Investigating insecticide resistance in UK populations of the cabbage stem flea beetle, *Psylliodes chrysocephala*

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Abstract

The cabbage stem flea beetle, *Psylliodes chrysocephala*, is a major pest of winter oilseed rape (OSR), particularly in the UK. Prior to December 2013, control of *P. chrysocephala* relied on the protection of OSR seedlings by neonicotinoid seed treatments, followed by the application of foliar pyrethroid sprays. However, in 2018, the EU-imposed a ban on the neonicotinoid seed treatments leaving just pyrethroid sprays for *P. chrysocephala* control. The increased selection pressure, caused by a lack of alternative control agents with different modes of action, has led to the emergence of pyrethroid resistance in this pest, resulting in widely reported control problems.

P. chrysocephala adult samples sourced from across the UK in 2018, 2019 and 2020 were found to exhibit high levels of resistance to lambda-cyhalothrin, with the mean resistance level increasing across the three years of monitoring. The target-site mutation L1014F, previously shown to contribute to pyrethroid resistance in samples of *P. chrysocephala,* was present across most of the samples tested; the L925I mutation was also present in some of the samples. Synergist bioassays found that pyrethroid resistance was suppressed in *P. chrysocephala* samples pre-treated with PBO, suggesting the presence of a metabolic-based mechanism for resistance.

RNA sequencing data from susceptible and resistant samples of *P. chrysocephala* was analysed and the cytochrome P450 *CYP6k1-like* identified as the most differentially expressed gene, with qPCR analysis showing that *CYP6k1-like* was significantly overexpressed in UK resistant samples. Delivery of *in vitro* synthesised dsRNA by both microinjection and oral delivery caused a knockdown effect of *CYP6k1-like.* After glass vial bioassays, mortality was significantly higher in the *P. chrysocephala* injected with *in vitro* synthesised ds*CYP6K1-like* relative to those injected with ds*GFP* suggesting the involvement of *CYP6k1-like* in conferring resistance to pyrethroids in *P. chrysocephala* adults.

Glass vial bioassays used to evaluate the efficacy of alternative insecticides against pyrethroid resistant *P. chrysocephala* adults and larvae identified cyantraniliprole as the most toxic compound against *P. chrysocephala* adults. Cyantraniliprole was also effective against eight *P. chrysocephala* samples collected across England, suggesting its potential in future control strategies. Of the compounds tested against *P. chrysocephala* larvae, acetamiprid and fipronil were identified as the most toxic.

Declaration

I declare that the work in this thesis was carried out in accordance with the regulations of the University of Gloucestershire and is original except where indicated by specific reference in the text. Some parts of this thesis have been previously submitted to the University of Gloucestershire as part of the postgraduate certificate mainly by research (research methods) academic award, in accordance with the requirements of the degree of Doctor of Philosophy. The thesis has not been presented to any other education institution in the United Kingdom or overseas. Any views expressed in the thesis are those of the author and in no way represent those of the University.

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List of Abbreviations

Abbreviations for nucleic acids

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

Abbreviations for amino acids

Amino acid	Three-letter abbreviation	One-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Additional abbreviations

ABC transporter	ATP-Binding Cassette transporter
ANOVA	Analysis of Variance
BLAST	Basic local alignment search tool
bp	base pair
ºC	Celsius
CCE	Carboxylesterase
cDNA	complementary deoxyribonucleic acid
CE	controlled environment
CI	confidence interval
CNS	central nervous system
CSFB	Cabbage Stem Flea Beetle
CT	Threshold cycle
DESeq2	Differential gene expression sequencing analysis 2
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
dsRNA	double stranded RNA
EdgeR	Empirical analysis of digital gene expression data
EU	European Union
FastQC	Fast Quality Control
g	gram
GFP	Green Fluorescent Protein
GO	Gene Ontology
GPCR	G Protein-Coupled Receptor

GST	Glutathione S-transferase
HISAT2	Hierarchical Indexing for Spliced Alignment of Transcripts 2
Hr	hour
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRAC	Insecticide Resistance Action Committee
kdr	Knockdown resistance
L	Litre
LB	Lysogeny Broth
LC	Lethal concentration
LD	Lethal dose
MCS	Multiple Cloning Site
MoA	Mode of Action
MRP4	Multidrug resistance-associated protein 4-like
MultiQC	Multi Quality Control
mL	Millilitre
nAChR	Nicotinic Acetylcholine receptor
NCBI	National Centre for Biotechnology Information
NGS	Next Generation Sequencing
OD	Optical Density
OSR	Oilseed Rape
OP	Organophosphates
P450	Cytochrome P450-dependent monooxygenase
PBO	Piperonyl Butoxide
PCA	Principle Component Analysis

PCR	Polymerase Chain Reaction
ppm	Parts per million
qPCR	quantitative Polymerase Chain Reaction
RNA	ribonucleic acid
RNAi	RNA interference
s	second
s-kdr	Super knockdown resistance
SOC	Super Optimal Broth with Catabolite Repression
Taq polymerase	*Thermus aquaticus* polymerase
Tm	Melting temperature
UDP	Uridine Phosphorylase
USA	United States of America
VGSC	Voltage gated Sodium Channel
µg	Microgram
µl	Microlitre
(v/v)	volume/volume
(w/v)	mass/volume

Chapter 1. Introduction

1.1 Oilseed rape as an agriculturally important crop

In a world where the population is predicted to increase by one billion within the next 10 years (FAO et al., 2020), the demand for vegetable oils is predicted to rise due to the growing demand for food and non-food products, particularly as a renewable energy source. Oilseed rape (OSR) (*Brassica napus*) is a major crop in Europe (Carré and Pouzet, 2014) and is now the second most important oilseed crop in the world after soybean (FAOSTAT, 2020). Since 1961, global production of OSR has increased by 5.4% per year (FAOSTAT, 2018). In 2019, worldwide production of rapeseed oil was estimated at 73.1 million tonnes (FAOSTAT, 2020), with Europe accounting for 21% of production (EUROSTAT, 2021).

OSR is a crop of significant economic value due to its many applications in agriculture, since it is rich in both oil and protein (Wanasundara, 2011; Matthaus, Özcan and Juhaimi, 2016). On a global scale, it is one of the most important crops for vegetable oil, protein-rich animal feed and biofuel production. The growing global demand for biofuel resulted in a 79% increase in the area of OSR grown worldwide from 1989 to 2009 (FAO, 2012). Since the introduction of the European Union (EU) Directive 2003/30/EC to promote the use of biofuels for transport, there has also been a large increase in the area of OSR grown across the EU, with 6.4 million hectares harvested in 2010, a 78% increase from 2003 (FAOSTAT, 2021). In 2004, just 27% of the oilseed rape produced in the EU was processed into biodiesel (Demirbas, 2009). In 2019, oilseed rape accounted for 39% of total biodiesel feedstock produced in the EU (USDA, 2019), with biodiesel representing 85% of the total transport biofuels market (USDA, 2020).

OSR is also an important break crop in cereal rotations in agriculture as it helps break the disease cycle of cereal pathogens and improve soil tilth (Hegewald et al., 2018). As OSR is important in cereal rotation, it was originally rotated in one-in-five-year cycles with cereals (ENDURE, 2007) due to these long rotations having often been found to result in increased yield (Zheng et al., 2020). However, as the value and demand for OSR has increased there has been a global trend to grow OSR in one-in-two or one-in-three-year rotations (Berry and Spink, 2006; Rusch et al., 2010). The shorter rotations have resulted in a 3-12% yield decline (Christen and Sieling, 1995).

This decline is concerning at a time when there is a rising demand for the crop as a source of biofuel and edible oil. Further, the movement from diversified agricultural landscapes to increased simplicity (due to shorter rotation cycles) also provides a higher availability of resources for insect pests. The pollen beetle (*Brassicogethes aeneus*) was identified as a key pest of OSR after 3-4 years of intensive OSR cultivation (Hokkanen, 2000).

Due to the increase in significance of OSR due the factors outlined above and the potential for it's straw being exploited as a renewable energy source (Chico-Santamarta et al., 2010; Wang et al., 2019), the area grown in Demark and Germany is predicted to increase in the future (Højland and Kristensen, 2018). However, the area of oilseed rape grown in the UK is now in decline. In 2012, the area grown in the UK was estimated at over 700,000ha (DEFRA, 2014), declining to 627,000ha in 2015 (DEFRA, 2015), 488,000ha in 2019 (DEFRA, 2019) and 345,000ha in 2020 (DEFRA, 2020). This significant decline is predicted to continue as oilseed rape continues to attract crucifer-specialist pests, such as the pollen beetle and cabbage stem flea beetle, making it increasingly difficult to grow (Dewar et al., 2016).

1.2 Pests of oilseed rape

Oilseed rape is targeted by a range of crucifer-specialist pests, all capable of inflicting damage by destroying either the pods or the stems, causing a reduced crop yield in heavily infested fields (Alford, Nilsson and Ulber, 2003; Williams, 2010). Major pests of OSR include the pollen beetle (*Meligethes aeneus*), cabbage stem flea beetle (*Psylliodes chrysocephala*), cabbage stem weevil (*Ceutorhynchus pallidactylus*), cabbage seed weevil (*Ceutorhynchus napi*), rape stem weevil (*Ceutorhynchus napi*) and the brassicae pod midge (*Dasineura brassicae*). Minor pests of oilseed rape include the cabbage aphid (*Brevicoryne brassicae*) and peach-potato aphid (*Myzus persicae*) (Williams, 2004). These pests attack the oilseed rape crop consecutively as it grows, targeting different stages and parts of the crop (Figure 1.1). For example, whilst the first damage to winter OSR is inflicted by the insect pest *P. chrysocephala* which attacks young emerging plants in the autumn (Ferguson et al., 2003), the adult pollen beetle (*M. aeneus*) is found to damage, through feeding, the green and yellow buds of the oilseed rape flowers (Williams, 2010). Spring oilseed rape is also attacked by some of the same insect pests as winter OSR, such as the cabbage seed weevil,

brassica pod midge and turnip flea beetles *Phyllotreta cruciferae* and *Phyllotreta nigripes* (Oakley, 2003; Ekbom, 2010; Dewar et al., 2016).

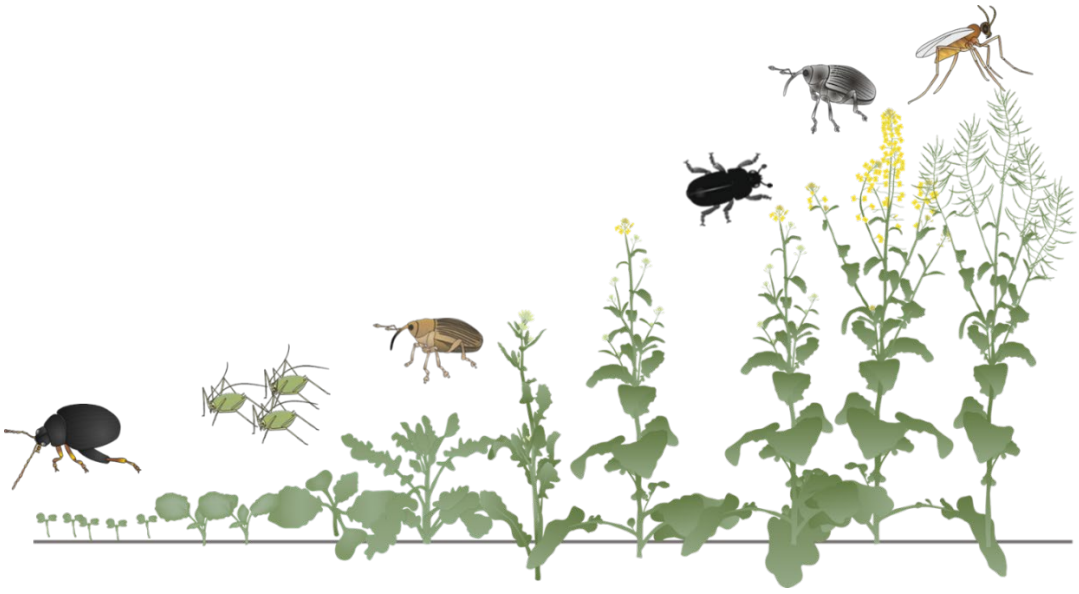


Figure 1.1: *Insect pests successively attack different growth stages and parts of the oilseed rape crop. The first damage to the emerging oilseed rape crop is inflicted by the cabbage stem flea beetle in September-October, followed by the peach-potato aphid, the cabbage seed weevil, the pollen beetle, the cabbage stem weevil and the brassicae pod midge. Source: Sam Cook, Rothamsted Research.*

1.2.1 Cabbage stem flea beetle (*Psylliodes chrysocephala*)

1.2.1.1 Description

Adult *P. chrysocephala* are approximately 3 – 5mm in length and are typically black in colour with a blue-green tinge (Alford, 1979) (Figure 1.2A); a brown variant has also been previously described (Williams, 2010). The adults have reddish legs and antennae and enlarged hind femora which allow them to jump powerfully; they also have rudimentary wings which enable them to fly (Hubble, 2010; Williams, 2010). The beetle has three larval instars. All three instars are milky white (Figure 1.2B), have a black head, a black dorsal plate at the hind end and three pairs of thoracic legs (Williams, 2010). The first instars are approximately 1mm in length, the second instars 3-4mm in length and the third instars 5-8mm in length. The first and second instars have numerous small black spots on their back.

***Figure 1.2**. A) An adult P. chrysocephala, B) A 2nd instar P. chrysocephala larva. C) 'Shot-holing' damage. Source: A) © Visual Communication Unit, Rothamsted Research.*

1.2.1.2 Distribution

Psylliodes chrysocephala is an established insect pest of winter oilseed rape (OSR) particularly in the UK (Graham and Alford, 1981; Winfield, 1992; Williams, 2010) and Germany (Zimmer *et al.*, 2014). It is also a significant pest of other Brassicaceae species in several European countries (Bromand, 1990), Asia, Canada, North Africa, the Middle East and the USA (reviewed in Williams, 2010) (Figure 1.3).

***Figure 1.3.** Map of the present (2020) distribution of P. chrysocephala. Source: cabi.org.*

1.2.1.3 Habitat

In mid-summer, *P. chrysocephala* adults feed on wild or cultivated crucifer plants before aestivating in sheltered areas such as hedgerows, under plant material, or within

plant stems (Bonnemaison and Jourdheuil, 1954). When they emerge from aestivation, they migrate to newly growing brassicae crops where they feed on the leaves and stems. Whilst *P. chrysocephala* has been found on 18 other crop species from the Brassicaceae family, it has also been recorded on three species outside this family: meadow-rue *Thalictrum majus*, (Ranunculaceae), common flax *Linum usitatissum* (Linaceae) and soybean *Glycine max* (Papilionaceae) (Bonnemaison and Jourdheuil, 1954).

1.2.1.4 Ecology

The life cycle of *P. chrysocephala* is well understood (Figure 1.4). *P. chrysocephala* is a univoltine insect. Upon emerging from summer aestivation in late August/early September, adult *P. chrysocephala* migrate to newly sown winter oilseed rape crops (Williams, 2010). Once they reach the crop their flight muscles begin to atrophy and they lose their ability to fly (Bonnemaison, 1995). The adults feed on the oilseed rape cotyledons and first true leaves and approximately two weeks after feeding, the females mate and lay pale orange eggs (Williams, 2010). The eggs are laid on either the lower part of the oilseed rape plant (Bonnemaison and Jourdheuil, 1954), or near the plant base in cracks in the soil (Såringer, 1984; Williams, 2004). Oviposition cannot occur at a temperature less than 2°C (Bonnemaison and Jourdheuil, 1954) and females can lay up to 1,000 eggs during their lifetime. After hatching, the neonate larvae move to a host plant where they then burrow into the lower leaf petioles near the attachment point to the main stem (Bonnemaison and Jourdheuil, 1954). They feed from October to April, subsequently moving into the main stem where they develop through their larval instars (Williams, 2010). From late April to early May, final instar larvae leave the plants and burrow into the soil to pupate (Williams, 2004, Ferguson *et al.,* 2006). New adults emerge from late May to early July where they feed on the stems and leaves of winter oilseed rape and other cruciferous plants. In mid-summer, after the oilseed rape harvest, the young adults feed on wild crucifers before finding a sheltered site and entering a period of aestivation for 1-2 months (Williams, 2004, 2010).

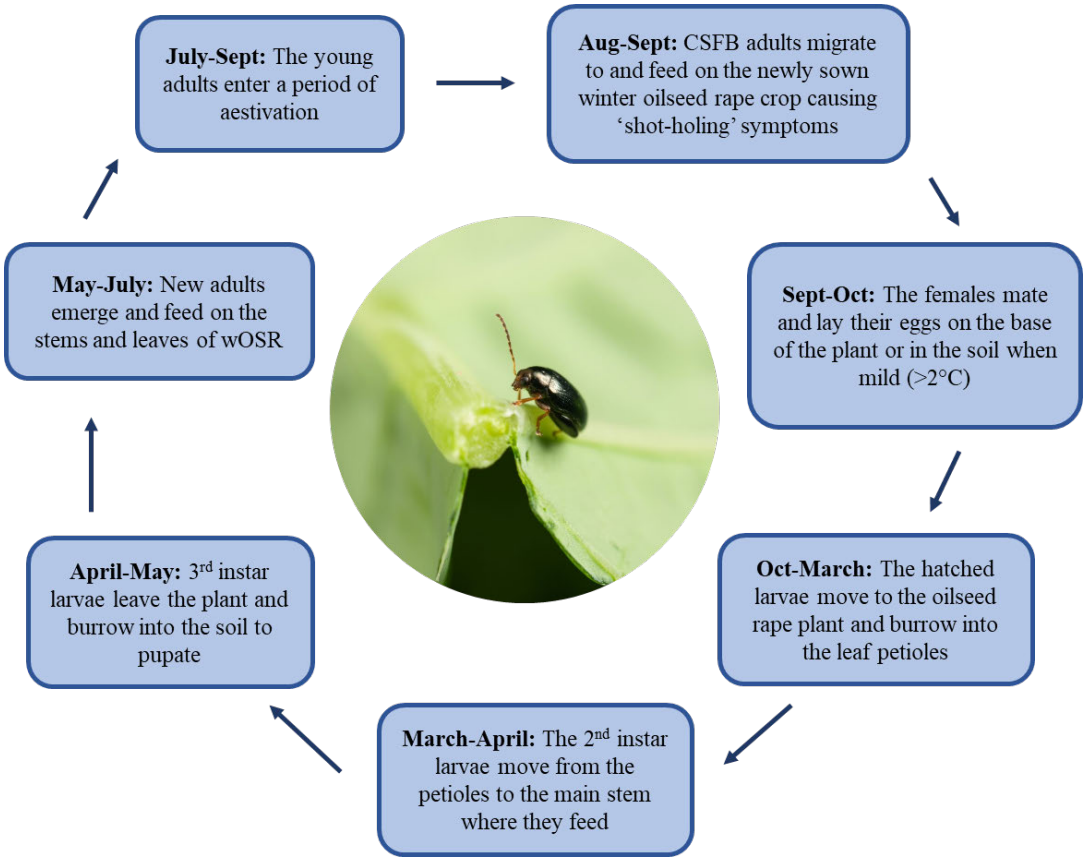


Figure 1.4. *P. chrysocephala life cycle. © Visual Communication Unit, Rothamsted Research.*

1.2.1.5 Damage

P. chrysocephala inflicts damage on OSR at both the larval and adult stage. Adult *P. chrysocephala* cause damage by feeding on the stems, cotyledons and the first true leaves of the emerging crop, resulting in characteristic holes called 'shot-holing' (Figure 1.2C) (Ellis, 2015). As the crop is the most vulnerable at this time, when fields are heavily infested with *P. chrysocephala* adults, feeding can result in seedling death before emergence, or poor crop establishment resulting in reduced plant density (Walters et al., 2001).

The tunnelling of feeding *P. chrysocephala* larvae into the leaf petioles and later the main stems can cause the most damage to the crop, through weakening of the upper section of the roots and lower part of the stem (Figure 1.2B) (Williams, 2004). When larval numbers are high, this can result in distortion of the plant tips, stem wilt and the infested plants becoming more susceptible to fungal pathogens such as stem canker *Leptosphaeria maculans* (Newman, 1984), the bacterial disease *Erwinia* sp. (Balaž, 2007) and frost damage (Williams, 2004). These symptoms can all result in early

senescence, yield loss or ultimately plant death (Lane and Cooper, 1989; Williams, 2004; Nicholls, 2016).

1.3 Control of insect pests

1.3.1 Biological control

Biological control of insect pests can be defined as the use of natural enemies to reduce a pest population (De Bach and Rosen, 1991) and has been widely used in pest management for the last two hundred years (De Bach, 1964). There are three main approaches to biological control: classical, augmentation and conservation. Classical biological control is used mostly against invasive insect pests which have become established in new habitats, regions, or countries. Natural enemies from the pest's place of origin are imported into the pest's new environment and built up to a level where they can control the pest (Caltagirone, 1981). This type of control is most successfully used with perennial crops and forests. Augmentation control involves either inoculative or inundative release of natural enemies and is most effective with greenhouse crops (van Lenteren, 2000). The introduction of large numbers of the natural enemy destabilises the natural pest-enemy relationship, resulting in a rapid reduction of the pest. Figueiredo et al (2015) found that three releases of the parasitoid *Trichogramma pretiosum,* a natural enemy of the fall armyworm (*Spodoptera frugiperda*), increased the yield of maize plots in Brazil by 19.4% per hectare due to a reduction in the *S. frugiperda* population. Whilst augmentation control looks to increase the number of natural enemies for pest control, conservation control manages pest numbers through the protection and stimulation of natural enemies (Rabb, Stinner and van den Bosch, 1976; Hoy et al., 1998; Barbosa, 1998; Ehler, 1998), for example, by providing additional food sources or overwinter refuges (Corbett and Rosenheim, 1996; Mensah, 1997). As this type of biological control is based on the permanent establishment of natural enemies, it works best in forests or on perennial crops (Bale, Van Lenteren and Bigler, 2008).

1.3.1.1 Biological control of *P. chrysocephala*

There are several species of parasitoid wasps (Hymenoptera) that attack either the larval or adult stage of *P. chrysocephala*. The parasitoid species belong to three families: Ichneumonidae, Braconidae and Pteromalidae and include *Diospilus*

oleraceus (Haliday), *Diospilus morosus* (Reinhardt), *Microctonus brassicae* (Haeselbarth), *Aneuclis melanaria* (Holmgren), *Tersilochus microgaster* (Szépligeti), *Tersilochus tripartitus* (Brischke) and *Trichomalus lucidus* (Walker) (Jourdheuil, 1960; Ulber et al., 2010).

Whilst these parasitoids have all been observed in the UK and Europe (de Jong et al., 2014), *Tersilochus microgaster* has been identified as the main parasitoid of *P. chrysocephala* larva (Ulber and Wedemeyer, 2004). Upon migrating to the OSR crop in March, the female *T. microgaster* lay their eggs in the *P. chrysocephala* larva (Barari et al., 2005; Ferguson et al., 2006). The univoltine endoparasitoid *Microctonus brassicae,* parasitizes the adult stage. These wasps oviposit into the *P. chrysocephala* body, either through the tip of the abdomen or mouthpart. Once the egg is laid, the *P. chrysocephala* continues to feed and the larvae develops inside. The larva exit the host's body through the anus once it has developed through its final instar (Jordan et al., 2020). Whilst Jordan et al. (2020) found the average parasitism rate for *P. chrysocephala* by *M. brassicae* within captivity was 44%, suggesting the potential of the parasitoid for biological control, a study by Patricia Ortega-Ramos (Rothamsted Research) in 2020 found that the parasitism rate was only approximately 7% in the field. Despite this, *M. brassicae* was present in 70% of the *P. chrysocephala* samples investigated (Patricia Ortega-Ramos, personal communication).

1.3.2 Synthetic insecticide control of insect pests

For the last 80 years, chemical compounds have been used as the principal method for the control of insect pests. The first synthetic insecticide, dichlorodiphenyltrichloroethane (DDT), was introduced in the 1940s to tackle malaria, typhus, and other insect-borne human diseases. This was quickly followed by the organophosphates (OPs) in the 1950s, methylcarbamates in the 1960s, pyrethroids in the 1970s, neonicotinoids in the 1990s, and diamides in the 2000s (Casida and Durkin, 2013). The most commonly applied insecticides in agriculture have a neurotoxic mode of action as this results in rapid kill, thus minimising crop damage. Whilst there are at least 11 target sites for neurotoxic insecticides in the insect nervous system, most insecticides target just four major sites comprising; the voltage-gated sodium channel (VGSC), the GABA-gated chloride channel (GABA), the nicotinic acetylcholine receptor (nAChR) and the acetylcholinesterase (AChE) (Casida and

Durkin, 2013). Figure 1.5 illustrates the location of the main target sites and the insecticide classes that target them.

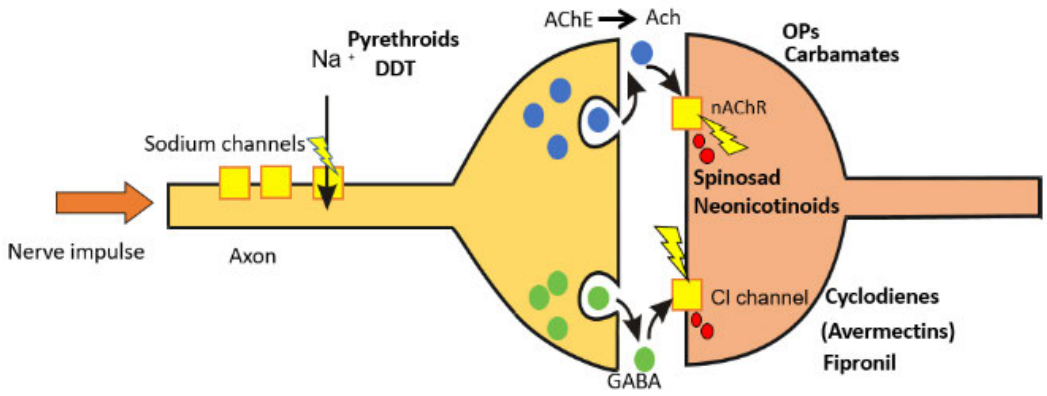


Figure 1.5. *Target sites of synthetic insecticides in the insect nervous system. Courtesy of Martin Williamson (Rothamsted Research).*

1.3.2.1 Chemical control of *P. chrysocephala*

Prior to December 2013, *P. chrysocephala* control relied on the protection of OSR seedlings by systemic neonicotinoid seed treatments such as imidacloprid, thiamethoxam and clothianidin, and pyrethroid sprays later in the season if pest infestation levels were high and the neonicotinoids were no longer able to effectively protect the crop (Højland and Kristensen, 2018). The neonicotinoids bind to the nicotinic acetylcholine receptors (nAChRs) (Jeschke, Nauen and Beck, 2013) whilst the pyrethroids target the voltage-gated sodium channels in the insect central nervous system (Khambay and Jewess, 2005). However, in December 2013 the European Commission introduced a regulatory restriction on the use of systemic neonicotinoids on crops attractive to bees including maize, oilseed rape and sunflower (EU Commission, 2013). The restriction was based on a risk assessment by the European Food Safety Authority (EFSA) in 2012 (EFSA, 2013b) which found that the neonicotinoids pose an unacceptably high risk to honey bees (Van der Sluijs et al., 2013). In May 2018 the regulatory restriction was converted to a complete ban on the outdoor use of imidacloprid, clothianidin and thiamethoxam on all crops (EU Commission, 2018).

Since the ban on neonicotinoid seed treatments the number of *P. chrysocephala* in the UK has increased and the damage caused has intensified (Collins, 2017). Prior to 2014, *P. chrysocephala* affected approximately 67% of the area of oil seed rape grown in the UK, causing an annual 1% yield loss (Clarke et al., 2009). However, in 2014, a

survey found that 2.7% of the national OSR crop was lost due to adult *P. chrysocephala*, the most serious losses (5–14%) being in Eastern and Southern England (Wynn, Ellis and Alves, 2014). In 2014 the damage caused by *P. chrysocephala* was valued at a loss of £23 million (Nicholls, 2016). In 2015/16, it was reported that 69% of crops were damaged. Whilst only 1% of the national OSR crop was lost, the damage caused by *P. chrysocephala* was more widely spread across the country than in 2014 (Alves, Wynn and Stopps, 2015). In the autumn of 2019, 50% of OSR crops had severe damage, with *P. chrysocephala* pressure spreading into the North and West of England (Jones, 2020b). Subsequent surveys have confirmed that the average numbers of larvae per plant have risen substantially in all regions since 2014 (as summarised in Dewar, 2017). An average of 0.2-27.8 larvae per plant was reported in 2015 (White, 2015), whilst an average of 8-30 larvae per plant was reported in 2019 (Agronomy Issues, 2021). Since the European regulatory ban on the use of systemic neonicotinoids, the pyrethroids (e.g. lambda-cyhalothrin) remain the only class of insecticide used for chemical control of *P. chrysocephala* in the UK.

1.3.2.2 Pyrethroids

The pyrethroid class of synthetic insecticides was developed in the mid-1970s, at Rothamsted Research, and they have been widely used for control of arthropod pests due their high insecticidal activities against a wide range of pests and low toxicity to mammals (Matsuo, 2019). Pyrethroids have their insecticidal activity by targeting the voltage-gated sodium channels in the central nervous system of insects (Narahashi, 1996, 2002), interfering with normal cellular communication (Davies et al., 2007). The voltage-gated sodium channels are crucial for the generation and propagation of action potentials in the central and peripheral nervous system, mediating the transient permeability of the neuronal cell membranes to sodium ions (Davies et al., 2007). Under normal conditions, the sodium channels are activated (opened) before being inactivated and deactivated (closed), with the transition between states being linked by the initiation and propagation of action potentials. However, when a pyrethroid insecticide binds, it binds preferentially to the activated insect voltage-gated sodium channels, interfering with nerve function by causing repeated discharges and inhibiting the transition between an activated (ion-conducting) and inactivated (non-conducting) state (Davies et al., 2007). As a result, cells become persistently depolarized and the sodium channels remain open. This causes paralysis and eventual

death in arthropod insects due to over-activation of nerve function (Narahashi, 1996, 2002).

Studies investigating the use of synthetic pyrethroids for the control of insect pests first began in the late 1940s, when Milton Schechter and colleagues found that the compounds allethrin and bioallethrin were approximately 20 times more effective against insect pests than DDT (Schechter, Green and LaForge, 1949; Laforge, Schechter and Green, 1956). In 1962, Michael Elliot developed resmethrin (Pickett, 2016) at Rothamsted Research (at that time known as Rothamsted Experimental Station) and found that whilst it was significantly more effective at killing the housefly (*Musca domestica*) than natural pyrethrin extracts, its use was limited due to its instability in sunlight (Ueda, Gauchan and Casida, 1974). The later discovery by Elliott of cypermethrin, deltamethrin and permethrin in the mid-1970s, a group of pyrethroids that combined a greater stability in light with significantly higher insecticidal activity, led to their commercialization and the subsequent establishment of pyrethroids as a dominant class of insecticide applied to agriculture (Elliott, Janes and Potter, 1978; Elliott, 1989). By 1995, pyrethroids commanded a significant share of the world insecticide market, representing 23% of the US dollar value (Casida and Quistad, 1998). By 2002, pyrethroids had become the most widely used insecticide, with deltamethrin being the highest selling pyrethroid at $208 million per year (Field et al., 2017). Since their initial discovery, further commercially successful pyrethroids such as lambda-cyhalothrin, tau-fluvalinate and beta-cyfluthrin have been developed.

Whilst the pyrethroid class of insecticides are based on the structure and insecticidal properties of pyrethrin, a natural pesticide found in chrysanthemum flowers, they are structurally highly diverse (Casida et al., 1983). Pyrethroids can be classified into two subclasses (type I and type II) based on their chemical structure. Whilst all pyrethroids contain a cyclopropane group linked to an alcohol moiety, type I pyrethroids do not contain an α-cyano group whilst type II pyrethroids do (Khambay and Jewess, 2005). When used as an insecticide, type I pyrethroids induce repetitive firing in axons causing hyperactivity and restlessness followed by paralysis, type II pyrethroids cause convulsion also followed by paralysis (Davies et al., 2007).

1.3.2.3 Diamides

Whilst not yet registered as a control agent for *P. chrysocephala*, the diamide class of insecticides offers a potential alternative for their chemical control. The diamides represent the most recent (major) class of insecticide available on the market for the control of a variety of insect pests, particularly being effective against species of the order Lepidoptera (Dong et al., 2017), as well as certain species of the order Coleoptera (Caballero et al., 2015), Hemiptera (Foster et al., 2012) and Diptera (Zhang et al., 2015). Diamides function by binding to the ryanodine receptors (RyR) in insect muscles causing the calcium channel to stay open, leading to the uncontrolled release of calcium into the cell (Lahm *et al.*, 2005; Cordova *et al.*, 2006). This prevents further muscle contraction causing lethargy, feeding cessation, paralysis and eventual death (Lahm et al., 2005; Tohnishi et al., 2005; Cordova et al., 2006). The phthalic diamide flubendiamide (Tohnishi et al., 2005) and the anthranilic diamides: chlorantraniliprole and cyantraniliprole (Lahm et al., 2005) were commercialised in 2006, 2007 and 2012 respectively and represented ~12% of the insecticide market in 2020 (Sparks et al., 2020).

The anthranilic diamides chlorantraniliprole and cyantraniliprole show low toxicity to vertebrates and non-target organisms (Sattelle, Cordova and Cheek, 2008), with the EPA classifying chlorantraniliprole as a reduced risk pesticide (EPA, 2008). Whilst chlorantraniliprole is especially effective for the chemical control of insect pests in the order Lepidoptera, cyantraniliprole shows a more extensive range of systemic insecticidal activity, providing good control towards a wide range of sucking and chewing insects (Sattelle, Cordova and Cheek, 2008; Foster et al., 2012; Grávalos et al., 2015). The alternative mode of action and effectiveness against some species of the order Coleoptera (Caballero et al., 2015; Plata-Rueda et al., 2019) suggests the potential of the diamide class of insecticide in controlling *P. chrysocephala.* However, further research is required to evaluate their efficacy against pyrethroid-resistant *P. chrysocephala*. As seen in the pyrethroids, resistance to diamide insecticides has been observed in a range of lepidopteran species (Roditakis et al., 2017; Troczka et al., 2012; Richardson et al., 2020).

1.4 Insecticide resistance

Resistance has been defined as 'a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species' (IRAC, 2016). Insecticide resistance is one of the key challenges in the chemical control of important arable crop pests (Georghiou and Taylor, 1986) and has influenced the strategies employed for chemical control of insect pests for more than 50 years (Sparks and Nauen, 2014). The problem of resistance is widespread (Georghiou and Lagunes-Tejeda, 1991), with at least 500 species of mites and insects now found to have developed resistance to at least one class of insecticide (Forgash, 1984; Georghiou and Taylor, 1986; Georghiou, 1990), due to the repeated and extensive use of insecticides with the same mode of action (Sparks and Nauen, 2014).

The first study documenting insecticide resistance was published in 1914 by Axel Melander who noted that the application of the insecticide sulphur-lime was less effective in controlling San José scale (*Quadraspidiotus perniciosus*), brown mite or orchard aphid than it had been in previous years (Melander, 1914). Further cases of insecticide resistance were reported in the following decades with Hough (1928) observing resistance in the codling month (*Laspeyresia pomonella*) against lead arsenate and Quayle (1938) reporting resistance in red scale (*Aonidiella aurantia*) against hydrogen cyanide. Since the initial discovery of insecticide resistance over 100 years ago, varying degrees of resistance against a range of the older insecticides, including the organophosphates, chlorinated hydrocarbons, pyrethroids and carbamates, has been observed (Ishaaya and Horowitz, 1998).

The mechanisms underlying insecticide resistance have been extensively studied. The two main mechanisms conferring insecticide resistance are: (i) target-site insensitivity, also known as target-site resistance, caused by point mutations in the insecticide target site reducing the binding affinity of insecticides and (ii) metabolic resistance, caused by the overexpression of enzymes in response to the presence of a xenobiotic compound which reduces the amount of insecticide able to bind to the target site. Whilst these two mechanisms are key in conferring insecticide resistance, other less common mechanisms of resistance include penetration resistance and behavioural resistance.

1.4.1 Target-site resistance

Target-site resistance occurs when point mutations in the insecticide target site prevent the insecticide from binding. Point mutations in the gene encoding the target site lead to a conformational change in the protein and the insecticide is unable to bind. As the main targets for insecticides are within the insect nervous system it is these transmembrane proteins that have developed such resistance. Target-site resistance can be observed in a range of insecticide classes.

Target-site resistance to pyrethroids, known as knock-down resistance or *kdr*, was first described in the house fly (*M. domestica*) (Williamson *et al.*, 1996) and results from point mutations in the voltage-gated sodium channel sequence affecting pyrethroid binding (Williamson et al., 1993; Davies et al., 2007; Soderlund, 2008). Whilst several point mutations in the VGSC conferring pyrethroid resistance have been described, a leucine (L) to phenylalanine (F) substitution at the amino acid residue 1014 (L1014F) (Figure 1.6) remains the most common and has been reported in a wide range of insect species (Davies et al., 2007; Rinkevich, Du and Dong, 2013). This mutation typically confers moderate (10-20-fold) levels of resistance. Elevated resistance levels (several 100-fold) to pyrethroids have also been seen in a range of pests when the L1014F mutation is associated with an additional substitution in the same domain. This mechanism of resistance has been termed *super-kdr* and is most often associated with a methionine (M) to threonine (T) substitution (M918T) (Ingles *et al.*, 1996; Williamson *et al.*, 1996).

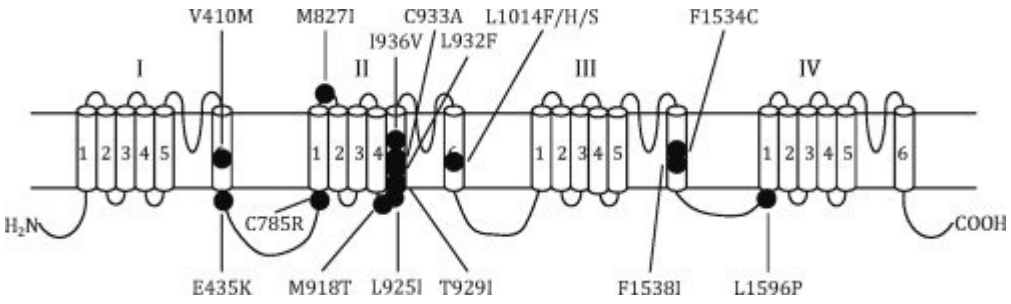


Figure 1.6. *The location of mutations in the sodium channel that confer pyrethroid resistance in Xenopus oocytes. Source: Rinkevich, Du and Dong (2013)*

The presence of *kdr* has been observed in populations of *P. chrysocephala*. Zimmer et al. (2014) reported the presence of the L1014F *kdr* mutation in pyrethroid resistant *P. chrysocephala* from Germany. Assays revealed high frequencies (90-100%) of the *kdr* allele in *P. chrysocephala* populations exhibiting resistance against a range of

pyrethroids, including lambda-cyhalothrin and tau-fluvalinate. More recently, a study by Højland and Kristensen (2018) showed that pyrethroid resistance as a result of target-site mutation has continued to spread, with resistance being observed in populations from Demark and across the United Kingdom. Samples from Germany showed spread of the *kdr* allele further south than reported by Zimmer in 2014.

1.4.2 Metabolic resistance

Metabolic resistance involves the detoxification of xenobiotic compounds (Li, Schuler and Berenbaum, 2007). There are three main groups of enzymes involved in metabolic resistance, the cytochrome P450 monooxygenases (P450s), glutathione *S*-transferases (GSTs) and esterases. Whilst these enzymes cause metabolic resistance primarily by overexpression, other mechanisms include sequence amplification of the enzyme encoding gene, upregulation through mutations in the *trans*-acting regulatory loci or *cis*-acting elements (Feyereisen, 1995; Liu and Scott, 1997), transcriptional enhancements, and mutations in gene coding sequences (Li, Schuler and Berenbaum, 2007). Whilst all three enzyme families are associated with metabolic resistance, P450 mediated resistance is thought to be the most common mechanism in insecticide resistance (Hodgson, 1983; Oppenoorth, 1985; Scott, 1999).

In insects, P450s play a key role in the detoxification of xenobiotic compounds such as plant toxins (Berenbaum, Cohen and Schuler, 1990; Schuler, 1996) and insecticides (Feyereisen, 2011, 2012). P450 mediated insecticide resistance results from P450 overexpression by upregulation of P450 genes (Liu and Scott, 1997, 1998; Feyereisen, 1999). Studies have found that the number of metabolic P450 enzymes in different insect species varies greatly, with the fruit fly (*Drosophila melanogaster*) found to have 85 cytochrome P450 genes (Tijet, Helvig and Feyereisen, 2001; Feyereisen, 2006), the silk moth (*Bombyx mori*) to have 81 and the red flour beetle (*Tribolium castaneum*) to have 143 P450 genes (Feyereisen, 2006). Within the insect order there are four clades of cytochrome P450 (CYP) genes, namely, CYP2, CYP3, CYP4 and the mitochondrial P450 clade (Feyereisen, 2006).

Although the function P450s play in insecticide resistance only became apparent in the 1960s, when Eldefrawi, Miskus and Sutcher (1960) showed that that the P450 inhibitor sesamin (the major ligand found in sesame oil) could prevent resistance to carbaryl, the first P450 was not reported in insects until 1967 (Ray, 1967). Today there

are numerous studies throughout the literature which provide examples of P450 mediated resistance in response to overexpression of either single or multiple P450 genes. For example, the cytochrome P450 *CYP6BQ9* was found to play a role in pyrethroid resistance in the red flour beetle (*T. castaneum*), showing more than a 200-fold higher expression in a deltamethrin resistant strain (Zhu et al., 2010) and the P450 *CYP6CM1* was found to be responsible for neonicotinoid resistance in both the B and Q biotypes of the whitefly (*Bemisia tabaci*), showing overexpression of ~17-fold in the imidacloprid resistant strains (Karunker et al., 2009).

Glutathione S-transferases (GSTs) have been found to confer resistance to pyrethroids, organophosphates (OP) and organochlorines. GSTs play a key role in protecting cells from oxidative stress by conjugating reduced glutathione (GSH) to the electrophilic centres of lipophilic compounds (Li, Schuler and Berenbaum, 2007). As with P450 mediated resistance, GST mediated resistance is a result of gene amplification or overexpression. Resistance as a result of GST overexpression has been documented for a range of species, with Tang and Tu (1994) finding a DDT resistant strain of *D. melanogaster* had elevated levels of GSTD1 and Syvanen, Zhou and Wang (1994) showing that an OP resistant strain of *M. domestica* had increased levels of *MdGSTD3* transcripts. Resistance to DDT due to increased GST expression has also been observed in a range of mosquito species. For example, resistant *Anopheles gambiae* displayed 5-fold higher levels of GST3-2 than the susceptible strains (Ranson et al., 2001) whilst resistant *Anopheles aegypti* had increased levels of GSTE2-2 (Lumjuan et al., 2005).

The carboxylesterases (CCEs) are a multi-gene family that hydrolyse a range of carboxylesters via the addition of water (Newcomb et al., 1997; Wheelock, Shan and Ottea, 2005). They confer insecticide resistance to xenobiotic compounds that contain ester bonds, such as the carbamates, pyrethroids and organophosphates (OPs), either by the overexpression of the enzymes or by a mutation within the gene coding sequences. In the peach-potato aphid (*M. persicae*), the amplification of the gene for the carboxylesterase E4/FE4 has been shown to cause resistance to OPs (Devonshire and Moores, 1982; Field and Devonshire, 1998). The esterases were found to be increased 4-fold in the resistant aphids, with 80 times more genes found in the R_3 clone (Field et al., 1999). In the sheep blowfly (*Lucilia cuprina*), Newcomb *et al.* (1997) found a G137D mutation in the carboxylesterase isozyme E3 caused it to reduce its

carboxylesterase activity and gain organophosphate hydrolase activity. An additional mutation in the E3 isozyme, W251L, was later found to confer increased hydrolysis activity against the organophosphate malathion (Campbell et al., 1998). The mosquito *Culex tritaeniorhynchus* has also been found to be resistant to OPs as a result of gene amplification, with the *Estβ1* gene showing elevated esterase activity (Karunaratne et al., 1998).

The ATP binding cassette (ABC) transporters are another superfamily of genes involved in metabolic resistance. These transporters play a role in phase III of the detoxification process. Phase I involves the P450 and CCE enzymes activating the toxic molecules, making them more reactive and water soluble; this increases their conjugation to the phase II enzymes, the GSTs and UPD-glycosyltransferases (Dermauw and Van Leeuwen, 2014). They can also be used without previous modification to the insecticides - phase 0 (Després, David and Gallet, 2007). These transporters use an ATP hydrolysis mechanism to transport insecticides and their metabolites out of the cells, preventing an intracellular build-up of these xenobiotic compounds (Broehan et al., 2013; Buss and Callaghan, 2008). The ABC transporters have been linked to resistance towards nine different insecticide classes including the carbamates, pyrethroids, neonicotinoids, organophosphates and phenylpyrazoles (Dermauw and Van Leeuwen, 2014). Pyrethroid resistance, as a result of ABC transporter-gene upregulation, has been documented in a range of different insect species. In the mosquito *Aedes aegypti,* the ABC transporter gene *ABCB4* and the P450 *CYP9J26* were found to be upregulated in the pyrethroid resistant strain, the gene-copy number of these genes having increased by ~7-fold (Bariami et al., 2012). Zhu et al (2013) observed the upregulation of the transporters ABC8-11 in the deltamethrin resistant strain of the bed bug *Cimex lectularius* and Bonizzoni et al (2012) reported the upregulation of an ABC transporter in a deltamethrin resistant strain of the mosquito *Anopheles gambiae.*

1.4.3 Penetration resistance

Penetration resistance involves minimising the amount of insecticide absorbed into the body. Within the insect cuticle there are three layers which can be altered in thickness or composition to give the resistant phenotype (Figure 1.7). In a pyrethroid resistant strain of the bed bug *Cimex lectularius,* Koganemaru, Miller and Adelman (2013)

found that a proportion of the cuticle protein-encoding genes identified in the transcriptome were substantially upregulated in the resistant strain when compared to the susceptible strain. A further study on *C. lectularius* found that cuticle thickness was positively correlated to the time it took to knock-down the bed bugs on exposure to the pyrethroid insecticide lambda-cyhalothrin (Lilly et al., 2016). As this mechanism of resistance can increase the time insecticides take to reach their target sites within the nervous system, detoxification enzymes have more time to respond, resulting in stronger resistant phenotypes. In the oriental fruit fly (*Bactrocera dorsalis*), β-cypermethrin resistant strains were shown to have both thicker cuticles and a greater number of chitin layers in the endocuticle when compared to the susceptible strains (Lin et al., 2012).

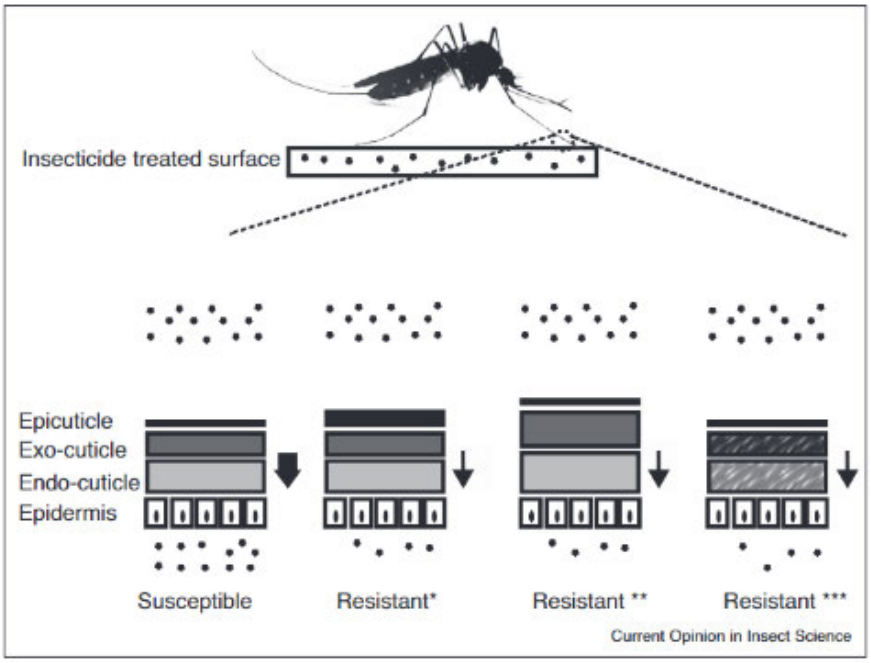


Figure 1.7. *Penetration resistance, either as a result of cuticle thickening or cuticular alteration, leading to a reduced absorption of insecticide. *Resistant: thickening of the epicuticle, **Resistant: thickening of the exo- and endo-cuticle, ***Resistant: alteration of the cuticle composition. Source: Balabanidou, Grigoraki and Vontas, 2018.*

1.4.4 Behavioural resistance

Behavioural resistance involves the modification of behaviour which facilitates the avoidance of toxic compounds. Sarfraz, Dosdall and Keddie (2005) found that under greenhouse conditions, diamondback moth *Plutella xylostella,* females laid a greater number of eggs on the stem than on the leaves of cruciferous plants. As foliar sprays are more likely to remain on the leaves than the stem, this ability to avoid high doses

of insecticides by oviposition site selection suggests the development of behavioural resistance in *P. xylostella*. Early studies into malaria control have demonstrated that mosquito behavioural adaptations may be emerging, facilitating the avoidance of insecticidal nets (Gatton et al., 2013; Thomsen et al., 2017). Whilst some behavioural resistance studies have been conducted, in general little is known about this mechanism as it is difficult to monitor in field populations and is more complex to quantify than target-site and metabolic mechanisms.

1.5 Literature cited

Where possible, the literature cited throughout this thesis refers to the primary source. As the topic of insect control and *P. chrysocephala* as an insect pest is a historic one, the literature cited within the introductory chapter dates back as far as the 1950s. Despite their age, these references remain relevant to the topic. Since the regulatory decision to restrict the use of neonicotinoids on outdoor crops (EU, 2013), *P. chrysocephala* have become a serious pest of oilseed rape. Consequentially the amount of research done on this pest has increased, resulting in more contemporary literature. It is important to note however, that the more contemporary papers still refer to the older literature, for example Ortega-Ramos et al (2022) refer to Bonnemaison and Jourdheuil (1954) and Såringer (1984). This newer literature is cited appropriately throughout the thesis. For example, the potential of parasitoid wasps as a biological control agent against *P. chrysocephala* (Jordan et al., 2020) is discussed in Chapter 1.

1.6 Research aims and objectives

Monitoring the geographical spread of pyrethroid resistance, elucidating the mechanism underlying resistance and identifying alternative insecticides efficacious against *P. chrysocephala* are all important aspects in implementing a sustainable and effective resistance management strategy for *P. chrysocephala* control.

The specific objectives for this thesis were:

1. To determine the extent and geographical spread of pyrethroid resistance in UK *P. chrysocephala* populations (Chapter 3)
2. To identify the enzyme(s) involved in metabolic resistance towards pyrethroids in UK *P. chrysocephala* (Chapter 4 and 5)

3. To evaluate potential alternative insecticides for controlling *P. chrysocephala* and determine their efficacy against pyrethroid resistant *P. chrysocephala* (Chapter 6)

Chapter 2. Materials and Methods

2.1 Insect material

2.1.1 Cabbage stem flea beetle (*Psylliodes chrysocephala*) adult collection

Live *P. chrysocephala* adults were collected from freshly harvested oilseed rape fields at Rothamsted Research, Hertfordshire, UK in July 2018, 2019, and 2020. Beetles were collected from trailers containing harvested rape pods using a hand-held battery-powered pooter (produced by the Rothamsted Research facilities team). Insects were maintained in a controlled environment (CE) cabinet at 15±1°C, with 65% relative humidity and a 12:12h light: dark photoperiod. The samples were kept in a mesh cage (housed within the CE cabinet) and fed on a diet of Chinese cabbage (*Brassica napus Wong Bok*) plants. *P. chrysocephala* samples received by post from oilseed rape fields elsewhere across the UK were kept in sealed plastic bags or plastic containers containing Chinese cabbage or oilseed rape leaves and moist tissue paper and maintained under the same environmental conditions as the Rothamsted samples until testing.

P. chrysocephala adult samples from Switzerland were provided by Syngenta Crop Protection, Stein. Samples from Germany were provided by Bayer CropScience AG, Monheim. The samples were snap-frozen in liquid nitrogen and shipped on dry ice to Rothamsted Research.

2.1.2 Cabbage stem flea beetle larvae collection

Live *P. chrysocephala* larvae were collected from oilseed rape plants grown on the farm at Rothamsted Research from December to March in 2018, 2019 and 2020. Larvae were extracted from the oilseed rape plants by dissection of the petioles and main stems using a scalpel (ThermoFisher Scientific, Hemel Hempstead, UK). *P. chrysocephala* were categorised into their larval stages (1st, 2nd, and 3rd instar) with the aid of a SZ40 Olympus microscope (Olympus Life Science, Southend-on-Sea, UK). Larvae were kept in 50ml Falcon tubes (ThermoFisher Scientific) containing moist tissue paper and maintained at 4°C until testing the following day.

2.2 Insect bioassays

2.2.1 Chemical reagents

Unless otherwise stated, all technical insecticides were supplied by either Sigma-Aldrich (St. Louis, MO, USA) or Greyhound Chromatography (Birkenhead, UK); all formulated insecticides were supplied by Syngenta Crop Protection (Stein, Switzerland) or BASF (Ludwigshafen, Germany). Acetone (technical grade) was supplied by Fisher Scientific (Waltham, MA, USA) and Agral supplied by Syngenta Crop Protection.

2.2.2 Toxicity of insecticide against Cabbage Stem Flea Beetle via glass vial assay

The test methodology used is based on IRAC (Insecticide Resistance Action Committee) Method 031 (www.irac-online.org/methods/weevils-and-flee-beetles/2014). Glass vials (14ml: 7cm tall/ 2cm diameter) (S Murray and Co, Surrey, UK) were prepared by coating the inner surface with different concentrations of insecticides. Initial stock solutions were prepared by diluting technical grade insecticide in technical-grade acetone. Glass vials treated with acetone only were used as controls. To coat, 500µl of prepared solution at the required concentration was pipetted into vials which were then placed horizontally without lids on a Luckham MultiMix Major roller (Denley Instruments Ltd, Colchester, UK) set at rolling speed setting 5. Vials were rotated at room temperature for at least 2 hours until all the acetone had completely evaporated. Vials were then left vertically at 4°C overnight in a fridge before attaching the screw cap tops the following day.

Adult beetles, collected from the oilseed rape harvest up to a few days before, were used in the bioassays. Only beetles capable of walking or jumping when released onto a tray inside a custom-made three-sided Perspex cage were selected for assay and these were collected using a hand-held battery-powered pooter. Ten beetles were transferred from the inverted pooter through a small funnel into each glass vial. The vials were then resealed with their screw cap lids and left at 18±1°C under a 16:8h light:dark photoperiod. After 24 hours, the beetles were transferred to untreated glass vials (7ml: 3.5cm tall/ 2cm diameter) (S Murray and Co) without lids, and each vial was placed under an inverted 200ml, transparent plastic disposable cup (VWR International Ltd,

Dublin, Ireland) to allow the beetles the opportunity to recover. Following a further 24 hours, the beetles in each vial were scored for mobility/mortality. This involved releasing the adult beetles onto a tray and individuals being categorised as 'mobile' (capable of jumping or walking in a coordinated way), 'affected' (incapable of jumping or coordinated movement) or 'dead' (no movement). After release, assessment of fitness for each replicate was monitored over 10 minutes to ensure that beetles potentially simulating death (a characteristic of this species) were correctly categorised. Following scoring, beetles in each category where transferred to correspondingly labelled Eppendorf tubes and snap-frozen using liquid nitrogen, before storing in a freezer at -80°C. Results were expressed as percentage mortalities.

2.2.3 Toxicity of insecticide against Cabbage Stem Flea Beetle via leaf dip assay

The test methodology used is based on IRAC Method 018 (www.irac-online.org/methods/plutella-xylostella-larvae/). Initial stock solutions of insecticides were prepared by diluting formulated insecticide in 0.01% (v/v) Agral solution. Agral was used as it is a spray surfactant that helps increase the surface area covered by the insecticide. Chinese cabbage leaf discs (7cm diameter) were made from 6-week-old leaves using a circular cutter. Discs were then individually dipped, using tweezers, into the appropriate concentration of insecticide solution for 20 seconds, with gentle agitation, before being left to surface-dry on paper towelling (abaxial surface facing upwards) for 30 minutes in a Fume Hood. Leaf discs dipped in 0.01% (v/v) Agral solution only were used as controls. Treated surface-dry discs were then transferred to the lids of 90mm Sterilin Petri dishes (ThermoFisher Scientific) each containing two discs of Whatman No1 (90mm diameter) filter paper (Sigma-Aldrich) moistened with approximately 2 ml of deionised water.

Both larvae and adult *P. chrysocephala*, extracted or collected from the oilseed rape crops up to a few days before, were used in the bioassays. For the adults, only beetles capable of walking or jumping when released onto a tray inside a custom-built three-sided Perspex cage were selected for the assay and these were collected using a hand-held battery-powered pooter. Ten adults were transferred from the inverter pooter through a small funnel into each Petri dish. For the larvae, ten larvae from the same developmental instar were transferred into a Petri dish using a fine damp paintbrush.

The Petri dishes were then sealed with their lids and maintained at 18±1°C under a 16:8h light: dark photoperiod for 24 – 72 hours, depending on the insecticide used. After this time, the adult *P. chrysocephala* were scored as described in section 2.2.2. The larvae were scored according to the three categories; 'mobile' (capable of moving in a coordinated way), 'affected' (reduced movement compared to the 'mobile' individuals) or 'dead' (no movement). Following scoring, the beetles were snap-frozen using liquid nitrogen, and stored in a freezer at -80°C. Results were expressed as percentage mortalities.

2.3 Standard molecular biological techniques

2.3.1 Biological and chemical reagents

Unless otherwise stated, all chemicals and reagents used were analytical grade and were supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.3.2 Media

Lysogeny Broth (LB) was prepared by adding 2.5g yeast extract, 5g tryptone, 2.5g NaCl and 400ml sterile distilled water to a 1l Duran™ bottle. The Duran bottle was shaken to dissolve the reagents before adding 1N NaOH to adjust the pH to 7.0. The solution was made up to 500ml with sterile distilled water and the Duran bottle autoclaved. To make LB agar, 7.5g of Bacto-agar was added per 500ml LB and the Duran bottle re-autoclaved. Once cooled to approximately 55°C the appropriate antibiotic was added to the LB agar solution, and the solution poured into standard 90mm Petri dishes. The LB solution and LB agar plates were stored at room temperature.

Super Optimal Broth with Catabolite Repression (S.O.C) media was prepared by adding 5g yeast extract, 20g tryptone, 0.6g 10mM NaCl and 0.2g 2.5mM KCl to 950ml sterile distilled water. The pH was adjusted to pH7 with 5M NaOH and the volume made up to 1l in a 1l Duran™ bottle. The Duran bottle was autoclaved and once cooled 3.6g 20mM glucose solution and 5ml 2M $MgCl_2$ were then added. The S.O.C medium was aliquoted into falcon tubes and stored at room temperature.

2.3.3 Total RNA extraction

Live *P. chrysocephala* adults were snap frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. Total RNA was extracted using the Isolate II RNA mini kit (Bioline Reagents Ltd, London, UK) according to the protocol supplied by the manufacturer. Using sterile forceps, three insects per sample were transferred to a sterile 1.5ml Eppendorf tube that had been chilled on dry ice. The insects were then ground to a powder using a sterile chilled pellet pestle (Sigma-Aldrich) before adding 350µl of Lysis buffer RLY and 3.5µl β-ME to ensure cell lysis. The contents of the tubes were mixed thoroughly by vortexing and the lysate transferred to a filter column within a 2ml collection tube. The tube was then centrifuged at 11,000 x g to filter out any insect debris. The filter unit was discarded and 350µl of 70% ethanol added to the homogenised lysate which was then mixed by pipetting. The lysate/ethanol mix was transferred to a new purification column and centrifuged at 11,000 x g for 30 seconds to bind the RNA to the column membrane. 350µl of membrane desalting buffer was then added to the column and centrifuged for 1 minute at 11,000 x g to dry the membrane. 95µl of a 1:10 solution of DNase I and reaction buffer for DNase I was applied to the silica membrane and incubated for 15 minutes at room temperature to remove any genomic DNA from the sample. The spin column membrane was subsequently washed three times, once with Wash buffer 1 and twice with Wash buffer 2, and RNA eluted in 30µl of RNase-free water.

2.3.4 Quantification of RNA

RNA was quantified using a NanoDrop™ 1000 Spectrophotometer (ThermoFisher Scientific). 1µl samples were loaded onto the Spectrophotometer and sterile distilled water used as a blank measurement. A Qubit Fluorometer (ThermoFisher Scientific) was used to substantiate Nanodrop readings in accordance with the Qubit assay kit manufacturers protocol. RNA quantity and quality were also determined by running on a 1% agarose gel.

2.3.5 Agarose gel electrophoresis

Gel electrophoresis was used for the separation of DNA/RNA fragments and PCR products. Gels comprised 1-1.5% (w/v) agarose with 1X TAE buffer (made by diluting 50X stock buffer (121g Tris base, 28.5ml Glacial Acetic acid, 50ml 0.5M EDTA pH 8.0 made up to 500ml with sterile distilled water)). Molecular biology grade agarose

was dissolved in 1X TAE buffer by heating in a microwave. Ethidium bromide was added to the agarose solution once cooled to approximately 50°C, to a final concentration of 0.5µg/ml. The agarose solution was then poured into a gel tray containing a comb and allowed to set; the comb introduced wells for the samples to be loaded into. Gel loading buffer II (0.05% (w/v) bromophenol blue, 40% (w/v) sucrose, 0.1M EDTA (pH 8.0), 0.5% (w/v) SDS) was added to each sample prior to loading onto the gel. 1µl of GeneRuler 1kb hyperladder (ThermoFisher Scientific) was run alongside samples to give an indication of sample fragment size. Gels were typically run at 80V and 200mA for 1 hour in tanks containing 1X TAE buffer. RNA was visualised and photographed using a GeneGenius UV cabinet (Syngene, MD, USA). Images were printed using a digital graphic UP-D895MD printer (Syngene).

2.3.6 Extraction of DNA from agarose gel

DNA was extracted and purified using the Wizard SV Gel and PCR Clean-up System (Promega, WI, USA) according to the protocol supplied by the manufacturer. The required DNA fragment was excised from the agarose gel using a clean, sharp scalpel. The gel fragment was placed in a 1.5ml Eppendorf tube and 10µl of membrane binding solution added per 10mg of gel slice. The tube was then incubated at 55ºC for 10 minutes with vortexing every 2 minutes to help the gel slice dissolve. The dissolved gel mixture was transferred to an SV Minicolumn in a collection tube and incubated at room temperature for 1 minute. The tube was then centrifuged at 16,000 x g for 1 minute, the flowthrough discarded and the Minicolumn reinserted into the collection tube. 700µl of Membrane Wash Solution (with ethanol added) was added and the tube centrifuged at 16,000 x g for 1 minute. The flowthrough was discarded, and the column washed again with 500µl of Membrane Wash solution. The flowthrough was once again discarded, and the tube centrifuged again for 1 minute to allow the evaporation of any residual ethanol. The Minicolumn with DNA bound to the matrix was transferred to a 1.5ml Eppendorf tube and 30µl of nuclease free water added. The Minicolumn was then incubated for 1 minute at room temperature before being centrifuged at 16,000 x g for 1 minute to elute the bound DNA. The concentration of the DNA was measured using a spectrophotometer as described in section 2.3.4.

2.3.7 Restriction endonuclease digestions

Plasmid vectors were linearised by digestion with the appropriate restriction enzyme using the ThermoFisher Scientific FastDigest system. Restriction digestion reactions were typically 20µl in volume. These reactions comprised 2 – 3µl of isolated DNA plasmid (1µg), 1µl of each FastDigest restriction endonuclease enzyme, 2X FastDigest buffer and sterile distilled water up to the final required volume. Reactions were incubated at 37ºC for 1 hour before heating to 65ºC for 5 minutes to stop the digestion reaction. Samples were run on an agarose gel alongside control samples to confirm linearization.

2.3.8 Ligation of DNA

Ligation reactions were typically 20µl in volume. These reactions comprised insert DNA and plasmid DNA in a 3:1 ratio, where amounts were based on DNA size and concentration, 2µl 10X ligase buffer, 2µl 10mM DTT, 2µl 10mM ATP, 1µl T4 ligase (ThermoFisher Scientific) and sterile distilled water up to the required 20µl volume. Reactions were left at 16ºC overnight to ensure complete ligation.

2.3.9 Bacterial transformation

DH5α (ThermoFisher Scientific) or HT115(DE3) (Sigma-Aldrich) Chemically Competent *E. coli* cells were removed from storage in the -80ºC freezer and thawed on ice. 1 – 2µl of isolated plasmid DNA (100ng) was added to the competent cells and mixed gently. The competent cell/DNA mixture was incubated on ice for 30 minutes before placing the bottom half of the Eppendorf tube into a 42ºC water bath for 40 seconds. The tube was then put on ice again for 2 minutes. 900µl of S.O.C media was added to the reaction and the tube was then placed in a 37ºC shaking incubator for 1 hour. The tube was then centrifuged for 1 minute at 8,000 x g to pellet the *E. coli* and 800µl of the reaction medium discarded. The remaining transformation mix was resuspended and 80µl plated onto a 10cm LB agar plate (1% (w/v) Trypticase Peptone, 0.5% (w/v) Yeast Extract, 1% (w/v) Sodium Chloride and 1.5% (w/v) agar)), under sterile conditions in a flow hood. Transformed DH5α cells were plated onto an agar plate containing 50µg/ml ampicillin and transformed HT115(DE3) cells onto an agar plate containing 50µg/ml ampicillin and 12.5µg/ml tetracycline. The plates were incubated at 37ºC overnight.

2.3.10 Colony PCR

Colony PCR was performed on *E. coli* colonies that showed sufficient growth after overnight incubation at 37ºC. Briefly, using 2µl pipette tips, cells from single colonies were picked from the transformation plate and streaked onto a new reference plate which was then incubated at 37ºC overnight. The pipette tips were subsequently placed into PCR tubes containing 10µl of sterile distilled water and the contents of the tubes mixed thoroughly by vortexing. The PCR tubes were then incubated at 95ºC for 10 minutes and 1µl of the reaction added to new PCR tubes containing 2X DreamTaq with *Taq* polymerase PCR mix (ThermoFisher Scientific) with 1µl of forward primer (10µM) and 1µl of reverse primer (10µM). 8 – 10 colonies were typically selected for colony screen PCR. The PCR cycle conditions consisted of an initial denaturation step at 95ºC for 2 minutes, then 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 50 – 65ºC for 30 seconds and extension at 72ºC for 30 – 60 seconds, and then a final extension step at 72ºC for 10 minutes. The PCR products were run on an agarose gel alongside untransformed plasmid DNA.

2.3.11 Inoculation of bacterial culture

For small-scale inoculation, bacterial clones from a previously streaked LB agar reference plate were transferred using a 2µl pipette tip to sterile 50ml falcon tubes containing 5ml LB broth. The broth was supplemented with 50µg/ml ampicillin alone or 50µg/ml ampicillin and 12.5µg/ml tetracycline depending on the bacterial clone, and the culture grown overnight at 37ºC on an orbital shaker at 200rpm. To make the LB agar selection plate, autoclaved LB agar was liquified by heating in a microwave and allowed to cool to approximately 55ºC before adding the appropriate antibiotic.

2.3.12 Plasmid DNA mini preparations

Plasmid DNA was isolated using the GeneJET Plasmid Miniprep kit (ThermoFisher Scientific), according to the manufacturers protocol. After incubation, 1.5ml of the bacterial culture was transferred to an Eppendorf tube and harvested by centrifugation at 6,800 x g for 2 minutes at room temperature. The supernatant was discarded, the pelleted cells resuspended in 250µl of resuspension solution and the mixture vortexed thoroughly until the pellet was dissolved. 250µl of lysis solution was then added and the Eppendorf tube inverted 4 – 6 times until the solution became homogenous. The lysis reaction was then terminated by the addition of 350µl of neutralisation solution

to the tube which was inverted 4 – 6 times. The tube was centrifuged for 12,000 x g for 5 minutes at room temperature and the supernatant transferred to the ThermoScientific GeneJET Spin Column and centrifuged for 1 minute to bind the DNA. The column was then washed twice by adding 500µl of wash solution and centrifuging for 1 minute. The empty column was centrifuged for a further 1 minute to remove any remaining wash solution and dry the membrane. To elute the purified DNA, the column was transferred to a new tube and 50µl of sterile distilled water added to the membrane. The column was left to incubate for 2 minutes before centrifuging for 2 minutes. The quantity of the DNA was determined using a spectrophotometer (see 2.3.4) and stored at -20ºC.

2.3.13 DNA sequencing and analysis

All DNA sequencing was carried out by Eurofins Genomics TubeSeq sequencing service (Eurofins Genomics, Ebersberg, Germany). Samples were sent premixed and consisted of 15µl of 100ng/µl PCR product and 2µl of 10µM primer specific to either the expression construct or vector. Geneious version 10.2.3 (Geneious Prime, Auckland, New Zealand) was used to view and check the sequence data obtained from Eurofins.

2.3.14 Glycerol stocks

Bacterial clones from a previously streaked LB agar reference plate were added to falcon tubes containing 5ml LB supplemented with appropriate antibiotic and grown overnight at 37ºC on an orbital shaker at 200rpm. 500µl of the overnight culture was added to 500µl of 50% (v/v) glycerol in sterile 1.5ml Eppendorf tubes. The tubes were vortexed and stored at -80ºC.

2.3.15 cDNA synthesis

Total RNA was used for cDNA synthesis using the ThermoScientific SuperScript III First-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). The kit was used according to the protocol supplied by the manufacturer. Reactions comprised; 400ng of total RNA, 1µl 10mM random hexamer primers, 1µl 10mM dNTP mix (10 mM each of dATP, dGTP, dCTP, dTTP at neutral pH) and sterile distilled water up to 13µl. The mixture was incubated in 0.2ml PCR tubes at 65ºC for 5 minutes followed by placing on ice for 1 minute. 4µl 5X First-Strand buffer, 1µl 0.1M DTT, 1µl

RNaseOUT™ Recombinant RNase Inhibitor (ThermoFisher Scientific) and 1µl of SuperScript III Reverse Transcriptase were then added and mixed by gently pipetting up and down. RNaseOUT was added to prevent RNA degradation and therefore improve cDNA yield. The mixture was incubated at 25ºC for 5 minutes, then 50ºC for 45 minutes and finally 70ºC for 15 minutes. The cDNA was stored at -20ºC.

2.4 Polymerase chain reaction (PCR)

2.4.1 Primer design

All oligonucleotide primers used in the synthesis of DNA constructs were designed using Geneious version 10.2.3 (Kearse et al., 2012) and synthesised by Sigma-Aldrich. Sterile distilled water was used to resuspend all primers to a concentration of 100µM in accordance with the specification sheet. Primers were subsequently diluted with sterile distilled water to a final concentration of 10µM.

2.4.2 Standard PCR

PCR was carried out using the C1000 Touch Thermal Cycler (Bio-Rad, Watford, UK). Reaction volumes were typically 25µl. These reactions comprised; 1µl of 10µM forward primer, 1µl of 10µM reverse primer, 1µl of DNA, 12.5µl of ThermoScientific 2X DreamTaq with *Taq* polymerase and sterile distilled water up to the required 25µl volume in 0.2ml PCR tubes. The cycling conditions varied according to the length of the amplicon and the Tm of the primers. Typically, reactions consisted of an initial denaturation step at 95ºC for 1 minute, 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 55-65ºC for 30 seconds, extension at 72ºC for 30-60 seconds and a final extension step at 72ºC for 5 minutes.

2.4.3 Quantitative PCR (qPCR)

Primers were designed to amplify regions of the candidate and housekeeping gene sequences with the parameters: primer size 18-23bp, primer Tm 57-62, primer GC% 40-60 and product size range 100-150bp. To increase the chance of finding a primer pair with adequate efficiency and specificity to the gene sequence, two pairs of primers were designed where possible. Primers were designed using transcripts from the *P. chrysocephala* genome (in house sequencing, not publicly available), the sequences are shown in Table 2.1.

Table 2.1. *Sequence of forward (F) and reverse (R) primers used for qPCR analysis.*

Primer	Sequence
20996 F1	AGTACGACGCGCAAGAATGA
20996 R1	CACGAAAAACTGCAGACCGG
b-actin F2	ACGAACTTCCCGATGGACAG
b-actin R2	CCAGCAGCTTCCATACCCAA
GAPDH F1	GCTTCCTGCACCACCAATTG
GAPDH R1	GTTTTGTCCAGCACCACGAC

Reactions were carried out in 15µl volume and comprised; 5µl of 4µg/µl cDNA, 7.5µl SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, UK), 0.5µl of 10µM forward primer, 0.5µl of 10µM reverse primer, and 1.5µl of sterile distilled water. qPCR was carried out using the Rotor-Gene Q (Qiagen, Venlo, Limburg, Netherlands). The cycling conditions varied according to the Tm of the primers. Typically, reactions consisted of an initial denaturation step at 95ºC for 2 minutes, followed by 45 cycles of denaturation at 95ºC for 10 seconds and annealing at 55-60ºC for 45 seconds. To check the specificity of the primers, a final melt-curve step at 95ºC was included. By melt-curve analysis, primers which displayed a single smooth peak were selected.

To validate the primers, standard curves for each primer pair were created. Reactions were as above but also included a series dilution of cDNA at four concentrations ranging from 10ng/µl to 0.01ng/µl. There were three technical replicates per concentration. The PCR program was the same as that used for the primer testing but without the final melt-curve step. Primers were chosen for gene expression analysis if their efficiency fell within the range of 0.9 to 1.1, with 1.0 representing optimal efficiency. Validation testing was also used to show that the efficiency of the candidate and housekeeping amplifications were approximately equal.

Finally, qPCR was used to compare candidate gene expression between insect populations. Control reactions used the housekeeping genes β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). These housekeeping genes were selected as they were stably expressed in both the susceptible and resistant populations. Three biological replicates and two technical replicates were used for each population.

The relative expression level of the candidate gene in the reference susceptible population and the resistant populations was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak

and Schmittgen, 2001). This method uses the threshold cycle (C_t) produced by the qPCR reaction to calculate relative changes in gene expression level using the formula below. Stably expressed housekeeping genes were used to normalise the qPCR reaction by correcting for differences in the amount of cDNA added to each sample and reducing variation resulting from the experimental set-up.

$\Delta\Delta C_t = \Delta C_t$ (resistant sample) – ΔC_t (susceptible sample)

And $\Delta C_t = C_t$ (gene of interest) – C_t (housekeeping gene).

Fold change in expression level between the resistant and susceptible populations was calculated using the formula: fold change=$2^{-\Delta Ct}$Resistant/$2^{-\Delta Ct}$Susceptible

Chapter 3. Resistance monitoring in *Psylliodes chrysocephala*

A substantial portion of the content of this chapter is based on the paper cited below, published in *Crop Protection* (see Appendix 1), but the data presented has been updated to include methodology and results relating to the 2020 resistance monitoring, *kdr/s-kdr* status, and the effect of lambda-cyhalothrin on *P. chrysocephala* larvae. The cited paper is chiefly the result of my own work (lead author), with co-authors contributing to the design and conceptualisation of the monitoring programme, the methodology used, and editing of the original draft (as per the CRediT authorship contribution statement). All the co-authors on the paper were actively involved in supervision and funding of this PhD.

Willis, C. E., Foster, S. P., Zimmer, C. T., Elias, J., Chang, X., Field, L. M., Williamson, M. S and Davies, T.G.E. (2020). 'Investigating the status of pyrethroid resistance in UK populations of the cabbage stem flea beetle (*Psylliodes chrysocephala*)', *Crop Protection, 138,* p. 105316

3.1 Aims

This chapter aims to examine how the extent and geographical spread of pyrethroid resistance in UK populations of *P. chrysocephala* has changed between 2018 and 2020. Bioassays, based on glass vial exposure of adult beetles to lambda-cyhalothrin, were carried out on samples collected in 2018, 2019 and 2020 to examine how the resistance profile has changed over this time across the UK. The presence of the *kdr* and super-*kdr* target-site mutations (to pyrethroids) in UK populations was also monitored, and the potential contribution of a metabolic resistance component in the beetles assessed using a pre-treatment method with the synergist Piperonyl butoxide (PBO), which is a cytochrome P450 inhibitor. The effect of lambda-cyhalothrin on *P. chrysocephala* larvae was also examined using leaf dip bioassays and the potential involvement of a metabolic resistance mechanism in this developmental stage assessed.

3.2 Introduction

For many years, control of the cabbage stem flea beetle, *Psylliodes chrysocephala* relied primarily on the use of systemic neonicotinoid, insecticide-based seed treatments containing either imidacloprid, thiamethoxam or clothianidin, followed by

the later application of foliar pyrethroid insecticide sprays (Højland *et al.*, 2015; Højland and Kristensen, 2018). These insecticide classes provide chemical control by acting on the insect nervous system causing paralysis (Davies et al., 2007), with the neonicotinoids targeting the nicotinic acetylcholine receptors (nAChRs) (Nauen and Denholm, 2005) and pyrethroids targeting the voltage-gated sodium channel (VGSC) (Narahashi, 1996, 2002). However, in December 2013, the European regulatory authorities (EU commission, 2013) restricted the use of neonicotinoid seed treatments on all outdoor flowering crops, preventing their use in oilseed rape, leading to an increase in *P. chrysocephala* and the increased use of pyrethroid sprays. As a result, at present, pyrethroids (e.g. lambda-cyhalothrin) are the only class of insecticide that remain available for chemical control of *P. chrysocephala* in the UK and other parts of mainland Europe.

The continuous use of pyrethroids to control *P. chrysocephala*, coupled with the lack of alternative insecticides with different modes of action, has led to a high selection pressure, driving the development and spread of resistance. Resistance to pyrethroids was first reported in 2008 in north-western Mecklenburg, Western Pomerania, a major oilseed rape growing area in Northern Germany (Heimbach and Müller, 2013). Zimmer *et al*. (2014) first reported the presence of the target-site L1014F *kdr* mutation (confering pyrethroid resistance) in the VGSC, with high frequencies of the allele (90-100%) being found in populations collected from across Northern Germany, with the beetles exhibiting low level resistance against a range of pyrethroids including lambda-cyhalothrin. More recently, studies by Højland *et al*. (2015) and Højland and Kristensen (2018) have shown that pyrethroid resistance resulting from the *kdr* mutation is also present in populations from both Denmark and the UK, whilst in Germany it has spread further south. Despite the presence of *kdr* in UK populations, Højland *et al*. (2015) found that the high pyrethroid resistance levels, with control failures being observed at the full field rate, did not completely correlate with the *kdr* genotype suggesting that another mechanism of resistance, such as metabolic resistance, is also present. Given the lack of alternative insecticides with different modes of action, the presence and spread of pyrethroid resistance is therefore concerning for the chemical control of *P. chrysocephala*.

As the development of resistance in *P. chrysocephala* is a matter of growing concern, with the increasing spread of insecticide resistance threatening the production of

oilseed rape, both in the UK and within Europe (Heimbach and Müller, 2013; Højland et al., 2015; Højland and Kristensen, 2018), continued monitoring for the extent and geographical spread of pyrethroid resistance in *P. chrysocephala* is crucial if adequate control of this pest is to be maintained. This monitoring program is particularly important at a time where synthetic pesticides are becoming less effective (and less favoured) as a pest control strategy and will improve decision making on how to best deploy the remaining pesticides effectively, thereby maintaining their efficacy for agriculture.

3.3 Materials and Methods

3.3.1 Insects

P. chrysocephala adults and larvae were collected and maintained as described in Chapter 2, section 2.1.1 and section 2.1.2 respectively. In 2018 and 2019, *P. chrysocephala* adult samples received from across the UK were the result of an outreach program/social media campaign. The campaign asked for *P. chrysocephala* samples, collected at the time of the OSR harvest, to be sent in to Rothamsted Research for insecticide resistance monitoring. Each sample received came with an accompanying 'sampling form', detailing the sample location, date sampled, and insecticide usage. In 2020, a more targeted approach was taken, samples were requested from a smaller number of specific farms with the aim of achieving an even distribution of samples from across the UK.

The beetles were maintained in mesh cages or plastic containers in a controlled environment cabinet at 15±1°C, with 65% relative humidity under a light: dark photoperiod of 12:12h, on a diet of Chinese cabbage (*Brassica napus Wong Bok*) plants until testing the following day. *P. chrysocephala* larvae were collected between December 2019 and March 2020 from oilseed rape plants also grown on the Rothamsted Research farm. Larvae were extracted from the oilseed rape by dissection of the plant as described in Chapter 2, section 2.1.2. The larvae were kept in falcon tubes with moist tissue paper and maintained at 4°C until testing.

3.3.2 Glass vial bioassays

P. chrysocephala samples were tested for resistance to the pyrethroid lambda-cyhalothrin using a glass vial bioassay previously described (see Chapter 2, section

2.2.2). Briefly, glass vials were prepared by coating the inner surface with three doses of lambda-cyhalothrin, equivalent to 4%, 20% and 100% of the of the recommended field application rate (7.5g ai/ha) (one replicate per concentration) (Table 3.1). Glass vials treated with acetone only were used as controls. To coat, 500µl of solution was pipetted into vials which were then placed on a roller and left to rotate at room temperature for 2 hours. Vials were then left at 4°C overnight before attaching the screw tops the next day for future use in bioassays.

Table 3.1. *Concentrations of lambda-cyhalothrin tested on P. chrysocephala via glass vial bioassay. Lambda-cyhalothrin was dissolved in acetone.*

Concentration		**% Field rate**
(g/ha)	**ppm**	
0.3	0.204	4
1.5	1.02	20
7.5	5.1	100

Healthy adult beetles, collected from oilseed rape harvest up to a few days before (see Chapter 2, section 2.2.2) were used in the bioassay. Ten beetles were transferred into each vial (n=10) (Figure 3.1); the vials were then sealed and stored upright at 18±1°C under a 16:8h light:dark photoperiod. After 24 hours, the beetles were transferred to untreated glass vials without lids under upturned plastic cups (Figure 3.2) to allow potential recovery. After a further 24 hours, the beetles were scored according to three categories: 'mobile', 'affected' or 'dead'. Results were expressed as percentage mortalities. Following scoring, beetles in each category were transferred to Eppendorf tubes and snap frozen using liquid nitrogen before being stored at -80°C.

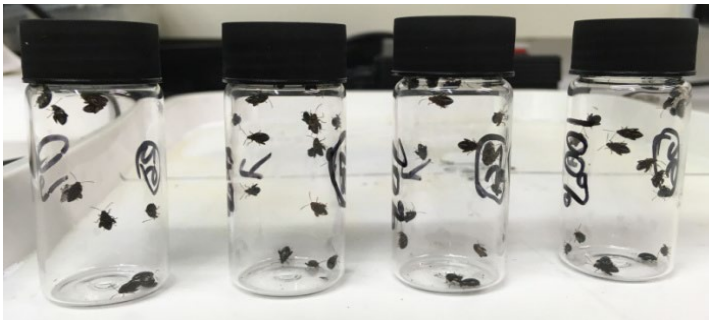


Figure 3.1. *Coated glass vials were used to assess* P. chrysocephala *resistance to lambda-cyhalothrin at 4%, 20% and 100% of the recommended field rate. 10 beetles were transferred to each vial.*

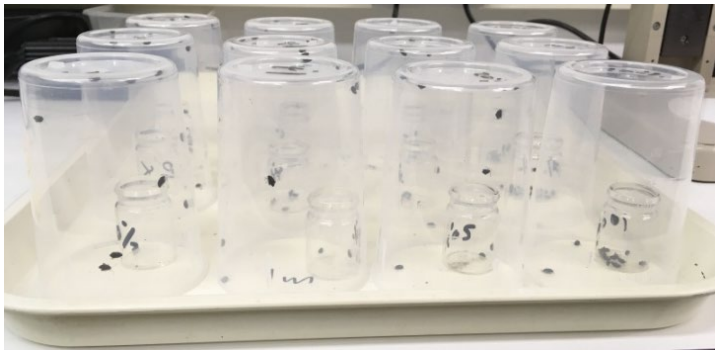


Figure 3.2. *P. chrysocephala were transferred into untreated glass vials without lids under upturned cups to allow potential recovery.*

3.3.3 TaqMan PCR assay

TaqMan genotyping assays were used to determine the presence of the L1014F *kdr* and L925I super-*kdr* mutation in the *P. chrysocephala* samples collected at sites across the UK (Livak, 1999). DNA was extracted from individual insects that had been stored at -80°C using sucrose buffer extraction. Using tweezers, single adults were transferred into each well of a Nunc MicroWell 96-Well Microplate (ThermoFisher, Scientific) before adding 50µl of 300mM sucrose solution to each well. The beetles were then ground with a Burkard 96-Well homogeniser (Burkard Scientific, UK) and an adhesive PCR plate seal (ThermoFisher Scientific) applied to the plate. The plate was subsequently placed in a shallow water bath at 99ºC for 10 minutes. The plate was then centrifuged at 3500rpm for 2 minutes before being placed onto ice for 5 minutes. 1.5µl aliquots were taken for *kdr* genotyping by TaqMan assay. Primer and probe sequences for the assay were previously designed by Martin Williamson (Rothamsted Research, UK) using Primer Express software v.2.0 (ThermoFisher Scientific) (Table 3.2). Primers were designed against sodium channel gene sequences neighbouring the *kdr* and *s-kdr* mutations. All primers are standard oligonucleotides with no modification. In both assays, VIC reporter dye-labelled probes were used to detect the wild-type susceptible allele and 6-FAM reporter dye-labelled probes to detect the resistant allele. Each probe contained a 3' non-fluorescent quencher dye.

Table 3.2. *Primers and probes used in the TaqMan assay for detection of the L1014F and L925I mutation in the P. chrysocephala sodium channel.*

Primer/Probe		Sequence
Primers	*kdr*-F	GGACTGTATGCTAGTCGGTGATGT
	kdr-R	GCAAAGCCAAGAAGAGATTCAGTA

	s-kdr-F	GCCAAGTCATGGCCAACTT
	s-kdr-R	TATAATGCACAGCACAAAGGTCA
Probes	*kdr*-VIC	TTACCACAAGATTACC
	kdr-FAM	TTACCACAAAATTACC
	s-kdr-VIC	TGGGTGCTTTAGGTAA
	s-kdr-FAM	TGGGTGCTATAGGTAA

PCR reaction volumes were typically 15µl and contained 1.5µl genomic DNA, 7.5µl SensiMix Probe mix (Bioline Reagents Ltd, London, UK), 800nM of each primer, 200nM of each probe and sterile distilled water up to 15µl. Reaction were run on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Cycling conditions were 50ºC for 2 minutes, 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 45 seconds. The increase in VIC and 6-FAM fluorescence was monitored in real time by acquiring each cycle on the yellow channel (530nm excitation and 555nm emission) and green channel (470nm excitation and 510 emission) of the 7900HT respectively.

3.3.4 Synergist bioassays (adults)

Pre-treatment with the insecticide synergist Piperonyl butoxide (PBO), obtained from Sigma-Aldrich (Missouri, USA), was used to detect potential metabolic resistance mechanisms *in vivo*. PBO was diluted in technical grade acetone to give an equivalent concentration of 4g ai/ha. Vials were coated with 500µl of PBO as described in section 2.2.2. Ten beetles per replicate were transferred to glass vials coated with PBO for 1 hour before being transferred to either untreated control vials or vials coated with lambda-cyhalothrin at 20% of the field rate (7.5g ai/ha). These beetles were bio-assayed in parallel to beetles of the same sample not pre-exposed to PBO following the method described in section 2.2.2.

3.3.5 Leaf dip assay (larvae)

P. chrysocephala larvae were tested for resistance to the pyrethroid lambda-cyhalothrin using a leaf dip bioassay previously described (see Chapter 2, section 2.2.3). Briefly, Chinese cabbage leaf discs were prepared by coating the surface with lambda-cyhalothrin; three doses, equivalent to 4%, 20% and 100% of the recommended field application rate were used (Table 3.1). Leaf discs dipped in 0.01% (v/v) Agral (Syngenta) solution only were used as controls. To prepare, Chinese

cabbage leaf discs (7cm diameter) were cut from 6-week-old leaves and dipped into insecticide solution for 20 seconds before being left to surface-dry for 30 minutes. The discs were then transferred to Petri dishes on top of filter paper moistened with deionised water.

P. chrysocephala larvae extracted from oilseed rape plants the day before (see Chapter 2, section 2.1.2) were used in the bioassay. Ten larvae from the same instar were transferred into a Petri dish (n=10) (Figure 3.3). The Petri dishes were then closed and maintained at 18±1°C under a 16:8h light: dark photoperiod for 24 hours. The larvae were scored as 'mobile', 'affected' or 'dead' at the end of the 24-hour period. Results were expressed as percentage mortalities. Following scoring, larvae in each category were transferred to Eppendorf tubes and snap frozen using liquid nitrogen before storing in a freezer at -80°C.

Figure 3.3. *Leaf dip assay were used to assess P. chrysocephala larvae resistance to lambda-cyhalothrin at 4%, 20% and 100% of the recommended field rate.*

3.3.6 Synergist studies (larvae)

PBO was dissolved in technical grade acetone to a concentration of 2g ai/ha. This concentration was chosen as it was the highest concentration which did not cause control mortality; a range of concentrations were tested (data not shown). Vials were coated with 500µl of PBO, as described in section 2.2.2. Ten larvae of the same instar were transferred to glass vials coated in PBO for 1 hour before being transferred to either untreated control leaves or leaves coated with lambda-cyhalothrin at the 100% field rate. These beetles were bio-assayed in parallel to beetles of the same sample that were not pre-exposed to PBO following the method described in section 2.2.2.

3.3.7 Statistical analysis

UK maps showing the extent and geographical spread of pyrethroid resistance in *P. chrysocephala* adults were produced using QGIS (version 3.0.3; QGIS Development Team, 2016; QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.osgeo.org). GenStat (20th edition, Payne, 2009) was used analyse the resistance monitoring data. GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com) was used to visualise the resistance data by the production of histograms.

Statistical analysis pertaining to changes in resistance level across the years/developmental stages, the impact of spatial variation, and the assessment of regional and county-level differences in 2019 were completed by Andrea Minter and Jess Evans (Statistics Department, Rothamsted Research). ANOVA and Chi-squared tests were performed to determine whether changes in resistance level across the three years of monitoring/developmental stages were significant. For the Chi-squared tests, resistance was categorised into 4 classes (0-25%, 25-50%, 50-75%, 75-100%) to test whether resistance was distributed between these classes differently in each year. ANOVA was performed on the original continuous resistance variable to test for differences between the average resistance level between years. This was repeated with Easting (x-coordinate) and Northing (y-coordinate) included as covariates (ANCOVA) to assess the impact of spatial variation on the resistance levels across the years. ANOVA was also performed on the 2019 data only incorporating a nested treatment structure (region/county) used to interpret regional and county-level differences in resistance level in 2019.

Larval developmental stage data was analysed using ANOVA with a factorial treatment structure, stage*dose, to test for differences in number of larvae affected due to different doses, different developmental stages and whether there is an interaction between dose and stage.

3.4 Results and discussion

3.4.1 Glass vial bioassays

Glass vial bioassays were used to monitor resistance levels in *P. chrysocephala.* Bioassays were used as they offer an approach for examining differences in the

phenotypic response following the application of the insecticide; this means resistant individuals can be identified without the need to understand the mechanism of resistance (Barres et al., 2016). By using this method, the application of lambda-cyhalothrin was standardized and biological characteristics of the pest such as developmental stage could be taken into consideration.

3.4.1.1 Resistance monitoring 2018

Resistance monitoring of *P. chrysocephala* adults towards the reference pyrethroid lambda-cyhalothrin began at Rothamsted Research in 2014. In 2018, a total of 41 *P. chrysocephala* samples were obtained from four different regions across England, primarily from the East of England (Figure 3.4). *P. chrysocephala* were tested to determine whether the extent and geographical spread of pyrethroid resistance in *P. chrysocephala* populations had changed with respect to resistance levels observed in previous years (2014-2017): studies done by Stephen Foster (2014 data published in Højland et al., 2015). *P. chrysocephala* were tested to assess resistance levels at 4%, 20% and 100% (7.5g ai/ha) of the recommended field rate of lambda-cyhalothrin, (one replicate per concentration). The samples were categorised as being either completely susceptible, or 0-25%, 25-50%, 50-75%, 75-99% or 100% resistant, depending on the percentage of beetles per sample surviving treatment with 7.5g ai/ha lambda-cyhalothrin. For example, in a sample of 10 beetles, if four beetles survived, this sample was categorised as 40% resistant. Although lambda-cyhalothrin was used as an exemplar in this study, other pyrethroids have also been found to contribute to the selection pressure in *P. chrysocephala* populations across Europe. The particular bioassay used was selected as it is an approved test method (Method 031) for determining resistance in *P. chrysocephala* (IRAC, 2014) and was used by Zimmer *et al*. (2014) to monitor the emergence and geographic spread of pyrethroid resistance in *P. chrysocephala* in Germany, by Højland *et al*. (2015) to determine the spread of pyrethroid resistance in Danish, British and German beetle samples and most recently by Højland and Kristensen (2018) when investigating lambda-cyhalothrin resistance in Danish populations. Similar bioassays have also been used to monitor the spread of pyrethroid resistance in European populations of pollen beetle (*Brassicogethes aeneus*), another major pest of oilseed rape (Zimmer and Nauen (2011), Slater *et al.,* (2011), Nauen *et al*., (2012)).

In 2018, of the 41 UK samples tested only five were found to contain no mobile beetles at 100% of the recommended field rate for lambda-cyhalothrin, which would be expected if the sample was fully susceptible. However, in these five samples, adult mortality was found to be <90% at 20% of the field rate, suggesting resistance is present as judged by the IRACs 'susceptibility rating scheme' (IRAC, 2014). The other 36 samples all showed some level of resistance with the highest resistance, at 89%, being a sample from Bishop Cannings (Wiltshire). Overall, variation in resistance level was relatively evenly spread with no resistance hotspots (Figure 3.4). However, as the number of beetles tested per sample at each concentration was small (n=10), any resistance trends may have been obscured by random effects.

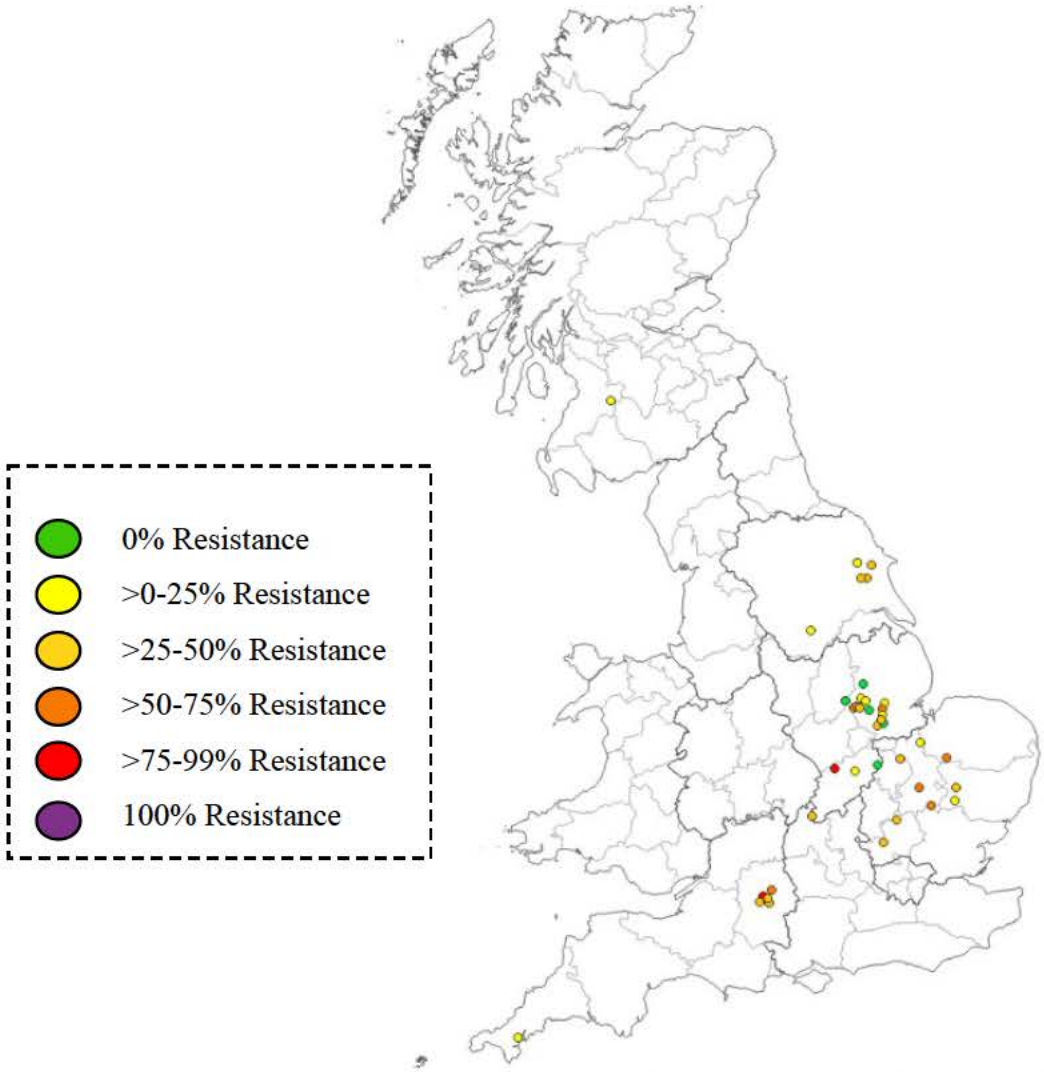


Figure 3.4. *Geographical spread of pyrethroid resistance in UK populations of P. chrysocephala adults in 2018. Assessment was by glass vial bioassays, control mortality was <20%. The map uses a 6-category colour scale to show the level of resistance. The map is divided into counties (light grey borders) and regions (dark grey borders).*

3.4.1.2 Resistance monitoring 2019

In 2019, a total of 146 UK *P. chrysocephala* samples were obtained from across England, representing a wider range of the country (Figure 3.5). In addition, two samples were received from Wales and one from Scotland. From each sample, 10 beetles (n=10) were tested to assess resistance levels predominantly at the recommended field rate of lambda-cyhalothrin. They were tested using the field rate because in 2018 most populations showed some degree of resistance to lambda-cyhalothrin at this dose and it was therefore decided that, in order to get a variation in resistance level, only the highest concentration needed to be tested. Of the 146 samples tested, only the Scottish sample was found to be fully susceptible to lambda-cyhalothrin, displaying 100% mortality at 20% of the recommended field rate. In contrast, several populations containing 100% resistant beetles were recorded for the first time in England. Overall, the distribution map for pyrethroid resistance in UK populations of *P. chrysocephala* (Figure 3.5) suggests that higher levels of resistance are starting to spread to the North and West of England and that resistance levels continue to remain high in the South-East.

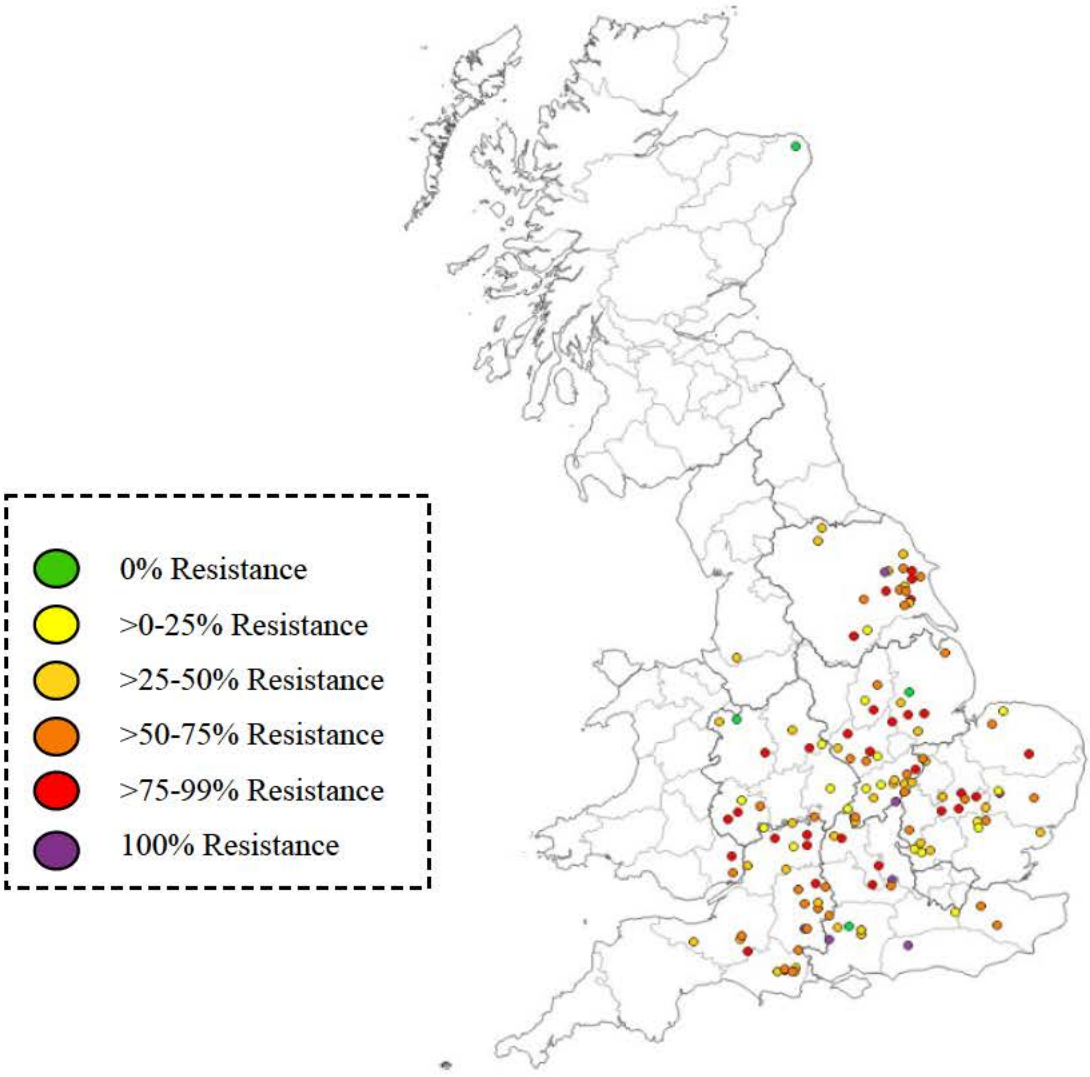


Figure 3.5. *Geographical spread of pyrethroid resistance in UK populations of P. chrysocephala adults in 2019. Assessment was by glass vial bioassays, control mortality was <20%. The map uses a 6-category colour scale to show the level of resistance. The map is divided into counties (light grey borders) and regions (dark grey borders).*

Further analysis was undertaken to assess regional and county-level differences. The mean pyrethroid resistance levels (Table 3.3A) were higher than the national average in the South East (60.39%), South West (57.83%), and Yorkshire and the Humber (63.32%). South-East Wales had the highest mean resistance (72.50%) but only two samples were tested. Analysis of variance (ANOVA), incorporating a nested treatment structure to reflect counties nested within regions, showed that mean resistance levels did not differ significantly between regions ($F_{8,113} = 1.28$, $p=0.262$). The standard errors of the differences between means (SEDs) at the regional level were also calculated (Table 3.3). There was also no significant difference in mean resistance levels between counties within the same region ($F_{24,113} = 1.06$, $p=0.395$). The absence

of statistically different mean resistance levels suggests there are no resistance 'hotspots' and that resistance is highly localised, almost on a farm-by-farm basis.

Despite the apparent lack of resistance 'hotspots', it is important to note that just 10 beetles were tested from each sample. Data on local resistance levels could therefore have been skewed and local trends missed. However, due to the large number of samples tested (n=147), it can be considered likely that the overall trend of higher levels of resistance spreading to the North and West of England and resistance levels remaining high in the South-East remains true.

Table 3.3. *A) Nested ANOVA showing the Region, County, number of samples and average resistance level and B) Standard error of differences.*

A

Region and County	Number of Samples	Average resistance level
East Midlands	**29**	**51.28**
Leicestershire	7	60.43
Northamptonshire	12	48.58
Nottinghamshire	3	49.67
Lincolnshire, Parts of Kesteven	6	45.33
Lincolnshire, Parts of Lindsey	1	60.00
East of England	**29**	**53.72**
Bedfordshire	3	57.33
Cambridgeshire	8	72.25
Essex	4	31.50
Hertfordshire	3	26.00
Huntingdonshire	2	57.00
Norfolk	3	53.33
Suffolk	6	55.00
North West	**1**	**40.00**
Lancashire	1	40.00
Scotland	**1**	**0.00**
Aberdeenshire	1	0.00
South East	**18**	**60.39**
Berkshire	2	80.00
Hampshire	6	50.67
Kent	3	61.67
Oxfordshire	5	64.00
Surrey	1	18.00
Sussex	1	100.00
South East Wales	**2**	**72.50**
Monmouthshire	2	72.50

South West	**35**	**57.83**
Dorset	7	53.57
Gloucestershire	8	57.88
Somerset	4	57.50
Wiltshire	16	59.75
West Midlands	**12**	**48.33**
Herefordshire	4	60.25
Shropshire	3	46.67
Staffordshire	3	46.33
Warwickshire	1	10.00
Worcestershire	1	50.00
Yorkshire and the Humber	**19**	**63.32**
East Riding of Yorkshire	14	68.93
North Riding of Yorkshire	3	43.33
West Riding of Yorkshire	2	54.00
Grand Total	**146**	**55.64**

B

Region		Standard Error of Differences								
East Midlands	1	*								
East of England	2	6.82	*							
North West	3	26.43	26.4	*						
Scotland	4	26.43	26.4	36.8	*					
South East	5	7.8	7.8	26.7	26.7	*				
South East Wales	6	19	19	31.8	31.83	19.4	*			
South West	7	6.53	6.53	26.4	26.36	7.54	18.89	*		
West Midlands	8	8.92	8.92	27.1	27.05	9.68	19.85	8.69	*	
Yorkshire and the Humber	9	7.67	7.67	26.7	26.66	8.55	19.32	7.41	9.58	* *
		1	2	3	4	5	6	7	8	9

3.4.1.3 Resistance monitoring 2020

In 2020, a total of 30 UK *P. chrysocephala* samples were obtained from across England, representing an even spread across the country (Figure 3.6). One sample was also received from Scotland. Ten beetles from each *P. chrysocephala* sample (n=10) were tested by glass vial bioassay to assess resistance levels at the recommended field

rate of lambda-cyhalothrin. Of the 30 samples tested, as in 2019, only the Scottish sample was found to be fully susceptible to lambda-cyhalothrin, displaying 100% mortality at 20% of the recommended field rate. Eight samples were found to be 100% resistant to lambda-cyhalothrin; only one sample was found to sit in the 0-25% resistance category. Overall, the 2020 distribution map for pyrethroid resistance in UK populations of *P. chrysocephala* (Figure 3.6) suggests that higher levels of resistance are continuing to spread to the North and West of England and that resistance levels continue to remain high in the South East of the country.

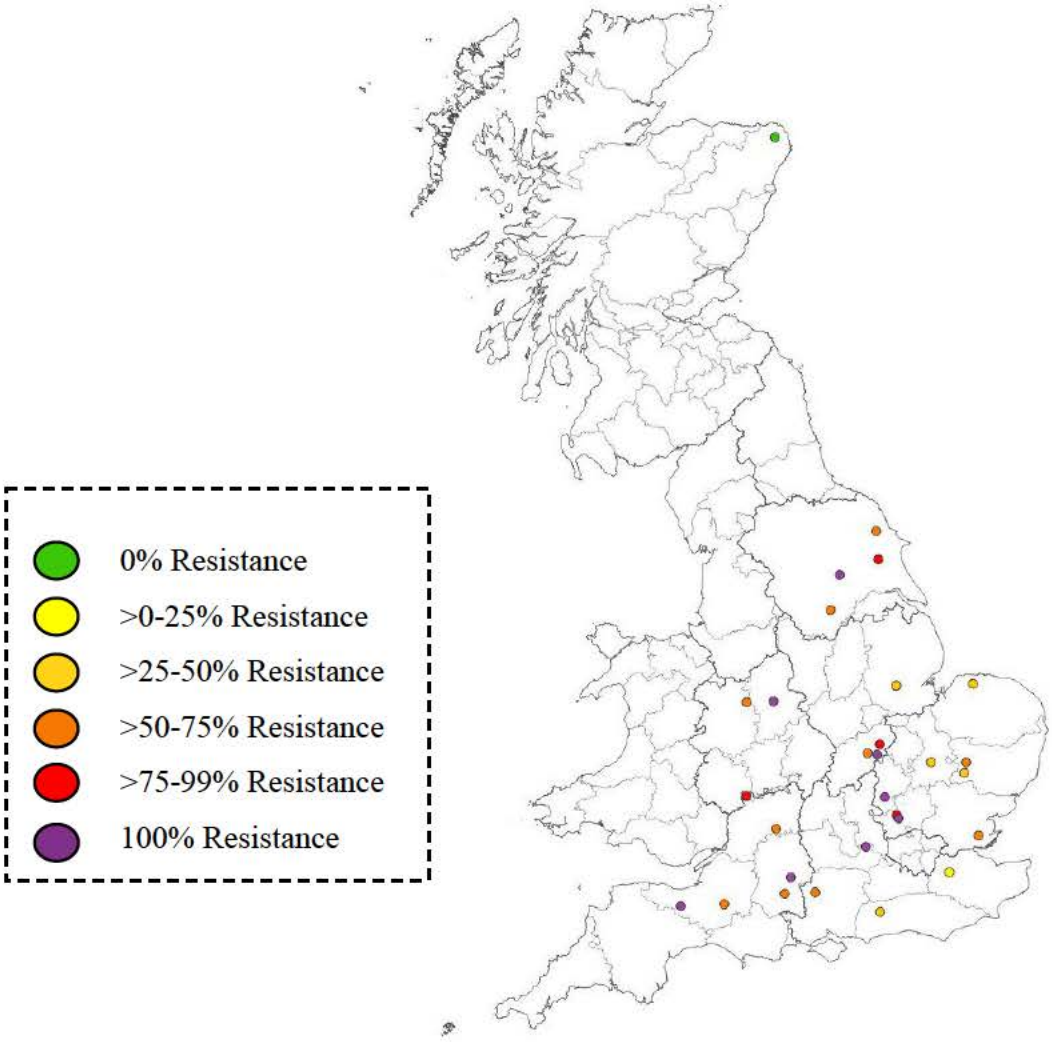


Figure 3.6. *Geographical spread of pyrethroid resistance in UK populations of* P. chrysocephala *adults in 2020. Assessment was by glass vial bioassays, control mortality was <20%. The map uses a 6-category colour scale to show the level of resistance. The map is divided into counties (light grey borders) and regions (dark grey borders).*

3.4.1.4 Comparison of resistance across years

There was a significant difference in mean resistance level across the three years of monitoring (two-way ANOVA, $F_{2,197} = 17.13$, $p < 0.001$). The standard errors of the differences between means (SEDs) were calculated (Table 3.4). The mean resistance level was significantly greater in 2020 (68.5%) compared to 2019 (55.6%) and significantly greater in 2019 compared to 2018 (32.9%). Over the three years, there was a significant difference in the distribution of measurements across the resistance categories, $X^2(6) = 28.36$, $p < 0.001$ (Figure 3.7). The residuals indicate that in 2018 the percentage of beetles in the low-level resistance categories was higher than expected, and lower than expected in the high-level resistance categories; in 2019 and 2020 there were more observations than expected in the higher resistance categories with this difference being larger in 2020. In 2018 the percentage of beetles in the 0-25% resistance category was 39%, this decreased to 16% in 2019 and 7% in 2020. In 2018 5% of samples were in the 75-100% resistance category, this increased to 28% in 2019 and 40% in 2020.

Table 3.4. *ANOVA showing the Standard error of differences.*

Year		Standard Error of Differences		
2018	1	*		
2019	2	4.65	*	
2020	3	6.24	5.26	*
		1	2	3

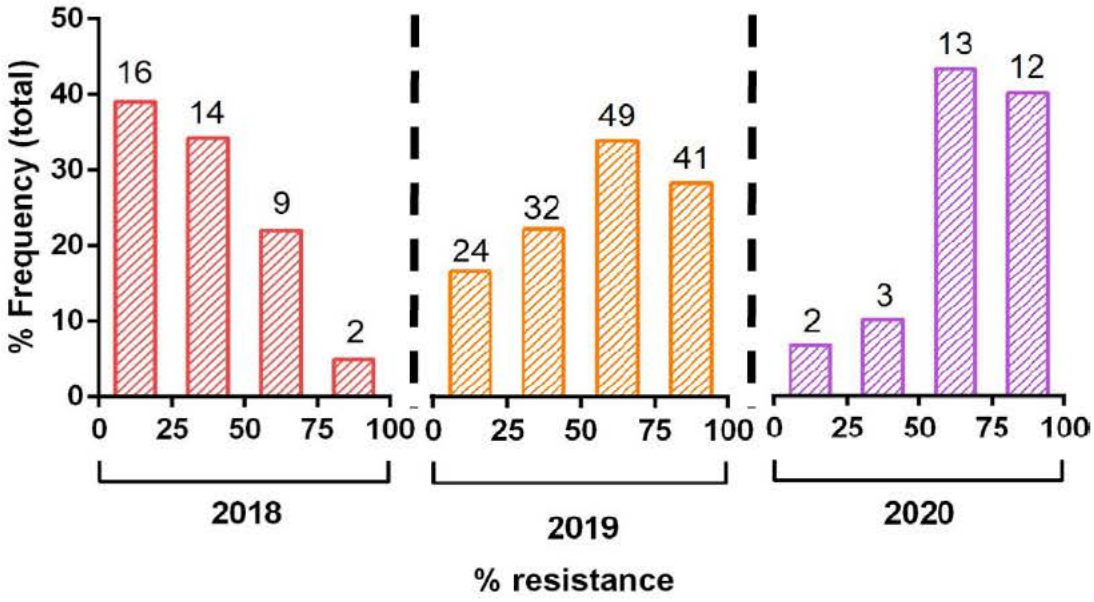


Figure 3.7. *Histograms showing the shift in the relative % frequency of pyrethroid resistant* P. chrysocephala *collected in 2018, 2019 and 2020. Numbers show the raw count data.*

To assess whether there was any impact of the spatial variation over which the samples were collected, analysis of covariance was undertaken based on year, adjusting for the easting and northing coordinates as covariates. There was no evidence of a linear association between the covariates and the outcome at the 5% significance level. The analysis was then repeated with 3 geographically extreme data values omitted (three observations in Scotland and one in Cornwall). Again, there was no evidence of a linear association between the covariates and the outcome. However, in all cases the analysis showed a significant difference in mean resistance across the three years ($p<0.001$). Scatter plots of the easting and northing coordinates, plotted against resistance, did not indicate any other non-linear association.

3.4.2 Detection of *kdr*/*s-kdr* alleles by TaqMan assay

TaqMan assays were used to detect the presence of the L1014F (*kdr*) and L925I (*s-kdr* -like) substitutions in the *para*-type sodium channel gene of *P. chrysocephala* samples. The L1014F mutation has been previously found in a range of pests including the peach-potato paid (*Myzus persicae*) (Martinez-Torres et al., 1999), diamondback moth (*Plutella xylostella*) (Schuler et al., 1998), pollen beetle (*Meligethes aeneus*) (Nauen et al., 2012), mosquito (*Culex pipiens*) (Tmimi et al., 2018) and *P. chrysocephala* (Højland et al., 2015) where it confers moderate resistance to pyrethroids (Davies et al., 2007). The L925I mutation confers higher levels of

resistance to pyrethroids and has been observed in pests such as the kissing bug (*Triatoma infestans*) (Capriotti et al., 2014), silverleaf whitefly (*Bemisia tabaci*) (Morin et al., 2002) and bed bug (*Cimex lectularius*) (Yoon et al., 2008).

3.4.2.1 TaqMan sequencing in 2018

In 2018, 40 beetles from seven UK samples of *P. chrysocephala* were tested using only individuals that had survived the 100% field rate of lambda-cyhalothrin, enabling the genotype associated with the resistant, mobile phenotype to be determined. These samples were from Great Saxham (Suffolk), Bishop Cannings (Wiltshire), Rothamsted Research (Hertfordshire), Linton (Cambridgeshire), Feltwell (Norfolk) and Horbling and Grantham (Lincolnshire). The L1014F target site mutation was present at all sites, with 47.5% of the beetles being homozygous for the resistant allele (RR), 37.5% heterozygous (SR) and the remaining 15% *kdr* SS, although this genotype was not present in the Suffolk or Wiltshire populations (Table 3.5). The detection of *kdr* SS genotypes in beetles that displayed the mobile phenotype after treatment with the label rate of lambda-cyhalothrin, suggests the presence of another resistance mechanism in *P. chrysocephala.* The *kdr* SS genotype was found in 20% of the mobile beetles from Lincolnshire, in 25% of the mobile beetles from Cambridgeshire, 16% of the mobile beetles from Norfolk and 25% of the mobile beetles from Hertfordshire. The *kdr* RR genotype was also identified in beetles that did not survive lambda-cyhalothrin treatment, confirming that this mutation on its own is not able to confer full protection to the field rate dose (results not shown).

In contrast to L1014F, the L925I mutation, which is predicted (based on studies in other insects) to be associated with higher resistance levels to pyrethroids than *kdr*, was much less common, with two samples (Norfolk and Hertfordshire) showing only the SS genotype and the overall percentage of beetles showing the homozygous L925I genotype (RR) being only 2.5%. Of the six beetles homozygous for the susceptible *kdr* allele (SS), one also displayed the homozygous resistant *s-kdr* allele (RR) and two displayed the heterozygous resistant *s-kdr* allele (SR) (data not shown). As three beetles were susceptible for both the *kdr* and *s-kdr* allele, this suggests the presence of another resistance mechanism. Direct sequencing of sodium channel fragments carrying the mutations showed that the L1014F (*kdr*) and L925I (*s-kdr*) mutations are

mutually exclusive and have arisen independently in different sodium channel alleles, thus limiting the number of genotypic combinations possible within individual beetles.

Table 3.5. *Detection of kdr/s-kdr alleles in 2018 samples of P. chrysocephala using TaqMan assay.*

Region	**County**	**Populations**	**No°**	***kdr status***			
				SS	**SR**	**RR**	**%RR**
East Midlands	Suffolk	1	4	0	3	1	25%
	Wiltshire	1	8	0	4	4	50%
	Lincolnshire	2	10	2	2	6	60%
East of England	Cambridgeshire	1	8	2	2	4	50%
	Norfolk	1	6	1	3	2	33%
	Hertfordshire	1	4	1	1	2	50%
	Total	7	40	6 (15%)	15 (37.5%)	19 (47.5%)	

Region	**County**	**Populations**	**No°**	***s-kdr status***			
				SS	**SR**	**RR**	**%RR**
East Midlands	Suffolk	1	4	3	1	0	0%
	Wiltshire	1	8	5	3	0	0%
	Lincolnshire	2	10	9	1	0	0%
East of England	Cambridgeshire	1	8	5	2	1	13%
	Norfolk	1	6	6	0	0	0%
	Hertfordshire	1	4	4	0	0	0%
	Total	7	40	32 (80%)	7 (17.5%)	1 (2.5%)	

3.4.2.2 TaqMan sequencing in 2019

In 2019, *P. chrysocephala* individuals were screened for *kdr* from sites close to those sampled in 2018 (a sample from Oxfordshire was also included) and again, only beetles that survived the 100% field rate of lambda-cyhalothrin were tested. The L1014F mutation was present at all sites. Compared to 2018, the percentage of beetles homozygous for the *kdr* resistance allele (RR) increased in three of the samples, Suffolk, Norfolk and Hertfordshire but decreased overall from 47.5% to 41.5% (Table 3.6). Given that the percentage of beetles resistant to lambda-cyhalothrin in each sample increased between 2018 and 2019, but there was an overall decrease in the homozygous and heterozygous L1014F mutation, this further suggests the presence of another, more potent, resistance mechanism in *P. chrysocephala*. Whilst the L925I (*s-kdr*) mutation was less common than the L1014F (*kdr*) mutation, it was found to be present in the Wiltshire and Hertfordshire samples, which contained only the wild-type metabolic genotype (SS) in 2018. In the Oxford sample, 15% of the beetles tested for the *s-kdr* mutation were homozygous for the resistant allele (RR). Overall, the percentage of beetles showing the homozygous genotype (RR) was 7.7%.

Table 3.6. *Detection of kdr/s-kdr alleles in 2019 samples of* P. chrysocephala *using TaqMan assay.*

Region	**County**	**Populations**	**No°**	***kdr status***			
				SS	**SR**	**RR**	**%RR**
East Midlands	Suffolk	1	4	0	1	3	75%
	Wiltshire	1	5	3	1	1	20%
	Lincolnshire	2	16	2	8	6	38%
East of England	Cambridgeshire	1	8	1	6	1	13%
	Norfolk	1	8	2	1	5	63%
	Hertfordshire	1	5	1	1	3	60%
SE England	Oxfordshire	1	19	7	4	8	42%
	Total	5	65	16 (24.6%)	22 (33.9%)	27 (41.5%)	

Region	**County**	**Populations**	**No°**	***s-kdr status***			
				SS	**SR**	**RR**	**%RR**
East Midlands	Suffolk	1	4	4	0	0	0%
	Wiltshire	1	3	1	1	1	33%
	Lincolnshire	2	16	11	5	0	0%
East of England	Cambridgeshire	1	8	5	3	0	0%
	Norfolk	1	8	7	1	0	0%
	Hertfordshire	1	6	4	1	1	17%
SE England	Oxfordshire	1	20	13	4	3	15%
	Total	5	65	45 (69.2%)	15 (23.1%)	5 (7.7%)	

To further investigate the suggestion that the *kdr* genotype does not correlate well with the resistance phenotype, the relationship between the resistance level and *kdr* frequency was assessed by plotting the resistance level value for each population with the corresponding *kdr* frequency (Figure 3.8). By linear regression there was no significant correlation, further indicating the presence of a potent metabolic-based resistance mechanism in *P. chrysocephala.* This result supports that found previously by Højland *et al.* (2015), who observed resistant UK beetles not possessing the *kdr* mutation.

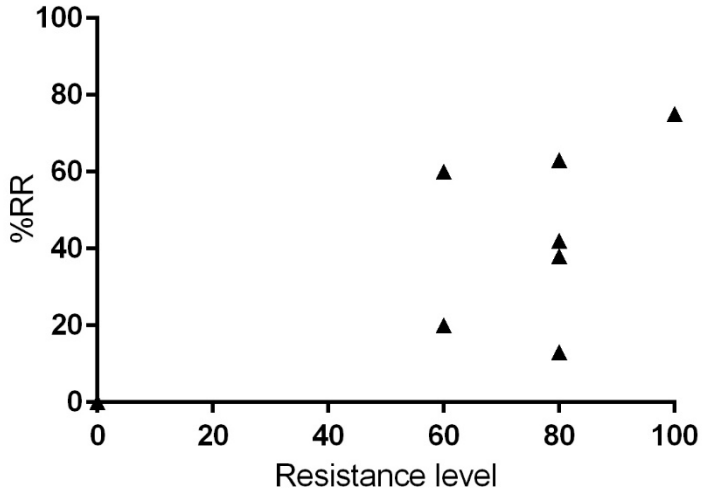


Figure 3.8. *Relationship of the resistance level to lambda-cyhalothrin and the kdr mutation.*

3.4.2.3 TaqMan sequencing in 2020

In 2020, *P. chrysocephala* individuals were screened for *kdr* from sites close to those sampled in 2018 and 2019; only beetles that survived the 100% field rate of lambda-cyhalothrin were tested. As in previous years, the L1014F mutation was present at all sites, with 45.4% of the beetles being homozygous for the resistant allele (RR), 18.2% heterozygous (SR) and the remaining 36.4% *kdr* SS (Table 3.7). Compared to 2019, the percentage of beetles homozygous for the *kdr* resistance allele (RR) decreased in four of the samples, from: Suffolk, Wiltshire, Lincolnshire, and Norfolk, but increased overall from 41% to 45.4%. Given that the percentage of beetles homozygous for the resistance allele (RR) remained similar across all three years, and the percentage of beetles homozygous for the susceptible allele (SS) increased, a rise in the mean resistance value from 32.9% in 2018 to 55.6% in 2019 to 68.5% in 2020 (see section

3.3.1.4) further shows a lack of correlation between the *kdr* genotype and the resistance phenotype, suggesting the presence of another resistance mechanism.

Whilst the L925I (*s-kdr*) mutation was again less common than the L1014F (*kdr*) mutation, it was found to be present at all sites. This contrasts with 2019 where the L925I mutation was only found at two sites. With respect to 2019, the percentage of beetles homozygous for the L925I mutation was also higher in all sites except in Hertfordshire, where 10% of the beetles tested for the *s-kdr* mutation were homozygous for the resistant allele (RR). Overall, the percentage of beetles showing the homozygous genotype (RR) was 29.6%.

Table 3.7. *Detection of kdr/s-kdr alleles in 2020 samples of P. chrysocephala using TaqMan assay.*

Region	**County**	**Populations**	**No°**	***kdr status***			
				SS	**SR**	**RR**	**%RR**
East Midlands	Suffolk	1	6	2	0	4	67%
	Wiltshire	1	4	4	0	0	0%
	Lincolnshire	2	11	5	4	2	18%
East of England	Cambridgeshire	2	8	1	1	6	75%
	Norfolk	1	5	3	0	2	40%
	Hertfordshire	1	10	1	3	6	60%
	Total	5	44	16 (36.4%)	8 (18.2%)	20 (45.4%)	

Region	**County**	**Populations**	**No°**	***s-kdr status***			
				SS	**SR**	**RR**	**%RR**
East Midlands	Suffolk	1	6	2	0	4	67%
	Wiltshire	1	4	2	0	2	50%
	Lincolnshire	2	11	5	2	4	36%
East of England	Cambridgeshire	2	8	6	1	1	13%
	Norfolk	1	5	2	2	1	20%
	Hertfordshire	1	10	8	1	1	10%
	Total	5	44	25 (56.8%)	6 (13.6%)	13 (29.6%)	

3.4.3 Synergist bioassays

The insecticide synergist piperonyl butoxide (PBO) has been shown to inhibit both cytochrome P450 monooxygenases and esterases, thereby acting as a tool for the identification of metabolic resistance in insect samples (Young, Gunning and Moores, 2006). To investigate the lack of correlation between lambda-cyhalothrin resistance and *kdr* frequency, and to determine whether P450 monooxygenases (and or esterases) may play a role in mediating pyrethroid resistance in UK *P. chrysocephala* populations, synergist bioassays with PBO pre-treatments were conducted on five *P. chrysocephala* samples collected in 2019.

When exposed to lambda-cyhalothrin at the recommended field rate, the percentage of beetles affected was 47% (North Yorkshire), 75% (Wiltshire), 8% (Wiltshire), 40% (Leicestershire) and 40% (Hertfordshire) (Figure 3.9). However, all adults pre-treated with PBO, prior to exposure to lambda-cyhalothrin at the same field rate, were killed (Figure 3.8). This strongly suggests that a metabolic-based mechanism for pyrethroid resistance is present in *P. chrysocephala,* although it must be acknowledged that PBO has many more effects than just inhibiting enzymes, aiding cuticular penetration and increasing the insect's susceptibility to environmental stressors. The results are in accordance with Xi et al. (2014) who found the toxicity of lambda-cyhalothrin towards a resistant strain of soybean aphid (*Aphis glycines* Matsummura) was dramatically increased by the synergists PBO, DEF and TPP, and Young, Gunning and Moores (2005) who found that in the cotton bollworm (*Helicoverpa armigera*), esterases could be inhibited by PBO, restoring pyrethroid efficacy.

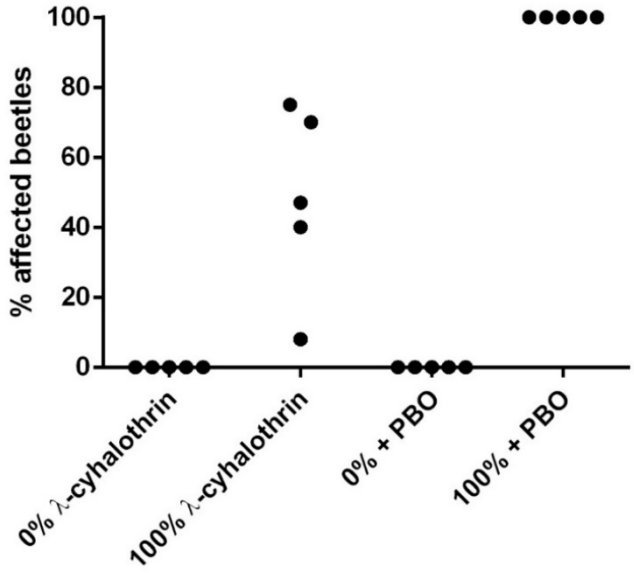


Figure 3.9. *Restoration of insecticide (pyrethroid) susceptibility in P. chrysocephala following pre-treatment with PBO. Samples tested were from North Yorkshire, Wiltshire (x2), Leicestershire and Hertfordshire.*

3.4.4 Larval bioassays

Whilst monitoring the geographical spread of pyrethroid resistance in *P. chrysocephala* adults started at Rothamsted Research in 2014, to date, little is known about the resistance levels of *P. chrysocephala* larvae towards pyrethroids. Until the EU imposed restriction in 2013, the larvae were predominantly targeted by the systemic neonicotinoid seed treatments. Since the neonicotinoid restriction, the pyrethroids remain the only class of insecticide for *P. chrysocephala* control. However, because the larvae develop inside the petioles and main stem, controlling them using pyrethroid sprays is difficult. If chemical control of *P. chrysocephala* larvae is to be effective, understanding how the pyrethroids affect them at all developmental stages is crucial.

3.4.4.1 Resistance across developmental stages

P. chrysocephala larvae were collected between December 2019 and March 2020 from a field on the Rothamsted Research farm (see section 2.1.2). A leaf dip assay (see section 3.2.5) was used to assess larval resistance levels at 4%, 20% and 100% of the recommended field rate of lambda-cyhalothrin. A leaf dip assay was used as it provides a more realistic delivery of the insecticide from the oilseed rape plant to the insect than glass vial assays, and has been previously used to assess the toxicity of

pyrethroids and organophosphates against the tobacco whitefly (*Bemisia tabaci*) (Cahill et al., 1995) and the toxicity of *Bacillus thuringiensis* (Bt) spores against the diamondback moth (*Plutella xylostella*) (Tang and Tu, 1994). The larvae were tested at all three developmental stages: 1st, 2nd, and 3rd instar.

The data required a logit transformation (log((affected+1)/(100-affected+1))) to satisfy the equal variance assumption of the ANOVA analysis. The back-transformed means and confidence intervals are shown in Table 3.8. Whilst there was a significant difference in mean percentage mortality across the developmental stages (two-way ANOVA, $F_{2,24} = 45.48$, $p < 0.001$), the mean percentage mortality at the recommended field rate of lambda-cyhalothrin was similar in the 1st (98.73%) and 2nd (98.55%) instar larvae (Figure 3.10). This result was unexpected as it was thought that the percentage of larvae surviving the assay would increase as the larvae developed through their instar stages. At the 100% field rate the 3rd instar larvae showed a significantly higher mean level of resistance to lambda-cyhalothrin (48.95%) than the 1st and 2nd instar larvae ($p < 0.001$). Despite this result, it was interesting to observe that the error bar at the 100% field rate (7.5g ai/ha) was larger than anticipated for the 3rd instar larvae. This large variation in number of larvae affected could be attributed to each larva ingesting different amounts of insecticide coated leaf, a potential disadvantage of the feeding assay method.

The near complete susceptibility of the 1st and 2nd instar larvae to the 100% recommended field rate of lambda-cyhalothrin was unexpected and could have interesting implications for chemical control methods. As the 1st instar larvae move from the soil into the leaf petioles after hatching, they are more likely to be exposed to insecticide than the more resistant 3rd instars, which usually stay within the stem (Sam Cook, personal communication). If the 1st instar larvae could be targeted at this time, the chances of killing the *P. chrysocephala* larvae could potentially be increased. Syngenta trials reported it possible to predict egg hatch and larvae movement based on weather data, with larvae emerging 240 degree days after egg laying (Issues, 2020). With this information it may be possible to more accurately time the application of pyrethroid sprays to target the 1st instar larvae. The decreased susceptibility of the 3rd instar larvae to lambda-cyhalothrin, compared to the 1st and 2nd instar larvae, could be due to a variety of reasons. As the 3rd instar larvae are much larger in size than the 2nd instar larvae, they might naturally be more resilient and therefore more likely to

survive the same concentrations of insecticide. This hypothesis is supported by Daly, Fisk and Forrester (1988) who found that in the cotton bollworm (*Helicoverpa armigera*), age-dependent mortality was due to changes in sensitivity to pyrethroid resulting from changes in larval size. An alternative explanation is that *P. chrysocephala* larvae have metabolic resistance mechanisms with the metabolic enzymes being expressed at a higher level in the 3rd instar larvae than in the 1st and 2nd instar larvae.

To compare insecticide resistance levels between *P. chrysocephala* larvae and adults, *P. chrysocephala* adults collected from a field at the Rothamsted Research farm in July 2020 were also tested at 4%, 20% and 100% of the recommended field rate of lambda-cyhalothrin. The adults were tested using the glass vial bioassay method. The *P. chrysocephala* adults were found to be significantly more resistant than the 3rd instar larvae ($p < 0.001$); all adults tested at the recommended field rate (7.5g ai/ha) survived. This result could be due to a variety of reasons. As different bioassay methods were used for the larvae and the adults, differences in resistance level could be due to the insecticide delivery method. In a leaf dip assay the insecticide is delivered by both contact and ingestion However, in a glass vial assay the insecticide is delivered by contact alone. In a study by Vandekerkhove and De Clercq (2004) it was found that in the southern green stinkbug (*Nezara viridula*), nymphs had five times greater mortality to lambda-cyhalothrin when exposed by ingestion than by residual contact. Differences in insect cuticle thickness may offer another explanation for differences in pyrethroid toxicity. If cuticle thickness affects the penetration rate of lambda-cyhalothrin, this may result in differences in toxicity between the adults which have a hard exoskeleton and the softer bodied larvae. Another explanation for the difference in resistance level between adults and larvae is stage-specific expression of resistance. For example, in the pyrethroid-resistant malaria vector *Anopheles funestus, CYP6P9* was not significantly overexpressed in the larvae, but it was overexpressed in the eggs and the adults (Amenya et al., 2008); the esterases α EST and β EST were more highly expressed in *Aedes aegypti* adults than larvae (Bisset et al., 2014). In the Colorado potato beetle (*Leptinotarsa decemlineata*), the 1st instar larvae were more sensitive to Bt toxins than the 3rd instar larvae (Wierenga, Norris and Whalon, 1996) and in the whitefly (*B. tabaci*), the susceptibility of nymphs to imidacloprid was 4 – 10 times greater than in the adults (Nauen et al., 2008).

Table 3.8. *ANOVA showing the average percentage of affected larvae and lower confidence interval (LCI) and upper confidence interval (UCI).*

Stage		1st	2nd	3rd	Adult
Dose					
0.3	**Mean**	23.89	24.93	1.27	0
	LCI - UCI	7.59 - 53.16	8.03 - 54.54	-0.34 - 6.55	-0.71 - 2.42
1.5	**Mean**	66.81	63.07	20	1.27
	LCI - UCI	35.85 - 88.18	32.16 - 86.27	6.02 - 47.58	-0.34 - 0.5
7.5	**Mean**	98.73	98.55	48.95	4.09
	LCI - UCI	93.45 - 100.34	92.89 - 100.29	20.91 - 77.64	0.5 - 14.86

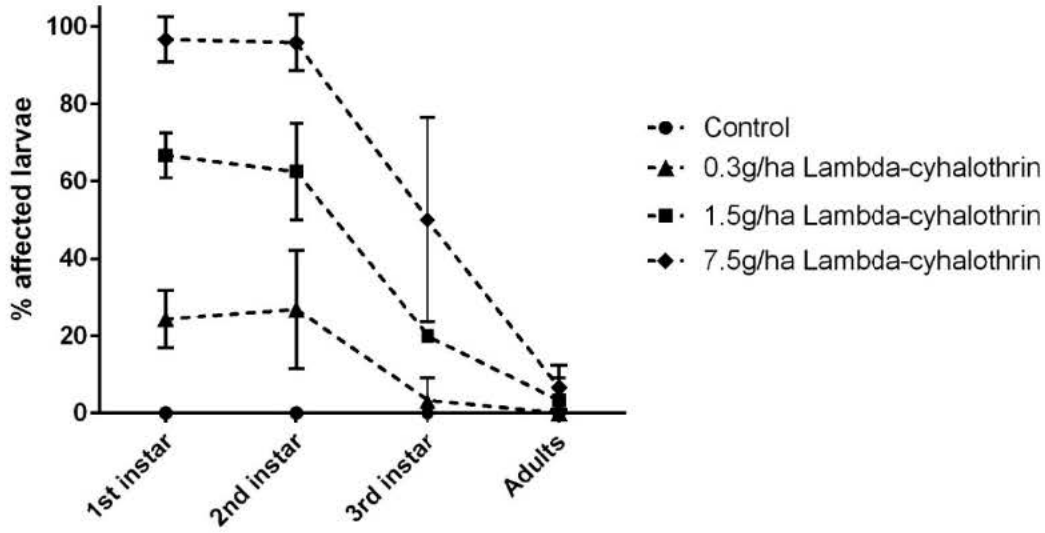
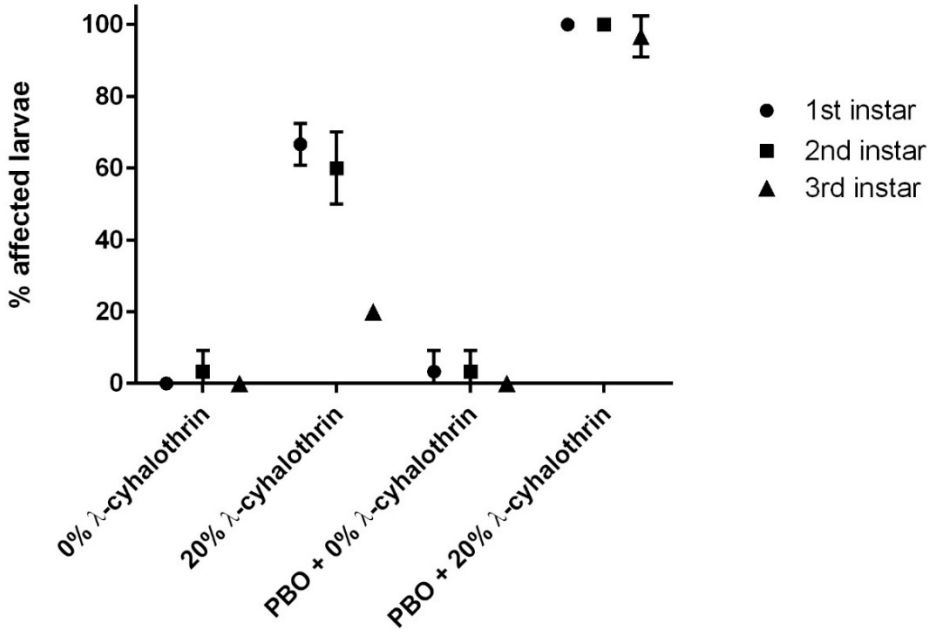


Figure 3.10. *Susceptibility of P. chrysocephala larvae and adults following treatment with lambda- cyhalothrin. P. chrysocephala larvae were tested at all three developmental stages at 4%, 20% and 100% of the recommended field rate using a leaf dip assay. Cabbage stem flea beetle adults were assessed using a glass vial assay at 4%, 20% and 100% of the recommended field rate.*

3.4.4.2 Synergist bioassays (larvae)

To determine whether the metabolic resistance mechanism thought to underlie pyrethroid resistance in *P. chrysocephala* adults is age-specific in expression, synergist bioassays with PBO pre-treatments were conducted on all larval stages. When exposed to 20% lambda-cyhalothrin alone the percentage of larvae affected was 70% (1st instar), 60% (2nd instar) and 20% (3rd instar) (Figure 3.11). However, when pre-treated with PBO prior to exposure to lambda-cyhalothrin at the same rate, nearly all larvae were killed. This suggests that metabolic enzymes are conferring some degree of protection in all developmental stages, and this is more pronounced in the 3rd instar larvae and adults. This result is in accordance with previous studies which

describe constitutive overexpression of enzymes associated with metabolic resistance. For example, the metabolic enzyme *CYP6PA1* was found to be overexpressed in



insecticide resistant housefly (*Musca domestica*) adults and larvae (Cariño et al., 1994); *CYP6AA7* was overexpressed in permethrin resistant mosquito (*Culex quinquefasciatus*) adults and larvae (Liu et al., 2015). Whilst target-site mutations could also confer a degree of resistance, there were not enough larvae to determine the presence of the L1014F (*kdr*) and L925I (*s-kdr*) mutations using TaqMan assays.

Figure 3.11. *Increase in susceptibility to insecticide (pyrethroid) in P. chrysocephala larvae following pre-treatment with PBO. Samples were from Rothamsted Research farm.*

3.5 Conclusion

Since the EU-imposed ban on neonicotinoid seed treatments on OSR, pyrethroid insecticides have been widely used for chemical control of *P. chrysocephala* in the UK. This has resulted in a high selection pressure and led to the development of resistance, particularly in the South East of England. In this chapter *P. chrysocephala* adults and larvae were tested for their susceptibility to lambda-cyhalothrin at a range of concentrations. This builds on the resistance monitoring work previously conducted at Rothamsted Research by Stephen Foster (Højland et al., 2015). Through glass vial bioassays, populations of *P. chrysocephala* from around the UK were found to exhibit high levels of resistance to lambda-cyhalothrin, with the mean resistance level

increasing from 32.9% in 2018, to 55.6% in 2019 to 68.5% in 2020. This resistance to pyrethroids has resulted in widely-reported control problems for this pest in the farming press (e.g. Clark 2014; Casswell, 2014; FarmingUK team, 2015; Hill, 2017; FarmingUK team, 2017; Case, 2018; Allison, 2019; Dyer, 2019; Gillbard, 2019) since the introduction of the neonicotinoid ban, demonstrating that lambda-cyhalothrin is now ineffective for the control of *P. chrysocephala*, in England.Despite the development of resistance in *P. chrysocephala*, pyrethroids continue to be used on UK farms as there remains a lack of available insecticides with alternative modes of action that can be deployed for resistance management. However, this continued reliance on pyrethroids is failing as a control strategy in many regions and is not sustainable in areas where resistance levels may appear low or non-existent. Since 2014, there has been a significant year by year decrease in the area of oilseed rape production in the UK, declining dramatically from 634,000 hectares in 2014 (DEFRA, 2014) to 497,000 hectares in 2019 (DEFRA, 2019) to 273,000 hectares in 2021 (DEFRA, 2021). This result suggests that unless alternative methods of control are identified, farmers will continue to struggle to grow OSR in the UK. This could have a negative impact on the supply of vegetable oils for a range of purposes (biofuels, food etc). It is therefore particularly important that the extent and geographical spread of pyrethroid resistance in *P. chrysocephala* continues to be monitored at a time when synthetic pesticides are becoming less favoured through EU legislation. Clearly, there needs to be informed decision making on how to best deploy pesticides effectively in the future. Alternative strategies, such as the potential of the parasitoid *Microctonus brassicae* for biological control (Jordan et al., 2020), trap cropping (Barari et al., 2005) and the use of insect-resistant varieties of oilseed rape (Hughes, 2019) also offer options for *P. chrysocephala* control, as well as reducing the amount of insecticide used. However, as insecticides are often an integral part of integrated pest management (IPM) strategies, understanding how pyrethroids affect natural enemy populations is important if biological control is to be effective.

Understanding the mechanism/s of resistance to pyrethroids is key in implementing effective resistance management strategies for the control of *P. chrysocephala*. Synergist bioassays have found that pyrethroid resistance was suppressed in both the adults and larvae by the cytochrome P450 inhibitor, PBO. This highly suggests that, as well as target site resistance, there is strong, P450 mediated-detoxification of

lambda-cyhalothrin, although further research is required to identify the specific P450(s) involved and elucidate the exact mechanism of resistance. As synergist bioassays resulted in increased mortality in both the beetle adults and larvae, this suggests the metabolic mechanism of resistance may be present in all life stages. The next chapter (Chapter 4) looks to identify the gene(s) underlying pyrethroid resistance in UK populations of *P. chrysocephala* by comparing gene expression between pyrethroid-susceptible and -resistant populations. Chapter 5 looks to validate the gene thought to confer metabolic resistance using RNA interference.

3.6 Key findings

- The mean resistance level of UK populations of *P. chrysocephala* to the pyrethroid lambda-cyhalothrin has increased across the three years of testing (2018 to 2020).
- Resistance has spread to the South West and North of England.
- The *P. chrysocephala* samples received from Scotland in 2019 and 2020 were fully susceptible to lambda-cyhalothrin.
- TaqMan assays showed that there were more homozygotes for the *kdr* allele in 2019 and 2020 than in 2018. This increase suggests that insecticides are creating a strong selection pressure, and that kdr is providing some advantage on top of the metabolic based mechanism.
- *Skdr* may also confer some evolutionary advantage.
- 1st and 2nd instar larvae showed near complete susceptibility to lambda-cyhalothrin at the recommended field rate, but the 3rd instar larvae were more resistant. This may be due to their larger size providing more resilience or metabolic resistance mechanisms being expressed at higher levels in the 3rd instars.
- *P. chrysocephala* adults were more resistant than the 3rd instar larvae, this may be due to cuticle thickness or stage dependent expression of resistance.
- Both adults and larvae became more susceptible to insecticide after pre-treatment with PBO suggesting the presence of a metabolic resistance mechanism.

Chapter 4. Using the *Psylliodes chrysocephala* genome to identify genes associated with insecticide resistance

4.1 Aims

The aim of the work reported in this chapter was to identify and validate the gene(s) involved in pyrethroid resistance in UK samples of *P. chrysocephala*. RNA sequencing was used to study the transcriptional profile of susceptible and resistant *P. chrysocephala* samples from across the UK and Europe, and bioinformatic analysis to identify the potential enzyme(s) involved in conferring metabolic resistance to pyrethroids in *P. chrysocephala*. qPCR was used to validate the candidate genes identified by bioinformatic analysis.

4.2 Introduction

Since the EU-imposed restriction on the outdoor use of neonicotinoid seed treatments in 2013 (EU commission, 2013), pyrethroids have become the main class of insecticide for chemical control of *Psylliodes chrysocephala* in the UK. However, the lack of alternative insecticides and therefore the continuous use of pyrethroid sprays has resulted in a strong selection pressure driving the the development of pyrethroid resistance. Today, this resistance is widespread in UK samples of *P. chrysocephala*, having advanced from the South East to the North and West of England (see Chapter 3). This increased spread of resistance has resulted in growers deciding to no longer grow oilseed rape and this has led to significant losses in the area of this crop grown in the UK over the last seven years (DEFRA, 2014, 2015, 2019, 2020). This scenario threatens the future of oilseed rape production in the UK. With no other insecticide classes registered for *P. chrysocephala* control to date, preservation of pyrethroid efficacy in Scotland, and other areas of Europe where samples appear to remain pyrethroid-susceptible, is of paramount importance. However, to effectively manage insecticide resistance, knowledge of the underlying basis of the resistance mechanism is key, particularly if resistance monitoring tools are to be used successfully. Although insecticide resistance, resulting from target-site insensitivity, is well documented in *P. chrysocephala* (Zimmer et al., 2014; Højland et al., 2015; Højland and Kristensen, 2018), it has been found that the *kdr* genotype (although known to be present in the UK) does not correlate well with the resistant phenotype seen in the UK, suggesting

another, more potent, mechanism of resistance (Højland et al., 2015; Højland and Kristensen, 2018). Whilst bioassays with the syngergist PBO implicate metabolic resistance as the main mechanism in the UK samples (see Chapter 3, section 3.3.5), the specific metabolic enzymes involved have yet to be identified.

At a time where the development of next generation sequencing (NGS) has significantly reduced the cost of DNA and RNA sequencing, the methods for studying insecticide resistance mechanisms have been drastically transformed (Behjati and Tarpey, 2013). The increased availability of annotated genome sequences means that studying the underlying mechanisms of insecticide resistance has become more accessible (Oakeshott et al., 2003). In organisms with no annotated genome the entire mRNA of specific species can be sequenced for the generation of a transcriptome, which can then be analysed for candidate genes associated with insecticide resistance. When individual genes of large enzyme families such as the cytochrome P450s (P450s), glutathione-S-transferases (GSTs) and carboxylesterases (CCEs), underlie the resistance mechanism, and the identity of the individual gene(s) that are overexpressed in insecticide resistant individuals are difficult to determine due to the large number of candidate genes (Ranson et al., 2002), genomic approaches are important in elucidating the genes involved in resistance mechanisms.

To identify the enzyme(s) involved in metabolic resistance to pyrethroids in *P. chrysocephala*, RNA sequencing was conducted on susceptible and resistant samples (assessed by bioassay) from across the UK and Europe. This RNA-seq method of analysis was chosen because it offers a good approach for the detection of novel transcripts without the need for transcript-specific probes, and it has a wide dynamic range in the quantification of gene expression. It can also detect low-abundance and rare transcripts, and additionally can detect a high percentage of differentially expressed genes (DEGs) whilst also quantifying the expression of isoforms (Zhao et al., 2014). In particular, RNA sequencing offers a way of detecting transcripts from organisms where little sequencing data is available in public databases, making it particularly useful when studying non-model organisms such as *P. chrysocephala* (Wang, Gerstein and Snyder, 2009; Mutz et al., 2013). qPCR was used to analyze candidate gene expression and validate the results obtained by bioinformatic analysis of the RNA-seq data.

4.3 Materials and methods

4.3.1 Insect material and bioassays

P. chrysocephala adults were collected from freshly harvested oilseed rape fields in Switzerland and Germany in 2018 and in the UK in 2018 – 2020, as described in Chapter 2 section 2.1.1. The beetles from the UK were maintained in a controlled environment cabinet at 15±1°C, 65% relative humidity, 12h:12h light: dark photoperiod, until tested for pyrethroid resistance. Glass vial bioassays were performed on live mobile beetles to determine resistance levels using a reference pyrethroid, lambda-cyhalothrin (see Chapter 2, section 2.2.2). Briefly, ten beetles were incubated for 24 hours in glass vials coated on the inner surface with 500µl of one of three doses of lambda-cyhalothrin, equivalent to 4%, 20% and 100% of the of the recommended field application rate (7.5g ai/ha). After 24 hours, the beetles were transferred to untreated glass vials, without lids, under inverted 200ml, transparent plastic disposable cups to allow an opportunity to recover from insecticide treatment. Mortality/coordination was determined after a further 24 hours. After resistance testing the beetles were snap frozen in liquid nitrogen and stored at -80ºC. The *P. chrysocephala* samples from Switzerland and Germany were tested for pyrethroid resistance in-house by Syngenta and Bayer respectively before being snap-frozen in liquid nitrogen and sent to Rothamsted Research on dry ice. These samples were also then stored at -80ºC in situ.

4.3.2 Selecting pyrethroid susceptible and resistant *Psylliodes chrysocephala* for RNA sequencing analysis

To examine differences in gene expression between pyrethroid-susceptible and resistant *P. chrysocephala* adults, the most susceptible and resistant samples, as judged from the bioassays, were used to extract RNA for the RNA sequencing experiment. In accordance with IRACs 'susceptibility rating scheme', samples were classed as susceptible if mortality was 100% at 1.5g h^{-1} (20% of the full field rate) or resistant if mortality was less than 90% at 20% of the full lambda-cyhalothrin field rate (100% is equivalent to 7.5g ai/ha). To circumvent the potential issue of post-mortem degradation of RNA in susceptible beetles, differences in gene expression level were compared in beetles from a susceptible sample vs beetles found to be resistant by glass vial bioassay. In total, seven samples of *P. chrysocephala* adults

from geographically distinct sites (Table 4.1) were selected for differential gene expression analysis by RNA sequencing.

***Table 4.1**. Location of sample sites with susceptible or resistant beetles.*

Country	Sample	Location
UK	susceptible	Fraserburgh, Aberdeenshire (2019)
	resistant A	Petworth, West Sussex
	resistant B	Bury St Edmunds, Suffolk
Switzerland	susceptible	Steinmaur, Zürich
	resistant	Uesslingen, Thurgau
Germany	susceptible	Bredenfelde, Mecklenburgische Seenplatte
	resistant	Bredenfelde, Mecklenburgische Seenplatte

4.3.3 RNA extraction and Illumina sequencing

For each sample site, total RNA was extracted from the pooled homogenate of three *P. chrysocephala* adults using the Isolate II RNA mini kit (see Chapter 2, section 2.3.3). From the UK there were three biological replicates per sample, one sample was susceptible and two were resistant (A, B). For the Swiss and German sites with susceptible beetles, there was one replicate per sample, and for resistant beetles two replicates per sample (n=15) (Table 4.2). To ensure all samples met quality requirements for sequencing, RNA quality was assessed as described in Chapter 2, section 2.3.4. The samples were then sent for standard RNA-seq by GENEWIZ (New Jersey, USA). GENEWIZ prepared the cDNA library and performed next generation sequencing on an Illumina HiSeq flowcell (2 x 150bp paired reads), producing 15 – 20 million reads per sample.

***Table 4.2.** The number of biological replicates used for each sample.*

Country	Sample	Number of biological reps
UK	susceptible	3
	resistant A	3
	resistant B	3
Switzerland	susceptible	1
	resistant	2
Germany	susceptible	1
	resistant	2
Total		**15**

4.3.4 Bioinformatic analysis

Once the sequence data were returned from GENEWIZ, bioinformatic analyses were conducted using Galaxy (Afgan et al., 2018), RStudio (R Core Team, 2017) and Blast2GO (Conesa et al., 2005). All parameters for the work packages used within Galaxy were the default unless otherwise specified. The RNA-seq processing pipeline used within Galaxy to identify differentially expressed genes is shown in Figure 4.1.

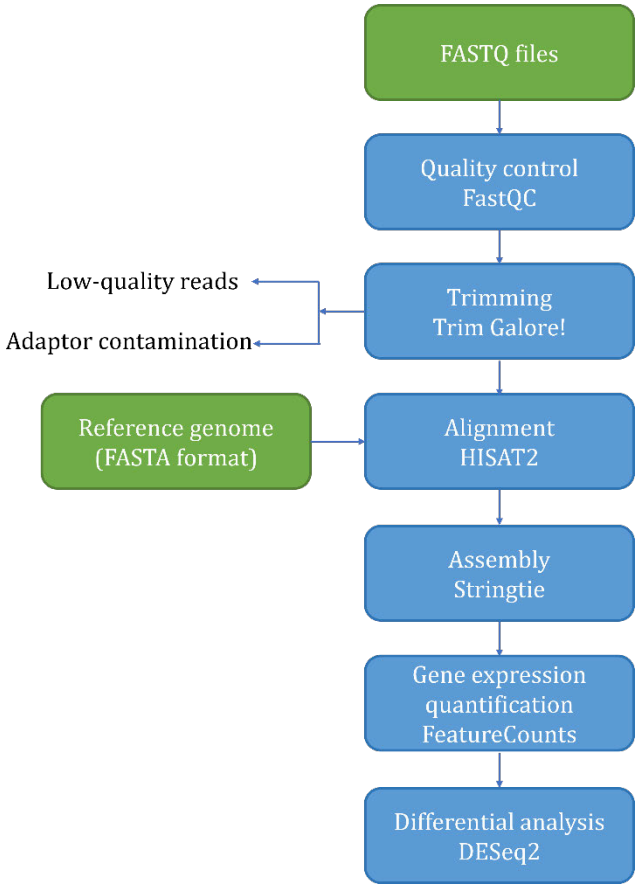


Figure 4.1. *RNA-seq pipeline to produce gene expression data in Galaxy. The arrows show the direction of the bioinformatic analysis and data flow.*

4.3.4.1 Assessment of Illumina RNA-Seq read quality

To check the quality of the raw sequence data FastQC (version 0.72; Andrews, 2010) was performed to identify any adaptor sequence contamination and low base read quality. If base read quality is low the observed read sequence is most likely wrong, which can lead to alignment errors. FastQC identified poor quality sequence reads from around 130-150bp (Figure 4.2a) and the presence of the Illumina universal adaptor sequence from around 90-137bp (Figure 4.3a). As there is often degradation in read quality over long runs, poor quality base calling towards the end of reads is not

uncommon. Trim Galore! (version 0.4.3.1; Krueger, 2017) was used to 'trim' (remove) low-quality portions of the reads from the 3' end of the sequence data, and the 3' end adaptor sequence. The FastQC analysis was then rerun to confirm the successful removal of the low-quality sequence reads (Figure 4.2b) and adapter sequences (Figure 4.3b). Table 4.3 shows the number of reads per sample pre- and post- trimming.

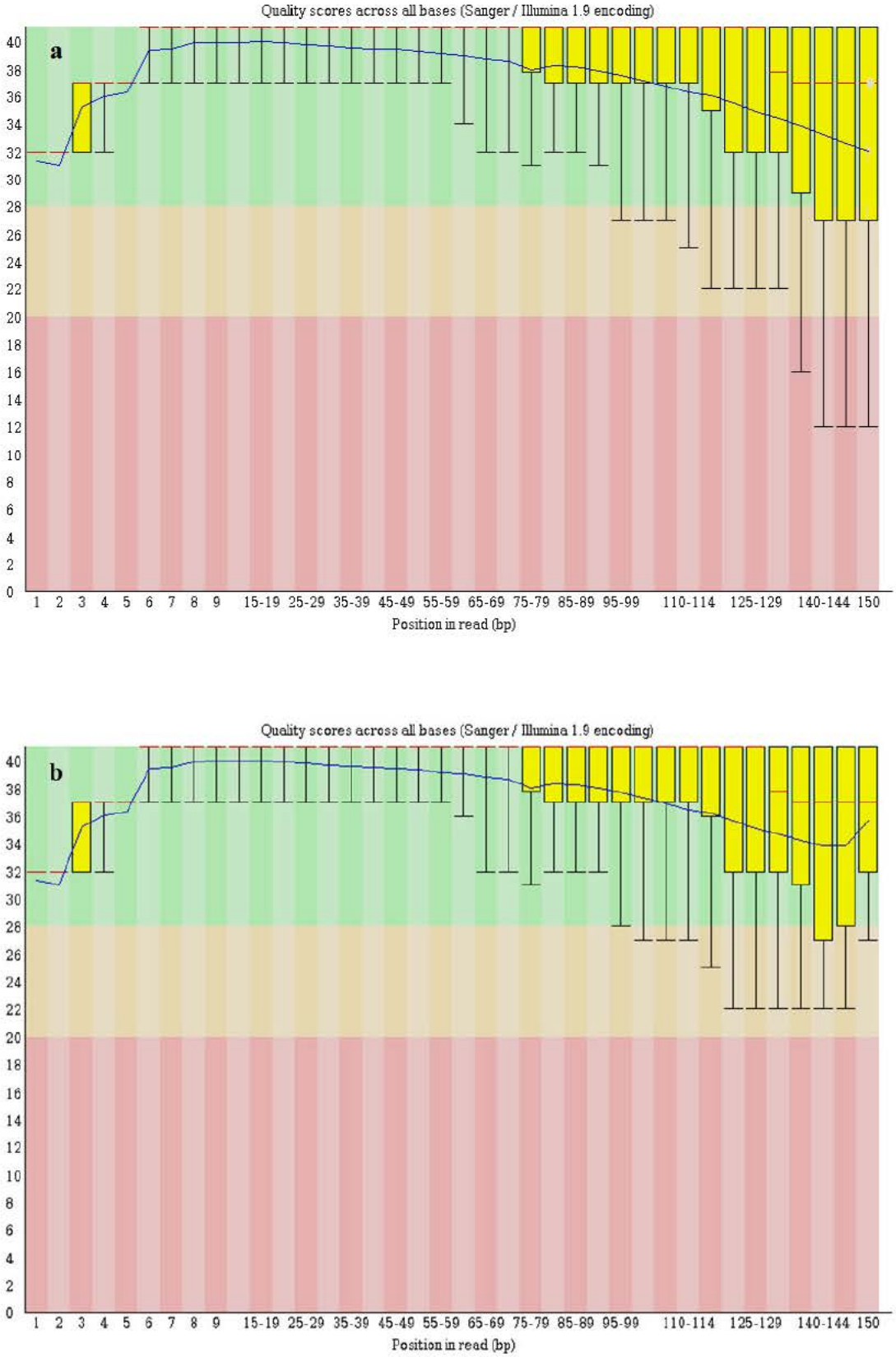


Figure 4.2. *Sequence quality paired-end reads for UK resistant beetle sample CW3 from FastQC analysis. The y-axis shows the quality score and the x-axis the position in the read (bp). The coloured background separates the y-axis into very good base quality (green), reasonable base quality (orange) and poor base quality (red). a) shows the sequence read before trimming and b) the sequence read after trimming.*

Figure 4.3. *Adaptor contamination for the UK resistant beetle sample CW3 from FastQC analysis. The y-axis shows the cumulative percentage count for the proportion of the run which has seen the adaptor sequence at each position. a) shows the sequence read before trimming and b) the sequence read after trimming.*

Table 4.3. *Pre-trimming & post-trimming number of reads and the percentage alignment of the transcripts for sequences of P. chrysocephala.*

Country	Sample	Number of Reads		Difference	% Alignment
		Pre-trimming	Post-trimming		
UK	susceptible	24231572	23860473	371099	85.68%
	susceptible	22344811	21997703	347108	86.31%
	susceptible	21781967	21484866	297101	85.99%
	resistant	21822952	21467529	355423	86.19%
	resistant	27884886	27485192	399694	86.70%
	resistant	24990273	24625239	365034	86.50%
	resistant	31095199	30630321	464878	86.03%
	resistant	22892792	22588227	304565	85.91%
	resistant	23147920	22812431	335489	86.07%
Switzerland	susceptible	24060219	24017400	42819	84.55%
	susceptible	21140399	21110907	29492	88.45%
	resistant	20274623	20239513	35110	85.16%
Germany	susceptible	21495523	21452170	43353	87.77%
	susceptible	19459403	19425188	34215	86.56%
	resistant	18816670	18789705	26965	88.25%

4.3.4.2 Alignment to the reference genome

HISAT2 (version 2.1.0; Kim, Langmead and Salzberg, 2015) was used to map the cleaned RNA sequence reads obtained against the *P. chrysocephala* PGI reference genome (sequenced by the Pest Genome Initiative https://www.rothamsted.ac.uk/projects/pest-genome-initiative, accessed via Syngenta & Rothamsted Research, UK and provided by Robert King, Rothamsted Research). The output summaries of the alignment statistics produced by HISAT2 were visualised using MultiQC (version 1.7; Ewels et al., 2016). For each sample >85% of the reads aligned to the reference genome and >15 million reads mapped uniquely (Figure 4.4).

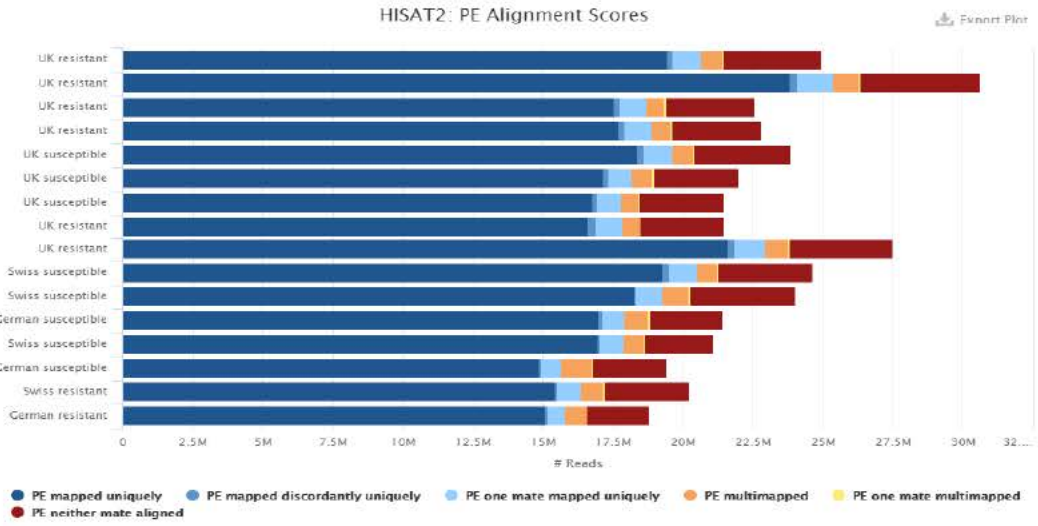


Figure 4.4. *Number of reads mapped to the P. chrysocephala reference genome. For each sample >15 million reads mapped uniquely.*

4.3.4.3 Identifying differentially expressed genes

Stringtie (version 1.2.3, Pertea et al., 2015) was used to assemble the RNA-seq alignments into potential transcripts. FeatureCounts (version 1.5.3; Liao, Smyth and Shi, 2014) was then used to measure gene expression levels, by counting the number of RNA sequence reads per gene from the HISAT2 output against the Stringtie assembly. The estimated counts were imported into DESeq2 (version 2.11.38; Love, Huber and Anders, 2014) which was then used to identify genes differentially expressed between the resistant and susceptible samples. DESeq2 uses a negative binomial model to determine differential expression. It was chosen over other software programmes such as Edge R (Robinson, McCarthy and Smyth, 2009) because it uses shrinkage estimators for dispersion and fold change for genes with low counts or high levels of variability. DESeq2 uses the distribution of the fold change values of all genes and uses this to shrink the fold change values of genes with less information (Love, Huber and Anders, 2014). The p-values outputted by DESeq2 were adjusted using the Benjamini-Hochberg method (Haynes, 2013) to decrease the false discovery rate and therefore control for the fact that sometimes small p-values occur by chance. The differentially expressed genes were filtered with the parameters; adjusted p-value <0.05 and log2foldchange >2.5. Multiple pairwise comparisons were then run using DESeq2 so that differences in gene expression as a result of country of origin alone could be identified and removed from further analysis.

4.3.4.4 Annotation analysis

Blast2GO (version 5.2.5, Conesa et al., 2005) was used to run a Blastp and InterPro search on the complete *P. chrysocephala* reference genome and the list of differentially expressed genes produced in Galaxy. The Blastp search was completed against the non-redundant protein database (NCBI, accessed 28/11/19) with the following parameters; taxonomy filter = (taxa: 7041, Coleoptera), blast expectation value (E-value) = 1 x 10^{-3}, and minimum number of blast hits = 20. The InterPro search was completed against the EMBL-EBI database.

4.3.4.5 Gene Ontology enrichment analysis

GO-term enrichment analysis was used to determine the potential properties (biological process, cellular component and molecular function) of the differentially expressed genes, and test whether any of the Gene Ontology (GO) terms were overrepresented among the DEGs vs the complete *P. chrysocephala* genome. The enrichment analysis was conducted in Blast2GO using Fisher's exact test with a False Discovery rate of <0.05.

4.3.4.6 Validation of genes of interest by qPCR

Quantitative PCR (qPCR) was used to validate possible differences in gene expression level between pyrethroid susceptible and resistant *P. chrysocephala* samples. As well as the seven samples of *P. chrysocephala* adults previously chosen for differential gene expression analysis by RNA sequencing, total RNA was extracted from five further samples (C, D, E, F, G) from the UK (Table 4.4), using the Isolate II RNA mini kit (Chapter 2, section 2.3.3). Total RNA was extracted from pooled homogenates of three *P. chrysocephala.* As before, resistance levels were determined by glass vial bioassay as described in section 4.2.1. The five additional samples analysed were all 100% resistant to lambda-cyhalothrin at the recommended field rate. cDNA was synthesised from approximately 400ng of RNA using the ThermoScientific SuperScript III First-strand cDNA synthesis kit (Invitrogen) (section 2.3.16). qPCR was carried out following the method described in Chapter 2 section 2.4.3. Briefly, PCR reactions were 15µl and comprised 7.5µl SYBR Green JumpStart Taq ReadyMix, 0.5µl of 10µM forward primer, 0.5µl of 10µM reverse primer (Table 4.5), 1.5µl of sterile distilled water, and 5µl of 4µg/µl cDNA. Sterile distilled water was used for the negative controls. qPCR was carried out using the Rotor-Gene Q with the

cycling conditions: 95ºC for 2 minutes, followed by 45 cycles of 95ºC for 10 seconds and 58ºC for 45 seconds. The susceptible sample was loaded onto the plate for each run to minimise variation across plates, along with β-actin and GAPDH housekeeping genes as reference controls.

Table 4.4. *Location of sample sites with 100% resistant P. chrysocephala adults.*

Country	Sample	Location
UK	susceptible	Fraserburgh, Aberdeenshire (2020)
	resistant C	Maidenhead, Berkshire
	resistant D	East Tytherley, Hampshire
	resistant E	Berwick St James, Wiltshire
	resistant F	Dulverton, Somerset
	resistant G	Stillingfleet, York

Table 4.5. *Forward (F) and reverse (R) primers used for qPCR analysis.*

Primer	Sequence
20996 F1	AGTACGACGCGCAAGAATGA
20996 R1	CACGAAAAACTGCAGACCGG
9127 F1	GGACAACGGCGAGGTTATAG
9127 R1	CTTGACCTGTTTCCTCCACC
b-actin F2	ACGAACTTCCCGATGGACAG
b-actin R2	CCAGCAGCTTCCATACCCAA
GAPDH F1	GCTTCCTGCACCACCAATTG
GAPDH R1	GTTTTGTCCAGCACCACGAC

4.3.5 Statistical analysis

The C_t values generated by the Rotor-Gene Q were imported into Microsoft Excel. The C_t values were normalised to the geometric mean of the β-actin and GAPDH housekeeping genes and the average $2^{-\Delta\Delta Ct}$ for each sample or life stage was calculated using the $2^{-\Delta\Delta Ct}$ method. The standard error and 95% confidence intervals of the $2^{-\Delta\Delta Ct}$ values were determined from the technical replicates of each sample or life stage. ANOVA and Student's t-test were used to identify significant differences in gene expression levels between samples. Graphs were produced using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

4.4 Results and discussion

4.4.1 Principle Component Analysis

Principal Component Analysis (PCA) plots produced by the DESeq2 analysis were used to visualise the data. These plots extract dominant patterns in the dataset, meaning samples with similar expression profiles should group together (Wold, Esbensen and Geladi, 1987), and any distinct groups should be well separated from other groups along the x-axis ('PC1': the first principle component). DESeq2 was initially performed using both country (UK, Switzerland, Germany) and resistance level (resistant or susceptible) as factors that influence the differences in gene expression observed. From the PCA plot showing this analysis (Figure 4.5) it is evident that there is a clear separation between the UK samples vs the Swiss and German samples. There is also a clear separation between the UK resistant and UK susceptible samples, whilst the German resistant and susceptible samples group together. This separation indicates differential gene expression between the UK and European samples as well as further differences in gene expression between the UK susceptible and resistant samples. DESeq2 analysis on the UK dataset alone further shows the separation between the susceptible and resistant samples (Figure 4.6). This separation suggests that there is a genetic difference between the susceptible and resistant samples, with further analyses needed to identify the specific metabolic enzymes involved.

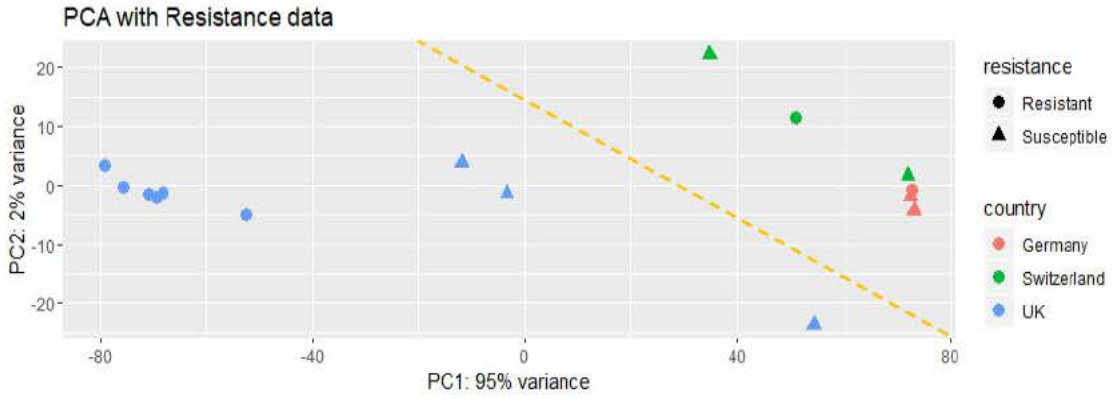


Figure 4.5. *Principle component analysis of samples by country of origin and susceptible versus resistant to lambda-cyhalothrin.*

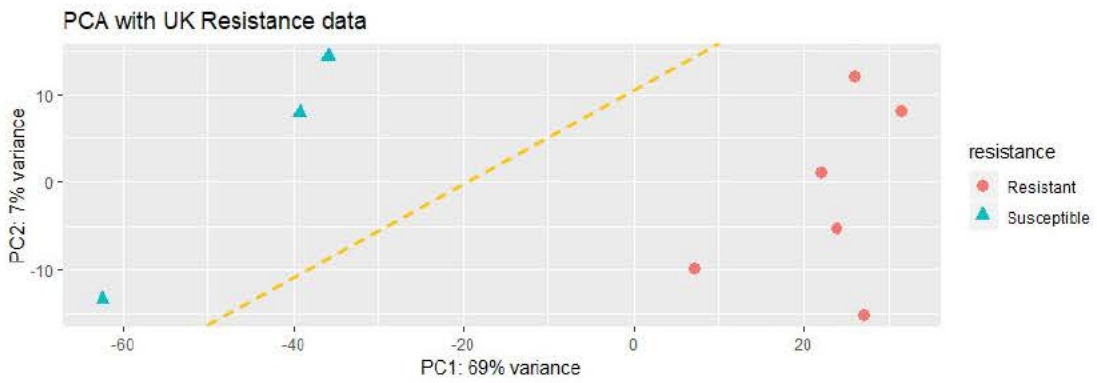
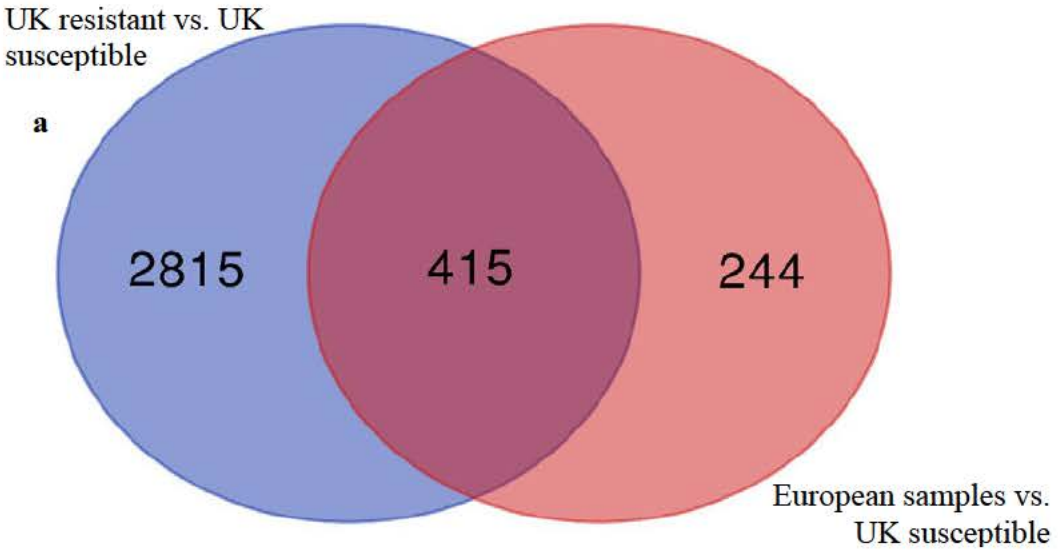


Figure 4.6. *Principle component analysis using UK samples alone.*

4.4.2 Identifying differentially expressed genes

To identify any differentially expressed genes (DEGs) potentially responsible for metabolic resistance in the UK samples, a pairwise comparison between (a) the UK resistant and UK susceptible samples and (b) the Swiss and German samples and the UK susceptible samples was conducted using DESeq2. The susceptible and resistant Swiss and German samples were grouped together because they were found to be genetically separated from the UK samples (Figure 4.5), and to date only UK samples of *P. chrysocephala* have been suggested to have metabolic resistance mechanisms (Højland et al., 2015; Højland and Kristensen, 2018). This means there should not be any upregulation of genes involved in metabolic resistance in the Swiss or German resistant samples. From the two normalised count files of differentially expressed genes (filtered adjusted p-value <0.05, log2foldchange >2), of the 3230 DEGs identified as up-regulated in the UK resistant vs UK susceptible beetles, 415 of these were also up-regulated in the European samples vs the UK susceptible beetles (and therefore were not considered to be involved in metabolic resistance). Based on the Venn diagram analysis, 2815 DEGs were identified as unique to, and up-regulated in, the UK resistant vs UK susceptible beetles (Figure **4.7**). The number of DEGs identified was much higher than anticipated. In previous studies, 47 microarray probes were differentially expressed between resistant mosquito (*Anopheles gambiae*) and controls (Mitchell et al., 2012), whilst 75 microarrays were differentially expressed between a resistant strain of the red flour beetle (*Tribolium castaneum*) and a susceptible strain (Zhu et al., 2010). The high number of DEGs could be due to a few reasons. The first being significant genetic variation between the resistant and

susceptible UK samples due to the geographic separation of the susceptible sample (Scotland), and the resistant samples (England). However, the number of DEGs between the European samples and the UK susceptible sample was just 659 and these samples are much more separated geographically. Another reason could be due to the low number of pairwise comparisons. Edi et al (2014) found that when using multiple pairwise comparisons, 145 microarray probes were identified to be differentially expressed in the bendiocarb resistant mosquito (*Anopheles gambiae*) compared to the susceptible sample. When a lower number of pairwise comparisons were used, 2453 microarray probes were differentially expressed.



b

Comparisons	Number of DEGs	Number of unique DEGs
UK resistant vs UK susceptible	3230	2815
Europe vs UK susceptible	659	244

Figure 4.7. *a) Differentially expressed genes in RNA-Seq comparisons between susceptible vs resistant* P. chrysocephala *samples, b) number of DEGs identified.*

4.4.3 Gene ontology over-representation analysis

The 415 overlapping DEGs identified above were removed from the UK resistant vs UK susceptible DESeq2 list to give the final list of 2815 DEGs. This list was then imported into Blast2GO for annotation and over-representation analysis. GO-term enrichment was performed to determine the biological properties (biological process,

cellular component, and molecular function) of the over-represented genes. The over-representation analysis was conducted using Fishers exact test with a False discovery rate of <0.05. To identify which GO terms were over-represented, the GO terms of the 2815 differentially expressed transcripts were compared to the GO terms of the PGI reference genome. In total, 40 GO terms were over-represented with p-value <0.05 (Table 4.6). Of these 40 overrepresented GO terms, 22 were classified as 'Biological process', 11 as 'Cellular component' and 7 as 'Molecular function'. Whilst identifying the overrepresented terms is interesting at a higher level, the GO names are too unspecific to be useful on their own.

Table 4.6. *List of GO IDs, GO names and the corresponding GO category of the 40 overrepresented GO terms identified from the Fishers exact test.*

GO ID	GO Name	GO Category
GO:0007017	microtubule-based process	Biological process
GO:0006928	movement of cell or subcellular component	Biological process
GO:0051674	localization of cell	Biological process
GO:0048870	cell motility	Biological process
GO:0060285	cilium-dependent cell motility	Biological process
GO:0001539	cilium or flagellum-dependent cell motility	Biological process
GO:0007018	microtubule-based movement	Biological process
GO:0040011	locomotion	Biological process
GO:0044782	cilium organization	Biological process
GO:0060271	cilium assembly	Biological process
GO:0030031	cell projection assembly	Biological process
GO:0120031	plasma membrane bounded cell projection assembly	Biological process
GO:0007606	sensory perception of chemical stimulus	Biological process
GO:0003341	cilium movement	Biological process
GO:0030030	cell projection organization	Biological process
GO:0120036	plasma membrane bounded cell projection organization	Biological process
GO:1901657	glycosyl compound metabolic process	Biological process
GO:0009116	nucleoside metabolic process	Biological process
GO:0007608	sensory perception of smell	Biological process
GO:0050877	nervous system process	Biological process
GO:0007600	sensory perception	Biological process
GO:0006090	pyruvate metabolic process	Biological process
GO:0030286	dynein complex	Cellular component
GO:0015630	microtubule cytoskeleton	Cellular component
GO:0005875	microtubule associated complex	Cellular component
GO:0005929	cilium	Cellular component
GO:0042995	cell projection	Cellular component
GO:0120025	plasma membrane bounded cell projection	Cellular component
GO:0005856	cytoskeleton	Cellular component
GO:0005930	axoneme	Cellular component

GO:0032838	plasma membrane bounded cell projection cytoplasm	Cellular component
GO:0097014	ciliary plasm	Cellular component
GO:0099568	cytoplasmic region	Cellular component
GO:0008569	minus-end-directed microtubule motor activity	Molecular function
GO:0003777	microtubule motor activity	Molecular function
GO:0004984	olfactory receptor activity	Molecular function
GO:0051959	dynein light intermediate chain binding	Molecular function
GO:0045505	dynein intermediate chain binding	Molecular function
GO:0004731	purine-nucleoside phosphorylase activity	Molecular function
GO:0048038	quinone binding	Molecular function

4.4.4 Genes with a potential involvement in metabolic resistance

As the aim of the RNA-seq experiment was to identify upregulated genes potentially involved in metabolic resistance towards pyrethroids, genes from the P450, CCE, GST, ABC transporter, GPCR and UDP enzyme families were manually extracted from the annotated list of DEGs. This identified five candidate genes (Table 4.7) comprising two P450s, two ABC transporters and one UDP. No CCEs, GSTs or GPCRs were found to be upregulated in the UK resistant vs susceptible samples. Whilst all five candidate genes were significantly overexpressed with respect to the Scottish sample, MSTRG.20996 was found to be the most differentially expressed, with a log2fc of 3.91. RStudio was used to visualise the normalised count data from the UK resistant vs UK susceptible pairwise comparison. The comparison showed MSTRG.20996 to be significantly upregulated in the resistant samples and expressed only at a low level in the susceptible one (Figure 4.8). MSTRG.20996 was also found to be expressed at low levels in the Swiss and German susceptible and resistant samples (Figure 4.9). As MSTRG.20996 was also identified as the most differentially expressed gene when the annotated list of DEGs was filtered by the adjusted p-value (data not shown), it is a promising candidate gene for causing metabolic resistance in *P. chrysocephala.*

Table 4.7. *Genes of interest (GOIs) after differential expression analysis. Sequence description was allocated via Blast2GO using Blastp. Protein families were identified using InterPro. Log2fc = log2 fold chance in expression from DESeq2 analysis.*

Gene ID	Sequence description	Protein family	log2fc	logfc	p-adjust
MSTRG.20996	Cytochrome P450 6k1-like	Cytochrome P450	3.91	15.03	1.88E-42
MSTRG.9127	Multidrug resistance-associated protein 4-like	ABC transporter	2.64	6.23	2.43E-09
MSTRG.33631	Uridine phosphorylase 1 isoform X2	Uridine phosphorylase	2.68	6.41	1.18E-05
MSTRG.19133	Cytochrome P450 301a1	Cytochrome P450	3.04	8.22	3.47E-04
MSTRG.21839	Multidrug resistance-associated protein 4-like	ABC transporter	2.67	6.36	1.24E-03

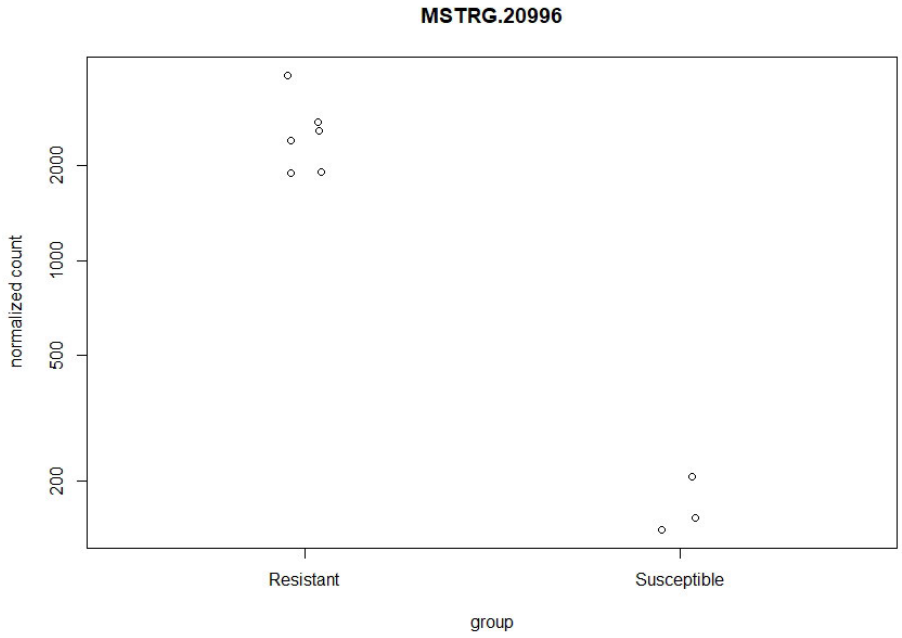


Figure 4.8. *Normalised count data for gene MSTRG.20996, a cytochrome P450, in a pairwise comparison between UK resistant and UK susceptible samples.*

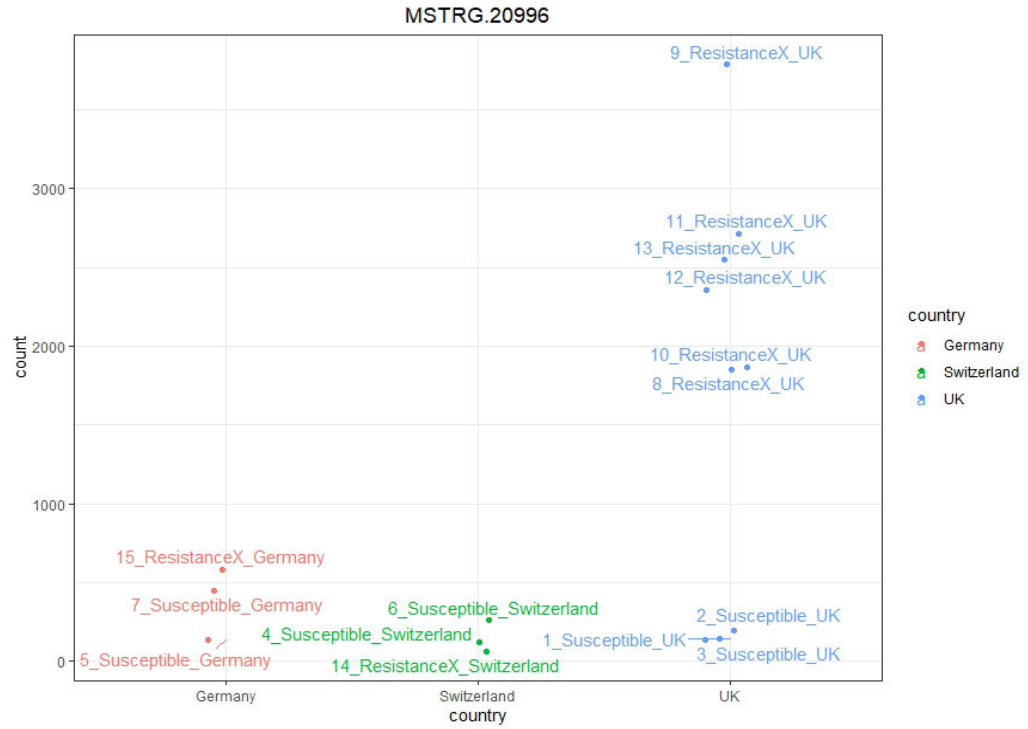


Figure 4.9. *Normalised count data for gene MSTRG.20996, in a pairwise comparison between all the UK, Swiss and German samples.*

Whilst all five candidate genes were from families encoding enzymes associated with metabolic resistance, the P450s were of particular interest because P450-mediated resistance is common in many insects (Hodgson and Kulkarni, 1983; Oppenoorth,

1985; Brattsten et al., 1986; Scott, 1999), and pre-treatment with the P450 inhibitor PBO was found to restore the toxicity of the reference pyrethroid lambda-cyhalothrin towards *P. chrysocephala* (see Chapter 3, section 3.3.5). Of the two P450 genes identified as potential candidates, MSTRG.20996 was of particular interest because it belongs to the cytochrome P450 monooxygenase 6 (*CYP6*) family. MSTRG.20996 is named *CYP6k1-like* as it shows 63% identity to the Western Corn Rootworm, (*Diabrotica virgifera virgifera*) cytochrome P450 *CYP6k1-like* (see Appendix 2). Previous studies have found that in situations where P450 overexpression confers insecticide resistance, the *CYP* gene involved most often belongs to the *CYP6* family. For example, constitutive overexpression of *CYP6CM1* has been found to correlate with imidacloprid resistance in the whitefly (*Bemisia tabaci*), with the resistant strains showing overexpression of ~17-fold (Karunker et al., 2009). In the malarial mosquito (*Anopheles gambiae*), *CYP6CM2* has been demonstrated to confer pyrethroid and DDT resistance (Stevenson et al., 2011; Mitchell et al., 2012) whilst *CYP6P3* has been found to correlate with both pyrethroid and carbamate resistance (Edi et al., 2014). The P450 *CYP6BQ9* has been found to play a role in pyrethroid resistance in the red flour beetle (*Tribolium castaneum*), showing more than 200-fold higher expression in the deltamethrin resistant strain (Zhu et al., 2010). Overexpression of *CYP6G1* is associated with DDT resistance in the fruit fly (*Drosophila melanogaster*) (Daborn et al., 2002). In the housefly (*Musca domestica*), overexpression of *CYP6D1* conferred resistance to pyrethroids (Kasai and Scott, 2000) and the overexpression of *CYP6A1* is linked to organophosphate resistance (Andersen, Utermohlen and Feyereisen, 1994).

4.4.5 Expression of *CYP6K1-like* in *P. chrysocephala* adults

As *CYP6K1-like* showed the most promise as a candidate gene conferring pyrethroid resistance in *P. chrysocephala* adults, the expression level of this gene was analysed by qPCR to validate the DESeq2 analysis. qPCR was carried out on the seven *P. chrysocephala* samples sent for RNA-seq analysis and a further six samples received from across the UK (see Chapter 2, section 2.1.1). For each additional sample site, total RNA was extracted from the pooled homogenate of three *P. chrysocephala* adults using the Isolate II RNA mini kit. By glass vial assay these additional samples were found to be either susceptible or 100% resistant to lambda-cyhalothrin at the recommended field rate (section 4.2.1 and 4.2.2). qPCR examined relative gene

expression levels in the UK resistant *P. chrysocephala* samples with respect to the UK susceptible samples and the susceptible and resistant European samples.

CYP6K1-like was found to be overexpressed in all the UK resistant samples (A-G) compared to the UK susceptible samples (Figure 4.10); overexpression ranged from ~80 fold to ~200 fold. Analysis of variance (ANOVA) showed that *CYP6K1-like* was significantly overexpressed in all UK resistant samples, with P values ranging from $P<0.05$ to $P<0.0001$. In contrast, there was no significant difference in gene expression between the European susceptible and resistant samples, and the UK susceptible samples. As *P. chrysocephala* adults from Switzerland and Germany are not yet thought to have developed a metabolic resistance mechanism, with Højland *et al.,* 2015 finding good correlation between the presence of the *kdr* allele and survival at high concentrations of lambda-cyhalothrin in German *P. chrysocephala*, the significant overexpression of *CYP6K1-like* in all of the UK resistant samples compared to the European and UK susceptible samples strongly suggests the involvement of this gene in conferring pyrethroid resistance in UK *P. chrysocephala* adults.

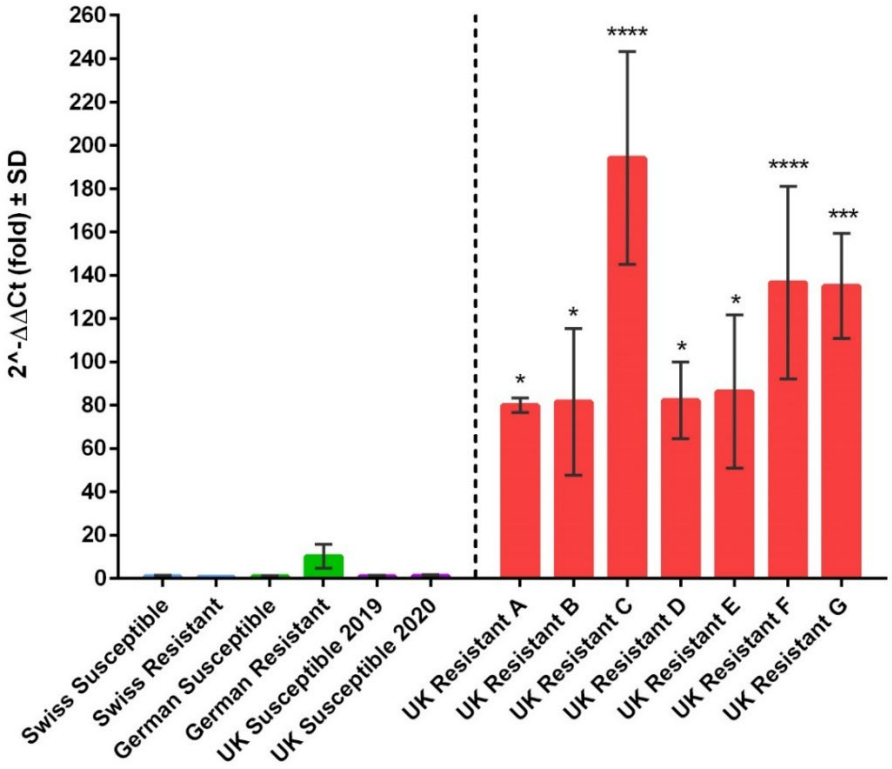


Figure 4.10. *Relative expression of CYP6K1-like in two UK susceptible (purple), seven UK resistant (red), one Swiss susceptible and resistant (blue) and one German susceptible and*

*resistant (green) sample(s) of P. chrysocephala. Error bars show the standard deviation. P values are shown as asterisks where * = P < 0.05, ** = P < 0.01, *** = P < 0.001 and **** = P < 0.0001. P values denoted by asterisks compare each mobile or affected sample to the 2018 UK susceptible sample.*

4.4.6 Expression of *CYP6K1-like* in *P. chrysocephala* larvae

As synergist bioassays with PBO pre-treatments were found to increase the susceptibility of 3rd instar *P. chrysocephala* larvae to lambda-cyhalothrin at 20% of the recommended field rate (section 3.3.6.2), the expression level of *CYP6K1-like* was analysed by qPCR to investigate whether overexpression of *CYP6K1-like* is specific to adult *P. chrysocephala*. qPCR was carried out on 3rd instar *P. chrysocephala* larvae collected from the Rothamsted Research farm in 2019. By leaf dip assay these larvae were found to be 50% resistant to lambda-cyhalothrin at the 100% field rate (see Chapter 3, section 3.3.6.1). Total RNA was only extracted from larvae that that survived the bioassay to overcome the problem of post-mortem RNA degradation. qPCR examined relative gene expression levels in *P. chrysocephala* larvae compared to *P. chrysocephala* adults also collected from the Rothamsted farm in 2019 and the UK susceptible sample.

CYP6K1-like was significantly overexpressed in the *P. chrysocephala* larvae with respect to the UK adult susceptible sample (two-sample t-test, $p<0.05$) (Figure 4.11). There was no significant difference in mean expression level between the Rothamsted larvae and the Rothamsted adults (two-sample t-test, $p= 0.098$). As the larvae showed a degree of resistance to lambda-cyhalothrin, with synergist bioassays previously showing increased susceptibility of 3rd instar *P. chrysocephala* larvae to lambda-cyhalothrin after pre-treatment with PBO (Chapter 3, section 3.3.6.2), the involvement of a metabolic resistance mechanism and therefore upregulation of *CYP6K1-like* was not entirely unexpected. This result contrasts with that previously reported by Jones et al (2011), who found overexpression of *CYP6CM1* was correlated with life-stage specific resistance in whitefly (*Bemisia tabaci*). Adult *B. tabaci* were found to be resistant to the neonicotinoid imidacloprid whilst the nymphs were found to be susceptible. Amenya et al (2008) found that in the pyrethroid-resistant malaria vector *Anopheles funestus, CYP6P9* was not significantly overexpressed in the larvae, but it was overexpressed in the eggs and the adults.

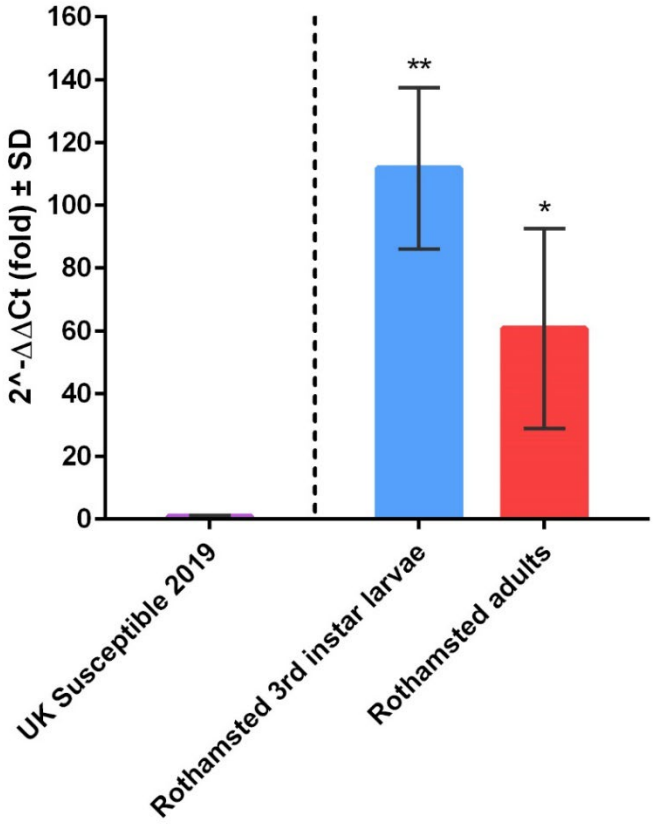


Figure 4.11. *Relative expression of MSTRG.20996 in a UK susceptible sample (purple), a sample of Rothamsted 3rd instar larvae (blue) and Rothamsted adult beetles (red). Error bars show the standard deviation. P values are shown as asterisks where * = P < 0.05 and ** = P < 0.01. P values denoted by asterisks compare each mobile or affected sample to the UK susceptible sample.*

4.4.7 Expression of *CYP6K1-like* in *P. chrysocephala* affected by pyrethroid

The significant overexpression of *CYP6K1-like* in both *P. chrysocephala* adult and larvae samples resistant to lambda-cyhalothrin suggests the possible role of this gene in a metabolic resistance mechanism. The involvement of this gene was therefore further validated by investigating its expression level in two of the *P. chrysocephala* samples that displayed lower levels of resistance. Differences in gene expression were examined between adults categorised as either mobile or affected within the same sample, after exposure to lambda-cyhalothrin by glass vial bioassay (Chapter 2, section 2.2.2). Due to RNA degradation post-mortem, gene expression level could not be examined and compared in *P. chrysocephala* that did not survive the assay. The two samples were collected from oilseed rape fields in the UK in 2019. Sample 1 (n=10) was found to be 50% resistant to lambda-cyhalothrin at the recommended field

rate and sample 2 (n=10) was 30% resistant. As there were only small numbers of beetles available in each category, total RNA was extracted from the pooled homogenate of just two *P. chrysocephala* adults. qPCR examined relative gene expression levels in *P. chrysocephala* from sample 1 and 2 compared to the UK susceptible sample.

The mean expression level of *CYP6k1-like* was significantly greater in both sample 1 and 2 compared to the UK susceptible sample (ANOVA, $F_{4,9}$ =190.5, p <0.0001) (Figure 4.12). *CYP6k1-like* was significantly overexpressed in the *P. chrysocephala* unaffected by lambda-cyhalothrin, in both sample 1 (two-sample t-test, p <0.0001) and sample 2 (two-sample t-test, p <0.0001), with respect to the UK adult susceptible sample. *CYP6k1-like* was also significantly overexpressed in *P. chrysocephala* affected by lambda-cyhalothrin in both sample 1 (two-sample t-test, p <0.05) and sample 2 (two-sample t-test, p<0.05), with respect to the UK susceptible sample. However, although the mean expression level of *CYP6k1-like* was significantly greater in both samples of *P. chrysocephala* affected by lambda-cyhalothrin with respect to the susceptible sample, there was also found to be a significant difference in *CYP6k1-like* expression level between the mobile and affected *P. chrysocephala* in sample 1 (two-sample t-test, p <0.05) and sample 2 (two-sample t-test, p <0.001). This difference in expression level between the mobile and affected *P. chrysocephala* was not entirely unsurprising. It was expected that the *P. chrysocephala* found to be mobile after exposure to lambda-cyhalothrin would express high levels of *CYP6k1-like* as observed in samples (A-G) whilst the *P. chrysocephala* affected by lambda-cyhalothrin would be either homozygous for the target-site resistant *kdr* allele (RR), conferring moderate resistant to pyrethroid (Davies et al., 2007), or overexpress *CYP6k1-like* to some degree. Although *s-kdr* has been found to confer higher levels of resistance to pyrethroids, the percentage of beetles homozygous for the *s-kdr* resistant allele (RR) was just 7.7% in 2019. Unfortunately, there were not enough beetles to determine the presence of the L1014F (*kdr*) and L925I (*s-kdr*) mutations in the mobile and affected *P. chrysocephala* samples using TaqMan assays.

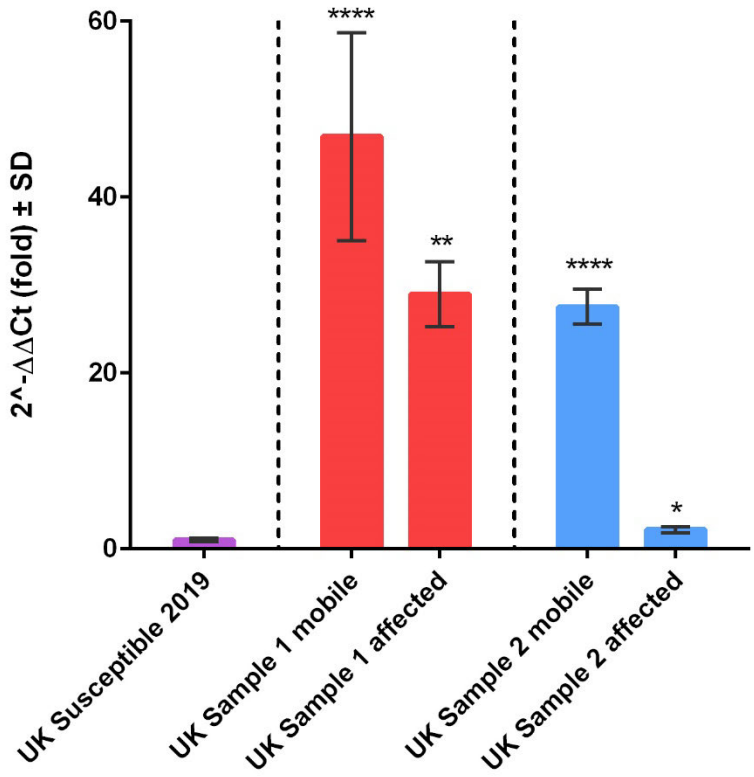


Figure 4.12. *Relative expression of CYP6k1-like in a UK susceptible (purple) and two UK resistant samples of P. chrysocephala. Error bars show the standard deviation. P values are shown as asterisks where * = P < 0.05, ** = P < 0.01, *** = P < 0.001 and **** = P < 0.0001. P values denoted by asterisks compare each mobile or affected sample to the UK susceptible sample.*

4.4.8 Expression of *MRP4-like* in *P. chrysocephala* adults

To determine whether any additional candidate genes were playing a role in conferring metabolic resistance, the expression level of the ABC transporter *MRP4-like* was analysed by qPCR. *MRP4-like* was selected because it was identified as the second most differentially expressed gene from the differential gene expression analysis. *MRP4-like* was expressed at very low levels in the two UK resistant samples (F and G). There was no statistically significant difference in gene expression in the UK resistant samples with respect to the UK susceptible sample (ANOVA, p=0.44).

CYP6k1-like had a p-adjusted value of 1.88E-42 after differential expression analysis and was found to be overexpressed in all UK resistant samples (A-G). In contrast *MRP4-like* had a larger p-adjusted value of 2.43E-09 and was found to be lowly expressed in two UK resistant samples. As the other three candidate genes had even

higher p-adjusted values than *MRP4-like,* their expression level in UK susceptible vs UK resistant samples was not analysed by qPCR.

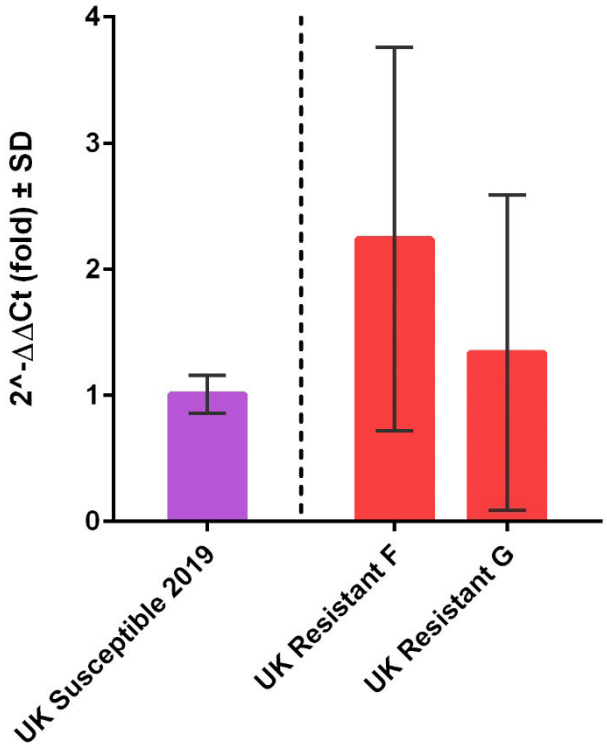


Figure 4.13. *Relative expression of MRP4-like in a UK susceptible (purple) and two UK resistant (red) samples of P. chrysocephala. Error bars show the standard deviation.*

4.5 Conclusions

Understanding the molecular basis of the mechanism underlying pyrethroid resistance in *P. chrysocephala* is important, in order to be able to effectively manage insecticide resistance through the development and application of appropriate resistance monitoring diagnostics, as well as the development and potential use of new insecticides. In this chapter, RNA sequencing was used to study the transcriptional profile of susceptible and resistant *P. chrysocephala* samples from across the UK and Europe, and bioinformatic analysis techniques were used to identify the potential enzyme(s) involved in conferring metabolic resistance to pyrethroids in *P. chrysocephala*. qPCR was used to validate the candidate genes identified.

By bioinformatic analysis, a total of 2,815 DEGs were identified as unique to, and up regulated in the UK resistant vs UK susceptible *P. chrysocephala* samples. Of these

2,815 DEGs, the cytochrome P450 *CYP6k1-like* was found to be the most differentially expressed. As genes from the CYP6 family have been previously implicated in pyrethroid resistance, *CYP6k1-like* was identified as a strong candidate gene for further investigation.

qPCR analysis showed that *CYP6k1-like* is over expressed in UK resistant samples, in both 3rd instar larvae and adult beetles, strongly suggesting its involvement in the metabolic resistance mechanism. The next chapter (chapter 5) looks to further validate the involvement of *CYP6k1-like* in the metabolic resistance mechanism using RNA interference techniques.

4.6 Key findings

- By bioinformatic analysis, *CYP6k1-like* was identified as the most differentially expressed gene in *P. chrysocephala* adults when the differentially expressed gene list was filtered by p-adjusted value.
- Bioinformatic and qPCR analysis showed that *CYP6k1-like* was highly expressed in the UK resistant samples of *P. chrysocephala* adults and moderately expressed in the UK susceptible and Swiss and German samples.
- qPCR analysis similarly found that *CYP6k1-like* was highly expressed in the UK sample of 3rd instar *P. chrysocephala* larvae.
- qPCR analysis found that *CYP6k1-like* was not as highly expressed in the affected *P. chrysocephala* adults as in the mobile *P. chrysocephala* adults but was still significantly higher than in the susceptible samples.

Chapter 5. Validation of a candidate gene for conferring metabolic resistance by RNA interference

5.1 Aims

This chapter aims to investigate the RNAi knockdown effect in *P. chrysocephala* adults and larvae following the delivery of dsRNA targeting the candidate resistance gene *CYP6k1-like*.

5.2 Introduction

RNA interference (RNAi), first demonstrated in the nematode *Caenorhabditis elegans,* describes the biological process of gene-specific degradation of endogenous mRNA resulting from the introduction of double-stranded RNA (dsRNA) (Fire et al., 1998; Waterhouse, Graham and Wang, 1998). In this mechanism, mRNA is targeted by an exogenous dsRNA sequence at least 60 base pairs (bp) in length (Bolognesi et al., 2012), that contains a strand complementary to a section of the target gene to be degraded (Mito et al., 2011). As RNAi results in the inactivation of specific genes it is being increasingly used for determining gene function (Tabara, Grishok and Mello, 1998; Kamath et al., 2001) and is proposed to be the next control strategy for managing insect pests (Gordon and Waterhouse, 2007; Price and Gatehouse, 2008; Huvenne and Smagghe, 2010; Joga et al., 2016). In 2007, two groups demonstrated the potential of dsRNA expression in transgenic plants for the control of insect pests. Baum et al (2007) demonstrated that feeding damage by the Western corn rootworm (WCR), (*Diabrotica virgifera virgifera*), was significantly reduced in transgenic corn plants expressing WCR dsRNA directed against V-ATPase A, whilst Mao et al (2007) showed that larval tolerance towards gossypol was reduced in the cotton bollworm (*Helicoverpa armigera*), when fed transgenic plants expressing cytochrome P450 *CYP6AE14* dsRNAs. Over the last decade, the increased understanding of dsRNA delivery systems and ability to identify target genes has further enhanced the potential of RNAi as a species-specific, environmentally friendly method of crop protection, both advantageous benefits over conventional chemical insecticides and biological control (Bramlett, Plaetinck and Maienfisch, 2020; Zhu and Palli, 2020; Mezzetti et al., 2020).

Whilst RNAi has been successfully applied in species from many orders such as Diptera (e.g. Li, Zhang and Zhang, 2011), Hemiptera (e.g. Araujo et al., 2006; Mao et al., 2007; Zha et al., 2011), Lepidoptera (e.g. Turner et al., 2006; Bautista et al., 2009; Bento et al., 2020), Dictyoptera (e.g. Revuelta et al., 2009; Guo et al., 2010) and Hymenoptera (e.g. Hunter et al., 2010), the level of efficacy has been found to be highly variable (Cooper et al., 2019; Zhu and Palli, 2020). However, in insects belonging to the order Coleoptera, RNAi has been found to work quite effectively, with small amounts of dsRNA delivered either orally or by microinjection often inducing a strong systemic response (e.g. Baum et al., 2007; Tomoyasu et al., 2008; Bolognesi et al., 2012; Powell et al., 2017). To date, successful induction of RNAi has been observed in a range of Coleopteran species including the Colorado potato beetle (*Leptinotarsa decemlineata*) (e.g. Baum et al., 2007; Zhu et al., 2011), ladybird beetle (*Adalia bipunctata* and *Coccinella septempunctata*) (Haller et al., 2019), African sweet potato weevil (*Cylas puncticollis*) (Prentice et al., 2017) and the Western corn rootworm (*D. virgifera virgifera*) (e.g. Baum et al., 2007; Rangasamy and Siegfried, 2012).

Over the last 20 years, multiple approaches have been used to deliver dsRNA to insects such as microinjection (e.g. Gatehouse et al., 2004; Tomoyasu and Denell, 2004), oral feeding of artificial diet (e.g. Timmons and Fire, 1998; Timmons, Court and Fire, 2001), soaking insects (e.g. Tabara, Grishok and Mello, 1998; Orii, Mochii and Watanabe, 2003; Yu, Gowda and Killiny, 2017) and transgenic plants (e.g. Mao et al., 2011; Pitino et al., 2011; Xiong et al., 2013). Of these, dsRNA is most often delivered by microinjection as it provides a more efficacious and robust knockdown effect than ingestion (Cao, Gatehouse and Fitches, 2018). Despite this, this delivery method is laborious, demands precision and cannot be used to control insect pests in the field. Conversely, oral delivery of dsRNA has the advantage of being non-invasive, less labour intensive (meaning it can be used to screen a large number of insects for gene function (Kamath et al., 2001)) and cheaper (as it does not require *in vitro* synthesized dsRNA (Kamath and Ahringer, 2003)). Delivery of dsRNA onto the crop by foliar sprays would allow the insect to naturally ingest the dsRNA (San Miguel and Scott, 2016) and fits with the current method of delivering a conventional insecticide (Huvenne and Smagghe, 2010; Zhu et al., 2011).

dsRNA for spraying onto a crop can be synthesized *in vitro* or produced in bacteria. In 1998, Timmons and Fire successfully induced RNAi in *Caenorhabditis elegans* using *Escherichia coli* to deliver the dsRNA (Timmons and Fire, 1998); Newmark et al induced RNAi in planarians (flatworms) using the same method in 2003 (Newmark et al., 2003). Compared to *in vitro* synthesis of dsRNA, bacterially expressed dsRNA is more cost-effective to produce, can be produced in large amounts and can therefore be more easily used to determine gene function in a large number of insects (Kamath et al., 2001).

Here, we determine whether microinjection of *in vitro* synthesised dsRNA, or feeding of dsRNA produced either *in vitro* or in bacteria, was able to silence the candidate metabolic resistance gene *CYP6K1-like* in *P. chrysocephala,* and whether pyrethroid insecticide toxicity could be restored after pre-treating with dsRNA.

5.3 Material and Methods

5.3.1 Insects

P. chrysocephala adults were obtained from freshly harvested oilseed rape fields at Rothamsted Research in July 2020 and 2021. The beetles were maintained in mesh cages in a controlled environment cabinet at 15±1°C, 65% relative humidity, 12:12h light: dark photoperiod on a diet of Chinese cabbage plants (see Chapter 2, section 2.1.1). Third instar *P. chrysocephala* larvae were collected in March 2021 from oilseed rape plants also grown on the Rothamsted Research farm. Larvae were extracted from the oilseed rape by dissection of the main stems as described in Chapter 2, section 2.1.2. The larvae were kept in falcon tubes with moist tissue paper and maintained at 4°C until testing.

5.3.2 Candidate gene selection

The cytochrome P450 *CYP6K1-like* was selected for further validation by RNAi studies based on the results discussed in Chapter 4.

5.3.3 Primer design

Primers (Table 5.1) were designed to amplify PCR products of 400 - 500bp for both the candidate gene (*CYP6k1-like*) (see Appendix 3 for the DNA sequence) and the control gene (*GFP*) (amplified from the L4440/GFP plasmid #11335, Addgene). For

in vitro dsRNA synthesis, the T7 promoter sequence was added to the forward and reverse primer for both the candidate gene and *GFP*. For the bacterial expressed dsRNA, the restriction site *Sal*I was added to the forward primer and *Nhe*I added to the reverse primer for the candidate gene; the restriction site *Kpn*I was added to the forward primer and *Xba*I added to the reverse primer for the *GFP* gene.

Table 5.1. *Sequence of forward (F) and reverse (R) primers used for dsRNA synthesis and cDNA cloning. Sequence in red shows the restriction site. Sequence highlighted in grey shows the T7 promoter sequence.*

Primer	Sequence
dsCYP6 SalI F	TAAGAGTCGACCGGTCTGCAGTTTTTCGTG
dsCYP6 NheI R	GAATGCTAGCGGGTGTCTGGGCACATCTTT
dsGFP KpnI F	CGAGGTACCTTTCAAGAGTGCCATGCCCG
dsGFP XbaI R	GCTCTAGAGGCATTGTGTGGACAGGT
dsCYP6 F	TAATACGACTCACTATAGGGCGGTCTGCAGTTTTTCGTGG
dsCYP6 R	TAATACGACTCACTATAGGGGGTGTCTGGGCATCTTT
dsGFP F	TAATACGACTCACTATAGGGGGCAGATTGTGTGGACAGGT
dsGFP R	TAATACGACTCACTATAGGGTTTCAAGAGTGCCATGCCCG
T7	TAATACGACTCACTATAGGG

5.3.4 Total RNA extraction and cDNA synthesis

Live *P. chrysocephala* adults were snap frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. RNA was extracted from the pooled homogenate of three *P. chrysocephala* adults per sample using the Isolate II RNA mini kit (see Chapter 2 section 2.3.3). The RNA was quantified using the NanoDrop™ 1000 Spectrophotometer. cDNA was then synthesised from approximately 400ng of total RNA using the ThermoScientific SuperScript III First-strand cDNA synthesis kit (see Chapter 2, section 2.3.16).

5.3.5 *In vitro* dsRNA synthesis

A ~500bp fragment of the target gene *CYP6k1-like* was PCR amplified from *P. chrysocephala* cDNA using hotshot PCR mastermix (Client Life Science, Stourbridge, UK) and the gene specific primers dsCYP6 F and dsCYP6 R (Table 5.1). The primers were designed using the genomic DNA as a template. A ~400bp fragment of the control gene Green Fluorescent Protein (GFP) was amplified from the L4440/GFP plasmid using the dsGFP F and dsGFP R primers. The PCR cycling conditions

consisted of an initial denaturation step at 95ºC for 5 minutes, 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 40 seconds, and a final extension step at 72ºC for 5 minutes. The PCR products were run on a 1% w/v agarose gel to confirm the sequences had been amplified. The products were then used as a template for *in vitro* dsRNA synthesis using the Megascript T7 kit (Ambion Inc., Waltham, MA, USA). Reaction volumes were typically 20µl and comprised 2µl 10X reaction buffer, 2µl T7 enzyme mix, 2µl ATP, 2µl CTP, 2µl GTP and 2µl UTP solution, 4µl PCR product and sterile distilled water up to the required 20µl volume. The reaction was mixed thoroughly by pipetting up and down before being incubated at 37ºC overnight. After the incubation period, 1µl of TURBO DNase (Ambion Inc) was added to the reaction. The mixture was then incubated again at 37ºC for an additional hour. The dsRNA was quality checked by running on an agarose gel.

Lithium chloride precipitation was performed on the dsRNA product to remove unincorporated nucleotides and proteins. $1/10^{th}$ volume of lithium chloride precipitation solution (7.5M lithium chloride, 50mM EDTA) and three volumes of 95% ethanol were added to the dsRNA and mixed thoroughly by vortexing. Reactions where then placed at -20°C for 30 minutes to allow for precipitation. The reaction was then centrifuged for 15 minutes at 11,000 x g to pellet the dsRNA. The supernatant was removed and discarded, and the remaining dsRNA pellet washed with 500µl of 75% ethanol. The reaction was then centrifuged again for 5 minutes at 11,000 x g to pellet the dsRNA once again. The ethanol was removed, and the tube left for 10 minutes to allow the remaining ethanol to evaporate. The dsRNA was then resuspended in 20µl of sterile distilled water and quantified using a spectrophotometer.

5.3.6 Plasmid constructs for bacterial expression of dsRNA

A ~500bp fragment of the target gene *CYP6k1-like* was amplified from *P. chrysocephala* cDNA by PCR using hotshot PCR mastermix and the gene specific primers dsCYP SalI F and dsCYP NheI R (Table 5.1). The primers were designed using the genomic DNA as a template. A ~400bp fragment of the control gene *GFP* was amplified using the dsGFP KpnI F and dsGFP XbaI R primers. The PCR cycling conditions consisted of an initial denaturation step at 95ºC for 5 minutes, 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 55ºC for 30 seconds, extension at

72ºC for 40 seconds, and a final extension step at 72ºC for 5 minutes. The PCR products were run on a 1% w/v agarose gel to confirm the complete sequence had been amplified. The L4440 plasmid (plasmid #1654, Addgene) was chosen as the cloning vector as it contains T7 promoter sites on either side of the multiple cloning site (MCS). For the target gene, the PCR product and expression vector L4440 were digested with the *Sal*I and *Nhe*I FastDigest restriction enzymes (ThermoFisher Scientific). For the control gene, the PCR product and expression vector L4440 were digested with the *Kpn*I and *Xba*I FastDigest restriction enzymes (see section 2.3.7). The products were then run on a 1% w/v agarose gel alongside the undigested PCR product and L4440 vector to confirm successful digestion. The digested target/control gene products and L4440 vector were then excised from the agarose gel and purified using the Wizard SV Gel and PCR Clean-up System (Promega) (see section 2.3.6). A ligation reaction containing ~25ng of the target/control gene DNA and ~75ng L4440 vector was carried out overnight (see section 2.3.8). The ligated plasmids were transformed into chemically competent DH5α *E. coli* (ThermoFisher Scientific) as described in Chapter 2, section 2.3.9. Colonies were picked and grown in 5ml LB media supplemented with 50µg/ml ampicillin (section 2.3.11), purified (section 2.3.12) and verified by restriction digestion analysis and DNA sequencing (section 2.3.13)

5.3.7 Bacterial expression of dsRNA

The purified L4440-CYP6k1-like plasmid and the control L4440-GFP plasmid were transformed into the *E. coli* bacterial strain HT115(DE3) (Nova lifetech Limited, Hong Kong) as described in Chapter 2, section 2.3.9. This strain of *E. coli* was used for the production of dsRNA as it contains the T7 polymerase encoding gene and is deficient in RNase III (Timmons, Court and Fire, 2001; Ongvarrasopone, Roshorm and Panyim, 2007) meaning it can produce large quantities of dsRNA specific to the target gene. Single colonies of HT115(DE3) bacteria containing the cloned L4440-Cyp6k1-like and L4440-GFP plasmid were picked from reference plates and inoculated in 5ml LB media with 50µg/ml ampicillin and 12.5µg/ml tetracycline. The cultures were incubated at 37°C with shaking at 220rpm overnight. The overnight cultures were then diluted 1:100 in 2x YT broth (Formedium, Hunstanton, UK) supplemented with 50µg/ml ampicillin and 12.5µg/ml tetracycline and grown for ~2 hours with shaking at 37°C to an optical density (OD) of 0.4 at 595nm. The OD was

determined using the Cary 50 probe UV-visible spectrophotometer (Varian, CA, USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich**)** was then added to a concentration of 0.4mM (to induce expression of the T7 polymerase) and the culture incubated at 37°C with shaking at 220rpm for 4 hours. As the T7 promoter sites are either side of the MCS in the L4440 plasmid, this results in the bidirectional transcription of complementary RNA strands, generating double stranded RNA. For both HT115(DE3) containing L4440-CYP6k1-like, and HT115(DE3) containing L4440-GFP, 1.5ml of overnight bacterial culture was used as a control and no IPTG was added.

5.3.8 Extraction of dsRNA (TRIzol® reagent extraction)

To analyse the dsRNA expressed in the bacteria HT115(DE3), total RNA was isolated from 1.5ml of the control and 1.5ml of the IPTG induced samples using the TRIzol® reagent (ThermoFisher Scientific) extraction method, according to the protocol provided by the manufacturer. The bacterial culture was harvested by centrifugation at 8,000 x g for 2 minutes at room temperature. The supernatant was then discarded, the pelleted cells resuspended in 1ml of TRIzol® reagent and incubated at room temperature for 15 minutes to allow the nucleoprotein complex to dissociate. 200µl of analytical grade chloroform (Thermo Fisher Scientific) was then added per 1ml of TRIzol®, the Eppendorf tube inverted 4 – 6 times and incubated at room temperature for 10 minutes. The sample was then centrifuged at 12,000 x g for 10 minutes at 4°C. The upper aqueous phase was transferred to a new Eppendorf tube and 500µl of 2-propanol (Sigma-Aldrich) added per 1ml of TRIzol®. The sample was then incubated at -20°C for 20 minutes before being centrifuged at 12,000 x g for 15 minutes at 4°C. The supernatant was discarded, the pellet washed in 1ml of 75% ethanol per 1ml of TRIzol® used and centrifuged at 7500 x g for 5 minutes at 4°C. The supernatant was then removed, the RNA pellet left to air dry for ~10 minutes and then resuspended in 50µl of sterile distilled water.

5.3.9 Analysis of dsRNA produced by bacterial expression

The total RNA was quantified using a spectrophotometer and approximately 2µg of each sample run on a 1% w/v agarose gel to determine whether the induction of dsRNA synthesis was successful. The bacteria culture was then heat killed by incubation in a water bath at 85°C for 15 minutes.

5.3.10 Delivery of dsRNA to *P. chrysocephala*

5.3.10.1 Experimental Workplan

<table>
<tr><td>hrs</td><td colspan="2">Microinjection of in-vitro synthesised dsRNA</td><td>Direct feeding assays with dsRNA</td></tr>
<tr><td></td><td>adults</td><td>larvae</td><td>adults</td><td>adults</td></tr>
<tr><td>0-24</td><td colspan="2" rowspan="2">Maintained at 18°C for 48hrs then subjected to qPCR analysis</td><td>Maintained at 18°C for 24hrs</td><td rowspan="2">Fed for 48hrs with either in vitro synthesised dsRNA or bacterially expressed dsRNA, then subjected to qPCR analysis</td></tr>
<tr><td>24-48</td><td rowspan="2">Subjected to pyrethroid bioassay</td></tr>
<tr><td>48-72</td><td colspan="2"></td><td></td></tr>
</table>

5.3.10.2 Microinjection bioassay method

The efficiency of dsRNA knockdown was investigated by microinjection bioassays. *In vitro* synthesised dsRNA was injected into both *P. chrysocephala* adults and third instar larvae. Third instar *P. chrysocephala* larvae were used as previous work showed that third instar larvae are more resistant to lambda-cyhalothrin than the other instars (see Chapter 3, section 3.3.6.1) and their bigger size makes them more amenable to injection than the smaller second instar larvae.

5.3.10.2.1 *P. chrysocephala* adults

P. chrysocephala adults were placed in a 50ml Falcon tube and, using a Flystuff pistol-grip blowgun (Scientific Laboratory Supplies, Nottingham, UK), anaesthetised by exposure to CO_2 for one minute. The beetles were then transferred to a Flypad connected to a Flowbuddy (Scientific Laboratory Supplies) set at 5 l/min to ensure they remained anaesthetised whilst being manually manipulated. The beetles were arranged laterally between two microscope slides using forceps and were secured in place by double sided sticky tape to minimise movement (see Figure 5.1 for the experimental set up). Injection of *in vitro* synthesised dsRNA into *P. chrysocephala* was carried out using a NanojectII™ injector (Drummond Scientific company, Broomall, PA, USA) fitted with a fine glass needle. The injection needles were pulled from glass capillaries (quartz with filament, O.D.: 1mm, I.D.: 0.7mm, 10cm length) (World Precision Instruments, Hitchin, UK) using a P-2000 micropipette puller (Sutter Instrument Co, Novato, USA) using the setting "Heat = 800, Fil = 4, Del = 145, Pull

= 175, Vel = 60". To prepare the injection needle, the pulled needle was first secured to a microscope slide using blue tack. Whilst looking through a SZ40 Olympus microscope (Olympus Life Science, UK), the needle was broken using forceps at the section where the tip bends. The glass needle was then backfilled with Halocarbon oil 27 (Sigma-Aldrich, Missouri, USA) using a 10ml Burkard syringe (Burkard Manufacturing, Rickmansworth, UK) to prevent bubbles forming in the dsRNA solution. The needle was then front filled with dsRNA solution using the 'fill' button on the NanoJectII. Injection of dsRNA into the beetles was carried out under a SZ40 Olympus microscope fitted with a Nikon 10x eyepiece. The needle was inserted at an angle of approximately 30°, aimed towards the head of the beetle, into the lateral side of the thorax to avoid damaging organs or the CNS. The needle was pulled back slightly after insertion into the beetle and the 'inject' button pressed to deliver the specified dose of dsRNA solution into the body cavity. dsRNA solution was injected at fast speed. 210nl of ds*CYP6k1-like* (or ds*GFP* control), diluted to a concentration of 1.2µg/µl using sterile distilled water, was injected to give a dose of 250ng per beetle. After injection the beetles were kept on ice for 30 minutes to prevent bleed back. The beetles were then maintained in Falcon tubes in a controlled environment room at 18±1°C, 16:8h light: dark photoperiod, on a diet of Chinese cabbage plants for 48 hours. After 48 hours the samples were snap frozen in liquid nitrogen for gene expression analysis by qPCR. Susceptibility of *P. chrysocephala* to lambda-cyhalothrin was investigated 24 hours post injection.

Figure 5.1. *Experimental set up for microinjection. P. chrysocephala adults were arranged laterally between two microscope slides and secured in place using double sided sticky tape. Holes in the tape allowed CO_2 to be supplied continuously by a Flypad, keeping the beetles anaesthetised whilst arranging them between the slides.*

5.3.10.2.2 *P. chrysocephala* larvae

The methodology used for larval injection is based on Linz et al. (2014). 1ml of 10x injection buffer was prepared by mixing 10µl of 0.1M sodium phosphate buffer (10ml made by mixing 8.5ml of 1M Na_2HPO_4 with 1.5ml of 1M NaH_2PO_4 and adjusting to pH 7.6) with 100µl of 0.5M KCl, 200µl red food dye and 690µl sterile distilled water. The 10x injection buffer was then diluted to make a 2x injection buffer (200µl of 10x injection buffer and 800µl sterile distilled water); both buffers were stored at 4°C until use. *P. chrysocephala* larvae were anaesthetised using ether. To make an etherisation basket, the plunger was removed from a 20ml disposable syringe and the syringe cut at the 15ml mark. A piece of mesh was then secured to the top half of the syringe using masking tape. 30ml of ether was then added to a 250ml Duran bottle containing several balls of cotton wool to help the ether evaporate (see Figure 5.2B for the experimental set up). To anaesthetise the *P. chrysocephala* larvae, the larvae were placed in the etherisation basket which was then placed into the ether bottle. The larvae were exposed to the ether for two minutes. After anaesthetisation, the larvae were arranged laterally between two microscope slides using forceps and secured in place by double sided sticky tape to minimise movement (see Figure 5.2D for the experimental set up). As with the *P. chrysocephala* adults, the injection needle was pulled using a P-2000 micropipette puller using the settings set out in section 5.2.10.2.1. The needle was prepared as described in section 5.2.10.2.1. To prepare the dsRNA injection solution, equal volumes of 2µg/µl *in vitro* synthesised dsRNA solution and 2x injection buffer were mixed. 1µl of the injection solution was then taken up into a 20µl microloader pipette tip (Eppendorf) which was used to backfill the glass needle; the solution was kept at the needle tip using backpressure. Injection of dsRNA into the larvae was carried out under a Leica M205 FCA microscope (Leica Microsystems, Wetzlar, Germany) and viewed on a computer screen using the Leica Application Suite X (version 3.3.3.16958) (Figure 5.2A). The needle was manipulated using the InjectMan4 (Eppendorf Ltd, Stevenage, UK) (Figure 5.2C) and was inserted at an angle of 30° into the lateral side of the thorax to avoid damaging organs or the CNS. The needle was pulled back slightly after insertion into the larvae to allow space for the dsRNA solution to enter the body cavity. 1µl of ds*CYP6k1-like* (or ds*GFP* control), diluted to a concentration of 250 ng/µl, was injected to give a dose of 250ng per beetle. When removing the needle, pressure was applied to the injection syringe to avoid dsRNA re-entering the needle. After injection the larvae were transferred to petri

dishes and maintained in a controlled environment room at 18±1°C, 16:8h light: dark photoperiod, on a diet of Chinese cabbage plants for 48 hours. After 48 hours the samples were snap frozen in liquid nitrogen.

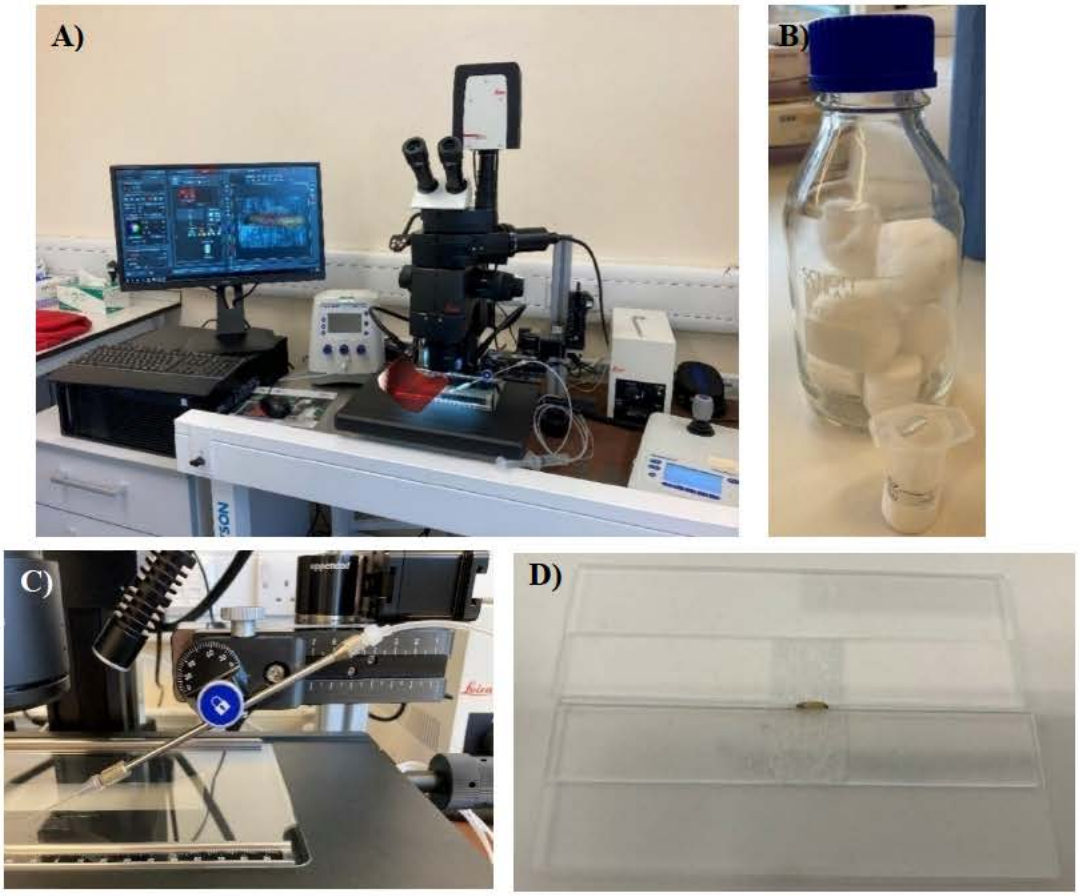


Figure 5.2. *Experimental set up showing A) the Leica M205 FCA microscope work station B) etherisation basket next to a Duran bottle containing 30ml ether and cotton balls to facilitate evaporation C) close up of the injection needle D) larvae secured laterally between two microscope slides using double sided sticky tape.*

5.3.10.3 Feeding bioassays

The efficiency of dsRNA knockdown by voluntary feeding was also assessed. *P. chrysocephala* adults were starved for 72 hours before starting the feeding assay. Assays were conducted using either *in vitro* synthesised dsRNA or bacterially expressed dsRNA. Chinese cabbage leaf discs were excised from 6-week-old leaves using circular cutters. For both assays, there were nine replicates per treatment. The bioassay was carried out for 48 hours and the petri dishes were kept at 18±1°C under a 16:8h light:dark photoperiod.

For the *in vitro* synthesised dsRNA, 5µl of dsRNA (diluted to 1µg/µl in sterile distilled water) was spread onto Chinese cabbage leaf discs (1cm in diameter) using a 10µl

pipette tip and allowed to dry for 15 minutes. The leaves were then transferred to the lids of 55mm Petri dishes (Thermo Scientific) each containing one disc of Whatman No1 (45mm Diameter) filter paper (Sigma-Aldrich) moistened with approximately 1 ml of deionised water. One adult was placed into each Petri dish and the dishes sealed with their base. The control beetles were fed 5µl of ds*GFP* (diluted to 1µg/µl in sterile distilled water). Only beetles confirmed to have eaten at least half of the leaf disc were included in qPCR analysis.

For the bacterially expressed dsRNA, Chinese cabbage leaf discs (7cm diameter) were individually dipped, using tweezers, into 100ml of the heat-killed bacterial culture for 20 seconds with gentle agitation before being left to dry on paper towelling (abaxial surface upwards) for 15 minutes in a fume hood. The leaves were then transferred to the lids of 90mm Sterilin Petri dishes (ThermoFisher Scientific) each containing two discs of Whatman No1 (90mm diameter) filter paper (Sigma-Aldrich) moistened with approximately 2 ml of deionised water. One adult was then placed into each Petri dish and the dishes sealed with their base. The control beetles were fed on leaves dipped in bacterial culture containing ds*GFP*. Only beetles that had obviously excreted leaf material were included in qPCR analysis.

5.3.11 Quantitative PCR (qPCR)

qPCR was used to examine the efficiency of microinjection and feeding bioassays on candidate gene knockdown in *P. chrysocephala* adults and larvae. *P. chrysocephala* categorised as 'mobile' 48 hours after beginning the microinjection or feeding assays were snap frozen in liquid nitrogen and stored at -80°C. Total RNA was then extracted from the pooled homogenate of three *P. chrysocephala* adults or larvae using the Isolate II RNA mini kit (see Chapter 2, section 2.3.3). RNA was quantified using the NanoDrop^{TM} 1000 Spectrophotometer and cDNA was synthesised from approximately 400ng of total RNA using the ThermoScientific SuperScript III First-strand cDNA synthesis kit (section 2.3.16). qPCR was carried out following the method described in Chapter 2, section 2.4.3.

5.3.12 Glass vial bioassays

The toxicity of lambda-cyhalothrin to *P. chrysocephala* adults subjected to RNAi was assessed using the glass vial bioassay previously described (see Chapter 2, section 2.2.2). Ten beetles were used for the treatment and the control, with three biological

replicates. 24 hours after injection of dsRNA the beetles were transferred to glass vials coated with the 100% recommended field rate of lambda-cyhalothrin. After 24 hours, the beetles were transferred to untreated vials without lids under upturned 200ml plastic disposable cups to allow potential recovery. After a further 24 hours, the beetles were scored as either mobile, affected, or dead. Results were expressed as percentage mortalities.

5.3.13 Leaf dip bioassays

The toxicity of lambda-cyhalothrin to *P. chrysocephala* larvae was assessed using the leaf dip bioassay previously described (see Chapter 2, section 2.2.3). Ten 3rd instar larvae were transferred into a Petri dish using a fine damp paintbrush. The Petri dishes were then sealed with their lids and maintained at 18±1°C under a 16:8h light: dark photoperiod for 24 hours. After this time, the larvae were transferred to untreated leaves to allow potential recovery. After an additional 24 hours the beetles were scored as described in section 2.2.2. There were three biological replicates per treatment.

5.3.14 Statistical analysis

To examine differences in gene expression level the C_t values generated by qPCR analysis were imported into Microsoft Excel. The C_t values were normalised to the geometric mean of the β-actin housekeeping gene and average gene expression in the control and treated beetles calculated using the $2^{-\Delta\Delta Ct}$ method (see Chapter 2, section 2.4.3). The standard error of the $2^{-\Delta\Delta Ct}$ values were determined from the technical replicates of each treatment. One-way ANOVA was used to compare gene expression levels between the control and treated samples. If the ANOVA result was significant, the mean expression values were compared further using Tukey's post hoc test. Graphs were produced using GraphPad Prism version 8.0.

5.4 Results

5.4.1 Glass vial and leaf dip bioassays to determine resistance level

Before starting RNAi experiments, glass vial bioassays were used to determine the resistance status of the *P. chrysocephala* adult sample used and leaf dip bioassays used to determine the resistance status of the larvae sample used. The adult sample was found to be 60% resistant to lambda-cyhalothrin at the recommended field rate (7.5gai/ha) and the larval sample 40% resistant.

5.4.2 The effect of microinjection and oral delivery of *in vitro* synthesised dsRNA on candidate gene expression in *P. chrysocephala* adults

To investigate the efficiency of dsRNA microinjection and feeding on knocking down the expression of the candidate gene in *P. chrysocephala* adults, qPCR analysis was conducted on beetles that were snap frozen 48 hours after starting the microinjection or feeding assay. For the microinjection assays, *P. chrysocephala* adults were injected with 250ng of *in vitro* synthesised dsRNA. For the feeding assays *P. chrysocephala* adults were exposed to 5µg of *in vitro* synthesised dsRNA coated onto the surface of 1cm Chinese cabbage leaf discs. Expression of the candidate gene *CYP6k1-like* in *P. chrysocephala* injected or fed with dsRNA targeting *CYP6k1-like* (ds*CYP6k1-like*) was compared to expression in *P. chrysocephala* injected or fed with dsRNA targeting *GFP* (ds*GFP*). All expression levels were normalised to the reference gene β-actin.

5.4.2.1 The effect of injected dsRNA on candidate gene expression

There was a significant difference in the mean $2^{-\Delta\Delta Ct}$ value across the *P. chrysocephala* injected with ds*CYP6k1-like* or ds*GFP,* and the un-injected susceptible sample (one-way ANOVA, $F_{2,4}$= 12.96, p =0.0179). A post hoc Tukey test revealed that the mean $2^{-\Delta\Delta Ct}$ value was significantly different between the *P. chrysocephala* injected with ds*CYP6k1-like* and the *P. chrysocephala* injected with ds*GFP* ($p < 0.05$). The mean expression level of *CYP6K1-like* was ~12 in *P. chrysocephala* adults injected with ds*CYP6k1-like* and ~102 in adults injected with ds*GFP* (Figure 5.3).

CYP6k1-like was knocked down to such a degree that there was found to be no significant difference in the mean $2^{-\Delta\Delta Ct}$ value between the *P. chrysocephala* injected with ds*CYP6k1-like* and the un-injected susceptible sample. Such highly efficient knockdown effect has been previously reported in other insects of the order Coleoptera. Willow et al (2021) reported an 82% reduction in mean expression of the target gene *αCOP* compared to the ds*GFP* control gene in pollen beetle (*Brassicogethes aeneus*) injected with ds*αCOP*, Zhao et al (2015) found the mean expression level of *AplaScrB-2* was reduced by 90% in emerald ash borer (*Agrilus planipennis*) adults injected with ds*AplaScrB-2* compared to the control gene ds*GFP* and Cao, Gatehouse and Fitches (2018) reported an >85% repression of V-type ATPase subunit E and inhibitor of apoptosis genes in red flour beetle (*Tribolium castaneum*) larvae injected with dsRNA.

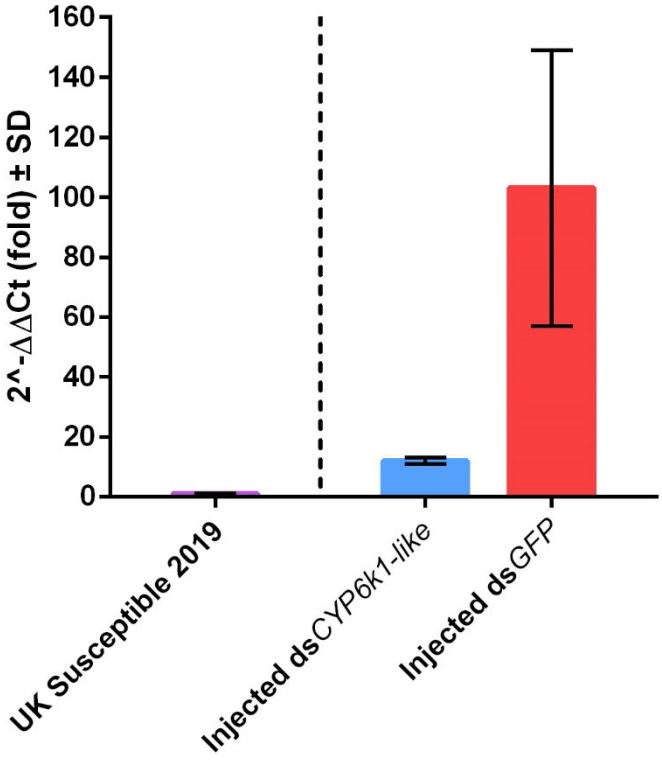


Figure 5.3. *Relative expression of CYP6k1-like mRNAs 48 hours after microinjection of target dsRNA (210µl at 1.2µg/µl) or control GFP into adult P. chrysocephala. Error bars show ± SE from three biological replicates (n = 3 beetles per treatment), each with three technical replicates.*

5.4.2.2 The effect of ingested dsRNA on candidate gene expression

There was also a significant difference in the mean $2^{-\Delta\Delta Ct}$ value across the *P. chrysocephala* fed ds*CYP6k1-like* or *dsGFP* and the susceptible control sample (one-way ANOVA, $F_{2,5}$= 579, p <0.0001). A post hoc Tukey test showed that the mean $2^{-\Delta\Delta Ct}$ value was significantly different between the *P. chrysocephala* fed ds*CYP6k1-like* and the *P. chrysocephala* fed ds*GFP* (p <0.01). The mean expression level of *CYP6K1-like* was ~76 in *P. chrysocephala* adults fed with ds*Cyp6k1-like* and ~100 in adults fed with ds*GFP* (Figure 5.4). Despite the knockdown effect observed, there was a significant difference in the mean $2^{-\Delta\Delta Ct}$ value of the *P. chrysocephala* fed ds*CYP6k1-like* compared to the susceptible control sample (post hoc Tukey test, p <0.0001).

Knockdown by ingestion has been previously observed in other Coleoptera. Ingestion of seedlings treated with arginine kinase dsRNA resulted in a significant knockdown

effect in the adult striped flea beetle (*Phyllotreta striolata*) (Zhao et al., 2008), and ingestion of diet treated with vacuolar ATPase dsRNA suppressed *vacuolar ATPase* expression in the Western corn rootworm (*D. virgifera virgifera*) (Rangasamy and Siegfried, 2012). Within Coleoptera, significant differences have however been reported in the efficacy of knockdown by ingestion. For example, knockdown by oral delivery was more effective in the Western corn rootworm (*D. virgifera virgifera*) and Colorado potato beetle (*L. decemlineata*), than in the red flour beetle (*T. castaneum*) (Baum et al., 2007; Whyard, Singh and Wong, 2009; Zhu et al., 2011).

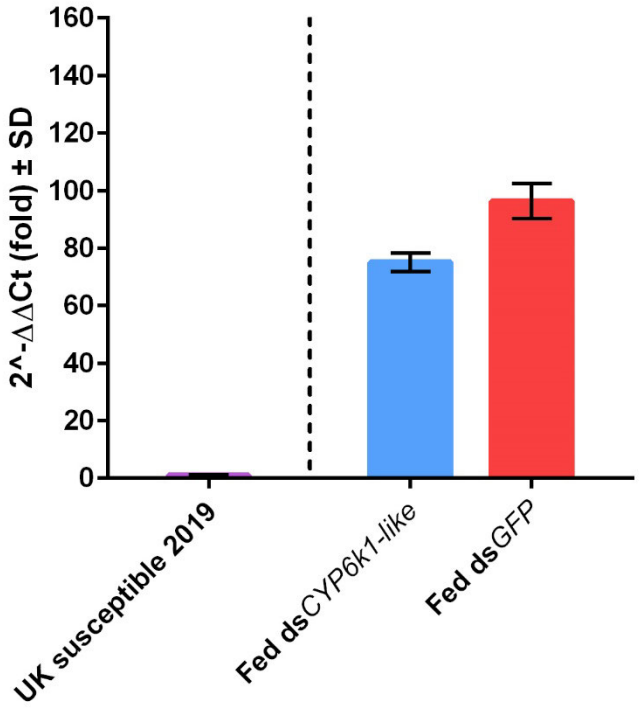


Figure 5.4. *Relative expression of CYP6k1-like mRNAs in adult P. chrysocephala 48 hours after feeding on leaf discs coated in in vitro expressed target dsRNA (5µg/leaf) or control GFP. Error bars show ± SE from three biological replicates (n = 3 beetles per treatment), each with three technical replicates.*

5.4.3 A comparison of the effect of microinjection and oral delivery of *in vitro* synthesised dsRNA on candidate gene expression in *P. chrysocephala* adults

Whilst *CYP6K1-like* was significantly knocked down in both the microinjection and feeding assays, the microinjection assays showed a more efficient knockdown than the feeding assays. Such a differential response was also previously observed in the red flour beetle (*T. castaneum*), where injection of target dsRNA was found to reduce the

mean expression level by >85% whilst oral delivery of target dsRNA only reduced the mean expression level by approximately 50% (Cao, Gatehouse and Fitches, 2018). A similar effect was also observed in the small hive beetle (*Aethina tumida*), where the expression level of the target gene was reduced by 25-45% 48 hours after injection whilst feeding had no knockdown effect (Powell et al., 2017).

This difference in knockdown efficacy could be attributed to insufficient amounts of dsRNA being ingested. Whilst only beetles that had eaten at least half of the coated leaf were used for qPCR analysis, as successful RNAi requires a certain amount of dsRNA to be taken up by the intestinal cells, the amount ingested from half a leaf may not have been sufficient, thereby limiting the RNAi response. The difference in knockdown efficacy could also be a result of dsRNA degradation due to the presence of dsRNA-degrading nucleases (dsRNases) in the midgut (reviewed in Cooper et al., 2019). dsRNA degradation assays found dsRNA was degraded in the gut extract of the African sweet potato weevil (*Cylas puncticollis*) (Prentice et al., 2017). As the persistence of dsRNA in the gut is key if cells are to take up the dsRNA, transmit it to other tissues and induce an RNAi effect, encapsulating dsRNA offers a potential approach for protecting dsRNA from such degradation (Silver, Cooper and Zhu, 2021).

5.4.4 Knockdown efficiency after oral delivery of bacteria synthesised dsRNA on candidate gene expression in *P. chrysocephala* adults

As oral delivery of *in vitro* synthesised dsRNA was found to significantly knockdown the expression of the candidate gene, the efficiency of candidate gene knockdown in *P. chrysocephala* adults fed bacterially expressed dsRNA (Figure 5.5) was also investigated. 400 - 500bp fragments of *CYP6k1-like* and *GFP* were amplified from *P. chrysocephala* cDNA and cloned into the L4440 expression vector; the recombinant vectors were then transformed into HT115(DE3) *E. coli* cells. IPTG was added to induce the expression of the T7 polymerase. Total RNA was extracted and 2µg analysed by gel electrophoresis on a 1% agarose gel to determine whether induction of ds*CYP6K1-like* and ds*GFP* synthesis was successful. As shown in Figure 5.5, dsRNAs of ~800bp were seen in the samples with IPTG added but not in the samples that did not have IPTG added. This shows that the IPTG was successful in T7 polymerase induction and subsequent dsRNA synthesis.

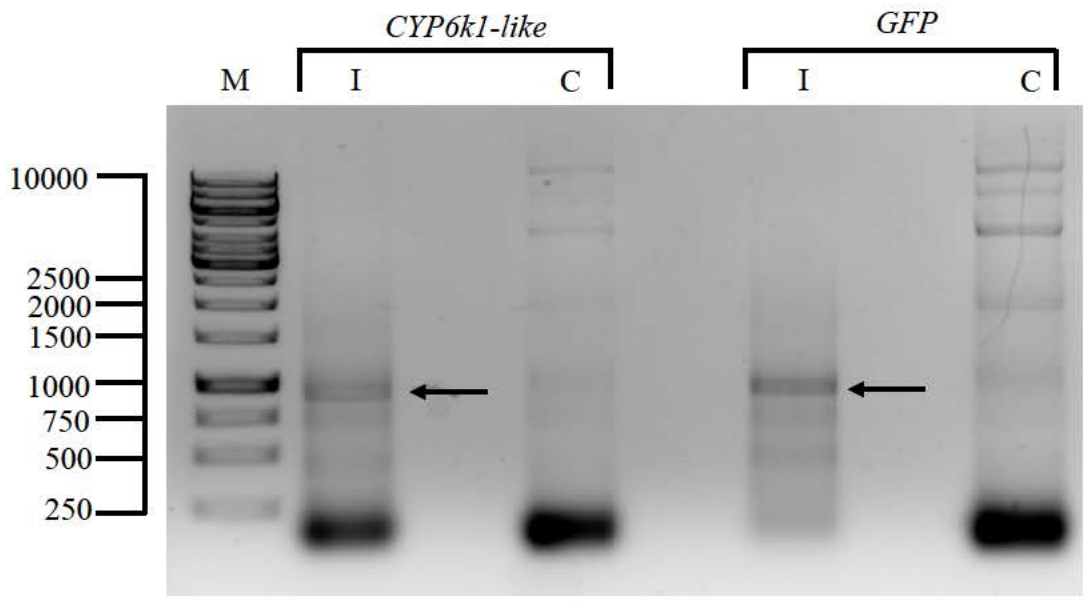


Figure 5.5. *Identification of dsRNA produced in RNase III deficient bacteria HT115(DE3). "I" denotes RNA samples extracted from bacterial culture treated with IPTG, "C" denotes the RNA samples extracted from bacterial culture not treated with IPTG, "M" denotes the 1kb DNA marker. Arrows indicate the dsRNA bands resolved at ~800bp.*

P. chrysocephala adults were exposed to Chinese cabbage leaf discs that had been dipped in 100ml of heat-killed bacterial culture. The culture had been previously induced to express either ds*CYP6k1-like* or ds*GFP*. qPCR analysis was conducted on beetles that were snap frozen 48 hours after starting the feeding assay. Expression of the candidate gene *CYP6k1-like* was compared in *P. chrysocephala* fed with ds*CYP6k1-like* with respect to *P. chrysocephala* fed with ds*GFP*. All expression levels were normalised to the reference gene β-actin.

Whilst there was a significant difference in the mean $2^{-\Delta\Delta Ct}$ value across the *P. chrysocephala* fed ds*CYP6k1-like* or ds*GFP* and the susceptible control sample (one-way ANOVA, $F_{2,6}= 14.94$, $p < 0.01$), a post hoc Tukey test revealed that the mean $2^{-\Delta\Delta Ct}$ value was not significantly different between the *P. chrysocephala* fed with ds*CYP6k1-like* and the *P. chrysocephala* fed with ds*GFP*. The mean expression level of *CYP6K1-like* was ~47 in *P. chrysocephala* adults injected with ds*CYP6k1-like* and ~57 in adults injected with ds*GFP* (Figure 5.6). This lack of gene knockdown contrasts with previous studies. For example, ingestion of bacterially expressed dsRNA was also found to silence five target genes in the leaf beetle (*Plagiodera versicolora*) (Zhang et al., 2019).

Whilst oral delivery of *in vitro* synthesised dsRNA caused significant gene knockdown in *P. chrysocephala* (Figure 5.4), the oral delivery of bacterially expressed dsRNA had no significant effect on *CYP6k1-like* expression (Figure 5.7). This differential response could be a result of insufficient amounts of dsRNA being consumed. Whilst only beetles that had obviously excreted leaf material were included in the qPCR analysis, the amount of dsRNA coated leaf tissue ingested may not have been sufficient to induce gene knockdown. A further disadvantage of the feeding assay method is that it is impossible to determine how much of the dsRNA has been ingested, with each insect most likely ingesting a different amount. The potential difference in amount ingested was apparent in the large variation in the $2^{-\Delta\Delta Ct}$ values across the biological repeats; the large error bars highlight this variation (Figure 5.6). Finally, as with the *in vitro* synthesised dsRNA, the lack of significant knockdown could be a result of dsRNA degradation in the midgut.

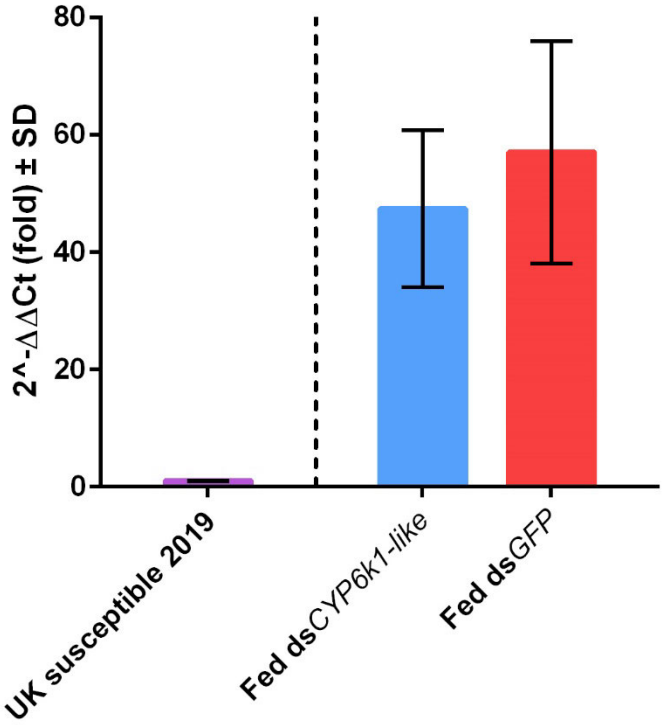


Figure 5.6. *Relative expression of CYP6k1-like mRNAs in adult P. chrysocephala 48 hours after feeding on leaf discs coated in bacterially expressed target dsRNA or control dsGFP. Error bars show ± SE from three biological replicates (n = 3 beetles per treatment), each with three technical replicates.*

5.4.5 Effect of CYP6k1-like knockdown on sensitivity to lambda-cyhalothrin

To establish whether a reduction in the expression of the candidate gene *CYP6k1-like* by RNAi restored pyrethroid toxicity, *P. chrysocephala* adults injected with 250ng of *in vitro* synthesised ds*CYP6k1-like* or ds*GFP* were treated with lambda-cyhalothrin by glass vial bioassay 24 hours post injection. dsRNA was delivered by injection as this method caused a greater RNAi response than ingestion. The glass vial bioassays were performed as described in section 5.2.12 and the results were expressed as percentage mortalities.

The mean percentage mortality was significantly greater in the *P. chrysocephala* injected with ds*CYP6K1-like* (80%) relative to those injected with ds*GFP* (57.5%) (two-sample t-test, $p<0.0001$) (Figure 5.7). The increase in percentage mortality in the ds*CYP6k1-like* injected beetles compared to the ds*GFP* injected beetles shows that the toxicity of lambda-cyhalothrin towards *P. Chrysocephala* adults was partially restored, suggesting the involvement of *CYP6k1-like* in conferring resistance to pyrethroids in *P. chrysocephala.*

Whilst the restoration of pyrethroid toxicity was not entirely unexpected, it was anticipated that the difference in percentage mortality between the beetles injected with ds*CYP6k1-like* and the beetles injected with ds*GFP* would have been somewhat greater. However, as the adult sample assayed was only 60% resistant to lambda-cyhalothrin at the recommended field rate (7.5gai/ha) prior to dsRNA injection, to truly validate the involvement of CYP6k1-like in conferring metabolic resistance, the assay could be repeated using a more highly resistant *P. chrysocephala* sample.

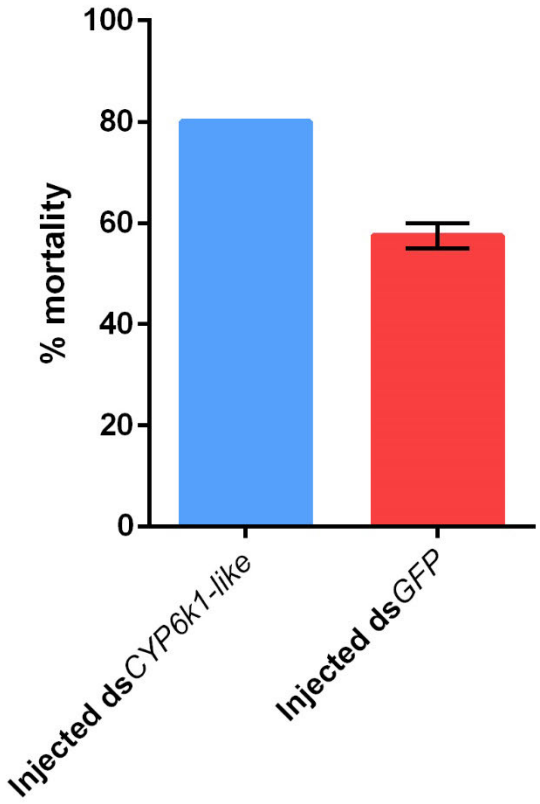


Figure 5.7. *Percentage of mobile* P. chrysocephala *adults 24 hours after microinjection of 250ng* CYP6k1-like *or* GFP *dsRNA followed by 48 hours of glass vial bioassay. Error bars show ± SE from three biological replicates (n = 10 beetles per treatment).*

5.4.6 The effect of microinjection of *in vitro* synthesised dsRNA on target gene expression in *P. chrysocephala* larvae

To investigate the efficiency of dsRNA microinjection on knocking down the expression of the candidate gene in third instar *P. chrysocephala* larvae, qPCR analysis was conducted on larvae that were snap frozen 48 hours after microinjection. Larvae were injected with 1µl of *in vitro* synthesised dsRNA at a concentration of 250ng/µl to give doses of 250ng per larvae. This dose was used as it was found to cause a significant knockdown effect in the *P. chrysocephala* adults. Expression of *CYP6k1-like* was compared in *P. chrysocephala* injected with ds*CYP6k1-like* with respect to *P. chrysocephala* injected with ds*GFP*. All expression levels were normalised to the reference gene β-actin.

There was a significant difference in the mean $2^{-\Delta\Delta Ct}$ value across the *P. chrysocephala* injected with ds*CYP6k1-like* or *dsGFP* and the susceptible adult control sample (one-

way ANOVA, $F_{2,6}$= 149.6, p <0.0001). A post hoc Tukey test showed that the mean $2^{-\Delta\Delta Ct}$ value was significantly different between the *P. chrysocephala* injected with ds*CYP6k1-like* and the *P. chrysocephala* injected with ds*GFP* (p <0.05). The mean expression level of *CYP6K1-like* was ~14 in *P. chrysocephala* larvae injected with ds*Cyp6k1-like* and ~18 in larvae injected with ds*GFP* (Figure 5.8).

Despite the significant knockdown effect, as the larvae showed a degree of resistance to lambda-cyhalothrin, with qPCR analysis previously showing that *CYP6K1-like* was significantly overexpressed in the larvae with respect to the UK adult susceptible sample (see Chapter 4, section 4.3.6), the low expression of *CYP6k1-like* in the larvae injected with ds*GFP* was unexpected. The moderate resistance observed could therefore be a result of the target-site mutation (*kdr or s-kdr*) and not overexpression of *CYP6k1-like*. Unfortunately, there were not enough larvae to determine the presence of the L1014F (*kdr*) and L925I (*s-kdr*) mutations in the mobile and affected *P. chrysocephala* samples using TaqMan assays.

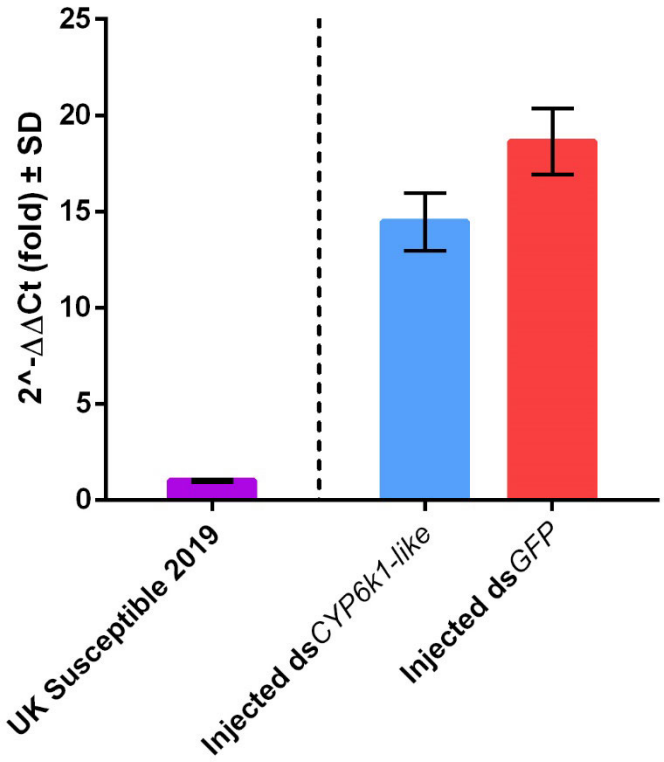


Figure 5.8. *Relative expression of CYP6k1-like mRNAs 48 hours after microinjection of target dsRNA (210µl at 1.2µg/µl) or control GFP into P. chrysocephala larvae. Error bars show ± SE from three biological replicates (n = 3 beetles per treatment), each with three technical replicates.*

5.5 Conclusion

Pyrethroids are no longer effective for the control of *P. chrysocephala* due to the development of insecticide resistance (see Chapter 3). As there remains a lack of insecticides with alternative modes of action that can be deployed for resistance management, RNAi offers a potential new control strategy for management of *P. chrysocephala*.

In this chapter, RNAi effects were investigated in *P. chrysocephala* adults and larvae following the delivery of dsRNA targeting the candidate gene *CYP6k1-like*, a cytochrome P450 thought to play a key role in conferring metabolic resistance towards pyrethroids (see Chapter 4). It was demonstrated that whilst knockdown of *CYP6k1-like* could be induced in *P. chrysocephala* by both injection and ingestion of *in vitro* synthesised dsRNA, the microinjection assays showed a more efficient knockdown. The effect of *CYP6k1-like* knockdown on sensitivity to lambda-cyhalothrin was investigated after delivering dsRNA by microinjection. After glass vial bioassays, mortality was significantly higher in the *P. chrysocephala* injected with *in vitro* synthesised ds*CYP6K1-like* relative to those injected with ds*GFP.* The result obtained also further confirms the likelihood of involvement of *CYP6k1-like* in conferring resistance to pyrethroids in *P. chrysocephala.*

A challenge of applying RNAi in the field is producing large enough quantities of dsRNA. Whilst dsRNA is expensive to produce using *in vitro* synthesis techniques, bacterially expressed dsRNA offers an alternative method for production. The effect of oral delivery of bacterially expressed dsRNA on *CYP6k1-like* expression was therefore examined in *P. chrysocephala* adults. Unfortunately, there was no significant knockdown effect observed in this instance. Although RNAi is promising in the development of novel insect control strategies, if dsRNA-based insecticides are to be successful commercially, understanding the limitations that underlie the differential RNAi response following injection or ingestion, such as dsRNA degradation, in *P. chrysocephala* is essential.

Another strategy to deliver dsRNA to insect pests in the field is through the production of transgenic plants. This has been achieved for insects of the order Coleoptera (Baum et al., 2007; Hussain et al., 2019), Lepidoptera (Mao et al., 2007, 2011) and Hemiptera (Zhao et al., 2018). An oilseed rape cultivar expressing dsRNA in the parts of the plant

where *P. chrysocephala* larvae and adults feed could offer an effective method for *P. chrysocephala* control. However, restrictions currently prevent the use of genetically modified crops expressing dsRNA in the field (Arpaia et al., 2020). For now, the delivery of well-timed dsRNA-based insecticide sprays onto the oilseed rape crop offers the most promise for *P. chrysocephala* control in the field.

Whilst this Chapter demonstrates the potential of RNAi to overcome insecticide resistance in UK populations of *P. chrysocephala,* further research is needed to determine potential adverse effects on non-target organisms and natural enemies. Baum et al (2007) reported that dsRNA designed to target the *vATPase A* of the Western corn rootworm (*D. virgifera virgifera*) also adversely affected the Southern corn rootworm (*D. undecimpunctata howardii*), and the Colorado potato beetle (*L. decemlineata*), whilst Haller et al (2019) found that dsRNA designed to target the same gene negatively affected the ladybird beetles *Adalia bipunctata and Coccinella septempunctata,* both natural enemies of *D. virgifera virgifera.* Understanding the effect of RNAi on the parasitic wasp *Microctonus brassicae,* a natural enemy of *P. chrysocephala* is therefore key if this control strategy is to be used in the field.

As with conventional insecticides, there is the possibility of insect pests developing resistance to dsRNA-based insecticides (Whyard, Singh and Wong, 2009; Khajuria et al., 2018). Further research is therefore needed into the possible mechanisms of resistance development, such as mutations of the target gene and RNAi core machinery genes. As the RNAi core machinery is important for processing endogenous dsRNA, the chance of insects developing resistance in these genes is low. However, resistance may occur through mutations in genes such as the RNA transporter SID-1 which facilitates the uptake of protein in many insects (Tomoyasu et al., 2008; Whyard, Singh and Wong, 2009). The next chapter (Chapter 6) aims to evaluate the efficacy of 17 alternative conventional insecticides for control against pyrethroid resistant *P. chrysocephala.*

5.6 Key findings

- ds*CYP6k1-like* and ds*GFP* products were successfully produced by *in vitro* and bacterial synthesis.
- Knockdown of *CYP6k1-like* was induced in *P. chrysocephala* adults by both injection and ingestion of *in vitro* synthesised dsRNA.

- The microinjection assays showed a more efficient knockdown of *CYP6k1-like* expression than the ingestion assays.
- There was no significant difference in *CYP6k1-like* expression in *P. chrysocephala* adults fed with bacterially expressed ds*CYP6k1-like* or ds*GFP*.
- Mortality was significantly higher in the *P. chrysocephala* adults injected with *in vitro* synthesised ds*CYP6K1-like* relative to those injected with ds*GFP* after pyrethroid glass vial bioassays.
- Restoration of lambda-cyhalothrin toxicity confirms the likelihood of *CYP6k1-like* involvement in conferring resistance to pyrethroids in *P. chrysocephala* adults.
- Knockdown of *CYP6k1-like* was induced in *P. chrysocephala* larvae by injection of *in vitro* synthesised dsRNA.

Chapter 6. Alternative compounds for the control of *Psylliodes chrysocephala*

6.1 Aims

The aim of the work reported in this chapter was to evaluate the efficacy of 17 alternative insecticides with different modes of action against pyrethroid resistant *P. chrysocephala* adults and larvae using glass vial and leaf dip bioassays.

6.2 Introduction

Between the early 1970s and late 1980s organophosphate sprays and organochlorine sprays and seed treatments were used for the control of insect pests on oilseed rape in the UK. In the 1990s, these insecticide classes were replaced by pyrethroid sprays because of their low toxicity towards mammals and high insecticidal activity against a wide range of insect pests. When the organochlorines were banned in 1999, due to their slow degradation and bioaccumulation in the environment, neonicotinoids such as imidacloprid, thiamethoxam and clothianidin became the main seed treatment for oilseed rape in the UK (FERA, 2015). However, in 2018 the EU imposed a complete ban (EU Commission, 2018) on the outdoor use of imidacloprid, thiamethoxam and clothianidin on all crops due to their effect on honeybees leaving just the pyrethroid sprays for *P. chrysocephala* control.

Given this recent ban by the European commission on outdoor use of most neonicotinoids, and the reduced effectiveness of pyrethroids as a pest control strategy, due to the development of resistance (Højland et al., 2015; Højland and Kristensen, 2018; Willis et al., 2020), identifying potential alternative compounds for *P. chrysocephala* control and evaluating their efficacy against pyrethroid resistant *P. chrysocephala* is critical if control of this insect pest is to be maintained in the future. This is particularly important at a time when knowledge about the susceptibility of *P. chrysocephala* towards alternative insecticides is scarce and the number of pesticides being commercialised is decreasing due to changes in the registration process and the introduction of the Biocidal Products Regulation (BPCA, 2020).

This Chapter aims to explore the potential of 17 alternative insecticidal compounds and evaluate their efficacy against pyrethroid resistant *P. chrysocephala*. The

compounds selected were chosen as they represent a range of different chemical classes (Sparks and Nauen, 2014) and were tested using glass and leaf dip bioassays against *P. chrysocephala* adults and larvae collected from oilseed rape fields at Rothamsted Research. Cyantraniliprole was further tested on nine *P. chrysocephala* samples received from across England.

6.3 Materials and Methods

6.3.1 Insects

P. chrysocephala adults were collected from oilseed rape fields at Rothamsted Research's farm in July 2019 and 2020 (see Chapter 2, section 2.1.1). Nine additional *P. chrysocephala* samples were received by post from samplers of oilseed rape fields elsewhere across England in 2020. The beetles were maintained in a controlled environment (CE) cabinet at 15±1°C, 65% relative humidity, 12:12h light: dark photoperiod. The beetles were kept in a mesh cage and fed continuously on a diet of Chinese cabbage *(Brassica rapa* spp*)* plants until tested.

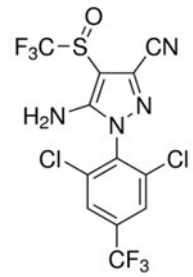
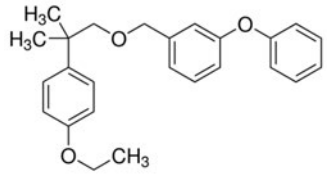
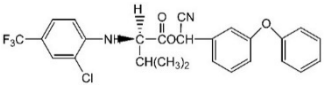
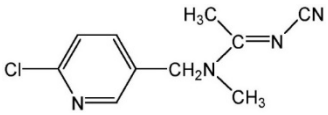
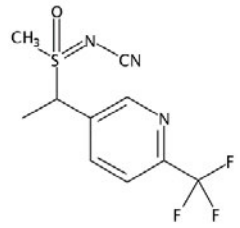
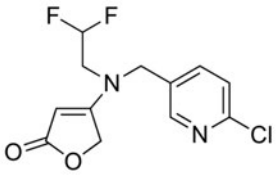
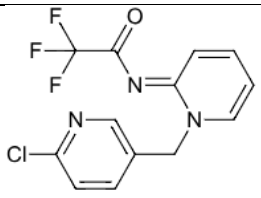
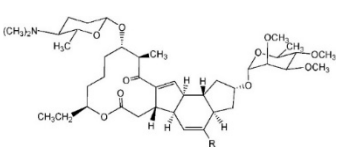
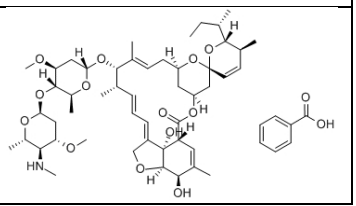
Second instar *P. chrysocephala* larvae were collected in March 2020 from oilseed rape plants at Rothamsted Research's farm. Larvae were extracted from the oilseed rape plants by dissection of the petioles as described in Chapter 2, section 2.1.2. The larvae were kept in Falcon tubes with moist tissue paper and maintained at 4°C until testing the following day.

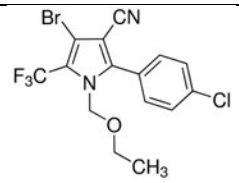
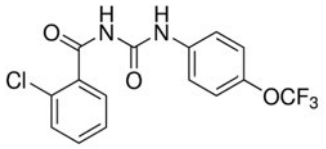
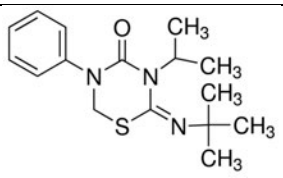
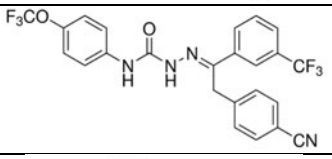
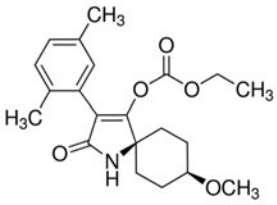
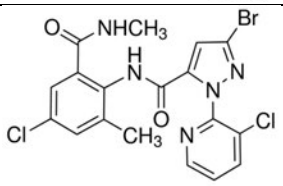
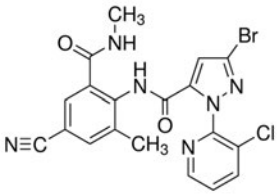
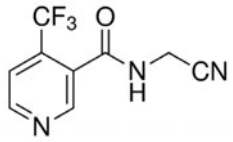
6.3.2 Insecticides

17 insecticides were tested for their efficacy against either *P. chrysocephala* adults or larvae. These compounds were chosen because they represent a range of insecticide groups spanning several Insecticide Resistance action Committee (IRAC) mode of action (MoA) classifications (Table 6.1). The technical grade insecticides were purchased from Sigma-Aldrich and Greyhound Chromatography; the formulated insecticides were obtained from Syngenta, Bayer and BASF.

Table 6.1. *IRAC MOA classification, main group, site of action, sub-group and structure of insecticidal compounds used in this study.*

Active ingredient	IRAC MOA group	Main group and Site of Action	Sub-group	Structure

Fipronil	2B	GABA-gated chloride channel blockers	Phenylpyrazoles	
Etofenprox	3A	Sodium channel modulators	Pyrethroids	
Tau-fluvalinate				
Acetamiprid	4A	Nicotinic acetylcholine receptor (nAChR) competitive modulators	Neonicotinoids	
Sulfoxaflor	4C	Nicotinic acetylcholine receptor (nAChR) competitive modulators	Sulfoximines	
Flupyradifurone	4D	Nicotinic acetylcholine receptor (nAChR) competitive modulators	Butenolides	
Flupyrimin	4F	Nicotinic acetylcholine receptor (nAChR) competitive modulators	Pyridylidenes	
Spinosad	5	Nicotinic acetylcholine receptor (nAChR) allosteric modulators	Spinosyns	
Emamectin Benzoate	6	Glutamate-gated chloride channel (GluCl) allosteric modulators	Avermectins	

Chlorfenapyr	13	Uncouplers of oxidative phosphorylation via disruption of the proton gradient	Pyrroles	
Triflumuron	15	Inhibitors of chitin biosynthesis affecting CHS1	Benzoylureas	
Buprofezin	16	Inhibitors of chitin biosynthesis, type 1	Buprofezin	
Metaflumizone	22B	Voltage-dependent sodium channel blockers	Semicarbazones	
Spirotetramat	23	Inhibitors of acetyl CoA carboxylase	Tetronic and Tetramic acid derivatives	
Chlorantraniliprole	28	Ryanodine receptor modulators	Diamides	
Cyantraniliprole				
Flonicamid	29	Chordotonal organ Modulators - undefined target site	Flonicamid	

6.3.3 Bioassays of *P. chrysocephala* adults

6.3.3.1 Glass vial bioassays

Glass vial bioassays were performed, as described in Chapter 2, section 2.2.2, to determine the efficacy of 14 alternative insecticidal compounds (Table 6.1) against *P. chrysocephala* adults. As acetamiprid and tau-fluvalinate have been previously used for the control of *P. chrysocephala* these compounds were tested at 4%, 20% and 100% of the 'recommended field rate'. These doses were chosen as they provide a good initial dose range. As Tau-fluvalinate is a pyrethroid it was also tested at the 200% 'field rate'. The '100% field rate' was the typical field rate used against the insect pests listed in Table 6.2. For all other compounds, preliminary range finder assays were performed using doses equivalent to 20%, 100% and 500% of the 'recommended field application rate' (Table 6.3). Initial stock solutions of 1,000ppm were prepared by diluting technical grade insecticide in technical grade acetone. Table 6.4 shows the conversion and working of the concentration of cyantraniliprole in gai/ha to parts per million (ppm). Glass vials were coated with 500µl of solution. Glass vials coated with acetone only were used as untreated controls. The bioassays were scored as described in Chapter 2, section 2.2.2 after 24, 48 and 72 hours. Three endpoints were scored because the time for the compound to take an effect was unknown. 10 beetles were used per glass vial. Three replicates were used for each concentration and the bioassays were carried out simultaneously under the same condition. Results are expressed as the percentage of affected and dead beetles (pooled).

Table 6.2. *The recommended field rate of each compound against a particular target pest. The compounds were tested against P. chrysocephala adults and larvae using the same field rate.*

Insecticide active ingredient	**100% field rate (gai/ha)**	**Pest**	**Literature**
Acetamiprid	72	Aphids	Assail 70 WP product label (DuPont)
Buprofezin	250	Tobacco whitefly	Applaud 25 SC product label (Certis)
Chlorfenapyr	27.5	Cabbage looper	Pylon product label (BASF)
Chlorantraniliprole	25		C. Zimmer, Per comms
Cyantraniliprole	25		C. Zimmer, Per comms
Emamectin benzoate	2.8	Fall armyworm	Denim product label (Syngenta)
Etofenprox	57.5	Pollen beetle	Trebon 30 EC product label (Certis)
Fipronil	25	Cotton thrips	Regent 200 SC product label (BASF)
Flonicamid	60	Aphids	Teppeki product label (Belchim)
Flupyradifurone	30	Aphids	Sivanto 200 SL product label (Bayer)
Flupyrimin	100		E. Davies, Per comms
Metaflumizone	240	Colorado potato beetle	Alverde 240 SC product label (BASF)
Spinosad	42.45	Willow leaf beetle	Conserve product label (Corteva)
Spirotetramat	75	Aphids	Movento product label (Bayer)
Sulfoxaflor	48	Aphids	Sequoia product label (Corteva)
Tau-fluvalinate	96	Cabbage stem flea beetle	Mavrik product label (ADAMA)
Triflumuron	5	Litter beetle larvae	Alsystin product label (Bayer)

Table 6.3. *Concentrations (ppm) of compounds tested against P. chrysocephala adults in the glass vial bioassay. Compounds were diluted in acetone.*

	Concentration used (ppm)				
Compound	**4%**	**20%**	**100%**	**200%**	**500%**
Acetamiprid	1.96	9.79	48.96		
Chlorantraniliprole		3.4	17		85
Chlorfenapyr		3.74	18.72		93.6
Cyantraniliprole		3.4	17		85
Emamectin Benzoate		0.35	1.75		8.75
Etofenprox		7.82	39.1		195.5
Fipronil		3.4	17		85
Flonicamid		8.16	40.8		204
Flupyradifurone		20.4	102		510
Flupyrimin		13.6	68		340
Metaflumizone		32.64	163.2		32.64
Spinosad		5.74	28.72		143.6
Sulfoxaflor		2	10		50
Tau-fluvalinate	1.92	9.6	48	96	

Table 6.4. *Concentrations of cyantraniliprole tested on P. chrysocephala via glass via bioassay and leaf dip bioassay. Cyantraniliprole was dissolved in acetone for the glass vial bioassay and in 0.01% (v/v) Agral for the leaf dip bioassay.*

Concentration			% Field rate
(gai/ha)	**Glass vial (ppm)**	**Leaf dip (ppm)**	
5	3.4	16.7	20
25	17	83	100
125	85	417	500

Glass vial conversion: gai/ha to ppm

Field rate: 25gai/ha = 25µgai/100cm^2 = 25µgai x 0.34/vial (glass vial 3.4cm^2) = 8.5µg/vial (in 500µl acetone) = 17µg/ml acetone = 17ppm

Leaf dip conversion: gai/ha to ppm

Field rate: 25gai/ha = 25,000pm in 1 litre of water. If it is assumed that the compounds would be diluted in 300 litres of water per hectare in the field: 25gai/300l = 25,000/300 = 83ppm

6.3.3.2 Leaf dip bioassays

Leaf dip bioassays were performed using the same method as described in Chapter 2, section 2.2.3, to investigate the efficacy of compounds found to be particularly toxic against *P. chrysocephala* adults by glass vial bioassay. The concentrations used were calculated by assuming that, in the field, the compounds would be diluted in 300 litres of water per hectare. Table 6.4 shows the conversion and working of the concentration of cyantraniliprole in g ai/ha to parts per million (ppm). Initial stock solutions of 1,000ppm were prepared by diluting technical grade insecticide in 1ml of technical grade acetone, and subsequently adjusting to 100ml with 0.01% (v/v) Agral. All further dilutions were made in 0.01% (v/v) Agral. The Chinese cabbage leaves were dipped in 100ml of solution. Leaf discs dipped in Agral alone were used as untreated controls. Where possible, commercial insecticide formulations were used instead of technical material as it is more realistic to the spray delivery route in the field. Cyantraniliprole was tested as the formulated product (Benevia 10 OD, Syngenta). Initial stock solutions of 1,000ppm were prepared by diluting 1mg of Benevia 10 OD formulation in 99ml 0.01% (v/v) Agral. All further dilutions were made in 0.01% (v/v) Agral. Bioassays consisted of three replicates at each concentration with 10 adult beetles used per leaf. The bioassays were scored as described in section 2.2.3 after 72 hours. Results are expressed as the percentage of affected and dead individuals.

6.3.4 Bioassays of *P. chrysocephala* larvae

6.3.4.1 Leaf dip bioassays

Leaf dip bioassays were performed, as described in Chapter 2, section 2.2.3, to determine the efficacy of the alternative compounds against 2nd instar *P. chrysocephala* larvae. 2nd instar larvae were used as they were easier to extract from the oilseed rape plants than the 3rd instar larvae. As the larvae develop within the petioles and main stem of the oilseed rape plant, the compounds tested were either systemic insecticides, or had a mode of action targeting the juvenile development stages. For each compound, preliminary range finder assays were performed using doses equivalent to 20% and 100% of the of the 'recommended field application rate' (S Foster, per comms) (Table 6.5). Cyantraniliprole was also tested at 4% of the 'field rate'. As with the adults, the '100% field rate' was the typical field rate used against the insect pests listed in Table 6.2. The concentrations used were calculated as

described in section 6.2.3.2. Initial stock solutions and dilutions were prepared as described in section 6.2.3.2. Cyantraniliprole was tested as the formulated product Benevia 10 OD (Syngenta), spirotetramat as the formulated product Movento (Bayer) and fipronil as the formulated product Regent 200 SC (BASF). Initial stock solutions and dilutions of formulated material were prepared as described in section 6.2.3.2. 10 larvae were used per leaf and three replicates were run per concentration. The bioassays were scored as described in Chapter 2 section 2.2.3 after 24, 48 and 72 hours. Results were expressed as the percentage of affected and dead larvae.

Table 6.5. *Concentrations (ppm) of compounds tested against P. chrysocephala larvae in the leaf dip bioassay. Compounds were diluted in 0.01% (v/v) Agral. *indicates compounds tested as the formulated product.*

	Concentration used (ppm)		
Compound	**4%**	**20%**	**100%**
Acetamiprid		30	120
Buprofezin		40	200
Cyantraniliprole*	3.4	16.7	83.4
Fipronil*		16.7	83.4
Spirotetramat*		50	250
Triflumuron		3.4	16.7

6.3.5 Bioassay data analysis

The range finder assay data are displayed graphically using GraphPad Prism version 8.0. The dose response data were analysed in GenStat (20th edition, (Payne, 2009)) using Probit analysis (Bliss, 1934). As Probit analysis is a type of regression analysis used to analyse binomial response experiments, and the *P. chrysocephala* were categorised as either alive, affected or dead, the affected or dead response was grouped together so there were only two outcomes, satisfying the requirement of a binomial response. The insecticide doses were log transformed and used to calculate the lethal concentration 50 (LC_{50}) and lethal concentration 95 (LC_{95}) values as well as the lower and upper 95% confidence intervals (95% CI). The LC_{50}, LC_{95} and 95% CIs were back transformed to the natural scale and used to compare the efficacy of each insecticide. The LC_{50} and LC_{95} values were considered significantly different if the respective confidence intervals did not overlap. Where there was a large variation in the data the 95% CI was not calculable (nc). The map of England showing the geographical spread

of *P. chrysocephala* samples collected for cyantraniliprole testing was produced using QGIS version 3.0.3.

6.4 Results and discussion

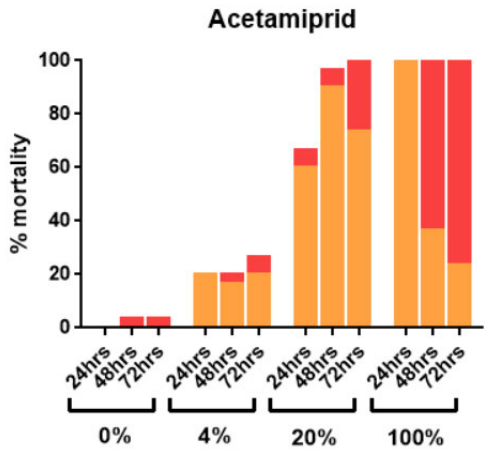
6.4.1 Susceptibility of *P. chrysocephala* adults to alternative compounds

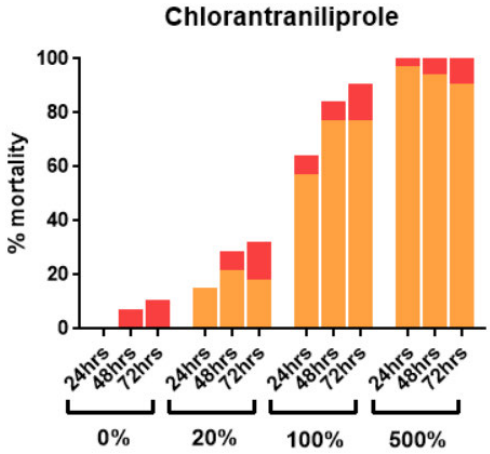
6.4.1.1 Glass vial bioassays

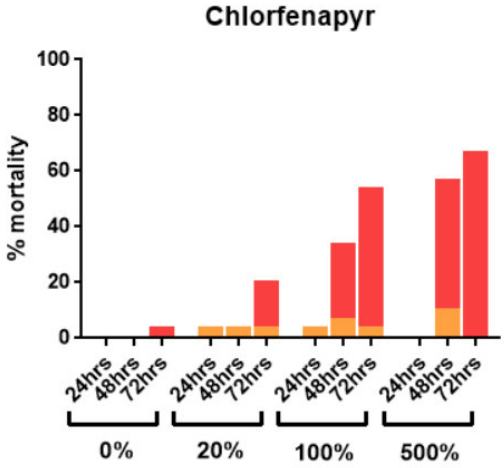
P. chrysocephala adults were tested for sensitivity to a range of alternative compounds. Acetamiprid and tau-fluvalinate were tested at 4%, 20% and 100% of the 'recommended field rate'; tau-fluvalinate was also tested at the 200% 'field rate'. All other compounds were initially tested at doses equivalent to 20%, 100% and 500% of the 'recommended field rate'.

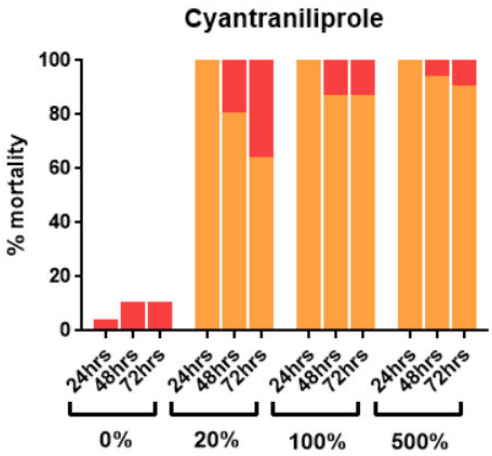
The results of the glass vial bioassays on *P. chrysocephala* are shown in Figure 6.1. The raw data can be found in Appendix 4 (Table 4A). From the graphs, it is evident that emamectin benzoate and metaflumizone showed little toxicity towards *P. chrysocephala* adults. Flonicamid showed no toxicity towards *P. chrysocephala* adults at the range of concentrations tested (data not shown).

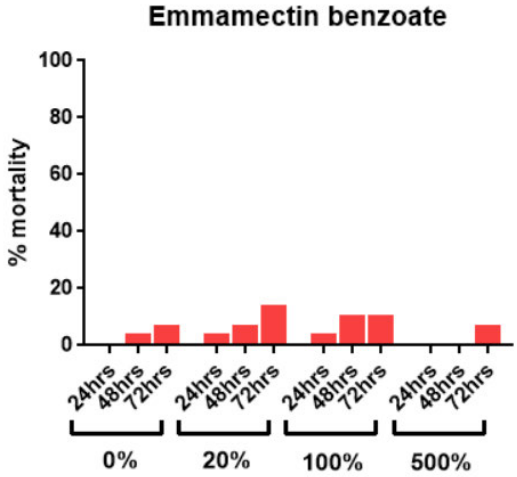
Acetamiprid, chlorantraniliprole, chlorfenapyr, cyantraniliprole, etofenprox, fipronil, flupyradifurone, flupyrimin, spinosad, sulfoxaflor and tau-fluvalinate all showed some degree of toxicity towards *P. chrysocephala* adults. Of these, acetamiprid, cyantraniliprole, fipronil, flupyrimin and spinosad were identified as the most toxic, causing complete mortality at 100% of the 'recommended field rate' after 72 hours. Whilst less effective at 100% of the 'recommended field rate', chlorantraniliprole, etofenprox, spinosad and sulfoxaflor all elicited strong responses at 500% of the 'recommended field rate' as the percentage of affected and dead beetles was 100% at the 24-hour time point. As the percentage of affected beetles in each sample was seen to decrease at both the 48- and 72-hour time point, and the number of dead beetles seen to increase, it was decided that subsequent assays using the compounds effective at either 100% or 500% of the 'field rate' would have a 72-hour end point. If cuticle thickness affects penetration rate, these insecticides may have been slow acting due to the hard exoskeleton making it more difficult for the compounds to penetrate the *P. chrysocephala* adults.

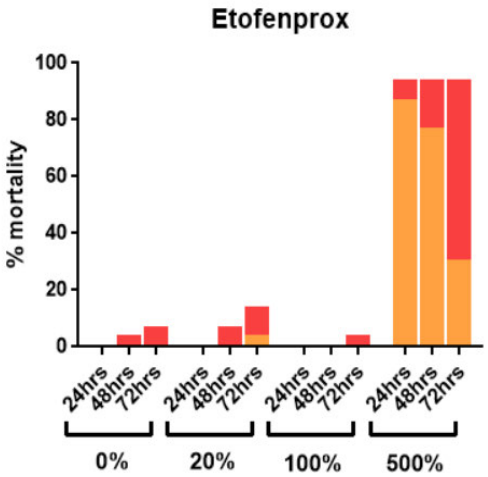

Acetamiprid
% mortality
100
80
60
40
20
0
24hrs 48hrs 72hrs
0%
4%
20%
100%

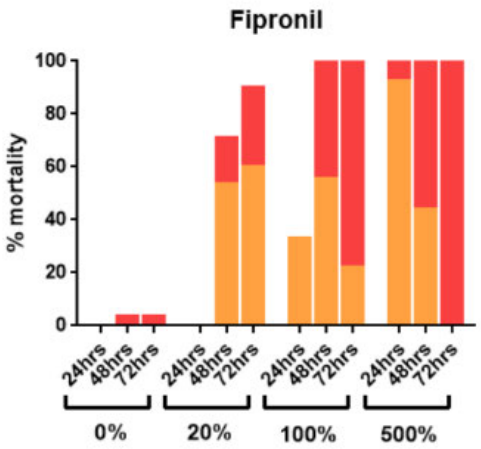

Chlorantraniliprole
% mortality
100
80
60
40
20
0
24hrs 48hrs 72hrs
0%
20%
100%
500%

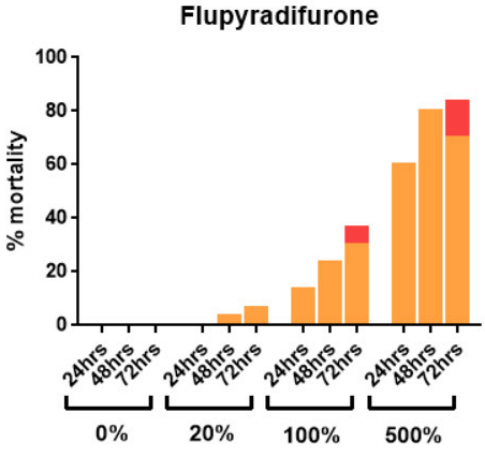

Chlorfenapyr
% mortality
100
80
60
40
20
0
24hrs 48hrs 72hrs
0%
20%
100%
500%

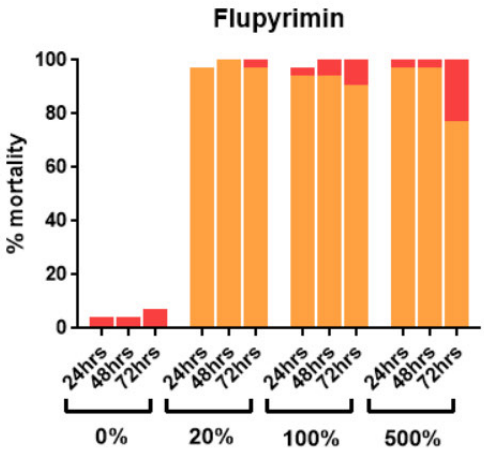

Cyantraniliprole
% mortality
100
80
60
40
20
0
24hrs 48hrs 72hrs
0%
20%
100%
500%

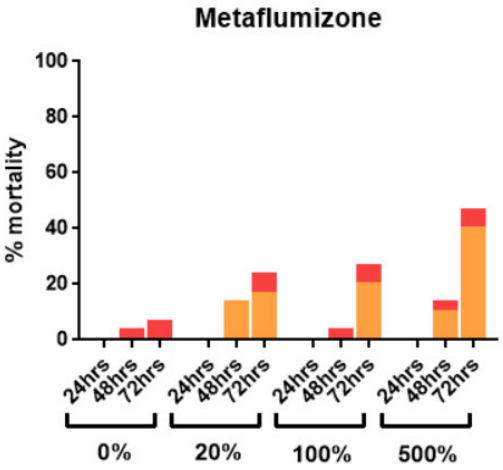

Emmamectin benzoate
% mortality
100
80
60
40
20
0
24hrs 48hrs 72hrs
0%
20%
100%
500%

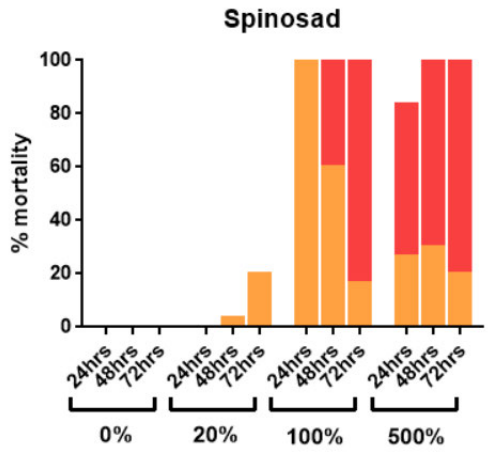

Etofenprox
% mortality
100
80
60
40
20
0
24hrs 48hrs 72hrs
0%
20%
100%
500%

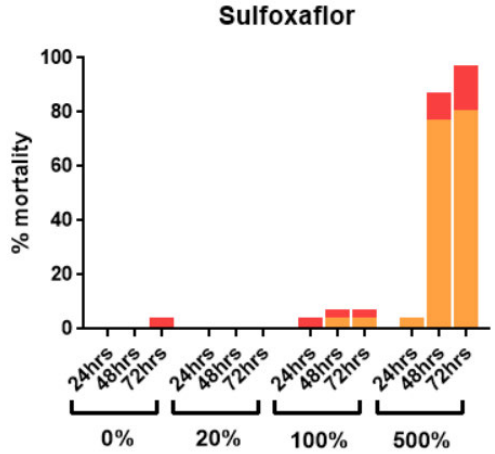

Fipronil
% mortality
100
80
60
40
20
0
24hrs
48hrs
72hrs
0%
20%
100%
500%


Flupyradifurone
% mortality
100
80
60
40
20
0
24hrs
48hrs
72hrs
0%
20%
100%
500%


Flupyrimin
% mortality
100
80
60
40
20
0
24hrs
48hrs
72hrs
0%
20%
100%
500%


Metaflumizone
% mortality
100
80
60
40
20
0
24hrs
48hrs
72hrs
0%
20%
100%
500%


Spinosad
% mortality
100
80
60
40
20
0
24hrs
48hrs
72hrs
0%
20%
100%
500%


Sulfoxaflor
% mortality
100
80
60
40
20
0
24hrs
48hrs
72hrs
0%
20%
100%
500%

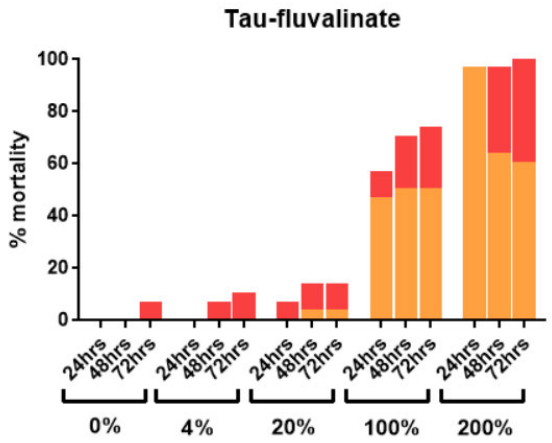


Figure 6.1. *The percentage of affected plus dead P. chrysocephala adults obtained in the glass vial range finder bioassays. Orange denotes the % affected and red the % dead.*

Acetamiprid, chlorantraniliprole, chlorfenapyr, cyantraniliprole, fipronil, flupyradifurone, flupyrimin and spinosad were investigated further using a full dose response assay with a 72-hour end point. For each compound, the results from the range finder assays were used to determine six or seven concentrations to be used in the standard glass vial bioassay. The doses were selected such that mortality should be 0% at the lowest concentration, approximately 50% at the middle concentrations and 100% at the highest concentration. Stock solutions of 1,000ppm were serially diluted with acetone to obtain the dilutions needed (Table 6.6). The susceptibility of *P. chrysocephala* adults to these nine compounds are expressed as LC_{50} values and are shown in Table 6.7.

Table 6.6. *Compound concentrations used (ppm) for P. chrysocephala adults by glass vial bioassay. All compounds were dissolved in acetone.*

Insecticide	Concentration used (ppm)
Acetamiprid	10, 3, 1, 0.3, 0.1, 0.03
Chlorantraniliprole	300, 100, 30, 10, 3, 1
Chlorfenapyr	1000, 300, 100, 30, 10, 3, 1
Cyantraniliprole	10, 3, 1, 0.3, 0.1, 0.03
Fipronil	30, 10, 3, 1, 0.3, 0.1, 0.03
Flupyradifurone	1000, 300, 100, 30, 10, 3
Flupyrimin	30, 10, 3, 1, 0.3, 0.1
Spinosad	100, 30, 10, 3, 1, 0.3

Table 6.7. *The LC_{50} and 95% confidence intervals for P. chrysocephala adults obtained using glass vial bioassays.*

Insecticide	LC_{50} (ppm)	95% CI (ppm)
Acetamiprid	15.79	4.278-2331
Chlorantraniliprole	472.6	318.7-1101
Chlorfenapyr	14.51	8.286-24.68
Cyantraniliprole	4.46	3.486-5.751
Fipronil	10.06	6.712-15.92
Flupyradifurone	55.5	32.41-95.09
Flupyrimin	18.61	8.433-79.51
Spinosad	8.46	nc

The LC_{50} values ranged from 4.5ppm (cyantraniliprole) to 423ppm (chlorantraniliprole). Cyantraniliprole was identified as the most toxic compound towards *P. chrysocephala.* As the 'recommended field rate' of cyantraniliprole was 25g ai/ha (17ppm) (C. Zimmer, per comms) it would be expected to provide good control against *P. chrysocephala*. Previous studies have tested the efficacy of cyantraniliprole against a range of insects of the order Coleoptera. Cyantraniliprole was found to be effective against the pepper weevil (*Anthonomus eugenii*) (Caballero et al., 2015) and the coffee borer beetle (*Hypothenemus hampei*) (Plata-Rueda et al., 2019). As cyantraniliprole was tested against *P. chrysocephala* resistant to pyrethroid, the high efficacy of this compound suggests it is not affected by the metabolic mechanism that confers resistance to the pyrethroid insecticide class in *P. chrysocephala*. This suggests cyantraniliprole could be a valuable tool for managing insecticide resistance in *P. chrysocephala* by rotation with pyrethroids.

The lack of efficacy of chlorantraniliprole towards *P. chrysocephala* adults in comparison to cyantraniliprole was an interesting finding as these are both diamide insecticides. Whilst cyantraniliprole is known to show an extensive range of systemic insecticidal activity across insect orders, chlorantraniliprole is found to be especially effective for the chemical control of insect pests in the order Lepidoptera (Selby, Lahm and Stevenson, 2017). Given that these two insecticides have the same mode of action and are similar in chemical structure differing in only one moiety, a nitrogen substituent for cyantraniliprole or a chlorine substituent for chlorantraniliprole (Figure 6.2), such a significant difference in efficacy was unexpected. Whilst Selby, Lahm and Stevenson (2017) reported no difference in efficacy between cyantraniliprole and

chlorantraniliprole against pests of the order Coleoptera, the difference in efficacy observed against *P. chrysocephala* is in accordance with Fettig et al (2011) who found cyantraniliprole to be more toxic towards the mountain pine beetle (*Dendroctonus ponderosaei*) than chlorantraniliprole and Koppenhöfer, Kostromytska and Wu (2018) who found cyantraniliprole to be more effective towards pyrethroid-resistant bluegrass weevil (*Listronotus maculicollis*) than chlorantraniliprole.

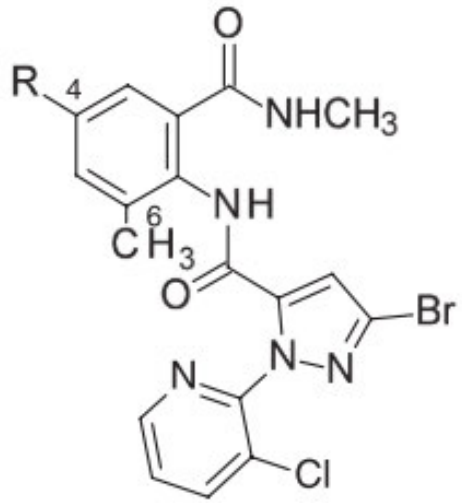


Figure 6.2. *The chemical structures of cyantraniliprole and chlorantraniliprole. Chlorantraniliprole, (R= Cl), Cyantraniliprole, (R= CN).*

Spinosad and fipronil were the second most toxic compounds towards *P. chrysocephala* with LC_{50} values of 8.5ppm and 10ppm respectively. The toxicity of spinosad towards *P. chrysocephala* is in accordance with previous studies, which have found this compound to be toxic towards other insects of the order Coleoptera including Colorado potato beetle (*Leptinotarsa decemlineara*) (Mota-Sanchez et al., 2006), lesser grain borer (*Rhyzopertha dominica*) (Daglish and Nayak, 2006) and the Asian lady beetle (*Harmonia axyridis*) (Galvan, Koch and Hutchison, 2005). A previous study found fipronil to be highly toxic against the Rhinoceros beetle (*Strategus aleous*) (Martínez et al., 2014). At the 'recommended field rate', spinosad and fipronil would be expected to provide good control against *P. chrysocephala*.

6.4.1.2 Leaf dip bioassays

P. chrysocephala adults from the Rothamsted farm were further tested for sensitivity to cyantraniliprole, fipronil, spinosad and flupyrimin by leaf dip assay. Whilst only the sixth most toxic compound towards *P. chrysocephala,* flupyrimin was tested by

leaf dip bioassay because it shows high safety towards beneficial organisms and pollinators (Onozaki et al., 2017). A leaf dip bioassay was employed as it provides a more realistic delivery of the insecticide from the surface of the oilseed rape leaf to the insect compared to glass vial assays, as the insecticide is delivered by both contact and ingestion. The six concentrations used for each insecticidal compound in the dose response assay were determined by the glass vial range finder assays (see section 6.3.1.1). Stock solutions of 1000ppm were serially diluted with 0.01% (v/v) Agral to get the dilutions needed (Table 6.8). The *P. chrysocephala* were scored as 'mobile', 'affected' or 'dead' after 72 hours. The susceptibility of *P. chrysocephala* adults to the four compounds were expressed as LC_{50} values (Table 6.9).

Table 6.8. *Compound concentrations used (ppm) for P. chrysocephala adults by leaf dip bioassay. All compounds were dissolved in acetone. *indicates compounds tested as the formulated product.*

Insecticide	Concentration used (ppm)
Cyantraniliprole*	417, 125.10, 37.53, 11.26, 3.38, 1.01
Fipronil	83, 25, 7.5, 2.25, 0.6, 0.2
Flupyrimin	490.20, 147.21, 49.07, 4.43, 1.33, 0.44
Spinosad	140, 42, 120.6, 3.78, 1.13, 0.34

The LC_{50} values ranged from 19.9ppm (cyantraniliprole) to 46.6ppm (spinosad) (Table 6.9). As observed in the glass vial bioassays, cyantraniliprole was identified as the most toxic compound towards *P. chrysocephala.* As the 'recommended field rate' of cyantraniliprole was 25 gai/ha (83.4ppm) by leaf dip bioassay (C. Zimmer, per comms) it would be expected to provide good control against *P. chrysocephala*. Cyantraniliprole was similarly toxic towards *P. chrysocephala* adults by glass vial and leaf dip bioassay. In both bioassays the LC_{50} values were approximately 4x less than the 'recommended field rate'; glass vial bioassay: LC_{50} = 4.46ppm, 'field rate' = 17ppm; leaf dip bioassay: LC_{50} = 19.9ppm, 'field rate' = 83.4ppm. This result is in contrast to a previous study which found cyantraniliprole to be >400 fold more toxic towards the cotton bollworm (*Helicoverpa armigera*) by ingestion than by residual contact (Bird, 2016). Flupyrimin was the second most toxic compound to *P. chrysocephala* with a LC_{50} value of 22.2ppm. As flupyrimin was only the sixth most toxic compound by glass vial bioassay this suggests that flupyrimin is more toxic by ingestion than by residual contact. The confidence intervals of cyantraniliprole and

flupyrimin overlapped indicating no significant difference in toxicity between these compounds.

Table 6.9. *LC_{50} and 95% confidence intervals.*

Insecticide	LC_{50} (ppm)	95% CI (ppm)
Cyantraniliprole	19.98	11.51-30.44
Fipronil	36.68	nc
Flupyrimin	22.17	15.61-29.17
Spinosad	46.59	nc

6.4.1.3 Bioassays with cyantraniliprole towards *P. chrysocephala* sourced from across England

Cyantraniliprole was identified as the most toxic compound towards *P. chrysocephala* by both glass vial and leaf dip bioassay. To investigate whether cyantraniliprole was as toxic towards *P. chrysocephala* samples collected from several farms across England with respect to the sample collected from the Rothamsted Research farm, nine further samples were tested by glass vial assay for their response to cyantraniliprole at the LC_{50}/10, LC_{50}, LC_{95} and 10x LC_{95} concentrations. The LC_{50} and LC_{95} values for each sample were calculated from the dose response assay using GenStat. The percentage mortality of the nine *P. chrysocephala* samples to cyantraniliprole after 72hrs at the LC_{50}/10, LC_{50}, LC_{95} and 10x LC_{95} values is displayed in Figure 6.3. The raw data can be found in Appendix 4 (Table 4B). The geographical spread of the nine samples of *P. chrysocephala* adults collected from across England is shown in Figure 6.4 and the LC_{50} and LC_{95} values for the nine samples shown in Table 6.10.

There was near complete mortality at the LC_{95} concentration in all samples apart from the sample from West Sussex. At the LC_{50} concentration, there was a significant variation both within and between samples. The large error bars (Figure 6.3) show the within sample variation, the lack of overlap of the error bars between some of the samples indicates the significant difference in mortality at this concentration. This variation may be due to the difficulty in categorising the response of the *P. chrysocephala* adults to cyantraniliprole: e.g. some of the *P. chrysocephala* were able to walk normally but only after a lot of encouragement with a paintbrush, some were able to jump but appeared unable to walk. As cyantraniliprole targets the ryanodine receptors, causing the release of calcium ion stores within the muscle cells (Selby et

al., 2013), the unwillingness of the *P. chrysocephala* to walk suggests cyantraniliprole was able to successfully target the ryanodine receptors within the beetle causing an inability to move and, potentially, feed or resist attack from beneficials such as parasitoid wasps.

The LC_{50} values for the nine samples ranged from 2.19ppm (Essex) to 9.62ppm (West Suffolk 2); the LC_{95} values ranged from 5.64 (Buckinghamshire) to 53.12 (West Sussex) (Table 6.10). Despite the variation in percentage mortality between the samples at the LC_{50} concentration (4.5ppm), the LC_{50} and LC_{95} values suggest that cyantraniliprole would still be expected to provide good control against all the *P. chrysocephala* samples collected from across England except from the sample from West Sussex at the 'recommended field rate'. The nine samples were pooled to give LC_{50} and LC_{95} values representative of the English population. The LC_{50} and LC_{95} values were: LC_{50} = 5.04ppm (95% CI = 4.57 – 5.57), LC_{95} = 10.19ppm (95% CI = 8.70 – 12.94). The LC_{50} and LC_{95} values for the sample from the Rothamsted farm were LC_{50} = 4.46ppm (95% CI = 3.49 – 5.75), LC_{95} = 10.31ppm (95% CI = 3.49 – 18.98). As the LC_{50} and LC_{95} values were not significantly different it can be concluded that cyantraniliprole was similarly toxic towards *P. chrysocephala* samples collected from across England and the Rothamsted Research farm sample.

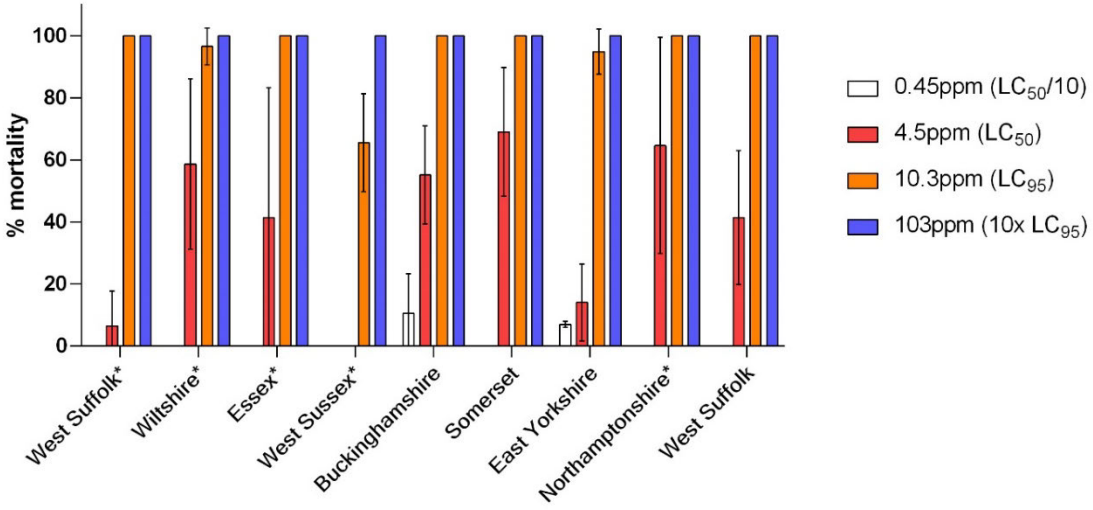


Figure 6.3. *Glass vial bioassays using technical grade cyantraniliprole on nine P. chrysocephala samples collected from farms across England at the LC_{50}/10, LC_{50}, LC_{95} and 10x LC_{95} concentrations. Error bars show standard error of the mean. *indicates the samples where the LC_{50}/10 concentration was not tested. Data graphed by C. Zimmer.*

Figure 6.4. *Geographical spread of nine populations of P. chrysocephala adults collected from across England in 2020.*

Table 6.10. *LC_{50} and LC_{95} values for nine P. chrysocephala samples collected from across England in 2020. Samples were tested for susceptibility to cyantraniliprole by glass vial bioassay. LCI = lower 95% confidence interval, UCI = upper 95% confidence interval, nc = not calculable.*

Sample location	Sample number	LC_{50} (ppm)	LCI - UCI	LC_{95} (ppm)	LCI - UCI
East Yorkshire	1	5.55	nc	6.98	nc
Northamptonshire	2	3.94	0.73 - 5.24	9.33	6.67 - 152.17
West Suffolk 1	3	4.65	nc	6.33	nc
West Suffolk 2	4	9.62	nc	12.5	nc
Essex	5	2.19	1.04 - 3.88	15.74	7.80 - 73.29
Buckinghamshire	6	4.05	nc	5.64	nc
West Sussex	7	5.4	1.47 - 26.28	53.12	15.10 - 186175
Wiltshire	8	4.17	nc	5.83	nc
Somerset	9	4.63	nc	6.15	nc

6.4.2 Bioassays of *P.* chrysocephala larvae

6.4.2.1 Leaf dip bioassays

2nd instar *P. chrysocephala* larvae were tested for sensitivity to six insecticidal compounds by leaf dip bioassay. Cyantraniliprole was included as it was the most toxic compound against the *P. chrysocephala* adults. The compounds were tested at doses equivalent to 20% and 100% of the 'recommended field rate' (Table 6.5). As cyantraniliprole was the most toxic compound towards the adults it was also tested at a dose equivalent to 4% of the 'field rate'. Stock solutions of 1,000ppm were serially diluted with 0.01% (v/v) Agral to get the dilutions needed. The larvae were scored after 72 hours.

The results of the leaf dip bioassays are shown in Figure 6.5. The raw data can be found in Appendix 4 (Table 4C). From the graphs, it is evident that growth regulators that inhibit chitin synthesis (buprofezin and triflumuron) had little effect, indicating that these compounds have little toxicity towards *P. chrysocephala* larvae. As these are chitin synthesis inhibitors it may take longer for these compounds to show an insecticidal effect than neurotoxic compounds (Reynolds, 1987). The 72-hour endpoint may therefore not have been long enough for an insecticidal effect to occur. When investigating the effect of triflumuron on *Tuta absoluta,* Silva et al (2011) used a seven-day endpoint. However, whilst increasing the bioassay endpoint to seven days may result in an insecticidal effect towards *P. chrysocephala* larvae, these slow-acting insecticides may not be practical in the field as the larvae may have already inflicted significant damage to the OSR plant before this point.

Acetamiprid and fipronil were identified as the most toxic compounds towards *P. chrysocephala* larvae, causing complete mortality at 20% of the 'recommended field rate' after 72 hours. A previous study found acetamiprid to be highly toxic against the juvenile stage of the ladybird beetle (*Eriopis connexa*) (Coleoptera: Coccinellidae) (Fogel et al., 2013). The susceptibility of *P. chrysocephala* larvae towards fipronil correlates with the findings of an HGCA report which noted a 58% reduction in damage caused by *P. chrysocephala* larvae following a fipronil seed treatment, with respect to the untreated control. *P. chrysocephala* larval numbers were also reported to have decreased by 36-40% following treatment with fipronil. Whilst this reduction in numbers was less than that caused by pyrethroid spray treatment with lambda-

cyhalothrin at 78%, the reduction caused by fipronil was not insignificant (Green, 2002).

Cyantraniliprole was the third most toxic compound towards *P. chrysocephala* larvae, causing 80% mortality at 100% of the 'recommended field rate'. This result is in accordance with a previous study which found cyantraniliprole to be effective against pyrethroid resistant annual bluegrass weevil (*Listronotus maculicollis*) larvae (Koppenhöfer et al., 2019).

Spirotetramat was less toxic towards *P. chrysocephala*, causing 60% mortality at 100% of the 'recommended field rate' after 72 hours. Spirotetramat has been previously reported to be particularly effective against juvenile stages of sucking pests such as aphids, and whitefly, causing death 2-10 days after application (Nauen et al., 2007). As the endpoint of the leaf dip assay was only 72 hours there may not have been enough time for insecticidal activity to occur. An alternative explanation is that the larvae did not ingest enough leaf material. Nauen et al (2007) reported that whilst spirotetramat shows good systemic activity, contact activity was low. The lack of insecticidal activity of spirotetramat towards *P. chrysocephala* could therefore be attributed to the larvae only ingesting small amounts of the insecticide coated leaf.

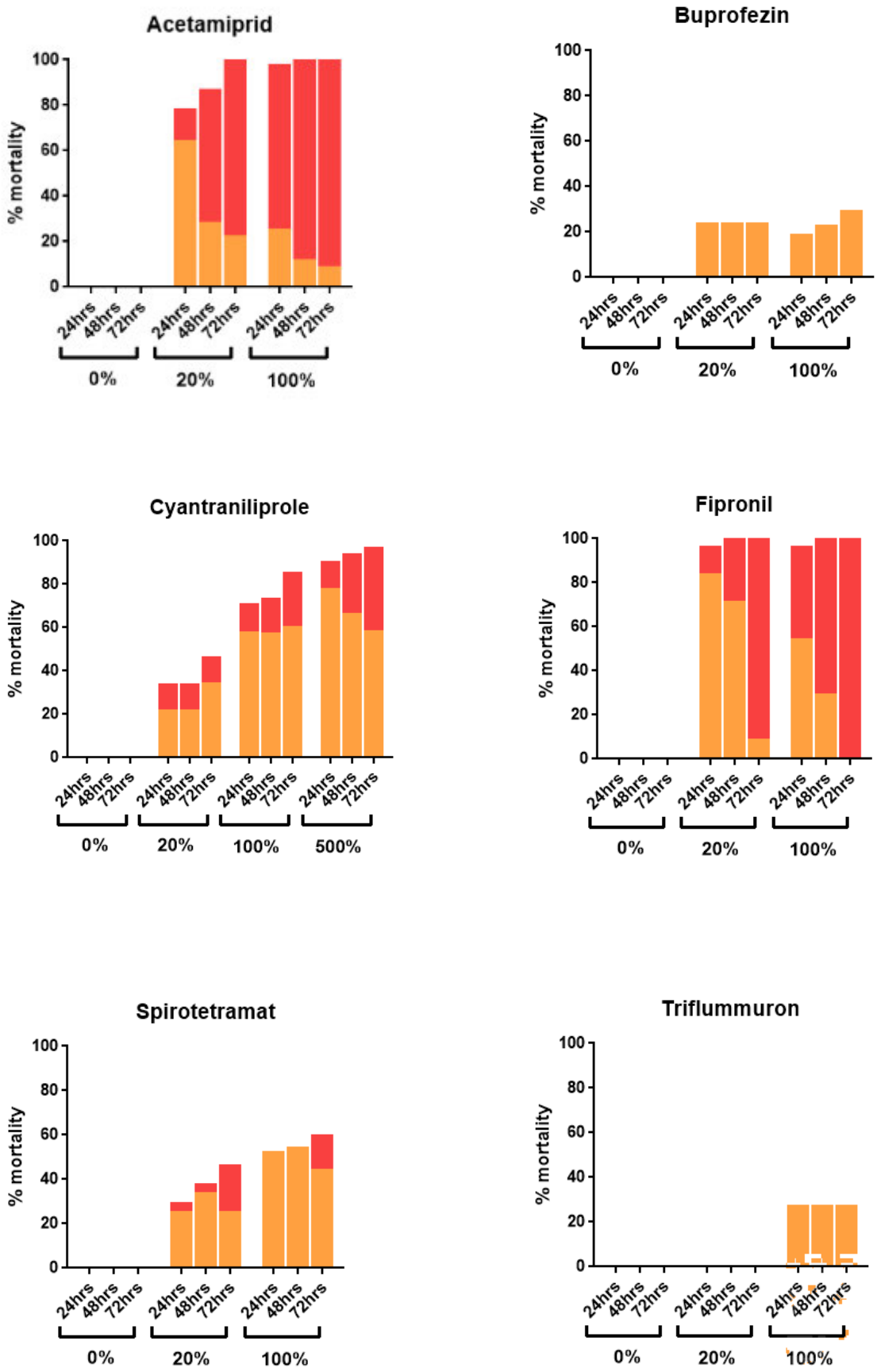


Figure 6.5. *The number of affected plus dead P. chrysocephala larvae obtained using leaf dip bioassays. Orange denotes the % affected and red the % dead.*

6.5 Conclusions

In this chapter, preliminary range finder assays were used to test the efficacy of 17 alternative insecticides with different modes of action against *P. chrysocephala* adults by glass vial bioassay. The range finder assays identified eight compounds which showed varying degrees of toxicity towards *P. chrysocephala* adults. Dose response assays were performed using these eight compounds. Cyantraniliprole (a Ryanodine receptor modulator) was identified as the most toxic compound against *P. chrysocephala* adults, followed by fipronil (a GABA-gated chloride channel blocker) and spinosad (a Nicotinic acetylcholine receptor (nAChR) allosteric modulator). *P. chrysocephala* adults were further tested for sensitivity to cyantraniliprole, fipronil, flupyrimin (a Nicotinic acetylcholine receptor (nAChR) agonist) and spinosad by leaf dip bioassay. Cyantraniliprole was again found to be the most toxic compound.

To determine whether cyantraniliprole was equally toxic to a range of *P. chrysocephala* samples, nine samples of *P. chrysocephala* adults from across England were tested for their susceptibility to cyantraniliprole by coated glass vial bioassay. Whilst there was variation, both within and between samples at the LC_{50} concentration (4.5ppm), this diamide compound was still highly effective at the LC_{95} concentration (10.3ppm) in all samples, apart from the sample from West Sussex (Figure 6.3a). As the 'recommended field rate' of cyantraniliprole is 17ppm, the LC_{50} and LC_{95} values for the nine samples (Table 6.10), coupled with the high mortality at the LC_{95} concentration, indicate that cyantraniliprole would be expected to provide good control against all the *P. chrysocephala* field populations collected from across England except from the sample from West Sussex, suggesting the potential of this diamide in the future of *P. chrysocephala* control, not only in the UK, but across mainland Europe.

Preliminary range finder assays were also used to test the efficacy of six insecticidal compounds against 2nd instar *P. chrysocephala* larvae by leaf dip bioassay. Whilst acetamiprid (a Nicotinic acetylcholine receptor (nAChR) competitive modulator) and fipronil were identified as the most toxic compounds towards *P. chrysocephala* larvae, cyantraniliprole was still relatively effective, causing 80% mortality at 100% of the 'recommended field rate'. Although the chitin synthesis inhibitors buprofezin and

triflumuron had little effect, the 72-hour endpoint may have limited the efficacy of these slow-acting insecticides.

The recent ban by the European commission on the outdoor use of most neonicotinoids has most probably resulted in the development of pyrethroid resistance in UK populations of *P. chrysocephala* (see Chapter 3). To prolong the use of pyrethroid sprays in areas such as Scotland, where *P. chrysocephala* populations appear to remain susceptible to pyrethroids, insecticides with alternative modes of action could be used in rotation with lambda-cyhalothrin. Cyantraniliprole, fipronil, flupyrimin and spinosad all showed toxicity towards *P. chrysocephala* adults. As these compounds all have different modes of action, they could be deployed in rotation with pyrethroid sprays in resistance management strategies. Alternatively, strategies such as using volunteer oilseed rape as a trap crop or defoliating winter OSR offer a way to control *P. chrysocephala,* as well as reduce the number of insecticide sprays used (White et al., 2020). By delaying the destruction of volunteer oilseed rape, rape that appears after the oilseed rape harvest, *P. chrysocephala* pressure on the newly emerging crop may be reduced. This may result if volunteer OSR is more attractive to migrating *P. chrysocephala* due to the large amounts of plant volatiles given off by this high-density crop (Bartlet et al., 1992; Pivnick, Lamb and Reed, 1992); it may also discourage the *P. chrysocephala* already in the volunteer crop from migrating. Defoliation by sheep grazing on oilseed rape crops could reduce *P. chrysocephala* larvae numbers by killing them either directly through ingestion, or indirectly by exposing them to the elements or natural enemies (White et al., 2020).

Whilst this Chapter shows the potential of a range of alternative insecticides for the control of *P. chrysocephala* adults and larvae, many of these insecticides are highly toxic to bees and are therefore unlikely be approved for use in the UK or Europe. However, developing insecticides within the classes which were found to be particularly effective (those that target the Nicotinic acetylcholine receptor, GABA-gated chloride channel and Ryanodine receptor), but show no adverse effect towards bees, offers a potential for future control methods. As cyantraniliprole was identified as the most toxic compound towards *P. chrysocephala* adults, understanding whether this compound is compatible with integrated pest management (IPM) strategies is key. For example, cyantraniliprole is reported to have sublethal effects on the non-target beneficial the seven spot ladybird (*Coccinella septempunctata*), reducing adult

longevity and reproductive rate (Jiang et al., 2020). To ensure the effectiveness of cyantraniliprole within IPM strategies, it is important to understand the impact of this insecticide on the wasp *Microctonus brassicae,* a parasitoid of *P. chrysocephala* adults (Jordan et al., 2020; Ortega-Ramos, pers comm). A previous study by Brugger et al (2010) found chlorantraniliprole to have no effect on several species of parasitic wasp making it a useful tool in IPM strategies.

As compounds that target the Nicotinic acetylcholine receptor and GABA-gated chloride channel were also found to be relatively toxic towards *P. chrysocephala* adults, understanding the impact of these compounds on honeybees, beneficial insects and parasitoids, and therefore their compatibility with IPM is also important if these compounds are to be considered to be used in the future. The nitro-containing neonicotinoids, imidacloprid, thiamethoxam and clothianidin are found to be highly toxic towards honeybees (EFSA, 2013b), causing both lethal and sublethal effects. Whilst acetamiprid, a cyano-containing neonicotinoid is reported to represent a low risk to honeybees (EFSA, 2016), sublethal doses are still found to disturb their development and shorten their lifespan (Shi et al., 2020b) as well as decrease the number of foraging flights of the worker bees (Shi et al., 2020a). Although acetamiprid is found to be only mildly toxic towards the parasitoid *Leptomastix dactylopii*, causing 20% mortality after 24 hours at the recommended field rate (Cloyd and Dickinson, 2006), the effect of acetamiprid on honeybees suggests acetamiprid may not be compatible with IPM strategies. Fipronil is reported to show high acute and sublethal toxicity towards honeybees, with sublethal doses impairing honeybee learning and memory (Pisa et al., 2014). In response, the European Commission restricted its use on maize and sunflowers in 2013 (EFSA, 2013a). Therefore, despite insecticides often playing an integral part of IPM strategies, as fipronil is frequently applied as a seed treatment and therefore acts systemically it is less compatible with IPM strategies as insecticides are applied first rather than as a last resort (Van Der Sluijs et al., 2015).

Given the reduced efficacy of pyrethroids for *P. chrysocephala* control in England, identifying alternative insecticides that may be effective against pyrethroid-resistant *P. chrysocephala* and evaluating their compatibility with IPM strategies is key in implementing sustainable resistant management strategies for future control of *P. chrysocephala*. Unfortunately, the availability of insecticides for controlling insect pests is being steadily eroded, not only through the selection of new forms of

resistance, but also recent EU legislation imposing losses of highly effective compounds. In Chapter 7, the main findings of this thesis are summarized and the implications for the future control of *P. chrysocephala* discussed.

6.6 Key findings

- By glass vial bioassay, cyantraniliprole was identified as the most toxic compound towards *P. chrysocephala* adults, followed by fipronil and spinosad.
- Cyantraniliprole was also the most toxic compound towards *P. chrysocephala* adults by leaf dip assay.
- The toxicity of cyantraniliprole was tested against nine *P. chrysocephala* samples collected from across England and was found to be highly effective at the LC95 concentration against all samples bar one. This suggests the potential of this compound in future control strategies against *P. chrysocephala.*
- Leaf dip assays against 2nd instar *P. chrysocephala* larvae found that acetamiprid and fipronil were the most toxic.
- Cyantraniliprole was also found to be relatively effective against 2nd instar *P. chrysocephala* larvae, causing 80% mortality at 100% of the 'recommended field rate'.

Chapter 7. General Discussion

This chapter provides a synopsis of the main findings of this thesis. The current resistance status of *P. chrysocephala* to the pyrethroid lambda-cyhalothrin is discussed and the implications of these findings regarding the future control of this pest species in the field touched upon. Potential future work is also outlined that could build upon the results reported.

7.1 Monitoring the extent and geographical spread of resistance in *P. chrysocephala*

The development of pyrethroid resistance in *P. chrysocephala* is a growing issue, with the increasing spread of resistance threatening the production of oilseed rape in the UK. Resistance monitoring is important because it allows for the tracking of trends in resistance level at a national scale. Understanding these trends is critical in informing a sustainable and effective resistance management strategy for *P. chrysocephala* control.

Prior to the start of this research in 2018, resistance to the pyrethroid lambda-cyhalothrin had already been reported in UK, German and Danish samples of *P. chrysocephala* adults (Højland et al., 2015). In Chapter 3, *P. chrysocephala* samples, sourced from across the UK, were tested for pyrethroid resistance and were found to show high levels of resistance to lambda-cyhalothrin, with the mean resistance value increasing across the three years of monitoring (2018 to 2020). During the three-year monitoring period, high levels of pyrethroid resistance spread from the South East to the North and West of England, with resistance remaining high in the South East. In both 2019 and 2020, a single sample from Fraserburgh, Aberdeenshire was found to be fully susceptible to lambda-cyhalothrin. *P. chrysocephala* larvae were also tested for pyrethroid resistance in 2020. Whilst the 1st and 2nd instar larvae showed near complete susceptibility at the recommended field rate of lambda-cyhalothrin (7.5g ai/ha), with mean percentage mortality values of 98.7% and 98.5% respectively, the 3rd instars showed a significantly higher level of resistance (48.9% mortality).

The results of the resistance monitoring program have implications for the future of *P. chrysocephala* control. In the UK, the current guidelines for *P. chrysocephala* control involve targeted pyrethroid sprays and IPM strategies (AHDB, 2021). The monitoring

results in this thesis show that *P. chrysocephala* remain a major pest of OSR in England, and that whilst pyrethroid resistance is spreading to the North and West of England, resistance appears to be highly localised (Chapter 3) making it difficult to predict where to spray for effective control. It is important to note however, that as just 10 beetles were tested from each sample, any trend in resistance level could have been masked. Therefore, whilst the maps of resistance (Chapter 3) provide a general overview of the geographical spread of resistance, local variation and annual migration can mean that farms with an average resistance level of 50% in year one can contain completely resistant beetles in year two. Overall, the results of the resistance monitoring program indicate that spraying with pyrethroids is likely to be ineffective for *P. chrysocephala* control in England and pyrethroid use will merely exacerbate and select for greater insecticide resistance. This result suggests that unless alternative methods of control are identified, farmers will continue to struggle to grow OSR. This is expected to lead to the continuation of the currently seen downward trend in the area of winter OSR grown in England since 2012. From 2020 to 2021 the total area of OSR fell by 21% (DEFRA, 2021). This could have a negative impact on the supply of vegetable oils for a range of purposes (biofuels, food etc). Further, as OSR is an important break crop in the UK, it's absence/reduced presence in crop rotations could have wider negative impacts on the health of farm systems (e.g. it helps break the disease cycle of cereal pathogens and improve soil tilth (Hegewald et al., 2018)).

The *P. chrysocephala* samples from Scotland remained susceptible from 2019 to 2020. Whilst caution should be taken over-extrapolating from this single data point, the absence of any fully susceptible samples in England in 2019 or 2020 suggests there are at least lower levels of resistance in Scotland than England. This result suggests that pyrethroid sprays could still be used in Scotland, but only if pest thresholds are met, and in conjunction with IPM strategies. Whilst the (single) yearly sample from Scotland was found to be susceptible to pyrethroid sprays, it is expected that resistance will spread to Scotland if a 'business as usual' approach is taken. The continued use of pyrethroid sprays without the deployment of wider IPM strategies is expected to result in high levels of resistance to spread to Scotland. Such a spread has already been seen across England during the three years of monitoring carried out as part this thesis. In order to reduce the likelihood of resistance spreading into Scotland, it is recommended that a similar approach to *P. chrysocephala* control is taken as was

recommended for England (above). The implementation of widespread IPM strategies and the use of insecticides with alternative modes of action in rotation with pyrethroids would reduce the likelihood of resistance to pyrethroids developing/spreading between populations. If such an approach is not taken, the reduction in the area of OSR grown in England is expected to be mirrored in Scotland. The corresponding reduction in the amount of OSR produced in the UK would exacerbate the issues described above.

The larvae also remain a major pest of OSR, causing damage by tunnel feeding in the petioles and main stem of the emerging crop. High larval numbers have been found to result in symptoms such as distortion of the plant tips, stem wilt and increased susceptibility to fungal pathogens (Newman, 1984); Williams, 2004; (Balaž, 2007), resulting in yield loss (Lane and Cooper, 1989; Williams, 2004; Nicholls, 2016). The differing levels of resistance across the *P. chrysocephala* developmental stages could also have interesting implications for resistance management. The near complete susceptibility of the 1st and 2nd instar larvae to the recommended field rate of lambda-cyhalothrin make them a good target for pyrethroid sprays (Chapter 3). Whilst *P. chrysocephala* larvae develop inside the petioles and main stem of the OSR plant, making them difficult to target using foliar sprays, there is an opportunity to target the 1st instar larvae when they move from the soil into the leaf petioles after egg hatch. As trials report it is possible to predict egg hatch and larva movement (Issues, 2021a), it may be feasible to accurately time the application of pyrethroid sprays and target the 1st instar larvae. This approach could reduce the size of the population before they enter the plant, thereby reducing the amount of damage caused to OSR crops by *P. chrysocephala*. Reduced damage could significantly increase crop yield, which is essential for working towards global food security, and the production of biofuel and animal fodder. Increasing crop yield is particularly important at a time when the anticipated yield is already insufficient to meet the growing demand for vegetable oils for both food and non-food products. Whilst the 1st and 2nd instars currently show near complete susceptibility to pyrethroids, to reduce the likelihood of resistance developing in these developmental stages, it is recommended that insecticides with alternative modes of action are used in rotation with pyrethroid sprays, as well as the implementation of widespread IPM strategies.

7.2 Elucidating the mechanism underlying pyrethroid resistance in *P. chrysocephala*

Without an understanding of the mechanism underlying pyrethroid resistance in *P. chrysocephala* it is difficult to track resistance trends quickly and efficiently. Understanding the molecular basis of this mechanism will enable the development and application of rapid resistance monitoring diagnostics. This will inform the implementation of a sustainable and effective resistance management strategy to protect OSR. Understanding of the mechanism of resistance would also allow any genes upregulated as part of a metabolic mechanism to be targeted with RNAi technology, informing the development and potential use of new insecticides.

As the target-site mutation L1014F (*kdr*) has been previously shown to contribute to pyrethroid resistance in UK and European samples of *P. chrysocephala* (Højland et al., 2015; Højland and Kristensen, 2018), the genotype of six UK samples was examined. The L1014F mutation was present across most of the samples tested (see Chapter 3), correlating with the results of previous studies. In addition to L1014F, some of the samples tested contained adult beetles homozygous for the *s-kdr* mutation L925I. Pyrethroid resistance-associated mutations at position 1014 and 925 in the *para*-type sodium channel have been observed across a wide range of insect species (reviewed in (Rinkevich, Du and Dong, 2013)). This parallel evolution of resistance across insect orders shows that these sites are important for pyrethroid action and there are potentially limited sites for resistance mutations across species.

Despite the presence of the target-site mutations, Højland et al (2015) found that some pyrethroid-resistant samples from the UK did not possess the *kdr* mutation. The restoration of pyrethroid toxicity following pre-treatment with PBO was reported in two of the UK samples of *P. chrysocephala* (Højland et al., 2015). In Chapter 3, the *kdr* genotype was confirmed to not correlate well with the resistant phenotype, and so the possibility of another, metabolic resistance mechanism/s conferring pyrethroid resistance in UK *P. chrysocephala* populations was explored. Using glass vial bioassays, the toxicity of lambda-cyhalothrin was seen to increase in all *P. chrysocephala* samples pre-treated with the synergist PBO, strongly suggesting that a metabolic-based mechanism for pyrethroid resistance is present in UK *P. chrysocephala.*

The role of cytochrome P450 monooxygenases in insecticide resistance has been reported in many insect pests (e.g. Karunker et al., 2009; Zhu et al., 2010). In Chapter 4, RNA was extracted from susceptible and resistant samples of *P. chrysocephala* sourced from across the UK, Switzerland, and Germany. RNA sequencing data was analysed to identify the gene(s) involved in pyrethroid resistance in these UK samples. By bioinformatic analysis, the cytochrome P450 *CYP6k1-like* was identified as the most differentially expressed gene ($p < 0.0001$). Whilst *CYP6k1-like* has not been previously associated with pyrethroid resistance, overexpression of *CYP6k1* was found to confer abamectin resistance in the diamondback moth (*Plutella xylostella*) (Yin et al., 2019). qPCR analysis showed that *CYP6k1-like* was overexpressed in UK resistant samples, in both 3rd instar larvae and adult beetles, further suggesting its involvement in the metabolic resistance mechanism.

As RNAi can result in significant inactivation of specific target genes, it is being increasingly used to determine gene function (Tabara, Grishok and Mello, 1998; Kamath et al., 2001). In Chapter 5, RNAi was used to validate the role of *CYP6k1-like* in conferring metabolic resistance towards pyrethroids in *P. chrysocephala.* Delivery of *in vitro* synthesised dsRNA by microinjection or oral delivery caused a knockdown effect on *CYP6k1-like,* with microinjection eliciting a stronger response. Microinjection followed by a glass vial bioassay increased the mortality of *P. chrysocephala* injected with *in vitro* synthesised ds*CYP6K1-like* relative to those injected with ds*GFP.* This result further suggests the involvement of *CYP6k1-like* in conferring resistance to pyrethroids in *P. chrysocephala* adults. To categorically confirm the involvement of *CYP6k1-like* in conferring metabolic resistance against pyrethroids in *P. chrysocephala,* the effect of *CYP6k1-like* knockdown on sensitivity to lambda-cyhalothrin should be tested in samples that are 100% resistant to lambda-cyhalothrin at the recommended field rate.

The results from the target-site resistance monitoring, and identification of the P450 enzyme involved in metabolic resistance have implications for the future of *P. chrysocephala* control. The TaqMan assays suggest that whilst *kdr* may not be the main mechanism conferring resistance against pyrethroids, it is providing some survival advantage on top of the metabolic P450-based mechanism (Chapter 3). As pyrethroid resistance continues to threaten *P. chrysocephala* control, screening populations for the L1014F mutation offers a way to monitor the frequency of

insecticide resistant alleles in UK populations. This information can be used to enact more effective and informed control strategies and prevent pyrethroids being sprayed in areas where they are likely to be less effective for *P. chrysocephala* control. As bioinformatic, qPCR and RNAi analysis suggest that overexpression of the cytochrome P450 *CYP6K1-like* confers metabolic resistance in pyrethroid resistant *P. chrysocephala* adults, a parallel screening approach could also be taken for *CYP6k1-like* resistance. As the regulatory mechanism that mediates P450 overexpression is currently unknown, elucidating this mechanism is key in developing an effective screening process. Resistance mechanisms could include mutations in the promoter region or overexpression of transcription factors. Development of a screening process would enable the resistance status of a population to be determined more quickly and without the need for visual assessments (used in glass vial assays) which have the potential for operator assessment bias. Again, the information obtained could be used in informing control strategies.

Given that the continuous use of pyrethroid sprays and lack of alternative insecticides has created a strong selection pressure, resulting in high levels of resistance in UK populations of *P. chrysocephala* (Willis et al., 2020), there is uncertainty over whether compounds in this insecticide class will be viable for *P. chrysocephala* control in the future. Establishing whether the target-site and metabolic resistance mechanisms carry a fitness cost in an insecticide-free environment (as seen in other pest species, such as aphids) is key in determining whether pyrethroids can be used as an element of future pest control strategies. Fitness costs due to the L1014F mutation (target-site resistance) have been found in other insect pests, suggesting it will also likely carry a fitness cost in *P. chrysocephala*. For example, Walsh et al (2021) reported that fecundity may be impacted in the English grain aphid (*Sitobion avenae*) homozygous for the L1014F mutation, and Foster et al (1999) reported that responsiveness to alarm pheromone was reduced in the peach-potato aphid (*Myzus persicae*) homozygous for the L1014F mutation. The frequency of the L1014F mutation was also found to decline in a mosquito (*Culex pipiens pallens*) population in the absence of insecticide selection pressure (Chen et al., 2010). In the case of metabolic resistance, it is likely that overexpression of *CYP6k1-like* also confers a fitness cost. A fitness cost was associated with the overexpression of *CYP6P9a* and *CYP6P9b* in pyrethroid resistant mosquito (*Anopheles funestus*), with the homozygous resistant larvae developing

significantly slower than the heterozygous and homozygous susceptible larvae. The proportion of susceptible individuals was also found to increase in the absence of insecticide pressure (Tchouakui et al., 2020, 2021). Establishing the fitness cost associated with the L1014F mutation and *CYP6k1-like* overexpression in *P. chrysocephala* is key in implementing a suitable resistance management strategy. If the target-site and metabolic resistance mechanisms do impose a high fitness cost, further spread of pyrethroid resistance could be slowed by employing a resistance management strategy based on insecticide rotation. Implementing such an approach would reduce the likelihood of resistance to pyrethroids developing/spreading in areas such as Scotland where the level of resistance appears to be lower than in England. Maintaining the efficacy of pyrethroids in these areas by using a rotation-based system is key in maintaining the area of OSR grown in the UK as well as crop yield, both of which are necessary if the growing demand for vegetable oil is to be met.

7.3 Identifying alternative insecticides efficacious against *P. chrysocephala*

The recent ban by the European commission on outdoor use of most neonicotinoids has caused an issue for *P. chrysocephala* control as it has resulted in an over-reliance on pyrethroid insecticides, resulting in a reduced effectiveness of pyrethroids due to increased and sustained selection pressure. Identifying potential alternative compounds for *P. chrysocephala* control and evaluating their efficacy against pyrethroid resistant *P. chrysocephala* is therefore critical for a sustainable and effective resistance management strategy incorporating an insecticide-based rotation system.

Whilst RNAi offers a potential control strategy for managing pyrethroid resistant *P. chrysocephala*, alternative compounds, not currently used against *P. chrysocephala*, offer another possible approach. In Chapter 6, bioassays were used to evaluate the efficacy of 17 alternative insecticides with different modes of action against pyrethroid resistant *P. chrysocephala* adults and larvae. By glass vial bioassay, cyantraniliprole was identified as the most toxic compound (leading to greatest mortality) at equivalent doses against *P. chrysocephala* adults, followed by fipronil and spinosad. As cyantraniliprole was also the most toxic compound by leaf dip assay, the toxicity of this compound was tested against nine *P. chrysocephala* samples collected from across

England. At the LC_{95} concentration, cyantraniliprole was highly effective against all samples bar one, suggesting the potential of this diamide for inclusion in future control strategies against this pest. Range finder assays used to assess the efficacy of six compounds against 2nd instar *P. chrysocephala* larvae found that acetamiprid and fipronil were the most toxic compounds.

The banning of neonicotinoid seed treatment use on OSR, EU-based legislation restricting the use of alternative compounds, and the spread of pyrethroid resistance, means chemical control options for *P. chrysocephala* are now severely limited. Therefore, the result of the alternative insecticide screening carried out in chapter 6 has important implications for the future of *P. chrysocephala* control. Cyantraniliprole was identified as the most toxic compound against *P. chrysocephala* adults in the insecticide screen. In the field, an insecticide seed treatment containing cyantraniliprole, Lumiposa (Corteva Agriscience), has been found to provide moderate control against *P. chrysocephala* adults, reducing OSR damage by 65% compared to untreated fields (van Nieuwenhoven, 2017), and increasing OSR yields by 10% (Jones, 2020a). The toxicity of cyantraniliprole by bioassay and in the field suggests the potential of this compound for chemical control of *P. chrysocephela*. As IRAC (2021) suggests rotating compounds with different modes of action to slow the development of further resistance, cyantraniliprole could be used in rotation with pyrethroids in areas such as Scotland where resistance levels remain lower than in England. This approach would help to maintain the efficacy of pyrethroids and reduce the likelihood of resistance to pyrethroids developing/spreading. Prolonging the efficacy of pyrethroids in these areas is key in maintaining the area of OSR grown in the UK as well as crop yield. IPM strategies should also be implemented in conjunction with the rotation of insecticides with the aim of maintaining insecticide efficacy for the longer term. To ensure the effectiveness of cyantraniliprole within IPM strategies, it is important to understand the impact of this insecticide on *Microctonus brassicae,* a parasitoid wasp of *P. chrysocephala* adults (Jordan et al., 2020). Any negative impacts on this parasitoid wasp would have a detrimental effect on wider IPM strategies.

Despite the potential of alternative MOA insecticides such as cyantraniliprole, the current lack of compounds registered for use against *P. chrysocephala* means future control strategies will need to rely on a wider range of control methods. As RNAi

shows promise as a species-specific method of crop protection (Price and Gatehouse, 2008), identification of the gene thought to underlie metabolic resistance in *P. chrysocephala* could have important implications for exploiting RNAi techniques in resistance management strategies against this pest (Chapters 4 and 5). Knockdown of *CYP6k1-like* was observed following injection and ingestion, with mortality significantly higher in *P. chrysocephala* adults injected with ds*CYP6k1-like* compared to those injected with ds*GFP* following treatment with lambda-cyhalothrin (Chapter 6). As the implementation of a dsRNA-based insecticide would help restore the toxicity of pyrethroids, this suggests the potential of using RNAi in a resistance management strategy based on insecticide rotation. Such an approach could contribute to the continued viability of growing OSR in the UK. dsRNA-based insecticides that target *CYP6k1-like* could be sprayed onto the OSR crop for *P. chrysocephala* control in the field. Alternatively, a dsRNA-based insecticide targeting an essential gene could be used as this may achieve more direct control; a dsRNA-based insecticide targeting *CYP6k1-like* would still require the subsequent use of a pyrethroid spray. Foliar application of ds*actin* was previously seen to be effective in protecting potato plants from the Colorado potato beetle (*L. decemlineata*) (San Miguel and Scott, 2016). If RNAi is to be incorporated into a sustainable resistance management strategy, understanding its effect on non-target organisms and natural enemies is crucial.

As well as RNAi techniques, there are currently a range of non-chemical control options under investigation for inclusion in IPM strategies for *P. chrysocephala*. Such non-chemical control options could help prolong the use of the existing chemical arsenal in areas such as Scotland, where *P. chrysocephala* populations appear to remain susceptible to pyrethroids, and ensure insecticides are only used when IPM strategies cannot provide sufficient control alone (White et al., 2020). Plant extracts such as Azadirachtin and biopesticides such as entomopathogenic nematodes and entomopathogenic fungi offer a potential novel approach to manage pyrethroid resistant *P. chrysocephala*, with entomopathogenic nematodes and entomopathogenic fungi show promising results against *P. chrysocephala* adults (Hoarau et al., 2020). Identifying OSR cultivars with greater resistance to *P. chrysocephala,* also offers a possible method for the management of pyrethroid resistant *P. chrysocephala.* To date, significant variation in feeding preferences of *P. chrysocephala* adults have been observed in different varieties of *Brassica napus,* with two lines identified as

unpalatable in the lab and, importantly, in field trials (Hughes, 2019). Given that pyrethroid sprays no longer provide adequate control against *P. chrysocephala* in the UK, there must be a concerted effort to develop sustainable IPM strategies that can limit the damage caused by this aggressive insect pest, protecting the future of OSR as a viable and important rotational crop in the UK and mainland Europe.

7.4 Future work relating to the research conducted in this thesis

1. The *CYP6k1-like* gene needs to be introduced into *D. melanogaster* and the resulting transgenic flies used to confirm the hypothesis that overexpression of this gene confers metabolic resistance in *P. chrysocephala.* Fly mortality assays with lambda-cyhalothrin should confirm whether *CYP6k1-like* confers resistance to pyrethroid compounds.
2. A potential fitness cost of the metabolic resistance mechanism should be examined by performing fitness assays on the transgenic *D. melanogaster* expressing *CYP6k1-like*.
3. The regulatory mechanism underlying overexpression of the P450 gene *CYP6k1-like* should be investigated to allow the development of a molecular diagnostic that could be used to screen for metabolic resistance in *P. chrysocephala.*
4. The *para*-type sodium channel in resistant *P. chrysocephala* adults needs to be sequenced to determine whether there are any other target-site mutations which could be contributing to pyrethroid resistance.
5. TaqMan assays should be performed on *P. chrysocephala* larvae to identify whether the moderate resistance observed is a result of target-site resistance mutations (*kdr* or *s-kdr*) and not the overexpression of *CYP6k1-like*.
6. Bioassays with the diamide, cyantraniliprole, could be conducted on European populations of *P. chrysocephala* to determine whether this compound is as effective as it is against UK populations.
7. The effect of cyantraniliprole on *Microctonus brassicae,* a parasitoid of *P. chrysocephala* adults and other natural enemies, should be determined using glass vial bioassays to understand whether this compound is compatible with IPM strategies. Indeed, it would be useful to measure whether this beneficial insect has resistance to pyrethroids.

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Appendix 1

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Investigating the status of pyrethroid resistance in UK populations of the cabbage stem flea beetle (*Psylliodes chrysocephala*)

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ABSTRACT

The cabbage stem flea beetle, *Psylliodes chrysocephala* L. is a major pest of winter oilseed rape in several European countries. Traditionally, neonicotinoid and pyrethroid insecticides have been widely used for control of *P. chrysocephala*, but in recent years, following the withdrawal of neonicotinoid insecticide seed treatments, control failures have occurred due to an over reliance on pyrethroids. In line with previous surveys, UK populations of *P. chrysocephala* were found to exhibit high levels of resistance to the pyrethroid lambda-cyhalothrin. This resistance was suppressed by pre-treatment with the cytochrome P450 inhibitor PBO under laboratory conditions, suggesting that the resistance has a strong metabolic component. The L1014F (kdr) mutation in the voltage-gated sodium channel, which confers relatively low levels (10–20 fold) of resistance to pyrethroids, was also found to be widespread across the UK regions sampled, whereas the L925I (s-kdr) mutation was much less common. The current survey also suggests that higher levels of pyrethroid resistance have spread to the North and West of England, and that resistance levels continue to remain high in the South East.

1. Introduction

The cabbage stem flea beetle, *Psylliodes chrysocephala* (Coleoptera: Chrysomelidae) is an established and key insect pest of winter oilseed rape, particularly in the UK (Graham and Alford, 1981) and Germany (Zimmer et al., 2014), and is a significant pest of other *Brassica* species in several European countries (Bromand, 1990; Bartlet and Williams, 1991; Bartlet et al., 1996). *P. chrysocephala* inflicts damage at both the larval and adult stage, with the tunnelling of the larvae into the leaf petioles and main stems causing the most damage through weakening of the upper section of the roots and lower parts of the stems (Williams, 2004). When infestation is high, the plant tips distort, the stems wilt and the infested plants become more susceptible to fungal infections such as *Phoma lingam* (Alford, 2003), the bacterial disease *Erwinia* sp. and frost damage (Højland et al., 2015; Højland and Kristensen, 2018). Adult *P. chrysocephala* cause damage by feeding on stems, cotyledons and the first true leaves during crop emergence resulting in 'shot-holing' symptoms, leading to poor plant vigour or potential seedling death before emergence when fields are heavily infested (Williams, 2010).

Prior to 2014, *P. chrysocephala* affected approximately 67% of the area of oilseed rape grown in the UK causing an annual 1% yield loss (Clarke et al., 2009). However, in 2014, serious crop losses due to adult beetles (2.7% of the national crop) were recorded, the most serious losses (5–14%) being in eastern and southern England (Wynn et al., 2014). In the autumn of 2015, a more extensive survey found that over 65% of crops had some damage, and that the damage was more widely distributed across the country than in 2014, although nationally only 1% of crops were lost (Alves et al., 2015). Subsequent surveys have confirmed that the average numbers of larvae per plant have risen substantially in all regions since 2014 (as summarised in Dewar, 2017).

Prior to December 2013, control of *P. chrysocephala* relied on the protection of oilseed rape seedlings by systemic neonicotinoid seed treatments containing either imidacloprid, thiamethoxam or clothianidin, followed by the application of foliar pyrethroid sprays later in the season if needed (Højland et al., 2015; Højland and Kristensen, 2018). However, in December 2013, the European regulatory authorities (EU Commission, 2013) banned the use of neonicotinoid seed treatments on oilseed rape, leading to the increase in *P. chrysocephala* and the

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increased use of pyrethroid sprays. Today, pyrethroids (e.g. lambda-cyhalothrin) are the only class of insecticide that remain for chemical control of *P. chrysocephala* in the UK and other parts of mainland Europe.

The continuous use of pyrethroids to control *P. chrysocephala*, coupled with the lack of alternative insecticides with different modes of action, has led to a high selection pressure, driving the development and spread of resistance. Resistance to pyrethroids was first reported in 2008, in north-western Mecklenburg, Western Pomerania, a major oilseed rape growing area in Northern Germany (Heimbach and Müller, 2013). Zimmer et al. (2014) reported the presence of the L1014F kdr mutation in the voltage-gated sodium channel, with high frequencies of the allele (90–100%) being found in populations collected from across Northern Germany, with the beetles exhibiting low level resistance against a range of pyrethroids including lambda-cyhalothrin. More recently, studies by Højland et al. (2015) and Højland and Kristensen (2018) have shown that pyrethroid resistance resulting from the kdr mutation is also present in populations from both Demark and the UK, whilst in Germany it has spread further south. Despite the presence of kdr in UK populations, Højland et al. (2015) found that the high pyrethroid resistance levels, with control failures being observed at the full field rate, did not completely correlate with the *kdr* genotype suggesting that another mechanism of resistance, such as metabolic resistance, is also present. Given the lack of alternative insecticides with different modes of action, the presence and spread of pyrethroid resistance is concerning for the chemical control of *P. chrysocephala*.

The present study has determined the current status, extent and geographical spread of pyrethroid resistance in UK populations of *P. chrysocephala*. Bioassays, based on glass vial exposure of adult beetles to lambda-cyhalothrin, were carried out on samples collected in 2018 and 2019 to examine how resistance had changed over this time across the UK. The presence of the *kdr* and super-*kdr* target-site mutations in UK populations was also monitored, and the potential contribution of a metabolic resistance component in the beetles assessed by pre-treatment with the synergist PBO, which is a cytochrome P450 inhibitor.

2. Methods

2.1. Collection of field samples of Psylliodes chrysocephala

In July/August 2018 and 2019, live *P. chrysocephala* adults were collected from oilseed rape pods freshly harvested from the fields at Rothamsted Research, Harpenden, Hertfordshire, using a hand-held battery-powered pooter. Insects were maintained at 15 ± 1 °C, with 65% relative humidity in a light:dark photoperiod of 12:12 h. Adults were kept in a mesh cage and fed continuously on a diet of Chinese cabbage *(Brassica rapa* spp*)*. Further samples were received by post from oilseed rape fields across the UK and were kept in sealed plastic bags or plastic containers containing Chinese cabbage or oilseed rape plant material and moist tissue paper, maintained in the same environmental conditions as the Rothamsted samples.

2.2. Bioassays to test the effect of pyrethroids on Psylliodes chrysocephala

P. chrysocephala samples were tested for resistance to the pyrethroid lambda-cyhalothrin using a glass vial bioassay based on IRAC (Insecticide Resistance Action Committee) Method 031 (www.irac-online.org/methods/weevils-and-flee-beetles/2014). Glass vials (14 ml: 7 cm tall/2 cm diameter) (S Murray and Co, Surrey, UK) were prepared by coating the inner surface with different concentrations of the insecticide. Initial stock solutions were prepared by diluting the technical grade insecticide in technical grade acetone. Three doses, equivalent to 4%, 20% and 100% of the recommended field application rate of lambda-cyhalothrin (7.5 g a.i./ha) were used. The controls were glass vials treated with acetone only. To coat vials, 500 µl of solution was pipetted into the vials which were then placed horizontally without lids on a roller in a fume hood. Vials were rotated at room temperature for at least 2 h until all the acetone had evaporated. Vials were then left vertically at 4 °C overnight before attaching the screw tops the following day.

The adult beetles (see 2.1) were used within a few days of collection and only those capable of walking or jumping when released onto a tray inside a three-sided Perspex cage were collected, using a hand-held battery-powered pooter. A minimum of ten beetles were transferred from the inverted pooter through a small funnel into each vial. The vials were then resealed and left at 18 ± 1 °C under a 16:8 h light:dark photoperiod. After 24 h, the beetles were transferred to untreated glass vials without lids under upturned 200 ml plastic disposable cups (VWR International Ltd, Dublin, Ireland), to allow for a potential recovery which can occur in insects with metabolic resistance. After a further 24 h, the beetles were released onto a tray and individuals scored using a fine paint brush according to three categories: 'mobile' (capable of jumping or walking in a coordinated way), 'affected' (incapable of jumping or coordinated movement) or 'dead' (no movement). Scoring of the beetles from each vial was done for 10 min to avoid adults that were simulating death, a behaviour shown by this species that has probably evolved through predation pressure. Results were expressed as percentage mortalities. Following scoring, beetles in each category were transferred to Eppendorf tubes and snap frozen using liquid nitrogen before being stored in a freezer at −80 °C.

2.3. TaqMan PCR assay to detect the presence of kdr/skdr in Psylliodes chrysocephala

TaqMan genotyping assays (Livak, 1999) were used to determine the presence of the mutations responsible for the kdr (L1014F) and super-kdr (L925I) sodium channel substitutions in individual adult beetles. Primer Express v.2.0 (Life Technologies) was used to design the primer and probe sequences for the assays (Table 1). In both assays, VIC reporter dye-labelled probes were used to detect the wild-type susceptible allele and 6-FAM reporter dye-labelled probes to detect the resistant allele. Each probe contained a 3' non-fluorescent quencher dye.

PCR reactions (15 µl) contained 1.5 µl (50 ng) genomic DNA, 7.5 µl SensiFast probe mix (Bioline Reagents Ltd, UK), 0.375 µl of kdr or skdr primer/probe mix (800 nM of each primer and 200 nM of each probe) and sterile water. Reactions were run on an Applied Biosystems 7900HT real-time PCR system, with initial incubations at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. The increase in VIC and 6-FAM reporter dye fluorescence was monitored in real time and an allelic discrimination analysis performed using the 7900HT Sequence Detection System software.

2.4. Use of a synergist to identify the presence of metabolic resistance in Psylliodes chrysocephala

Pre-treatment with the insecticide synergist Piperonyl butoxide (PBO), obtained from Sigma-Aldrich (Missouri, USA), was used to detect potential metabolic resistance mechanisms *in-vivo*. PBO was diluted in technical grade acetone to give an equivalent concentration of 0.011 mg cm^{-2} (Højland et al., 2015). This dose was chosen because it did not

Table 1
Primer and probe sequences used for TaqMan assays to detect the L1014F (kdr) and L925I (skdr) mutations in *Psylliodes chrysocephala*.

Primer/Probe		Sequence
Primers	kdr-F	GGACTGTAGGCTAGTGRGTGAYAT
	kdr-R	GCAAAGCCAAGAAGAGATTCAGTA
	skdr-F	GCCAAGTCAYGGCCAACTT
	skdr-R	TATAATGCACAGCACAAAGGTCA
Probes	kdr-VIC	TTACCACAAGATTACC
	kdr-FAM	TTACCACAAAATTACC
	skdr-VIC	TGGTGCTTAGGTAA
	skdr-FAM	TGGTGCTACAGGTAA

cause control mortality when tested. 500 μl of solution was then used to coat glass vials (see 2.2). Ten beetles per replicate were transferred to the PBO-coated vials for 1 h before being transferred to either untreated control vials or vials coated with lambda-cyhalothrin at the 100% field rate (7.5 g a.i./ha). The beetles were then bio-assayed in parallel to beetles from the same sample not pre-exposed to PBO.

3. Results and discussion

3.1. Survey of pyrethroid resistance in Psylliodes chrysocephala across the UK

To determine the current extent and geographical spread of resistance to pyrethroid insecticides in UK *P. chrysocephala* populations, and how this compares to previous reports (Højland et al., 2015), bioassays with lambda-cyhalothrin were conducted on adult beetle samples from Rothamsted Research's farm in Hertfordshire and oilseed rape fields located across England, Scotland and Wales. The bioassays allowed the samples to be categorised as being either completely susceptible, or to contain beetles that were 0–25%, 25–50%, 50–75%, 75–99% and 100% resistant, depending on the percentage of beetles per sample surviving treatment with 7.5 g a.i. ha^{-1} lambda-cyhalothrin. Although lambda-cyhalothrin is used as an exemplar in these studies, other pyrethroids also contribute to the selection pressure in *P. chrysocephala* populations across Europe. The bioassay is an approved test method (method 031) for determining resistance in *P. chrysocephala* (IRAC, 2104) and was used by Zimmer et al. (2014) to monitor the emergence and geographic spread of pyrethroid resistance in *P. chrysocephala* in Germany, by Højland et al. (2015) to determine the spread of pyrethroid resistance in Danish, British and German samples and most recently by Højland and Kristensen (2018) when investigating lambda-cyhalothrin resistance in Danish populations. Similar bioassays have also been used to monitor the spread of pyrethroid resistance in European populations of pollen beetle (*Brassicogethes aeneus*), another major pest of oilseed rape (Zimmer and Nauen, 2011; Slater et al., 2011; Nauen et al., 2012).

In 2018, a total of 41 *P. chrysocephala* samples, obtained from four different regions across England, but primarily from counties in the East (Fig. 1), were tested. Of these only five samples were found to contain no mobile beetles at 100% of the recommended field rate for lambda-cyhalothrin, which would be expected if the sample was susceptible. However, for these five samples mortality was found to be < 90% at 20% of the field rate, suggesting resistance is present as judged by the IRACs 'susceptibility rating scheme' (IRAC, 2104). The other 37 samples all showed some level of resistance with the highest resistance, at 89% being the sample from Bishop Cannings (Wiltshire).

In 2019 a total of 146 *P. chrysocephala* samples were obtained from across England, representing more of the country (Fig. 1), two samples were received from Wales and one from Scotland. Only the Scottish sample was found to be truly susceptible to lambda-cyhalothrin, displaying 100% mortality at 20% of the recommended field rate. Worryingly, several populations containing 100% resistant beetles were recorded for the first time in the UK. Overall, the distribution maps for pyrethroid resistance in UK populations of *P. chrysocephala* (Fig. 1) suggest that higher levels of resistance have spread to the North and West of England and that resistance levels continue to remain high in the South East.

Over the two years of monitoring, the percentage of highly pyrethroid-resistant beetles in the samples increased. The mean resistance level was significantly greater in 2019 (55.64%) compared to

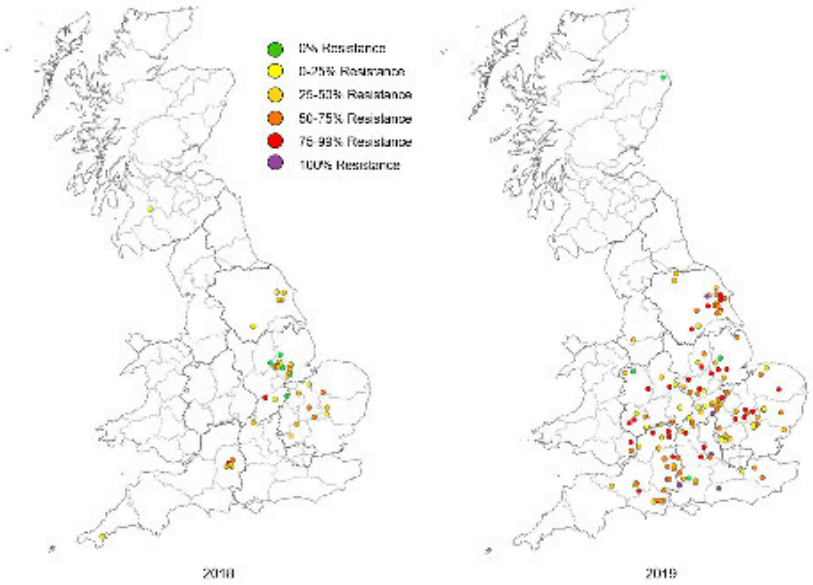

Fig. 1. Pyrethroid resistance in *P. chrysocephala* in the UK for 2018 and 2019. The maps were created using QGIS (version 3.0.3) and use a 6-category colour scale to show the level of resistance. The map is divided into counties (light grey borders) and regions (dark grey borders).

2018 (32.9%) (two-sample *t*-test, $t_{185} = -5.02$, $p < 0.001$, SED = 4.529). Over the two years there was found to be a significant difference in the distribution of measurements across the resistance categories, $\chi^2(3) = 18.47$, $p < 0.001$ (Fig. 2). In 2018 the percentage of beetles in the 0–25% resistance category was 39%, whereas in 2019 this decreased to 16%. In 2018 5% of samples were in the 75–100% resistance category whereas in 2019 this increased to 28%.

To assess whether there was any impact of the spatial variation over which the samples were collected, analysis of covariance was undertaken based on year, adjusting for the easting and northing coordinates as covariates. There was no evidence of a linear association between the covariates and the outcome at the 5% significance level. The analysis was then repeated with 3 geographically extreme data values omitted (two observations in Scotland and one in Cornwall). Again, there was no evidence of a linear association between the covariates and the outcome. However, in both cases the analysis showed a significant difference in mean resistance between the two years ($p < 0.001$). Scatter plots of the easting and northing coordinates plotted against resistance did not indicate any other non-linear association.

Further analysis was undertaken to assess regional and county-level differences for the 2019 data. The mean resistance levels (Table 2a) were higher than the national average in the South East (60.39%), South West (57.83%), and Yorkshire and the Humber (63.32%). South-East Wales had the highest mean resistance (72.50%) but only two samples were tested. Analysis of variance (ANOVA), incorporating a nested treatment structure to reflect counties nested within regions, showed that mean resistance levels did not differ significantly between regions ($F_{8,113} = 1.28$, $p = 0.262$). The standard errors of the differences between means (SEDs) at the regional level were also calculated (Table 2b). There was also found to be no significant difference in mean resistance levels between counties within the same region ($F_{24,113} = 1.06$, $p = 0.395$). The residual mean square from the ANOVA was 675.3. The absence of statistically different mean resistance levels suggests there are no resistance 'hotspots' and that resistance is highly localised, almost on a farm-by-farm basis.

3.2. Pyrethroid resistance mechanism(s) in *P. chrysocephala*

The TaqMan assays (see 2.3) were used to detect the presence of the L1014F (kdr) and L925I (*s*-kdr -like) substitutions in the 2018 and 2019 *P. chrysocephala* samples (Table 3). In 2018, 40 beetles from seven UK samples of *P. chrysocephala* were tested using only individuals that had survived the 100% field rate of lambda-cyhalothrin, enabling the genotype associated with the resistant, mobile phenotype to be determined. The samples were from Great Saxham (Suffolk), Bishop Cannings (Wiltshire), Rothamsted (Hertfordshire), Linton (Cambridgeshire), Feltwell (Norfolk) and Horbling and Grantham (Lincolnshire). The L1014F mutation was present at all sites, with 47.5% of the beetles being homozygous for the resistant allele (RR), 37.5% heterozygous (SR) and the remaining 15% *kdr* SS, although this genotype was not present in the Suffolk or Wiltshire populations. The detection of *kdr* SS genotypes in beetles that displayed the mobile phenotype after treatment with the label rate of lambda-cyhalothrin, strongly suggests the presence of another resistance mechanism in *P. chrysocephala*. We also identified *kdr* RR (homozygote) genotypes in beetles that did not survive lambda-cyhalothrin treatment, confirming that the L1014 mutation on its own is not able to confer protection to the field rate dose (results not shown). In contrast to L1014F, the L925I mutation, which is predicted (based on studies in other insects) to be associated with higher resistance levels to pyrethroids than kdr, was much less common, with two samples (Norfolk and Hertfordshire) showing only the SS genotype and the overall percentage of beetles showing the homozygous L925I genotype (RR) being only 2.5%. Of the six beetles homozygous for the susceptible *kdr* allele (SS), one also displayed the homozygous resistant *s-kdr* allele (RR) and two displayed the heterozygous resistant *s-kdr* allele (SR) (data not shown). As three beetles were susceptible for both the *kdr* and *s-kdr* allele this suggests the presence of another resistance mechanism. Direct sequencing of sodium channel fragments carrying the mutations showed that L1014F (kdr) and L925I (*s*-kdr) are mutually exclusive and have arisen independently in different sodium channel alleles, thus limiting the number of genotypic combinations possible within individual beetles.

In 2019, *P. chrysocephala* individuals were screened for kdr from sites close to those sampled in 2018 (a sample from Oxfordshire was also included) and again, only beetles that survived the 100% field rate of lambda-cyhalothrin were tested. The L1014F mutation was present at all sites except the one from Scotland. The percentage of beetles homozygous for the *kdr* resistance allele (RR) increased in three of the samples, Suffolk, Norfolk and Hertfordshire but decreased overall from 47.5% to 36%. Given that the percentage of beetles resistant to lambda-cyhalothrin in each sample increased between 2018 and 2019, but there was an overall decrease in the homozygous and heterozygous L1014F mutation, this further suggests the presence of another resistance mechanism in *P. chrysocephala*. Whilst the L925I (*s*-kdr) mutation was less common than the L1014F (kdr) mutation, it was found to be present in the Wiltshire and Hertfordshire samples which contained only the wild-type metabolic genotype (SS) in 2018. In the Oxford sample 15% of the beetles tested for the *s*-kdr mutation were homozygous for the resistant allele (RR). Overall the percentage of beetles showing the homozygous genotype (RR) was 6.6%.

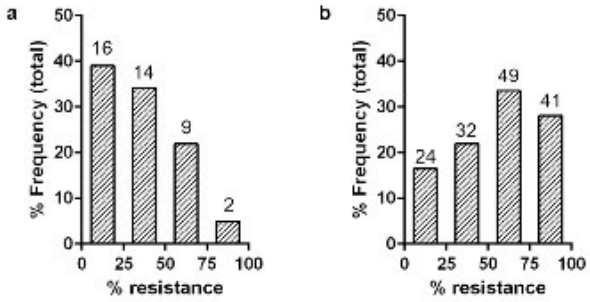

Fig. 2. Histograms showing the shift in the relative % frequency of pyrethroid resistant *P. chrysocephala* in (a) 2018 and (b) 2019. Numbers show the raw count data.

Table 2
(a) Summary of average resistance levels by region and county and (b) Standard error of differences between means at the regional level.

A

Region and County	Number of Samples	Average resistance level (%)
East Midlands	29	51.28
Leicestershire	7	60.43
Northamptonshire	12	48.58
Nottinghamshire	3	49.67
Lincolnshire, Parts of Kesteven	6	45.33
Lincolnshire, Parts of Lindsey	1	60
East of England	29	53.72
Bedfordshire	3	57.33
Cambridgeshire	8	72.25
Essex	4	31.5
Hertfordshire	3	26
Huntingdonshire	2	57
Norfolk	3	53.33
Suffolk	6	55
North West	1	40
Lancashire	1	40
Scotland	1	0
Aberdeenshire	1	0
South East	18	60.39
Berkshire	2	80
Hampshire	6	50.67
Kent	3	61.67
Oxfordshire	5	64
Surrey	1	18
Sussex	1	100
South East Wales	2	72.5
Monmouthshire	2	72.5
South West	35	57.83
Dorset	7	53.57
Gloucestershire	8	57.88
Somerset	4	57.5
Wiltshire	16	59.75
West Midlands	12	48.33
Herefordshire	4	60.25
Shropshire	3	46.67
Staffordshire	3	46.33
Warwickshire	1	10
Worcestershire	1	50
Yorkshire and the Humber	19	63.32
East Riding of Yorkshire	14	68.93
North Riding of Yorkshire	3	43.33
West Riding of Yorkshire	2	54
Grand Total	146	55.64

B

Region		Standard Error of Differences								
East Midlands	1	*								
East of England	2	6.82	*							
North West	3	26.43	26.43	*						
Scotland	4	26.43	26.43	36.75	*					
South East	5	7.8	7.8	26.7	26.7	*				
South East Wales	6	19	19	31.83	31.83	19.37	*			
South West	7	6.53	6.53	26.36	26.36	7.54	18.89	*		
West Midlands	8	8.92	8.92	27.05	27.05	9.68	19.85	8.69	*	
Yorkshire and the Humber	9	7.67	7.67	26.66	26.66	8.55	19.32	7.41	9.58	**
		1	2	3	4	5	6	7	8	9

3.3. Bioassays of *P. chrysocephala* using lambda-cyhalothrin and the synergist PBO

The insecticide synergist piperonyl butoxide has been shown to inhibit both P450 monooxygenases and esterases, thereby acting as a tool for the identification of metabolic resistance in insect samples (Young et al., 2006). To investigate the lack of correlation between lambda-cyhalothrin resistance and *kdr* frequency, and to determine whether P450 monooxygenases (and/or esterases) may play a role in mediating pyrethroid resistance in UK *P. chrysocephala* populations, synergist bioassays with PBO pre-treatments were conducted on five *P. chrysocephala* samples.

When exposed to lambda-cyhalothrin at the recommended field rate, the percentage of beetles affected was 47% (North Yorkshire), 75% (Wiltshire), 8% (Wiltshire), 40% (Leicestershire) and 40% (Hertfordshire) (Fig. 3). However, all adults pre-treated with PBO, prior to exposure to lambda-cyhalothrin at the same field rate were killed. This strongly suggests that a metabolic-based mechanism for pyrethroid resistance is present in *P. chrysocephala*, although it must be acknowledged that PBO has many more effects than just inhibiting enzymes, aiding cuticular penetration and increasing the insect's susceptibility to environmental stressors.

4. Conclusions

Since the EU-imposed ban on neonicotinoid seed treatments,

Table 3
Detection of kdr/skdr alleles in *P. chrysocephala* using TaqMan assay.

Region	County	Populations	No°	SS	SR	RR	%RR
2018				*kdr* status			
East Midlands	Suffolk	1	4	0	3	1	25%
	Wiltshire	1	8	0	4	4	50%
	Lincolnshire	2	10	2	2	6	60%
East of England	Cambridgeshire	1	8	2	2	4	50%
	Norfolk	1	6	1	3	2	33%
	Hertfordshire	1	4	1	1	2	50%
	Total	7	40	6 (15%)	15 (37.5%)	19 (47.5%)	
				skdr status			
East Midlands	Suffolk	1	4	3	1	0	0%
	Wiltshire	1	8	5	3	0	0%
	Lincolnshire	2	10	9	1	0	0%
East of England	Cambridgeshire	1	8	5	2	1	13%
	Norfolk	1	6	6	0	0	0%
	Hertfordshire	1	4	4	0	0	0%
	Total	7	40	32 (80%)	7 (17.5%)	1 (2.5%)	
2019				*kdr* status			
East Midlands	Suffolk	1	4	0	1	3	75%
	Wiltshire	1	5	3	1	1	20%
	Lincolnshire	2	16	2	8	6	38%
East of England	Cambridgeshire	1	8	1	6	1	13%
	Norfolk	1	8	2	4	5	63%
	Hertfordshire	1	5	1	1	3	60%
SE England	Oxfordshire	1	19	7	4	8	42%
Scotland	Aberdeenshire	1	10	10	0	0	0%
	Total	9	75	26 (34.7%)	25 (33.3%)	27 (36%)	
				skdr status			
East Midlands	Suffolk	1	5	5	0	0	0%
	Wiltshire	1	3	1	1	1	33%
	Lincolnshire	2	16	11	5	0	0%
East of England	Cambridgeshire	1	8	5	3	0	0%
	Norfolk	1	8	7	1	0	0%
	Hertfordshire	1	6	4	1	1	17%
SE England	Oxfordshire	1	20	13	4	3	15%
Scotland	Aberdeenshire	1	10	10	0	0	0%
	Total	5	76	56 (73.7%)	15 (19.7%)	5 (6.6%)	

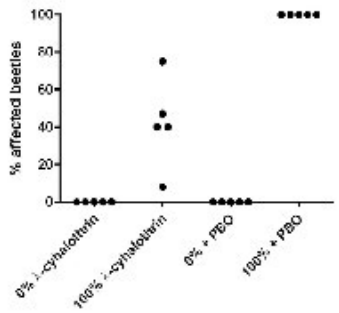

Fig. 3. Restoration of insecticide (pyrethroid) susceptibility in *P. chrysocephala* following pre-treatment with PBO. Samples tested were from North Yorkshire, Wiltshire (x2), Leicestershire and Hertfordshire.

pyrethroid insecticides have been widely used for chemical control of *P. chrysocephala* in the UK. This has resulted in a high selection pressure and led to the development of resistance, particularly in the South East of England. In the current study, populations of *P. chrysocephala* from around the UK were found to exhibit high levels of resistance to lambda-cyhalothrin, but this resistance was suppressed by the cytochrome P450 inhibitor PBO. This suggests that, as well as target site resistance, there may be P450 mediated-detoxification of lambda-cyhalothrin, although further research is required to identify the specific P450(s) involved and elucidate the exact mechanism of resistance. This resistance to pyrethroids has resulted in widely-reported control problems for this pest in the farming press (e.g. Clarke, 2014; Caswell, 2014; FarmingUK Team, 2015; Hill, 2017; FarmingUK Team, 2017; Case, 2018; Allison, 2019; Dyer, 2019; Gillbard, 2019) since the introduction of the neonicotinoid seed treatment ban.

Despite the development of resistance in *P. chrysocephala*, pyrethroids continue to be used on UK farms as there remains a lack of insecticides with alternative modes of action that can be deployed for resistance management. However, this continued reliance on pyrethroids is failing as a control strategy in many regions and is not sustainable in areas where resistance levels may appear low or non-existent. Since 2014, there has been a significant year by year decrease in the area of oilseed rape production in the UK, declining from 634,000 ha in 2014 (DEFRA, 2014) to 497,000 ha in 2019 (DEFRA, 2019). It is therefore particularly important that the extent and geographical spread of pyrethroid resistance in this pest continues to be monitored at a time when synthetic pesticides are becoming less favoured through EU legislation. Clearly there needs to be informed decision making on how to best deploy pesticides effectively in the future. Alternative strategies, such as the potential of the parasitoid *Microctonus brassicae* for biological control

(Jordan et al., 2020), trap cropping (Barari et al., 2005) and the use of insect-resistant varieties of oilseed rape also offer options for *P. chrysocephala* control and are being further explored.

CRediT authorship contribution statement

Caitlin E. Willis: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Stephen P. Foster:** Conceptualization, Methodology, Writing - review & editing, Supervision. **Christoph T. Zimmer:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision. **Jan Elias:** Resources, Writing - review & editing, Project administration. **Xianmin Chang:** Writing - review & editing, Supervision. **Linda M. Field:** Writing - review & editing, Supervision. **Martin S. Williamson:** Conceptualization, Methodology, Writing - review & editing, Supervision. **T.G. Emyr Davies:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix 2

Protein BLAST output of the *P. chrysocephala CYP6k1-like* protein sequence against the Western corn rootworm (*Diabrotica virgifera virgifera*) *CYP6k1-like* protein sequence.

cytochrome P450 6k1-like isoform X1 [Diabrotica virgifera virgifera]

Sequence ID: XP_028141403.1 Length: 499 Number of Matches: 1

Range 1: 1 to 499 GenPept Graphics ▼ Next Match ▲ Pre

Score	Expect	Method	Identities	Positives	Gaps
676 bits(1744)	0.0	Compositional matrix adjust.	319/503(63%)	396/503(78%)	6/503(1%)

```
Query  1    MLLTSSWIPDLVLFLATISLVSYLYATRKFDYWKKRNVLYQKPVPFFGNFKEVITLKKQI  60
            MLLTSSWI D++ F  T+S + Y+Y TR F YWKKRNV Y+KP PFFGNFK+V ++K  I
Sbjct  1    MLLTSSWIVDVLAFSVTLSTLLYIYFTRHFSYWKKRNVFYRKPTPFFGNFKDVASMKTTI  60

Query  61   GIWLREAYNAAKDTPYFGMFVFDEPYLVLKSPQLIKHVMIKDFNNFSDRTVACPDHNNVT  120
            G WLR+ YN A   PYFG+FVFDEP LV+KSP++IK++MIKDFNNF DRT A P+HN V
Sbjct  61   GEWLRDEYNKAAKQPYFGIFVFDEPKLVIKSPEIIKNIMIKDFNNFCDRTFASPEHNEVE  120

Query  121  KNFLFFMRNPEWKTNRVQLTPVFTSGKLKYMFPTAVLVSDRLQKYLANNLGVLEAKEIAA  180
             NFLF M NP+WK +R QL+P FTSGKLK M P    V   LQKYL++N  VLEAKE+ A
Sbjct  121  SNFLFMMHNPKWKKHRTQLSPAFTSGKLKAMLPIIHEVGQSLQKYLSDNQTVLEAKEVMA  180

Query  181  KYSTEAMARSFFGINAHCFDDENAMFRVLGRKIFDFSVRNGLVNTAYFSMQKLVRLFRIN  240
            KYST+ + + F GIN HCFD+++A+FRVLGR +FDFS++N L  TAYFS   +V+ F+I+
Sbjct  181  KYSTDVIGKCFLGINPHCFDNDDALFRVLGRAMFDFSIKNALKQTAYFSTPGIVKFFKIS  240

Query  241  IFEKWVFEYFIDSFTKAFEARENSTTRKNDFIDILIELNKKDGGNGLFDMD--KINGTGL  298
             FE+WV ++F+D F+K++EARE S  RKNDFIDIL ++ +K    G+ D D   I G+ +
Sbjct  241  FFEQWVTDHFVDVFSKSYEAREKSKIRKNDFIDILRDMERK----GVIDKDIGSIQGSSM  296

Query  299  QFFVAGFETTSSTISYTLHELCLNKTVQNKVRQEILANMKENDGLTYEGVMGMKYLDMCV  358
            QFF AGFETTSST S+TL+ELCLNKT+QNKVR EILAN++EN G+TYEGVM MKYLD C+
Sbjct  297  QFFAAGFETTSSTTSFTLYELCLNKTIQNKVRSEILANIRENKGITYEGVMSMKYLDQCI  356

Query  359  KETLRKYPAVPFLDRKCVNECKLPGTDLILEKGASVYIPLVGLHYDEKYFPEPDKYDPER  418
            KETLRKYP +PFLDR+C+N   +PGTDL++EKG+SVYIP+ GLHYDE YFPEP KY PER
Sbjct  357  KETLRKYPVLPFLDRRCINNYTIPGTDLVIEKGSSVYIPMFGLHYDETYFPEPMKYMPER  416

Query  419  FANPKCNSEGLVYLPFGEGPRMCIGERLGMLTTKLGIISVLTKFEVERCAQTPDPVIFEA  478
            F N   N+ GLVY PFGEGPR+CIGER G++++KL +I VLTKFEVE+C  TPDP+ FE
Sbjct  417  FENANYNTNGLVYFPFGEGPRICIGERFGLMSSKLALIYVLTKFEVEKCESTPDPLEFEP  476

Query  479  KSLVLQSTVGLPMTFKYITPPPA  501
            KSLVLQS VG+PM FK++ P PA
Sbjct  477  KSLVLQSKVGVPMRFKHLVPSPA  499
```

Appendix 3

DNA sequence for *P. chrysocephala CYP6k1-like.*

ATGTTGCTGACTTCATCGTGGATTCCAGATTTAGTACTTTTTCTTGCAACGATATC
GTTGGTGTCATACTTGTATGCAACTAGAAAATTCGATTATTGGAAAAAACGCAATG
TCCTCTACCAAAAACCTGTACCATTCTTTGGCAATTTCAAAGAAGTAATTACTTTG
AAAAAACAAATTGGTATATGGTTAAGAGAAGCGTACAATGCAGCCAAAGATACGCC
ATATTTTGGAATGTTCGTCTTTGATGAACCGTATTTGGTATTGAAATCTCCGCAGT
TGATCAAGCATGTCATGATTAAAGATTTTAACAATTTCAGTGATAGAACTGTTGCT
TGTCCAGATCACAATAACGTTACAAAAAACTTTCTGTTTTTTATGAGAAATCCCGA
GTGGAAAACTAATAGAGTTCAGTTGACACCGGTTTTTACTTCTGGAAAATTGAAGT
ATATGTTTCCGACTGCTGTTTTAGTGAGTGACCGTTTACAAAAATATCTAGCCAAC
AATCTTGGAGTGTTGGAGGCCAAAGAAATAGCTGCCAAATATTCCACTGAAGCCAT
GGCCAGAAGCTTTTTCGGCATAAATGCGCATTGTTTCGACGACGAAAACGCCATGT
TCAGAGTATTGGGAAGGAAAATTTTTGATTTCAGTGTTAGAAACGGCCTCGTAAAC
ACAGCCTACTTTTCAATGCAAAAATTGGTTAGACTGTTTCGCATTAATATATTCGA
AAAATGGGTGTTTGAGTATTTTATTGATAGTTTTACGAAAGCATTTGAGGCAAGAG
AAAATAGTACGACGCGCAAGAATGATTTTATTGATATCTTGATTGAATTAAACAAA
AAGGATGGCGGAAATGGATTATTTGATATGGACAAAATTAATGGTACCGGTCTGCA
GTTTTTCGTGGCTGGATTTGAAACCACAAGTTCAACTATTTCGTATACCCTGCACG
AATTATGCCTAAACAAGACTGTGCAGAATAAAGTGAGACAAGAAATCCTAGCCAAC
ATGAAAGAAAATGATGGATTAACATACGAAGGCGTCATGGGAATGAAATATTTGGA
TATGTGTGTCAAAGAGACACTCAGGAAATATCCAGCGGTACCATTTTTGGACAGAA
AATGTGTAAACGAATGCAAATTACCCGGAACTGATTTAATATTAGAAAAAGGAGCA
TCAGTTTATATTCCGTTGGTCGGCTTGCACTATGACGAAAAATATTTTCCAGAACC
GGACAAGTACGATCCAGAAAGATTTGCTAATCCAAAATGCAACAGCGAAGGGTTGG
TTTATCTACCATTTGGAGAAGGACCAAGAATGTGTATAGGCGAGAGACTAGGCATG
TTAACGACGAAACTTGGAATTATTTCTGTCCTAACTAAATTTGAAGTCGAAAGATG
TGCCCAGACACCCGATCCAGTAATATTTGAAGCAAAGAGTCTTGTTCTTCAATCCA
CAGTTGGCCTTCCAATGACATTTAAATATATCACTCCACCACCAGCTTGA

Appendix 4

Table 4A. Percentage mortalities (±SD) of P. chrysocephala adults at 4%, 20%, 100%, 200% and 500% of the 'field rate' scored at 24, 48 and 72 hours.

Compound	End-point	4% Field rate	20% Field rate	100% Field rate	200% Field rate	500% Field rate
Acetamiprid	24hrs	20.00 (±15.28)	96.67 (±3.33)	100.00 (±0.00)		
	48hrs	20.00 (±15.28)	96.67 (±3.33)	100.00 (±0.00)		
	72hrs	26.67 (±16.67)	100.00 (±0.00)	100.00 (±0.00)		
Chlorantraniliprole	24hrs		14.43 (±9.87)	63.33 (±18.56)		100.00 (±0.00)
	48hrs		28.13 (±8.13)	96.67 (±3.33)		100.00 (±0.00)
	72hrs		28.53 (±13.84)	100.00 (±0.00)		100.00 (±0.00)
Chlorfenapyr	24hrs		3.33 (±3.33)	3.33 (±3.33)		0.00 (±0.00)
	48hrs		6.67 (±3.33)	33.33 (±8.82)		46.67 (±3.33)
	72hrs		26.70 (±4.46)	53.33 (±8.82)		63.33 (±8.82)
Cyantraniliprole	24hrs		100.00 (±0.00)	100.00 (±0.00)		100.00 (±0.00)
	48hrs		100.00 (±0.00)	100.00 (±0.00)		100.00 (±0.00)
	72hrs		100.00 (±0.00)	100.00 (±0.00)		100.00 (±0.00)
Emamectin Benzoate	24hrs		3.33 (±3.33)	3.33 (±3.33)		0.00 (±0.00)
	48hrs		6.67 (±0.00)	10.00 (±5.77)		0.00 (±0.00)
	72hrs		17.03 (±2.97)	10.37 (±5.79)		7.40 (±3.70)
Etofenprox	24hrs		0.00 (±0.00)	0.00 (±0.00)		96.67 (±3.33)
	48hrs		8.33 (±4.16)	0.00 (±0.00)		96.67 (±3.33)
	72hrs		19.07 (±9.53)	3.33 (±3.33)		96.67 (±3.33)

Fipronil	24hrs		0.00 (±0.00)	33 (±16.98)		100.00 (±0.00)
	48hrs		71.10 (±6.75)	100.00 (±0.00)		100.00 (±0.00)
	72hrs		90.00 (±5.77)	100.00 (±0.00)		100.00 (±0.00)
Flupyradifurone	24hrs		0.00 (±0.00)	13.33 (±3.33)		60.00 (±15.28)
	48hrs		3.33 (±3.33)	23.33 (±13.33)		80.00 (±11.55)
	72hrs		6.67 (±3.33)	36.67 (±17.64)		88.33 (±7.26)
Flupyrimin	24hrs		96.67 (±3.33)	96.67 (±3.33)		100.00 (±0.00)
	48hrs		100.00 (±0.00)	100.00 (±0.00)		100.00 (±0.00)
	72hrs		100.00 (±0.00)	100.00 (±0.00)		100.00 (±0.00)
Metaflumizone	24hrs		0.00 (±0.00)	0.00 (±0.00)		0.00 (±0.00)
	48hrs		13.33 (±3.33)	3.33 (±3.33)		13.33 (±3.33)
	72hrs		16.67 (±6.67)	26.67 (±14.53)		46.67 (±12.02)
Spinosad	24hrs		0.00 (±0.00)	100.00 (±0.00)		83.00 (±16.67)
	48hrs		3.33 (±3.33)	100.00 (±0.00)		100.00 (±0.00)
	72hrs		20 (±15.28)	100.00 (±0.00)		100.00 (±0.00)
Sulfoxaflor	24hrs		0.00 (±0.00)	3.33 (±3.33)		3.33 (±3.33)
	48hrs		0.00 (±0.00)	6.67 (±3.33)		86.67 (±8.82)
	72hrs		0.00 (±0.00)	6.67 (±3.33)		96.67 (±3.33)
Tau-fluvalinate	24hrs	0.00 (±0.00)	6.67 (±5.77)	56.67 (±5.77)	96.67 (±5.77)	
	48hrs	6.67 (±5.77)	13.33 (±5.77)	70.00 (±26.46)	96.67 (±5.77)	
	72hrs	10.10 (10.00)	13.33 (±5.77)	73.33 (±23.09)	100.00 (±0.00)	

Table 4B. Percentage mortalities (±SEM) of P. chrysocephala adults at 0.45ppm, 4.5ppm, 10.3ppm, 103ppm at 72 hours. nt = not tested.

Compound	Sample location	0.45ppm (±SE)	4.5ppm (±SE)	10.3ppm (±SE)	103ppm (±SE)
Cyantraniliprole	West Suffolk	nt	6.50 (±6.50)	100.00 (±0.00)	100.00 (±0.00)
	Wiltshire	nt	58.60 (±15.81)	96.60 (±3.45)	100.00 (±0.00)
	Essex	nt	41.40 (±24.14)	100.00 (±0.00)	100.00 (±0.00)
	West Sussex	nt	0.00 (±0.00)	65.50 (±9.12)	100.00 (±0.00)
	Buckinghamshire	10.60 (±7.38)	55.20 (±9.12)	100.00 (±0.00)	100.00 (±0.00)
	Somerset	0.00 (±0.00)	69.00 (±11.95)	100.00 (±0.00)	100.00 (±0.00)
	East Yorkshire	7.00 (±0.60)	14.10 (±7.61)	94.80 (±5.17)	100.00 (±0.00)
	Northamptonshire	nt	64.60 (±20.13)	100.00 (±0.00)	100.00 (±0.00)
	West Suffolk	0.00 (±0.00)	41.10 (±12.43)	100.00 (±0.00)	100.00 (±0.00)

Table 4C. Percentage mortalities (±SD) of P. chrysocephala larvae at 4%, 20% and 100%, of the 'field rate' scored at 24, 48 and 72 hours.

Compound	End-point	4% Field rate	20% Field rate	100% Field rate
Acetamiprid	24hrs		86.00 (±12.77)	100.00 (±0.00)
	48hrs		94.33 (±9.81)	100.00 (±0.00)
	72hrs		100.00 (±0.00)	100.00 (±0.00)
Buprofezin	24hrs		23.33 (±5.77)	18.33 (±7.64)
	48hrs		26.67 (±5.77)	22.50 (±13.92)
	72hrs		26.67 (±5.77)	29.17 (±8.78)
Cyantraniliprole	24hrs	31.23 (±13.69)	75.33 (±10.95)	90.00 (±17.32)
	48hrs	31.23 (±13.69)	74.83 (±6.18)	96.67 (±5.77)
	72hrs	43.97 (±6.93)	87.57 (±5.01)	100.00 (±0.00)
Fipronil	24hrs		95.83 (±7.21)	100.00 (±0.00)
	48hrs		100.00 (±0.00)	100.00 (±0.00)
	72hrs		100.00 (±0.00)	100.00 (±0.00)
Spirotetramat	24hrs		29.17 (±7.22)	51.87 (±12.91)
	48hrs		37.50 (±12.50)	54.17 (±14.43)
	72hrs		45.83 (±7.22)	58.33 (±19.09)
Triflumuron	24hrs		0.00 (±0.00)	27.00 (±8.19)
	48hrs		0.00 (±0.00)	27.00 (±8.19)
	72hrs		0.00 (±0.00)	27.00 (±8.19)