacte i per via sistèmica en laboratori. L'spinetoram va causar la major mortalitat en ous (80-90%); mentre que dimetoat, lambda cihalotrin, spinetoram, ciantraniliprol i oli parafínic van causar més d'un 90% de mortalitat en nimfes. Dimetoat, spinetoram i oli parafínic també van demostrar una alta eficàcia en adults. Posteriorment, es va avaluar l'impacte de sis d'aquests insecticides (acetamiprid, dimetoat, ciantraniliprol, flupiradifurona, spirotetramat i sulfoxaflor) sobre el parasitoide del cotonet del cítrics *Anagyrus vladimiri*. Quan el parasitoide s'alimentava de solucions d'aquests insecticides, tots ells, excepte el spirotetramat, van provocar una mortalitat significativa. També van reduir la fertilitat de les femelles de *A. vladimiri*, excepte la flupiradifurona. Després, es van tractar per irrigació plantes de *Fagopyrum esculentum* amb els insecticides, i es va observar una mortalitat més elevada (entre 50 i 96%), en les femelles d'*A. vladimiri* després d'alimentar-se del nèctar d'aquestes plantes durant 6 dies, excepte amb la flupiradifurona. La fertilitat només es va veure reduïda en el cas del dimetoat i l'spirotetramat. Per altra banda, per tal de determinar quins depredadors han incorporat *T. erythrae* en la seva dieta, es van dissenyar un parell d'encebadors específics per detectar-la en el contingut estomacal de depredadors. Després, es van recol·lectar depredadors potencials en cítrics infestats de *T. erythrae* a les illes Canàries i a Galícia, i es van analitzar per PCR amb els encebadors específics dissenyats. Un ampli rang de taxons depredadors havia ingerit *T. erythrae*, entre ells, espècies de Coccinellidae, Anthocoridae, Chrysopidae, Hemerobiidae, Formicidae, Miridae, Syrphidae, Formicidae, Erythraeidae i Araneae, amb percentatges de detecció entre el 20 i el 100%. Finalment, es van mostrejar set espècies d'arbres ornamentals localitzats prop de zones cítriques i infestats per psíl·lids per tal de catalogar els enemics naturals presents. Les famílies Anthocoridae i Coccinellidae, juntament amb l'ordre Araneae van ser les més abundants. Les associacions psíl·lid-arbre amb més diversitat de depredadors van ser *Acizzia jamaonica* sobre *Albizia julibrissin*, *Cacopsylla alaterni* sobre *Rhamnus alaternus*, i *Lauritrioza alacris* sobre *Laurus nobilis*. Els resultats d'aquesta Tesis Doctoral aporten eines rellevants pel control de *T. erythrae* que poden ser incloses en futurs programes de Gestió Integrada de Plagues.

Resumen

El psílido *Trioza erythrae* es uno de los vectores de la enfermedad de los cítricos Huanglongbing (HLB), la principal amenaza de este cultivo. Desde su detección en el noroeste de la península Ibérica en 2014, su contención y erradicación han sido una prioridad para evitar su propagación. Esta Tesis Doctoral pretende aportar nuevos conocimientos para el control químico y biológico de este vector. Se evaluó la eficacia de varios insecticidas con diferentes modos de acción sobre los diferentes estados de desarrollo de *T. erythrae*, aplicados por contacto y por vía sistémica en laboratorio. Spinetoram causó la mayor mortalidad en huevos (80-90%) y dimetoato, lambda cihalotrin, spinetoram, ciantraniliprol y aceite parafínico causaron más de un 90% de mortalidad en ninfas. Dimetoato, spinetoram y aceite parafínico también mostraron una alta eficacia en adultos. Así mismo, se evaluó el impacto de seis de estos insecticidas (acetamiprid, dimetoato, ciantraniliprol, flupiradifurona, spirotetramat y sulfoxaflor) sobre el parasitoide del cotonet de los cítricos *Anagyrus vladimiri*. Hubo mortalidad significativa cuando el parasitoide se alimentó de soluciones de insecticida en todos los casos excepto con spirotetramat. Todos los insecticidas ensayados excepto la flupiradifurona también afectaron a la fertilidad de las hembras. Cuando se trataron por irrigación plantas de *Fagopyrum esculentum* con estos insecticidas se observó una mayor mortalidad (entre 50 y 96%) en *A. vladimiri* después de alimentarse del néctar de las flores de estas plantas durante 6 días, excepto en flupiradifurona. La fertilidad solo se vio reducida en el caso del dimetoato y spirotetramat. Con el fin de determinar qué depredadores incorporan *T. erythrae* en su dieta, se diseñaron un par de cebadores específicos para detectar al psílido en el contenido estomacal de depredadores. Así mismo se recolectaron depredadores en cítricos infestados de *T. erythrae* en las islas Canarias y en Galicia, y se analizaron por PCR con los cebadores específicos diseñados. Un gran rango de taxones depredadores había ingerido *T. erythrae* (20-100% detección), entre ellos, especies de Coccinellidae, Anthocoridae, Chrysopidae, Hemerobiidae, Forficulidae, Miridae, Syrphidae, Formicidae, Erythraeidae y Araneae. Además, se muestrearon siete especies de árboles ornamentales localizados cerca de zonas citrícolas e infestados por psílicos con el fin de catalogar los enemigos naturales presentes. Las familias Anthocoridae y Coccinellidae, juntamente con el orden Araneae fueron las más abundantes. Las asociaciones psila-árbol con más diversidad de depredadores fueron *Acizzia jamatonica* sobre *Albizia julibrissin*, *Cacopsylla alaterni* sobre *Rhamnus alaternus* y *Lauritrioza alacris* sobre *Laurus nobilis*. Los resultados de esta Tesis Doctoral aportan herramientas relevantes para el control de *T. erythrae* que podrán ser incluidas en futuros programas de Gestión Integrada de Plagas.

General introduction

Citrus in Spain

Citrus is one of the main crops grown in the Mediterranean basin. The main cultivated citrus are oranges, mandarins, lemons and grapefruits, destined to fresh consumption, and this kind of production implies high quality standards, including both aesthetics and organoleptic characteristics. Spain is the first larger producer in the European Union (60%) and the sixth larger producer worldwide (5%) (FAO, 2021). Citrus production in Spain is around 6,850,000 tons per year, of which 60% is exported, becoming the most important exporter of fresh fruit worldwide (MAPA, 2022).

Pest management on citrus in the Mediterranean basin is mainly based on Integrated Pest Management (IPM) strategies, which involves the combination of biological, biotechnological, chemical and plant selection approaches to maintain pests below economic injury levels (EILs) (Kogan, 1998). One of the IPM objectives is the reduction of the pesticide use and its impact on human health, the environment, and the biocontrol agents used (Ehler, 2006). In Europe, IPM is mandatory since 2014, according to the European Directive 2009/128/CE. In fact, IPM programs in Spanish citrus have been implemented for decades, and the number of chemical treatments per season has been reduced (Garcia-Marí, 2012). Pesticide treatments per season in the Mediterranean citrus are restricted to key pests, which are those that their populations exceed EILs because their control by natural enemies is not enough.

As a perennial crop, citrus agroecosystems host a diverse community of phytophagous species and their natural enemies. Although a lot of phytophagous species can be present in citrus, only some of them are considered pests (Garcia-Marí, 2012). Among them, secondary pests are those that predators and parasitoids are able to maintain their populations below EILs, such as *Icerya purchasi* Maskell (Hemiptera: Monophlebidae) controlled by *Rodolia cardinalis* Mulsant (Coleoptera: Coccinellidae), or *Panonychus citri* (McGregor) (Acari: Tetranychidae) controlled by *Euseius stipulatus* Athias-Henriot (Acari: Phytoseiidae) (Jacas et al., 2006). Some citrus pests are not always satisfactorily controlled by natural enemies, being categorized as occasional pests, such as *Aleurothrixus floccosus* (Maskell) (Hemiptera: Aleyrodidae), *Planococcus citri* Risso (Hemiptera: Pseudococcidae) and *Phyllocnistis citrella* Station (Lepidoptera: Gracillariidae). Nevertheless, in the Mediterranean basin, the key pests are *Aonidella aurantii* (Maskell) (Hemiptera: Diaspididae), *Aphis spiraecola* Patch (Hemiptera: Aphididae), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae) and *Tetranychus urticae* Koch (Acari: Tetranychidae) (Jacas & Urbaneja, 2010; Urbaneja et al., 2020).

As other crops, citrus are also threatened by the arrival of exotic pests, which have been increasing in the last decades. Two of the main causes of this increase are the intensification of the international trade, and the rise of temperatures because of the global warming (Peña, 2013). Nevertheless, one of the highest threats for Mediterranean citrus is the arrival of the devastating citrus disease known as citrus greening or Huanglongbing (HLB) (Halbert & Manjunath, 2004).

The citrus disease Huanglongbing (HLB)

Huanglongbing is the most serious worldwide citrus disease which affects some plants of the Rutaceae family, mainly those species of the *Citrus* genus. The susceptibility to HLB varies among the host species, and all commercial citrus varieties are susceptible. The first report of HLB was in China back in 1919 (Reinking, 1919), where one of its main symptoms, the appearance of yellow shoots, gave it the name of Huanglongbing, meaning “yellow dragon disease”. Currently, HLB is present almost in all citrus growing areas of Asia, Africa, America, and Oceania (CABI, 2019). Until now, the Mediterranean basin and Australia are the only citrus growing areas free of HLB (CABI, 2019).

The pathogen agent of HLB is a phloem-restricted bacterium which is associated with three species of gram-negative proteobacteria: *Candidatus Liberibacter asiaticus* (Las), *Candidatus Liberibacter americanus* (Lam) and *Candidatus Liberibacter africanus* (Laf) (Bové, 2006).

Among the characteristic symptoms of this disease, there is a blotchy mottle of the leaves that results in yellow shoots, because leaves become partly yellow and partly green, with several shades of yellow, pale green and dark green (Bové, 2006). Fruits can be affected by being small and lopsided. Also, a characteristic colour inversion is observed when the fruit change the colour from green to yellow/orange, the peduncle end becomes yellow/orange, and the stylar end is still green. In lopsided fruits, the asymmetry is also evident inside the fruit, and some dark aborted seeds can also be observed. Furthermore, HLB can induce a premature fruit drop and a bitter taste in infected fruits (Ghosh et al., 2018; Gottwald et al., 2007). The disease eventually weakens the tree and causes its death.

Huanglongbing has no cure, so management strategies are based on the control of its vector, and the removal of infected trees to reduce the amount of inoculum available to the vectors (National Academies of Sciences, Engineering and Medicine, 2018). Additionally, the remove of infected material is hampered by the fact that this disease

has a latency period (6 to 12 months or longer) in which the tree is infected but asymptomatic (Bové, 2006).

This bacterium is mainly transmitted by the psyllids *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) and *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). Also, Cen et al. (2012) demonstrated that another psyllid species, *Cacopsylla citrisuga* (Yang & Li) (Hemiptera: Psyllidae), was also carrying Las in lemon trees in China. *Trioza erytreae*, commonly known as the African citrus psyllid, is responsible to spread Laf in Africa (Garnier et al., 2000; Haapalainen, 2014), and it has also been reported to transmit Las in Ethiopia (Ajene et al., 2019). On the other hand, *D. citri*, commonly known as the Asian citrus psyllid, is associated with the spread of Las and Lam in Asia and America (Halbert & Manjunath, 2004).

The detection of *T. erytreae* in mainland Europe in 2014 (Pérez-Otero et al., 2015) supposed that a potential risk of HLB was even closer (Arenas-Arenas et al., 2018).

The African citrus psyllid, *Trioza erytreae*

Trioza erytreae is native from south-eastern Africa (Del Guercio, 1918; van der Merwe, 1923), being distributed in most countries of the Afrotropic zone, including the Arabian Peninsula, and the islands of Saint Helena, Mauritius, Reunion, and Madagascar (Fig.I.1) (EPPO, 2022). In Europe, *T. erytreae* is present in the Macaronesia islands since 1994, when it was also detected in Madeira (Portugal) (Carvalho and Aguiar, 1997). Later, in 2002, it was detected in the Canary Islands (Spain) (González-Hernández, 2003). In mainland Europe, it is present in the Iberian Peninsula since 2014, when it was detected in Pontevedra (Galicia, Spain), spreading through the north coast of the Iberian Peninsula until the Basque Country (Spain), and through its western coast until the Algarve (Portugal) (EPPO, 2020 and 2021). This psyllid has moved through the citrus trees present in backyards and urban areas, and small citrus groves until it reached the citrus growing area of the Algarve, being very closed to the Spanish citrus growing area of Huelva.

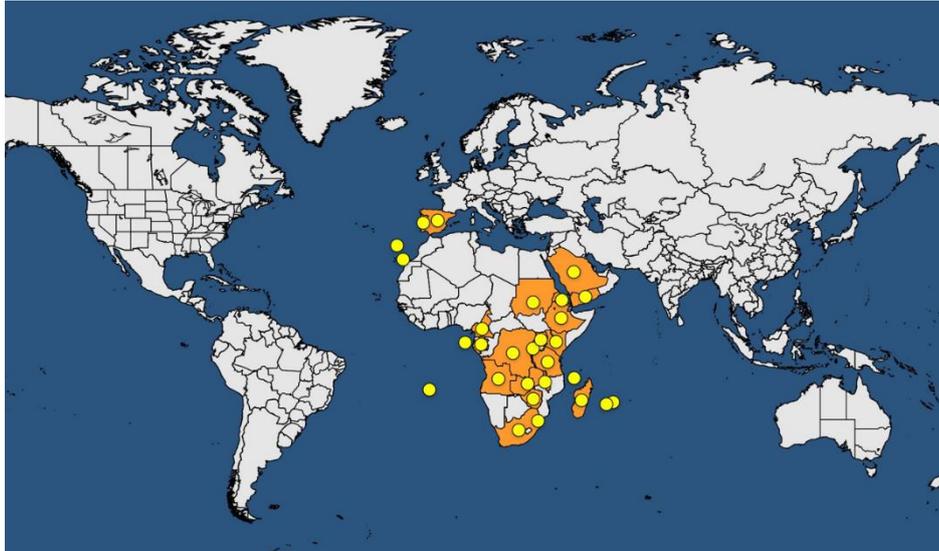


Figure I.1. Distribution of *Trioza erytreae*. Yellow spots indicate those locations where the psyllid is present nowadays (EPPO, 2022).

Its biological cycle passes through an egg and five nymphal instars to become an adult (Hodkinson, 1974). Adults are 3-4 mm long, being females longer than males (Fig. I.2C). They have transparent wings, and the body is first green, turning brown after few days. Females lay eggs on young shoots of the citrus trees. Eggs are yellow/orange, smooth, cylindrical with an anterior sharp point, and about 0.5 mm long (Fig. I.2A). Nymphs are oval and dorsally flattened with waxy white filaments (Fig. I.2B). Colour varies from pale orange to olive green with maturity, and their size can be between 0.25 to 1.13 mm from the first to the fifth instar (Moran & Blowers, 1967). Adults can live between 2-3 months on mature leaves when no young shoots are available (Catling, 1972).



Figure I.2. *Trioza erytreae* eggs (A), nymph (B) and adult (C).

Trioza erytreae biology is influenced by the weather conditions and the flushing rhythm of the citrus where they are present (Catling, 1972; Tamesse & Messi, 2004). Psyllid populations are limited by the extreme weather, since all development stages are particularly sensitive to high temperatures and low humidity (Catling, 1969a; Green &

Catling, 1971). The optimal conditions for its development are between 20-24°C and around 70% of relative humidity (Moran & Blowers, 1967). Nymphs cannot develop on mature leaves, so their survival depends on the availability of soft leaves on the flushes (Moran & Buchan, 1975). It has been described that *T. erytreae* populations are well correlated with the citrus flushing rhythm, and the number of psyllid generations per year is related with the annual flushing cycles (Catling, 1969b).

The feeding activity of *T. erytreae* causes direct and indirect damages on citrus. After the egg emergence, nymphs move to the underside of the leaves, producing a characteristic leaf deformation that results in open galls (Fig. I.3). When infestations are severe, the abundant honeydew excreted by their nymphs may be easily covered by a sooty mould. When heavy populations are present on the leaves, they become chlorotic, curly, and distorted (Catling, 1973). The major threat caused by *T. erytreae* is its capability to transmit the HLB. In fact, in areas where HLB is not present, it is considered a secondary pest (Cocuzza et al., 2017). Both nymphs and adults can acquire the bacterium, but HLB transmission is carried out by the adults, since only a small percentage of fourth and fifth instar nymphs can transmit it (Halbert & Manjunath, 2004; van den Berg et al., 1992). The transmission occurs through the salivary secretion (Aubert, 1987), and depending on the feeding time of the psyllid on HLB-infected tree, the transmission to healthy plants can happen from 24 h onwards (Buitendag & von Broembsen, 1993; van Vuuren & van der Merwe, 1992). Once the psyllid acquires the bacteria, it remains infective for life (Cocuzza et al., 2017). The efficiency rate transmitting the bacterium also varies according to the acquisition feeding time and the days after feeding (van Vuuren & van der Merwe, 1992).



Figure I.3. Deformation of leaves in form of open galls caused by *Trioza erytreae* nymphs.

Control methods of *Trioza erytreae*

Nowadays, eradication of *T. erytreae* has been impossible in those areas where the psyllid is widespread. Among the factors to consider when planning its containment and eradication, the presence of HLB is a determining one. Fortunately, in Europe, even if *T. erytreae* is present in some parts of the Iberian Peninsula and the Canary Islands for years, up to now this has not led to the HLB arrival (Siverio et al., 2017).

Since now, in the case that this psyllid vector is present together with the disease, chemical control measures are recommended, being usually applied with a high frequency of treatments per season and the use of broad-spectrum pesticides (Gottwald, 2010; Grafton-Cardwell et al., 2013). Broad information about *T. erytreae* chemical control is based on studies conducted more than 30 years ago in its native region and surrounding countries (Catling, 1969c; Pyle, 1977; van den Berg et al., 1983; Wortmann & Schafer, 1977). Successful control using pesticides has not been reported in these areas, nor in Madeira and Canary Islands, probably due to the difficult application in some infested areas, such as backyards and urban areas where the psyllid may take refuge (Fernandes & Aguiar, 2001; González-Hernández, 2003). Instead, more information about *D. citri* chemical control is available in large citrus growing areas, such as in Brazil or Florida (USA). These examples indicate a decline in citrus IPM because of the *D. citri* and HLB arrival (Belasque et al., 2010; Hall et al., 2013). In these citrus growing areas, the high frequency of broad-spectrum pesticides application makes difficult to combine it with other strategies to manage *D. citri* and other pests, such as biological control (Monzó et al., 2014; Qureshi & Stansly, 2009).

Chemical control against HLB vectors known to date is difficult to integrate in citrus with IPM strategies of the Mediterranean basin. On one hand, many of the pesticides that have been used for the vector control are no longer allowed in the EU, or their use is becoming increasingly restrictive. On the other hand, a seasonal application of pesticide treatments could disrupt the biological control of other citrus pests. For these reasons, it is crucial to study the efficacy of selective pesticides on *T. erytreae*, and their effects on natural enemies used in current citrus IPM programs.

Many predators have been described in Africa feeding on *T. erytreae*, contributing to its biological control, including some species of ladybird beetles, brown and green lacewings, hoverflies, anthocorids bugs and spiders among others (Catling, 1970; van den Berg et al., 1987). In Europe, some predator species have been found in *T. erytreae* infested citrus in the Canary Islands, which also belonged to the families described in

Africa, such as the ladybirds *Harmonia axyridis* Pallas and *Cryptolaemus montrouzieri* Mulsant, the green lacewing *Chrysoperla carnea* (Stephens) and the anthocorid bug *Orius laevigatus* (Fieber) (Estévez et al., 2018). In this study, the feeding activity conducted by those predators was estimated as not enough to control *T. erythrae* populations. Nevertheless, the use of molecular markers to study predator gut content in real predation impact in field conditions can be used to disentangle their potential effect, as it has been previously done for assessing prey-predator interactions in citrus agroecosystems (Agustí, Shayler et al., 2003; Furlong, 2015; Monzó et al., 2010; Romeu-Dalmau et al., 2012). Because parasitoids were not found naturally parasitizing *T. erythrae* nymphs in the Canary Islands (Estévez et al., 2018), a classical biological control program has been conducted there since 2018, and since 2019 in Iberian Peninsula (Hernández-Suárez et al., 2020; Tena et al., 2021). The main parasitoid of *T. erythrae* in Africa, *Tamarixia dryi* Waterson (Hymenoptera: Encyrtidae) (van den Berg & Greenland, 2000; Tamesse et al., 2002) was then introduced in both cases.

The natural enemies complex present in those areas where an exotic pest arrives can have a predatory/parasitic action providing a fortuitous biological control, implying the natural control of the exotic species without human manipulation (DeBach & Rosen, 1991). Although, as mentioned above, some predatory species have been reported in *T. erythrae* infested trees in Canary Islands, it is unknown which natural enemies are feeding on this psyllid populations in Iberian Peninsula.

Objectives

The main objective of this Doctoral Thesis was to find solutions for the potential presence of *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) on the Mediterranean citrus growing area. To achieve this, four specific objectives were addressed:

1. To study the efficacy of several pesticides under laboratory conditions to develop an integrated control strategy against *T. erytreae* (Chapter 1).
2. To evaluate the potential side effects of the previously selected pesticides on the mealybug parasitoid *Anagyrus vladimiri* Triapitsin (Hymenoptera: Encyrtidae), parasitoid of one of the most important pests in Mediterranean citrus, *Planococcus citri*. (Chapter 2).
3. To develop a field-collected predator molecular analysis to evaluate the natural predation on *T. erytreae* present on Spanish citrus, in order to show potential candidates for its biological control (Chapter 3).
4. To evaluate the potential of some natural enemies affecting other Psylloidea present on ornamental trees situated close to citrus growing areas of the Spanish Mediterranean coast for a potential fortuitous biological control of *T. erytreae* (Chapter 4).

CHAPTER 1

Efficacy of selected pesticides for the chemical control of
Trioza erytreae

Introduction

The African citrus psyllid, *Trioza erytreae* (Del Guercio, 1918) (Hemiptera: Triozidae), is a vector of the phloem-limited bacteria *Candidatus Liberibacter africanus* and *Candidatus Liberibacter asiaticus*. These bacteria are the causal agents of African citrus greening disease (Aubert et al., 1988; Jagoueix et al., 1996), and Asian citrus greening disease (Ajene et al., 2019), respectively. Both bacteria are etiological agents causing Citrus greening or Huanglongbing (HLB), which is currently the most devastating citrus disease worldwide (Cocuzza et al., 2017; Duran-Vila & Bové, 2015). HLB has been associated with the collapse of several citrus industries in Asia, America (Bové, 2006) and Africa (Pole et al., 2010; Richard et al., 2018).

Trioza erytreae is mainly found in Sub-Saharan Africa and its neighbouring islands (CABI, 2019; EPPO, 2020). In 1994, *T. erytreae* was found in the archipelago of Madeira (Portugal) (Carvalho & Aguiar, 1997) and in 2002 in the Canary Islands (González-Hernández, 2003). In 2014, *T. erytreae* was detected in north-western Spain (Pontevedra, Galicia) and northern Portugal (Pérez-Otero et al., 2015), which was the first time it was detected in the mainland Mediterranean basin. Control measures applied during the outbreak did not stop the spread of the pest, and currently it is present in several key citrus-producing areas of Portugal, including the region of the Algarve, next to Huelva, a very important citrus producing area of Spain (Arenas-Arenas et al., 2018, 2019; EPPO, 2020). Therefore, a major effort must be made to contain the spread of this pest, which now threatens the nearby citrus production of Andalusia. Indeed, it presents a great risk for the entire Mediterranean citrus sector, since the vector's arrival in an area is usually followed by the arrival of the bacteria (Bové, 2006; Duran-Vila & Bové, 2015).

Since *T. erytreae* was detected on the Canary Islands none of the generalist predators have been effective in reducing psyllid populations. Recently, a classical biological control programme was launched and the parasitoid *Tamarixia dryi* Waterson (Hymenoptera: Encyrtidae) was imported and released in the area (Hernández-Suárez et al., 2020).

Therefore, chemical control is a crucial strategy to prevent the spread of this vector and reduce its impact where the disease is already established (Monzó & Stansly, 2017). However, little information on *T. erytreae* chemical control is available in the literature. Most studies on its control have been carried out in South Africa and were published before the 1990s (Catling, 1969c; Deacon et al., 1989; Pyle, 1977; Wortmann & Schafer, 1977). The main chemical strategy has focused on neonicotinoids, thiamethoxam and imidacloprid, but nowadays they cannot be used in the Mediterranean citrus area, since

they are banned in Europe (European Commission, 2018b; MAPA, 2021). Moreover, there are serious concerns about resistance development in *T. erytreae* populations. Thus, any chemical control programmes against this pest should consider the rotation of active ingredients to be sustainable in the long term (Tiwari et al., 2011). For this reason, there is considerable urgency to develop an effective strategy for the control of *T. erytreae* using with different alternatives.

Thus, with the aim to provide useful information for the development of an integrated control strategy against *T. erytreae*, the present study compares the efficacy of various pesticides with distinct modes of action on different stages of development of *T. erytreae* under laboratory conditions.

Materials and Methods

Pesticides

The eleven pesticides used in the assays are listed in Table 1.1. Pesticides were selected based on their different modes of action, as well as for being effective at controlling other citrus sucking pests or other psyllids in other crops. Selected active ingredients (ai) have either contact action, systemic action, or both. The pesticides were applied at the maximum field recommended concentration (MFRC).

Insects

Trioza erytreae eggs, nymphs, and adults used in laboratory bioassays were collected directly from pesticide-free lemon *Citrus limon* (L.) Osbeck orchards on Tenerife (28°29'21.6" N, 16°21'20.3" W) and Gran Canaria (28°03'45.9" N, 15°34'28.9" W) (Canary Islands, Spain) less than 2 h before performing the experiments.

Laboratory bioassays

These bioassays were conducted under laboratory conditions in the Canary Institute of Agricultural Research (ICIA) facilities in Valle de Guerra (Tenerife, Spain).

Table 1.1. Insecticides used in the contact (C) and systemic (S) laboratory bioassays.

Trade Name	Active Ingredient	Formulation ^a	IRAC Subgroup _b	Rate (%)	Manufacturer	Bioassay	
						C	S
Gazel Plus [®]	Acetamiprid	20% SG	4A	0.025	BASF	x	x
Exirel [®]	Cyantranilprole	10% SE	28	0.1	FMC	x	x
Perfekthion Top [®]	Dimethoate	40% EC	1B	0.1	BASF	x	x
Teppeki [®]	Fonicamid	50% WG	29	0.005	Belchim Crop Protection	x	x
Sivanto [®]	Flupyradifurone	20% SL	4D	0.05	Bayer CropScience	x	x
Kenotrin [®]	Lambda cyhalothrin	2.5% WG	3A	0.08	Kenogard	x	
Ovitex [®]	Paraffin oil	83% EC	UN	1.5	Belchim Crop Protection	x	
Plenum [®]	Pymetrozine	50% WG	9B	0.04	Syngenta	x	x
Delegate [®]	Spinetoram	25%WG	5	0.04	Corteva Agriscience	x	
Movento 150 O-TEC [®]	Spirotetramat	15% OD	23	0.04	Bayer CropScience	x	x
GF-2626 [®]	Sulfoxaflor	12% SC	4C	0.04	Dow Agroscience	x	x

^a SG: water soluble granules; EC: emulsifiable concentrate; SL: soluble concentrate; SE: sus-po-emulsion; WG: water dispersible granules; OD: oil dispersion; SC: suspension concentrate. ^b IRAC (Insecticide Resistance Action Committee): 4A: neonicotinoids; UN: compounds of unknown or uncertain IRAC subgroup; 28: diamides; 1B: organophosphates; 29: flonicamid; 4D: butenolides; 3A: pyrethroids, pyrethrins; 9B: pyridine azomethine derivatives; 5: spinosyns; 23: tetrone and tetramic acid derivatives; 4C: sulfoximines.

Contact bioassays

Contact assays were conducted with *T. erytrae* eggs, nymphs, and adults. The protocol used was modified from Srinivasan et al. (2008). Citrus shoots heavily infested with *T. erytrae* eggs and nymphs collected from citrus orchards were transported to the laboratory in a portable fridge (approx. 10 °C). Leaves with more than 20 eggs and leaves with 20–60 1st–3rd instar nymphs per leaf were selected. Each replicate consisted of one selected leaf, and four replicates were performed for each treatment and developmental stage. All selected leaves, either with eggs or nymphs, were dipped in the different pesticide solutions (Table 1.1) for 10 s and placed on a filter paper disc until the complete evaporation of the pesticides. Distilled water was used as a control treatment. After that, each leaf with eggs was placed in a Petri dish (90 mm diameter) containing an agar-agar solution (1.5%) for 96 h, where petioles were inserted to keep leaf turgidity. These Petri dishes had small holes in the lids to allow ventilation and were sealed with Parafilm© (Bemis Company, Inc., Neenah, USA) to prevent the emerging nymphs from escaping. The number of non-hatched eggs and nymphs were counted after this period. Additionally, each leaf with nymphs was placed in a Petri dish (50 mm

diameter) with a wet filter paper disc at the bottom, and the numbers of dead and live psyllids nymphs were recorded after 24 h.

Trioza erytreae adults were collected in groups of ten with an insect pooter from citrus groves and taken to the laboratory in a portable fridge. There, each group was chilled for 90 s and then transferred to a Petri dish (50 mm diameter) with a filter paper disc on the bottom. Each pesticide (0.5 mL) was then topically applied to each group of adults with a micropipette. Four replicates per treatment were performed. After two minutes of the application, adult psyllids were transferred to a ventilated plastic cage ($\varnothing = 60$ mm, $h = 32$ mm) containing a single citrus leaf, as a food source, and a small water vial (2 mL) as a humidity source. After 24 h, the numbers of dead and live adults were recorded.

Systemic bioassays

The toxicity of eight systemic pesticides (Table 1.1) was assessed against nymphs and adults of *T. erytreae* using an uptake bioassay technique for systemic insecticides, as described in Prabhaker et al. (2007). Citrus shoots of 15 cm, with at least two terminal leaves infested with psyllid nymphs, were collected. Each main stem of the shoot was placed in a glass tube (50 mL) with one pesticide solution for 24 h. Distilled water was used as a control treatment. Four replicates were performed per treatment. To prevent nymphs from escaping, each stem was protected with a paper cone with glycerine in the corners. After that, shoots were transferred to a glass tube with water, where nymph mortality was checked after 24 h.

For the adult experiment, citrus shoots were placed in each pesticide solution for 24 h. After that, shoots were transferred to a clean 1.5 mL tube with water. Each tube was placed inside a plastic container (1 L) with a filter paper disc on the bottom, a 2 mL water vial as a humidity source, and ten adults of *T. erytreae*. Four replicates per pesticide were performed. After 48 h, the numbers of dead and live psyllids adults were counted.

Statistical analysis

Percentage mortality was calculated in all contact and systemic bioassays for each *T. erytreae* stage treated. This percentage was compared with the control using the t-test ($p < 0.05$) proc TTEST (SAS, 2009, SAS Institute Inc, Cary, NC, USA) in the laboratory contact bioassays. In the laboratory, systemic bioassays percentage mortality was analyzed using a one-way analysis of variance with the treatment as a factor and

mortality as the dependent variable proc ANOVA (SAS, 2009, SAS Institute Inc, Cary, NC, USA) followed by a Tukey post-hoc test.

Mortality percentages of the contact bioassay and systemic with nymphs were corrected by the Henderson-Tilton formula (Henderson & Tilton, 1955), since the initial number of individuals was not uniform among treatments. On the contrary, since the population of adults in the systemic bioassays was uniform among treatments, we applied Schneider-Orelli's formula (Schneider-Orelli, 1947).

Results

Contact bioassays

In the contact bioassays, only four active ingredients were effective on eggs: dimethoate, lambda cyhalothrin, spinetoram, and flupyradifurone (Table 1.2). The efficacy on eggs ranged from 64.2% for flupyradifurone to 81% for spinetoram. In the case of nymphs, three pesticides were ineffective (flupyradifurone, spirotetramat, and pymetrozine), whereas efficacy ranged from 60% for sulfoxaflor to 100% for paraffin oil. Four active ingredients (paraffin oil, dimethoate, lambda cyhalothrin, and spinetoram) showed efficacies > 93%. Six active ingredients were effective on adults, ranging from 54.2% for flupyradifurone to 100% for dimethoate, paraffin oil, spinetoram, and dimethoate presented efficacies > 95%.

Table 1.2. Percentage of corrected mortality (%) in *Trioza erytreae* eggs, nymphs and adults in contact bioassays. The mortality was recorded 96 h after treatment for eggs, and 24 h after treatment for nymphs and adults.

Active Ingredient	Eggs	t	Nymphs	t	Adults	t
Acetamiprid	0	2.44	69.4	6.12 *	78.4	8.99 *
Cyantraniliprole	0	1.44	65.1	8.97 *	0	0.38
Dimethoate	71.6	3.23 *	93.7	15.41 *	100	19.66 *
Flonicamid	0	0.90	62.9	6.20 *	0	0.10
Flupyradifurone	64.2	2.69 *	0	2.02	54.2	4.39 *
Lambda cyhalothrin	65.8	3.06 *	99.2	7.86 *	79	6.47 *
Paraffin oil	0	0.80	100	20.45 *	95.8	2.85 *
Pymetrozine	0	0.43	0	0.86	0	0.60
Spinetoram	81	4.29 *	95.8	18.18 *	95.8	2.85 *
Spirotetramat	0	1.50	0	0.41	0	0.75
Sulfoxaflor	0	0.50	60	10.39 *	0	0.90

df = 6. * Indicate significant differences ($p < 0.05$; t-test) between treatments and the control.

The active ingredients dimethoate, lambda-cyhalothrin, and spinetoram were significantly effective on the three development stages of *T. erytrae*. Dimethoate was 100% effective in adults, over 90% in nymphs, and 71.6% in eggs. Lambda cyhalothrin showed high efficacy against nymphs, almost 100%, 78.9% in adults, and 65.7% in eggs. Regarding spinetoram, the percentage of efficacy was the same for nymphs and adults, 95.8%, and 81% in eggs.

Paraffin oil was highly effective against nymphs with 100% mortality and 95.7% mortality in adults, but it was not significantly effective against eggs. The same scenario was repeated for acetamiprid, though mortality was lower, 69.4% in nymphs and 78.3% in adults. Flupyradifurone was effective in eggs and nymphs, but with lower mortality in comparison with the other active ingredients, 64–21% in eggs and 54.1% in nymphs. Cyantraniliprole, sulfoxaflor, and flonicamid were only significantly effective for nymphs; with around 60% mortality. Finally, there were two active ingredients, spirotetramat and pymetrozine, which were not effective against any of the three stages of *T. erytrae* in contact bioassays.

Systemic bioassays

In the systemic bioassays (Table 1.3), five of the eight active ingredients tested were significantly effective against nymphs, however, only dimethoate was effective against adults (90.6%). For nymphs, dimethoate and cyantraniliprole led to mortality of more than 90%, while with sulfoxaflor and spirotetramat mortality was higher than 75% and flupyradifurone led to 51.7% mortality. Dimethoate was effective on all the developmental stages, while cyantraniliprole and sulfoxaflor showed efficacy only against nymphs, both applied by contact and systemically.

Table 1.3. Mean percentage of *T. erytrae* nymph mortality (%) \pm standard error in systemic bioassays. In brackets the mean percentages of corrected mortality (%). The mortality was recorded 48 h after shoots were treated.

Active Ingredient	Nymphs	Adults
Control	19.4 \pm 7.5 a	7.5 \pm 4.8 A
Acetamiprid	49 \pm 3.9 ab (0)	27.5 \pm 8.5 A (0)
Cyantraniliprole	95.4 \pm 1.9 c (93.7)	5 \pm 5 A (0)
Dimethoate	96 \pm 3.4 c (94.5)	80 \pm 4.1 B (90.6)
Flonicamid	46.6 \pm 6.7 ab (0)	12.5 \pm 6.3 A (0)
Flupyradifurone	57.9 \pm 5.1 b (51.8)	15 \pm 8.7 A (0)
Pymetrozine	50.9 \pm 8.2 ab (0)	5 \pm 5 A (0)
Spirotetramat	73.9 \pm 13.3 bc (77.1)	5 \pm 2.9 A (0)
Sulfoxaflor	79.3 \pm 7.8 bc (78.8)	22.5 \pm 8.5 A (0)
F	12.35	14.36
df	8, 27	8, 27
<i>p</i> -value	<0.001	<0.001

Means followed by different letters in the same column differ at the 5% significance level when compared using the Tukey test (lowercase for nymphs, capital letters for adults).

Discussion

In this study, we have investigated the efficacy of eleven products that could be candidate pesticides in IPM programs to control *T. erytrae* populations. Such insecticidal vector control is considered one of the main strategies to slow the spread of HLB once the disease is established (Monzó & Stansly, 2017).

Key factors to consider when attempting to control *T. erytrae* populations are the flushing rhythm and flushing quality of the citrus plants, as reported in South Africa (Catling, 1969b). For this reason, the management of this pest must focus on the main flushes in spring, summer, and fall. Although pesticide application in winter effectively decreased nymph density for a short period, it failed to maintain populations at low levels in the long term, such as in the following spring flushes. Therefore, pesticide applications in winter must be applied just to adults, when no new flushes are present that could allow *T. erytrae* adults to overwinter hidden/protected in the tree canopy and feed on mature leaves (Catling, 1972). In adult experiments, six pesticides exceeded 50% efficacy, and three of them (dimethoate, spinetoram, and paraffin oil) even reached 90%. The

application of these pesticides in this season can also control other citrus pests. For example, paraffin oil applied in winter can control red spider mites, citrus leafminers, and wax scales (Beattie et al., 1991; Jacas & Garcia-Marí, 2001). In addition, application in winter also reduces the potential negative impact of the pesticide treatments on the beneficial entomofauna (Jacas & Garcia-Marí, 2001).

Spring flushes tend to be the most abundant and concentrated, and psyllid populations grow very fast during these flushes. Sometimes, a second flushing period occurs in summer and autumn. At these times, treatments should focus on eggs, and especially on nymphs. In our results, four active ingredients showed more than 50% efficacy against *T. erytreae* eggs, but only one, spinetoram, achieved 80–90%. Several of the tested pesticides were very effective in the control of nymphs. All the pesticides tested, except pymetrozine, were effective, and five of them (dimethoate, lambda cyhalothrin, spinetoram, cyantraniliprole, and paraffin oil) produced more than 90% nymph mortality. For *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) nymphs and adults, spinetoram, spirotetramat, cyantraniliprole, sulfoxaflor, lambda cyhalothrin and paraffin oil (only for nymphs) have also been reported as highly effective (Khan et al., 2013; Qureshi et al., 2014; Rae et al 1997; Tiwari & Stelinski, 2013). In addition, cyantraniliprole showed antifeeding activity on *D. citri* adults, which leads to a reduction in HLB transmission (Ammar et al., 2015).

Even though sulfoxaflor, flonicamid, flupyradifurone, and spirotetramat showed medium efficacy (50–80%) on *T. erytreae*, they can also be used in IPM programs, since they act on other citrus pests such as aphids, citrus leafminer, or whiteflies (Bacci et al., 2018; Nauen et al., 2008, 2015). Moreover, citrus entomofauna is rich and diverse, thus, giving these natural enemies an important role to play in conservation biological control of citrus pests (Urbaneja et al., 2020). Owing to this, the toxicity of selected pesticides for *T. erytreae* populations on predators and parasitoids present on citrus must also be considered (Jacas et al., 2010). Dimethoate, which showed the highest efficacy against *T. erytreae* in our work, reported the most negative effect on *T. erytreae* parasitoid, *T. dryi* (Dionisio et al., 2021). However, some of the tested pesticides, such as cyantraniliprole were reported to be much less toxic to *T. dryi* than to the target psyllid (Dionisio et al., 2021). Similarly, this pesticide showed less toxicity to the *D. citri* parasitoid, *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) than to the target psyllid (Tiwari & Stelinski, 2013). Additionally, sulfoxaflor was reported as less toxic for this parasitoid than for *D. citri* adults (Brar et al., 2017). Moreover, in Florida, lacewing abundance was not affected by pesticide treatments against *D. citri*, even when broad-spectrum pesticides were applied (Monzó et al., 2014). In the same study, however, the

reduction of spider predation on *D. citri* in spring could be explained by the broad-spectrum pesticides used in winter, though pesticides used later such as spinetoram and spirotetramat were not considered toxic to spiders.

Resistance of *D. citri* to different chemical families has been reported for chlorpyrifos (organophosphates), fenpropathrin (pyrethroids), imidacloprid, and thiamethoxam (neonicotinoids). Therefore, it is crucial to contemplate the need to rotate between different modes of action to maintain effective control of psyllid pests (Tiwari et al., 2011).

In conclusion, the first tool to slow the spread of *T. erytreae* in a new citrus growing area is the application of chemical treatments. In this work, we have shown that several phytosanitary products with different modes of action have high efficacy and can be used on citrus trees for the control of *T. erytreae*. Specifically, for the best results from chemical control of *T. erytreae* consideration must be given to the plant phenology, the season of the year, and the main developmental stages of the pest. Such chemical control integrated into pest management programs of citrus fruits could also be effective for the control of other pests, yet preserving, as far as possible, the natural enemies that are present naturally or released in citrus groves.

CHAPTER 2

Side effects of six systemic candidate pesticides to control *Trioza erytreae* on the parasitoid *Anagyrus vladimiri*

Introduction

Citrus orchards, as perennial tree crops, can host a wide range of pests along the year, as well as their natural enemies. Most of the citrus pests are successfully controlled by a guild of parasitoids and predators, remaining below their economic threshold level (Jacas & Urbaneja, 2010; Urbaneja et al., 2020). Conservation biological control (CBC) is a key tool in citrus orchards where Integrated Pest Management (IPM) programs are applied. On the other hand, resident or sown vegetation covers in citrus orchards may act as food source, provides shelter for beneficial insects, and contribute to the conservation of natural enemies (Gómez-Marco et al., 2016; IOBC/WPRS, 2022; Silva et al., 2010).

Natural enemies are directly exposed to pesticides when they are directly applied or through their residues. The first toxicity impact is by contact and/or inhalation when the tree canopy is treated. There, natural enemies can be affected by topically exposure of pesticides or by walking on dried residues (Barros et al., 2018; Suma et al., 2009). Other ways of exposure may be by feeding on pesticide-treated prey (Martinou et al., 2014; Rugno et al., 2021) or by the ingestion of food sources such as pollen, nectar, honeydew or guttation drops (Calvo-Agudo et al., 2019; Cloyd & Bethke, 2011; Martínez-Ferrer et al., 2019). Furthermore, since a 29% of the sprayed pesticides applied to the tree canopy reaches the ground (Garcerá et al., 2017), pesticide residues can also be present on the flowers of the ground cover, and they can eventually translocate to pollen and nectar (David et al., 2016; García-Vacárcel et al., 2022). It has been reported that neonicotinoid residues can be detected in wildflowers nectar more than 200 days after their application in citrus orchards (García-Vacárcel et al., 2022). Consumption of contaminated nectar of wildflowers may lead to lethal or sublethal side effects on natural enemies fitness (Desneux et al., 2007), and consequently reducing or limiting their regulatory capacity of pest population.

The parasitoid *Anagyrus vladimiri* Triapytsin (Hymenoptera: Encyrtidae) is one of the main natural enemies present in Mediterranean citrus orchards, playing an important role in the control of the citrus mealybug *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) (Jacas & Urbaneja, 2010). In fact, this pest is currently considered as occasional in the Mediterranean basin (Urbaneja et al., 2020), due to the efficient control done by this parasitoid.

One of the main threats in the Mediterranean citrus industry is the arrival of the citrus disease Huanglongbing (HLB). One of its vectors, the African citrus psyllid, *Trioza erytrae* (Hemiptera: Triozidae) was detected in the Iberian Peninsula in 2014 (Pérez-

Otero et al., 2015). Currently, *T. erytraea* is present in South Portugal (Algarve), very close to the Mediterranean citrus growing area (EPPO, 2021). Similarly, to other regions where HLB vectors have been detected, its arrival would imply an increase in the use of synthetic pesticides, which could alter the current IPM programs in citrus. According to recent studies, acetamiprid, dimethoate, cyantraniliprole, flupyradifurone, spirotetramat and sulfoxaflor are potential pesticide candidates for the chemical control of *T. erytraea* (Molina et al., 2022). The biological control of citrus pests could be then disrupted if these pesticides are applied and the number of pesticide applications per season increases. Therefore, to plan a control strategy for *T. erytraea*, it is crucial to study which pesticides are less harmful for the natural enemies used in citrus IPM programs, considering the different exposure routes.

The aim of this study was to assess the potential side effects on the survival, longevity, and fertility of the described systemic pesticides on the parasitoid *A. vladimiri*. First, we studied their toxicity by direct ingestion of pesticides, and secondly their indirect toxicity by providing flowers of treated plants to *A. vladimiri*.

Materials and Methods

Plants and insects

Buckwheat, *F. esculentum*, was selected for the morphology of its flowers, with an easy access to the nectaries (Cawoy et al., 2008). Plants were seeded directly on pots (3L) and grown up to a height between 40 and 60 cm high.

Planococcus citri females were obtained from IRTA-Amposta's rearing, raised on potato shoots at 24.4 ± 0.5 °C, $66 \pm 6.5\%$ relative humidity (RH) and 16:8 photoperiod conditions. *Anagyrus vladimiri* individuals were first purchased at Koppert® (Citripar, Koppert Biological Systems, The Netherlands), and then raised on *P. citri* females. All parasitoid females used in the experiments were between 0 to 72 h days old.

Direct ingestion of pesticides: solutions experiment

Six systemic pesticides were selected according to their efficacy against *T. erytraea* (Molina et al., 2022): acetamiprid, cyantraniliprole, dimethoate, flupyradifurone, spirotetramat and sulfoxaflor. They were used at the maximum application rate (Table 2.1), to investigate their side effects on the parasitoid *A. vladimiri*. Water was used as control treatment. Sugar (25%) was added in all solutions to ensure parasitoid

consumption. Each solution was placed in a plastic vial (50 mL) with a permeable wick in the lid to allow parasitoid feeding. This vial was placed in a bigger one ($\varnothing = 11$ cm, $h = 10.5$ cm) with a ventilated lid, where 10 females of *A. vladimiri* starved for 24 h were introduced per replicate. Four replicates per treatment were conducted. To assess mortality, the number of alive and dead females were recorded after 3 h and 6 h.

After that, the surviving *A. vladimiri* females were individualized in ventilated plastic vials (3 cm diameter and 7 cm high) with a sugar-watered solution (25%) as food source offered in a cotton swab, which was replaced twice a week. The number of alive and dead parasitoids was recorded daily for 15 days. A maximum of 20 replicates were conducted per treatment.

Table 2.1. Pesticide solutions used in the direct ingestion experiment.

Active Ingredient	Trade Name	Manufacturer	Formulation ^a	Rate (%)
Acetamiprid	Carnadine®	Nufarm	20% SL	0.025
Cyantraniliprole	Exirel®	FMC	10% SE	0.1
Dimethoate	Perfekthion® Top	BASF	40% EC	0.1
Flupyradifurone	Sivanto®	Bayer CropScience	20% SL	0.05
Spirotetramat	Movento® 150 O-TEC	Bayer CropScience	15% OD	0.04
Sulfoxaflor	Closer®	Corteva Agriscience	11.3% SC	0.04

^a SL: soluble concentrate; SE: suspoemulsion; EC: emulsifiable concentrate; OD: oil dispersion; SC: suspension concentrate.

The effect of pesticides on *A. vladimiri* fertility was also studied. For this, parasitoids were allowed to feed on pesticide solutions for 6 h. Each female was placed in a ventilated plastic vial (6 cm diameter and 3 cm high) with 2 *A. vladimiri* males, and a piece of potato shoot with at least 30 young females of *P. citri*. Additionally, some honey drops were added in each cage as food source for the parasitoids. Four days later, parasitoids were removed from the cages and the number of alive and dead individuals was recorded. Females of *P. citri* were kept until the emergence of the parasitoids, and then gender and progeny was recorded. The experiment was conducted under controlled conditions (24.41 ± 0.5 °C, $66 \pm 6.5\%$ RH and 16:8 photoperiod).

Indirect ingestion of pesticides: plants experiment

The six systemic pesticides were applied by watering fully bloomed buckwheat plants. The maximum allowed rate per hectare (ha) was applied for each pesticide (Table 2.1). For those pesticides where this rate was not indicated, a standard application volume of 1500 L/ha was used. Plants were daily irrigated, avoiding percolation. Treated

buckwheat plants were placed on shelves and covered with a mesh to prevent insects feeding on flowers. After 3 days, each plant was placed in a cage (60 x 60 x 60 cm) with 10 females of *A. vladimiri* for 6 days. After this period, their mortality was assessed by recording the number of dead and alive *A. vladimiri* females per cage. The substrate was covered with expanded polystyrene to hinder parasitoids access. Three sets of trials (August, September, and October) were carried out, each with 4 replicates per treatment. The experiment was conducted under semi-field conditions: August (26.16 ± 0.23 °C and 80.32 ± 0.72 % HR), September (21.3 ± 0.19 °C and 70.78 ± 0.95 % HR), and October (17.88 ± 0.2 °C and 78.66 ± 0.64 % HR). The climatic conditions were recorded with a data logger (Onset HOBO® MX2300 Temperature/RH Data Loggers, Onset Computer Corporation, Bourne, Massachusetts, USA) located near the cages. Surviving females of each treatment were allowed to parasitize young females of *P. citri*, and the progeny was recorded after 21 days, as described in the previous section.

Statistical analysis

Mortality produced by the different pesticide solutions was analysed by the General Linear Model (GLM) (Binomial distribution) for each treatment at 3h and 6h, followed by the Tukey post-hoc test. For those pesticides that were significantly different to the control treatment, the Abbott's formula (Abbot, 1925) was applied for obtaining the corrected mortality percentage. In addition, for those significant treatments, t-test was performed to find differences between 3h and 6h mortality. Kaplan-Meier analysis was performed to estimate survival curves of the *A. vladimiri* females, which were compared with Log-rank test, and the pairwise analysis was performed. Finally, fertility was analysed by GLM (Poisson distribution) for each treatment using the total number of adults emerged. Replicates where females were dead before finalizing the experiment were not considered. Sex ratio was calculated for each replicate, and a GLM (Gaussian distribution) was performed to compare all treatments.

In the plants experiment, females' survival was analysed by GLM (Binomial distribution) considering the number of live individuals per cage as the response variable, and the pesticide treatment and the trial month as explanatory variables. Then, Tukey post-hoc tests were conducted for multiple comparisons between pesticide treatments. When it was significant, mortality was corrected by the Abbott's formula. The fertility trial was analysed as described in the previous solutions experiment. All statistical analysis were conducted with R (version 4.2.0) (R Core Team, 2022), and they were carried out with a significance level of 5%.

Results

Direct ingestion of pesticides: solutions experiment

The number of survival *A. vladimiri* females after feeding directly on the pesticide solutions for 3h ($\chi^2= 46.6$, $df =6$, $p<0.001$) and 6h ($\chi^2= 120$, $df =6$, $p<0.001$) was significantly different among treatments (Table 2.2). After 3h, *A. vladimiri* survival in dimethoate (35.14% corrected mortality), flupyradifurone (43.24% corrected mortality) and sulfoxaflor (32.43% corrected mortality) treatments were significantly lower than in the control treatment. The corrected mortality in the 6h feeding experiment reached 72.97% for flupyradifurone and 70.27% for dimethoate and sulfoxaflor. After 6h, females which fed on acetamiprid also showed lower significant survival (62.16% corrected mortality) than females feeding on the control treatment. Regarding the four pesticides which caused significant mortality on *A. vladimiri*, dimethoate and flupyradifurone caused significant higher mortality after 6h feeding, than after 3 h ($t=2.8$, $df=6$, $p=0.0333$; $t=2.8$, $df= 6$, $p=0.0320$, respectively). In sulfoxaflor and acetamiprid treatments, although the parasitoid survival was lower after 6 h than after 3 h feeding, no significant differences were observed ($t=1.9$, $df= 6$, $p=0.1020$; $t=1.8$, $df= 6$, $p=0.1250$, respectively).

Table 2.2. Number of alive *Anagyrus vladimiri* at 3h and 6h after feeding on the different pesticide solutions. Also shown the number of *A. vladimiri* (Mean \pm SE) and sex ratio emerged per treatment.

Treatment	3h	6h	Emerged adults	Sex Ratio
Control	9.25 \pm 0.75 a	9.25 \pm 0.75 a	31.75 \pm 3.06 a	0.34 \pm 0.04
Acetamiprid	6.50 \pm 1.19 abc	3.50 \pm 1.19 b	21.92 \pm 3.62 b	0.33 \pm 0.06
Cyantraniliprole	8.75 \pm 0.63 ab	7.50 \pm 0.65 a	21.50 \pm 2.39 b	0.34 \pm 0.05
Dimethoate	6.00 \pm 0.82 bc	2.75 \pm 0.85 b	22.92 \pm 3.77 b	0.38 \pm 0.06
Flupyradifurone	5.25 \pm 0.48 c	2.50 \pm 0.87 b	28.50 \pm 2.53 a	0.49 \pm 0.06
Spirotetramat	9.75 \pm 0.25 a	9.25 \pm 0.25 a	31.40 \pm 4.61 a	0.39 \pm 0.07
Sulfoxaflor	6.25 \pm 1.31 bc	2.75 \pm 1.25 b	22.00 \pm 4.27 b	0.38 \pm 0.07

Different letters in each column indicate differences among treatments with Tukey test (5% of significance level).

After 6h feeding on the different pesticide solutions, only those females which had fed on cyantraniliprole showed a significantly different survival rate than the control treatment ($\chi^2= 22.9$, $df =6$, $p<0.001$) (Fig. 2.1), since more than 50% of them died during the first two days.

Fertility of the surviving females was negatively affected on those that fed on acetamiprid, cyantraniliprole, dimethoate and sulfoxaflor solutions ($\chi^2= 65.7$, $df =6$, $p<0.001$), since

the progeny was significantly lower in those treatments than in the control treatment (Table 2.2). No differences among treatments were found in the sex ratio of the progeny for any of the tested pesticides ($\chi^2 = 7.73$, $df = 6$, $p = 0.26$).

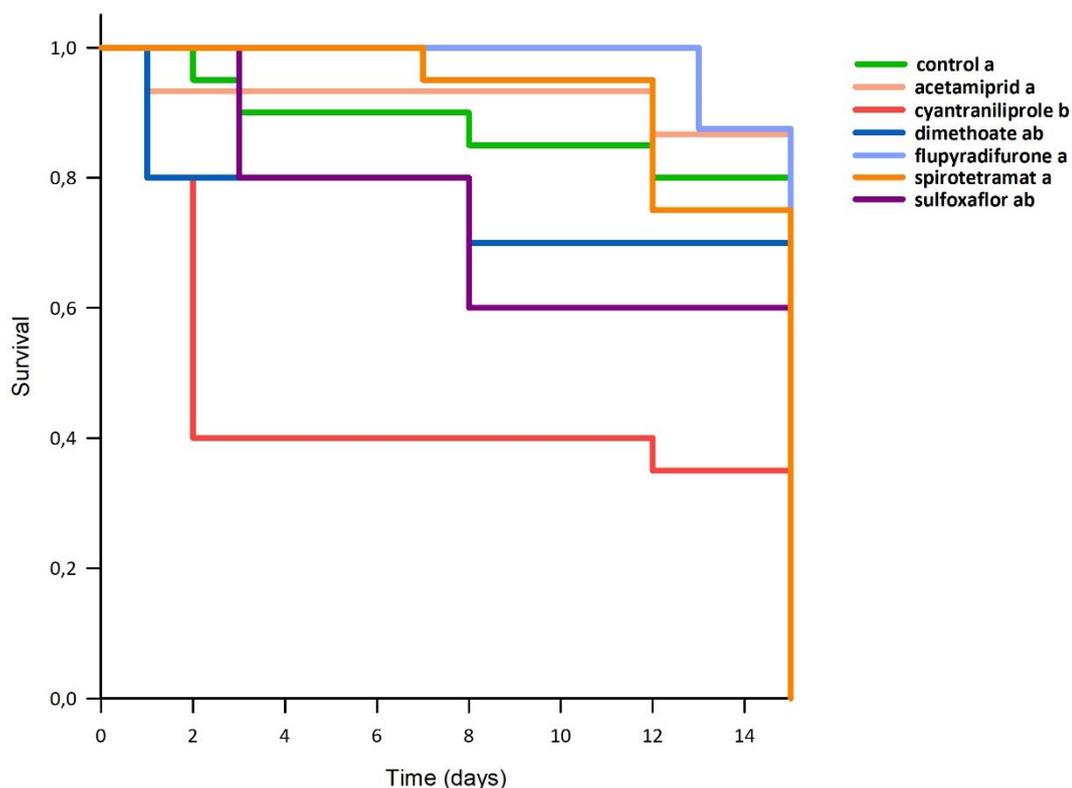


Figure 2.1. Kaplan – Meier estimated survivorship of *A. vladimiri* females feeding on different pesticide solutions for 6h up to 15 days. Different letters in the legends indicate significant differences among treatments (5% of significance level).

Indirect ingestion of pesticides: plants experiment

Because an interaction between treatments and trial sets was found ($\chi^2 = 47.6$, $df = 12$, $p < 0.001$), the three trials were separately analysed (Table 2.3). In August, *A. vladimiri* females that fed on buckwheat plants watered with three of the six tested pesticides showed a significantly lower survival than in the control treatment ($\chi^2 = 54$, $df = 6$, $p < 0.001$). Dimethoate caused 96.3% mortality, spirotetramat 81.48% and acetamiprid 62.96%. In September, treatments significantly different from the control treatment were acetamiprid (62.07% mortality), cyantraniliprole (62.07% mortality), spirotetramat (82.76% mortality) and sulfoxaflor (51.72% mortality) ($\chi^2 = 39.4$, $df = 6$, $p < 0.001$). Finally, in October, no treatment was significantly different to the control ($\chi^2 = 16.7$, $df = 6$, $p = 0.01$).

Table 2.3. Number of alive *Anagyrus vladimirii* females per treatment and month after feeding on nectar of treated buckwheat plants. In addition, the number of *A. vladimirii* (Mean \pm SE) that emerged, and the sex ratio are shown.

Treatment	August	September	October	Emerged adults	Sex Ratio
Control	6.75 \pm 1.31 a	7.25 \pm 1.55 a	7 \pm 0.41 ab	23.68 \pm 1.69 ab	0.55 \pm 0.04
Acetamiprid	2.5 \pm 2.18 bc	2.75 \pm 1.8 bc	6.5 \pm 0.87 ab	22.87 \pm 2.38 abc	0.52 \pm 0.05
Cyantraniliprole	3.5 \pm 1.85 abc	2.75 \pm 2.14 bc	8.25 \pm 0.48 a	26.11 \pm 2.06 a	0.52 \pm 0.04
Dimethoate	0.25 \pm 0.25 c	4.25 \pm 1.55 abc	5.75 \pm 0.48 ab	14.36 \pm 2.82 d	0.55 \pm 0.06
Flupyradifurone	3.5 \pm 1.94 abc	5 \pm 1.47 ab	6 \pm 0.71 ab	20.89 \pm 2.15 bc	0.56 \pm 0.04
Spirotetramat	1.25 \pm 0.95 bc	1.25 \pm 0.95 c	5.5 \pm 1.32 ab	19.13 \pm 2.30 cd	0.63 \pm 0.04
Sulfoxaflor	3.75 \pm 2.39 ab	3.5 \pm 1.32 bc	4.25 \pm 1.65 b	22.43 \pm 2.63 abc	0.54 \pm 0.04

Different letters in each column indicate differences between mean values, compared with Tukey test (5% of significance level).

Regarding fertility, the number of individuals emerged from those females fed on buckwheat nectar of plants watered with spirotetramat and dimethoate was significantly lower than from the control treatment ($\chi^2= 63.5$, $df =6$, $p<0.001$) (Table 2.3). As in the solutions experiment, no differences were found in the sex ratio of the different treatments ($\chi^2= 4.02$, $df =6$, $p=0.67$).

Discussion

When *A. vladimirii* females were allowed to feed on pesticide solutions, acetamiprid, dimethoate, flupyradifurone and sulfoxaflor caused mortalities higher than 60 %. Except acetamiprid, the other three pesticides also had a significant effect on the survival of *A. vladimirii* females after 3h of being fed. The neonicotinoid acetamiprid has been reported harmful for the citrus mealybug parasitoid, *Leptomastix dactylopii* Howard (Hymenoptera: Encyrtidae) (Cloyd & Dickinson, 2006). Dimethoate, flupyradifurone and sulfoxaflor have also shown side effects in other parasitoid species after being in contact with them or their residues (Brar et al., 2017; Brunner et al., 2001; Tabebordbar et al., 2020). Flupyradifurone was the only one that did not affect the progeny of female parasitoids. Our results also showed a delayed toxicity of cyantraniliprole on *A. vladimirii*, since the survival was not immediately affected, but within the following two days it was reduced to the half. In fact, it was the only treatment that reduced the longevity of surviving *A. vladimirii* females few days after having fed on the pesticide solutions. Reduced fertility on parasitoid females was also recorded for cyantraniliprole, which had been described as toxic for other parasitoids, being its use barely compatible with natural

enemies (Amarasekare et al., 2016; Radrigán-Navarro et al., 2021). However, for other parasitoids, such as *Tamarixia radiata* Waterson (Hymenoptera: Eulophidae) it was found less toxic than for their host *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Tiwari & Stelinski, 2013). Finally, spirotetramat had no toxic effect on *A. vladimiri* females by direct ingestion. As previously shown, this pesticide needs to penetrate plant tissues to have toxic properties (Nauen et al., 2008).

When *A. vladimiri* females fed on nectar of *F. esculentum* treated with pesticides, their survival and fertility were affected by some of them as well. The effect of the pesticides differed from the month when it was applied, except for flupyradifurone, which was always harmless to *A. vladimiri* females. These different responses could be due to the different temperatures of each trial. In October, the coldest month, no pesticide had an impact on the parasitoid survival, whereas in the other two months several pesticides showed toxic effects on *A. vladimiri*. Interactions between temperature and pesticide have been reported in other studies where the pesticide toxicity was reduced or increased with the temperature, and showing that some pesticides were more affected to temperatures than others (Abbes et al., 2015; Musser & Shelton, 2005; Nauen et al., 2008). The neonicotinoid acetamiprid and the keno-enol spirotetramat reduced the survival of *A. vladimiri* females in August and September, and the second one had a negative impact on the parasitoid fertility as well. The reported side effects of neonicotinoids on the beneficial arthropod fitness through the nectar route was the main reason why this pesticide family has been tested in the present study. For this reason, in some countries their use has been restricted or forbidden (European Commission, 2018a, 2018b). A reduction on the survival of *Anagyrus pseudococci* (Girault) (Hymenoptera: Encyrtidae) when fed on flowers of buckwheat treated with the neonicotinoid imidacloprid was also recorded (Krischik et al., 2007). Although acetamiprid was considered less harmful than other neonicotinoids, such as imidacloprid or thiamethoxam, it may also have an impact on beneficial arthropods. As previously mentioned, spirotetramat did not have a toxicity on *A. vladimiri* in the solutions experiment, but after uptake on the buckwheat plants, it had a toxic effect on the parasitoid females. This side effect was not observed in October, probably due to the lower temperatures, since its pesticide action is reduced when temperatures decrease (Nauen et al., 2008). Fertility of females fed on dimethoate treated plants was also reduced, as well as their survival in August. In September, cyantraniliprole and sulfoxaflor also affected the survival of *A. vladimiri* females through the nectar. The fact that these pesticides reached the nectar have been cited on a wide range of cultivated and non-cultivated plant species (Jiang et al., 2020; Zioga et al., 2020). The

concentration of these products could be related to others parameters than temperature, such as the solubility of the pesticide (Edwards, 1975). These factors could explain the variability of the toxic effect of the pesticide residues throughout the three months tested.

Overall, five of the six systemic pesticides tested caused mortality on *A. vladimiri* after direct ingestion of pesticide solutions. In addition, four of them also reduced females fertility. Spirotetramat did not have a direct side effect on the parasitoid, but it had an impact on the survival and the fertility of *A. vladimiri* females when this pesticide was soil-applied on buckwheat plants, simulating the drift after canopy-tree application. Acetamiprid, cyantraniliprole, dimethoate and sulfoxaflor also reduced the parasitoid survival after feeding on treated buckwheat plants. These results highlight the importance of minimizing the fraction of the spray that reaches the crop vegetation. It is necessary to focus on the efficiency of the application equipment to avoid losses on the ground due to runoff and drift. This risk needs to be considered when designing a pesticide application programme to control new invasive pests in citrus, as *T. erytrae*, to minimize the impact on the biological control of the citrus pests.

CHAPTER 3

Development of a PCR-based method for the screening of potential predators of the African citrus psyllid *Trioza erytreae*

Introduction

The African citrus psyllid, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae) is a pest mainly known for being one of the main vector species of Huanlongbing (HLB) (Urbaneja, et al., 2020), the main global threat for citrus groves (Halbert & Manjunath, 2004), as the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). While *D. citri* is native from the South of Asia, *T. erytreae* is a south-east African native species that has been present in Madeira (Portugal) and Canary Islands (Spain) since 1994 and 2002, respectively (Carvalho & Aguiar, 1997; González-Hernández, 2003). In mainland Europe, it was firstly detected in the north-western Iberian Peninsula (Galicia) in 2014 (Pérez-Otero et al., 2015). Then, it was rapidly spread along the Atlantic coast from the North to the South of Portugal, and along the Cantabrian coast from the West to the East of Spain (EPPO, 2020), threatening the Spanish citriculture located in the South of the country and in the Mediterranean coast. Although Spain is nowadays free of HLB (Wang, 2020), the contention and eradication of *T. erytreae* is extremely important to reduce transmission risks under the potential HLB presence (Urbaneja-Bernat et al., 2020).

Until now, *T. erytreae* has been mainly managed with chemical treatments with a high frequency of broad-spectrum pesticide applications per season, but its control has not been successful (Cocuzza et al., 2017; Gottwald, 2010). Chemical control of this pest is difficult to combine with integrated pest management (IPM) strategies in citrus groves in Spain, because of the high number of pesticide applications, which could negatively affect natural enemies, disrupting biological control strategies being applied for the management of various pests (Jacas & Urbaneja, 2010). Moreover, the fact that *T. erytreae* is present in private and urban gardens, make even more necessary a less harmful alternative.

Several polyphagous predators have been described to be potentially useful to manage *T. erytreae* in citrus groves in South Africa, as the families Coccinellidae, Anthocoridae, Miridae, Chrysopidae, Hemerobiidae, Syrphidae and Formicidae, together with the order Arachnida (Catling, 1970; van den Berg et al., 1987). In the Canary Islands, the presence of some of them (coccinellids, anthocorids, lacewing and spiders) in citrus trees infested with *T. erytreae* has also been reported, but even if some species of these families had been observed feeding on *T. erytreae* (Estévez et al., 2018; González-Hernández, 2003), the real impact of these predators in the field, avoiding laboratory artifacts, remains still unknown. Even if they have contributed to reduce *T. erytreae* populations, they have not succeeded to contain it. A successful classical biological control of this pest was achieved in Reunion Island with the host-specific parasitoid *Tamarixia dryi*

(Waterson) (Hymenoptera: Eulophidae) (Etienne & Aubert, 1980). For this reason, *T. dryi* was introduced in Tenerife (Canary Islands, Spain) in spring of 2018, showing ratios of parasitism higher than 70% (Hernández-Suárez et al., 2020) and rapidly spreading to other Canary Islands, as Gran Canaria. After that, *T. dryi* was released in three sites in Pontevedra (Galicia, Spain) in autumn of 2019 and in spring and summer of 2020. Until now, the parasitoid has spread more than 30 km, and up to 75% of parasitism rate has been reported (Tena et al., 2021).

Given the risk of the potential arrival of *T. erytreae* to the main Spanish citrus area, the biological control of *T. erytreae* is necessary to be approached in several aspects and, in particular, to find out which native generalist predators where the pest is currently located better contribute to reduce this psyllid populations. A suitable approach for identifying predator–prey interactions in an agroecosystem is the use of molecular markers with prey-specific primers for gut content analysis of generalist predators (Agustí, Shayler et al., 2003). This method has been previously used to study trophic relationships between some predators and some citrus pest, like *Forficula auricularia* L. (Dermaptera: Forficulidae) to feed on aphids (Romeu-Dalmau et al., 2012), or several predator species to feed on the California red scale *Aeonidella aurantii* (Maskell) (Hemiptera: Diaspididae) (Bouvet et al., 2019).

In the present study, a *T. erytreae*-specific pair of primers has been designed and a conventional PCR protocol has been developed for the detection of *T. erytreae* within field-collected generalist predators. This tool allows tracking *T. erytreae* frequencies of predation by the predator assemblage present in citrus groves in Spain under natural field conditions, showing potential candidates for biological control further strategies.

Materials and Methods

Primer design, DNA extraction and amplification

A pair of *T. erytreae* specific primers was designed from the mitochondrial cytochrome *c* oxidase subunit I (COI) region. To design them, sequences from the GenBank database (www.ncbi.nlm.nih.gov) were used (Table 3.1), including: *T. erytreae*, other citrus pest species, other Psylloidea than *T. erytreae*, and some predators present in citrus crops in Spain. Sequences were aligned using ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2) and primers were designed as described in Agustí, Unruh & Welter (2003).

Table 3.1. Species used for primer design (GenBank accession number indicated), and species tested for specificity of the *T. erytrae*-specific primer pair Te2F/Te3R, as well as with the universal pair of primers 16SLR-J-12961/16SLR-N-13398. Also indicated the origin of the samples, all in Spain. NA= not applicable.

Group	Order	Family	Species	Primer design	Specificity test					
				GenBank accession number	Origin	Te2F/Te3R PCR detection	16SLR-J-12961 /16SLR-N-13398 PCR detection			
Citrus pests	Hemiptera	Triozidae	<i>Triozia erytrae</i> Del Guercio	KU517195	Tenerife	+	+			
				KY754656						
				KY754588						
				KY754594						
				<i>Triozia urticae</i> (L.)	KY011195	Barcelona	-	+		
				<i>Lauritriozia alacris</i> (Flor)	MG988839	Barcelona	-	+		
			Aleyrodidae	<i>Aleurothrixus floccosus</i> (Maskell)	KF059956	Tenerife	-	+		
			Aphidini	<i>Aphis gossypii</i> Glover	EU930154	Tarragona	-	+		
				<i>Aphis spiraecola</i> Patch	JX844415	Tarragona	-	+		
				Diaspididae	<i>Aeonidiella aurantii</i> (Maskell)	HM474070		NA	NA	
				Coccidae	<i>Saissetia coffeae</i> (Walker)	NA	Tenerife	-	+	
				Monophlebidae	<i>Icerya purchasi</i> Maskell	NA	Tarragona	-	+	
				Pseudococcidae	<i>Planococcus citri</i> (Riso)	JQ085543		NA	NA	
			Lepidoptera	Gracillariidae	<i>Phyllocnistis citrella</i> Stainton	KF492017	Tarragona	-	+	
Other Psylloidea	Hemiptera	Homotomidae	<i>Macrohomotoma gladiata</i> Kuwayama	MG988795		NA	NA			
					Psyllidae	<i>Cacopsylla alaterni</i> (Foerster)	AY100431		NA	NA
					<i>Euphyllura olivina</i> Costa	KR052011		NA	NA	
			<i>Psyllopsis fraxinicola</i> Foerster	KU517186		NA	NA			
Predators	Hemiptera	Anthocoridae	<i>Orius laevigatus</i> Fieber	NA	Lleida	-	+			
				<i>Orius majusculus</i> (Reuter)	NA	Lleida	-	+		
				<i>Orius niger</i> Wolff	NA	Lleida	-	+		
	Coleoptera	Coccinellidae	<i>Cryptolaemus montrouzieri</i> Mulsant	FM210142	commercial	-	+			
	Neuroptera	Chrysopidae	<i>Chrysoperla carnea</i> (Stephens)	AY743793	commercial	-	+			
Parasitoid	Hymenoptera	Eulophidae	<i>Tamarixia dryi</i> (Waterston)	NA	Tenerife	-	+			

DNA from individual insects was extracted using SpeedTools Tissue DNA Extraction Kit (Biotools, Madrid, Spain), eluted in 100 μ L of BBE buffer provided by the manufacturer and stored at -20°C . Negative controls were added to each DNA extraction set. The whole body was used for all insects, except for coccinellids, from which the elytra were removed, and earwigs, from which only the abdomen was used for DNA extraction, as done by Romeu-Dalmau et al. (2012). PCR reaction volumes (20 μ L) contained 2 μ L of resuspended DNA, 10 μ L of Master Mix (Biotools, Madrid, Spain) and 0.4 μ L of each primer [10 μ M]. Samples were amplified for 35 cycles at 94°C for 30 s, 63°C for 30 s and 72°C for 45 s in a 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). A first cycle of denaturation at 94°C for 2 min and a final extension at 72°C for 2 min was carried out. *Trioza erytreae* DNA and water were always included as positive and negative controls, respectively. PCR products were analysed by electrophoresis in 2.4% agarose gels stained with GelRed® (Biotium, Hayward, CA) and visualized under UV light.

Species specificity

The specificity of the designed primers was tested by attempting to amplify the DNA of other psyllids species, some other citrus pests, some potential predators and one parasitoid of *T. erytreae* liberated in Spain and Portugal as biocontrol agent (Table 3.1). Three to five individuals of each species were tested, except for *T. erytreae*, which we tested 10 (5 adults and 5 nymphs). To ensure the presence of DNA in specimens that were not amplified with specific primers, we double-checked with a pair of universal arthropod primers (16SLR-J-12961 and 16SLR-N-13398) (Simon et al., 1994). For this amplification, PCR reaction volumes (25 μ L) contained 2 μ L of DNA template, 0.2 mM of each primer, 1.25 U of Taq DNA polymerase (Invitrogen), 0.2 mM dNTPs (Promega) and 2.5 mM of MgCl_2 in the manufacturers' reaction buffer. Samples were amplified for 40 cycles at 95°C for 30 s; at 45°C for 30 s; and at 72°C for 60 s. The first cycle of denaturation was done at 95°C for 15 s, and a final extension was done at 72°C for 5 min.

Prey DNA decay rates

Chrysoperla carnea and *C. montrouzieri* larvae (2nd instar) feeding trials were carried out to determine the decay rates of *T. erytreae* within their gut. They were conducted in the Canary Institute of Agrarian Research (ICIA) in Tenerife (Canary Islands, Spain)

where the pest was present. Nymphs of *T. erytreae* were collected on infested leaves in citrus groves (*Citrus lemon* (L.) Osbeck) in northern Tenerife and transferred to the laboratory in a portable fridge. Predator larvae were purchased from Koppert© and they were individually placed in 1.5 mL tubes with a cotton soaked in water as a lid and humidity source, where they were in starvation for 48 h at controlled conditions of 24 °C and 16:8 (L:D) photoperiod. After that, each predator larva was transferred to a plastic container (2.5 cm diameter × 1.5 cm high) with a piece of infested citrus leaf containing 10 nymphs of *T. erytreae* (1st-3rd instar) for 1 h at room temperature. After this period, consumed *T. erytreae* nymphs were counted and only those larvae that fed on 2 to 6 nymphs were immediately frozen ($t = 0$) at -20 °C or maintained for 4 h and 12 h, at 24 °C and 16:8 (L:D) photoperiod, and then frozen to be analysed. Other predator larvae used as negative controls were previously starved for 48 h at the same controlled conditions and immediately frozen without ingestion. Ten individuals were tested for each period of time. Each predator was tested up to 3 times and considered positive if *T. erytreae* DNA was detected in one, meaning that if the first PCR was negative, we conducted a second one, and up to third one if the second was negative. This method was conducted to avoid false PCR negatives, as done in Gomez-Polo et al. (2015, 2016), and Monzó et al. (2010). The number of positive predators was recorded, and the percentage of positives was calculated for each post-ingestion period. The time interval associated with 50% positive responses (i.e. detectability half-life) was calculated by reverse prediction from best-fitted equations.

Field sampling and analysis of field-collected predators

Potential predators of *T. erytreae* were collected in three sampling locations (and dates) on infested citrus trees where eggs, larvae and adults of *T. erytreae* were observed. The first sampling was in October 2018 in Tenerife (Canary Islands, Spain), in three small (<1.5 ha) citrus groves (28° 29' 21.6" N, 16° 21' 20.3" W; 28° 22' 42.1" N, 16° 32' 10.9" W; and 28° 23' 34.6" N, 16° 32' 14.6" W). The second sampling was in June 2019 in one lemon grove in Gran Canaria (Canary Islands, Spain) (28° 03' 45.9" N, 15° 34' 28.9" W). The third sampling was in November 2019 on isolated citrus trees located in private gardens or urban areas in Pontevedra (Galicia, NW Spain) (42° 30' 0" N, 8° 48' 0" W).

Collection of these predators was conducted by beating only those flushes observed to have a high infestation of *T. erytreae* to ensure the prey presence was not limited. These flushes were beaten three times on a white tray. Each predator was collected from the tray, placed in a 1.5 mL tube and transferred to the laboratory in a portable fridge. Once

in the laboratory tubes were stored at $-20\text{ }^{\circ}\text{C}$ up to DNA extraction. Each field-collected predator was also tested up to 3 times and considered positive if *T. erytreae* DNA was detected in one of them to avoid false PCR negatives.

Before gut content analysis by PCR, predators were morphologically identified using taxonomic keys and bibliographic references (Albouy & Caussanel, 1990; Barrientos, 1988; Eizaguirre, 2007; Gómez & Espadaler, 2007; Noualhier, 1893; F. García-Marí, personal communication). Specimens of the genus *Orius* and hoverfly larvae were identified by molecular methods previously developed (Gomez-Polo et al., 2013, 2014, respectively).

Results

Primer design

A pair of *T. erytreae* specific primers was successfully designed from the COI region, which amplified a fragment of 194 bp. Primer sequences were: 5' GAGGATATTCAGTAGATACTGC 3' (Te2F) and 5' CTGCTAAAACAGGTAATGCC 3' (Te3R). None of the species tested for specificity with this pair of primers was amplified (Table 3.1), showing their high specificity. When we double-checked with the universal primers, all of them were amplified, indicating the presence of insect DNA in all samples.

Prey DNA decay rate

PCR analysis of the feeding trials of *C. carnea* larvae, showed a 100% detection of the tested larvae for *T. erytreae* DNA immediately after feeding ($t = 0$) (Fig. 3.1). At 4 h after feeding, detection decreased to 50%, and dropped to 20% at 12 h after ingestion. Feeding trials of *C. montrouzieri*, showed a 70% detection of the tested larvae at $t = 0$, decreasing to 50% at $t = 4$ h, as happened with *C. carnea*, and to 30% at 12 h after ingestion. Detection of *T. erytreae* DNA in both predators was better fitted to an exponential decay, with an R^2 value of 0.9879 for *C. carnea*, and 0.9948 for *C. montrouzieri*. Detectability half-life calculated from these equations (Fig. 3.1) was situated at 4.78 h and 4.48 h, respectively.

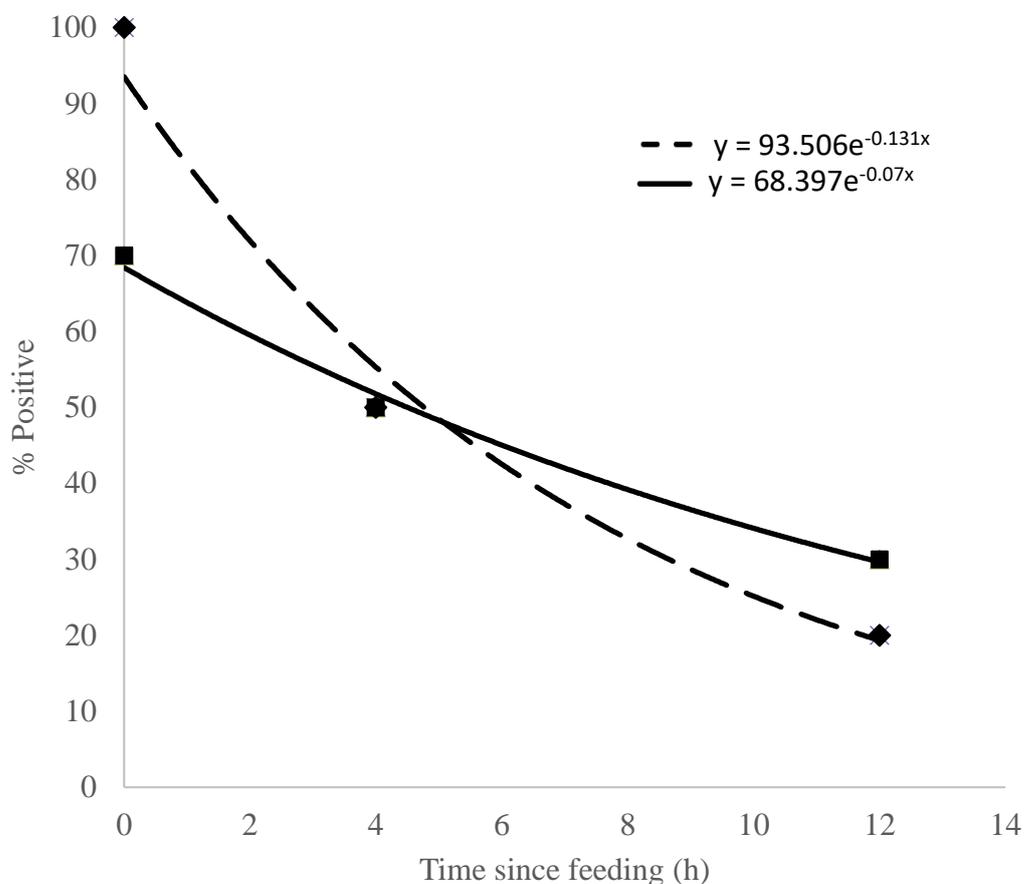


Figure 3.1. Detection of *Trioza erytreae* DNA ingested by *Chrysoperla carnea* (◆, discontinued line) and *Cryptolaemus montrouzieri* (■, continued line) larvae at different times after ingestion.

Field sampling and analysis field-collected predators

A total of 479 potential predators were collected in the three sampling dates (Table 3.2), which were all identified to species or genus level, except spiders, which were identified to family level. In some taxa, the number of collected individuals was very low, but they were still analysed in order to better characterize the range of potential predators of *T. erytreae*. In the PCR analysis, 45.7% of them were tested only once, because they were positive at the 1st PCR. Those negative predators were tested a second time, having 12.9% of positives, and 11.5% of them were positives at the 3rd PCR. Therefore, 29.9% of the analysed predators were negative after 3 chances.

Anthocoridae was the most abundant taxon, with the highest collection of individuals (N = 202), and with the species *Orius laevigatus* Fieber (Hemiptera: Anthocoridae) as the most abundant (N = 168, all collected in Galicia); followed by Coccinellidae (N = 84),

with *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) as the most abundant species (N = 32, all collected in Tenerife) (Table 3.2).

Table 3.2. Number of field-collected analysed individuals (N) and percentage of PCR detection (Detection (%)) of each arthropod taxa. The location of collection in Spain and the developmental stage are also indicated. NI = non identified.

Order	Family	Genus/ Species	Stage	Location	N	Detection (%)	
Araneae	Anyphaenidae		adult	Galicia	3	100	
		Araneidae	adult	Tenerife	3	100	
					Gran Canaria	1	0
					Galicia	15	46.7
	Dictynidae		adult	Gran Canaria	1	100	
	Linyphiidae		adult	Tenerife	1	0	
					Gran Canaria	4	0
	Mimetidae		adult	Tenerife	1	0	
	Philodromidae		adult	Galicia	1	0	
	Salticidae		adult	Tenerife	3	66.7	
					Gran Canaria	4	100
	Theridiidae		adult	Tenerife	1	0	
	Thomisidae		adult	Gran Canaria	1	0	
				Galicia	1	100	
Coleoptera	Coccinellidae	<i>Adalia bipunctata</i> (L.)	adult	Galicia	17	76.5	
		<i>Adalia decempunctata</i> (L.)	adult	Galicia	1	100	
		<i>Clitostethus arcuatus</i> (Rossi)	adult	Galicia	4	0	
		<i>Coccinella miranda</i> Wollaston	adult	Gran Canaria	1	100	
		<i>Coccinella septempunctata</i> L.		adult	Gran Canaria	1	100
					Galicia	1	100
		<i>Cryptolaemus montrouzieri</i> Mulsant		larva	Gran Canaria	2	100
				adult	Gran Canaria	4	100
		<i>Exochomus quadripustulatus</i> (L.)	adult	Galicia	1	100	
		<i>Harmonia axyridis</i> (Pallas)	adult	Tenerife	52	40.6	
		<i>Hippodamia variegata</i> (Goeze)	adult	Gran Canaria	1	0	
		<i>Propylea quatuordecimpunctata</i> (L.)	adult	Galicia	1	100	
		<i>Rhyzobius chrysomeloides</i> (Herbst)	adult	Galicia	1	100	
		<i>Rhyzobius forestieri</i> (Mulsant)	adult	Galicia	7	42.9	
		<i>Rhyzobius litura</i> (Fabricius)	adult	Gran Canaria	1	0	
		<i>Rodolia cardinalis</i> (Mulsant)		adult	Tenerife	1	0
					Galicia	5	20
		<i>Scymnus canariensis</i> Wollaston	adult	Gran Canaria	1	0	
		<i>Scymnus rubromaculatus</i> (Goeze)	adult	Gran Canaria	1	100	
		<i>Stethorus punctillum</i> Weise	adult	Gran Canaria	1	0	
Dermaptera	Forficulidae	<i>Forficula auricularia</i> L.	nymph	Galicia	7	71.4	
			adult	Galicia	4	100	

Table 3.2. (Continued).

Order	Family	Genus/ Species	Stage	Location	N	Detection (%)
Diptera	Syrphidae	NI	larva	Galicia	6	100
		<i>Meliscaeva auricollis</i> (Meigen)	larva	Galicia	10	100
Hemiptera	Anthocoridae	<i>Anthocoris</i> sp.	nymph	Galicia	3	100
			adult	Galicia	18	83.3
		<i>Orius albidipennis</i> Reuter	adult	Galicia	1	100
		<i>Orius laevigatus</i> Fieber	adult	Galicia	168	82.1
		<i>Orius majusculus</i> Reuter	adult	Galicia	7	85.7
		<i>Orius niger</i> Wolf	adult	Galicia	1	100
		<i>Orius</i> spp.	adult	Tenerife	3	66.7
				Galicia	1	100
	Miridae	<i>Aetorhinella parviceps</i> Noualhier	adult	Gran Canaria	6	83.3
Himenoptera	Formicidae	<i>Lasius grandis</i> Forel	adult	Galicia	14	21.4
		<i>Linepithema humile</i> (Mayr)	adult	Gran Canaria	19	31.6
				Galicia	6	0
Neuroptera	Chrysopidae	<i>Chrysoperla carnea</i> (Stephens)	larva	Tenerife	13	38.5
				Gran Canaria	20	100
				Galicia	1	100
	Hemerobiidae	<i>Hemerobius eatoni</i> Morton	larva	Gran Canaria	6	83.3
			adult	Gran Canaria	11	100
Trombidiformes	Erythreidae	<i>Leptus</i> spp.	adult	Gran Canaria	4	100
				Galicia	6	83.3
Total					479	68.7

The percentage of positive predators for the detection of *T. erytreae* DNA in their gut grouped by family (or order in the case of the spiders) is shown in Fig. 3.2. In total, 70% of the analysed predators gave a positive detection of *T. erytreae* DNA. A certain percentage of positive individuals was detected in all groups, ranging from 100% in syrphids to 23% in ants (Fig. 3.2). Also, Hemerobiidae with 94% of positive individuals showed a high detection, followed by other five families: Erythreidae, Anthocoridae, Miridae, Forficulidae and Chrysopidae, which showed more or around 80% of detection. Araneae and Coccinellidae showed around 50% of detection both.

Considering the sampling location of the collected predators, 311 of them were collected in Galicia and 74.6% were positive for *T. erytreae* DNA. In the Canary Islands, 90 individuals were collected in Gran Canaria, with a 72.2% of positive, and 58 individuals were collected in Tenerife, with 43.1% of positive of *T. erytreae* DNA.

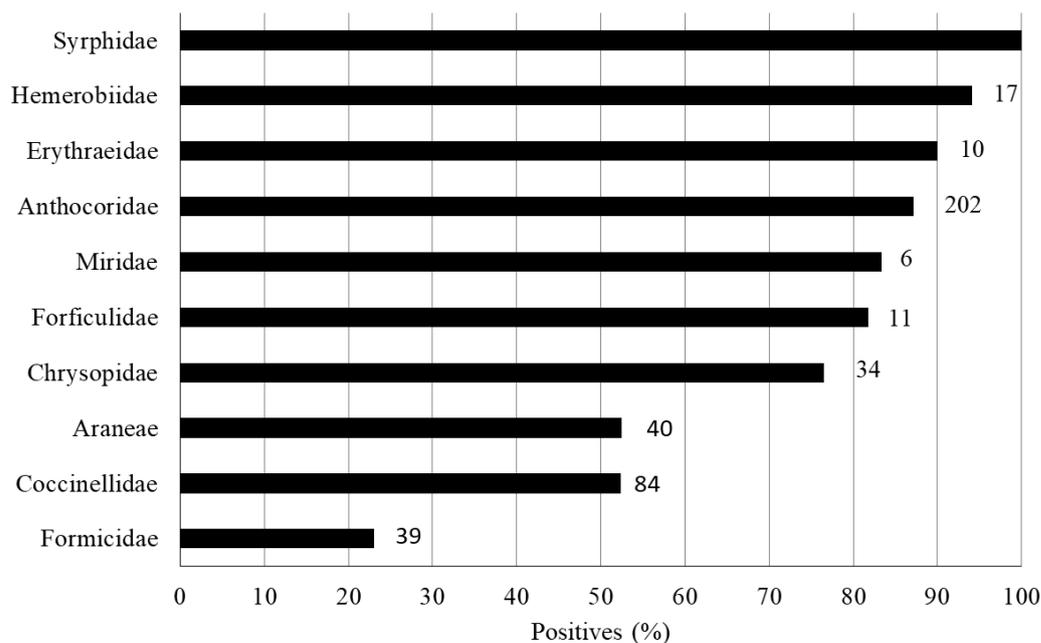


Figure 3.2. Percentage of PCR positive detection using the *T. erytraeae*-specific primers showed by family, except for the order Araneae. The number at the end of the bar indicates the number of individuals tested per taxa.

Discussion

The pair of primers designed to detect *T. erytraeae* in predator gut contents was specific enough to detect the target species avoiding the detection of other pests, predators and even a parasitoid potentially present in citrus crops. This validates the potential use of these molecular markers for monitoring interactions between *T. erytraeae* and some predator species. On the other hand, the amplified COI fragment is the same for all *T. erytraeae* developmental stages, as it happens in all DNA-based predation studies, meaning that is not possible to know whether a predator had been feeding on eggs, nymphs or adults. Because many of the tested predators might feed on eggs, it would be interesting to conduct laboratory feeding trials of *T. erytraeae* eggs in further studies and to calculate the half-life detection of this developmental stage. In the present study, we have shown that the half-life detection of 1st–3rd instar larvae of this psyllid by two generalist predators showed that *T. erytraeae* detection is possible in 50% of the cases up to 4.5 h under the conditions tested, then showing the most recent feeding episodes. This half-life detection time is similar to the Asian citrus psyllid *D. citri* DNA half-life detection ingested by the lacewing *Chrysoperla externa* (Hagen) (Neuroptera:

Chrysopidae) and the ladybird *Hippodamia convergens* Guérin-Ménéville (Coleoptera: Coccinellidae) obtained by Nanini et al. (2019), which were 5.5 h and 6.1 h, respectively.

Our field sampling study demonstrated that a wide range of generalist predators include *T. erytrae* in their diets, being *O. laevigatus* the most abundant predator collected in Galicia, the highest consumer of *T. erytrae* (82% of them). We have also demonstrated that other anthocorid species ingested *T. erytrae* (Table 3.2). Among them, *O. laevigatus* and *Anthocoris* sp. have been occasionally observed feeding on *T. erytrae* in citrus groves in the Canary Islands (Estévez et al., 2018). It is important to note that almost all the analysed anthocorids were collected in Galicia, where they were found in isolated citrus trees in gardens, instead of in citrus orchards. In the Mediterranean basin, *Orius* spp. are not frequently found in citrus groves, but the fact that they were detected with this abundance on those citrus trees make them potential candidates for the biological control of this psyllid species. In South Africa, non-identified anthocorid individuals were also observed feeding on *T. erytrae* nymphs (van den Berg et al., 1987).

Coccinellidae was the family with the highest number of species collected and analysed (Table 3.2). Even though only one specimen was collected in most of these species, only in five of them *T. erytrae* DNA was not detected. *Harmonia axyridis* was the most abundant coccinellid in one of the citrus groves sampled in Tenerife, and 40% of them were positive for *T. erytrae* DNA. This coccinellid species had been observed to feed on eggs and nymphs of *T. erytrae* in laboratory experiments (Estévez et al., 2018), as well as to feed on *D. citri* in Florida and Brazil (Michaud et al., 2004; Monzó et al., 2014; Nanini et al., 2019). The second coccinellid regarding abundance was *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae), also showing ingestion of *T. erytrae* in 76% of the cases, which could also be interesting for IPM programs in Spain, because is very common in Mediterranean citrus agroecosystems (Boukhris-Bouhachem, 2011; Kavallieratos et al., 2004). Khan et al. (2016) have described this coccinellid as good commercial predator for *D. citri* nymphs. Other analysed coccinellids, like *C. montrouzieri*, *Rodolia cardinalis* (Mulsant), *Rhyzobius* spp. and *Coccinella* spp., in which we have detected the target psyllid with different percentages (Table 3.2), had been also found in citrus groves in Valencia (Spain) (Alvis, 2003). It is well known that some of them have an important role reducing populations of various citrus key pests (Jacas & Urbaneja, 2010).

Predation of *T. erytrae* by Neuroptera was previously described in citrus groves in South Africa (van den Berg et al., 1987), and in the case of *C. carnea* was also frequently

observed in citrus groves in Tenerife (Estévez et al., 2018). The present study confirmed the consumption of *T. erytrae* by *C. carnea*, as well as by the brown lacewing *Hemerobius eatoni* Morton (Neuroptera: Hemerobiidae), an endemic species of the Canary Islands. The mirid bug *Aetorhinella parviceps* Noualhier (Hemiptera: Miridae) was another endemic species of the Canary Islands which has also been recorded preying on *T. erytrae* in the present study. More than 80% of the analysed individuals of both endemic species were positive for the target DNA, demonstrating how two native predators were able to feed on a new invasive species as *T. erytrae*.

All analysed syrphid larvae were positive for the target DNA, making them also potential candidates for the biological control of *T. erytrae*. The molecular method used to identify the syrphid species allowed the identification of only ten of them (10 specimens of *Meliscaeva auricollis* (Meigen) (Diptera: Syrphidae)). The remaining six larvae were not identified with the method used, indicating that they might be other syrphid species than those identified with this multiplex PCR.

The earwig *F. auricularia* was collected in Galicia as nymphs and adults, and most of them (82%) fed on *T. erytrae*. This species was also recorded as predator in citrus orchards by Romeu-Dalmau et al. (2012), since the DNA of the main citrus aphids was detected in their gut. Although earwigs could have a potential predator role in citrus orchards, it remains a controversial subject, particularly in young trees, where earwigs can cause damages due to their phytophagous behaviour (Grafton-Cardwell et al., 2003; Kallsen, 2006).

Regarding Araneae, individuals of nine families were analysed, and five of these families fed on *T. erytrae* (Table 3.2). In Spain, some studies emphasize the abundance of these predators in citrus groves and their relationship with some pests, like aphids, the mussel scale *Lepidosaphes beckii* (Newman) (Hemiptera: Diaspididae), and the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Alvis, 2003; Monzó et al., 2010). In South Africa they have been cited to contribute in the reduction of *T. erytrae* populations, mostly of species belonging to Salticidae (van den Berg, Dippenaar-Shoemang et al., 1992). For *D. citri*, spiders are reported as predominant predator group on *D. citri* colonies (Qureshi & Stansly, 2009), and the families Anyphaenidae and Salticidae have been reported feeding on this psyllid in Florida (Michaud, 2002), as it happens in the present study for *T. erytrae*.

As cited by Estévez et al. (2018), we observed larvae of *Leptus* spp. parasitizing adults of *T. erytrae* in the field. The analysis of some adults of these erythraeid mites showed

a 90% of adults positive for the target DNA. Therefore, they could contribute to the biological control of *T. erytrae*.

Two ant species were also analysed for the presence of the target DNA in their gut, *Lasius grandis* Forel and *Linepithema humile* (Mayr) (Hymenoptera: Formicidae), showing the lowest percentage of positive detection of *T. erytrae* DNA (Table 3.2).

The most frequent species in citrus trees in Spain is *L. grandis*, that together with *L. humile*, were the most abundant ants in the sampled citrus trees. Both species feed mainly on carbohydrates and have been reported attending several honeydew-producing hemipterans in citrus crops (Calabuig et al., 2014; Martínez-Ferrer et al., 2003; Martínez-Ferrer & Campos-Rivela, 2017; Pekas et al., 2011; Zina, 2008). However, both ant species are omnivorous. Predation on honeydew-producing hemipterans by ants has been reported for some species, depending on their population density and the availability of honeydew (Billick et al., 2007; Sakata, 1994). The detection of *T. erytrae* in their gut revealed that both analysed ant species fed on the target insect, and therefore, its predatory role in the biological control of this pest should not be underestimated. Even if ingestion has not been detected in some of them, sometimes ants transport the prey to the nest without having ingested them (Cerdà & Dejean, 2011).

In summary, the pair of primers designed in this study and the PCR method developed allowed the detection of *T. erytrae* in the gut content of field collected predators in citrus trees with tender flushes infested by the psyllid. Some of these generalist predator species are not common in citrus-growing areas, such as *O. laevigatus*. Since this species is commercially available, its inundative release could be considered in some particular cases. Most of them are commonly present in citrus agroecosystems of the Mediterranean coast of Spain, which means that they might contribute to the biological control of this citrus pest if it will arrive to this area.

CHAPTER 4

Potential natural enemies for a fortuitous biological control of *Trioza erytreae* in the Mediterranean basin

Introduction

The African citrus psyllid, *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) is one of the vectors of the devastating citrus disease Huanglongbing (HLB) (Bové, 2006; Gottwald et al., 2007). Its detection in a north-western region of Spain (Galicia) in 2014 (Pérez-Otero et al., 2015) meant that the threat was closer to the Mediterranean basin citrus industry. Since its arrival, *T. erytreae* has spread to northern Spain and southern Portugal (Algarve), currently located very close to the main citrus growing Spanish area (EPPO, 2020, 2021). This spreading came from small citrus groves or even citrus trees present in backyards or urban areas (Molina et al., 2021). The control of this psyllid is crucial to avoid its spread and reduce the potential risk associated with an HLB arrival (Arenas-Arenas et al., 2018; Bassanezi et al., 2020). One of the key strategies to manage this vector is conservation biological control.

The natural biological control that does not involve a manipulation by humans is known as fortuitous biological control (DeBach & Rosen, 1991). This includes any unintentional reduction of pest populations when any of the agents, pest or natural enemy, is not native. The fortuitous introductions of exotic parasitoids and predators have reduced populations of indigenous pest, some cases were about hemipteran parasitoids which parasitize non-target species (DeBach, 1971; Fand et al., 2013; Nechols, 2003). On the other hand, as *T. erytreae* situation, when an exotic pest arrives to a new area, it could be attacked and controlled by an indigenous complex of predators and parasitoids. For example, Nechols (2003) reported that the spherical mealybug, *Nipaecoccus viridis* (Newstead) (Hemiptera: Pseudococcidae) remained at low or undetectable levels due to the action of several indigenous predators and the parasitoid *Anagyrus indicus* Shafee (Hymenoptera: Encyrtidae) in Guam and Mariana Islands. In the Mediterranean basin, native parasitoids (eulophids and braconids), together with predatory mirid bugs were described as control agents of the invasive pest *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in tomato crops (Abbes et al., 2014; Urbaneja et al., 2012; Gabarra et al., 2014). Furthermore, native predators present in citrus growing areas invaded by the other HLB vector *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), like the ladybird *Olla v-nigrum* (Coleoptera: Coccinellidae) in Florida (USA), feed on this psyllid nymphs (Grafton-Cardwell et al., 2013; Michaud, 2002).

Psyllids are associated to a wide range of host plants, including cultivated and non-cultivated species, such as ornamental trees and shrubs. In Spanish gardens and urban areas, psyllids are considered pests of common trees such as laurel, Judas tree and silk tree, among others (MAPA, 2020; De Alfonso et al., 2014; Pons et al., 2006). Those trees

also host predators and parasitoids of these psyllid species that could be natural enemies of *T. erythrae*. The aim of this study is to know the potential biological control agents of *T. erythrae* present in ornamental trees and shrubs situated close to citrus growing areas in the Spanish Mediterranean coast.

Material and methods

Sampling

A total of seven ornamental plant species were sampled in different locations of Tarragona (Catalonia, Spain), and Barcelona (Catalonia, Spain) near the main citrus growing areas. Samplings were conducted between March and October 2018 and 2019, depending on the presence of each psylla species. They were performed fortnightly whenever each psyllid populations were present on the sampled host plant (Table 4.1).

Table 4.1. Host plants sampled for psyllids and their natural enemies. Locations: Tarragona (T); Barcelona (B). In brackets [] it is indicated the number of points sampled in each location different from one.

Host plant	Locations	Sampling period		Total samples
		2018	2019	
<i>Albizia julibrissin</i> Durazz.	Amposta (T) Bítem (T) La Ràpita (T) Santa Bàrbara (T)	July-October	-	169
<i>Cercis siliquastrum</i> L.	Amposta (T) L'Aldea (T) [2]	May-July	-	108
<i>Ficus microcarpa</i> L.F.	Alcanar (T) [3]	May-October	-	276
<i>Laurus nobilis</i> L.	Cabrils (B) Pineda de Mar (T)	-	April-September	162
<i>Olea europaea</i> L.	Alcanar (T) L'Ampolla (T) Ulldecona (T)	April-July	-	124
<i>Pistacia lentiscus</i> L.	Bítem (T) L'Aldea (T) L'Ampolla (T) Santa Bàrbara (T)	May-October	-	274
<i>Rhamnus alaternus</i> L.	Cabrils (B) Pineda de Mar (B)	-	March-September	160

Two different kinds of samplings were performed: beat samplings (for psyllids and predators), and evolutionary samplings (for psyllid parasitoids). In the first case, psyllids and predators were collected by beating branches infested with psyllid colonies on a funnel (30 cm Ø) with a plastic pot (3,7 cm Ø x 6,4 cm) at the bottom. Up to four samples for each plant species at each point and date were conducted. After that, arthropods were frozen and kept until identification. In the case of the evolutionary samplings, four samples of 10-15 cm length shoots infested with nymphs were collected at each point and date. These shoots were maintained in water inside a ventilated cage (11 cm Ø x 15 cm) placed in the laboratory, until adult psyllids or parasitoids emerged. Then, adults were preserved in 70% ethanol until identification. In case that some predators appeared in these samples, they were also collected and recorded.

Psyllids, predator and parasitoid adults were identified at least up to family level, being identified up to genus or even species level using taxonomic keys if possible (Barrientos, 2004; Grissell & Schauff, 1990; Hodkinson & White, 1979, Péricart, 1972).

Statistical analysis

The average of individuals obtained per sampling point and month in each sampling method (beat or evolutionary) was calculated. For the most abundant predator taxa (Anthocoridae, Coccinellidae and Araneae), a correlation coefficient was calculated to study the relationship between the psylla species, and the predator abundances present in the beat samplings. Since abundances were not parametric, Spearman's rank correlation was performed. For the obtained parasitoids, the correlation between the parasitoid and psyllid abundance was calculated. Data were analysed with R (version 4.2.0) (R Core Team, 2022).

Results

A total of 1273 samples were collected in the beat (659) and evolutionary (614) samplings. The psyllid species found in the sampled plants were the following: *Acizzia jamatonica* (Kuwayama) on *Albizia julibrissin*; *Cacopsylla pulchella* (Löv) on *Cercis siliquastrum*; *Macrohomotoma gladiata* Kuwayama on *Ficus macrocarpa*; *Lauritrioza alacris* (Flor) on *Laurus nobilis*; *Euphyllura olivina* (Costa) on *Olea europea*; *Agonoscena cisti* (Puton) on *Pistacia lentiscus*; and *Cacopsylla alaterni* (Foerster) on *Rhamnus alaternus*. Psyllid population abundances were also different depending on the sampled month (Fig. 4.1). *Cacopsylla alaterni* showed the highest population in early spring,

whereas *E. olivina* and *M. gladiata* in May. In summer, *A. jamatonica* and *L. alacris* showed their highest populations (July and August, respectively), and *A. cisti* population peaked in September. In autumn, the psyllids species present in the sampled host plants were *A. jamatonica*, *A. cisti* and *M. gladiata*, being the abundance of *M. gladiata* the same as in spring.

A total of 1568 predators were collected in the beat samplings, mainly belonging to Anthocoridae and Coccinellidae families, and the Araneae order. However, some individuals of other families were also collected, such as mirid bugs, green lacewings, dustywings, hoverflies and predatory thrips (Fig. 4.2). Anthocoridae bugs, ladybird beetles and spiders were present in all plants, although the abundance was not the same. In most sampled trees, predators belonged mostly to Anthocoridae family and spiders, except for *F. microcarpa*, where ladybird beetles were the most collected.

The total number of predators collected from the evolutionary samples were 252. Anthocoridae, Coccinellidae and Araneae were also the most abundant, as in the beat samplings (Fig. 4.3). The highest number of Anthocoridae bugs was collected in shoots of *L. nobilis* infested with *L. alacris*. Ladybird beetles were mostly collected on shoots of *F. microcarpa* infested with *M. gladiata*, and the number of spiders was higher on shoots of *R. alaternus* infested with *C. alaterni*. Hoverflies, lacewings and mirid bugs were mostly collected on *A. julibrissin*. Anthocoridae species found belonged to the genus *Anthocoris* (82.7%) and *Orius* (17.3%). Sixteen Coccinellidae species were identified (Table 4.2), founding the highest diversity on *R. alaternus* and *L. nobilis*.

In the beat samplings, the abundance of Anthocoridae bugs were positively correlated to the abundance of *A. cisti*, *C. alaterni*, *L. alacris* and *M. gladiata* adults (Table 4.3). Ladybirds' abundance was positively correlated to *M. gladiata* abundance, and negatively correlated to *C. pulchella* abundance. The abundance of spiders was positively correlated only to the abundance of *A. cisti*.

Considering the parasitoids emerged from the evolutionary samplings, the species *Prionomitus mitratus* (Dalman) (Hymenoptera: Encyrtidae) was obtained from *M. gladiata* nymphs. A total of 348 individuals of this species emerged from all *M. gladiata* nymphs found, showing a positive correlation between the parasitoid and the number of adults psyllids emerged in the evolutionary samplings ($R=0.69$, $p=0.0014$, $S=296.77$). Eleven adults of *Psyllaephagus euphyllurae* (Masi) (Hymenoptera: Encyrtidae) emerged from *E. olivina* nymphs, and the hyperparasitoids *Apocharips trapezoidea* (Hartig) (Hymenoptera: Figitidae) ($N=3$) and *Pachyneuron muscarum* (L.)

(Hymenoptera: Pteromalidae) (N=2) were also detected from these nymphs collected on *O. europaea*.

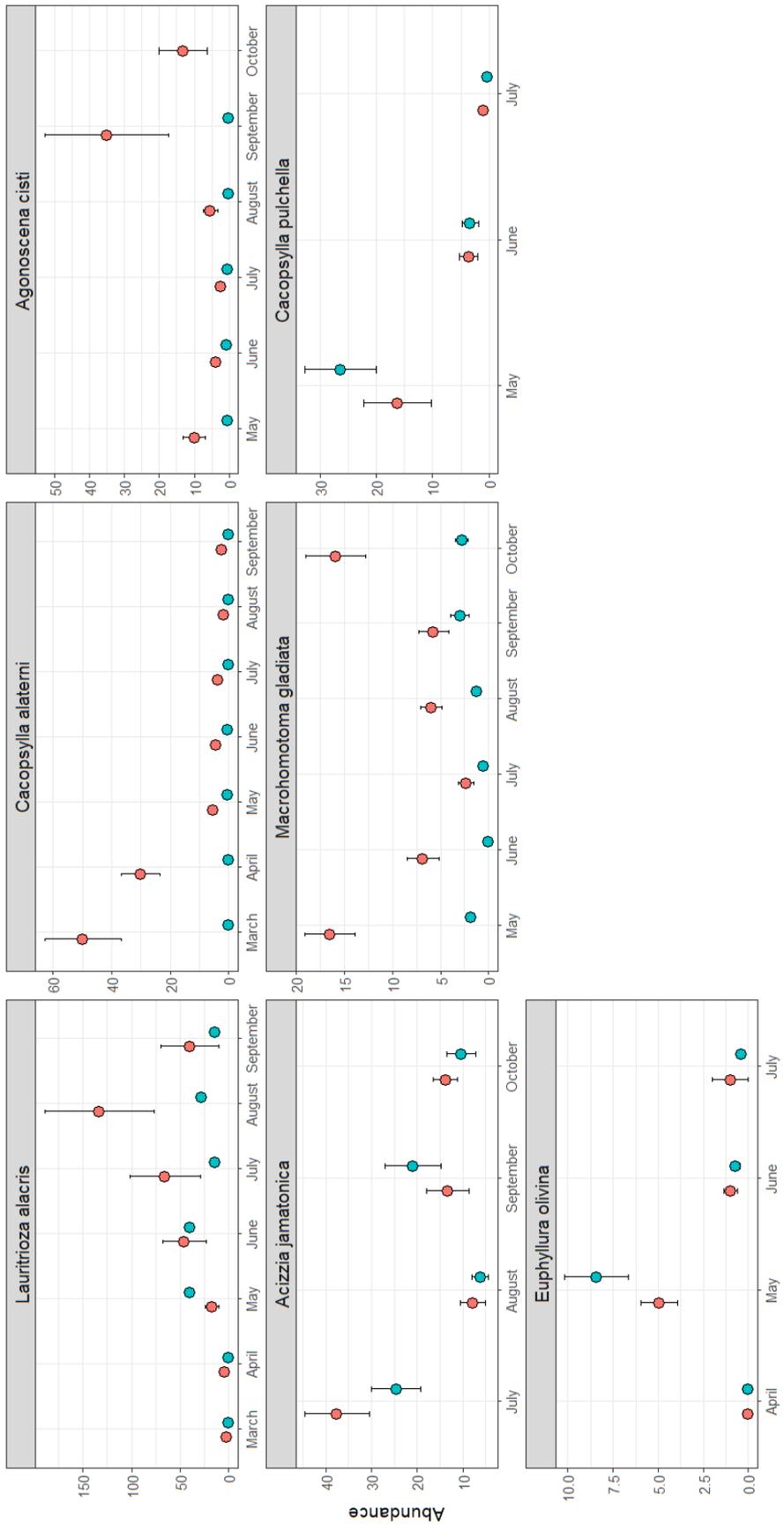


Figure 4.1. Psyllid abundances (mean of psyllid adults per month) found in the evolutionary samplings (in blue) and the beat samplings (in red) of each host plant species. Note that the y-axis values are different in each graphic to better observe the abundances per month.

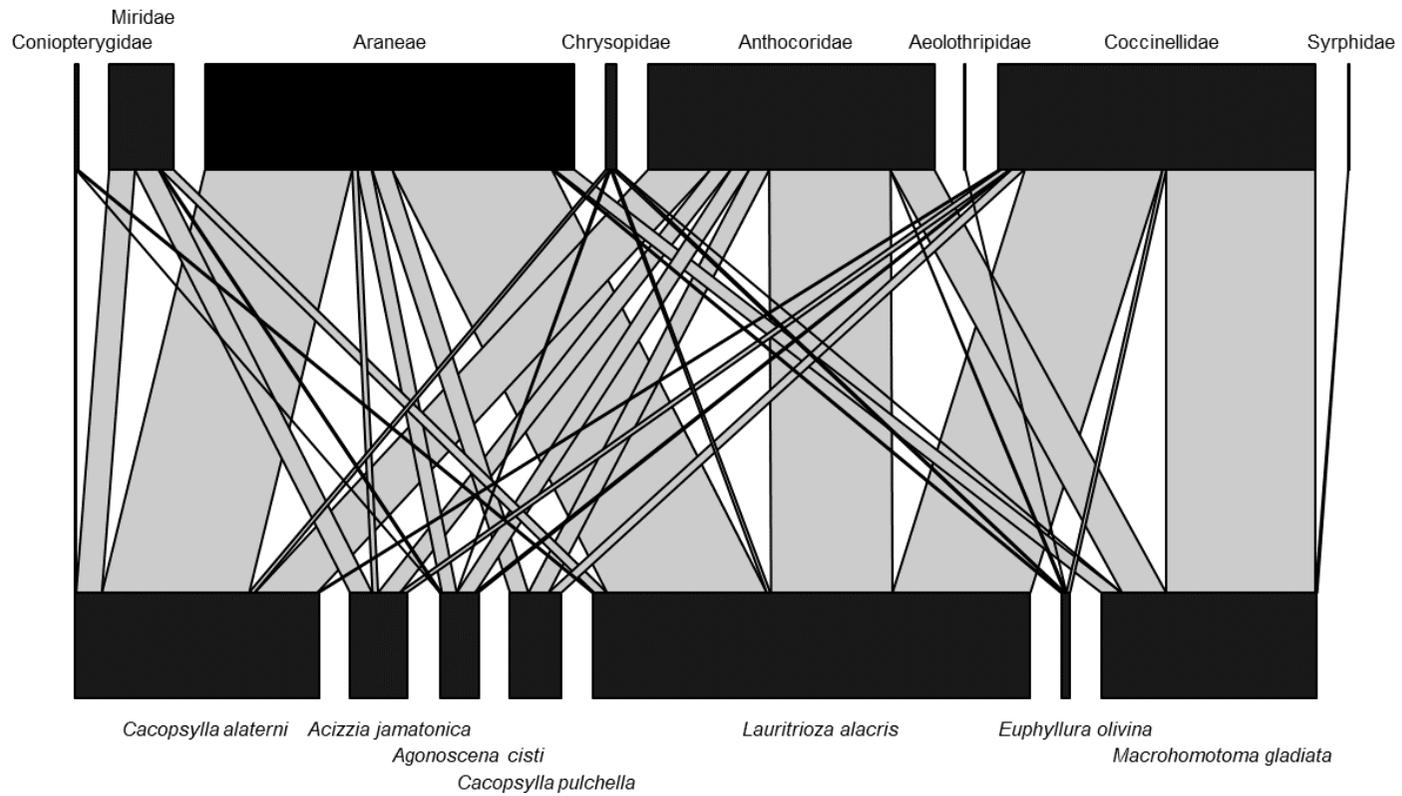


Figure 4.2. Plot web of the average number of predators per family and the order Araneae sampled by beating each host plant infested by psyllids.

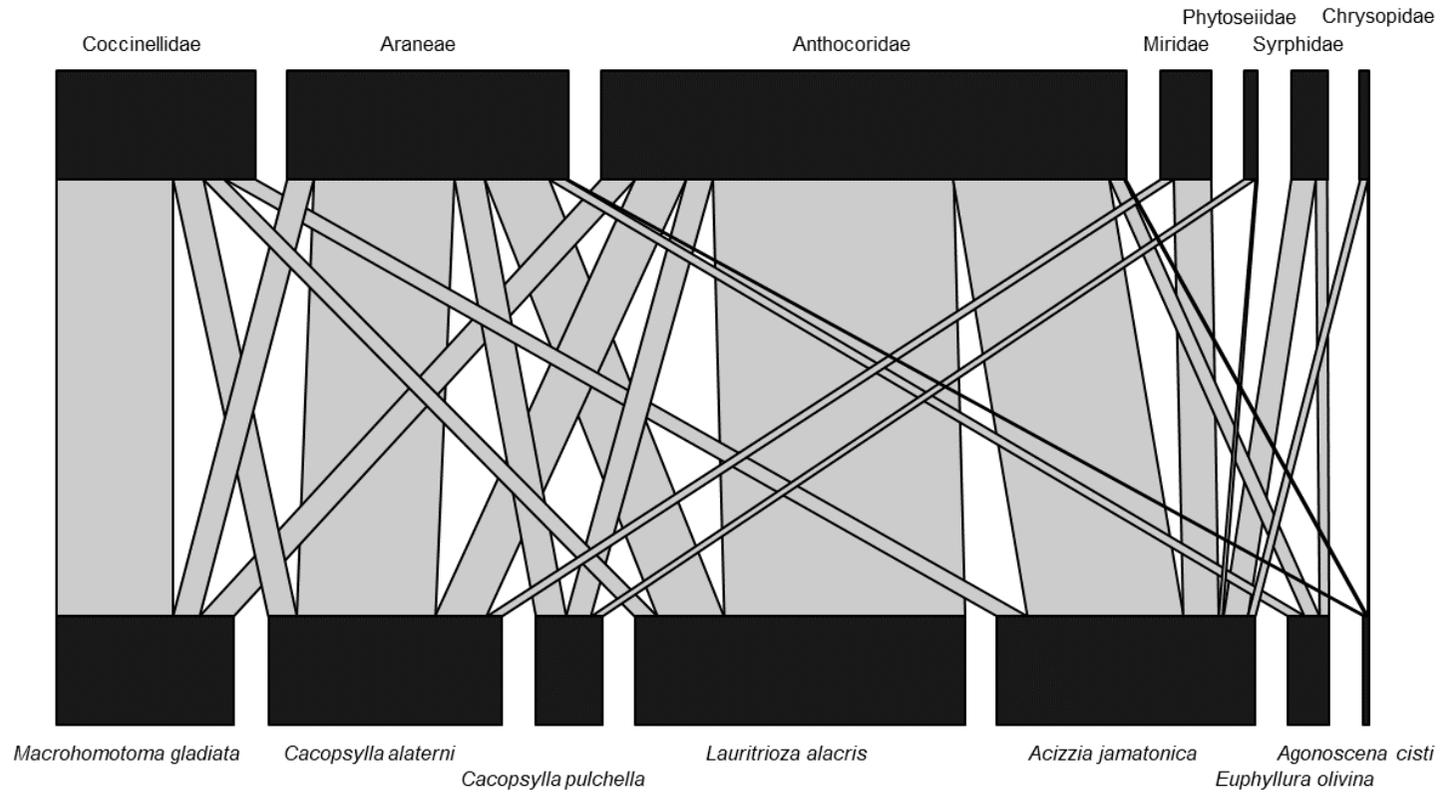


Figure 4.3. Plot web of the average number of predators per family and the order Araneae from the evolutionary samples of psyllid infested shoots.

Table 4.2. Coccinellidae species present (+) or absent (-) on each psyllid studied species in evolutionary (E) and beat (B) samplings.

Species	<i>Acizzia jamatonica</i>		<i>Agonoscena cisti</i>		<i>Cacopsylla alaterni</i>		<i>Cacopsylla pulchella</i>		<i>Euphyllura olivina</i>		<i>Lauritrioza alacris</i>		<i>Macrohomotoma gladiata</i>	
	E	B	E	B	E	B	E	B	E	B	E	B	E	B
<i>Adalia bipunctata</i> (L.)	-	-	-	-	-	+	-	-	-	-	-	-	-	-
<i>Adalia decempunctata</i> (L.)	-	-	-	-	-	-	-	-	-	-	-	+	-	-
<i>Chilocorus bipustulatus</i> (L.)	-	+	-	+	-	-	-	-	-	-	-	+	-	-
<i>Cryptolaemus montrouzieri</i> Mulsant	+	+	-	+	-	-	-	-	-	-	+	+	+	+
<i>Exochomus nigromaculatus</i> (Goeze)	-	-	-	-	-	-	-	-	-	-	-	+	-	-
<i>Hippodamia variegata</i> (Goeze)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Propylea quatuordecimpunctata</i> (L.)	+	-	-	-	-	+	-	+	-	-	-	+	-	-
<i>Psyllobora vigintiduopunctata</i> (L.)	-	-	-	-	-	-	-	+	-	-	-	+	-	-
<i>Rhyzobius chrysomeloides</i> (Herbst)	-	-	-	-	+	+	-	-	-	-	-	-	-	-
<i>Rhyzobius forestieri</i> (Mulsant)	-	-	-	-	-	-	-	-	-	-	-	+	-	-
<i>Rhyzobius litura</i> (Fabricius)	-	-	-	-	-	+	-	-	-	-	-	+	-	-
<i>Rhyzobius lophantae</i> Blaisdell	-	-	-	-	-	+	-	-	-	+	-	+	-	-
<i>Rodolia cardinalis</i> (Mulsant)	+	-	-	-	-	-	-	-	-	-	+	+	+	-
<i>Scymnus interruptus</i> (Goeze)	-	+	-	-	-	+	-	-	-	-	-	+	-	-
<i>Scymnus mediterraneus</i> lablokoff-Khnzorian	-	-	-	-	-	+	-	-	-	-	-	-	-	-
<i>Scymnus</i> spp.	+	-	-	-	-	-	-	-	-	-	-	-	-	+
<i>Scymnus subvillosus</i> (Goeze)	-	-	-	-	+	+	-	-	-	+	-	+	-	-
<i>Stethorus</i> sp.	-	-	-	-	-	+	-	-	-	-	-	+	-	-

Table 4.3. Spearman's rank correlations between predator (Anthocoridae, Araneae and Coccinellidae) and psylla species abundances in beat samplings.* indicates significant correlation ($p \leq 0.05$).

	Anthocoridae			Araneae			Coccinellidae		
	R	<i>p</i>	S	R	<i>p</i>	S	R	<i>p</i>	S
<i>Acizzia jamatonica</i>	0.04	0.8891	436.3	-0.28	0.3363	581.38	0.33	0.2492	304.85
<i>Agonoscena cisti</i>	0.79	0.0001*	203.96	0.68	0.0019*	311.09	0.33	0.1872	653.42
<i>Cacopsylla alaterni</i>	0.71	0.0090*	81.57	-0.56	0.0628	446	0.43	0.0521	878.87
<i>Cacopsylla pulchella</i>	-0.01	0.9798	121.19	-0.48	0.1918	177.51	-0.67	0.0496*	200.08
<i>Euphyllura olivina</i>	0.28	0.4719	86.859	0.28	0.4719	86.86	0.48	0.1875	62.004
<i>Lauritrioza alacris</i>	0.85	0.0002*	53.647	0.31	0.2967	249.84	0.54	0.0553	166.41
<i>Macrohomotoma gladiata</i>	0.60	0.0104*	323.7	-0.25	0.3341	1019.6	0.60	0.0115*	329.51

Discussion

In this study, the psyllid species found on each ornamental tree/shrub were those expected as main hostplant. Some of them are native from the Mediterranean basin, such as *C. pulchella*, *E. olivina*, *L. alacris* (Mifsud et al., 2010; Ouvrard, 2022). Others, like *A. jamatonica* and *M. gladiata*, are exotic in the sampled region but present in other regions of the Iberian Peninsula for few years ago (Sánchez, 2012; Sánchez & Burckhardt, 2009). The high specificity to their host plant is a characteristic of psyllids, since although they may visit other plant species, the completed life cycle is usually associated with one or very few closely related plant species (Burckhardt et al., 2014). The psyllids abundance in the sampled months was variable according to the species and location, and they may have overlapping generations with one or more peaks of abundance per season (Hodkinson, 1974; Jiménez-Alagarda et al., 2014).

Generalist predators were also found in the sampled psyllid-infested shoots and were present on different host plants throughout the year. Anthocoridae was the most abundant family in beat and evolutionary samplings, and they were collected on all sampled plant species. This family has already been reported preying on psylla species in the Mediterranean basin, mainly the species *Anthocoris nemoralis* (Fabricius) (Bella & Rapisarda, 2014; Kamel et al., 2021; Onillon, 2016, Pedata et al., 2012). In our study, they were found related with the psyllid species *A. cisti*, *C. alaterni*, *C. pulchella*, *L. alacris* and *M. gladiata*. Some anthocorid bugs have been previously found associated with several psyllid species (Laborda et al., 2015; Paredes et al., 2019).

Ladybird beetles were also abundant, and this abundance was correlated to *C. pulchella* and *M. gladiata* abundances. Some of the ladybird species found have been previously recorded as psyllid predators, like *Adalia bipunctata* (L.), *Hippodamia variegata* (Goeze) and *Scymnus* spp. on *A. jamatonica* and *M. gladiata* (Bella & Rapisarda, 2014; Harizanova et al., 2012). Among the collected ladybird beetles, there were some commonly found in citrus growing areas of the Mediterranean basin, such as the genera *Rhyzobius* and *Scymnus* (Alvis, 2003).

Spiders were also one of the most abundant taxa in the samplings, predominantly on *C. alaterni* and *L. alacris*, and then related to the psyllid *A. cisti*. It is very interesting that a positive correlation between spiders and *T. erythrae* abundance was reported by van den Berg, Dippenaar-Shoemang et al. (1992) in citrus orchards in South Africa.

As mentioned above, some of the collected psylla species, as *A. jamatonica* and *M. gladiata*, are not native from the Mediterranean basin. Hence, the predatory activity on these psyllas may be an example of fortuitous biological control, showing the potential

of native predators in incorporating exotic species in their diets, and reducing psyllid populations. The predatory groups reported in our study, such as green and brown lacewings, ladybirds, hoverflies, anthocorid bugs, mirid predatory bugs, spiders and predatory mites, have been recorded as natural enemies of *T. erytreae* in southern Africa, where it is native, and in sub-Saharan Africa and Arabian Peninsula, where it is also present (Catling, 1970; van den Berg et al., 1987). Predators from these families were also described in *T. erytreae* infested citrus orchards in Canary Islands (Spain), where the pest is present since 2002 (Estévez et al., 2018; González-Hernández, 2003). Furthermore, *T. erytreae* was detected in the gut content of several predators collected on citrus trees of Canary Islands and Galicia (Spain) (Molina et al., 2021). Although some of the collected families are not common predators found in Mediterranean citrus orchards, *T. erytreae* arrival may imply their movement into the citrus trees. This could be the case of Anthocorid bugs, since *Orius* spp. were detected feeding on *T. erytreae* in infested citrus trees in Galicia (Spain) (Molina et al., 2021). In this context, Shaltiel & Coll (2004) proposed planting *R. alaternus* near pear orchards in Israel, after observing that *Cacopsylla bidens* (Sulc) (Hemiptera: Psyllidae) populations were low in those pear orchards adjacent to natural woodlands because of the higher reproduction of the predator *A. nemoralis* in *R. alaternus* than in pear trees. Therefore, plant species with high abundance of this anthocorid bug may be good candidates to host this predator, providing alternative prey when crop pest densities are low. Population dynamics of *T. erytreae* is regulated, among other factors, by the availability of young flushes (Catling, 1972). Therefore, depending on the flushing rhythm of the cultivated citrus, available prey may be not enough, and the presence of some trees infested with psyllids is an alternative option to be further studied. For example, in the spring citrus flushing, the presence of *R. alaternus*, *C. siliquastrum* or *F. macrocarpa* close to citrus orchards could attract generalist predators, whereas in the summer citrus flushing, *A. julibrissin* and *L. nobilis* could keep predators near citrus orchards since their associated psylla species were abundant in those months. Finally, in autumn when citrus might have another flushing, the tree species which may host psyllids in this period could be *F. macrocarpa*, where the psyllid *M. gladiata* was again abundant, and *P. lentiscus*.

Parasitism on psyllids was not observed in all evolutionary samples. We were able to verify parasitism only on *M. gladiata*, parasitized by *P. mitratus*, and on *E. olivina*, parasitized by *Psyllaephagus* sp. The parasitoid *P. mitratus* has a wide range of psyllid host in comparison of other psyllid parasitoids, and it is able to parasitize various species of *Cacopsylla* and *Psylla* genera, as well as the species *Trioza baemeri* Tuthill (Hemiptera: Triozidae) (Jerinić-Prodanović et al., 2019; Noyes, 2019). The average

parasitism rate by *P. mitratus* reported in Spain was 7% (Laborda et al., 2015), but sometimes it could reach 32% (Rodrigo et al., 2016). Associated with *E. olivina*, the parasitoid species *P. euphyllurae* has been described in the study area from specimens collected in Spain by Triapitsyn et al. (2014) and, as in our study, they also reported the presence of the hyperparasitoid *A. trapezoidea* associated with Chalcidoidea (Hymenoptera) (Menke & Evenhuis, 1991) and a specimen of *Pachyneuron* sp. emerged from parasitized *E. olivina* nymph. Other psyllid parasitoids of the studied species have not been reported in the Mediterranean basin, except the case of the parasitoid *Psyllaephagus provincialis* Panis & Onillon (Hymenoptera: Encyrtidae) on *C. pulchella* nymphs, which could reach 23% in some months of the year (Onillon, 2016). Concerning *T. erytrae*, the parasitoid *Tamarixia dryi* Waterson (Hymenoptera: Eulophidae) has been reported as the main parasitoid of this psyllid in South Africa, together with *Psyllaephagus pulvinatus* Waterson (Hymenoptera: Encyrtidae) and another species of the genus *Tamarixia* (Pérez-Rodríguez et al., 2019).

As a conclusion, there is a wide range of generalist predators associated to psyllid species present on ornamental trees and shrubs that could potentially feed on *T. erytrae* in the Spanish Mediterranean region. Some of them, as the anthocorid bugs, are not commonly found in citrus orchards of the Mediterranean basin, but they are strongly associated with psyllid populations and present in trees and shrubs located close to citrus growing areas. Therewith, in the hypothetical situation that *T. erytrae* spreads to citrus growing areas of Spain, those generalist predators may provide a fortuitous biological control of this pest. This strategy, that need to be further studied, could complement others to control this pest, such as classical biological control with the parasitoid *T. dryi*, already used in other areas of mainland Spain where no citrus are grown.

General discussion

The psyllid *T. erytrae* is vector of HLB, the most devastating disease attacking citrus worldwide, and its presence in the Iberian Peninsula threatens the current IPM programs in these crops. IPM prioritizes the use of biological control and other kind of alternatives to chemical strategies because of their strong impact, either by the emergence of pest resistances through their repeated applications, or by their effect on the control agents used, such as predators and parasitoids. Under this context, the most hazardous pesticides have been forbidden in the last years in the European Union (EU). The European Commission aims to reduce by 50% the use of chemical pesticides by 2030 (European Commission, 2020).

Most of the knowledge about *T. erytrae* biology and control dates from early 90s, being conducted under subtropical conditions, as shown in the Introduction of this Thesis. Its arrival on mainland Europa entailed the need to provide a more extended knowledge for its control to contain its spread. Some aspects of the chemical control of *T. erytrae* have been approached in the first part of this Thesis (Chapters 1 and 2), being the second part (Chapters 3 and 4) involved with *T. erytrae* biological control.

The chemical control laboratory experiments conducted in Chapter 1 showed that several contact and systemic pesticides could be included in citrus IPM programs to control *T. erytrae*. Among them, there are some pesticides that are not yet authorized in Spanish citrus orchards, such as spinetoram, which is effective on other psyllids attacking other crops, for example in pear orchards against the pear psylla (Boselli & Scannavini, 2014). This Thesis shows that spinetoram has been effective against *T. erytrae* in laboratory experiments. Moreover, given the rapid movement of the inclusion or prohibition of active substances for pest control in Europe, during the realization of this Thesis some pesticides tested in Chapter 1 have been banned or restricted in the EU as it is the case of dimethoate and sulfoxaflor (European Commission, 2019 & 2022, respectively). Even so, the results obtained in this Thesis about these pesticides could be considered in other citrus growing areas under the potential risk of *T. erytrae* arrival, including some African countries of the Mediterranean basin.

In the citrus agroecosystem, biological control agents manage to maintain pests under EILs, and if its action is undisturbed, allow to reduce the number of chemical treatments per season. Most of the pests are naturally controlled by their natural enemies. The introduction of a new pest in the citrus agroecosystem could alter the existing pest/natural enemy balances. Under this scenario, it is essential to know pests' biology, their natural enemies, their behaviour, and the efficacy of the used pesticides and their

side effect on the natural enemies also present in the agroecosystem (Barzman et al., 2015; Urbaneja et al., 2022). The use of new or more frequent pesticides to control *T. erytrae* in IPM programs in citrus must also consider the side effects on natural enemies and, consequently, a possible disruption in the biological control of other citrus pests. The effect of the systemic pesticides that resulted effective against *T. erytrae* in Chapter 1 was assessed in Chapter 2 on the parasitoid *A. vladimiri*, parasitoid that control *P. citri*, an important pest of citrus. In this Chapter it was demonstrated that the pesticides that killed *A. vladimiri* while feeding on pesticide solutions had not an impact on the surviving parasitoids the following days after feeding. Otherwise, in the case of cyantraniliprole, even if it had not affected *A. vladimiri* survival while feeding on it, it has been demonstrated that the mortality of the parasitoid occurred in the following days after feeding on it. Parasitoids can also ingest pesticides through the available food sources (nectar and pollen of flowers, honeydew or gutation drops) present at that moment on citrus trees, since these sources can be dropped by the pesticide solution. A previous study has reported pesticide residues in wildflowers of citrus ground cover after applying treatments on citrus trees canopy (García-Vacárcel et al., 2022). In Chapter 2, buckwheat plants were irrigated with the pesticides selected from Chapter 1 and offered to *A. vladimiri* females so that its nectar could serve as food. The obtained results have shown that *A. vladimiri* survival can be affected by this exposure route, and the impact had been different according to temperature. Therefore, the effects of pesticide drifts on the plant cover and their effect on natural enemies may be further considered. Among pesticides tested in Chapter 2, the only one that did not cause mortality on *A. vladimiri* females after feeding on nectar of treated plants was flupyradifurone. This pesticide was effective against *T. erytrae* eggs and adults by contact, and by the systemic way against nymphs (Chapter 1). Regarding these results, the application of flupyradifurone in *T. erytrae* chemical control may not disrupt the biological control of *citrus* mealybug, but further exposure routes should be assessed between this pesticide and other biological control agents.

The neonicotinoid acetamiprid was selected in Chapter 1 as alternative to the banned neonicotinoids in Europe, imidacloprid and thiamethoxam, which have been widely used in citrus growing areas where HLB vectors are present (Qureshi et al., 2014). Interestingly, it was effective against *T. erytrae* nymphs and adults when applied topically, but not when it was applied systemically. In Chapter 2, acetamiprid caused mortality on *A. vladimiri* females after feeding on nectar of treated buckwheat plants in two of the three conducted trials. Thus, the use of acetamiprid, as other neonicotinoids, could have an impact on the auxiliary fauna through food sources (Calvo-Agudo et al.,

2021; Goulson, 2013; Jones et al., 2020). Therefore, its use must be assessed, since neonicotinoids are considered harmful to auxiliary fauna such as pollinators, whose abundance has been greatly reduced when neonicotinoids were included in chemical pest management programs (Woodcock et al., 2016).

Hence, the results from Chapter 2 highlight the importance of high efficiency of the application machinery to avoid losses to the ground due to runoff and drift, and consequently reduce the impact of pesticide on biocontrol agents by this exposure route. This allows the possibility of using those pesticides that are effective against *T. erytraeae* and could be compatible with its biological control, such cyantraniliprole which was highly effective against *T. erytraeae* nymphs (Chapter 1), and it has been reported to be much less toxic for the main parasitoids of HLB vectors, *T. dryi* and *T. radiata*, than to the target psyllid (Dionisio et al., 2021; Tiwari & Stelinski, 2013).

In Chapter 3, I have studied the possibility of using a conservation biological control strategy for *T. erytraeae*. A specific molecular marker of *T. erytraeae* has been designed and tested in Chapter 3 to evaluate predation on *T. erytraeae* in field-collected potential predators. This pair of *T. erytraeae*-specific primers has allowed to identify some predators that have fed on this vector in Spanish infested trees located in small citrus orchards in the Canary Islands and in backyards and urban areas in Galicia. This tool could also be used to study predation in commercial citrus orchards of the Mediterranean basin, considering that the environmental conditions of agroecosystems and predator range may slightly differ depending on the region. In Chapter 4, different psyllid-infested plant species, mostly ornamental trees and shrubs, located near citrus orchards were sampled for a prospection of potential natural enemies. Among the predator species that have been feeding on *T. erytraeae*, the Anthocoridae family, a predatory taxon that is not common in citrus orchards, was very abundant in Galician citrus trees, as mentioned in Chapter 3. The presence of this predatory family may be explained by its attraction to some psyllid species (Artigues et al., 1996; Juan & Bravaccini, 2009; Paredes et al., 2019; Shaltiel & Coll, 2004). In fact, this family was one of the most collected on psyllid infested ornamental trees in the samplings conducted in Chapter 4. In both Chapters, we have found a wide range of generalist predators that may also prey on *T. erytraeae*, such as ladybird beetles, lacewings, and spiders. Some of the species from the Coccinellidae family collected in psylla-infested ornamental trees and shrubs (Chapter 4) such as *A. bipunctata*, *H. variegata*, *Scymnus* spp. and *Rhyzobius* spp. were also detected preying on *T. erytraeae* (Chapter 3). These species have also been reported as psyllid predators, and they are present in Mediterranean citrus orchards (Alvis, 2003; Bella & Rapisarda, 2014; Harizanova et al., 2012). Concerning green lacewings, *C. carnea*

larvae can prey on different *T. erytrae* stages: on nymphs, as shown in the feeding trials conducted in Chapter 3; and on eggs, as reported by Aguiar et al. (2019). In this study, this predator reduced the *T. erytrae* attack severity in those citrus trees treated with commercial *C. carnea* in Portugal. In addition, in north-western Portugal, web spiders (Theridiidae family) were recorded capturing *T. erytrae* adults in lemon orchards (Benhadi-Marín et al., 2021). In Chapter 3, only 1 individual of this family was collected and no *T. erytrae* DNA was detected in its gut content. Nevertheless, as shown in this Chapter, other spider families had been fed on *T. erytrae*. Furthermore, it is noteworthy that in Chapter 3 the ants *L. grandis* and *L. humile* has found feeding on *T. erytrae*. Until now, both species have not been considered as predators in citrus agroecosystems, but disrupters of biological control of honeydew-producing hemipters, so that further studies may determine the role of these ants with *T. erytrae* in citrus agroecosystems.

In California a key factor to contain the spread of HLB has been the biological control of its vector *D. citri*. General predators, such as hoverflies and green lacewings, together with its parasitoid *T. radiata* were responsible of *D. citri* mortality in this citrus growing area (Kistner et al., 2016). Furthermore, other general predators, such as ladybird beetles, brown lacewings and spiders, have been reported in USA and Brazil on *D. citri*, and some laboratory and field studies have assessed their capability of feeding on this psyllid (Gómez-Marco et al, 2022; Khan et al., 2016; Michaud, 2002; Michaud et al., 2004; Monzó et al., 2014; Qureshi & Stansly 2009). In this Thesis, all these predatory taxa have been detected feeding on *T. erytrae* using the designed molecular tool (Chapter 3). The next step would be to assess who is better contributing to reduce *T. erytrae* populations, and how to enhance conservation biological control. For example, Irvin et al. (2021) have evaluated buckwheat and alyssum as insectary plants to enhance de biological control of *D. citri* by hoverflies. The presence of these insectary plants may also act as a food source of other natural enemies of other pest, such as the mealybug parasitoid *A. vladirimi*, which is able to feed on buckwheat nectar (Chapter 2).

Future Prospects

Trioza erytrae has not yet reached the Mediterranean citrus growing area but is currently present in the Portuguese citrus growing area of the Algarve. The management of this pest in isolated trees in mainland Spain, where no citrus crops are grown, and in citrus orchards in the Canary Islands is nowadays based on a classical biological control program using the parasitoid *T. dryi*, where it has been successful in reducing *T. erytrae* populations. Nevertheless, the psyllid has spread through the Cantabrian and Atlantic

coast of the Iberian Peninsula. Unfortunately, despite the good biological control exerted by *T. dryi*, it is unknown what could happen when *T. erytreae* will arrive to the Spanish citrus orchards area, since the parasitoid will be under different weather conditions and may have the pressure of other factors, like chemical control. The approaches addressed in the four Chapters of this Thesis to control *T. erytreae* improve the knowledge to apply novel strategies to manages this pest when arriving to Spanish citrus orchards. The use of selective pesticides may be a good strategy, particularly combined with the natural biological control present. The designed molecular markers can identify predators consuming the target vector in the field and provide a basis for future IPM strategies. It is very important to content the presence of *T. erytreae* in citrus trees of backyards and urban areas to prevent its spread to commercial citrus orchards. For that, the presence of ornamental trees and shrubs that are often infested with other psyllids near the citrus trees could act as host of psylla predatory species.

Conclusions

The conclusions of this Doctoral Thesis are the following:

- I. There are several effective pesticides on the *Trioza erytreae* developmental stages under laboratory conditions. Most of them are more effective against nymphs than for adults and eggs.
- II. The proved efficacy of those pesticides on the different development stages of *T. erytreae*, together with their contact and/or systemic action, will permit the design of efficient chemical control strategies to be included in current citrus IPM programs. This fact would allow the rotation of pesticides to avoid the development of resistances, and to minimize the impact on the entomofauna of citrus agroecosystems.
- III. The application of those pesticides may contaminate potential food sources for natural enemies present in citrus orchards. The ingestion of pesticides dropped on these food sources immediately after their application may affect the parasitoid *Anagyrus vladimiri*, causing adult death or reducing fertility. *Anagyrus vladimiri* females fed on nectar of *Fagopyrum esculentum* plants treated by irrigation have also shown higher mortality than when feeding on untreated plants. These results prove the need to avoid runoff and drift to the ground to prevent that the systemic pesticides used could reach the nectar of the wildflowers.
- IV. The designed *T. erytreae*-specific primers allow the detection of *T. erytreae* remains in the gut content of predators collected on citrus trees. This tool has been very useful to identify predatory species. After the potential arrival of *T. erytreae* to Mediterranean citrus growing area, it will be of a great help to promote the biological control of this vector.
- V. A wide range of predatory taxa are feeding on *T. erytreae* in infested citrus trees in Spain. These potential candidates to control *T. erytreae* include some species commonly found in citrus crops, like some ladybird beetles, lacewings, hoverflies, spiders, earwigs and ants, together with other species not commonly found in citrus orchards, like anthocorid bugs.

- VI. Ornamental trees and shrubs found near citrus orchards in the Mediterranean basin are often infested by psyllids and harbour a wide range of potential predators of *T. erytrae*. Ladybirds, anthocorid bugs and spiders were the most abundant taxa found. These predators would provide a fortuitous biological control of *T. erytrae* in the citrus growing areas of Spain, with the potential risk arrival of this vector. The highest number of psyllid predators were collected on *Cercis siliquastrum*, *Laurus nobilis* and *Rhamnus alaternus*. Thus, these plant species should be considered as part of ecological infrastructures in citrus orchards.

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