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Monitoring, mechanisms and management of insecticide resistance and insecticide mode of action in coleopteran pests of winter oilseed rape with special reference to neonicotinoid insecticides under laboratory and applied aspects

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The challenge of resistance
will be with us for eternity
GEORGE P. GEORGHIOU (2000)

Zusammenfassung

Der Winterraps, *Brassica napus* L., hat sich in Europa zu einem festen Bestandteil der maßgeblich von Getreide dominierten Fruchtfolgen entwickelt. Winterraps wird von einer Vielzahl an Schädlingen befallen welche in der Praxis fast ausschließlich durch den Einsatz von chemischen Insektiziden (maßgeblich Pyrethroide) bekämpft werden. Verglichen mit anderen Großkulturen ist der Insektizideinsatz im Rapsanbau relativ hoch. Der seit mehr als 20 Jahren wiederkehrende Einsatz von Pyrethroiden zur Kontrolle von Rapsschädlingen in Europa resultierte in einem enormen Selektionsdruck. Vor diesem Hintergrund ist es nicht verwunderlich, dass drei Schädlinge der Ordnung Coleoptera gegenwärtig eine Resistenz gegen Pyrethroide aufweisen. Bei den drei coleopteren Vertretern handelt es sich um den Rapsglanzkäfer, *Meligethes aeneus* F., den Rapserdfloh, *Psylliodes chrysocephala* L. und den Kohlschotenrüssler, *Ceutorhynchus assimilis* PAYK..

Mittels einem auf insektizidbeschichteten Glasröhrchen beruhenden Biotestsystem („adult vial tests“) wurde die Ausprägung (Stärke und Kreuzresistenzmuster) sowie die geographische Verteilung der Pyrethroidresistenz bei Rapsglanzkäfern und Rapserdflöhen untersucht. Unter Verwendung des identischen Testsystems wurde die „Baseline Susceptibility“ (Ausgangsempfindlichkeit) von Kohlschotenrüsseler gegen *lambda*-Cyhalothrin, ein in der Praxis sehr häufig eingesetztes Pyrethroid, erfasst. Resistente Rapserdflöhe reagierten im Test mit verschiedenen Pyrethroiden gleichermaßen unempfindlich und zeigen eine absolute Kreuzresistenz gegen die Wirkstoffklasse der Pyrethroide, wohingegen beim Rapsglanzkäfer zwei resistente Phänotypen gefunden wurden. Der am häufigsten auftretende Phänotyp weist eine moderate Kreuzresistenz innerhalb der Wirkstoffgruppe auf während ein weiterer Phänotyp ähnlich wie beim Rapserdfloh eine absolute Kreuzresistenz aufweist. Unter Verwendung von Thiacloprid, ein Wirkstoff der chemischen Klasse der Neonikotinoide, im Glasröhrchen Testsystem wurde die Baseline Susceptibility vom Rapsglanzkäfer und Kohlschotenrüssler erfasst. Für den Rapsglanzkäfer wurden von 2009-2012 Daten erfasst und dadurch eine kontinuierliche Überwachung der Sensibilität über die Jahre gewährleistet, für den Kohlschotenrüssler wurden Daten im Jahr 2012 erfasst die eine hohe Empfindlichkeit gegenüber dem Wirkstoff Thiacloprid nachweisen.

In Synergismus-Experimenten mit Rapsglanzkäferpopulationen die hauptsächlich in Mitteleuropa gesammelt wurden, steigerte Piperonyl-Butoxid (PBO) ein Synergist der Cytochrom P450-abhänigen Monooxygenasen, die Toxizität von *lambda*-Cyhalothrin in resistenten Populationen *in vivo* erheblich. Im Folgeexperiment zum Metabolismus von Deltamethrin in Inkubationen mit nativen mikrosomalen Membranpräparationen bestätigte sich der Befund, dass die Resistenz durch Cytochrom P450-abhänigen Monooxygenasen vermittelt wurde, durch den Nachweis von 4-Hydroxy-Deltamethrin. Die Metabolismusrate von

Deltamethrin zu 4-OH-Deltamethrin *in vitro* war mit der erfassten Pyrethroidresistenz *in vivo* positiv korreliert und konnte mit PBO inhibiert werden. Darüber hinaus ließ sich die Hydroxylierung von Deltamethrin durch Zugabe von *tau*-Fluvalinat bzw. *lambda*-Cyhalothrin kompetitiv inhibieren, dieser Befund deckt sich somit mit der *in vivo* erfassten Kreuzresistenz. Mit einer PCR Strategie basierend auf degenerierten Oligonukleotiden wurden Genfragmente der P450 Genfamilie aus Rapsglanzkäfern isoliert und deren Expression mittels qRT-PCR (quantitative Real-Time PCR) in Populationen mit verschiedenen Resistenzausprägungen quantifiziert. Ein P450 Gen, welches CYP6BQ23 kodiert, war in resistenten Populationen signifikant hochreguliert (bis zu 900-fach). Die Überexpression war signifikant korreliert mit der Resistenzausprägung *in vivo* und der Umsetzung von Deltamethrin *in vitro*. Mittels RACE-PCR (Rapid Amplification of cDNA Ends) wurde die komplette mRNA/cDNA isoliert welche ein Leseraster kodierend für 522 Aminosäuren enthält. CYP6BQ23 wurde anschließend in einer Insektenzellkultur rekombinant funktionell exprimiert und der CYP6BQ23 abhängige Deltamethrinmetabolismus bewiesen. Darüber hinaus wurde gezeigt, dass CYP6BQ23 auch *tau*-Fluvalinat umsetzt, allerdings mit einer geringeren Effizienz im Vergleich zu Deltamethrin. Die Metabolismusrate von beiden Wirkstoffen deckt sich mit dem Resistenzmuster vom moderat Kreuzresistenten Phänotyp. Mithilfe eines Computermodels von CYP6BQ23 wurde die Substratbindung simuliert und eine bessere Bindung für Deltamethrin im Vergleich zu *tau*-Fluvalinat prognostiziert.

Der Einfluss von wirkortspezifischer Resistenz (target-site resistance) wurde ebenfalls untersucht. Vom *para*-locus, welcher den spannungsabhängigen Natriumkanal (Voltage Gated Sodium Channel, VGSC) im zentralen Nervensystem der Insekten kodiert, wurde ein Genfragment (Domäne IIS4-6) PCR-amplifiziert, sequenziert und auf Einzelnukleotidpoli-morphismen (SNPs, Single Nucleotide Polymorphisms) untersucht. Ein SNP wurde identifiziert der in einem Aminosäureaustausch von Leucin zu Phenylalanin an Positon 1014 (*Musca domestica* L. Nummerierung) resultiert. Diese Mutation ist in einer Vielzahl von Insekten als knock down resistance (*kdr*) beschrieben und vermittelt eine Kreuzresistenz gegenüber Pyrethroiden und DDT. Im Rapsglanzkäfer wurde diese Resistenzmutation fast ausschließlich in skandinavischen Populationen gefunden wo eine absolute Kreuzresistenz im Feld beobachtet wurde, wohingegen in Mitteleuropa der moderat kreuzresistente Phänotyp vorherrscht. Im Rapserdfloh wurde die gleiche Region im orthologen Locus untersucht mit dem identischen Befund. Die L1014F *kdr* Mutation im Rapserdfloh ist korreliert mit Bekämpfungsproblemen von Rapserdflöhen beim Einsatz von Pyrethroiden und mit dem *in vitro* erfassten Kreuzresistenzmuster.

In den meisten Studien zur Aufklärung von Resistenzmechnismen wurde der Fokus auf eine spezielle Enzym-/Genfamilie gelegt. Um einen holistischeren Weg zu gehen wurde eine Transkriptomstudie beim Rapsglanzkäfer durchgeführt. Ziel war das Assemblieren des

de novo Transkriptoms, die Isolierung von Genen die insektizide Wirkorte kodieren und deren Analyse auf SNPs sowie eine komparative Analyse der Genregulierung zwischen pyrethroid–resistenten und –sensitiven Populationen. Die putativ komplette mRNA des VGSC's konnte assembliert werden. Eine SNP Analyse mittels „Illumina-read mapping" bestätigte die L1014F Mutation in einer schwedischen Population, brachte aber keine zusätzlichen bzw. neuen Mutationen hervor. Die Analyse der Genexpression zwischen resistenten und sensitiven Populationen untermauert den Beitrag von CYP6BQ23 in der Pyrethroidresistenz in allen resistenten Populationen, darüber hinaus wurden noch weitere Kandidatengene, wie z.B. Glutathione S-transferasen und Carboxylesterasen, identifiziert.

Die Konsequenzen der Ergebnisse für ein nachhaltiges Resistenzmanagement in coleopteren Schädlingen in Winterraps and die Möglichkeiten für weiterführende Studien werden in der vorliegenden Arbeit diskutiert.

Abstract

Winter oilseed rape, *Brassica napus* L., has become a vital part of cereal-based crop rotations in Europe. It is attacked by numerous insect pests and their control relies on the intensive use of insecticides (compared to other broad acre crops). The exclusive and continuous use of pyrethroid insecticides for almost twenty years led to an enormous selection pressure and facilitated the development of resistance in oilseed rape pests in Europe. Unsurprising three out of the five major pests of the order Coleoptera are reported to be pyrethroid resistant at present: the pollen beetle, *Meligethes aeneus* F.; the cabbage stem flea beetle, *Psylliodes* c*hrysocephala* L. and the cabbage seed weevil, *Ceutorhynchus assimilis* PAYK..

An adult vial bioassay, which is based on insecticide coated glass vials, was used to monitor the spread and strength of pyrethroid resistance and to determine cross-resistance pattern in pollen beetle and cabbage stem flea beetle. Furthermore, baseline susceptibility towards *lambda*-cyhalothrin (a widely used pyrethroid) was also established for the cabbage seed weevil. Whereas the resistant phenotype of the cabbage stem flea beetle expresses an absolute cross-resistance, the cross-resistance pattern observed in pollen beetle was of two main phenotypes one characterized by moderate cross-resistance and one by absolute cross-resistance. The vial bioassay methodology was adapted to thiacloprid, a neonicotinoid insecticide, to determine baseline susceptibility and to provide a methodology to allow long-term susceptibility monitoring of pollen beetle and cabbage seed weevil. Thiacloprid monitoring revealed that pollen beetle and cabbage seed weevil populations collected across Europe in 2009-2012 and 2012 respectively were highly susceptible to this insecticide class.

Synergism experiments on pollen beetle populations collected mainly in central Europe revealed a high synergistic potential for piperonyl butoxide (PBO) *in vivo* suggesting the involvement of enhanced metabolism by cytochrome P450 monooxygenases. Metabolism studies using native microsomal preparations as the enzyme source and deltamethrin as substrate revealed metabolism of deltamethrin with 4-OH-deltamethrin being the major metabolite. Metabolite formation *in vitro* was correlated with the observed pyrethroid resistance level *in vivo* and was suppressible by PBO. Furthermore *tau*-fluvalinate and *lambda*-cyhalothrin competitively inhibited deltamethrin hydroxylation suggesting the involvement of the same P450(s) in the cross-resistance observed *in vivo.* A degenerate PCR approach was used to identify partial P450 gene sequences from pollen beetle. qRT-PCR screening covering a range of pollen beetle populations differing in levels of pyrethroid resistance identified a single P450, CYP6BQ23, as significantly and highly overexpressed (up to ~900-fold) in resistant strains compared to susceptible strains. The expression of CYP6BQ23 was significantly correlated with both the level of resistance and with the rate of

deltamethrin metabolism in microsomal preparations of these populations. The full length open reading frame of CYP6BQ23 encoding 522 amino acids was isolated using RACE-PCR. Recombinant expression of this P450 in an insect cell line demonstrated that it is capable of hydroxylating deltamethrin and *tau*-fluvalinate, albeit the latter at lower efficiency. The turnover of these pyrethroids by CYP6BQ23 is in line with the observed moderate cross-resistant phenotype. Molecular modeling suggested a better fit of deltamethrin into the active site of CYP6BQ23 compared to *tau*-fluvalinate also supporting the biochemical results.

The occurrence of target-site resistance was investigated by single nucleotide polymorphism (SNP) analysis of the *para*-locus encoding the voltage-gated sodium channel (VGSC) in insects. To achieve this goal a partial fragment (domain IIS4-6) encoding an important region of the pyrethroid binding site was PCR amplified and screened for non-synonymous SNPs. One SNP was identified causing a leucine to phenylalanine substitution at amino acid residue number 1014 (*Musca domestica* L. numbering), well known as knock down resistance (*kdr*) conferring an absolute cross-resistance to pyrethroids and DDT in various insect species. Interestingly this target site mutation was found almost exclusively in samples obtained from Scandinavia where absolute cross-resistant pollen beetles may be found in the field, but not in central Europe where the moderate cross-resistant phenotype dominates. Sequencing of the very same gene region in the cabbage stem flea beetle also revealed the presence of the L1014F *kdr* mutation in pyrethroid resistant flea beetle populations, thus explaining the strong cross-resistance pattern observed *in vitro*.

Most mechanistic studies of resistance have focused on elucidating the contribution of particular genes/gene families to pyrethroid resistance. To generate a comprehensive sequence resource and to elucidate global changes in gene regulation related to insecticide resistance in pollen beetle a *de novo* transcriptome was assembled from sequence pools generated by next-generation sequencing. RNA-sequencing of three pyrethroid resistant and one highly susceptible reference population allowed a global gene expression analysis by short read mapping against the generated transcriptome, as well as a SNP analysis. The putative full length mRNA of the VGSC was represented in the *de novo* transcriptome, a SNP analysis confirmed the presence of the *kdr* resistance in a Swedish population but did not provide novel findings in regards to target-site resistance. The gene expression analysis underpinned the massive overexpression of CYP6BQ23 in all resistant samples and identified additional candidate resistance genes belonging to detoxification related gene families such as P450s, glutathione S-transferases and carboxylesterases.

The implications of these results for resistance management in coleopteran pests in winter oilseed rape and opportunities for future work are discussed.

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Abbreviations

AA	Amino acid
ABC transporter	ATP binding cassette transporter
ABT	1-aminobenzotriazole
ACh	Acetylcholine
AChE	Acetylcholinesterase
AI	Active ingredient
ATP	Adenosine-triphosphat
BFC	7-benzyloxy-4-trifluoromethyl coumarin
bp	Base pair(s)
BOMFC	7-benzyloxymethoxy-4-trifluoromethyl coumarin
BOMCC	7-benzyloxymethoxy-3-cyano coumarin
BOMR	7-benzyloxymethoxy resorufin
BR	7-benzyloxyresorufin
C	Celcius
CCE	Carboxylesterases
CDS	Coding sequence
cDNA	complementary deoxyribonucleic acid
cm	Centimeter
CL	Confidence interval
CYP	Cytochrome P450-dependent monooxygenase
DCJW	Active metabolite of indoxacarb
DDT	Dichlorodiphenyltrichloroethane
DEF	S.S.S-tributyl phosphorotrithioate
DEM	Diethyl maleate
DNA	deoxyribonucleic acid
EC	7-ethoxy coumarin
EFC	7-ethoxy-4-trifluoromethyl coumarin
e.g.	Example give
EI	Efficacy index
ER	7-ethoxyresorufin
EST(s)	Expressed sequence tag(s)
Fig	Figure
FL	Fiducial limits
g	Relative centrifugation force (g-force)
g	Gram
GABA	*gamma*-aminobuteric acid receptor
gamma-HCH	*gamma*-hexachlorocyclohexane
GM	Genetically modified
GMO	Genetically modified organism
gDNA	genomic deoxyribonucleic acid
GO	Gene onthology
GOI	Gene(s) of interest
GST	glutathione S-transferases
H	Hour
Ha	Hectare
HPLC	Highl performance liquid chromatography
HSP	High-scoring segment pairs
i.e.	*id est*
kdr	Knock down resistance
K_m	Michaelis constant
λ_{em}	Emmission wavelength
λ_{ex}	Excitation wavelength
LCT	*lambda*-cyhalothrin

LPR	Learn Pyrethroid Resistance strain of *M. domestica*
LC	Lethal concentration
L	Liter
logP	Logarithm of partition coefficient, a measure of lipophilicity
MFC	7-methoxy-4-trifluoromethyl coumarin
MS	Mass spectrometry
m/z	Mass-to-charge ratio
mg	Milligram
mL	Milliliter
mM	Millimolar
min	Minute
µg	Microgram
µL	Microliter
µm	Micrometer
mito	Mitochondrial
M	Molar
MR	7-methoxyresorufin
ng	Nanogram
NADPH	Nicotinamide adenine dinucleotide hydrogen phosphate
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
nm	nanometer
nAChR	Nicotinic acetylcholinereceptor
nt	Nucleotide
OD	Oil dispersion
ORF	Open reading frame
P450(s)	Cytochrome P450-dependent monooxygenase(s)
PBO	Piperonyl butoxide
PC	7-n-pentoxy coumarin
PCR	Polymerase chain reaction
Pfam	Protein families database
Pg	Picogram
PR	7-n-pentoxyresorufin
qRT-PCR	Quantitative real time polymerase chain reaction
r	Correlation coefficient
RACE	Rapid amplification of cDNA ends
RDL	Resistant to dieldrin
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Seconds
SNP(s)	Single nucleotide polymorphism(s)
SD	Standard deviation
SE	Standard error
SEM	Standard error mean
SC	Suspension concentrate
skdr	Super knock down resistance
TCZ	Tebuconazole
UK	United Kingdom
VGSC	Voltage-gated sodium channel
V_{max}	Maximum velocity

Chapter 1

Introduction

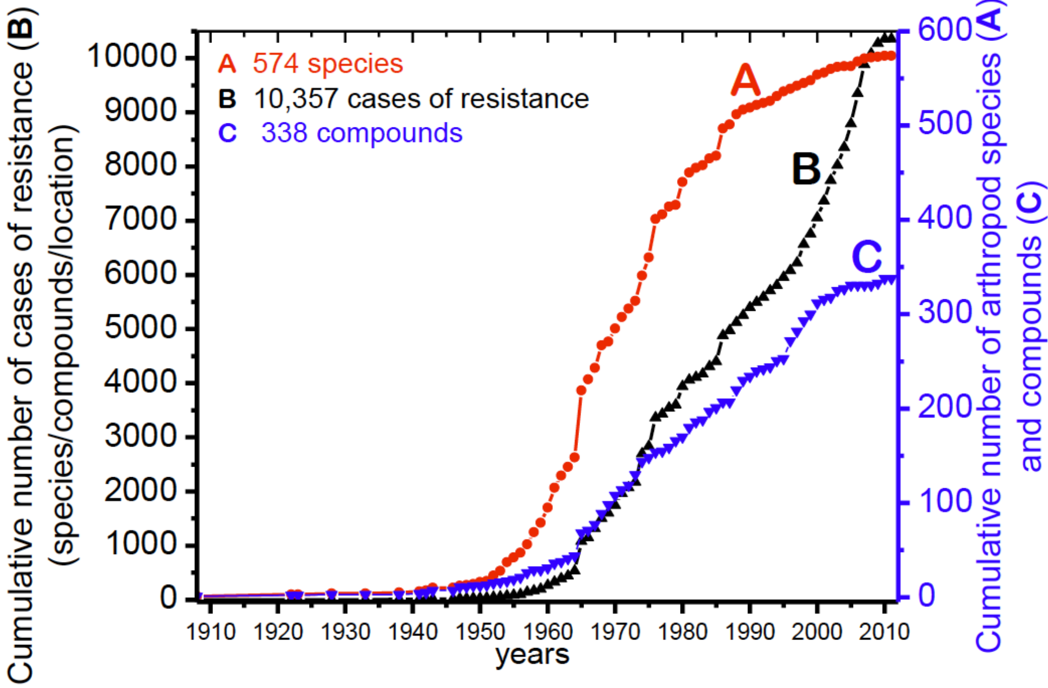
The problem of insecticide resistance represents an important man-made example of natural selection and is one of the major threats of sustainable agriculture in our time. The loss of insecticide effectiveness results in crop losses and products of poor quality, it invariably leads to increased application frequencies and dosages thus increasing the environmental footprint of agriculture. Insecticide resistance is not limited to the agricultural sector but affects the control of vector-borne diseases and veterinary applications. The economic and human costs of resistance are significant but can be hard to precisely quantify.

The history of insecticide resistance dates back to the late 19th century. In 1897 two reports in "Garden Forest" complain about the efficacy of control measures taken to control codling moth, *Cydia pomonella* L., and San Jose scale, *Quadraspidiotus perniciosus* COMSTOCK in apple orchards [1,2] cited in [3]. In 1914, A.L. MELANDER published the first scientific study on insecticide resistance covering the field failures of sulfur-lime to control the San Jose Scale, dating back to 1908 [4]. The "epidemic outbreak" of insecticide resistance occurred in the 1950s due to the broad availability and large scale usage of synthetic insecticides, i.e. organophosphates and DDT [3]. The challenge to combat insects became a challenge focused on combating resistance by maintaining the finite resource "susceptibility", a strategy known as "resistance management" [5]. One century after the finding of A.L. MELANDER more than 10,357 cases of resistance in 574 arthropod species have been recorded [6,7] thus underpinning the challenge of resistance management on a global scale (Fig. 1).

The finite resource "insecticide susceptibility" was not treated with the appropriate respect in many cropping systems; unfortunately, the winter oilseed rape, *Brassica napus* L., cropping system in Europe has been no exception. At present we are facing resistance problems in three major pests of oilseed rape. The development of resistance is a result of natural selection, the massive increase in the cropping area of winter oilseed rape in Europe in the past 50 years (Fig. 2) and the reliance on only one chemical class of synthetic insecticides, i.e. pyrethroids, in the more recent past (see section 1.2) providing optimal conditions for the emergence of insecticide resistance.

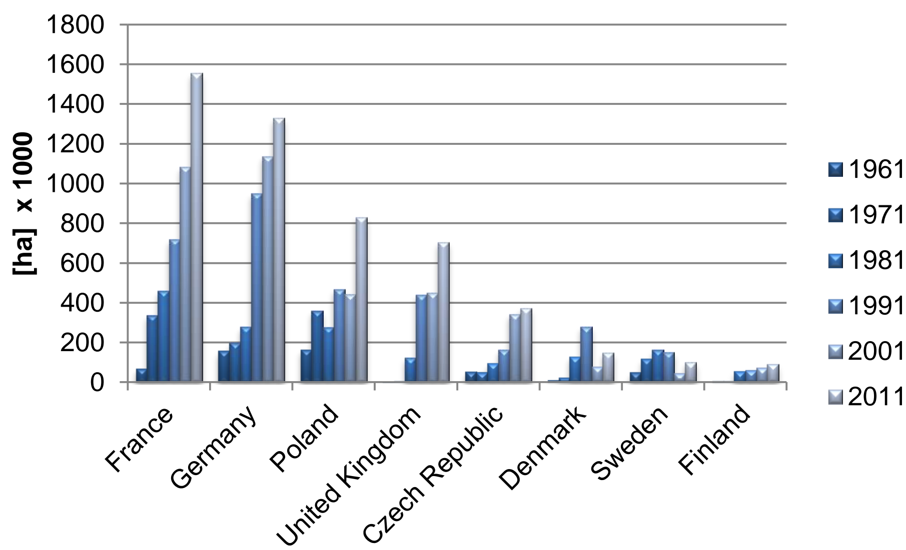
In the following sections of the introductory chapter an overview of important coleopteran pests of oilseed rape is given followed by a review of insecticide use in oilseed

rape in Europe. The nature of insecticide resistance is then reviewed and the objectives of the presented thesis are outlined.



WHALON *et al.* (2013) [7]

Fig. 1 *Evolution of arthropod insecticide resistance from 1908 to 2012 (species (A), total number of cases (B) and compounds (C)).*

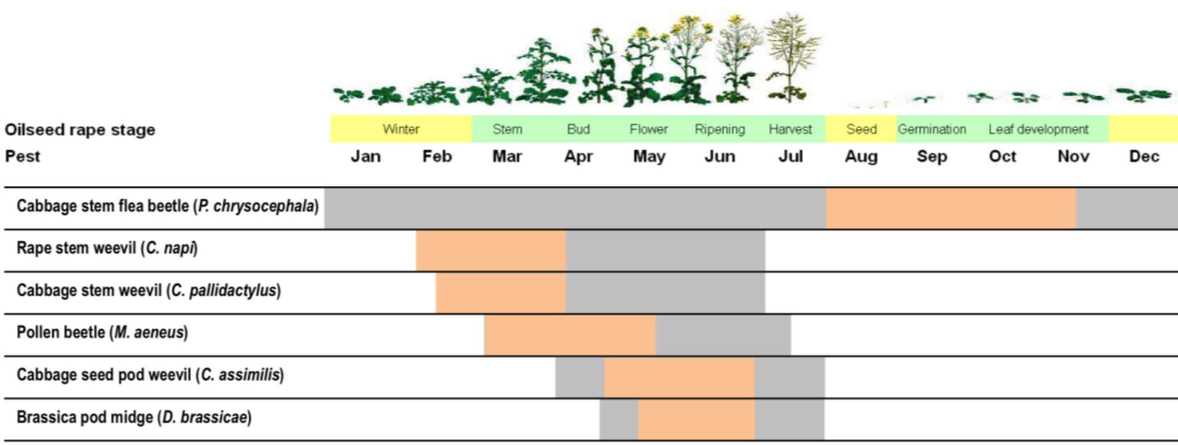


Source: Data obtained from FAO 2013 [8]

Fig. 2 *The development of winter oilseed rape cropping in the main oilseed rape cropping countries in Europe from 1961-2011.*

1.1 Insect pests of oilseed rape

Oilseed rape is attacked by a number of insect pests including coleopteran, dipteran and hemipteran species. ALFORD *et al.* [9] highlighted six species to be major pests of oilseed rape crops in Europe (Fig. 3), one dipteran species i.e. *Dasineura brassicae* WINN., and five coleopteran species which are described in more detail below.



Source: modified from IRAC 2011 [10]

Fig. 3 *Seasonal abundance (grey) of the major winter oilseed rape insect pests and crop stage where chemical control measures may be required (orange).*

1.1.1 Coleopteran insect pests of oilseed rape

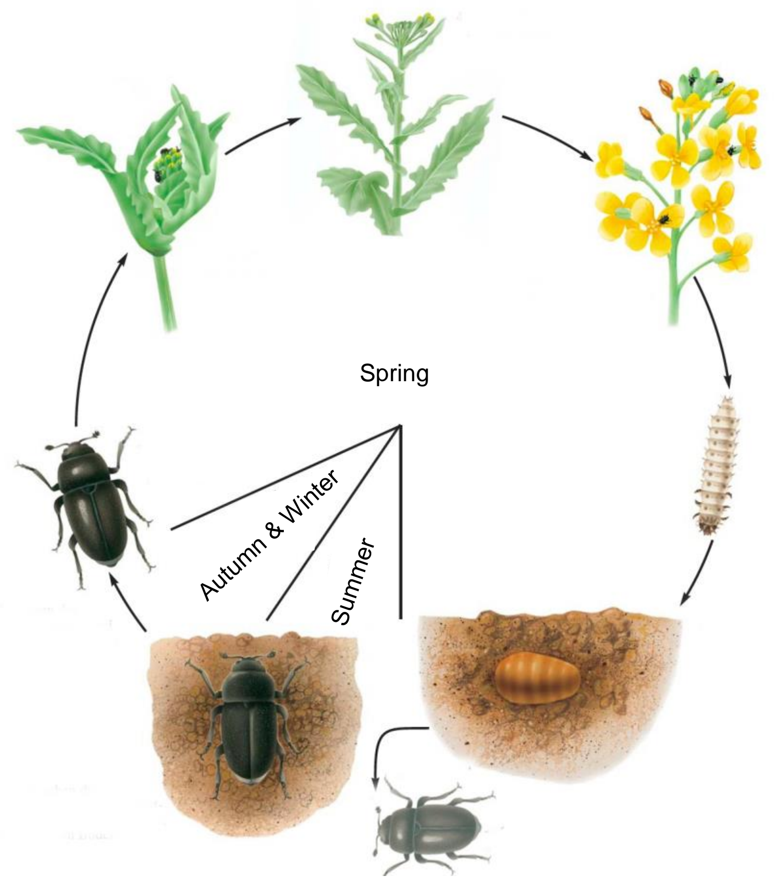
1.1.1.1 *Pollen beetles (*Brassicogethes *spp. syn.* Meligethes *spp.)*

Pollen beetles, widely known under the genus *Meligethes* STEPHENS 1830 were recently re-examined and re-named *Brassicogethes* AUDISIO & CLINE, gen. nov. [11]. Within this thesis the older genus term *Meligethes* is used. Throughout Europe pollen beetles can regularly be found at high levels on winter and spring oilseed rape [12]. The dominant species and the most important insect pest of oilseed rape is *Meligethes aeneus* F. (Fig. 4) [12–17]. Out of several other *Meligethes* spp. that may appear in oilseed rape *M. viridescens* F. is, after *M. aeneus*, the second most abundant pollen beetle [13,15,16]. *M. viridescens* depends on higher temperatures for emerging thus it is more abundant in spring oilseed rape [9,12,13,15]. Hereafter the term pollen beetle(s) refers to *M. aeneus* only.

Source: Pictures kindly provided by the Visual Communicaion Unit of Rothamsted Research

Fig. 4 *Adult pollen beetle,* M. aeneus, *feeding on pollen in an oilseed rape flower (left) and larval instars in an oilseed rape flower (right).*

Pollen beetles overwinter as the adult stage in the soil, they emerge in spring once the temperatures exceed 9 °C [14,18] and start infesting oilseed rape fields once temperatures reach 12-15 °C [15,18]. Emergence depends mainly on temperature, but CO_2 concentration and humidity at the hibernation site are influencing factors [15]. Newly emerged females are reproductively immature and feed either on pollen of various plants close to the hibernation sites or directly on oilseed rape if temperatures remain above 12 °C for a week or two [15,16,18,19]. Pollen beetles arrive in oilseed rape fields for mating and oviposition when the crop reaches the early bud stage (Fig. 5) [15,20]. They are able to locate oilseed rape at this stage by olfactory stimuli [21]. Females prefer buds 2-3 mm in length for oviposition [16] and lay their eggs through a hole chewed at the base of the bud [22,23]. Each female produces 100-300 eggs [15,16,22], and usually lays 2-3 eggs per bud [16]. The eggs hatch after about 4 days at 21 °C [15,16] or 1510 accumulated degree hours above 4 °C [24]. Pollen beetles have two larval instars [16,25,26]. The first instar develops in closed buds [27] and the second instar feeds on pollen in buds and open flowers before it drops to the ground to pupate in the soil [15,28]. In 22-30 days [16,22] or 2770 accumulated degree hours above 4 °C [24] the development progresses from first instar to pupae. The pupal diapause lasts 10-18 days [15], the new generation emerges early in summer and feeds on pollen of various plants [28]. The adult beetles seek their hibernation sites off the fields in perennial vegetation, leaf litter of woodlands and hedgerows from August [14–16,20].



Source: modified from PAUL (2003) [29]

Fig. 5 *Lifecycle of the pollen beetle,* M. aeneus.

The pollen beetle, *M. aeneus* is the major oilseed rape pest throughout Europe [30]. Pollen beetle damage the crop through feeding activity on immature flower parts in the early bud stage of the crop often destroying the ovary [15,31]. As the plants develop the impact of pollen beetle attacks on crop yield losses reduce [14,28,31–34]. In the light of this economic thresholds are adapted to crop stages in many countries ranging from one beetle/plant in France and Poland to 3-4 beetles/plant in Germany in BBCH 50-51 (BBCH code see [35]) and 2-3 beetles/plant in France to 7-8 beetles/plant in Germany in BBCH ≥52 [9]. The actual damage potential of pollen beetle in winter oilseed rape is controversial. In some years the crop compensation capacity can overcome even relatively heavy attacks [13,34,36]. Unfortunately the factors influencing crop compensation are not fully understood and therefore deciding when control is required is not an exact science. Whereas the condition of the crop e.g. plant density per square meter can be assessed at a certain time point the future weather conditions cannot be controlled. The latter is relevant as water supply is known to be a crucial factor determining compensation capacity [14,36]. Despite the ability of the crop to compensate for pollen beetle damage yield losses ranging from 20 to 100 % on acreage of

200,000 ha winter oilseed rape in Germany in 2006 due to the limited control of pyrethroid resistant pollen beetles clearly demonstrate the enormous damage potential of this particular pest [37].

1.1.1.2 *Stem and pod weevlis (*Ceutorhynchus *spp.)*

Stem and pod weevils attacking oilseed rape belong to the genus *Ceutorhynchus* and are univoltine [9,38–40]. Three weevils are considered to be major pests of oilseed rape [9] and may cause significant yield reduction: *C. napi* GYLL. (rape stem weevil) and *C. pallidactylus* MARSH. (cabbage stem weevil) and *C. assimilis* PAYK. syn. *C. obstrictus* MARSH. (cabbage seed weevil) (Fig. 6). *C. napi* is of particular importance in continental Europe, especially in Germany, France, Switzerland, Austria and Poland but is not a pest in northern Europe or the UK, whereas *C. pallidactylus* and *C. assimilis* are major rape crop pests throughout Europe [9,12].

Source: Pictures kindly provided by the Visual Communicaion Unit of Rothamsted Research

Fig. 6 *Adult* Ceutorhynchus *spp. on oilseed rape, from left to right:* C. napi, C. pallidactylus *and* C. assimilis.

The lifecycle of *C. napi* and *C. pallidactylus* is broadly similar, adults migrate into winter oilseed rape fields in early spring to feed and oviposit into stems (*C. napi*) [39,41,42] or petioles as well as mid-ribs of leaves (*C. pallidactylus*) [9,38,42] of the oilseed rape plants. Eggs hatch in 1-2 weeks [12,43]. Larvae of *C. napi* and *C. pallidactylus* feed within the pith for about 5 weeks [9,39] and in the petioles, stem and lateral shoots for 3-6 weeks [12,40], respectively. On maturity larvae exit the plant, drop to the ground and pupate in the soil [38–40]. 3-4 weeks later adult stages are reached, adults of *C. pallidactylus* emerge and feed on various cruciferous plants prior to entering hibernation outside the crop in compost, plant debris or bushes [38] whereas adult *C. napi* remain *in situ* to emerge the following spring [9,39].

The damage caused by stem weevils is various. Larval feeding weakens the plants and generates entry paths for plant diseases i.e. fungal and bacterial diseases, through feeding and oviposition activities [9,41,43–46]. Substantial damage to the plant itself is caused by the oviposition activity of rape stem weevil. Secretions by the egg-laying female lead to reductions in stem growth with swelling, longitudinal splitting and twisting [9,14,41,43].

The third member of *Ceutorhynchus* genus that causes considerable damage is *C. assimilis*, unlike the other two species this species is abundant in the crop from early flowering until ripening of the seeds. The females are reproductively immature while they migrate into crops in the early bud stage [47,48], after two weeks feeding on different parts of cruciferous plants the females start to oviposit into young seedpods [9]. Usually the eggs are laid singly in pods through a hole bored with the mouthparts [28,49]. A pheromone is released by the female subsequently after oviposition to defer further oviposition in the same pod [50,51]. The eggs hatch 1-2 weeks later and during larval development which covers three instars about five seeds are destroyed per larvae [9,49]. Mature third instar larvae chew a hole in the pod wall and drop to the ground to pupate for up to 23 days [49]. The adults emerge during July and feed on a range of cruciferous plants before seeking overwintering sites in leaf litter of perennial vegetation [9,28].

The main damage caused by *C. assimilis* is due to larval feeding [9,30,49]. Furthermore, the damaged pods of the crop serve another insect pest the brassica pod midge, *D. brassicae*, as oviposition sites and are an entry path for fungal diseases [9,12,52].

1.1.1.3 *Flea beetles*

Flea beetles of the genus *Phyllotreta* and *Psylliodes* feed on cruciferous plants [12]. Adults of both genus' mainly damage the emerging oilseed rape plants due to feeding on cotyledons and the first true leaves. Whereas *Psylliodes chrysocephala* L. is only present in winter oilseed rape *Phyllotreta* spp. are more important pests of spring oilseed rape and brassicaceous vegetables [9,12,53,54]. In North America and Scandinavian countries *Phyllotreta* spp. dominate whereas the cabbage stem flea beetle, *P. chrysocephala (Fig. 7),* is the most prevalent flea beetle in countries of central and northern Europe with a maritime climate and the most economically important flea beetle species [12,55].

Source: Pictures kindly provided by the Visual Communicaion Unit of Rothamsted Research

Fig. 7 *Adult flea beetle,* P. chrysocephala *on oilseed rape.*

P. chrysocephala is a univoltine species [9], after arrival in the crop their flight muscles atrophy [40]. After about two weeks of feeding activity on the crop the ovaries of the females become mature and the beetles start mating [56]. Eggs are preferably laid on the stem of newly emerged plants or close to rape plants in the soil [14,57]. The accepted temperature conditions for egg-laying are 4-16 ^oC [57], thus oviposition takes place mainly in autumn but may continue throughout mild winters [58]. 240 accumulated day degrees above 3.2 ^oC are required for eggs to hatch and larvae can be found in the field from September onwards [58]. Larvae bore into the petioles of leaves and proceed towards the stem where they feed until they reach the third and final larval stage [59,60]. Mature larvae leave the oilseed rape plant to pupate in the soil, and after a pupal diapause of about three month the new generation emerges in the late spring [56]. The new generation feeds on various tissues of oilseed rape and other cruciferous plants [12,56].

The damage caused by cabbage stem flea beetle is mostly caused by larval feeding activity and it is usually not necessary to take control measures to prevent the feeding of adults [12]. Larvae may damage the plants (1) directly especially when plant density is low and the number of larvae per plant increases resulting in insufficient food source for the larvae and, (2) indirectly as the boring and tunneling of the larval feeding activity makes plants more susceptible to frost damage and provides a portal of entry for plant pathogens such as *Phoma lingam* [44,53].

1.2 Insecticides used to control oilseed rape insect pests in the present and the past

Historically a variety of chemicals were used on rape crops to control insect pests. WEIß [61] reviewed several studies carried out in the early 1920s regarding the efficacy of arsenic compounds such as copper arsenite and lead arsenite. However, most studies on such compounds revealed unsatisfactory results because of low efficacy and undesirable phytotoxic effects [62,63]. Pyrethrum powders were reported to be frequently used for control [61,62] and were even used in some areas prior to the First World War [61]. Although aqueous tobacco leaf extracts water mixtures did not show efficacy in the field satisfactory results were achieved using nicotine-extracts in the laboratory [61]. In contrast BLUNCK & MEYER [64] presented good results using nicotine-dust and rotenone-dust for pollen beetle control in the field. FREY [65] showed a high efficacy of rotenone based products but high costs of the product [64] and the limited availability of the natural resource after the Second World War limited their widespread use in the field [14].

After decades of use of inorganic chemistry and natural extracts the era of synthetic insecticides dawned. Dichlorodiphenyltrichloroethane (DDT) was introduced in the 1940s and was the most frequently used insecticide in cruciferous crops in the early 1950s [14]. As the efficacy of DDT against weevils was not sufficient parathion, an organophosphate, was often used instead or applied in mixtures with DDT [14,40,66]. With toxaphene, an organochlorine, the first compound that was non-toxic to bees became available, and was thus suitable for use in the flowering stage of crops [40,67]. For flea beetle and stem weevil control seed treatments with *gamma*-hexachlorocyclohexane (*gamma*-HCH), also known as lindane, were used [38,40]. Spray application of *gamma*-HCH and dieldrin were found to be effective on weevils as well [40]. With compounds such as DDT, *gamma*-HCH, dieldrin, toxaphen, chlordane, endosulfan, diazinon, parathion and pyrenone (6 % pyrethrin, 60 % piperonylbutoxide) a broad range of insecticides was available from the 1960s [40]. Unintentionally at this time a basis for resistance management was provided by the available chemistry which acted on different target sites in the insect i.e. DDT and pyrethrin act on the voltage-gated sodium channel (VGSC) [68–71], *gamma*-HCH, dieldrin, toxaphen, chlordane and endosulfan target the *gamma*-aminobutyric acid receptor (GABA)-gated chloride channels [72], and diazinon and parathrion bind irreversibly to the acetylcholinesterase (AChE) [73].

Even though the level of control of insect pests reached an efficacy never known previously, with CASIDA & QUISTAD calling it the first victory over insects [74], the massive use of synthetic insecticides also had very significant negative side effects. Most early synthetic compounds were relatively persistent and displayed minimal selectivity with sometimes highly

toxicity to mammals and non-target arthropods. In the late 1970s synthetic pyrethroids were introduced [69,75] and displayed a combination of exceptional activity with a superior toxicological profile [69,75–79]. With the introduction and widespread adoption of the pyrethroids many of the older insecticides were withdrawn due to concerns relating to human health and environmental impact [80,81]. As a result in the more recent past, i.e. before 2007, just a single chemical class, the pyrethroids, was relied on as a mainstay for the control of various insect pests in oilseed rape in most European countries [80–83]. These circumstances led to exceptional selection pressure on this particular chemical class of insecticides and to the eventual development of pyrethroid resistance in at least two different oilseed rape pest species (see Chapter 2, Chapter 7 and Chapter 8). The development of pyrethroid resistance in pollen beetle (see Chapter 2) led to the registration of alternative chemistry thus today a variety of chemistry with distinct modes of action is available throughout Europe to be used in oilseed rape crops. Currently compounds belonging to four different chemical classes i.e. pyrethroids, the neonicotinoids thiacloprid and acetamiprid, indoxacarb (an oxadiazine) and pymetrozine (a pyridine azomethine) are registered in Germany [84], France [85], Poland [86], and the UK [87]. Among oder chemistry organophosphates are still registered in France and Poland. Switzerland has registered spinosad as well as all other insecticides mentioned above except for indoxacarb [88]. However, the situation becomes more difficult at the species level. The main driver for the new and re-registration of alternative compounds was the development of pyrethroid resistance in pollen beetle which spread rapidly throughout Europe [89], thus many compounds such as indoxacarb, pymetrozine and chlorpyrifos are only registered for pollen beetle control [84,85]. Hence, most newly and re-registered chemistry is limited to pollen beetle control and the options for controlling pests other than pollen beetle are currently restricted to two chemical classes i.e. pyrethroids and neonicotinoids.

Insecticide classes currently registered to control invertebrate oilseed rape arthropod pests in Europe, their target sites and modes of action are described in more detail hereafter with an emphasis on the pyrethroid insecticides as the most widely used class.

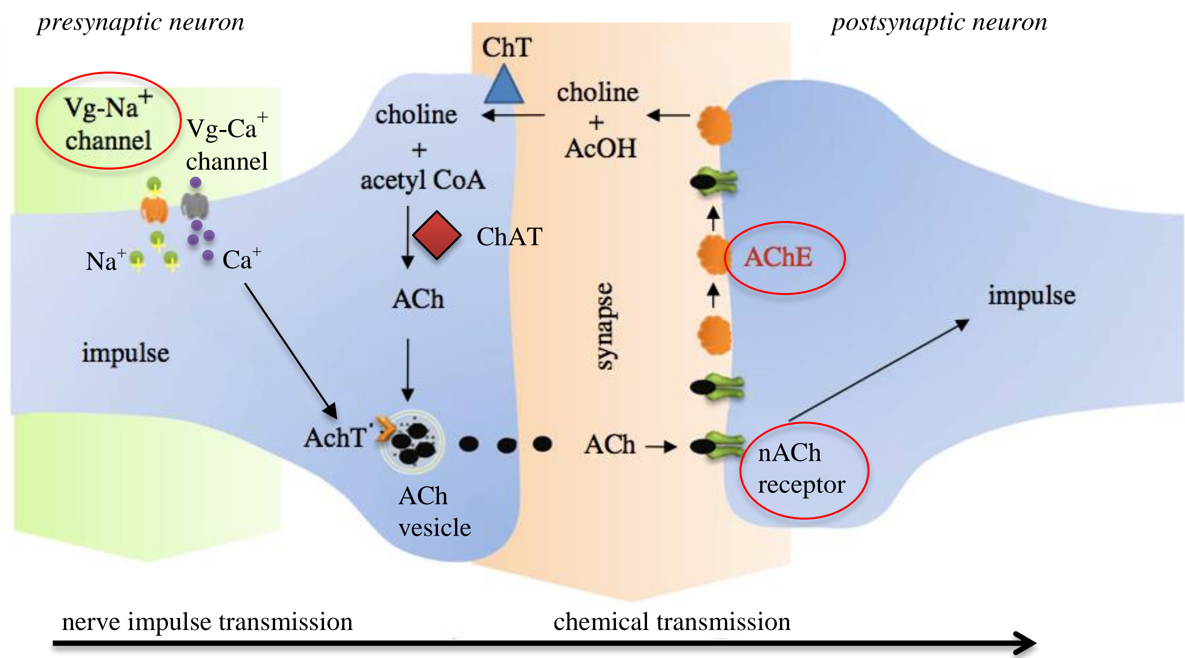
1.2.1 Insecticides targeting voltage-gated sodium channels

The voltage-gated sodium channel (VGSC) (Fig. 8) is a protein targeted by natural as well as synthetic insecticides in the present and the past [69,90]. The VGSC as an insecticide target is addressed by pyrethroids and indoxacarb, even though these chemistries share the same target site they have distinct modes of action (see below for details) and are therefore classified in individual IRAC classes (Table 1). Historically the VGSC is one the oldest and well established target sites as it has already been addressed more than 100 years ago by botanical pyrethrins and later on by DDT, one of the first synthetic insecticides [91] (Fig. 8 and Fig. 11).

Table 1 *Mode of action and IRAC classification of insecticide classes currently available for control of certain oilseed rape pests.*

Chemical class	Mode of action	IRAC classification
Pyrethroids	Sodium channel modulator	3A
Indoxacarb (oxadiazines)	VGSC channel blocker	22
Neonicotinoids	nAChR agonists	4A
Spinosyns	nAChR allosteric activators	5
Organophosphates	AChE inhibitors	1B
Pymetrozine (pyridine azomethine)	Chordotonal organ modulator*	9B

* see section 1.2.4 Source: http://www.irac-online.org/eClassification/



Source: modified from DAVID *et al.* (2013) [92]

Fig. 8 *Target sites of insecticides in the central nervous system of arthropods. Red ellipses indicating target sites currently addressed by insecticides used in oilseed rape; ACh, acetylcholine; AchT, ACh transporter; AcOH, acetic acid; ChT, choline transporter; ChAT, choline acetyltransferase; Vg-Na$^+$ channel, voltage-gated sodium channel; Vg-Ca$^+$ channel, voltage-gated calcium channel; nACh receptor, nicotinic acetylcholinereceptor; AChE, acetylcholinesterase.*

1.2.1.1 *Synthetic pyrethroids*

Pyrethroids are synthetic analogues of the naturally occurring pyrethrins [69,78,79]. Pyrethrins are natural insecticides occurring in flowering parts of the pyrethrum daisy (*Tanacetum cinerariaefolium* (TREVIR.) SCH. BIP.*;* syns. *Chrysanthemum cinerariaefolium* and *Pyrethrum cinerariaefolium*) [69]. Pyrethrum extract contains six insecticidal esters i.e. pyrethrin I, II; jasmonine I, II and cinerin I, II, they share a common structure and differ only in the terminal substitutions (Fig. 9) [69]. The I and II series contain a chrysanthemic acid and pyrethric acid, respectively [93]. Although pyrethrum extract is highly active against a broad spectrum of arthropods the photo-instability of the insecticidal compounds prevented their successful large-scale use [79,94]. The photo-instability of the natural compounds was a driving factor for discovering alternatives, and the lead structure leading to development of successful synthetic pyrethroids was pyrethrin I [78,94]. However two of the initial promising compounds resmethrin and bioresmethrin which displayed more than 50-fold higher efficacy against houseflies compared to pyrethrin I were also not suitable for large-scale use in agriculture due to rapid photolysis [78,79]. This hurdle was finally overcome with the synthesis of permethrin in 1973 [79,95], the first synthetic pyrethroid to be used on a broader scale within agriculture [91,96]. Permethrin contains the chrysanthemum acid moiety, a central ester bond and the 3-phenoxybenzyl alcohol moiety and became the lead structure for many following compounds of commercial value (Fig. 9) [97]. Finally, the insecticidal activity of pyrethroids was further enhanced by the introduction of an α-cyano group e.g. cypermethrin and deltamethrin (Fig. 9) [69,79,93]. Deltamethrin is 1400-fold more active against houseflies and ~8-fold less toxic to mammals compared to pyrethrin I and displayed a level of activity against insects that had never been seen before [93]. Typically ester pyrethroids have three chiral centres resulting in a possibility of eight isomeres. Only two of them (1R cis and trans, with αS configuration) are active [78]. Deltamethrin represents a single isomer pyrethroid (1R cis) [75], whereas cypermethrin is a mixture of all eight possible isomeres and the optimized *alpha*-cypermethrin contains only two isomeres (1R cis, αS and 1R cis, αR) [93].

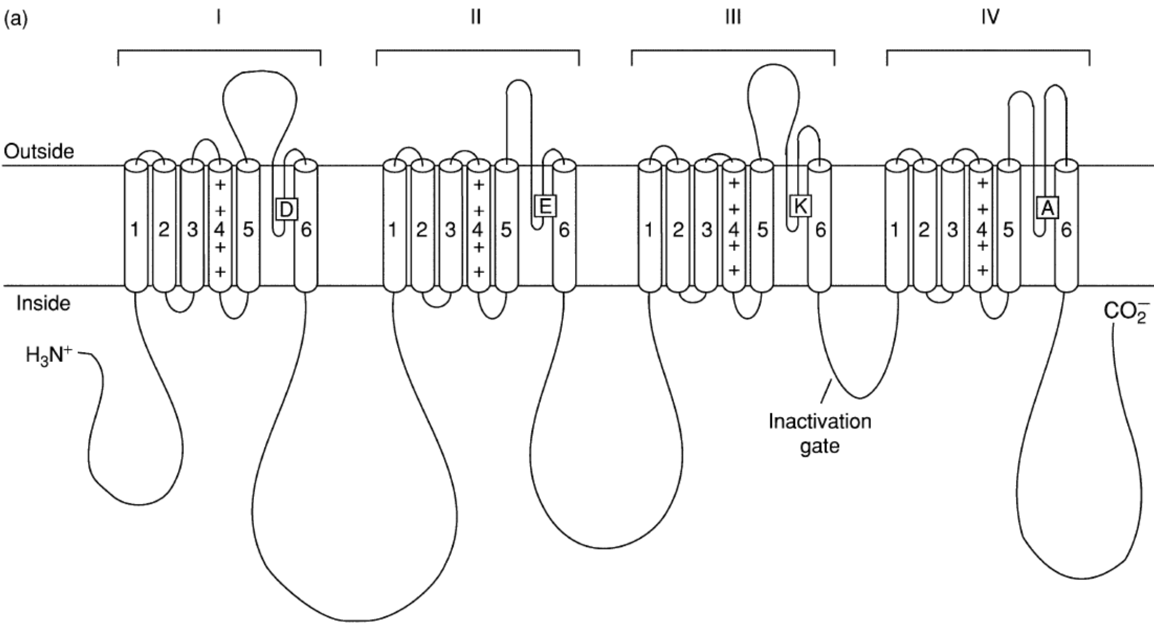
In respect to the currently registered pyrethroids for the control of insect pests in oilseed rape three further compounds should be highlighted i.e. bifenthrin, etofenprox and *tau*-fluvalinate. Most of the pyrethroids which have frequently been used in oilseed rape have a permethrin-like structure in common (see above) e.g. *lambda*-cyhalothrin, deltamethrin, cypermethrin and cyfluthrin [82]. The three compounds named above are structurally different: bifenthrin containing a biphenyl moiety instead of the common 3-phenoxybenzyl alcohol moiety; etofenprox a non-ester pyrethroid and *tau*-fluvalinate where the chrysanthemum acid moiety is substituted by an aromatic amino acid moiety (see Fig. 25, Chapter 3).

Source: KHAMBAY (2005) [78]

Fig. 9 *Chemical structures of natural occuring pyrethrins, the early synthetic analog bioresmethrin and some important commercialized synthetic pyrethroids.*

Synthetic pyrethroids, natural pyrethrins and DDT act on insect VGSCs located in the central nervous system [90,98,99]. VGSCs generate action potentials and are critical for electrical signaling in most excitable cells [100]. In response to changes in the membrane potential the channels open and then close [101]. The open conformation of the channel is the active stage where sodium ions are allowed to flow into the cell resulting in depolarization of the membrane potential, this stage usually only lasts a few milliseconds before the channel pore is occluded [100]. VGSCs in insects are encoded by a single gene named *para* after the temperature-sensitive paralytic phenotype of *Drosophila melanogaster* MEIGEN [102]. In contrast, in mammals several sodium channel α-subunits are expressed by at least 10 genes [103]. Prior to the identification of *para* the *dsc1* locus was thought to be the VGSC encoding locus in *D. melanogaster* [104,105]. However, as recently shown the *dsc1* locus encodes a distinct calcium channel [106]. Orthologs of *para* were reported in more than 15 arthropod species [105]. The single polypeptide chain encoded by *para* is a pore-forming α-subunit with four internally homologous transmembrane domains (I-IV), each containing six transmembrane helices (S1-S6) (Fig. 10) [99,105]. However, one gene does not necessarily mean only one gene product, due to splice variants and RNA editing one gene can express

several pharmacologically distinct channels [100,107]. Detailed pharmacological analysis of interactions between pyrethroids and sodium channels are rather limited due to the hydrophobic nature of pyrethroids and the complexity of the target site [108], but mutations may facilitate a deeper understanding of these interactions. O'REILLY *et al.* [99] published a model of the *M. domestica* sodium channel pore region derived from a crystal structure of the homologous voltage-gated potassium channel. Based on this model they predicted a single "putative" binding site for pyrethroids within the pore in a region where several amino acid residues have been implicated in pyrethroid resistance i.e. the domain IIS4-S5 linker and domain IIIS6 (see section 1.3.1.2 for more details). DU *et al.* [109] predicted a second putative binding site based on electrophysiological experiments on *Xenopus* oocyte-based functional expression of the *Aedes aegypti* L. AaNa$_v$1-1 sodium channel.

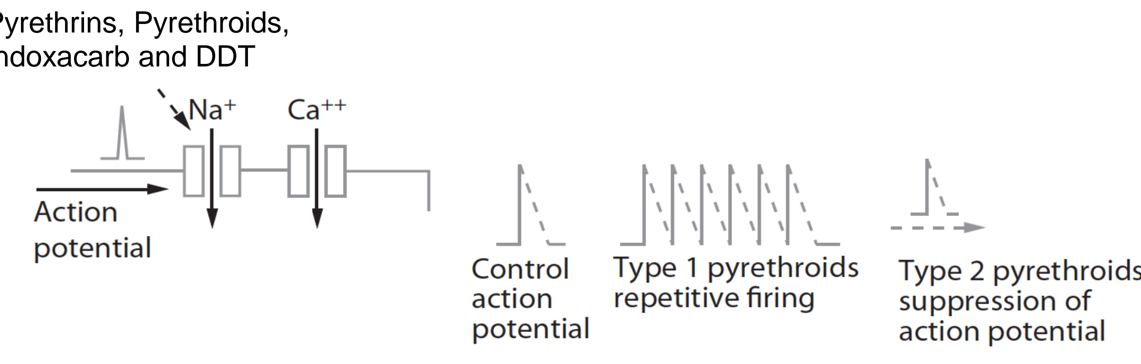


Source: SODERLUND (2005) [105]

Fig. 10 *A schematic illustration of a voltage-gated sodium channel. The alpha-subunit forming the pore consists of a single polypeptide chain comprising four internally homologous domains (I-IV), each containing six hydrophobic transmembrane helices S1-S6. Multiple positively charged residues (+) in the four S4 helices constitute the voltage sensor, the "DEKA amino acid motif" constituting the selectivity filter.*

Pyrethroids can be grouped into type I and type II compounds based on their chemical structures, distinct poisoning symptoms and effects on nerve preparations [96,110–114]. Based on the poisoning symptoms of cockroaches and rats two syndromes were characterized, the type I poisoning syndrome in the cockroach is restlessness, incoordination, prostration and paralysis [98,115] and a whole body tremor followed by prostration in the rat,

the latter syndromes were named T syndrome [116]. Type II symptoms were convulsion, rapid paralysis and incoordination in the cockroach [110] and coarse tremors, burrowing behavior, clonic seizures, sinuous writhing and profuse salivation without lacrimation in rats [116]. The type II symptoms in rats were referred to as CS (choreoathetosis/salvation) syndrome [98,116]. Chemically both types can be distinguished by the absence (type I) or presence (type II) of the α-cyano group [98,100,113]. Neurophysiological differences in the modification of the action of VGSC can be determined in voltage-clamp experiments. Repetitive discharges (also known as repetitive firing), and membrane depolarization accompanied by a suppression of the action potential are caused by type I, and type II pyrethroids, respectively (Fig. 11) [96]. The classification based on the α-cyano group holds true for most compounds, but exceptions have been reported. CASIDA *et al.* [98] described the symptomology of fenpropathrin, a pyrethroid containing an α-cyano group, as a mixture between type I and II effects. Roughly 25 years later fenpropathrin and esfenvalerate have been described as intermediates between type I and II in a study considering both symptomology and neurophysiology [112].



Source: CASIDA & DURKIN (2013) [90]

Fig. 11 *Target site of insecticides acting on the VGSC (presynaptic nerve terminal) and neuroactive action of pyrethroids.*

A recognized efficacy feature of pyrethroids is their rapid knock-down action on insects [117]. However, pyrethroids often do not kill insects as rapidly as they knock them down and they may recover fully under controlled conditions within a few days [78]. However, long-term recovery under field conditions may not actually reduce control as death may occur by secondary processes such as predation and desiccation [78].

The continued research on pyrethroid structures led to the development of more than 30 compounds [78]. Pyrethroids show a high selectivity for insects over mammals with the VGSCs of insects at least 1000-fold more sensitive to pyrethroids than their mammalian counterparts [118]. They are potent broad-spectrum insecticides and extremely lipophilic (logP

4-9 [119]), thus they show excellent contact activity and no systemic activity [79]. Due to their favorable selectivity combined with a broad insecticidal spectrum they are widely used not only in agriculture but also to combat vector borne disease and in human and veterinary health medications [117]. Introduced in the late 1970s, the adoption of pyrethroids grew rapidly reaching peak sales in 1985 with a market share of more than 20 % [75]. The fact that the market share has remained around 20 % for many years and 30 years after their introduction was still at 18 % in 2010 [90] reflects both their importance as a insecticides class and the lack of alternatives.

1.2.1.2 *Indoxacarb*

Indoxacarb belongs to the chemical class of oxadiazines and is the first commerialized pyrazoline-type insecticide [120,121]. Pyrazoline-type insecticides were known since the early 1970s but were not of commercial relevance because of their unfavorable environmental properties and toxicological profiles [122,123]. The active metabolite of indoxacarb i.e. DCJW (Fig. 12) is a potent sodium channel blocker of both vertebrate and invertebrate sodium channels [120,124,125]. Indoxacarb is a pro-insecticide, after metabolic activation by an insect hydrolase, putatively an esterase or amidase-type of enzyme, its *N*-decarbomethoxylated form (DCJW) is a potent sodium channel blocker [121,125,126]. Like pyrethroids and DDT the molecular target site is the VGSC, but with a different mode of action [100,121]. The blocking of the VGSC is stage dependent, DCJW acts on the inactivated state of the sodium channel by shifting the voltage dependence of inactivation to more hyperpolarized potentials [127,128]. Indoxacarb itself shows a high selectivity for insects over mammals due to its pro-insecticide mode of action [121]. Whereas insects convert indoxacarb into DCJW it is converted into non-toxic metabolites in higher organisms such as mammals via different metabolic pathways [100,121,122].

Indoxacarb: R = CO_2CH_3
DCJW: R = H

Source: von Stein *et al.* (2013) [129]

Fig. 12 *Chemical structure of the pro-insecticide indoxacarb and its active metabolite DCJW.*

Pyrazoline-type insecticides cause pseudo-paralysis symptoms, and insects are immobilized but show violent convulsions when disturbed [121]. Oral toxicity of indoxacarb is about 9-times higher compared to its contact activity [121]. The pest spectrum of indoxacarb covers mainly lepidopteran, hemipteran and homopteran species and to some extent coleopterans [121]. The high efficacy of indoxacarb against Lepidoptera is related to the rate of indoxacarb bio-activation [125,126,130]. Indoxacarb was firstly used in pollen beetle control in 2010 in Germany on a restricted area with an emergency registration while applying for a full registration [131]. It is now available in different European countries for the control of pyrethroid resistant pollen beetles (see section 1.2).

1.2.2 Insecticides targeting nicotinic acetylcholine receptors

1.2.2.1 *Neonicotinoids*

The first commercialized compound of the neonicotinoid insecticides was imidacloprid; it was launched in 1991 and has become the best-selling insecticide worldwide [132,133]. The term neonicotinoid was proposed to differentiate the newer insecticides acting on the nicotinic acetylcholine receptor (nAChR) from the older naturally occurring (*S*)-nicotine sharing the same mode of action [134]. The first compound considered to be the lead structure of neonicotinoids was synthesized in the early 1970s by Shell, named nithiazine [134,135]. Even though nithiazine showed good initial results in the laboratory the field efficacy of the

compound was rather limited due to rapid degradation under both photolytic and hydrolytic conditions [136]. Today seven neonicotinoids are commercially well-established and they can be classified based on structural motifs such as cyclic and noncyclic structures (Fig. 13), the cyclic structures can be further divided in five-membered-ring (imidacloprid, thiacloprid) and six-membered-ring (thiamethoxam) systems [132,137,138]. Noncyclic compounds such as acetamiprid have a similar insecticidal activity compared to their cyclic counterparts by forming a so-called quasi-cyclic conformation while interacting with their molecular target site [134]. Neonicotinoids can also be classified by their pharmacophore system in nitroenamines, *N*-nitro-guanidines and *N*-cyano-amidines, noteworthy is the fact that the two *N*-cyano-amidines, thiacloprid and acetamiprid are nearly non-toxic to bees in contrast to other neonicotinoids [134].

Ring systems:

imidacloprid thiacloprid thiamethoxam

Noncyclic structures:

nitenpyram acetamiprid clothianidin

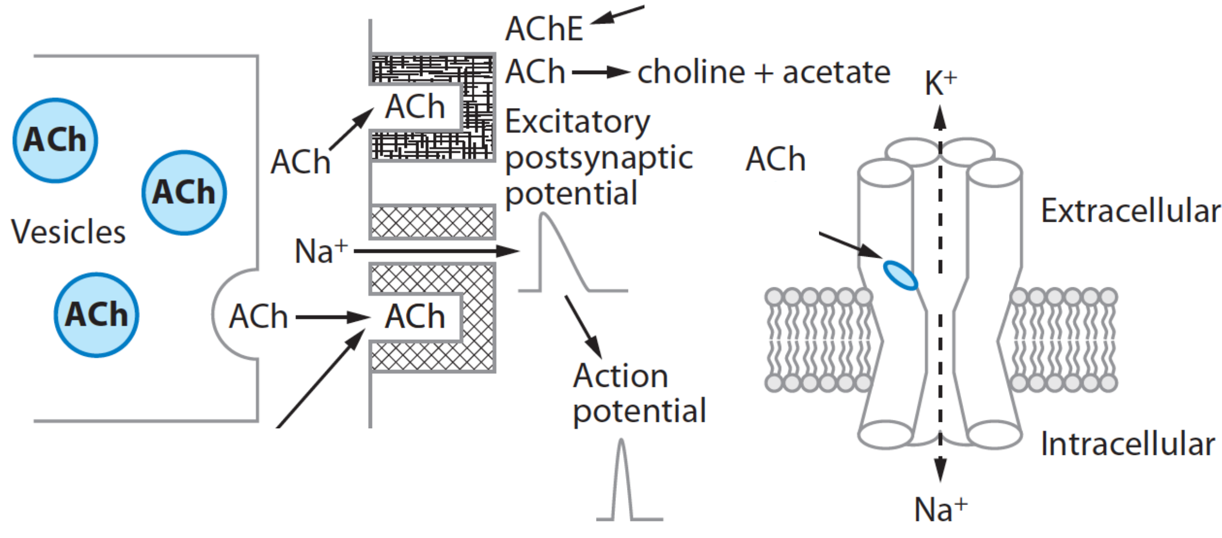
dinotefuran

Source: JESCHKE *et al.* (2013) [132]

Fig. 13 *Chemical structure of seven commerical well-established neonicotinoids with ring systems and noncyclic structures.*

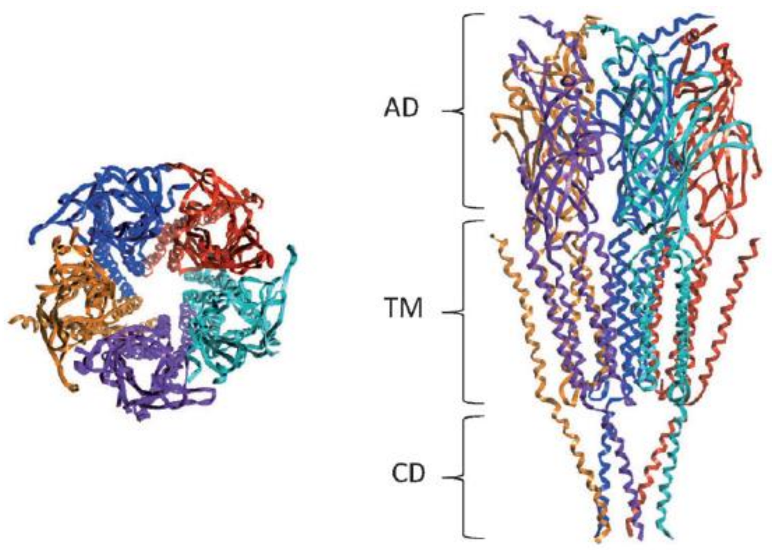
All neonicotinoids act selectively as agonists on the insect post-synaptic nAChRs (Fig. 8 & Fig. 14) in the central nervous system [90,133,139–144]. The excitatory neurotransmitter acetylcholine (ACh) is the natural endogenous ligand of the cholinergic nervous system; it interacts with the extracellular binding site. ACh binding triggers conformational changes of the receptor/ion channel complex (Fig. 15) leading to channel opening and ion flux, thus disrupting the equilibrium state of the membrane potential (Fig. 14) [145,146]. [^{3}H]-imidacloprid displacement experiments revealed the neonicotinoid-binding site is the same or closely linked to the binding site of ACh in invertebrates [146]. All neonicotinoids are highly selective insecticides sharing a favorable toxicological profile in contrast to nicotine [147]. The low mammalian toxicity of neonicotinoids is based on target-site selectivity due to differences in the architecture of mammalian nAChRs compared to insects [90,132,146,148]. TOMIZAWA & CASIDA [146] indicated that cationic amino acid residues(s) in insect nAChR are responsible for successful neonicotinoid binding whereas the anionic environment in mammalian nAChR prohibits an interaction. A recently discovered a target-site mutation in the *Myzus persicae* SULZER ß1 subunit confirmed the importance of a positively charged amino acid at a corresponding position in ligand binding [149].

Neonicotinoid insecticides are widely used in agriculture and veterinary medicine [134]. They are potent systemic insecticides acting through both contact and feeding used foliarly, by soil applicationa and as seed [134,147,150]. A wide range of insect species belonging to the orders of Hemiptera, Homoptera, Coleoptera, Diptera and Lepidoptera are controlled by neonicotinoids [151,152]. Since the introduction of imidacloprid in 1991 the market share of neonicotinoid insecticides grew steadily reaching almost 30 % in 2010 mainly due to replacing older, environmentally begnin insecticides such as organophosphates [90,152]. The two compounds registered for foliar spray in oilseed rape in Europe are thiacloprid and acetamiprid (see section 1.2). In addition seed treatment with neonicotinoids such as thiamethoxam and clothianidin have been used to control *P. chrysocephala* and aphid species in oilseed rape, however the recent two year suspension of neonicotinoids for seed treatment in oilseed rape will limit the insecticide portfolio for flea beetle control [153].



Source: CASIDA & DURKIN (2013) [90]

Fig. 14 *Neuroactive action of AChE inhibitors and nAChR agonists. Left, presynaptic nerve terminal and postsynaptic membrane; right, cartoon of nAChR integrated in lipid membrane. ACh, acetylcholine; AChE, acetylcholinesterase; nAChR, nicotinic acetylcholinreceptor; NNI, neonicotinoid.*



Source: JESCHKE *et al.* (2013) [132]

Fig. 15 *nAChR 3D Structure of* Torpedo marmorata RISSO; *side view (left) and along the channel direction (right); AD, agonist binding domain; TM, transmembrane domain; CD, cytoplasmatic domain.*

1.2.2.2 *Spinosyns*

Spinosyns are a class of macrocyclic lactones with insecticidal activity derived from fermentation broth of two species of *Saccharopolyspora spinosa* [154]. The naturally occurring bio-insecticide spinosad is a mixture of spinosyns A and D (Fig. 16) [155]. The molecular target site of spinosyns is the nAChR (Fig. 14 & Fig. 15) but they act at a unique site different from neonicotinoids and are therefore classified in a indivudual IRAC class (Table 1) [154]. Spinosad is available in oilseed rape only in Switzerland for the control of pollen beetle [88]. Due to its limited contribution to the overall situation of insect control in oilseed rape in Europe this group is not described further in this thesis, however the spinosyns insecticide family is thoroughly reviewed in [154].

Spinosyn A (R = H)
Spinosyn D (R = CH_3)

Source: KIRST (2010) [154]

Fig. 16 *Structure of spinosyn A and spinosyn D.*

1.2.3 Insecticides targeting acetylcholinesterases

Principally two chemical classes are known to act on acetylcholinesterase, i.e. carbamates and organophosphates. However particularly for pollen beetle control only organophosphates are still used in European winter oilseed rape. Organophosphate insecticides were first introduced in 1944, and some very early compounds e.g. tetraethyl phosphate were soon abandoned due to their high mammalian toxicity [156]. Organophosphates show a wide range of structural diversity, they are either amide derivates of phosphoric acid or a natural ester that contain a phosphoryl or thio-phosphoryl group [157]. Some organophosphates, such as chlorpyrifos are phosphorothionate ester pro-insecticides and are activated to their respective P=O derivatives (Fig. 17) [158]. The mode of action of organophosphates is though inhibition of acetylcholinesterase (AChE) [73,90,157–162]. AChE is located at synaptic regions of cholinergic nerves and is responsible for the hydrolysis of the

excitatory neurotransmitter acetylcholine (ACh) (Fig. 8) [156,159,163]. Organophosphates are irreversible inhibitors of AChE and act as competitive inhibitors (Fig. 14) [158]. The hydrolysis of organophosphate insecticides by AChE results in a very stable phosphorylated AChE complex [164]. In contrast, AChE is naturally acetylated after the degradation of ACh and rapidly regenerates though the formation of acetic acid (Fig. 8) [164]. Organophosphates cause ACh to accumulate which results in hyper-excitation of cholinergic receptors leading to tremors, paralysis, exhaustion and death of poisoned insects [73,90,165].

R^1-R^2= CH_3 or CH_2-CH_3

R^2-O-P(=S)(-O-R^1)-O-*<Leaving group>

organophosphate (general structure)

chlorpyrifos

P450 catalyzed desulfurization

chlorpyrifos

chlorpyrifos-oxon

Source: modiefied from National Pesticide Information Center (http://npic.orst.edu/mcapro/opbiomarkers.html)

Fig. 17 *General structure of organophosphate insecticides and chemical structure of chlorpyrifos and its P450 catalyzed activation; *leaving group is specific to individual compounds and can either be aliphatic or based on benzene.*

More than 100 organophosphates were developed, most of which have an unfavorable toxicological profile due to the fact they are acting on a fairly conserved target site [73,163–165]. Due to their structural diversity they may have different physicochemical properties [166] with logP-values rangeing from 1 - 5.5 [119], but only a minority of organophosphates are systemic [73] e.g. dimethoate [156]. They control a broad range of insect pests such as Lepidoptera, Coleoptera, Diptera, Hemiptera and Homoptera [73]. Organophosphate insecticides replaced the organochlorines in many applications after resistance issues had

reduced their effectiveness by the mid-1960s [73]. They soon dominated the market and by 1990 had a market share of 43 % [152]. As a consequence of the development of new chemistry with novel modes of action the use of organophosphates dropped continuously since then to a market share of 23 % in 2004 [167] and below 15 % in 2010 [90]. Organophosphates were used to control oilseed rape pests in the past (see section 1.2) and particular to control pollen beetles in the recent past, mostly in eastern European countries [82]. Since the development of pyrethroid resistance in pollen beetles chlorpyrifos was used on a restricted area with an emergency registration in Germany in 2007 [168] and are still available in some European countries for the control of pyrethroid resistant pollen beetles (see section 1.2).

1.2.4 Other insecticides (pymetrozine)

Another insecticide recently introduced to control pyrethroid resistant pollen beetles is pymetrozine (Fig. 18). It is a neuroactive pyridine azomethine and is known as a selective homopteran feeding blocker modulationg chordotonal organs by binding to an unknown molecular target site [169–172]. However, it was proven effective in field trials in controlling pyrethroid resistant pollen beetles [173,174]. A reasonable explanation for the high efficacy against pollen beetle in the field is still outstanding, a report on the side effects of pymetrozine in aphid field trials described a decrease in the abundance of Colorado potato beetle, *Leptinotarsa decemlineata* SAY in the field and may describes an identical phenomena [175]. Pymetrozine was first approved for pollen beetle control in the United Kingdom (UK) in 2011 and has since became approved in many European countries including the big three oilseed rape producers France, Germany and Poland [173] (see section 1.2).

N
HN
N
N
O
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Source: www.chemicalbook.com

Fig. 18 *Chemical structure of pymetrozine.*

1.3 Insecticide resistance

"Can insects become resistant to sprays?" headlined the first scientific paper which discussed the declined efficacy of the inorganic insecticide sulfur-lime published in 1914 by A.L. MELANDER [4]. In this publication MELANDER mentioned some uncertainties about the field efficacy of sulfur-lime to control the San Jose scale, *Quadraspidiotus perniciosus* in 1908 and explained later experiments where a 10-fold dose of the normal application rate of sulfur-lime failed to kill San Jose scale.

While reviewing the literature several definitions for the term resistance may be found, and three frequently cited definitions are:

Resistance is…

"…the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species" [176];

"…the microevolutionary process whereby genetic adaptation through pesticide selection results in populations of arthropods which present unique and often more difficult management challenges" [177];

"…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" [178].

Whereas the older WHO definition [176] focuses more on the population level, later definitions for example by IRAC [178] consider them from an applied perspective resulting from selection pressure by repeated applications. Resistance development is the result of natural selection and DOBZHANSKY [179] cited the emergence of insecticide resistant populations as "probably the best proof of the effectiveness of natural selection yet obtained". Resistant insects outnumber susceptible ones in the presence of permanent selection pressure [74,178,180]. The speed of resistance development depends on several factors/risks and these can be divided into: (1) the agronomic risk such as the number, the rate, and the timing of applications in the lifetime of one generation as well as the specificity of the crop protection product, and (2) the inherent risk including the migration and host range of the pest, its reproduction capacity and speed and the availability of nearby susceptible populations [178,181].

The genetic background of insecticide resistance may be of monogenic or polygenic nature [182,183], in fact most of the cases are caused by a single resistance allele [183–187]. Changes at the genetic level are diverse and include: single nucleotide polymorphisms (SNPs) [70], gene amplification [188] crossing over events [189], DNA methylation [190], regulatory tandem repeats [191], transposable elements [192], alternative and/or mis-splicing [193,194] and RNA editing [195]. The changes to DNA/RNA result in complex downstream physiological changes within arthropod species and can be further classified into four main resistance mechanisms as outlined in the section below.

1.3.1 Mechanisms of resistance

The principal mechanisms involved in insecticide resistance are (1) metabolic detoxification, e.g. the enhanced expression of metabolism enzymes, (2) target-site alteration e.g. single amino acid changes at the binding site affects the interaction of insecticide with its target, (3) penetration resistance e.g. changes in the architecture of the cuticle may change the penetration rate of an insecticide, and (4) behavioral resistance e.g. insects avoid exposure by avoiding the treated plant parts. Of these the most important/common mechanisms are target-site and metabolic resistance [165,178,183,185,186,188,196–198].

1.3.1.1 *Metabolic resistance*

In general metabolism can be divided into two phases: Phase I metabolism includes the initial biotransformation of a xenobiotic mainly by oxidative, hydrolytic and reductive processes, whereas Phase II metabolism refers to the bioconjugation of the Phase I metabolites or the parent compound with naturally occurring compounds such as sugars, sugar acids, amino acids or glutathione to produce polar compounds to facilitate excretion [78,186]. Metabolic resistance is one of the most common types of resistance in insects [198]. Enzymes commonly involved in metabolic resistance are the cytochrome P450-dependent monooxygenases (P450s), the carboxylesterases (CCEs) and the glutathione S-transferases (GSTs). The Phase I metabolism enzymes are not only known for their contribution to resistance but also for activation of pro-insecticides [121,158]. The enzyme classes most frequently involved in pyrethroid resistance are the P450s [78,186,198,199], followed by CCEs [200] and GSTs [201].

Many examples of P450-mediated pyrethroid resistance may be found in the literature covering several insect species including the housefly, *Musca domestica* L. [202], the fruit fly *D. melanogaster* [203] several mosquito species [92,204–206], the red flour beetle, *Tribolium castaneum* HERBST [207] and many more [199]. In addition to their role in detoxification of xenobiotics such as pesticides and plant secondary metabolites P450s have crucial roles in

many biosynthetic and developmental processes [198,208]. In regards to insecticide toxicology P450s can also play a role in the species specificity of insecticides, e.g. the use of *tau*-fluvalinate in beehives for the control of the varroa mite, *Varroa destructor* ANDERSON & TRUEMAN [209].

The mechanisms leading to P450-mediated insecticide resistance can be complex at both the genetic and biochemical level. A common pattern observed in metabolic-mediated resistance is the constitutive overexpression of a particular enzyme, thus increasing the abundance of the enzyme and the metabolism of the compound it degrades [210]. Overexpression might be age and/ or tissue specific [198,211]. The reasons causing overexpression are diverse, mechanisms such as regulatory cis- and trans-elements and gene amplification have been described [188,191,212,213]. Good examples where overexpression of a P450 correlates with resistance to insecticides are CYP6CM1 a P450 causing imidacloprid and pymetrozine resistance in *Bemisia tabaci* GENNADIUS [214–216] and CYP6BQ9 a P450 conferring pyrethroid resistance in *T. castaneum* [207]. Interestingly, the simple overexpression of a P450 can result in remarkably resistant phenotypes. For example, laboratory selection of the housefly strain “Learn Pyrethroid Resistance” (LPR) with the pyrethroid permethrin resulted in a resistance level of > 5000-fold against pyrethroids containing a phenoxybenzyl moiety [202,217] which is correlated with a ~10-fold and ~8-fold, increase in expression and protein level, respectively, of CYP6D1 which was shown to cause the resistance in this strain [213,217–223]. P450s may also confer resistance through qualitative rather than quantitative changes in the underlying P450 gene(s). A well-characterized example of change in function in P450s related to resistance against DDT is CYP6A2 from *D. melanogaster.* While the expression level of CYP6A2 remains similar between susceptible and a resistant strain, three amino-acid substitutions were observed. Functional expression of both the wild-type and the mutated allele revealed a substrate shift to DDT boosting the formation of the non-insecticidal dicofol [224]. A recent example of P450 evolution regarding pyrethroid resistance is the emergence of a chimeric P450, CYP337B3, caused by a crossing over event in *Helicoverpa armigera* HÜBNER [189]. Several more cases are known and their description is beyond the scope of my thesis, theirfore I would like to refer to some comprehensive reviews for further reading [199,208,223,225].

Carboxylesterases hydrolyze a broad spectrum of endogenous and exogenous compounds containing an ester linkage [200]. Compounds such as pyrethroids, organophosphates and carbamates (cumulative market share > 40 % in 2010 and > 60 % in 2000 [90]) are esters and vulnerable to hydrolysis. The hydrolysis of the ester group results in a significant decrease or even the total loss of insecticidal activity [186]. The pyrethroid chemical structure influences the rate of hydrolysis; type II pyrethroid esters containing an α-

cyano group adjacent to the ester are less efficiently metabolized by CCEs compared to pyrethroids lacking this motif [226]. Hydrolysis of pyrethroid esters in insects is mostly mediated by soluble non-specific B-type CCEs [78]. CCEs are thought to play a more minor role in pyrethroid resistance and are often described as a secondary resistance mechanism [78,227]. Resistance is not only conferred by hydrolysis but may also occur due to sequestration. A case that is particularly well described in the literature is the carboxylesterase E4/FE4 mediated resistance affecting organophosphates through sequestering and to a lesser extent pyrethroids in *M. persicae* [228,229]. In this case the E4 gene or its paralog FE4 is amplified (up to ~80 copies) resulting in CCE-protein of up to 1 % of the total body weight of the aphid [228,230]. In the absence of selection pressure the E4 gene may be silenced by des-methylation [190]. The E4 CCE-mediated mechanism provides the aphids with an efficient resistance mechanism against organophosphates but not against pyrethroids where the FE4 background confers a modest ~4-fold resistance factor. However, the combination of amplified esterase gene with target site resistance boosts the resistance level to pyrethroids up to 150-540-fold [229].

Glutathione S-transferases are mainly involved in the detoxification of organophosphorous insecticides [201], abamectin [231,232] and to a lesser extent in DDT and pyrethroid resistance [78,233]. The molecular mechanisms of GSTs in pyrethroid resistance are not well understood, sequestration has been reported [234] and tissue protection from pyrethroid-induced lipid peroxidation products was hypothesized [235].

Very recently ATP binding cassette transporter (ABC transporter) have been implicated in pyrethroid resistance in *H. armigera* [236], *Ae. aegypti* [237], *Cimex lectularius* L. [238] and *Rhipicephalus microplus* CANESTRINI [239]. ABC transporters are responsible for ATP-dependent translocation of substances across membranes and represent one of the largest gene families of transporters covering a variety subrates such as ions, amino acids, sugars, peptides, hormones, polysachharides, lipids, sugars and xenobiotics [240,241].

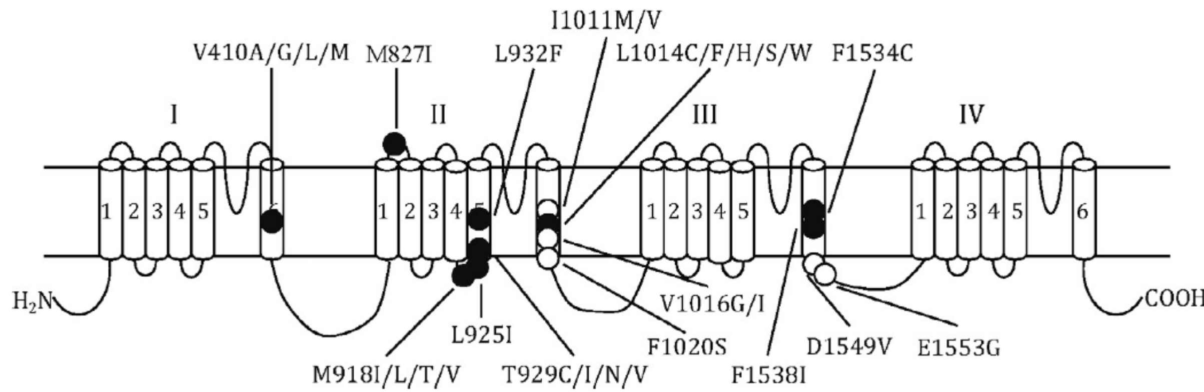
1.3.1.2 *Target-site resistance*

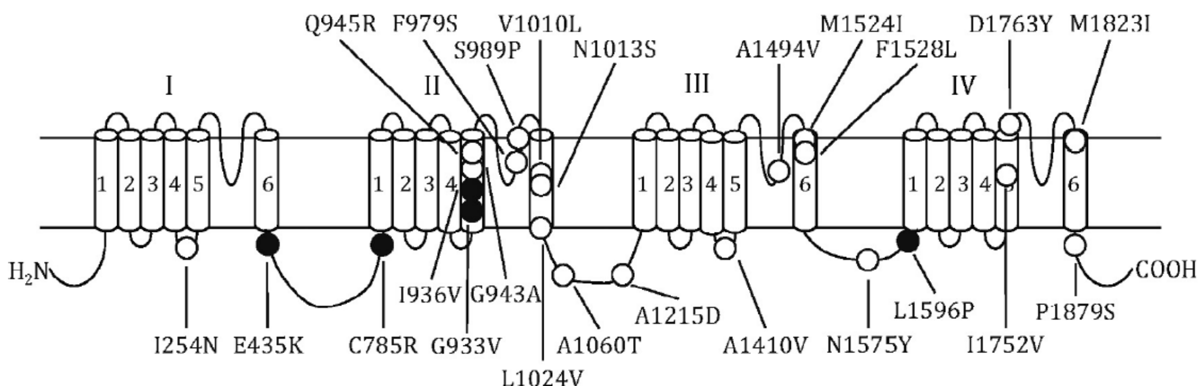
Target-site resistance (also known as altered target-site resistance, target-site modification or mutation) usually refers to qualitative changes in the target protein. Target-site resistance is thought to be the most important resistance mechanism affecting pyrethroids [78]. The majority of target-site resistance cases involves the substitution of a single amino acid and has been described for several insects and a broad range of older chemistries including the pyrethroids [70,242], cyclodienes [243], organophosphates (and carbamates) [160], and some newer classes such as the neonicotinoids [149], diamides [244] and spinosyns [245]. Focusing on pyrethroid resistance cases, mutations underlying the

knockdown resistance (*kdr*) trait(s) have now been described in a remarkable number of insect species [246]. *Kdr* confers resistance to DDT, pyrethrins and pyrethroids, and its phenotypic description in *M. domestica* in 1951 predates the discovery of the pyrethroids [247]. In 1988 direct evidence for the *kdr* trait was provided by reduced neuronal sensitivity in electrophysiological assays with various nerve preparations from *kdr* insects [248]. After the linkage of the *kdr* trait to the *para*-orthologous gene in *M. domestica* [68] the (*Vssc1*) gene was cloned and the resistance associated mutation causing a leucine to phenylalanine substitution at amino acid position 1014 (L1014F) was identified (Fig. 19) [70,242]. Functional evidence was provided by expressing functional sodium channels (*Vssc1* from *M. domestica* and *para*^{CSMA} from *Blatella germanica* L.) carrying the L1014F mutation in *Xenopus* oocyte revealing a 10-fold less sensitive response compared to wild type channels [249,250]. A trait conferring an even stronger resistance to pyrethroids termed *super-kdr* (*s-kdr)* was characterized in 1978, again in *M. domestica* [251]. The *s-kdr* trait maps to the same allele and causes a methionine to threonine substitution at amino acid position 918 (M918T) (Fig. 19). Whereas *kdr* results in ~10-fold resistance to pyrethroids [249] the *s-kdr* trait confers resistance levels of > 500-fold [108,252]. Based on a model of the *M. domestica* sodium channel pore region derived from a crystal structure of the homologous voltage-gated potassium channel the putative ligand binding site was modeled (Fig. 20) [99]. This work has suggested that the huge differences in the resistance levels obtained for the M918T and L1014F mutations may be explained by the direct involvement of amino acid 918 in the pyrethroid interaction, which is in contrast to the amino acid 1014 located in the primary binding site (Fig. 20). The L1014F mutation modifies presumably the action of channel opening [99,246]. The model is in line with biological data showing that the M918T mutation affects only pyrethroids but not DDT due to direct interaction between this residue and pyrethroids but not DDT, whereas the L1014F mutation affects both chemistries [91,252,253].

Different variants of the *kdr* trait i.e. L1014F, L1014H and L1014S were subsequently discovered and it has been hypothesized that individual variants arose due to specific selection pressure with either DDT or pyrethroids [254–256]. The *kdr* trait is in many species a result of a SNP resulting in an amino acid substitution at the corresponding position [105]. However, as shown in *M. domestica* and *B. germanica* the genomic DNA is not necessarily the only source of *kdr* sodium channels. The fly strain “ALHF” and the cockroach strain “Apyr-R” were shown to express *kdr* sodium channels by RNA editing [195]. Another case involves a combination of genetic changes and epigenetic regulation. In diamondback moth, *Plutella xylostella* L. alternative splicing of the VGSC was described resulting in the expression of native sodium channels and sodium channels containing a T929I mutation [193], which is known to confer strong resistance to pyrethroids [193,257,258]. *Kdr* resistance has been

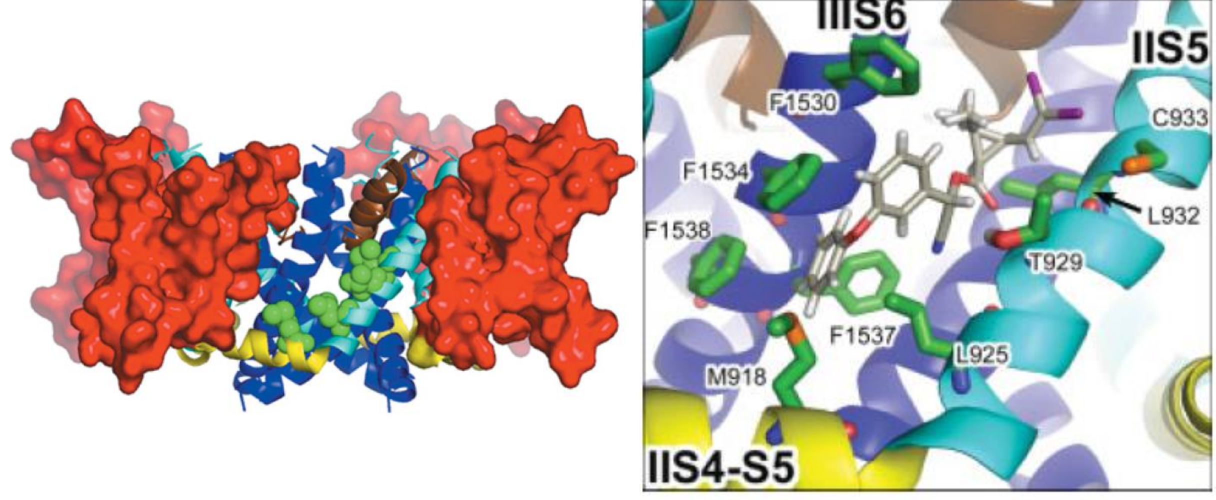
reported in at least 20 insect species [246]. Many more amino acid substitutions in the predicted pore region of the VGSC have been reported (Fig. 19), and are comprehensively reviewed in [108,246,259].





Source: RINKEVICH *et al.* (2013) [246]

Fig. 19 *Location of sodium channel mutations associated with pyrethroid resistance that are detected in more than one species (top) and that are detected in only one species (bottom). Solid circles represent mutations which have been confirmed to reduce insect sodium channel sensitivity to pyrethroid insecticides when functionally expressed in* Xenopus *oocytes. Mutations indicated by open circles have not been examinded yet.*



Source: O'REILLY *et al.* (2006) [99] and DAVIES *et al.* (2008) [260]

Fig. 20 *Model showing the activated-state of* Musca domestica *VGSC (left), voltage sensor domains shown in surface representation (red); S4-S5 linkers (yellow), pore helices (brown) and helices of S5 (cyan) and S6 (blue) are shown in cartoon; Residues implicated in pyrethroid resistance in various pest species (M918, L925, T929 and L932) are shown in space fill (green). Predicted docking of deltamethrin with the VGSC (right).*

Despite the *kdr*-like resistance mechanisms affecting pyrethroids and often DDT a recent case of target site resistance affecting the fairly new spinosyn class provides a good example of another target-site resistance mechanism. Spinosyns, share their target the nAChR with other chemical classes such as neonicotinoids, sulfoximines and butenolids but act at a distinct site at the nAChR [132]. Resistance to this particular insecticide class is frequently conferred by loss of the target site i.e. the alpha6 subunit of nAChR [194,261,262]. Alternatively, an amino acid substitution (G275E) in the alpha6 subunit was recently shown to confer resistance against spinosyns [245].

1.3.1.3 *Penetration resistance*

Penetration resistance is characterized by "a much slower entry of an insecticide into the resistant insect than that into a comparable susceptible insect" [178]. Examples are the reduced penetration of S-fenvalerate in resistant strains of *P. xylostella* [263], *trans*-cypermethrin in a resistant strain of *H. armigera* [264] and imidacloprid in a resistant clonal culture of *M. persicae* [265]. Penetration resistance is often not an efficient mechanism on its own and normally just contributing to other resistance mechanisms [264].

1.3.1.4 *Behavioral resistance*

Behavioral resistance occurs when arthropods minimize contact with or completely avoid the insecticide [178]. This type of resistance is difficult to detect, a well-described case is the “bait avoidance” of *B. germanica* [266–270]. Two examples related to pyrethroid insecticides are the tobacco budworm, *Heliothes virescens* F. and the horn fly, *Haemotobio irritans* L. [271].

1.3.2 Insecticide resistance in oilseed rape insect pests

This thesis is cumulative and includes chapters made of papers published (and submitted) on insecticide resistance monitoring, mechanisms and management in several coleopteran pests of oilseed rape. Due to the fact that each individual chapter includes its own introduction to insecticide resistance history and issues in specific oilseed rape pests no further introduction is given here. Please refer to Chapter 2 (Chapter 3, Chapter 4, Chapter 5 and Chapter 6) for pollen beetle; to Chapter 7 and Chapter 8 for cabbage seed weevil and cabbage flea beetle, respectively.

1.4 Objectives

The recent development of pyrethroid resistance in pollen beetle and other coleopteran oilseed rape pests highlights the importance of resistance management strategies. In order to implement strategically sustainable resistance management strategies it is of utmost importance to describe the geographical extent of the resistance problems, to understand the molecular mechanisms of resistance involved, and to assess alternative chemical control options not yet affected by insecticide resistance in order to avoid control tactics compromised by cross-resistance issues. Therefore the thesis covers the following aspects in a number of individual chapters:

Chapter 2 aimed to assess the level of pyrethroid resistance in individual pollen beetle populations collected in Europe and to provide an overview of its geographical distribution. Furthermore the baseline susceptibility against thiacloprid, a European wide registered compound belonging to the chemical class of neonicotinoids, was determined to allow the early detection of resistance development against this compound in the future.

The aim of Chapter 3 was to determine the cross-resistance pattern in pollen beetle and the identification of the enzyme family involved in pyrethroid resistance.

The objective of Chapter 4 was the identification of resistance alleles confering *kdr*-like target-site resistance in pollen beetle and their distribution in populations collected across Europe.

Chapter 5 aimed to identify the cytochrome P450 monooxygenase encoding gene(s) involved in pyrethroid resistance in pollen beetle and to clarify its contribution to cross-resistance patterns observed in bioassays.

In Chapter 6, next generation sequencing was used to generate a comprehensive sequence resource and allowing the *de novo* assembly of the first pollen beetle transcriptome. This dataset was used to identify genes encoding insecticide target sites and detoxification enzymes and the global gene expression profiling of three resistant and one susceptible strain allowed the identification of genes putatively involved in pyrethroid resistance.

The aim of Chapter 7 was the monitoring of thiacloprid susceptibility in pollen beetle and cabbage seed weevil populations collected across Europe; furthermore the susceptibility of cabbage seed weevil against *lambda*-cyhalothrin was assessed. The practicality of supplying external testing sites with thiacloprid test kits was examined.

Chapter 8 aimed to identify the molecular mechanism causing field failures of pyrethroids in cabbage flea beetle control in Germany and to derive implications for future resistance management.

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Chapter 2

Pyrethroid resistance and thiacloprid baseline susceptibility of European populations of *Meligethes aeneus* (Coleoptera: Nitidulidae) collected in winter oilseed rape

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Abstract

BACKGROUND: Pollen beetle, *Meligethes aeneus* (Coleoptera: Nitidulidae) are a major pest in European winter oilseed rape. Recently control failures with pyrethroid insecticides commonly used to control this pest were reported in many European countries. For resistance management purposes the neonicotinoid insecticide thiacloprid was widely introduced as a new mode of action for pollen beetle control.

RESULTS: A number of pollen beetle populations collected in Germany, France, Austria, Great Britain, Sweden, Denmark, Finland, Poland, Czech Republic and Ukraine were tested for pyrethroid resistance using *lambda*-cyhalothrin-coated glass vials (adult vial test). Most of the populations tested exhibited substantial levels of resistance to *lambda*-cyhalothrin, and resistance ratios ranged from < 10 to > 2000. A similar resistance monitoring bioassay for the neonicotinoid insecticide thiacloprid was developed and validated by assessing baseline susceptibility data for 88 European pollen beetle populations. A variation of less than 5-fold in response to thiacloprid was detected. The thiacloprid adult vial bioassay is based on glass vials coated with an oil-dispersion based formulation of thiacloprid, resulting in a much better

bioavailability compared with technical material. Analytical measurements revealed a > 56d and 28d stability of thiacloprid and *lambda*-cyhalothrin in coated glass vials at room temperature, respectively. No cross-resistance between thiacloprid and *lambda*-cyhalothrin based on log-dose probit-mortality data was detected.

CONCLUSION: Pyrethroid resistance in many European populations of *M. aeneus* was confirmed, whereas all populations are susceptible to thiacloprid when tested in a newly designed and validated monitoring bioassay based on oil dispersion formulated thiacloprid-coated glass vials. Based on the homogenous results it is concluded that thiacloprid could be an important chemical tool for pollen beetle resistance management strategies in European winter oilseed rape.

2.1 Introduction

Winter oilseed rape is one of the most important crops in several European countries. The four main oilseed rape growing countries in Europe are France (1.58 mio ha in 2009), Germany (1.55 mio ha), Poland (0.8 mio ha) and the United Kingdom (0,68 mio ha) [1]. The pollen beetle, *Meligethes aeneus* F. (Coleoptera: Nitidulidae), is one of the major pests in European oilseed rape and known to be quite destructive once infestation thresholds are exceeded and no chemical control measures taken [2]. After emerging from overwintering sites adults start to infest oilseed rape plants in mid-March until May, and can damage the flowering parts by feeding and oviposition, and particularly feeding larvae cause bud abscission. The consequence of these infestations are pod-less stalks and dramatically reduced yields, so the farmers need to control pollen beetles to keep numbers low and to avoid economic damage. All over Europe, pyrethroid insecticides have a long history in pollen beetle control [3]. In many countries the common practice is more than one insecticide application against pollen beetle per season [4]. The requirement for control and limited availability of compounds from other chemical classes have conspired, resulting in intense selection pressures being imposed by pyrethroid insecticides [5]. In 2005 almost a 100 % of all insecticide applications in oilseed rape in Germany accounted to pyrethroid insecticides [3,6].

The first case of reduced susceptibility of pollen beetle to pyrethroids was reported in 1999 in the Champagne region in North-Eastern France [7,8]. Confirmed cases of pyrethroid resistance in Germany were documented in 2002, and in 2006 more than 50 % of the winter oilseed rape acreage in Germany was affected [3]. First cases of pyrethroid resistance in Denmark were described in 2000 and 2001 and confirmed in a larger study in 2003 [7,8]. Since then pyrethroid resistance data from several other countries in Europe such as Switzerland, France and Poland have been published [9-11]. UK is one of the major oilseed rape growing countries in the EU, which seemed to be less affected by pyrethroid resistance

until recently, only in 2007 were the first resistant populations discovered [12]. Pollen beetle pyrethroid resistance monitoring carried out by the Insecticide Resistance Action Committee (IRAC) in 2008 confirmed that pyrethroid resistance is widespread in Europe, particularly in France, Germany and Poland [13,14]. Resistance to pyrethroids in pollen beetle is not limited to individual compounds, but affected the whole chemical class of pyrethroid insecticides, although some of them seem to show higher activity at recommended field rates than others [3]. The problem of pollen beetle resistance to pyrethroids in European winter oilseed rape was also covered in a recent workshop organized by the European and Mediterranean Plant Protection Organisation (EPPO) [15].

In 2007 the first resistance management strategy for pollen beetle in winter oilseed rape was recommended in Germany and is mainly based on alternations with thiacloprid, belonging to the chemical class of neonicotinoids, known to target insect nicotinic acetylcholine receptors [16]. Thiacloprid has been fully registered for pollen beetle control since 2007, and since its introduction also insecticides with different modes of action have been investigated for their potential on pollen beetle, in order to increase diversity for resistance management purposes [17,18]. The pollen beetle resistance management strategy implemented in Germany includes a well-defined application scheme based on the occurrence of pollen beetle before and during flowering, and additionally taking into account other oilseed rape pests such as weevils [19-21]. The strategy also considers as an emergency exemption the use of organophosphate insecticides such as chlorpyrifos-methyl at high infestation levels before flowering.

The objectives of the current study was to develop a robust, reliable, rapid and validated method to effectively assess pollen beetle susceptibility to thiacloprid and to establish baseline data with populations collected in several European countries, which can be used in future monitoring campaigns to detect early shifts in susceptibility. Furthermore the pyrethoid resistance status of all collected populations was tested in parallel to check for cross-resistance issues.

2.2 Material and Methods

2.2.1 Insects

In April/May 2009 and 2010 pollen beetle populations were collected in winter oilseed rape fields from different European countries, including the most important oilseed rape producing countries France, Germany, Poland and Great Britain (Fig. 21). The adult insects were packed in plastic bags with some rape buds and foliage and shipped to Bayer CropScience in Monheim, Germany. After arrival in the laboratory, beetles were stored for

24 h at 4 °C. Two hours before bioassay the insects were removed from the refrigerator and equilibrated to room temperature (20 ± 2° C). Those beetles of lower fitness remain on the bottom of the bag and are not used for the bioassays.

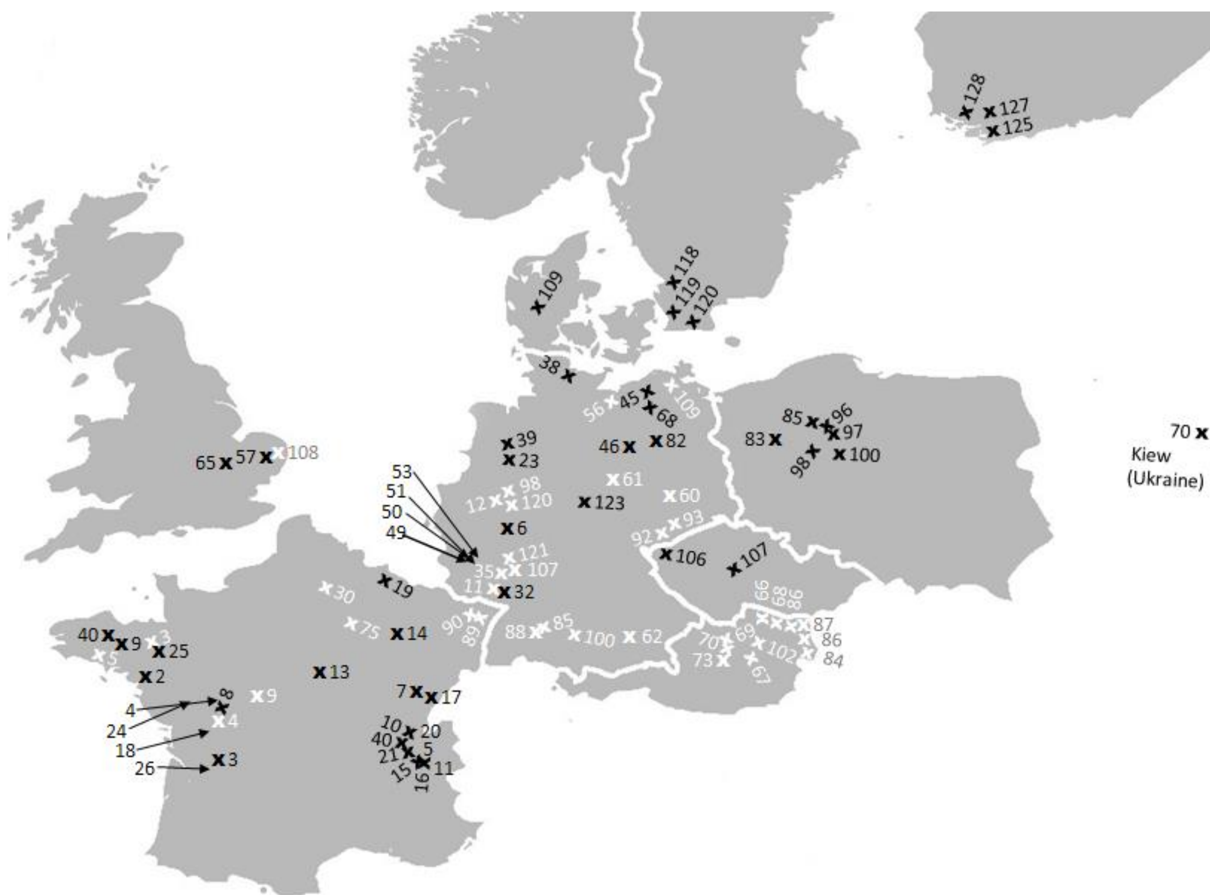


Fig. 21 *Pollen beetle sampling sites in Europe (white and black numbers mark collection sites in 2009 and 2010, respectively).*

2.2.2 Pyrethroid resistance monitoring bioassay

All pyrethroid resistance monitoring bioassays were conducted according to instructions outlined in IRAC´s Susceptibility Method No. 11 “Pollen Beetle Susceptibility Monitoring Bioassay – Synthetic Pyrethroids” [22]. The method is based on glass vials (Zinsser Analytics, Germany) coated with defined concentrations of *lambda*-cyhalothrin. Beetles confined to glass vials were assessed for mortality after 24 h. The IRAC method was slightly modified in two points: (1) The assessment was done by directly scoring affected beetles in the vials rather than using the recommended filter disc assessment arena; (2) Instead of the two pyrethroid concentrations, five concentrations were used to generate dose-response curves, i.e. 0.375 µg AI cm^{-2} inner glass surface (500 % of common field application rate of 7.5 g ha^{-1}), 0.075 µg cm^{-2} (100 %), 0.015 µg cm^{-2} (20 %), 0.003 µg cm^{-2} (4 %) and 0.0006 µg cm^{-2} (0.8 %). For one highly sensitive population obtained from Ukraine (strain 70-

10) two more concentrations were added, i.e. 0.00012 and 0.000024 µg cm^{-2}. Two of the tested concentrations (100 % and 20 % of the field rate) were used to classify the degree of pyrethroid resistance in tested populations by using an IRAC recommended rating scheme (Table 2) [22].

Table 2 *IRAC pyrethroid resistance classification scheme for pollen beetles.*

Concentration (% of label rate)	Affected beetles	Classification	Code
100 % 20 %	100 % 100 %	Highly susceptible	1
100 % 20 %	100 % < 100 %	Susceptible	2
100 %	< 100 % to ≥ 90 %	Moderately resistant	3
100 %	< 90 % to ≥ 50 %	Resistant	4
100 %	< 50 %	Highly Resistant	5

2.3 Thiacloprid adult vial bioassay

In order to check pollen beetle populations for thiacloprid baseline susceptibility the above-mentioned adult vial bioassay method for pyrethroids was slightly modified. Vials were coated using the commercially available formulation “Biscaya® 240OD” (240 g thiacloprid L^{-1} in oil dispersion), since preliminary trials revealed that technical material is not appropriate, even when applied with adjuvants (results not shown). Stock solutions were prepared by dissolving 140.4 mg OD 240 formulation (containing 32.4 mg a.i. thiacloprid) in 2 mL distilled water, subsequently adjusted to 100 mL with acetone. All further dilutions were made in acetone. For coating purposes glass vials (20 ml volume, 45 cm^2 internal surface) were filled with 500 µL of solution. For all bioassays five concentrations were used: 1.44 µg cm^{-2} internal surface area (corresponds to 200 % of the field-recommeded rate of 72 g ha^{-1}), 0.72 µg cm^{-2} (100 %), 0.144 µg cm^{-2} (20 %), 0.0288 µg cm^{-2} (4 %) and 0.00576 µg cm^{-2} (0.8 %). The vials have to rotate for a minimum of 2 h and subjection to a further evaporation phase without rotation for a minimum of 2 h (or overnight) is obligatory before capping and storing the vials. The prepared vials can be stored at room temperature (dark) for a minimum of 4 weeks without a significant loss of thiacloprid (see section 2.4.1 and 2.5.2).

Some trials using an identical procedure as described above were done with blank formulation in order to check for mortality possibly caused by exceeding a maximum level of oil formulation in coated vials.

For testing purposes ten pollen beetles were placed in each vial, using three replicates per concentration and population (plus an acetone control). Capped vials are then stored upright at 20 ± 2 °C for 24 h. Prior to assessment vials are briefly shaken to differentiate alive and affected beetles more easily.

2.3.1 Storage stability tests and analytics

In order to investigate the storage stability of *lambda*-cyhalothrin and thiacloprid in coated glass vials, three replicates per concentration were analyzed for active ingredient after 0, 14, 28 and 56 days. Coated glass vials were stored in the dark at both 4 °C and 20 °C to check for temperature effects. Stored vials were washed two times with 500 µL acetonitrile, and combined volumes and subjected to quantitative HPLC-MS/MS measurements. The samples were measured on an Applied Biosystems API4000 QTrap MS/MS system running in positive electrospray MRM mode with a capillary voltage of 4kV and Turbo V gas temperature of 500 °C. The HPLC-system was a Waters Acquity UPLC consisting of Binary Solvent Manager, Column Manager and Sample Manager. The samples were run on a Waters Acquity HSS T3 1.8 µm column (size: 50 x 2.1 mm) running in reverse phase gradient mode. For the determination of thiacloprid acetonitrile/water/0.1 % formic acid was used as eluent, whereas for the determination of *lambda*-cyhalothrin methanol/2mM NH_4OAc/1 % acetic acid was used.

For the quantitation the MRM transitions 253.1 > 126.0 (thiacloprid) and 467.1 > 225.1 (*lambda*-Cyhalothrin) were monitored. The peak integrals were calibrated externally against a standard calibration curve with a correlation coefficient of r > 0.99. The limits of quantitation (S/N > 10) are 10 pg mL^{-1} for thiacloprid and 100 pg mL^{-1} for *lambda*-cyhalothrin.

2.3.2 Data analysis

The lethal concentration-values (LCs) were calculated by probit analysis using Polo Plus Software Version 1 (LeOra Software, California). All mortality figures were corrected for control mortality by using ABBOTT´S formula [23]. Further statistical analyses were performed with Graphpad Prism 5 software (GraphPad Software Inc., California). Analysis of variance (ANOVA) procedures, student t-tests and appropriate post-tests (e.g. Tukey Kramer) were performed to test for significant diffrences between strains, resistance classes, treatments and insecticides.

2.4 Results

2.4.1 Validation of the adult vial test based on thiacloprid OD240 formulation

The trials with oil dispersion blank formulation in glass vials revealed an upper limit of 200 % of the field-recommended rate based on thiacloprid content (absent in blank formulation) not affecting pollen beetle after 24 h (Fig. 22). The high percentage of affected beetles at rates above 200 % is probably a consequence of the oil film on the internal surface area of the vials. In all cases the observed mortality is linked to pollen beetles which sticked to the internal glass vial surface, rather than symptoms of poisoning as observed with thiacloprid.

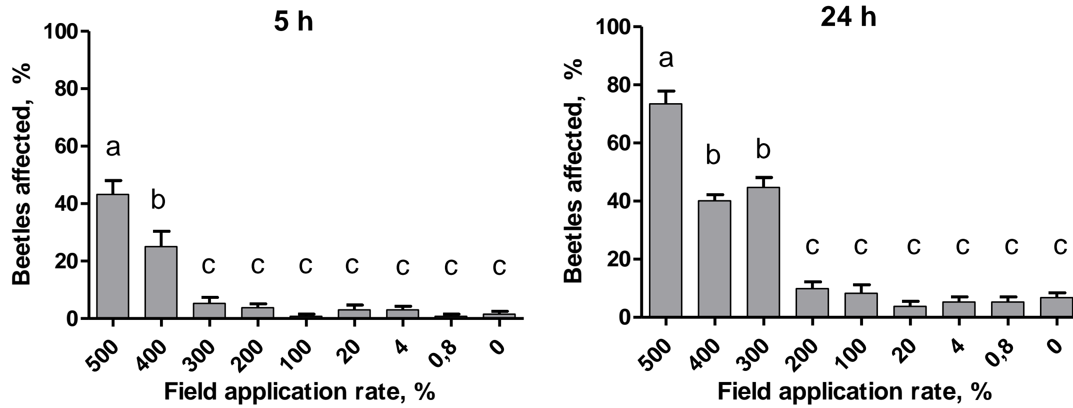


Fig. 22 *Effect of blank oil dispersion formulation of "Biscaya" on pollen beetles in an adult vial test after 5 h and 24 h. Data are mean values ± SEM (n=4), and different letters indicate significant differences (p < 0.05, t-test).*

2.4.2 Stability of insecticides in coated vials

The concentration of thiacloprid in glass vials coated for resistance monitoring purposes remains stable at both 4 °C and 20 °C, and even after a storage period of 56 days no significant differences compared with directly analysed samples (0 days) were observed (p > 0.05) (Fig. 23). Although the analytical results revealed a stable and unchanged concentration of thiacloprid over a period of 56 days, we observed a somewhat lower efficacy against pollen beetle in such vials (data not shown), therefore it is suggested to store vials no longer than 28 days.

The concentration of lamda-cyhalothrin also did not change significantly up to 4 weeks after storage at both 4 °C and 21 °C. However, after 8 weeks of storage at 20 °C a slight, but significant decrease in the concentration of *lambda*-cyhalothrin was observed (p < 0.05) (Fig. 23).

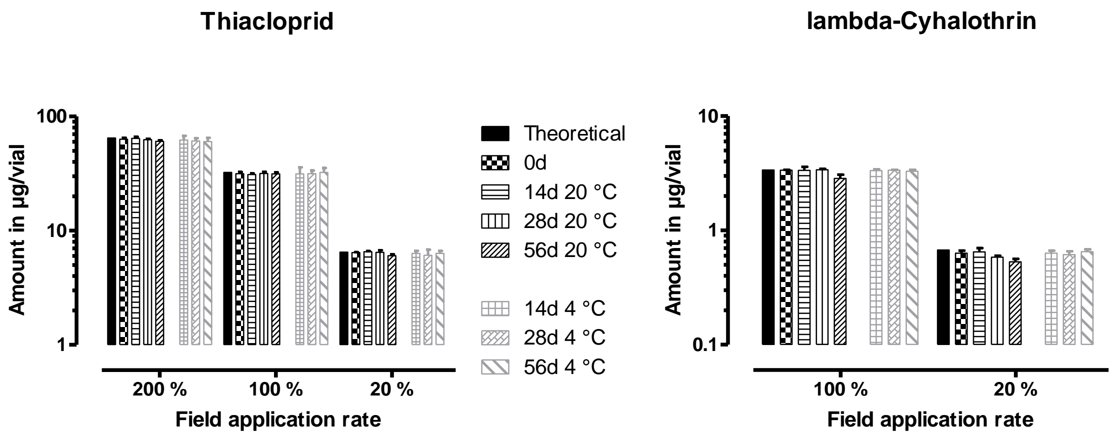
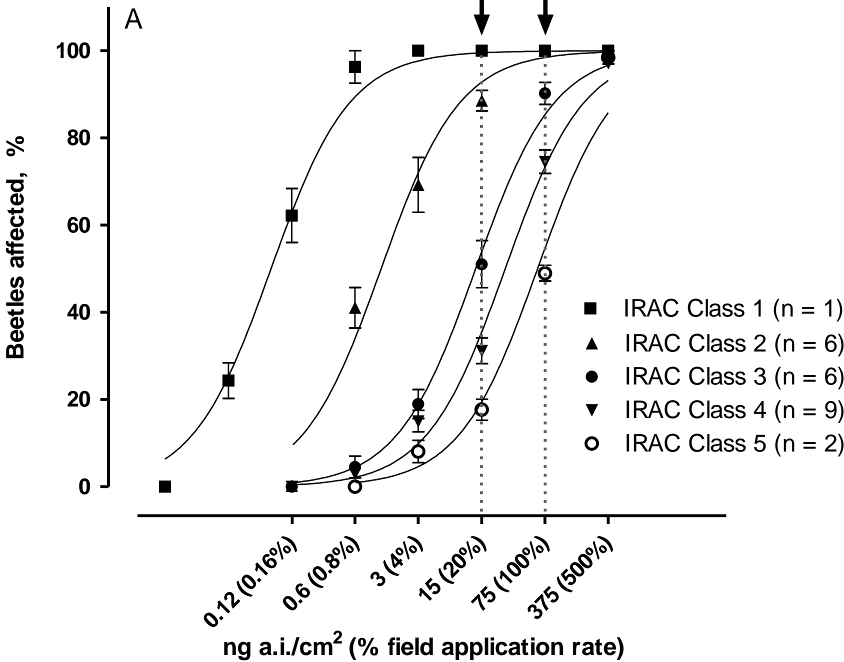
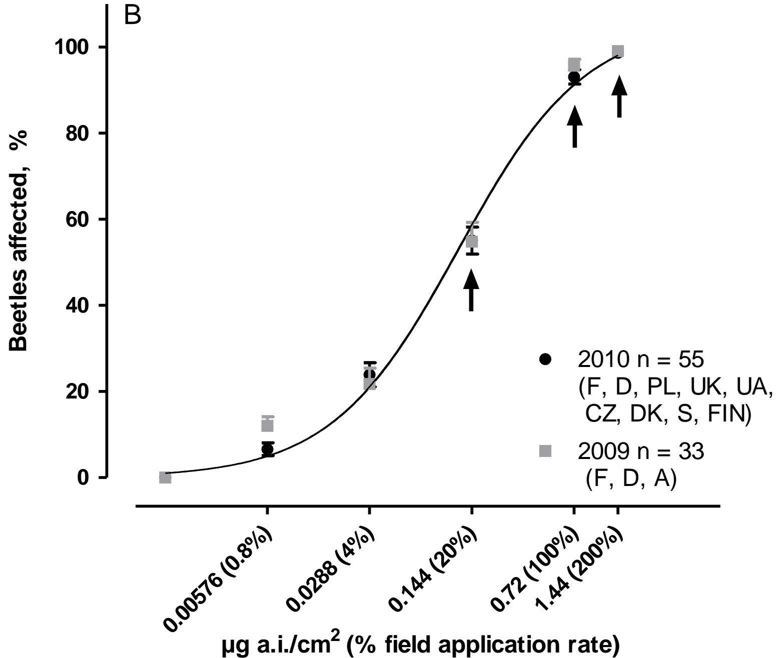


Fig. 23 *Stability of active ingredients in coated glass vials stored at different temperatures and analyzed after different elapsed time intervals. Data are mean values ± SD (n=3).*

2.4.3 Pyrethroid resistance monitoring

Susceptibility to *lambda*-cyhalothrin of 25 European pollen beetle populations collected in each 2009 and 2010 was tested, and calculated LC_{50}-values range from as low as 0.0001 µg cm^{-2} (0.1 % of field rate) to 0.051 µg cm^{-2} (67 % of field rate), leading to resistance ratios of up-to 500-fold (Table 3). However based on extrapolated LC_{95}-values, resistance factors even exceeded 1000-fold in some populations collected in Germany (5) and France (2). Several populations show less than 95 % mortality at 5-fold of the recommended field rate (Table 3). The samples collected in 2010 included one highly susceptible population (LC_{95} 0.00042 µg cm^{-2}) collected in the central Ukraine, which was taken as the reference to calculate all resistance ratios given in Table 2. All dose-response bioassays performed included both concentrations (i.e. 100 % and 20 % of field dose) recommended by IRAC for resistance class determination, and all classes from 1 to 5 are present in the European populations collected, at least in 2010 (Table 3). Fifteen out of 50 tested populations (i.e. 30 %) could be classified as pyrethroid susceptible. All IRAC resistance classes determined and based on two concentrations per population could be well separated by their dose-response relationship when including all tested concentrations, thus supporting the IRAC proposed classification (indicated by arrows and dotted lines given in Fig. 24). However dose-response relationships for populations assigned to classes 3 to 5 (moderately to highly resistant) are not as well separated as those belonging to classes 1 to 3 (highly susceptible to moderately resistant) (Fig. 24).





Error bars = standard error mean; F = France, D = Germany, PL = Poland, UK = United Kingdom, UA = Ukraine, CZ = Czechia, DK = Denmark, S = Sweden, FIN = Finland, A = Austria)

Fig. 24 *(A) Response of pollen beetle populations collected in 2010 to different concentrations of* lambda-*cyhalothrin and their classification to different pyrethroid resistance groups as proposed by IRAC [22]. The arrows and dotted lines mark the IRAC recommended discriminating rates. (B) Baseline susceptibility to thiacloprid of pollen beetle populations collected in 2009 and 2010. Dose response curves represent mean values of combined data ± SEM. The arrows mark our proposed discriminating rates for future monitoring initiatives.*

2.4.4 Baseline susceptibility of thiacloprid

Baseline susceptibility to thiacloprid was determined based on log-dose probit-mortality results of 33 and 55 field populations of pollen beetle collected in 2009 and 2010, respectively. The calculated LC_{50}-values ranged from 0.038 µg cm^{-2} to 0.122 µg cm^{-2} and from 0.04 µg cm^{-2} to 0.196 µg cm^{-2} in 2009 and 2010, respectively (Table 4). So the LC_{50}-values for both years show a maximum variation of 5-fold between all 88 populations tested, compared to approx. 500-fold determined for the pyrethroid *lambda*-cyhalothrin. The LC_{95}-values ranged from 0.47 µg cm^{-2} to 1.48 µg cm^{-2} and from 0.3 µg cm^{-2} to 2.22 µg cm^{-2} in 2009 and 2010, respectively (Table 4). Again the variation in response is quite low, so the method is considered to provide reliable data in future resistance monitoring campaigns. Combining all data from 2009 and 2010 revealed non-significantly differing composite LC_{95}-values based on field rates of 134 % to 146 %, respectively (Table 4).

The very low variation in pollen beetle response to thiacloprid in both years 2009 and 2010 resulted in overlapping dose-response-curves indicating no shift in susceptibility from one year to the other (Fig. 24). Based on the obtained results it is suggested to use 200 %, 100% and 20% of the field rate as discriminating doses in adult vial tests for future monitoring purposes (see arrows Fig. 24). The mean mortality obtained in adult vial tests for the rates 200 %, 100 % and 20 % is 98.5 ± 2.9 %, 95.7 ± 7.2 % and 54.8 ± 12 %, respectively.

2.4.5 Cross-resistance investigations

In all populations tested in both 2009 and 2010 no trends of cross-resistance is observed between *lambda*-cyhalothrin and thiacloprid. With regard to the IRAC pyrethroid resistance classification, which clearly and significantly separates populations based on their allocation to different resistance classes, it is demonstrated that thiacloprid does not follow the same trend (Table 3 and Table 4). Even those populations classified as highly resistant to pyrethroids are not showing any lower susceptibility to thiacloprid, suggesting the complete lack of cross-resistance. This is also statistically validated by regression analysis revealing no correlation between LC_{50}- and LC_{95}-values for both compounds ($p > 0.05$).

Table 3 *Log-dose probit-mortality data for* lambda*-cyhalothrin against pollen beetle populations collected in 2009 and 2010 (adult vial test).*

Please refer to the following two pages.

Strain	Country	P-RC[a]	LC_{50} µg cm^{-2} (Field rate %)	95 % CL µg cm^{-2}	LC_{95} µg cm^{-2} (Field rate %)	95 % CL µg cm^{-2}	Slope (±SE)	RR LC_{50}	RR LC_{95}
67-09	Austria	1	0.001 (1.4)	0.0008-0.0014	0.005 (7.2)	0.004-0.011	2.27 (±0.2)	10	13
84-09	Austria	1	0.0008 (1.1)	0.0006-0.0012	0.004 (5.6)	0.003-0.009	2.36 (±0.21)	8	10
109-09	Germany	1	0.0008 (1)	0.0006-0.0009	0.003 (4.3)	0.003-0.005	1.53 (±0.1)	8	8
102-09	Germany	2	0.0011 (1.5)	0.0004-0.0028	0.013 (17.4)	0.004-0.105	1.46 (±0.11)	11	33
68-09[b]	Austria	2	0.003 (3.9)	0.0015-0.0057	0.02 (26.3)	0.009-0.140	1.99 (±0.16)	30	50
87-09	Austria	2	0.0008 (1.1)	0.0004-0.0018	0.006 (8.1)	0.003-0.06	1.93 (±0.16)	8	15
90-09	France	2	0.0011 (1.5)	0.0007-0.0017	0.007 (9.1)	0.004-0.018	2.10 (±0.17)	11	18
108-09	G. Britain	2	0.0013 (1.7)	0.0009-0.0017	0.009 (12)	0.006-0.018	1.94 (±0.16)	13	23
56-09	Germany	3	0.005 (6.7)	0.0017-0.0141	0.177 (236)	0.046-4.109	1.03 (±0.07)	50	443
61-09	Germany	3	0.009 (12.1)	0.0058-0.0147	0.186 (248.5)	0.089-0.580	1.25 (±0.09)	90	465
66-09	Austria	3	0.0048 (6.4)	0.0053-0.0078	0.192 (256)	0.011-0.066	2.72 (±0.24)	48	480
69-09[b]	Austria	3	0.0067 (8.9)	0.0053-0.0084	0.075 (99.9)	0.052-0.119	1.57 (±0.11)	67	188
70-09[b]	Austria	3	0.0061 (8.1)	0.0048-0.0078	0.104 (138.5)	0.069-0.173	1.33 (±0.09)	61	260
73-09	Austria	3	0.0053 (7.1)	0.0031-0.0093	0.108 (144.7)	0.048-0.409	1.26 (±0.09)	53	270
86-09	Austria	3	0.0037 (4.9)	0.0023-0.0059	0.074 (98.3)	0.037-0.215	1.09 (±0.08)	37	185
88-09	Germany	3	0.0084 (11.2)	0.0047-0.0153	0.189 (251.9)	0.079-0.794	1.22 (±0.08)	84	473
107-09	Germany	3	0.0087 (11.6)	0.0032-0.0251	0.147 (196.2)	0.043-3.365	1.34 (±0.09)	87	368
120-09	Germany	3	0.0056 (7.5)	0.0027-0.0114	0.144 (191.9)	0.053-0.903	1.17 (±0.08)	56	360
35-09	Germany	4	0.0088 (11.7)	0.002-0.0419	0.559 (745.2)	0.086-176.01	0.92 (±0.06)	88	1398
30-09	France	4	0.0149 (19.8)	0.0056-0.0441	0.254 (338.7)	0.073-6.15	1.34 (±0.09)	149	635
60-09	Germany	4	0.0104 (13.8)	0.0022-0.0566	0.355 (473.4)	0.063-181.13	1.07 (±0.07)	104	888
62-09	Germany	4	0.0123 (16.4)	0.0058-0.0274	0.29 (387.3)	0.097-2.43	1.2 (±0.081)	123	725
85-09	Germany	4	0.0167 (22.3)	0.0057-0.0586	0.562 (749.4)	0.125-28.46	1.08 (±0.08)	167	1405
100-09	Germany	4	0.0181 (24.1)	0.0144-0.0229	0.227 (302.6)	0.16-0.367	1.5 (±0.10)	181	568
121-09	Germany	4	0.0173 (23)	0.0077-0.042	0.454 (604.9)	0.138-5.482	1.16 (±0.08)	173	1135
70-10	Ukraine	1	0.0001 (0.1)	0.0001-0.0001	0.0004 (0.5)	0.0003-0.0008	2.93 (±0.29)	1	1

2-10	France	2	0.0035 (4.7)	0.0017-0.0073	0.062 (83.3)	0.024-0.393	1.31 (±0.09)	35	155
4-10	France	2	0.0053 (7.1)	0.002-0.0139	0.092 (122.5)	0.029-1.193	1.34 (±0.09)	53	230
3-10	France	2	0.0034 (4.5)	0.0023-0.0051	0.032 (42.4)	0.018-0.079	1.69 (±0.13)	34	80
125-10	Finland	2	0.0012 (1.6)	0.0004-0.0028	0.019 (24.9)	0.006-0.253	1.36 (±0.12)	12	48
128-10	Finland	2	0.0012 (1.6)	0.0004-0.0028	0.02 (26.1)	0.007-0.237	1.36 (±0.1)	12	50
127-10	Finland	2	0.0008 (1.1)	0.0004-0.0014	0.006 (7.6)	0.003-0.024	1.92 (±0.16)	8	15
5-10	France	3	0.0047 (6.2)	0.0032-0.0066	0.064 (85.4)	0.037-0.139	1.4 (±0.1)	47	160
109-10	Denmark	3	0.017 (22.7)	0.0095-0.0294	0.135 (180.3)	0.068-0.458	1.83 (±0.12)	170	338
119-10	Sweden	3	0.016 (21.3)	0.0104-0.0246	0.114 (151.5)	0.064-0.297	1.9 (±0.14)	160	285
107-10	Czechia	3	0.0037 (5)	0.0023-0.0061	0.053 (70.5)	0.026-0.158	1.43 (±0.1)	37	133
118-10	Sweden	3	0.0281 (37.5)	0.0195-0.0401	0.265 (353.2)	0.161-0.539	1.69 (±0.11)	281	663
120-10	Sweden	3	0.0162 (21.6)	0.0119-0.0219	0.121 (161.7)	0.078-0.226	1.88 (±0.14)	162	303
7-10	France	4	0.0177 (23.6)	0.0073-0.0407	0.517 (689.6)	0.169-4.487	1.12 (±0.07)	177	1293
32-10	Germany	4	0.0284 (37.8)	0.0125-0.063	0.58 (773)	0.204-4.489	1.26 (±0.11)	284	1450
96-10	Poland	4	0.0231 (30.8)	0.0088-0.0575	0.237 (316)	0.086-2.491	1.63 (±0.13)	231	593
98-10	Poland	4	0.021 (28.1)	0.0104-0.0414	0.214 (285)	0.093-1.055	1.63 (±0.11)	210	535
8-10	France	4	0.0155 (20.6)	0.0049-0.045	0.896 (1194.2)	0.204-248.2	0.93 (±0.06)	155	2240
39-10	Germany	4	0.0046 (6.1)	0.0007-0.0209	0.1 (133.3)	0.022-1.842	1.23 (±0.08)	46	250
85-10	Poland	4	0.0299 (39.9)	0.0251-0.0355	0.123 (164)	0.091-0.173	1.96 (±0.09)	299	308
97-10	Poland	4	0.0212 (28.2)	0.0064-0.0669	0.152 (203.2)	0.053-5.225	1.91 (±0.13)	212	380
100-10	Poland	4	0.0148 (19.8)	0.0072-0.0292	0.245 (326.8)	0.1-1.261	1.35 (±0.09)	148	613
106-10	Czechia	4	0.0369 (49.2)	0.0188-0.0694	0.232 (309.3)	0.111-1.059	2.04 (±0.14)	369	580
68-10	Germany	5	0.0383 (51.1)	0.0131-0.1088	0.578 (770)	0.18-12.086	1.3 (±0.16)	383	1445
82-10	Germany	5	0.0506 (67.4)	0.0255-0.0938	0.315 (420)	0.152-1.493	1.99 (±0.16)	506	788
Composite 2009			0.0045 (6)	0.0026-0.0075	0.113 (150.1)	0.053-0.356	1.17 (±0.075)		
Composite 2010			0.0098 (13.1)	0.0063-0.0153	0.237 (315.8)	0.119-0.639	1.19 (±0.08)		

[a] P-RC = pyrethroid resistance index after IRAC susceptibility method No. 11; [b] RR = resistance ratio based on strain 70-10; [c] Including ≥ 20 % *M. viridescens*, in all others ≤ 5 %

Table 4 *Log-dose probit-mortality data for thiacloprid against pollen beetle populations collected in 2009 and 2010 (adult vial test).*

Strain	Country	P-RC[a]	LC_{50} µg cm^{-2} (Field rate %)	95 % CL µg cm^{-2}	LC_{95}-µg cm^{-2} (Field rate %)	95 % CL µg/cm^{-2}	Slope (±SE)	RR LC_{50}	RR LC_{95}
67-09	Austria	1	0.05 (7)	0.02-0.1	0.92 (127.8)	0.38-4.39	1.3 (±0.08)	1	2
84-09	Austria	1	0.05 (6.9)	0.03-0.09	0.73 (101.5)	0.34-1.91	1.41 (±0.09)	1	2
5-09	France	2	0.07 (9.2)	0.05-0.09	0.59 (81.5)	0.36-1.17	1.74 (±0.13)	2	1
102-09	Germany	2	0.09 (12)	0.04-0.17	0.83 (115.5)	0.36-4.28	1.67 (±0.11)	2	2
68-09[b]	Austria	2	0.04 (5.5)	0.02-0.07	0.51 (70.9)	0.24-1.81	1.48 (±0.1)	1	1
87-09	Austria	2	0.07 (10.3)	0.04-0.14	0.88 (122.5)	0.4-3.57	1.53 (±0.1)	2	2
98-09	Germany	2	0.09 (12.3)	0.03-0.25	1.19 (165.3)	0.38-20.65	1.46 (±0.9)	2	3
89-09	France	2	0.04 (5.6)	0.02-0.07	0.51 (70.1)	0.26-1.73	1.7 (±0.13)	1	1
108-09	G. Britain	3	0.1 (13.8)	0.05-0.19	1.1 (152.5)	0.49-4.72	1.58 (±0.1)	3	3
13-09	Germany	3	0.04 (5.3)	0.02-0.07	0.47 (65.8)	0.22-1.66	1.51 (±0.1)	1	1
56-09	Germany	3	0.08 (11.1)	0.04-0.14	0.66 (91.6)	0.32-2.46	1.79 (±0.12)	2	2
61-09	Austria	3	0.06 (8.3)	0.04-0.1	0.71 (99.2)	0.36-2.12	1.53 (±0.1)	2	2
66-09	Austria	3	0.06 (7.6)	0.03-0.1	0.87 (121.2)	0.4-3.68	1.37 (±0.08)	2	2
69-09[b]	Austria	3	0.06 (8.7)	0.03-0.13	0.86 (118.9)	0.34-4.99	1.45 (±0.09)	2	2
70-09[b]	Austria	3	0.04 (6)	0.02-0.09	0.67 (92.5)	0.25-4.51	1.38 (±0.09)	1	2
73-09	France	3	0.05 (7.1)	0.02-0.11	1.01 (140.1)	0.37-6.74	1.27 (±0.08)	1	2
75-09	Austria	3	0.09 (11.9)	0.05-0.13	0.87 (120.8)	0.47-2.27	1.63 (±0.11)	2	2
86-09	Germany	3	0.09 (12.7)	0.05-0.16	0.68 (93.7)	0.34-2.3	1.9 (±0.13)	2	2
88-09	Germany	3	0.08 (11.3)	0.02-0.22	1.23 (170.8)	0.4-17.17	1.39 (±0.09)	2	3
92-09	Germany	3	0.07 (10.2)	0.04-0.12	0.98 (135.5)	0.48-3.05	1.46 (±0.09)	2	2
93-09	Germany	3	0.05 (6.9)	0.03-0.09	0.76 (105.9)	0.33-3.16	1.39 (±0.09)	1	2
107-09	Germany	3	0.10 (14.5)	0.06-0.17	0.85 (117.8)	0.44-2.62	1.81 (±0.12)	3	2
120-09	France	4	0.1 (13.9)	0.04-0.23	1.26 (175.6)	0.47-10.47	1.49 (±0.09)	3	3
3-09	France	4	0.10 (14.2)	0.06-0.18	1.1 (152.2)	0.53-3.78	1.59 (±0.1)	3	3

4-09	France	4	0.08 (12.1)	0.04-0.17	1.01 (140.9)	0.43-5.14	1.54 (±0.1)	2	2
9-09	Germany	4	0.07 (10)	0.02-0.19	1.22 (169.1)	0.38-18.11	1.34 (±0.08)	2	3
11-09	Germany	4	0.12 (16.9)	0.05-0.3	1.01 (139.6)	0.39-10.51	1.62 (±0.1)	3	2
12-09	France	4	0.05 (7.3)	0.03-0.1	0.68 (95)	0.28-3.61	1.47 (±0.09)	1	2
30-09	Germany	4	0.07 (9.3)	0.04-0.12	0.84 (116.5)	0.39-3.05	1.5 (±0.1)	2	2
35-09	Germany	4	0.12 (16.1)	0.05-0.25	1.49 (206.1)	0.59-9.44	1.49 (±0.09)	3	3
62-09	Germany	4	0.05 (6.4)	0.02-0.09	0.76 (105.7)	0.31-3.98	1.35 (±0.08)	1	2
85-09	Germany	4	0.08 (11.1)	0.06-0.11	0.58 (74.7)	0.35-0.99	1.98 (±0.01)	2	1
100-09	Germany	4	0.11 (15.3)	0.05-0.24	1.02 (141.2)	0.41-7.32	1.71 (±0.11)	3	2
70-10	Ukraine	1	0.12 (16.4)	0.1-0.14	1.17 (162.8)	0.86-1.7	1.65 (±0.11)	3	3
127-10	Finland	2	0.04 (6)	0.04-0.05	0.57 (79)	0.42-0.71	1.7 (±0.13)	1	1
125-10	Finland	2	0.07 (10.1)	0.04-0.12	0.71 (98.7)	0.41-1.43	1.56 (±0.09)	2	2
128-10	Finland	2	0.11 (14.8)	0.06-0.15	1.28 (178)	0.7-3.26	1.78 (±0.14)	3	3
2-10	France	2	0.11 (15.5)	0.07-0.18	1.61 (223.3)	0.87-4.05	1.42 (±0.09)	3	4
4-10	France	2	0.05 (7.3)	0.03-0.1	0.45 (62.5)	0.2-2.31	1.77 (±0.12)	1	1
25-10	France	2	0.06 (8.6)	0.04-0.1	1 (138.6)	0.48-3.08	1.36 (±0.08)	2	2
3-10	France	2	0.11 (15)	0.05-0.22	1.04 (144.2)	0.45-5.27	1.67 (±0.11)	3	2
15-10	France	2	0.06 (8.8)	0.04-0.11	0.65 (90.5)	0.31-2.41	1.63 (±0.11)	2	2
24-10	France	2	0.06 (8.6)	0.05-0.08	0.76 (105.1)	0.49-1.34	1.51 (±0.1)	2	2
26-10	France	2	0.12 (16.2)	0.08-0.16	1 (138.7)	0.62-1.94	1.76 (±0.12)	3	2
107-10	Czechia	3	0.13 (17.3)	0.07-0.2	1.74 (242.1)	0.9-4.83	1.43 (±0.09)	3	4
109-10	Denmark	3	0.06 (7.9)	0.05-0.07	0.59 (81.3)	0.42-0.87	1.62 (±0.11)	2	1
12-10	France	3	0.06 (8.4)	0.03-0.13	0.95 (131.5)	0.36-6	1.38 (±0.09)	2	2
14-10	France	3	0.09 (12.9)	0.04-0.2	1.02 (142.1)	0.41-6.71	1.58 (±0.1)	2	2
5-10	France	3	0.04 (5.3)	0.02-0.07	0.43 (60.1)	0.23-1.8	1.48 (±0.1)	1	1
13-10	France	3	0.10 (14.4)	0.07-0.15	0.97 (134)	0.56-2.2	1.7 (±0.11)	3	2
19-10	France	3	0.05 (6.3)	0.04-0.06	0.45 (62.3)	0.26-0.55	1.82 (±0.13)	1	1

Table 4 *continued*

Strain	Country	P-RC[a]	LC_{50}-µg cm^{-2} (Field rate %)	95 % CL µg cm^{-2}	LC_{95}-µg cm^{-2} (Field rate %)	95 % CL µg cm^{-2}	Slope (±SE)	RR LC_{50}	RR LC_{95}
23-10	Germany	3	0.10 (14.2)	0.05-0.2	1.29 (178.8)	0.54-6.54	1.5 (±0.09)	3	3
38-10	Germany	3	0.08 (10.5)	0.04-0.15	0.68 (93.9)	0.29-3.8	1.73 (±0.12)	2	2
40-10	France	3	0.07 (9.4)	0.05-0.09	0.47 (65.8)	0.3-0.92	1.95 (±0.14)	2	1
46-10	Germany	3	0.12 (16)	0.07-0.18	1.67 (231.3)	0.87-4.48	1.42 (±0.09)	3	4
123-10	Germany	3	0.05 (6.8)	0.04-0.06	0.52 (72)	0.35-0.73	1.8 (±0.14)	1	1
119-10	Sweden	3	0.09 (12.4)	0.06-0.13	0.65 (90.7)	0.38-1.54	1.9 (±0.14)	2	2
118-10	Sweden	3	0.07 (10.3)	0.03-0.16	0.82 (114)	0.32-5.86	1.57 (±0.1)	2	2
120-10	Sweden	3	0.09 (12.7)	0.05-0.16	0.68 (93.7)	0.34-2.3	1.89 (±0.13)	2	2
106-10	Czechia	4	0.14 (19.3)	0.09-0.21	1.29 (179)	0.73-3.12	1.7 (±0.11)	4	3
8-10	France	4	0.05 (7.5)	0.02-0.12	1.77 (245.7)	0.61-12.14	1.09 (±0.07)	1	4
10-10	France	4	0.09 (12.5)	0.06-0.13	1.45 (200.9)	0.85-2.97	1.37 (±0.08)	2	3
16-10	France	4	0.09 (12.7)	0.05-0.16	1.11 (154)	0.52-4.06	1.52 (±0.1)	2	3
18-10	France	4	0.07 (10.1)	0.04-0.12	0.93 (128.9)	0.49-2.47	1.49 (±0.09)	2	2
20-10	France	4	0.10 (13.7)	0.06-0.15	1.06 (147.6)	0.58-2.65	1.59 (±0.01)	3	2
7-10	France	4	0.07 (9.8)	0.04-0.12	0.95 (132.2)	0.46-3.14	1.46 (±0.09)	2	2
9-10	France	4	0.08 (11.1)	0.04-0.15	1.49 (206.8)	0.65-5.91	1.29 (±0.08)	2	3
11-10	France	4	0.05 (6.7)	0.02-0.09	1.11 (154.5)	0.46-4.79	1.21 (±0.07)	1	3
17-10	France	4	0.08 (10.8)	0.04-0.14	0.67 (93.5)	0.33-2.45	1.75 (±0.12)	2	2
21-10	France	4	0.12 (16.7)	0.07-0.2	0.96 (133.3)	0.49-3.02	0.12 (±0.1)	3	2
6-10	Germany	4	0.10 (14.4)	0.06-0.16	1.27 (176.5)	0.69-3.19	1.51 (±0.1)	3	3
32-10	Germany	4	0.18 (24.9)	0.12-0.27	2.22 (308.6)	1.26-5.12	1.55 (±0.1)	5	5
39-10	Germany	4	0.08 (10.9)	0.05-0.12	0.60 (83.7)	0.33-1.69	1.86 (±0.13)	2	1
45-10	Germany	4	0.06 (8.5)	0.03-0.14	0.94 (130.9)	0.36-6.32	1.39 (±0.09)	2	2
50-10	Germany	4	0.2 (27.3)	0.16-0.24	1.35 (186.9)	1.03-1.87	1.97 (±0.14)	5	3

53-10	Germany	4	0.17 (23.7)	0.14-0.21	1.16 (161.2)	0.89-1.61	1.98 (±0.14)	4	3
83-10	Poland	4	0.11 (15.2)	0.06-0.21	0.94 (130)	0.43-4.33	1.77 (±0.12)	3	2
49-10	Germany	4	0.12 (17)	0.06-0.24	1.27 (176)	0.55-6.2	1.62 (±0.1)	3	3
51-10	Germany	4	0.13 (17.6)	0.07-0.22	1.84 (255.8)	0.79-5.32	1.49 (±0.1)	3	4
65-10	G. Britain	4	0.18 (24.3)	0.13-0.23	0.86 (119.7)	0.6-1.43	2.38 (±0.18)	5	2
57-10	G. Britain	4	0.15 (20.8)	0.12-0.19	2.04 (283.4)	1.47-3.05	1.45 (±0.09)	4	5
96-10	Poland	4	0.09 (11.8)	0.07-0.1	0.63 (87.4)	0.47-0.91	1.89 (±0.13)	2	1
98-10	Poland	4	0.09 (12.8)	0.08-0.11	0.52 (72)	0.39-0.74	2.2 (±0.17)	2	1
85-10	Poland	4	0.10 (14.5)	0.05-0.21	0.89 (124.1)	0.39-4.58	1.76 (±0.12)	3	2
97-10	Poland	4	0.11 (15.3)	0.07-0.16	1.28 (178)	0.74-2.86	1.55 (±0.1)	3	3
100-10	Poland	4	0.08 (11.2)	0.03-0.18	1.14 (157.7)	0.43-8.55	1.43 (±0.09)	2	3
68-10	Germany	5	0.07 (9.4)	0.05-0.1	0.66 (91.2)	0.39-1.38	1.67 (±0.12)	2	2
82-10	Germany	5	0.11 (15.2)	0.06-0.21	0.94 (130)	0.43-4.33	1.77 (±0.12)	3	2
Composite 2009			0.07 (9.8)	0.04-0.13	0.96 (133.7)	0.44-3.71	1.45 (±0.09)		
Composite 2010			0.09 (12.3)	0.06-0.12	1.05 (145.6)	0.67-1.91	1.53 (±0.1)		

[a] P-RC = pyrethroid resistance index after IRAC susceptibility method No. 11; [b] RR = resistance ratio based on strain 5-10; [c] Including ≥ 20 % *M. viridescens*, in all others ≤ 5 %

Table 5 *Relationships between field-recommended rates and log-dose probit-mortality data for different insecticides against pyrethroid susceptible pollen beetles (strain 84-09; adult vial test).*

Compound	100 % Field rate (g AI ha^{-1})	LC_{50} – g AI ha^{-1} (Field rate %)	95 % CL[a]	Quotient 100% Field rate /LC_{50}	LC_{95} – g AI ha^{-1} (Field rate %)	95 % CL	Quotient 100% Field rate / LC_{95}
Thiacloprid	72	4.9 (6.8)	2.98-9.03	15	73.1 (101.5)	34.1-191.1	0.98
lambda-cyhalothrin	7.5	0.08 (1.1)	0.06-0.12	94	0.41 (5.5)	0.26-0.92	18
Chlorpyrifos-methyl[b]	337.5	0.41 (0.1)	0.18-0.92	823	6.26 (1.9)	2.23-58.78	54

[a] 95% Confidence limits; [b] Data obtained by conducting an adult vial bioassay according to IRAC method No.11

2.5 Discussion

2.5.1 Pyrethroid resistance monitoring

Our study confirmed earlier data of widespread pyrethroid resistance in pollen beetles collected in winter oilseed rape in many European countries in 2009 and 2010. The data collected are based on an IRAC recommended adult vial bioassay employing *lambda*-cyhalothrin as a reference pyrethroid [22]. The vials are usually coated with *lambda*-cyhalothrin and either stored in the laboratory until use, or shipped to other laboratories or field stations, and as our studies have shown they are stable for at least 8 weeks when stored at 4°C in the dark, suggesting a single production cycle before the season is sufficient. The use of a standard pyrethroid as suggested by IRAC [22] was shown to be justified since pollen beetle populations collected all-over Europe (n = 42) were shown to be cross-resistant to other pyrethroids such as deltamethrin, alpha-cypermethrin, bifenthrin and etofenprox in an earlier study [3]. However the extent of cross-resistance seems to differ between pyrethroids, with a tendency of some compounds being less, but still significantly affected, e.g. bifenthrin [3]. The high resistance factors reported resulted from a reference population taken from Ukraine (strain 70-10, Table 3), and displaying the highest susceptibility to pyrethroids detected in 2009 and 2010. The population turned out to be 5-8 fold more susceptible than those usually assigned to IRAC pyrethroid susceptibility class 1, therefore we subjected a number of individuals to a more detailed morphometric species determination based on leg morphology. The population indeed consisted to 100 % of *M. aeneus* and additionally it shows no higher susceptibility to thiacloprid when compared to the other populations (Table 4). The majority of the tested populations, i.e. 70 % turned out to be moderately to highly resistant to pyrethroids according to the IRAC rating scheme. Close to 90 % of the randomly collected German populations (n = 17) exhibited pyrethroid resistance, and similar values have been reported earlier [3]. Reliable control of pollen beetle by pyrethroids is only possible in those regions where resistance monitoring data before application confirm susceptibility, i.e. LC_{95}-values not greater than approx. 10 % of the recommended field rate as shown in our investigations (Table 3).

Apart from the lack of any resistance management considerations in oilseed rape, there are three main reasons among others which most likely contribute to the rapid spread of pollen beetle pyrethroid resistance in many European countries: (1) The dramatic expansion of winter oilseed rape cultivation in many countries, e.g. in Germany the cultivated winter oilseed rape acreage was doubled within the last 15 years and thus providing unlimited breeding sites and food sources for pollen beetles [24]. (2) Low treatment thresholds result in an increased number of applications in some countries (the compensation ability of winter

oilseed rape is often neglected), e.g. the threshold in Poland is reported to be 1-3 beetles per plant (BBCH 50-52) compared to 15 beetles per plant in the UK [4]; (3) A politically and environmentally driven ban of older classes of insecticides addressing other biochemical modes of action such as organophosphates, without having available appropriate alternatives other than pyrethroids [3,6,17].

The evolution of insecticide resistance in pollen beetles is likely to be a rather old story considering reports which date back to 1921, and claim disappointing performance of chemicals in pollen beetle control in Germany [25]. These early reports triggered the re-invention of other methods to control pollen beetle mainly based on mechanical trapping devices [26]. However such methods also provided no means of control when high infestation levels were monitored, thus resulting in repeated spraying and dusting of natural insecticides such as nicotine, rotenone and also pyrethrum, the forerunner of the synthetic pyrethroids [27]. Later on DDT and organophosphates were introduced and provided good control of pollen beetles [28]. DDT resistance development was first reported in Polish pollen beetle populations in 1967, and confirmed in trials in 1969 [28]. DDT as well as pyrethroids acts on voltage-gated sodium channels and it would have been interesting to know whether it selected for kdr (knock-down resistance, a well-known target site mutation) [30] in pollen beetles these days, since resistance ratios reported were quite high, i.e. exceeding factors of 1000-fold [29].

2.5.2 Thiacloprid method validation and baseline-susceptibility

With the introduction of the neonicotinoid insecticide thiacloprid in 2006 the first new mode of action since decades was introduced for pollen beetle control in Germany. In the first year of its introduction thiacloprid just received an emergency registration in Germany and the use was limited to 100,000 ha, but since 2007 it has a full registration also in other European countries. The compound is well known and considered to be safe to honey bees, so it can be applied during flowering [31-32].

The adult vial test we developed is based on glass vials coated with the commercial oil-dispersion formulation of thiacloprid, Biscaya® OD240. The bioassay system required considerable fine-tuning and lots of experiments until validation due to the fact an OD formulation is used for coating. The highest possible rate which could be applied to the inner glass vial surface equals 200 % of the field recommended rate (1.44 µg cm^{-2}). Higher concentrations resulted in physical trapping of beetles on the oily surface and unpractically long evaporation times. The shortest estimated evaporation time producing reliable and repeatable results is at least 4 hours (incl. 2 hours rotation time). Shorter intervals result in high beetle mortality as a consequence of combination of physical and biological action, even at rather low doses definitely representing unrealisitic exposure scenarios. The mean

thiacloprid concentration resulting in 50 % pollen beetle mortality is around 0.080 µg cm^{-2} when combining composite data of 2009 and 2010 (Table 4). This is in strong contrast to very low LC_{50}-values of approx. 0.0001 µg cm^{-2} recently published for thiacloprid using a similar methodology [33]. Such a low value is difficult to explain considering thiacloprids much lower intrinsic toxicity compared to a pyrethroid insecticide (c. 60-fold), therefore we think its likely that evaporation times were too short (no checks with blank formulation were included), although the method itself is considered very practical and similar to ours. Furthermore the analytical results presented here suggest a good stability of the active ingredient in coated glass-vials, so their production in advance of a resistance monitoring campaign is not being considered problematic.

The very homogenous efficacy results we obtained with thiacloprid against pollen beetle populations collected in 2009 and 2010 revealed no shifting yet, and importantly no cross-resistance to pyrethroids. However, possibly not surprising considering the fact that so far in none of the neonicotinoid-targeted agricultural pest insects has cross-resistance to pyrethroids been described [31]. Neonicotinoid insecticides such as thiacloprid and acetamiprid are intrinsically less active than *lambda*-cyhalothrin and chlorpyrifos-methyl, which is reflected by the fact that recommended rates are not consistently providing a 100 % mortality of pollen beetles. The variation in response to thiacloprid we detected in pollen beetles is less than 5-fold, and comparable to baseline studies conducted on other invertebrate pest species such as aphids and whiteflies [31,32]. Resistance monitoring studies with acetamiprid in 2004 in three pollen beetle populations collected in Poland suggested low resistance to neonicotinoid insecticides, and were based on the fact that mortality figures at recommended field rates were below 100 % [34]. With reference to the lower intrinsic activity mentioned above and the lack of any presented baseline data, it is rather unlikely that the investigated Polish populations indeed show resistance. This is confirmed by another study by the same authors showing only slight variation in LC_{50}-values with mostly overlapping 95 % fiducial limits for acetamiprid in pollen beetle populations, again collected in Poland, but in 2005-2007 [11]. However we definitely support the view of WEGOREK *et al.* [11] to continue to monitor for the development of resistance against different classes of insecticides in pollen beetles, particularly neonicotinoids. Neonicotinoids such as thiacloprid and acetamiprid show a much lower intrinsic activity and are used at relatively low rates when compared to the organophosphate chlorpyrifos-methyl (Table 5). Once resistance occurs these facts are likely to affect their field performance much faster than pyrethroids or organophosphates, i.e. based on LC_{95}-values even resistance ratios as low as 5- to 10-fold are suggested to convert into neonicotinoid field failure against pollen beetle, whereas such low resistance ratios would never affect the efficacy of *lambda*-cyhalothrin at recommended rates. Considering the data

presented in this study we presume that a 10-fold and 50-fold resistance to a pyrethroid such as *lambda*-cyhalothrin and an organophosphate such as chlorpyrifos-methyl, respectively is unlikely to be of practical significance at recommended rates under field conditions (Table 5). Therefore it is strongly suggested to use thiacloprid in alternation with other pollen beetle insecticides of different modes of action in order to sustain its efficacy as a valuable tool in resistance management strategies. The fragile use-rate/efficacy relationship suggests to rather annually than bi-annually monitoring for neonicotinoid susceptibility in pollen beetle populations, particularly in those regions where this class of chemistry is heavily used due to the lack of appropriate alternative chemical means.

2.6 Conclusion

Pyrethroid resistance in *Meligethes* ssp. is at moderate to high levels in many European countries, therefore resistance management strategies based on mode-of-action rotation need to be implemented and have already been recommended [19-21,35]. In order to implement such a strategy several chemical options not affected by resistance or representing new modes of action are necessary. Taken together, and summarizing all results presented, it can be concluded that thiacloprid among other new chemical classes of insecticides is a valuable option for future pollen beetle control without any signs of resistance yet detected. Thiacloprid belongs to the chemical class of neonicotinoid insecticides, which are only systematically used for pollen beetle control since a few years. Neonicotinoid insecticides such as thiacloprid form an essential part in resistance management strategies [21,35]. Therefore their performance should be carefully monitored in the future in order to detect early shifts in pollen beetle susceptibility. For this purpose the adult vial bioassay based on a thiacloprid OD240 formulation (Biscaya®) was developed and validated using pollen beetle populations collected in several European countries in 2009 and 2010. The variation in response to thiacloprid of all populations tested is less than 5-fold, and not related to pyrethroid resistance, suggesting full thiacloprid baseline susceptibility of all populations tested by the proposed monitoring method. The method was also considered by IRAC for inclusion in their methods list [36]. For future neonicotinoid resistance monitoring initiatives with thiacloprid we are suggesting to employ 200 % (1.44 µg cm^{-2}), 100 % (0.72 µg cm^{-2}) and 20 % (0.144 µg cm^{-2}) of the field-recommended rate as diagnostic doses providing a mean mortality of 98.5 ± 2.9 %, 95.7 ± 7.2 % and 54.8 ± 12 %, respectively. It has been taken into account that thiacloprid is intrinsically less active than pyrethroids and organophosphates, so the mean mortalities mentioned above indeed represent the real baseline activity, even though they are not consistently providing 100 % mortality.

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Chapter 3

Cytochrome P450 mediated pyrethroid resistance in European populations of *Meligethes aeneus* (Coleoptera: Nitidulidae)

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Abstract

Pollen beetle, *Meligethes aeneus* (Coleoptera: Nitidulidae) is a major pest on several million hectares in European winter oilseed rape cultivation. Synthetic pyrethroids have been successfully used for many years to keep them under economic damage thresholds. Recently wide-spread resistance development to pyrethroids in pollen beetle populations was described in many European countries, including Germany, France, Poland, Denmark and others. Resistance monitoring is conducted by incubating beetles for 24 h in glass vials coated with different concentrations of *lambda*-cyhalothrin. Using such an assay format we were able to show cross-resistance to other pyrethroids, such as deltamethrin, cypermethrin, and to a somewhat lower extent bifenthrin, etofenprox and *tau*-fluvalinate. Here we also investigated in more detail in 27 different populations the biochemical mechanism of pyrethroid resistance. Synergism experiments revealed a high synergistic potential for piperonyl butoxide *in vivo*, whereas other compounds such as S,S,S-tributylphosphorotrithioate and diethylmaleimide failed to suppress pyrethroid resistance. Incubating microsomal fractions of pollen beetle with deltamethrin and subsequent LC-MS/MS analysis revealed 4-OH-deltamethrin as the major metabolite. Metabolite formation *in vitro* and pyrethroid resistance *in vivo* is correlated and inhibition trials with piperonyl butoxide, tebuconazole and aminobenzotriazole suggest the

involvement of cytochrome P450s. Furthermore we were able to show cross-resistance to *tau*-fluvalinate which is supported by the competitive inhibition of 4-OH-deltamethrin formation by increasing concentrations of *tau*-fluvalinate in microsomal hydroxylation assays. Although we provided clear experimental evidence for an oxidative mechanism of resistance in numerous populations, other mechanisms might be involved based on the data discussed.

3.1 Introduction

The pollen beetle, *Meligethes aeneus* F. (Coleoptera: Nitidulidae) is one of the major pests in European oilseed rape and known to be quite destructive once infestation thresholds are exceeded and no chemical control measures are taken [1]. In the past, the control of pollen beetles in Europe was mainly based on pyrethroid insecticides [2]. As a consequence of the limited availability of insecticides of other chemical classes with different modes of action, the selection pressure on pyrethroid insecticides was enormous [2-5].

Pyrethroid resistance in pollen beetles was first reported in 1999 in the Champagne region in North-Eastern France [4]. Based on the evidence of reduced pyrethroid susceptibility in pollen beetles in France, resistance monitoring activities were initiated in many other European countries. Resistance to pyrethroids was discovered in 2000 in Switzerland [6] and in Sweden [7], in 2001 and 2003 in Denmark [3,8], in 2002 in Germany [2,4,5], in 2003 in Finland [9] and in 2006 in Poland as well [10]. A few countries in Europe seemed to be less affected by pyrethroid resistance development until recently, however in 2007 the first resistant populations were also discovered in the United Kingdom and Austria [11]. A pollen beetle pyrethroid resistance monitoring carried out by the Insecticide Resistance Action Committee (IRAC) confirmed that pyrethroid resistance is widespread in Europe, particularly in France, Germany and Poland [12]. The first coordinated resistance monitoring project was organized by IRAC and carried out in 2007. Based on the monitoring results of 2007, and those obtained in subsequent years the IRAC Pollen Beetle Working Group concluded an ongoing shifting to high pyrethroid resistance in central Europe and a spread of resistance to Eastern Europe [12].

In 2007 thiacloprid, belonging to the chemical class of neonicotinoid insecticides, and known to target insect nicotinic acetylcholine receptors [13], was the first fully registered chemical tool with a different mode of action for pollen beetle control in Germany, subsequently registered in other European contries [14]. Since its introduction also other modes of action were investigated for their potential to control pollen beetles, in order to increase chemical diversity for resistance management purposes [15-17]. Resistance to pyrethroids in pollen beetle affects the whole chemical class of pyrethroids and is not limited to individual compounds [2], however some pyrethroids such as *tau*-fluvalinate and etofenprox

seem to be less affected by cross-resistance and are still used in many countries. Cross-resistance to *tau*-fluvalinate in pollen beetles is documented at a moderate level for Denmark [8] and Sweden [18], and HEIMBACH *et al*. [19] showed a decrease in susceptibility of pollen beetles in Germany to etofenprox over a period of 5 years.

It must be assumed that the resistance mechanism is mainly based on an oxidative degradation of pyrethroids by cytochrome P450-dependent monooxygenases (P450), because a highly synergistic effect of the potent monooxygenase inhibitor piperonyl butoxide on several populations/locations in Europe has recently been described [10,16,20,21]. Apart from metabolic resistance also target-site resistance (*kdr*) in the voltage-gated sodium channel was discovered in 2008 in some populations sampled in Denmark, but not in populations collected in central Europe at this time [22].

The aim of this study was to investigate more detailed the biochemical mechanism of pyrethroid resistance in pollen beetle populations collected in several European countries.

3.2 Material and methods

3.2.1 Insects

In April/May 2006 and 2010 pollen beetle populations were collected in winter oilseed rape fields from different European countries, including the most important oilseed rape producing countries. In 2006 beetles were sampled in Germany, France, Belgium, Austria, Great Britain and Poland; 2010 in Germany, France, Great Britain, Poland, Czechia, Sweden, Finland and Ukraine. The adult insects were packed in plastic bags with some rape buds and foliage and shipped to Bayer CropScience in Monheim, Germany. After arrival in the laboratory, beetles were stored for 24 h at 4 °C. Two hours before bioassay the insects were removed from the refrigerator and equilibrated to room temperature (20 ± 2° C). Those beetles of lower fitness remain on the bottom of the bag and were not used for the bioassays.

3.2.2 Insecticides and synergists

The insecticides and synergists used were of technical grade. *Lambda*-cyhalothrin, deltamethrin, cypermethrin, bifenthrin, etofenprox and *tau*-fluvalinate were obtained from Fluka Chemicals (Buchs, Switzerland). Piperonyl butoxide (PBO) and diethyl maleate (DEM) were purchased from Arcos organics (Geel, Belgium), (DEF) was obtained from Chem Service (West Chester, PA, USA).

3.2.3 Bioassays

All bioassays were conducted according to instructions outlined in IRAC´s Susceptibility Method No. 11 “Pollen Beetle Susceptibility Monitoring Bioassay – Synthetic Pyrethroids” [23]. In brief: The method is based on glass vials (Zinsser Analytics, Germany) coated with defined concentrations of *lambda*-cyhalothrin. Other compunds of the pyrethroid class of chemistry were used similarly. Beetles confined to glass vials were assessed for mortality after 24h. The IRAC method was slightly modified, i.e. the assessment was done by directly scoring affected beetles in the vials rather than using the recommended filter disc assessment arena. Pyrethroid concentrations used were equivalent to 20 % and 100 % of the typical field application rate, i.e. *lambda*-cyhalothrin and deltamethrin both at 75 and 15 ng AI cm^{-2} inner glass surface, bifenthrin at 100 and 20 ng cm^{-2}, etofenprox at 575 and 115 ng cm^{-2} , and *tau*-fluvalinate at 480 and 96 ng cm^{-2}.

For testing purposes ten pollen beetles were placed in each vial, using three replicates per concentration and population (plus an acetone control). Capped vials are then stored in up-right position at 20 ± 2°C for 24 h. Prior assessment vials are briefly shaken to differentiate alive and affected beetles more easily. Populations are considered resistant to pyrethroids if 100 % of the field-rate is not resulting in 100 % affected beetles [12]. An efficacy index can be calculated by adding the mortality figures obtained at both concentrations tested.

3.2.4 Synergist Studies

Three synergists were used to detect possible metabolic resistance mechanisms *in vivo*: the cytochrome P450-dependent monooxygenase-inhibitor PBO, the esterase-inhibitor DEF, and the glutathione depleter DEM. Several concentrations of each synergist were tested against some strains to be sure that the concentration chosen was below toxic levels.

The synergists were dissolved in acetone (PBO and DEM 4 g L^{-1}, DEF 1 g L^{-1}) and 500 µL were transferred to a vial and coated while horizontally rotating. Ten beetles were incubated in synergist-coated vials 1 h prior to transferring them into *lambda*-cyhalothrin coated vials. The concentration of *lambda*-cyhalothrin used to detect synergistic effects was 3 ng AI cm^{-2} inner glass surface except for the highly susceptible population obtained from Ukraine (strain 70-10) [14], a concentration of 0.024 ng cm^{-2} was applied.

3.2.5 Deltamethrin metabolism

Deltamethrin metabolism was assayed by incubation of native microsome preparations from 27 pollen beetle populations including highly resistant and susceptible strains. For this purpose 300 – 400 adult pollen beetles were homogenized in ice-cold sodium phosphate buffer (0.1 M, pH 7.6, 1 mM EDTA, 1 mM DTT, and 200 mM sucrose). The homogenate was

centrifuged at 5,000 g for 5 min and 4 °C. The supernatant was transferred to another centrifugation vial and the remaining pellet was resuspended and centrifuged again. The combined supernatants were centrifuged at 15,000 g for 15 min and 4 °C. The resulting supernatant was then centrifuged at 100,000 g for 1 h at 4 °C. The last centrifugation step was repeated once again and the microsomal pellet resuspended in sodium phosphate buffer (0.1 M, pH 7.6, and 5 % glycerol). The protein content was determined according to Bradford [24]. The enzyme source was diluted to a protein content of 400 µg mL^{-1} and stored at -80 °C until use. The microsomes were incubated with deltamethrin in 0.1 M sodium phosphate buffer pH 7.6 containing a NADPH-regenerating system (Promega, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, and 0.4 U mL^{-1} glucose-6-phosphate dehydrogenase) for 1 h at 22 °C. Microsomes without NADPH served as a control. The total assay volume of 200 µL contained 30 µg microsomal protein and the reaction was stopped by the addition of 800 µL acetonitrile. After centrifugation at 3,000 g for 10 min, 250 µL of the supernatant were analyzed by HPLC-MS/MS for quantification of 4-OH-deltamethrin (m/z 539.2) as described in ZHU *et al.* [25]. Recovery rates of deltamethrin from microsomal incubations without NADPH were usually close to 100 %. Other hydroxylated deltamethrin metabolites than 4-OH-deltamethrin were not detected.

3.2.6 Inhibition of deltamethrin metabolism

Inhibition experiments were conducted with well known inhibitors of microsomal monooxygenases, i.e. PBO, 1-amonibenzotriazole (ABT) and tebuconazole (TCZ) [26-28]. PBO, ABT and TCZ were used in equimolar concentrations of deltamethrin (10 µM) and incubated and analysed as described above (2.5). Furthermore the effect on the velocity of deltamethrin hydroxylation of different concentrations of two pyrethroids, *lambda*-cyhalothrin and *tau*-fluvalinate was measured in microsomal preparations by Michaelis-Menten kinetics. Results were expressed by double-reciprocal plots in order to investigate the inhibition mode.

3.2.7 7-ethoxycoumarin O-deethylation assay

The 7-ethoxycoumarin fluorescence assay was conducted in 96 well plates as described in STUMPF & NAUEN [46]. The enzyme source was purified as described above and incubated with 50 µM 7-ethoxycoumarin in 0.1 M sodium phosphate buffer pH 7.6 containing 1 mM NADPH for 30 min at 30 °C while shaking (400 rpm) in a thermomixer (Eppendorf). The total assay volume of 200 µL contained 20 µg of microsomal protein. The reaction was stopped, and the excessive NADPH was degraded to the non-fluorescent $NADP^+$ by the addition of 100 µL oxidation/stopp-solution containing 5mM oxidized L-glutathione, 160 U glutathione reductase in DMSO:TRIZMA-base buffer 50 % vol:vol, 0.05 M, pH 10. The

microplate was then incubated for 15 min at 20-22 °C before the fluorescence was measured on a SpectraMax M2 (Molecular Devices) (λex = 390 nm, λem = 465 nm).

3.2.8 Data analysis

All mortality figures were corrected for control mortality by using ABBOTT´S formula [29]. Results of bioassays were compared using the efficacy index. The efficacy index is the sum of the ABBOTT corrected values of the assessment of both concentrations tested. Analyses of enzyme kinetics (nonlinear fitting and Lineweaver-Burk transformation) and further statistical analyses were performed with Graphpad Prism 5 software (GraphPad Software Inc., California).

3.3 Results

3.3.1 Cross-resistance of pyrethroid insecticides

The data obtained from bioassays using 6 different pyrethroid insecticides (Fig. 25) showed a significant cross-resistance between the reference compound *lambda*-cyhalothrin and all other compounds used (Table 6). The correlation coefficient indicated a strong cross-resistance between *lambda*-cyhalothrin and deltamethrin ($r > 0.8$), as well as cypermethrin ($r > 0.8$). A moderate level of cross-resistance is seen between *lambda*-cyhalothrin and etofenprox ($r = 0.52$), *tau*-fluvalinate ($r = 0.51$) and bifenthrin ($r = 0.66$). All strains ($n = 27$) tested in 2010 were used to study the biochemical mechanisms of pyrethroid resistance.

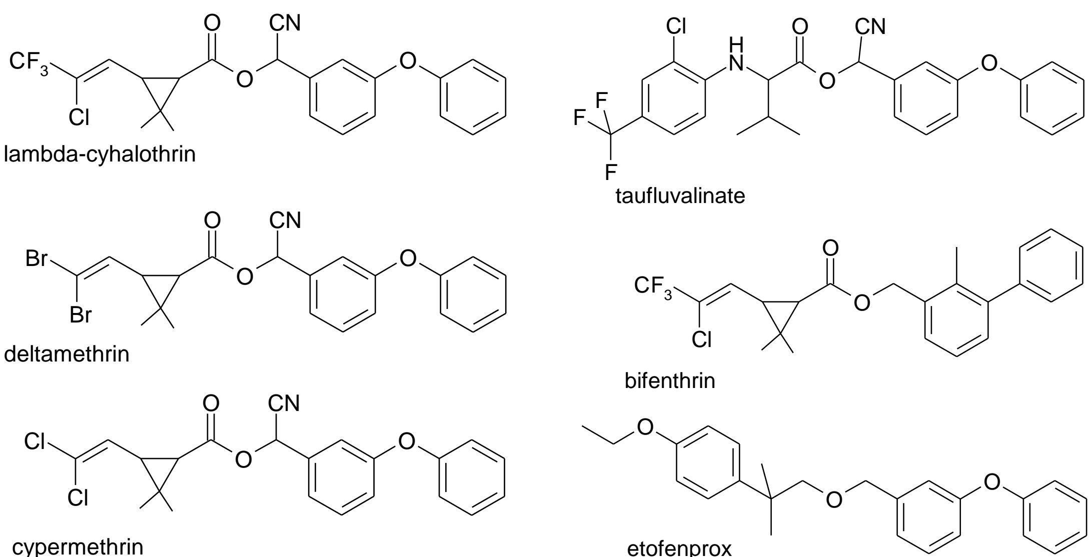

Fig. 25 *Chemical structures of pyrethroids used in bioassays.*

Table 6 *Analysis of cross-resistance between* lambda-*cyhalothrin vs. deltamethrin, cypermethrin, etofenprox, and bifenthrin (data obtained from bioassays conducted*

in 2006), and lambda-*cyhalothrin vs.* tau-*fluvalinate (data obtained from bioassays conducted in 2010).*

	2006				2010
1	**Deltamethrin**	**Cypermethrin**	**Etofenprox**	**Bifenthrin**	**τ-Fluvalinate**
Number of XY Pairs	42	42	42	42	27
Pearson r	0.842	0.836	0.519	0.658	0.509
95 % confidence interval	0.722 - 0.912	0.713 - 0.909	0.255 - 0.711	0.442 - 0.805	0.159 - 0.745
P value (two-tailed)	< 0.0001	< 0.0001	0.0004	< 0.0001	0.0068
P value summary	***	***	***	***	**
Is the correlation significant?	Yes	Yes	Yes	Yes	Yes
R squared	0.710	0.700	0.270	0.433	0.259

3.3.2 Synergism bioassays

Three common synergists were chosen to investigate possible metabolic resistance towards *lambda*-cyhalothrin in 27 pollen beetle populations. The pre-treatment of pollen beetles with DEF or DEM did not influence the efficacy of *lambda*-cyhalothrin, whereas PBO reduced the pyrethroid-resistance dramatically in resistant strains, but showed no synergistic effect in the highly susceptible strain 70-10 collected in Ukraine (Fig. 26). These results suggest the primary involvement of monooxygenases in pyrethroid resistance in pollen beetles.

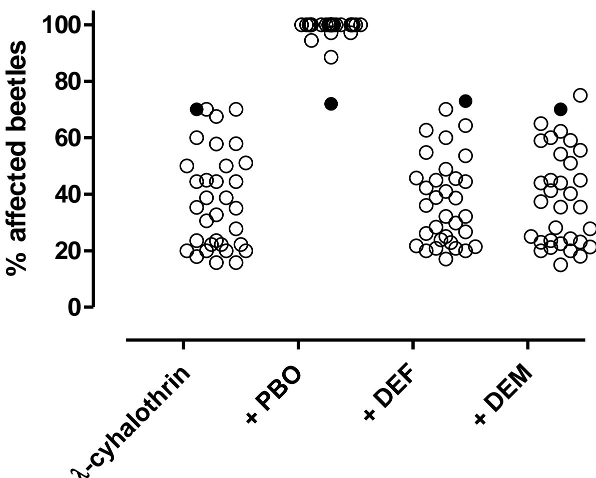

Fig. 26 *Synergistic effect of PBO, DEF and DEM on several* M. aeneus *populations expressing different levels of pyrethroid-resistance (open circles) and a highly susceptible population from Ukraine (strain 70-10; closed circles) tested with* lambda-*cyhalothrin concentrations of 3 ng cm^{-2} and 0.03 ng cm^{-2}, respectively.*

3.3.3 Deltamethrin metabolism

Experiments were conducted to show the potential of microsomal preparations of resistant pollen beetles to metabolise deltamethrin. Pilot tests on 5 pollen beelte populations including highly resistant and susceptible strains indicated an increased formation of 4-OH-deltamethrin in resistant populations as shown by HPLC-MS/MS analysis. The mass spectrum clearly revealed the formation of a metabolite matching the m/z value of 539.2 for hydroxylated deltamethrin (Fig. 27). The metabolite shows a much lower activity against pollen beetles in an adult vial test (LC_{50} > 500 % of field rate of deltamethrin) (Fig. 28). The highest amount of metabolite was formed by strain 80-10 (3.6 ng h^{-1}, V_{max}^{app} 17.84 nmol min^{-1} mg^{-1} protein) whereas strain 70-10 from Ukraine showed the lowest rate of metabolite formation (Table 7). The velocity of metabolite formation is strongly correlated to pyrethroid resistance. As shown in Fig. 29 efficacy indices of both *lambda*-cyhalothrin and *tau*-fluvalinate are significantly correlated with the rate of deltamethrin-hydroxylation.

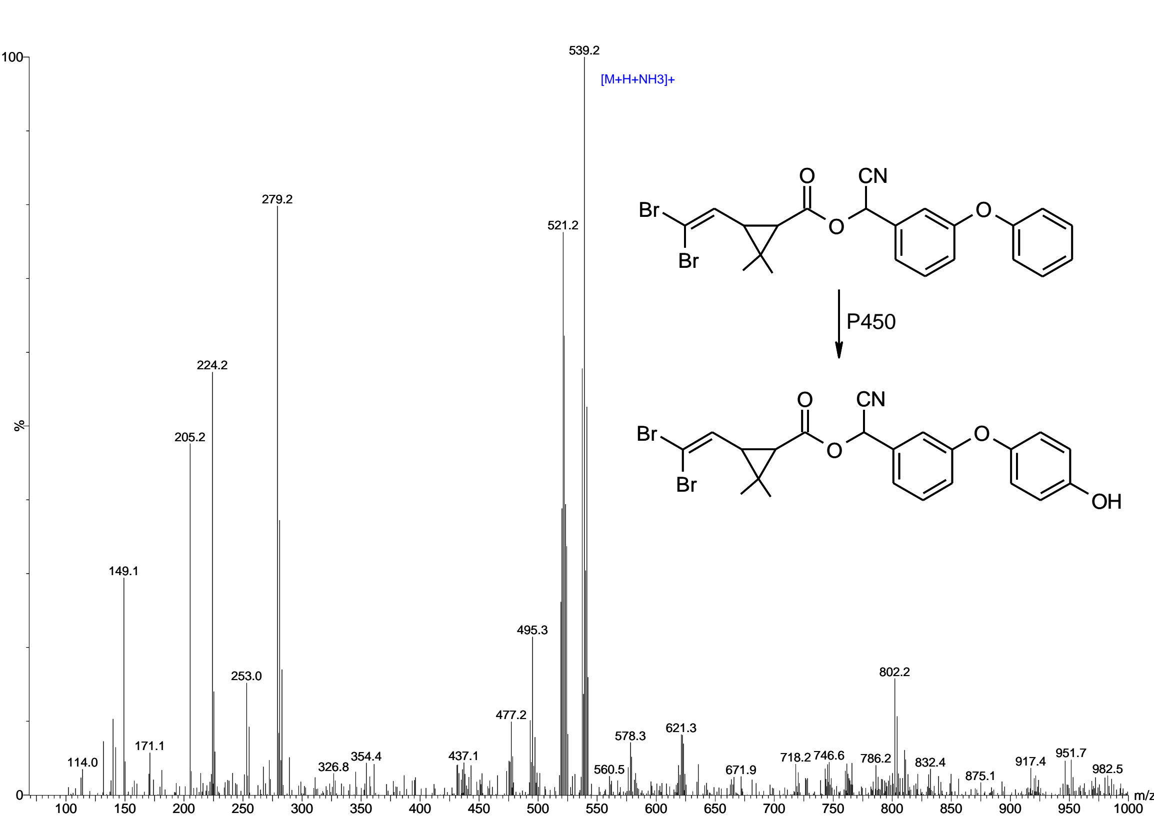

Fig. 27 *Mass spectrum of 4-OH-deltamethrin. No other metabolites were detected in microsomal fractions of pollen beetle incubated with deltamethrin.*

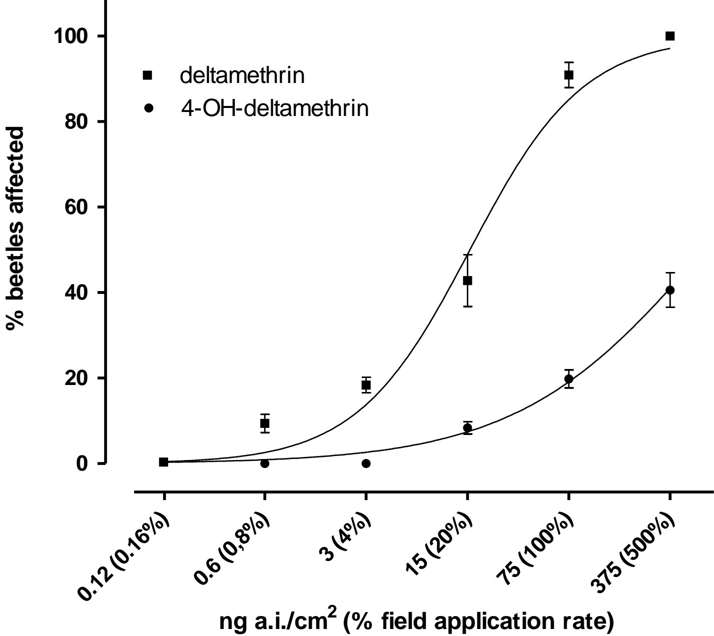

Fig. 28 *Mean response of three resistant pollen beetle populations collected in 2010 to different concentrations of deltamethrin and its metabolite 4-OH-deltamethrin in adult vial tests.*

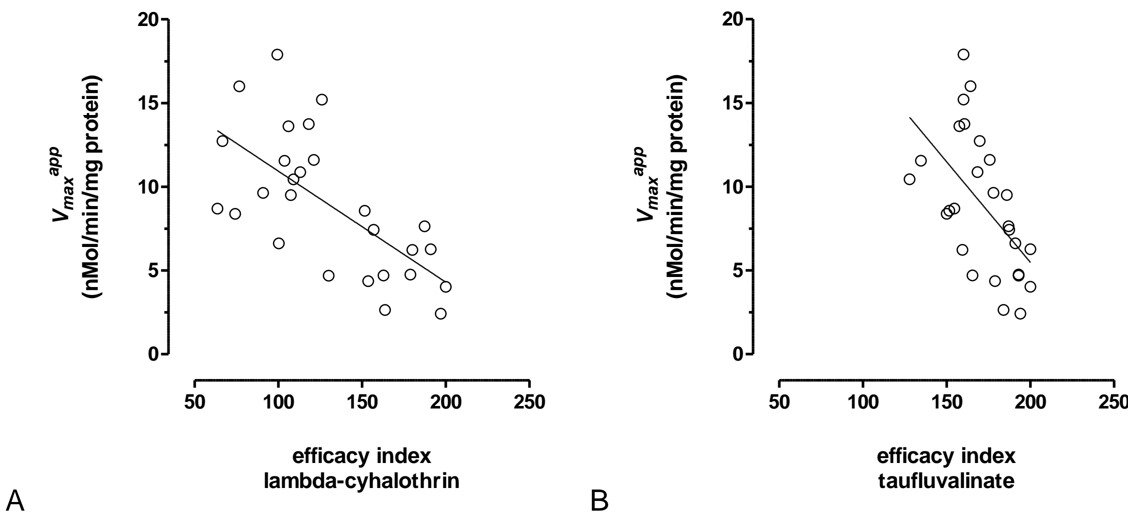

Fig. 29 *Pearson correlation of maximal apparent velocity (*V_{max}*) of deltamethrin hydroxylation in microsomal fractions* in vitro *vs. pyrethroid efficacy indices obtained from adult vial tests* in vivo *of (A)* lambda*-cyhalothrin and (B)* tau*-fluvalinate.*

3.3.4 Inhibitors of deltamethrin metabolism

The influence on deltamethrin metabolism of known cytochrome P450 inhibitors, i.e. PBO, ABT and TCZ was investigated in microsomal fractions. Heat inactiviation of microsomes resulted in complete inhibition of deltamethrin metabolism, indicating the lack of any non-enzymatic reactions triggering hydroxylation. PBO and TCZ turned out very efficient in inhibiting the microsomal oxidation of deltamethrin since metabolite formation decreased by more than 80 % (Fig. 30). In contrast the incubation of microsomes with ABT resulted in a rather weak inhibition of deltamethrin oxidation.

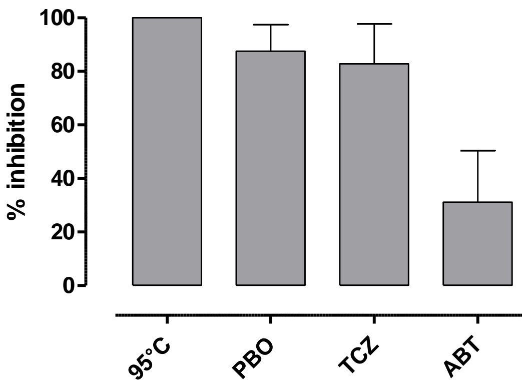

Fig. 30 *Inhibition of 4-OH-deltamethrin-formation by heat treatment of microsomes (5 min for 95° C) and by equimolar concentrations of different monooxygenase inhibitors (PBO, piperonyl butoxide; TCZ, tebuconazole; ABT, aminobenzotriazole (10µM each)). Data are mean values ± SD (n=5).*

In a second set of experiments the influence on deltamethrin hydroxylation of other pyrethroids was analysed using a classical Michaelis-Menten kinetics approach. For this purpose different concentrations of *lambda*-cyhalothrin and *tau*-fluvalinate were chosen and revealed a competitive inhibition pattern shown by an unchanged V_{max}-value for 4-OH-deltamethrin formation when graphically analyzed by double-reciprocal Lineweaver-Burk plots (Fig. 31 and Fig. 32). In the presence of 100 µM *lambda*-cyhalothrin the apparent K_m values in microsomal preparations of both strains 57-10 and 96-10 changed considerably (Fig. 32). A similar trend was seen when co-incubating deltamethrin with microsomes in the presence of different concentrations of *tau*-fluvalinate (Fig. 31).

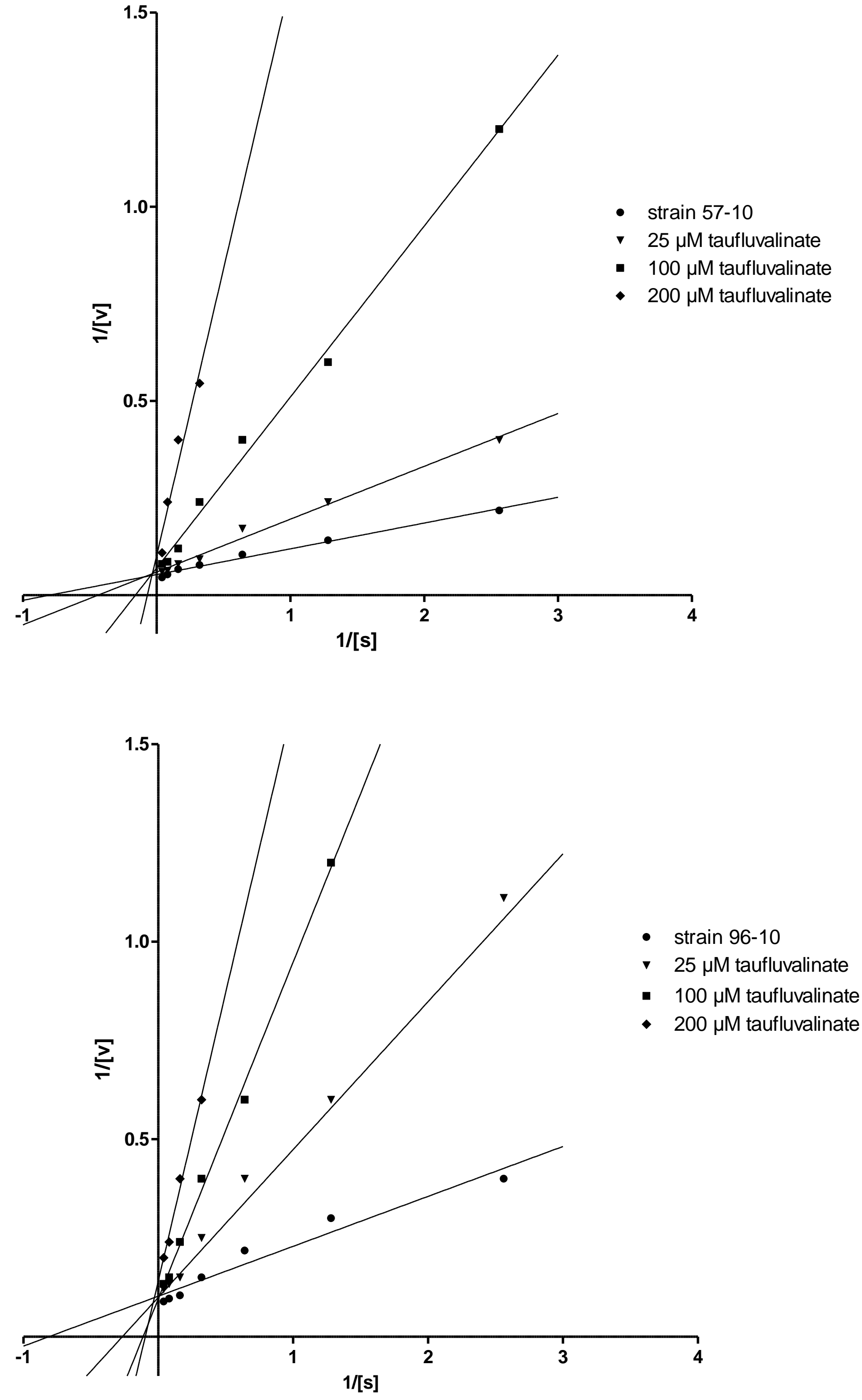

Fig. 31 *Lineweaver-Burk plots showing competitive inhibition of deltamethrin hydroxylation by different concentrations of* tau-*fluvalinate in microsomal preparations of two pyrethroid resistant pollen beetle strains. [s] = deltamethrin.*

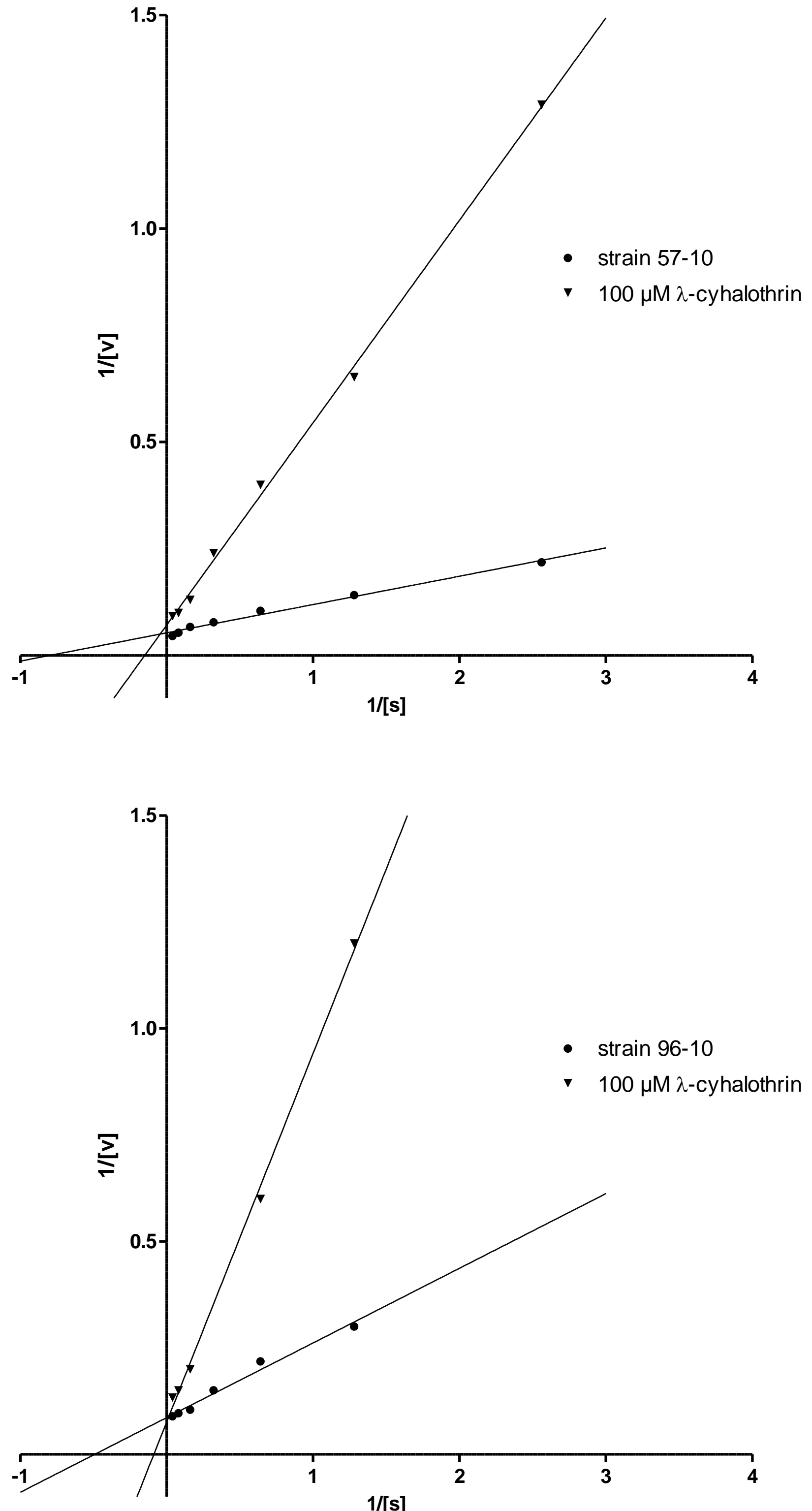

Fig. 32 *Lineweaver-Burk plots showing competitive inhibition of deltamethrin hydroxylation by* lambda-*cyhalothrin in microsomal preparations of two pyrethroid resistant pollen beetle strains. [s] = deltamethrin.*

3.3.5 Artificial substrate testing

When incubating microsomal preparations of all pollen beetle strains tested in the vial assay as well as in the microsomal deltamethrin hydroxylation assay with the well-known artificial model substrate 7-ethoxycoumarin, we were not able to find any correlation with pyrethroid resistance levels (Fig. 33). Hence such an assay cannot displace the more laborious microsomal hydroxylation asssay coupled with subsequent LC-MS/MS analysis.

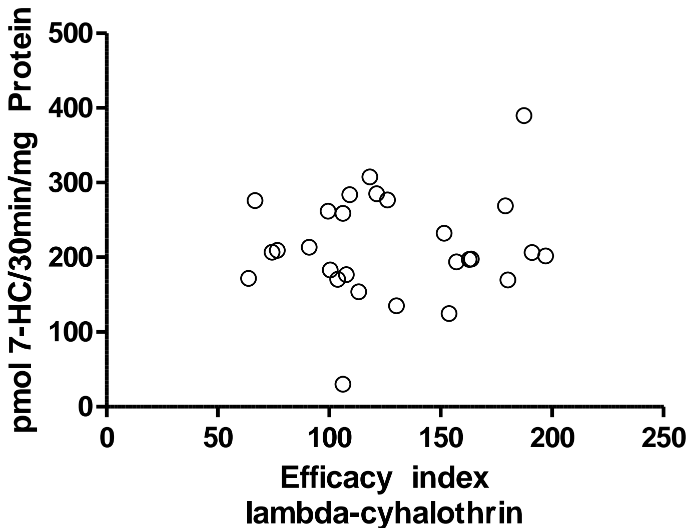

Fig. 33 *Pyrethroid resistance and 7-ethoxycoumarin O-deethylase activity in pollen beetles is not correlated. Each dot represents a single population.*

Table 7 *Efficacy indices of* lambda-*cyhalothrin (EI LCT) and* tau-*fluvalinate (EI TFV) as well as kinetic properties of microsomes with regard to 4-OH-deltamethrin formation of different European pollen beetle populations sampled in 2010.*

Strain	Country	EI LCT	EI TFV	ng 4-OH-deltamethrin formed / h	V_{max}^{app} (nmol $min^{-1} mg^{-1}$ protein)	95% CL	R^2
70-10*	Ukraine	200	200	0.25	-	-	0.814
127-10	Finland	196.97	193.94	0.45	2.19	1.62 - 3.4	0.951
39-10	Germany	163.64	183.94	0.6	2.39	1.98 - 3	0.971
38-10	Germany	162.73	165.45	0.65	4.74	3.99 - 6.02	0.969
107-10	Czechia	178.89	192.96	0.8	4.88	3.42 - 8.47	0.99
120-10	Sweden	130	192.96	0.8	3.97	2.24 - 16.95	0.958
9-10	France	153.64	178.79	0.85	4.05	3.59 - 4.63	0.999
13-10	France	180	159.39	1.25	7.69	5.52 - 12.66	0.995
25-10	France	190.91	200	1.25	6.13	4 - 12.99	0.995
6-10	Germany	156.97	187.27	1.3	7.3	5.97 - 9.43	0.997
8-10	France	100.3	190.91	1.4	6.21	4.55 - 9.62	0.973
3-10	France	187.27	186.97	1.5	7.33	6.52 - 8.5	0.971
66-10	Germany	74.24	150	1.6	8.13	6.56 - 10.75	0.988
82-10	Germany	63.64	154.55	1.75	7.94	5.8 - 12.5	0.975
63-10	Germany	151.52	151.52	1.8	7.75	5.74 - 11.9	0.973
83-10	Poland	107.5	185.83	1.85	9.35	5.08 - 58.82	0.936
32-10	Germany	90.91	177.88	2	11.11	6.05 - 66.67	0.97
51-10	Germany	109.09	127.88	2.1	10.99	9.47 - 12.99	0.999
57-10	UK	113.1	168.38	2.25	10.99	7.92 - 17.86	0.988
96-10	Poland	103.64	134.55	2.3	11.26	10.05 - 13.01	0.988
68-10	Germany	66.67	169.7	2.4	10.64	8.34 - 14.71	0.992
62-10	Germany	121.21	175.76	2.4	11.24	9.75 - 13.16	0.997
7-10	France	106.06	157.58	2.55	12.5	10.11 - 16.13	0.995
97-10	Poland	118.18	160.61	2.55	14.44	12.16 - 18.1	0.994
106-10	Czechia	126.06	160	2.8	13.33	8.38 - 33.33	0.954
80-10	Germany	76.67	164.24	3.2	13.89	11.57 - 16.95	0.997
79-10	Germany	99.33	160	3.6	17.84	16.34 - 19.8	1

*Strain 70-10 does not fit the assumptions of analysis
EI = efficacy index, LCT = *lambda*-cyhalothrin, TFV = *tau*-fluvalinate, CL = confidence limits

3.4 Discussion

Failure in pollen beetle control can result in high economic damage and sometimes to complete yield loss as recently happened in Germany due to pollen beetle feeding on the reproductive organs of oilseed rape [2]. One of the major classes of insecticides used for pollen beetle control are the pyrethroids, and after 20 years control failures were shown to be the result of resistance development in many European countries [12, 14]. Our study confirmed the presence of high levels of pyrethroid resistant pollen beetles in 2010 in many European countries based on results of bioassays, and for the first time with a clear correlation to an identified biochemical marker. Only one sample collected in Ukraine (strain 70-10) was fully susceptible and showed no synergistic effect upon pre-exposure with PBO, DEM or DEF in adult vial bioassays. All other populations responded strongly to deltamethrin after incubation with PBO, but not to DEM and DEF. Although it is known that PBO could also inhibit esterases [30], we focused our work on the biochemical validation of a cytochrome P450-driven metabolic oxidation of pyrethroids. A very recent study conducted on a smaller number of populations also suggested the involvement of oxidative metabolism based on synergist bioassays conducted *in vivo* and *in vitro* (with the model substrate 7-ethoxycoumarin) [31]. However as the authors pointed out there was no obvious correlation of their *in vitro* results with the observed levels of pyrethroid resistance [31]. A similar observation was made in this study, since we also failed to correlate 7-ethoxycoumarin O-deethylase activity in microsomes of 27 populations tested with pyrethroid resistance levels. It would be interesting in future studies to seek for artificial substrates which can be used in a fluorescent assay as a molecular tool to screen large numbers of individuals and populations, because the microsomal hydroxylation assay described in this study is quite laborious.

The mortality of strains 80-10 and 79-10 increased after PBO pre-treatment to over 90 %. These two strains were the most active ones in the microsomal hydroxylation assay of deltamethrin, indicating that the observed synergism is linked to monooxygenase inhibition. However our screening of all 27 populations for their ability of forming the lesser toxic metabolite 4-OH-deltamethrin confirmed biochemically the presence of a cytochrome P450-based resistance mechanism in all populations obtained from several European countries, excluding the reference strain 70-10. Until 2007 no serious cases of resistance in pollen beetle were observed in the UK [11], but recently two larger resistance monitoring projects reported that resistance is on the move [12, 14]. Strain 57-10 sampled in the UK in 2010 showed low mortality figures in adult vial assays for both *lambda*-cyhalothrin and *tau*-fluvalinate, and additionally a high hydroxylation activity on deltamethrin in the metabolism assay, indicating the presence of the same detoxification mechanism observed in continental populations. In contrast some resistant strains from Germany (38-10, 39-10, 68-10), France (9-10, 13-10) and

one of Sweden (120-10) showed a lower activity in the metabolism assay as expected based on the results of bioassays, suggesting the presence of additional mechanisms of resistance. Michaelis-Menten kinetics on deltamethrin hydroxylation velocity in microsomal preparations revealed a strong and significant correlation between V_{max}-values and resistance levels to *lambda*-cyhalothrin. Similar to *lambda*-cyhalothrin the resistance levels to *tau*-fluvalinate albeit structurally somewhat different also correlate significantly with deltamethrin hydrxylation. This result confirmed the cross-resistance pattern of a range of pyrethroids observed in adult vial bioassays. However the bioassay data of those pyrethroids which are structurally very similar show a high correlation coefficient (e.g. Pearson r = 0.84; efficacy of *lambda*-cyhalothrin vs. deltamethrin). Whereas the correlation between results of *tau*-fluvalinate and *lambda*-cyhalothrin is less pronounced (r = 0.5). Lineweaver-Burk plots revealed a competitive inhibition of deltamethrin hydroxylation by *tau*-fluvalinate, so it is fairly likely that both compounds are competing for the same binding site in monooxygenase(s) involved in pyrethroid resistance. Co-incubation of deltamethrin with increasing concentrations of *tau*-fluvalinate does not change the V_{max}-value of the hydroxylation reaction, but increases K_m-values as known for competive inhibitors. Metabolic experiments with *tau*-fluvalinate as well as *lambda*-cyhalothrin haven´t yet been conducted in pollen beetle microsomal preparations due to the lack of reference metabolites for LC-MS quantification. Similar to *tau*-fluvalinate, *lambda*-cyhalothrin also competitively inhibits deltamethrin metabolism.

The hydroxylation of the 3-phenoxybenzyl-alcohol moiety in para-position is one of the most prominent detoxification pathways for pyrethroids [27,32-36], but also the 3-phenylbenzyl-alcohol moiety in bifenthrin is known to be hydroxylated in that position [37]. Although the microsomal oxidation of pyrethroids via cytochrome P450-mediated hydroxylations is the most common detoxification route, specific data for *tau*-fluvalinate is not available. For *tau*-fluvalinate the metabolism in mammals is mainly based on esterases (as also described for other pyrethroids) and the major metabolite is anilinic acid. Nonetheless, at least two studies also reported the 4-hydroxylation of the phenoxybenzyl ring system in fluvalinate [38, 39]. From studies with *Apis mellifera* it is known, that honeybee safety of *tau*-fluvalinate is based on oxidative degradation mediated by monooygenases [40]; three different cytochrome P450s were described yet to degrade *tau*-fluvalinate [41].

In coincidence with the observed synergist effects on adult beetles *in vivo* the hydroxylation reaction in microsomal preparations was also strongly inhibited by PBO and two other monooxygenase inhibitors, i.e. tebuconazole and 1-aminobenzotriazole (ABT). The inhibition rates were equal between the strains for the different synergists use, but not the extent of the inhibition. The blockage was lowest when ABT is used as an inhibitor. Scott [42] reported for CYP6D1 isolated from pyrethroid resistant *Musca domestica* (LPR-Strain) also a

low inhibitory potency of ABT. Further experiments are necessary to describe the resistance mechanism to pyrethroids in pollen beetle in more detail, in particular the molecular identification of the cytochrome P450(s) involved.

3.5 Conclusions

Due to the fact that cross-resistance to pyrethroid insecticides in pollen beetle is spread all-over Europe these days and as shown here quite likely being confered by the same oxidative mechanism, the importance of the implementation of adequate resistance management strategies - based on mode of action rotation – has been highlighted recently [12, 43]. Pyrethroids are still an important group of insecticides for controlling insect pests in oilseed rape, i.e. stem and pod weevils, but applications against resistant pollen beetles should be avoided in order reduce the selection pressure, and the expression of additional resistance mechanisms such as target-site resistance. Even though some pyrethroids such as *tau*-fluvalinate and bifenthrin seemed to perform somewhat better against pollen beetle under field-conditions, it is strategically questionable to use them in areas known for pyrethroid resistance [8, 18]. It has been shown in other pest species that also with structurally different pyrethroids such as *tau*-fluvalinate resistance levels could be > 100,000-fold due to oxidative mechanisms covering the entire class of chemistry [44]. With the acreage of winter oilseed rape still growing in some European countries, it has also been shown that the number of pollen beetles continuously increased [45], in other words the frequency of new resistance genes is also likely to increase. Therefore it is important to learn from the past and introduce new modes of action to control pollen beetles in the future and to guarantee sustainable yields.

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Chapter 4

Molecular and functional characterization of CYP6BQ23, a cytochrome P450 conferring resistance to pyrethroids in European populations of pollen beetle, *Meligethes aeneus*

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Abstract

The pollen beetle (*Meligethes aeneus*) is widespread throughout much of Europe where it is a major coleopteran pest of oilseed rape (*Brassica napus*). The reliance on synthetic insecticides for control, particularly the pyrethroid class, has led to the development of populations with high levels of resistance. Resistance to pyrethroids is now widespread throughout Europe and is thought to be mediated by enhanced detoxification by cytochrome P450s and/or mutation of the pyrethroid target-site, the voltage-gated sodium channel. However, in the case of cytochrome P450 mediated detoxification, the specific enzyme(s) involved has (have) not yet been identified. In this study a degenerate PCR approach was used to identify ten partial P450 gene sequences from pollen beetle. Quantitative PCR was then used to examine the level of expression of these genes in a range of pollen beetle populations that showed differing levels of resistance to pyrethroids in bioassays. The study revealed a single P450 gene, *CYP6BQ23*, which is significantly and highly overexpressed (up

to ~900-fold) in adults and larvae of pyrethroid resistant strains but not in susceptible strains. *CYP6BQ23* overexpression is significantly correlated with both the level of resistance and with the rate of deltamethrin metabolism in microsomal preparations of these populations. Functional recombinant expression of full length CYP6BQ23 along with cytochrome P450 reductase in an insect (Sf9) cell line showed that it is able to efficiently metabolise deltamethrin to 4-hydroxy deltamethrin. Furthermore we demonstrated by detection of 4-hydroxy *tau*-fluvalinate using ESI-TOF MS/MS that functionally expressed CYP6BQ23 also metabolizes *tau*-fluvalinate. A protein model was generated and subsequent docking simulations revealed the predicted substrate-binding mode of both deltamethrin and *tau*-fluvalinate in CYP6BQ23 protein. Taken together these results strongly suggest that the overexpression of *CYP6BQ23* is the primary mechanism conferring pyrethroid resistance in pollen beetle populations throughout much of Europe.

4.1 Introduction

Oilseed rape (*Brassica napus*) is a crop of global economic importance and particularly winter oilseed rape, is grown on several million hectares in Europe and indispensable in many crop rotations. The main winter oilseed rape growing countries in Europe are France, Germany, Poland and UK with a total cropping area exceeding 4 mio ha [1]. Oilseed rape is attacked by a number of invertebrate pests of the order Coleoptera, such as flea beetles (*Psylliodes* ssp., *Phyllotreta* ssp.), stem weevils and seed pod weevils (*Ceutorhynchus* ssp.) and pollen beetle (*Meligethes ssp.*) [2]. Although the genus *Meligethes* STEPHENS, 1830 (Coleoptera: Nitidulidae, Meligethinae) comprises globally more than 600 pollen eating species [3], *Meligethes aeneus* is by far the most destructive species attacking oilseed rape crops throughout Europe [2]. Adult beetles emerging from overwintering sites feed almost exclusively on pollen and cause damage during the early (green to yellow) stages, whereas feeding larvae cause bud abscission resulting in podless stalks. Pollen beetle infestations exceeding economic damage thresholds can for example result in yield reductions as high as 70% in spring oilseed rape [4,5].

The main method of crop protection against pollen beetle infestations is the large scale application of chemical insecticides, and a recent survey revealed that in many European countries 100 % of the crop area received treatments against pollen beetle [1]. Over the last two decades pollen beetle control has mainly relied on the pyrethroid class of insecticides, e.g. *alpha*-cypermethrin, bifenthrin, cypermethrin, deltamethrin, *lambda*-cyhalothrin, *tau*-fluvalinate and *zeta*-cypermethrin [6]. Pyrethroid insecticides are neurotoxic and bind to voltage-gated sodium channels in the insect central nervous system providing a fast knock-down of pests at low application rates [7]. This intensive use of pyrethroids has led to the development of

resistance in pollen beetle, which was first reported in 1999 in North Eastern France [4,8,9]. Concerted large scale pollen beetle pyrethroid resistance monitoring campaigns between 2007 and 2011 revealed that resistance has become widespread and is at high levels in several European countries including France, Germany, UK, Poland, Czech Republic, Denmark and Sweden [9,10].

Insecticide resistance most commonly evolves by two main mechanisms, i.e. increased levels of detoxification enzymes resulting in metabolic resistance, and target-site mutations resulting in lower binding affinity of the respective insecticides [11–13]. Pyrethroid resistance can be conferred by both mechanisms, but target-site insensitivity caused by mutations in the voltage-gated sodium, known as knock-down resistance (*kdr*), is a common mechanism in many pests [7,14–16]. Target-site resistance to pyrethroids was first described in *Musca domestica* and linked to two mutations in the housefly voltage-gated sodium channel gene (*Vssc1*) leading to amino acid changes at positions L1014F (*kdr*) and M918T (*s-kdr*) in domain II of the channel protein [14]. Subsequently, many more mutations in voltage-gated sodium channels conferring pyrethroid target-site resistance have been described, but L1014F remains the most common mutation described in almost 20 different pest species [16], including in pollen beetle [17]. However the lack of *kdr* in a large number of highly resistant pollen beetle populations collected in European countries other than Denmark and Sweden suggests enhanced metabolic detoxification of pyrethroids may be the main driver of resistance [17]. A recent study has provided several lines of evidence that support this assumption [18]. Firstly, the authors were able to show that the efficacy of pyrethroids can be synergized by the cytochrome P450 (P450) inhibitor piperonyl butoxide (PBO), but not S,S,S-tributyl phosphorotrithioate (DEF, an esterase inhibitor) and diethyl maleate (DEM, a glutathione depleter affecting glutathione S-transferases). The synergistic effect of PBO against resistant pollen beetle populations was also shown by other authors to be primarily correlated with oxidative metabolism [19]. Furthermore, microsomal preparations from resistant strains showed a significantly increased rate of deltamethrin degradation in the presence of NADPH, which was inhibited by PBO and other well-known P450-inhibitors such as tebuconazole and 1-aminobenzotriazole [18]. Finally, LC-MS/MS analyses of 4-hydroxy deltamethrin formation in microsomes from several pollen beetle strains correlated with the level of pyrethroid resistance. Some pyrethroids such as bifenthrin and *tau*-fluvalinate were shown to be less affected by resistance, but shown to competitively inhibit the formation of 4-hydroxy deltamethrin, which correlates to some extent with the cross-resistance profile observed in resistant strains [10,20]. Although all these findings strongly suggest that pyrethroid (cross-) resistance in pollen beetle is based on oxidative degradation by P450s, the specific enzyme(s) involved has (have) not been identified. Individual members of the

arthropod cytochrome P450 gene superfamily are well known for their involvement in the detoxification of synthetic insecticides as well as toxic secondary plant metabolites [13,21,22]. Recent progress in genome and transcriptome sequencing facilitated the discovery and functional characterization of several insect P450s involved in pyrethroid resistance [23–28]. However, in Coleoptera only a single P450 from the red flour beetle, *Tribolium castaneum*, has been identified and functionally characterized for its involvement in pyrethroid resistance [24]. In this study the authors exploited microarray technology and reverse genetic approaches to demonstrate that a single brain-specific P450, CYP6BQ9, is overexpressed 200-fold in a deltamethrin resistant strain of *T. castaneum* and confers high levels of deltamethrin resistance. CYP6BQ9 was shown to metabolize deltamethrin to its 4-hydroxy derivative *in vitro*, similar to CYP6M2 of *Anopheles gambiae* and members of the CYP9J subfamily of *Aedes aegypti* [25,29].

The formation of 4-hydroxy deltamethrin by microsomal preparations has recently also been described to be correlated with pyrethroid resistance in pollen beetle [18]. Therefore the aim of this study was to identify the P450(s) involved in pyrethroid resistance in pollen beetle populations sampled across Europe. Due to the lack of available genomic and transcriptomic data on pollen beetle P450 genes, a PCR approach employing degenerate primers based on conserved helix I and heme binding regions was used to identify P450 genes involved in pyrethroid resistance. We isolated a full-length cDNA clone of a single P450, CYP6BQ23 which is several hundred fold overexpressed in pyrethroid resistant pollen beetle adults and larvae. qRT-PCR data shows that its overexpression is significantly correlated with pyrethroid resistance. We characterized the substrate profile of recombinant CYP6BQ23 expressed in insect Sf9 cells as well as its ability to hydroxylate deltamethrin and *tau*-fluvalinate. Finally a protein homology model of CYP6BQ23 was generated and subsequent docking simulations used to provide insights into the possible orientation of pyrethroid substrates in its active site.

4.2 Material and Methods

4.2.1 Insect populations and bioassays

European pollen beetle (*Meligethes aeneus* F.) populations were collected in oilseed rape fields between April and June in 2009 and 2010, except adults of strain 91-11 from Ukraine collected in 2011. All beetles were shipped to the authors´ laboratory and maintained at 4 °C until testing for pyrethroid resistance (within 24h after arrival). Each sample was subjected to morphometric analysis to check for *M. aeneus* based on markers described by SCHERNEY [30], FRITZSCHE [31] & FREUDE *et al.* [32]. No other *Meligethes* ssp. was found in

those samples used in this study. In May 2011 pollen beetle 2nd instar larvae were collected in winter oilseed rape fields from three different regions in Germany. Larvae can easily be collected in the late flowering stage of winter oilseed rape (BBCH 67-69) by tapping the plants into a close-meshed insect net. Larvae were also stored at 4 °C until testing (within 24 h after sampling). From each adult and larval strain collected, a sample was flash frozen in liquid nitrogen and stored at -80 °C for subsequent molecular analysis. Pyrethroid resistance levels in adults were recently assayed using *lambda*-cyhalothrin as a reference pyrethroid insecticide in an adult vial test [10]. Briefly: Ten beetles each were incubated at room temperature in 30 ml glass vials coated on the inner surface with 5-6 different concentrations of *lambda*-cyhalothrin dissolved in acetone (concentration range between 0.6 ng cm^{-2} and 375 ng cm^{-2}). Beetles were scored for mortality after 24 h and each concentration was replicated thrice as recently described [10]. Second instar larvae of pollen beetle strains 40-11, 59-11 and 72-11 were recently tested and shown to be resistant to pyrethroids [33].

4.2.2 Chemicals

All chemicals and solvents used in this study were of analytical grade unless otherwise stated. Technical *lambda*-cyhalothrin and NAPDH were obtained from Sigma Aldrich. Deltamethrin was a Bayer CropScience internal analytical standard (purity > 99%). Fluorescent artificial P450 substrates such as BFC, 7-benzyloxy-4-trifluoromethyl coumarin; MFC, 7-methoxy-4-trifluoromethyl coumarin; EFC, 7-ethoxy-4-trifluoromethyl coumarin; BOMFC, 7-benzyloxymethoxy-4-trifluoromethyl coumarin; BOMCC, 7-benzyloxymethoxy-3-cyano coumarin; PC, 7-n-pentoxy coumarin; EC, 7-ethoxy coumarin; BOMR, 7-benzyloxymethoxy resorufin; ER, 7-ethoxyresorufin; BR, 7-benzyloxyresorufin; MR, 7-methoxyresorufin; PR, 7-n-pentoxyresorufin, and all consumables for recombinant expression were purchased from Life Technologies.

4.2.3 DNA/RNA extraction and cDNA synthesis

Adults and 2nd instar larvae of individual pollen beetle samples were flash frozen in liquid nitrogen and kept at -80 °C prior to nucleic acids extraction. Nucleic acids were extracted from 15 to 20 pooled insects using either Agencourt DNAdvance kit (Beckman Coulter, USA) for DNA extraction or TRIzol reagent (Invitrogen, CA, USA) followed by Agencourt RNAdvance Tissue kit (Beckman Coulter, USA) for RNA extraction. Pools of insects were placed into pre-cooled (-80 °C) 1.5 ml Eppendorf vials including two 3 mm tungsten beads (Qiagen, Germany). Tubes containing samples and beads were deep frozen in liquid nitrogen and afterwards subjected to a Retsch TissueLyser (Qiagen) for 1 min at a frequency of 18 Hz for sample disruption. For total RNA extraction tubes were briefly centrifuged to collect the resulted powdery content at the bottom and 0.5 ml TRIzol reagent

was added. Subsequently the tubes containing TRIzol, sample and beads were subjected again to Retsch TissueLyser and homogenized for 1 min at a frequency of 20 Hz and subsequently briefly centrifuged to collect the sediment. After 3 min incubation at room temperature 0.1 ml chloroform was added to each sample and mixed on a Vortex for 15 sec. After 5 min incubation at room temperature the samples were centrifuged at 4 °C and 12,000 g for 15 min. The aqueous phase was transferred to a 96 deep well plate and processed on a Biomek NXp liquid handling platform (Beckman Coulter) according to Agencourt RNAdvance Tissue kit protocol. DNase I (Ambion, TX, USA) digestion to remove any genomic DNA was included in the purification process on the liquid handling platform. The quality and quantity of RNA was determined by spectrophotometry (NanoQuant Infinite 200, Tecan, Switzerland) and by running 1 µg RNA per sample on a 1.5 % agarose gel using a denaturing RNA loading dye (Thermo-Fermentas, MS, USA). Intact RNA is indicated by a single bright band without any smear because of a specific 28S RNA as known from other insect species [34]. The RNA content of all samples was normalized to 500 ng μL^{-1} on a Biomek 3000 liquid handling platform (Beckman Coulter) and 2.5 µg total RNA was used in 20 µL reactions for cDNA synthesis using Superscript III (Invitrogen) and random hexameres equally mixed (v/v) with oligo dT primers (Invitrogen) according to manufacturer's instructions.

For DNA extractions, the powdery content of the tubes was suspended in lysis buffer provided with Agencourt DNAdvance kit and processed on a Biomek NXp liquid handling platform according to Agencourt DNAdvance kit protocol. After purification the samples were treated with RNase A (Ambion) in Tris-HCl buffer pH 8.0, and re-purified using Agencourt AMPure reagent (Beckman Coulter). The DNA was quantified by spectrophotometry; quality was assessed by running an aliquot on a 1.5 % agarose gel and samples were diluted to 2.5 ng μL^{-1}.

4.2.4 Isolation of P450 and reference genes

A degenerate primer PCR strategy was used to amplify CYP4 and CYP6 family specific regions of P450 genes as well as coding sequence regions of α-tubulin and actin. For this approach the same CYP specific primer pairs and PCR cycling conditions were used which recently resulted in the identification of eleven P450 genes including *CYP6CM1* in the cotton whitefly, *Bemisia tabaci* [35]. For α-tubulin and actin degenerate primer pairs (supplemantary file1) were designed based on highly conserved regions among insect species determined by multiple alignment of several amino acid sequences (GenBank accession numbers for tubulin: AAF54067, AAF54433, EEZ99348, NP_001036884, XP_001120096, XP_001870369; and for actin: AAA28318, BAA74592, NP_001165844, XP_966960,

XP_976003). Degenerate PCR was conducted in 25 µL reaction mixtures containing 1 µL cDNA (20 ng), 10 µM of each degenerate primer and 12.5 µL DreamTaq PCR Master Mix (Thermo-Fermentas) containing Taq polymerase, 2x PCR buffer and 4 mM $MgCl_2$ (2 mM final concentration). For actin and tubulin a nested PCR strategy was used, repeating the PCR with an inner primer pair using 0.5 µL of the primary PCR as a template. Samples were separated on a 1.2 % (w/v) Agarose/TAE gel and PCR products of the expected size (440bp and 390 bp for CYP4 and CYP6 sequences, and ~600bp and ~890 bp for actin and tubulin, respectively) were extracted from the gel. The nucleic acid was isolated from the gel slices by Wizard SV Gel kit (Promega, WI, USA) and cloned into pSC-A-amp/kan vector using Strataclone PCR cloning kit (Agilent Technologies, CA, USA). Resulting plasmids were purified from Minipreps using GeneJET plasmid kit (Thermo-Fermentas), and sequenced with an automated DNA sequencer ABI model 3700 using the ABI BigDye Terminator Cycle Sequencing kit and M13 primers. Degenerate PCR was carried out on cDNA extracted from both pyrethroid resistant and susceptible pollen beetle populations. Amino acid sequence alignments were done by using Geneious v5.5 (www.geneious.com).

4.2.5 Real time qRT-PCR and determination of P450 gene copy number

Real time qRT-PCR was performed on a CFX-96 real time cycler (Bio-Rad Laboratories, CA, USA). Primer pairs were designed using Primer3 program [36] to amplify a fragment of 90-150 bp in size for each gene (supplemantary file2). Reaction mixtures (20 µL) contained 4 µL cDNA (5 ng), 10 µL of iQ SYBR Green Supermix (Bio-Rad Laboratories) and 0.25 µM of each primer. Thermocycling conditions were 3 min at 95 °C followed by 40 cycles of 95 °C for 15 s, 57 °C for 15 s and 72 °C for 20 s. A final melt-curve step was included post-PCR (ramping from 65°C-95°C by 0.5 °C every 5 s) to check for nonspecific amplification. Data analysis was performed with Bio-Rad CFX Manager 3.0 built in gene expression analysis module. Two reference genes, i.e. α-tubulin (GenBank KC840056.1) and actin (GenBank KC840045.1), were used for normalization according to $_{\Delta\Delta}$Ct method [37]. Both reference genes were stably expressed in all strains as they had acceptable M-values and a low covariance, α-tubulin (0.671; CV 0.2409) and actin (0.614; CV 0.2236). For each primer pair used, a standard curve was made using triplicate dilutions covering a 1000 fold range (20-0.02 ng), only primer pairs were used with a $R^2 \geq 0.99$ and a PCR efficiency > 90 % (see Table S2). PCR efficiency of individual targets was taken into account for gene expression analysis. Four independent biological replicates containing 15-20 beetles each were run in triplicate in each qRT-PCR experiment. A similar experimental design with gDNA as a template (10 ng per reaction) was used to determine the gene copy number of *CYP6BQ23* in different pollen beetle strains.

4.2.6 Rapid amplification of cDNA ends (RACE)

Five and three prime RACE was carried out using Invitrogen's 5'RACE and 3'RACE System for Rapid Amplification of cDNA Ends and RLM-RACE kit (Ambion) following manufacturer's protocols. For RACE purposes a cDNA pool of both pyrethroid resistant and susceptible strains was used. The details of the gene-specific primers used for RACE are listed in supplemantary file1. Single PCR products were extracted from the gel, purified, cloned and sequenced as described above. In order to prove the assembly the full length coding sequence of *CYP6BQ23* was amplified by nested PCR using primers *CYP6BQ23* F1, R1 and R2, respectively (Table S1). PCR reactions (20 µL) contained 1 µL cDNA (20 ng), 0.5 µM of each primer and 10 µl Phusion Flash High-Fidelity PCR Master Mix (New England Biolabs, MA, USA) were subjected to cycling conditions of: 15 s at 98 °C followed by 30 cycles of 98 °C for 10 s, 62 °C for 15 s and 72 °C for 30 s and a final extension step at 72 °C for 1 min. PCR products were purified using Agencourt AMPure reagent (Beckman Coulter) and directly sequenced using primers as detailed in supplemantary file1.

4.2.7 Functional expression of CYP6BQ23 in Sf9 cells

Meligethes aeneus full length *CYP6BQ23* coding sequence of strain 79-10 (GenBank KC840055.1) and *Musca domestica* NADPH cytochrome P450 reductase (CPR) coding sequence (GenBank Q07994) were obtained by gene synthesis (Geneart, CA, USA). *CYP6BQ23* and *CPR* sequence was inserted in pDEST8 expression vector (Invitrogen). PFastbac1 vector containing no foreign DNA was used to produce a control virus. The recombinant baculovirus DNA was constructed and transfected to Sf9 insect cells (Gibco) using Bac-to-Bac baculovirus expression system (Invitrogen) according to manufacturer's instructions. The titer of the recombinant viruses was determined following standard protocols of the supplier. Sf9 cells were maintained in suspension culture under serum-free conditions (SF-900 II SFM, Gibco) at 27 °C containing 25 µg mL^{-1} gentamycin (Gibco). Insect cells grown to a density of 2×10^6 cells mL^{-1} were co-infected with recombinant baculoviruses containing *CYP6BQ23* and *CPR* with various MOI (multiplicity of infection) ratios to check out best conditions. Control cells were co-infected with the baculovirus containing vector with no insert (ctrl-virus) and the recombinant baculovirus only expressing CPR using the same MOI ratios as described above. Ferric citrate and δ-aminolevulinic acid hydrochloride was added to a final concentration of 0.1 mM at the time of infection and 24 h after infection to compensate the low levels of endogenous heme in the insect cells. After 60 h cells were harvested and washed with PBS, and the microsomes of the membrane fraction were prepared according to standard procedures and stored at −80 °C [38]. CYP6BQ23 expression and functionality was estimated by measuring CO-difference spectra in reduced samples [39].

4.2.8 Enzyme activity determination

CYP6BQ23 enzymatic activity was confirmed by its O-dealkylation and O-dearylation activity on a range of fluorescence model substrates (50 µM, 0.1 M Na-phosphate buffer pH 7.6, 0.1 % DMSO) using CYP6BQ23 microsomes in a 96-well plate with the prepared microsomes of CYP6BQ23/NADPH CPR (10 µg in 50 µL assay volume, containing 1 mM NADPH; 30 min incubation at 27 °C while shaking at 800 rpm). After adding 100 µl DMSO:TRIZMA-base buffer 50 % (v/v), 0.05 M, pH 10 into the wells the fluorescence was measured with a Spectra Max M2 reader (Molecular Devices) at the appropriate excitation/emission wavelength settings according to manufacturer instructions (Invitrogen). The activity of CYP6BQ23 microsomes was compared to control microsomes obtained from Sf9 cells infected with recombinant baculovirus containing only CPR. Protein content of samples was determined according to BRADFORD [40] using bovine serum albumin as a reference.

4.2.9 Pyrethroid metabolism and UPLC MS/MS analysis

Deltamethrin metabolism was assayed by incubation of the recombinant CYP6BQ23/CPR (2 pmol P450 per assay) or ctrl-virus/CPR microsomes in 0.1 M potassium phosphate buffer with an NADPH-regenerating system (Promega; 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, 0.4 U mL^{-1} glucose-6-phosphate dehydrogenase) and 12.5 µM deltamethrin (0.8 – 25 µM for Michaelis-Menten kinetics) at 27 °C for 1 h. The metabolism auf *tau*-fluvalinate was measured under the same conditions but incubated at 27 °C for 240 min due to its slower degradation. The total assay volume was 200 µL using three replicates for each data point. Initial experiments were repeated twice with different batches of recombinant CYP6BQ23/CPR microsomes. Microsomes incubated without NADPH served as a control. The assay was quenched by the addition of ice-cold acetonitrile (to 80 % final concentration), centrifuged for 10 min at 3000 g and the supernatant subsequently analyzed by tandem mass spectrometry as recently described in ZHU [24]. Recovery rates of deltamethrin using microsomal fractions without NADPH were normally close to 100 %. Deltamethrin turnover from two independent reactions were plotted vs. controls. Michaelis-Menten kinetic was analyzed using GraphPad Prism version 5 (GraphPad Software, CA, USA).

4.2.10 P450 modelling and substrate docking

The molecular model of CYP6BQ23 was created using the Orchestrar Suite module within the molecular modelling software SYBYLx2.0 (Certara, L.P., St. Louis, USA) based on the crystal structure of human CYP3A4 (Yano et al., 2004; PDB-ID:1TQN). This template structure was chosen because of its close homology to the CYP6BQ23 sequence. The

resulting raw model was subjected to an energy minimization using the AMBER force field 2002 within SYBYLx2.0, to remove distortions and unallowed van der Waals contacts resulting from the molecular modeling process. The docking of the pyrethroids deltamethrin and *tau*-fluvalinate into the active site of CYP6BQ23 was realized by using the software LeadIt Release 2.1.3 from BioSolveIT (St. Augustin, Germany). A pharmacophoric constraint was applied which allowed only positions, where any heavy atom of the ligands is within a 3.5Å radius distance of the CYP6BQ23 heme iron centre. Thus, for deltamethrin in total 191 possible orientations were obtained, whereas for *tau*-fluvalinate 112 orientations were calculated. All obtained orientations were additionally assessed with the HYDE post-scoring function as recently described [41]. These results allowed to predict the most likely ligand orientations of both deltamethrin and *tau*-fluvalinate for 4-hydroxylation in the active site of CYP6BQ23.

4.3 Results

4.3.1 Identification of pollen beetle P450 and reference genes

The degenerate CYP4 and CYP6 primer PCR approach resulted in ten gene fragments of approximately 440bp and 390bp in length, respectively. Subsequent cloning in sequencing revealed that all fragments are assumed to represent real P450 sequences as they all contain the conserved EXXR helix K-motif and the PXRF motif in the deduced amino acid sequence. The amino acid identity between the obtained three CYP6-like sequences ranged from 49.6 to 52.8 % (60.6 to 64.1 % nucleotide identity). The amino acid identity between the seven CYP4-like sequences ranged from 42.3 to 68.5 % (26.6 to 68.8 % nucleotide identity). This clearly indicates that all isolated sequences represent unique gene fragments, rather than allelic variants. The BLASTp alignment received for all sequences revealed P450s of three different coleopteran species as best hits. Most of the hits returned for P450s from *Tribolium castaneum* (red flour beetle), followed by *Dendroctonus ponderosae* (mountain pine beetle) and *Brontispa longissima* (coconut leaf beetle). All CYP6-like sequences seemed to be related to only one subfamily, CYP6B, whereas the CYP4-like sequences are related to three subfamilies, i.e. CYP4B, CYP4H and CYP4Q. The best sequence identity scores based on deduced amino acid sequences were obtained for P450s from *T. castaneum* (Table 8). PCR products of the degenerate PCR strategy for actin- and tubulin-like sequences were 509 bp and 602 bp in length, respectively. After cloning and sequencing the most similar BLASTp hits for the isolated *M. aeneus* actin- and tubulin fragments were partial sequences of *beta*-actin of *Nasutitermes takasagoensis* (Isoptera: Termitidae) with 98.8 % amino acid identity (GenBank BAI22849) and *alpha*-tubulin of *Teleopsis dalmanni* (Diptera: Diopsidae) showing 100 % amino acid identity (GenBank

AFM80094) respectively. The partial sequences for *M. aeneus* actin (GenBank KC840045.1) and *M. aeneus* tubulin (GenBank KC840056.1) were submitted to GenBank.

Table 8 *Sequence identity calculated from BLASTp alignments of* M. aeneus *partial (helix I to heme binding region) CYP4, CYP6 and CYP6BQ23 deduced amino acid sequences to their most similar cytochrome P450 in* T. castaneum.

P450 family[a]	Most similar P450 in *T. castaneum* (GenBank accession no.)	E-value	% Pairwise AA Identity	Assigned *M. aeneus n*ame (GenBank accession no.)
CYP6	CYP6BQ10 (NP_001164249)	1e -166	53.0	CYP6BQ23 (KC840055.1)
	CYP6BK5 (EFA12633)	1.91e -50	59.2	CYP6-like 1 (KC840047.1)
	CYP6BQ13 (EEZ99338)	8.75e -51	63.1	CYP6-like 2 (KC840046.1)
CYP4	CYP4BN1 (NP_001123993)	1.52e -51	59.3	CYP4-like 1 (KC840051.1)
	CYP4H10 (NP_001107836)	3.07e -53	56.5	CYP4-like 2 (KC840054.1)
	CYP4Q6 (XP_970404)	4.04e -50	57.1	CYP4-like 3 (KC840048.1)
	CYP4Q9(NP_001107850)	2.59e -62	64.4	CYP4-like 4 (KC840053.1)
	CYP4BN1 (NP_001123993)	2.19e -53	59.4	CYP4-like 5 (KC840049.1)
	CYP4BN1 (NP_001123993)	4.04e -54	62.8	CYP4-like 6 (KC840052.1)
	CYP4Q2 (NP_001107846)	4.12e -57	61.3	CYP4-like 7 (KC840050.1)

[a] CYP family was assigned based on BLASTp hits (except for CYP6BQ23)

4.3.2 Expression of P450 genes in different strains of M. aeneus

The gene expression level of each P450 gene was checked by qRT-PCR on mRNA isolated from a number of pollen beetle strains collected from different regions in Europe. Two of the strains were completely susceptible (s) to pyrethroids (70-10 and 127-10) and all others were recently described as highly resistant (r) to pyrethroids showing resistance ratios >100-fold [10]. Only a single P450, *CYP6BQ23* was significantly overexpressed in all pyrethroid resistant strains, but not in the two susceptible strains (Table 9). In strain 127-10 (s) some P450 genes are significantly down-regulated, but no difference in *CYP6BQ23* expression compared to the original pyrethroid susceptible reference strain 70-10. A more detailed secondary screening was carried out to correlate the extent of pyrethroid resistance (based on *in vivo* bioassay results) and the expression level of *CYP6BQ23* mRNA in the same strains (Table 10). In total 13 pollen beetle strains from 8 different European countries were re-analyzed and we obtained a significant correlation between the recently calculated resistance ratio based on LC_{50}-values for *lambda*-cyhalothrin and fold change in expression level ($_{\Delta\Delta}Cq$) for *CYP6BQ23* ($r = 0.79$, $F = 18.7$, p-value = 0.0012). The remarkably high expression level of *CYP6BQ23* in pyrethroid resistant strains of *M. aeneus* was confirmed using a second primer set (Table S2). In conclusion our results suggest a significant link between overexpression of

CYP6BQ23 and pyrethroid resistance level irrespective of the geographic origin of the strain. In a subsequent analysis we also correlated the expression level of *CYP6BQ23* in a different set of strains recently analyzed for pyrethroid resistance based on adult vial tests and formation of 4-hydroxy deltamethrin in their microsomal preparations [18]. Once again we obtained a clear correlation between *in vivo* bioassays, *in vitro* deltamethrin metabolism and fold change in *CYP6BQ23* expression (Fig. 34). Finally we investigated the expression level of *CYP6BQ23* in pollen beetle adults and larvae of individual strains collected in Germany and known to be resistant to pyrethroids [33]. Again *CYP6BQ23* expression was significantly elevated in all three strains in both adults ($_{\Delta\Delta}$Cq 174.99-365.47) and 2nd instar larvae ($_{\Delta\Delta}$Cq 130.66-251.27) (Fig. 35).

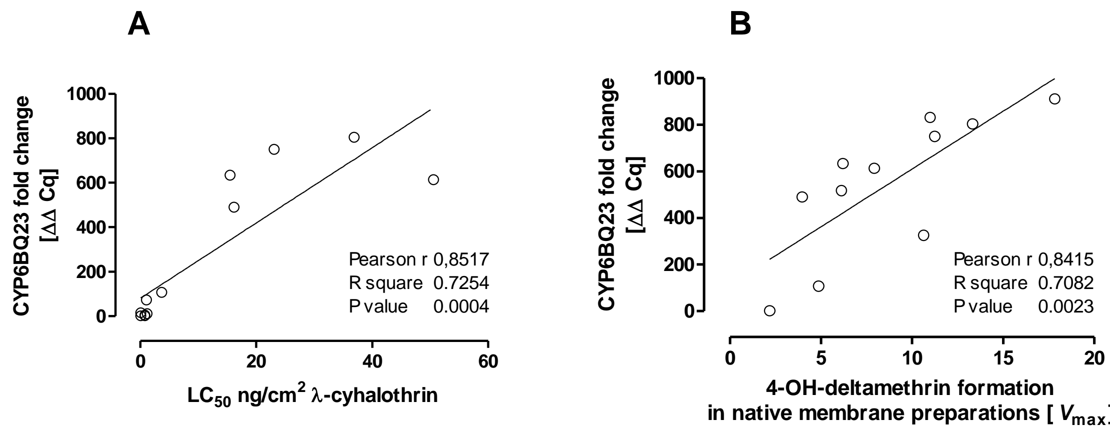

Fig. 34 *Pearson correlation between CYP6BQ23 fold change in expression level and pyrethroid resistance in different pollen beetle populations (A) in vivo based on LC_{50}-values for* lambda-*cyhalothrin obtained in an adult vial test (24 h), and (B) in vitro based on 4-OH-deltamethrin formation in microsomal preparations. The level of CYP6BQ23 transcripts was determined in this study in pollen beetle populations frozen at -80 °C and recently characterized for pyrethroid resistance in another study (see Chapter 2).*

Table 9 *Fold change in expression of ten P450 genes in pyrethroid resistant (r) and susceptible (s) strains of* Meligethes aeneus.

Strain		**ΔΔCq (fold) ± SEM**									
		CYP6BQ23	CYP6-like 1	CYP6-like 2	CYP4-like 1	CYP4-like 2	CYP4-like 3	CYP4-like 4	CYP4-like 5	CYP4-like 6	CYP4-like 7
70-10[a]	s	1 ± 0.501	1 ± 0.122	1 ± 0,218	1 ± 0.362	1 ± 0.119	1 ± 0.272	1 ± 0.163	1 ± 0.249	1 ± 0.229	1 ± 0.163
127-10	s	1.45 ± 0.224	0.382 ± 0.08	0.159 ± 0.029*	0.455 ± 0.2	0.204 ± 0.013*	0.469 ± 0.166	0.102 ± 0.016**	2.26 ± 0.583*	0.52 ± 0.063	0.185 ± 0,004**
8-10	r	491 ± 169***	1.44 ± 0.173	0.503 ± 0.04	1.07 ± 0.669	0.687 ± 0.181	0.625 ± 0.073	0.495 ± 0.06	1.67 ± 0.403	1.02 ± 0.023	0.678 ± 0.042
57-10	r	548 ± 82.0***	0.874 ± 0.052	0.632 ± 0.039	1.18 ± 0.526	1.10 ± 0.247	0.705 ± 0.096	0.503 ± 0.04	2.42 ± 0.682	1.22 ± 0.122	0.743 ± 0.045
68-10	r	337 ± 87.0***	0.513 ± 0.105	0.725 ± 0.253	0.307 ± 0.042	1.10 ± 0.302	0.69 ± 0.133	1.19 ± 0.064	1.80 ± 0.288	1.08 ± 0.205	0.501 ± 0.171
79-10	r	938 ± 62.6***	1.33 ± 0.22	0.911 ± 0.127	2.29 ± 1.33	0.81 ± 0.119	1.30 ± 0.104	1.49 ± 0.341	1.51 ± 0.476	0.669 ± 0.079	0.711 ± 0.051
96-10	r	493 ± 81.5***	1.58 ± 0.559	0.433 ± 0.124	4.39 ± 1.72	0.979 ± 0.115	0.834 ± 0.05	0.862 ± 0.105	3.23 ± 1.63	1.59 ± 0.5	1.06 ± 0.22
106-10	r	549 ± 117***	0.878 ± 0.288	0.584 ± 0.111	2.57 ± 1.24	0.861 ± 0.095	1.21 ± 0.382	0.682 ± 0.07	126 ± 0.325	0.742 ± 0.03	0.807 ± 0.155
120-10	r	269 ± 85.2***	2.15 ± 0.943	0.627 ± 0.187	2.33 ± 0.995	0.63 ± 0.193	1.74 ± 0.26	1.03 ± 0.064	1.19 ± 0.492	1.04 ± 0.125	1.02 ± 0.192

[a] Reference strain 70-10 (Ukraine, susceptible);

Significance: *** P value < 0.001; ** P value < 0.01; *P value < 0.05 (all other changes in gene expression are not significantly different from reference strain 70-10)

Table 10 *Relation between CYP6BQ23 expression and pyrethroid resistance in Meligethes aeneus.*

Strain	Country	LC_{50}, ng cm^{-2} (field rate %)[b]	95% FL[c]	RR[d]	MCq_E[e]	$_{\Delta\Delta}Cq$[f]	Expression SEM	P-value
70-10[a]	Ukraine	0.1 (0.1)	0.1-0.1	1	27.52	1	0.164	N/A
91-11	Ukraine	Not tested	-	-	27.32	1.085	0.192	> 0.05
127-10	Finland	0.8 (1.1)	0.4-1.4	8	26.76	1.630	3.869	> 0.05
84-09	Austria	0.8 (1.1)	0.6-1.2	8	25.18	4.541	0.677	< 0.001
67-09	Austria	1.0 (1.4)	0.8-1.4	10	24.39	15.633	5.153	< 0.01
128-10	Finland	1.2 (1.6)	0.4-2.8	12	25.51	10.482	0.190	< 0.01
107-10	Czechia	3.7 (5)	2.3-6.1	37	21.89	107.172	7.207	< 0.0001
8-10	France	15.5 (20.6)	4.9-45	155	18.59	633.539	107.105	< 0.0001
120-10	Sweden	16.2 (21.6)	11.9-21.9	162	19.66	490.672	37.573	< 0.0001
96-10	Poland	23.1 (30.8)	8.8-57.5	231	18.17	750.482	8.741	< 0.0001
106-10	Czechia	36.9 (49.2)	18.8-69.4	369	18.49	803.836	54.880	< 0.0001
68-10	Germany	38.3 (51.1)	13.1-108.8	383	19.75	325.105	14.796	< 0.0001
82-10	Germany	50.6 (67.4)	25.5-93.8	506	18.68	613.662	37.549	< 0.0001

[a] Susceptible reference strain

[b] Rounded LC_{50} values for *lambda*-cyhalothrin in an adult vial test (data taken from [10]); the manufacturer recommended field rate for lambda-cyhalothrin is 7.5 g ha^{-1}

[c] 95% fiducial limits

[d] resistance ratio (LC_{50} value of strain x divided by LC_{50} value of strain 70-10)

[e] MCq_E = Mean Efficiency Corrected Cq

[f] $_{\Delta\Delta}Cq$ = fold change in expression.

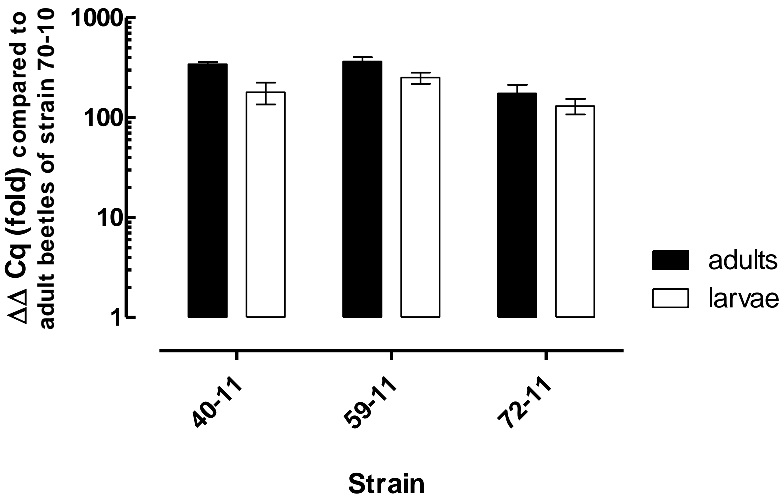

Fig. 35 *CYP6BQ23 fold change expression in adults and larvae of pyrethroid resistant pollen beetle (collected in Germany in 2011) compared to a highly susceptible strain 70-10 (Ukraine, data not shown). Data are mean values ± SEM (n=4).*

4.3.3 Gene copy number

Quantitative PCR was used to compare *CYP6BQ23* gene copy number in six pollen beetle strains, i.e. 70-10 (s), 25-10 (r), 79-10 (r), 106-10 (r), 120-10 (r) and 127-10 (s) using genomic DNA as a template. Data were normalized using two genes: *M. aeneus* actin and tubulin as described above. Even though the exact copy number for these reference genes is not known they have been used due to the lack of other sequence information, because the method is based on relative (s vs. r) rather than absolute quantification. No significant difference between pyrethroid susceptible and resistant strains was observed (fold change in copy number range from 0.985 to 1.461 for tubulin, and 0.956 to 1.522 for actin), indicating that the *CYP6BQ23* gene is most likely not amplified or duplicated.

4.3.4 CYP6BQ23 cDNA characterization

The partial sequence of ~390bp of *CYP6BQ23* obtained by degenerate PCR and represented by 10 clones of the resistant strain 79-10 was subjected to 3' and 5' RACE to obtain the complete cDNA sequence. The resulting CYP6BQ23 cDNA sequence (GenBank KC840055.1) contains an open reading frame of 1566 bp open reading frame (ORF) encoding a putative protein containing 522 amino acid residues (Fig. 36). Its calculated molecular weight is 59,460 Da and the predicted isoelectric point is 8.2. A BLASTp search indicated that CYP6BQ23 shows highest pairwise amino acid similarity with *T. castaneum* CYP6BQ10 (GenBank NP_001164249), i.e. 53 % identity. The amino acid alignment revealed that the encoded protein contains a hydrophobic N- terminal domain that likely acts as a transmembrane anchor typical for microsomal monooxygenases. Furthermore the deduced amino acid sequence contains a number of conserved domains characteristic for P450

proteins such as the WxxxR motif (helix C), the oxygen binding motif of helix I (A/GGxE/DTT/S), the helix K motif (ExLR), the PxRF motif located after helix K and the heme-binding ‘‘signature’’ motif (PFxxGxxxCxG), as well as 6 predicted substrate recognition sites (SRS). Variation in the sequence of *CYP6BQ23* in several pollen beetle strains was examined by both direct sequencing and by cloning and sequencing a ~2040 bp fragment containing the full length ORF encoding for *CYP6BQ23* and most of the 5’ and 3’UTR. The obtained fragments showed a 98.7 % and 99.6 % pairwise identity at nucleotide and amino acid level, respectively. In total seven non-synonymous SNP´s were detected resulting in amino acid changes at positions 96, 156, 160, 174, 251, 420 and 443, but no obvious link to pyrethroid resistant or susceptible phenotypes could be found (Fig. 36; supplemantary file 3).

Fig. 36 *Deduced amino acid sequence of* Meligethes aeneus *CYP6BQ23. Amino acid substitutions are indicated by a grey box below the sequence. The predicted transmembrane region is indicated by an arrow (position 12). Conserved domains common to cytochrome P450s such as the helix C motif (position 135), the helix I motif (position 324), the helix K motif (position 382), the PXRF motif (position 438) and the heme binding motif (position 456) as well as proposed substrate recognition sites (SRS) are indicated. Boxed amino acid residues constitute the binding site of the CYP6BQ23 protein model, and are defined by a distance of ≤3.5Å from any atom of both deltamethrin and tau-fluvalinate substrates docked to the active site.*

4.3.5 Functional expression of CYP6BQ23 and metabolism studies

CYP6BQ23 was recombinantly co-expressed with cytochrome P450 reductase (CPR) of *M. domestica* in Sf9 cells using a baculovirus expression system. The reduced CO-difference spectrum of CYP6BQ23 microsomes showed a distinct peak at 450.3nm. The prepared microsomes contained a P450 concentration of 193 nmol mg^{-1} protein (data not shown). The functional activity of CYP6BQ23 was tested with a broad range of fluorescent model substrates and revealed that the recombinantly expressed protein is catalytically active (Fig. 37). The specific activity of CYP6BQ23 was highest with the artificial substrate BOMFC, i.e. 50.5 ± 0.13 pmol min^{-1} mg^{-1} protein (equal to 0.52 ± pmol min^{-1} $pmol^{-1}$ P450 ± 0.0231). The results seem to suggest a preference of CYP6BQ23 for bulkier molecules such as BOMFC, BFC and BOMR, whereas well-known standard substrates such as 7-ethoxycoumarin were practically not metabolized (Fig. 37).

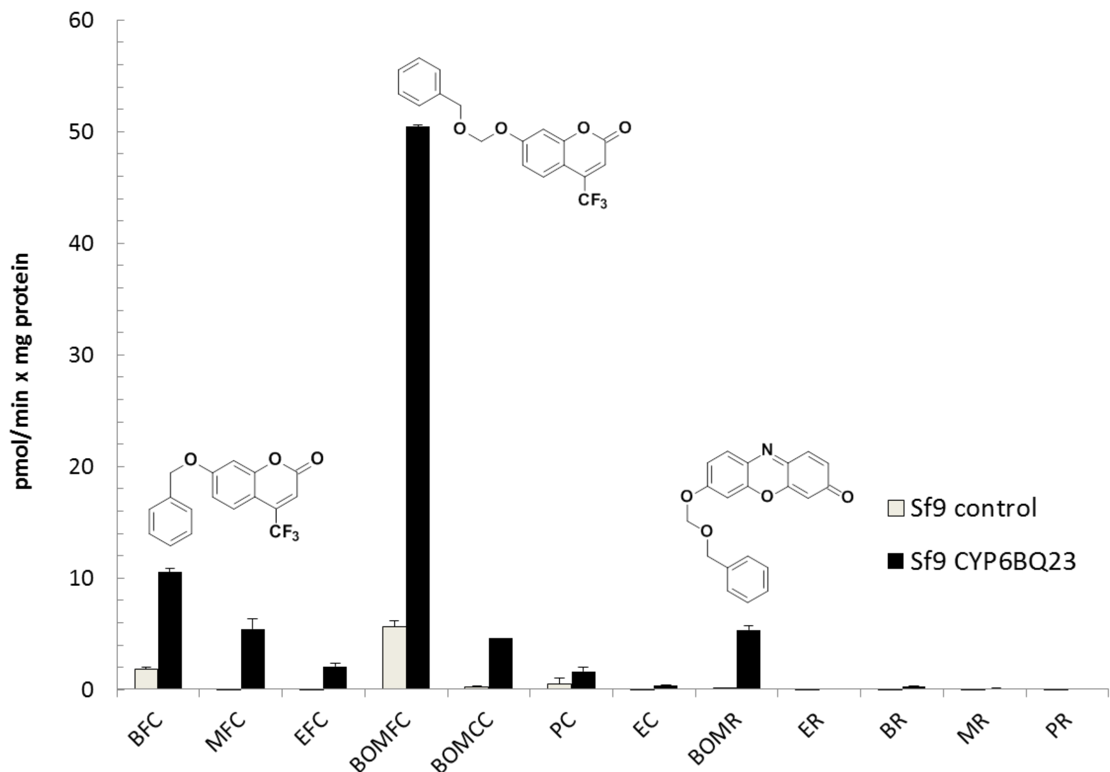

Fig. 37 *Metabolism (O-dealkylation/dearylation) of different artificial coumarin and resorufin substrates by functionally expressed CYP6BQ23. Data are mean values ± SD (n=4). Abbreviations: BFC, 7-benzyloxy-4-trifluoromethyl coumarin; MFC, 7-methoxy-4-trifluoromethyl coumarin; EFC, 7-ethoxy-4-trifluoromethyl coumarin; BOMFC, 7-benzyloxymethoxy-4-trifluoromethyl coumarin; BOMCC, 7-benzyloxymethoxy-3-cyano coumarin; PC, 7-n-pentoxy coumarin; EC, 7-ethoxy coumarin; BOMR, 7-benzyloxymethoxy resorufin; ER, 7-ethoxyresorufin; BR, 7-benzyloxyresorufin; MR, 7-methoxyresorufin; PR, 7-n-pentoxyresorufin.*

The metabolism of deltamethrin was measured by LC-MS/MS detection and quantification of its metabolite 4-hydroxy deltamethrin. The detection limit under the analytical conditions chosen for both deltamethrin and its 4-hydroxy metabolite was 0.1 ng ml^{-1}, so even very low metabolic rates could be detected. CYP6BQ23 microsomes incubated with deltamethrin in the absence of NADPH (even for prolonged times) did not show any metabolic activity as no 4-hydroxy deltamethrin was detected (Fig. 38), whereas incubations in the presence of NADPH resulted in the formation of 4-hydroxy deltamethrin (0.494 ± 0.039 pmol min^{-1} $pmol^{-1}$ P450). Based on protein content Sf9 microsomes containing CYP6B23/CPR metabolized deltamethrin at a rate of approx. 60 pmol min^{-1} mg^{-1}, whereas virus-control (+CPR) Sf9 microsomes show a fairly low turnover of 2.05 ± 0.14 pmol min^{-1} mg^{-1} protein, i.e. 30-fold lower efficiency. This result confirms the metabolism of deltamethrin by recombinantly expressed CYP6BQ23.

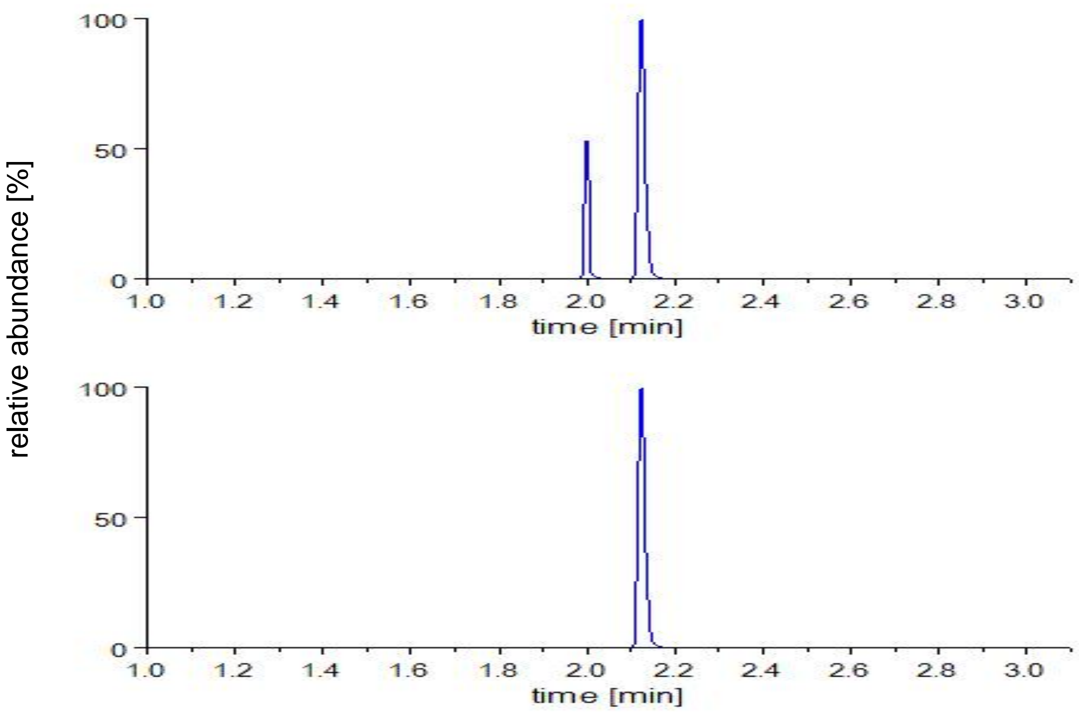

Fig. 38 *UPLC-MS analysis of deltamethrin and its 4-hydroxy metabolite after incubation with microsomes isolated from Sf9-cells recombinantly expressing CYP6BQ23: Incubation of 2.5 nmol deltamethrin for 60min in the presence of NADPH (top); incubation of 2.5 nmol deltamethrin for 240 min in the absence of NADPH (bottom).*

The rate of deltamethrin hydroxylation by recombinantly expressed CYP6BQ23 is time-dependent and followed Michaelis–Menten kinetics in response to deltamethrin concentration resulting in a K_m value of 9.51 ± 1.35 µM and a catalytic activity K_{cat} of 0.917 ± 0.057 pmol min^{-1} $pmol^{-1}$ P450 (Fig. 39). In a second set of experiments utilizing Michaelis–Menten kinetics we were able to show that both *tau*-fluvalinate and *lambda*-cyhalothrin competitively inhibit the formation of 4-hydroxy deltamethrin when co-incubated with different concentrations of deltamethrin, and as a result K_m-values change dramatically (Fig. 40). The finding that *tau*-fluvalinate is likely to compete with deltamethrin binding to the catalytic site of recombinantly expressed CYP6BQ23 is supported by the fact that we were able to detect 4-hydroxy *tau*-fluvalinate, when incubating CYP6BQ23 microsomes with *tau*-fluvalinate. Analysis of samples subjected to ESI-TOF high resolution MS/MS clearly revealed the presence of 4-hydroxy *tau*-fluvalinate and several characteristic fragments (Fig. 41), providing confirmation that CYP6BQ23 is capable of metabolizing both deltamethrin and *tau*-fluvalinate by 4-hydroxylation of the pyrethroid alcohol moiety.

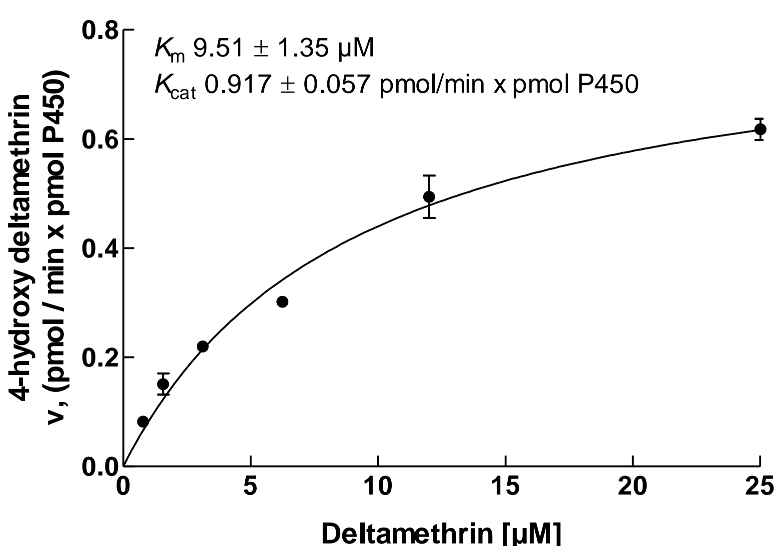

Fig. 39 *Michaelis-Menten kinetics of deltamethrin hydroxylation by recombinantly expressed CYP6BQ23 analyzed by non-linear regression. Data points are mean values ± SEM (n=3).*

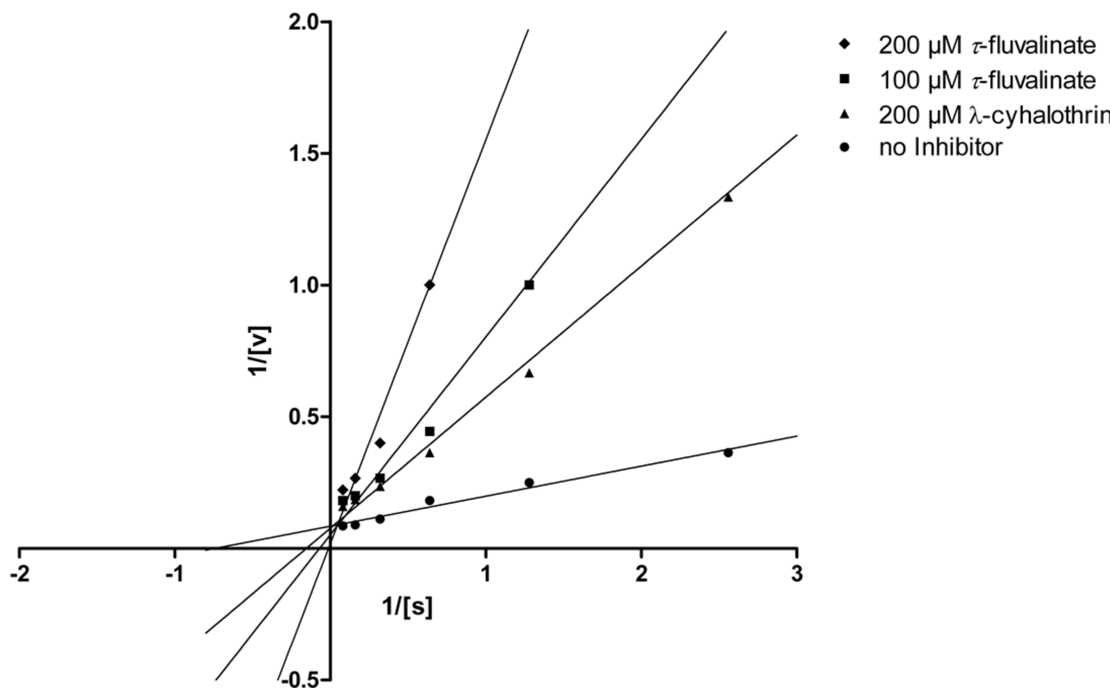

Fig. 40 *Lineweaver–Burk plot showing the competitive inhibition of CYP6BQ23-catalyzed deltamethrin hydroxylation by different concentrations of* tau-*fluvalinate and* lambda-*cyhalothrin (1h incubation at 27°C). [s] = µM deltamethrin.*

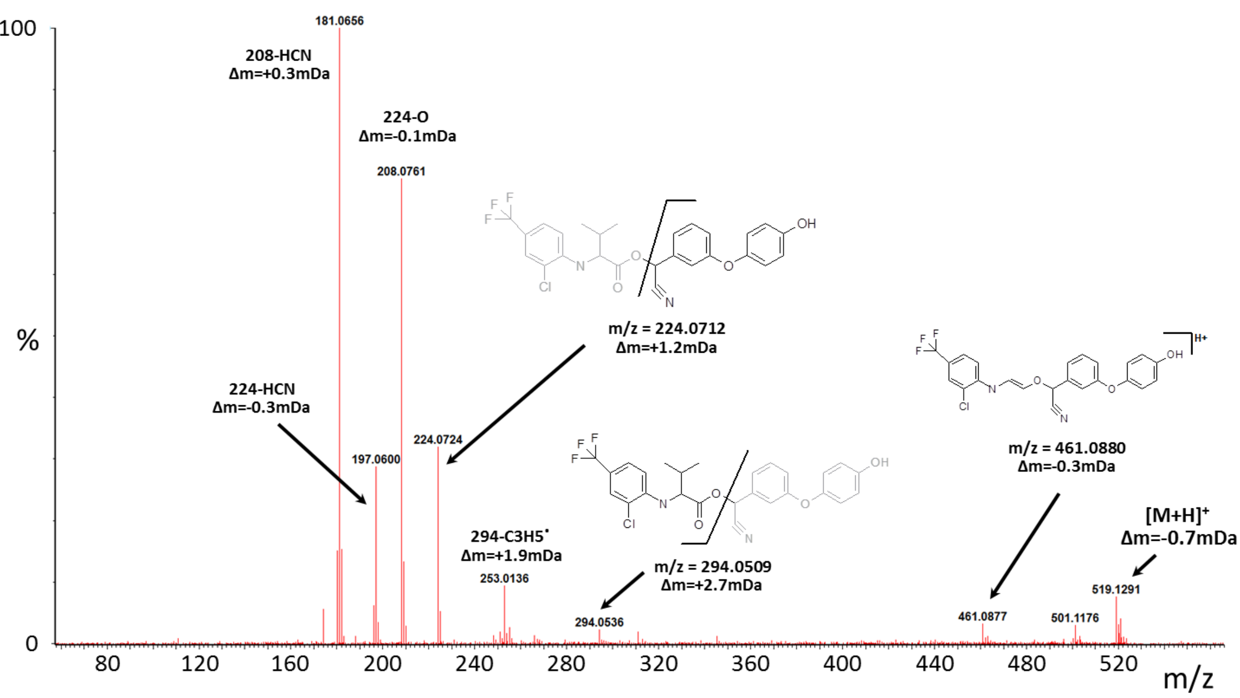

Fig. 41 *ESI-TOF high resolution MS/MS-spectrum of 4-hydroxy* tau-*fluvalinate resulting from incubations of tau-fluvalinate with recombinantly expressed CYP6BQ23. The molecular ion [M+H]$^+$ is detected at m/z 519.13. Δm is the mass difference in milli-Dalton of the detected fragment vs. its theoretical molecular mass.*

4.3.6 CYP6BQ23 modelling and substrate docking

The results obtained from microsomal incubations containing recombinantly expressed CYP6BQ23 are supported by molecular modelling studies. A protein model of CYP6BQ23 based on the crystal structure of CYP3A4 was generated and *in silico* docking studies with both deltamethrin and *tau*-fluvalinate helped to rationalize the experimental findings with regard to their hydroxylation of the phenoxybenzyl 4´ site (Fig. 42). The predicted binding site of CYP6BQ23 easily accommodates both pyrethroids and Arg112 and Phe128 of SRS1 are supposed to be most important in relation to the correct orientation of both substrates. However docking simulations support 4-hydroxylation as a major mechanism of deltamethrin as well as *tau*-fluvalinate metabolism, albeit the catalytic reactivity is potentially greater with deltamethrin as it docks closer to the oxygen coordinated heme center of the active site of CYP6BQ23 (Fig. 42B).

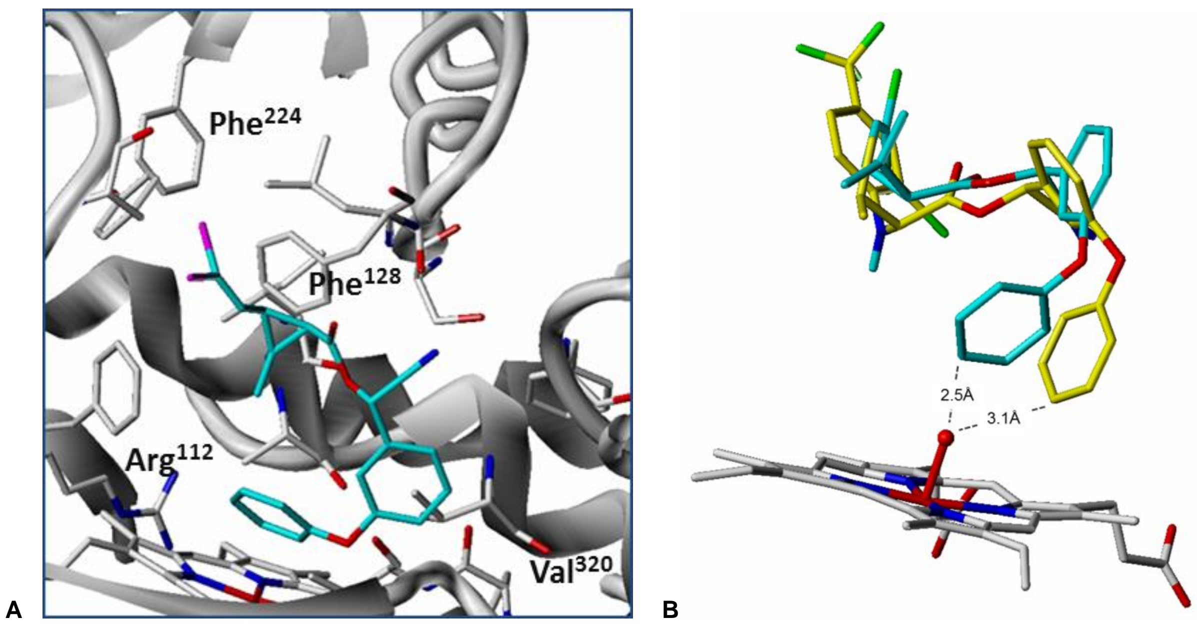

Fig. 42 *(A) Binding site model showing the predicted substrate-binding mode of deltamethrin in* Meligethes aeneus *CYP6BQ23 protein. The predicted structure of CYP6BQ23 is based on the crystal structure of human CYP3A4 (PDB-ID: 1TQN). Amino acid residues shown constitute the binding site defined by a distance of ≤3.5Å from any atom of deltamethrin shown in light blue elemental stick format on the ribbon backbone. Amino acids predicted to be most critical for the observed substrate orientation and docking are R112/F128 (SRS1), F224 (SRS2) and V320 (SRS3). (B) Docking models of CYP6BQ23 with both deltamethrin and* tau*-fluvalinate in light blue and yellow elemental stick format, respectively. For the sake of clarity the amino acid residues forming the active site within a distance of ≤3.5Å from any atom of both substrates are not shown, but the predicted binding mode and best-ranked docking for 4-hydroxylation with the distance to O coordinated to Fe indicated.*

4.4 Discussion

The main aim of this study was to identify the molecular mechanism(s) driving metabolic resistance to pyrethroid insecticides in *M. aeneus*, a destructive coleopteran pest of European oilseed rape production. As a consequence of increased resistance problems with pyrethroids, insecticides with different modes of action were tested [42], and subsequently introduced such as the neonicotinoid thiacloprid, acting agonistically on insect nicotinic acetylcholine receptors [43].

Several resistance monitoring projects revealed the spread of pyrethroid resistance in pollen beetle all over Europe [9,10]. However, it was only recently that elevated levels of microsomal monooxygenase activity coupled with increased deltamethrin metabolism was described as a mechanism of resistance which significantly correlates with pyrethroid resistance in pollen beetle collected from a wide geographic range [18]. Pyrethroid resistance in pollen beetle often easily exceeds resistance ratios of 500-fold based on pyrethroid contact bioassays [10], so the metabolic mechanism expressed by pollen beetles seems well able to confer strong levels of resistance. Similar cases of P450-mediated resistance have recently been described in other insect pests including *B. tabaci* [35,44,45], *Myzus persicae* [46], *Nilaparvata lugens* [47], *T. castaneum* [24], *M. domestica* [48] and *Trialeurodes vaporariorum* [49]. In these cases a single P450 of the CYP6 family was shown to be associated with resistance to insecticides belonging to different chemical classes, and in some cases functional evidence was provided that the candidate gene identified encodes a P450 protein which metabolizes the insecticide of interest, e.g. CYP6CM1 of *B. tabaci* was shown to metabolize imidacloprid and CYP6BQ9 of *T. castaneum* detoxifies deltamethrin [24,44].

Out of the ten P450 partial gene sequences identified in this study only one, CYP6BQ23 could be correlated with high levels of pyrethroid resistance in *M. aeneus*. Despite this finding we cannot exclude the possibility that other CYP6BQ enzymes are overexpressed in resistant strains in addition to CYP6BQ23 but were not identified by the degenerate primers used in our PCR approach. Due to the lack of transcriptomic data for *M. aeneus* we resorted to an approach recently used to successfully identify CYP6CM1 as the major P450 conferring neonicotinoid resistance in *B. tabaci* [35]. However, based on pairwise amino acid similarity CYP6BQ10 of *T. castaneum* shows the highest identity to CYP6BQ23, followed by CYP6BQ9 recently described as a brain-specific P450 conferring deltamethrin resistance [24]. Similar to recombinantly expressed CYP6BQ23, CYP6BQ9 was shown to metabolize deltamethrin to 4-hydroxy deltamethrin. Considering deltamethrin metabolism in insects, the phenoxybenzyl 4´ site is also described in other studies to be the major site of hydroxylation in deltamethrin

metabolism, for example CYP6M2 catalyzes 4-hydroxylation as a main route of deltamethrin detoxification in the malaria vector *Anopheles gambiae*, whereas hydroxymethyl deltamethrin was described as a minor metabolite [25]. The CYP6M2 K_m and K_{cat} values of 2.0 µM and 1.2 min^{-1} reported for deltamethrin are similar to those values obtained for CYP6BQ23. CYP6P9 of *Anopheles funestus* was also shown to metabolize deltamethrin as well as other pyrethroids such as permethrin and bifenthrin as measured by substrate depletion [28]. However no primary metabolites were included as standards or elucidated by structural analysis, so it remains unclear whether the observed substrate depletion of highly hydrophobic pyrethroids provided by CYP6P9 is due to sequestration or indeed metabolism. Furthermore a series of CYP9J P450s of *Aedes aegypti* were also shown to metabolize pyrethroids with CYP9J32 showing the strongest detoxification of deltamethrin and again kinetic parameters were in a similar range to those we described in this paper for CYP6BQ23 [29]. CYP6BQ23 was shown to hydroxylate the phenoxybenzyl 4´ site of deltamethrin and it would be interesting in future studies to examine its capability to sequentially metabolize deltamethrin and other pyrethroids as recently shown for CYP6M2 [25]. Other P450s such as CYP6Z8 of *A. aegypti* was recently shown to play a pivotal role in processing deltamethrin metabolites resulting from esterase mediated metabolism, i.e. phenoxybenzyl-alcohol and –aldehyde [50]. Apart from CYP6BQ23 there is only one more P450 from an agricultural pest, which was shown to metabolize pyrethroids when functionally expressed: CYP337B3 in Australian *Helicoverpa armigera* which resulted from an equal crossing-over of two parental P450s and was recently shown to 4-hydroxylate fenvalerate when functionally expressed in Ha2302 cells [27].

Only a few P450s were yet shown to metabolize tau-fluvalinate, a pyrethroid not used for vector control due to its lower overall efficacy and limited knockdown properties compared to other pyrethroids [7]. Our study presents the first description of 4-hydroxylation of *tau*-fluvalinate by a functionally expressed P450 derived from a major agricultural pest. However 4-hydroxylation was recently described by CYP9Qs from *Apis mellifera* in order to investigate the selectivity of *tau*-fluvalinate as it is particularly used for *Varroa* mite control in bee hives without affecting honeybees [51]. The metabolic fate of *tau*-fluvalinate is well understood in vertebrates such as rats and P450 mediated formation of 4-hydroxy tau-fluvalinate is an important initial step in its degradation [52]. It is interesting that our CYP6BQ23 modelling coupled with substrate docking simulations for both deltamethrin and *tau*-fluvalinate suggests to some extent a lower catalytic activity towards the latter due to the higher distance of the phenoxybenzyl 4´site to the heme iron. It has been shown that *tau*-fluvalinate is less affected by microsomal oxidation in pollen beetles expressing high resistance to *lambda*-cyhalothrin, however, cross-resistance between these insecticides has been described, albeit at a

somewhat lower level than between deltamethrin and *tau*-fluvalinate [18]. It would be interesting therefore to look for additional P450s in *M. aeneus* being more specific for tau-fluvalinate, as increasing resistance to this pyrethroid was recently reported in some German populations [20]. In relation to this it has been recently shown that Danish and Swedish *M. aeneus* populations compromise field efficacy of tau-fluvalinate against pollen beetle by expressing *kdr*-like target-site resistance in combination with elevated levels of oxidative detoxification [17].

The metabolism profile of fluorescent model substrates by CYP6BQ23 suggests a preference for bulkier substrates being O-dearylated rather than smaller ones being O-dealkylated. CYP6BQ23 shows the highest activity with BOMFC, followed by BFC and BOMR. However it is not clear yet if these substrates could serve as non-pyrethroid probes to monitor for the presence of elevated P450 activity conferring pyrethroid resistance. BOMR was recently also shown an excellent substrate for functionally expressed CYP6BQ9 metabolizing deltamethrin in *T. castaneum* [24]. STEVENSON *et al.* [29] also investigated the metabolism of a number of fluorescent substrates by *A. aegypti* and *A. gambiae* P450s and concluded that non-pyrethroid metabolizing CYP6Z2 showed a marked preference for smaller probe substrates which was in contrast to pyrethroid-metabolizing CYP6P3 and CYP6M2 which preferred bulkier substrates. Recently BFC was also demonstrated to be a preferred substrate for CYP6Z8 of *A. aegypti* [50].

CYP6BQ23 is several hundred folds overexpressed suggesting a fairly high concentration in pollen beetle microsomes resistant to pyrethroids. This may be a mechanism of compensation for the somewhat lower catalytic rate of deltamethrin detoxification of this P450 compared to other insect P450s especially those from mosquitoes.. The molecular mechanisms explaining the evolutionary origin of CYP6BQ23 and those driving the constitutive overexpression of CYP6BQ23 in resistant *M. aeneus* are not known, but we show that overexpression does not result from gene amplification, a mechanism shown to drive overexpression of CYP6CY3 in neonicotinoid resistant *M. persicae* [46]. Similarly tandemly duplicated CYP6P9 P450s in *A. funestus* were shown to drive pyrethroid resistance [30]. An interesting case of gene duplication and parallel evolution of *cis*-acting genomic changes by insertion of the retrotransposon *accord* resulting in the upregulation of *Drosophila melanogaster* Cyp6g1 expression, which confers resistance to DDT, has recently been reviewed [53]. Another *cis*-regulatory motif was recently described that enhances the expression of CYP9M10 in larvae of *Culex quinquefasciatus* and is associated with pyrethroid resistance. In this case the regulatory element was only present upstream of *CYP9M10* in resistant strains where it was shown to drive 10-times higher expression of a fluorescent reporter gene [54].

It is possible that the CYP6BQ23 mediated resistance mechanism in *M.aeneus* evolved independently in different geographic areas since the dispersal rate of *M. aeneus* is limited as they normally overwinter close to the sites where they feed during spring/summer time [2]. To examine this possibility and to check for overexpression of P450 genes other than CYP6BQ23 it would be useful to carry out a transcriptomic analysis of pollen beetle populations from different areas. Such an analysis may also disclose other P450s that together with CYP6BQ23 contribute to the sequential detoxification of pyrethroids in pollen beetle populations throughout Europe. In combination with the present work such studies will provide a better understanding of how to manage insecticide resistance in *M. aeneus* and so contribute to sustainable oilseed production in Europe.

Supplementary files

The supplementary files mentioned in this chapter can be found in appendix A.

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Chapter 5

Target-site resistance to pyrethroids in European populations of pollen beetle, *Meligethes aeneus* F.

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Abstract

Pollen beetle, *Meligethes aeneus* F. (Coleoptera: Nitidulidae) is a major univoltine pest of oilseed rape in many European countries. Winter oilseed rape is cultivated on several million hectares in Europe and the continuous use of pyrethroid insecticides to control pollen beetle populations has resulted in high selection pressure and subsequent development of resistance. Resistance to pyrethroid insecticides in this pest is now widespread and the levels of resistance are often sufficient to result in field control failures at recommended application rates. Recently, metabolic resistance mediated by cytochrome P450 monooxygenases was implicated in the resistance of several pollen beetle populations from different European regions. Here, we have also investigated the possible occurrence of a target-site mechanism

caused by modification of the pollen beetle *para*-type voltage-gated sodium channel gene. We detected a single nucleotide change that results in an amino acid substitution (L1014F) within the domain IIS6 region of the channel protein. The L1014F mutation, often termed *kdr*, has been found in several other insect pests and is known to confer moderate levels of resistance to pyrethroids. We developed a pyrosequencing-based diagnostic assay that can detect the L1014F mutation in individual beetles and tested more than 350 populations collected between 2006 and 2010 in 13 European countries. In the majority of populations tested the mutation was absent, and only samples from two countries, Denmark and Sweden, contained pollen beetles heterozygous or homozygous for the L1014F mutation. The mutation was first detected in a sample from Denmark collected in 2007 after reports of field failure using *tau*-fluvalinate, and has since been detected in 7 out of 11 samples from Denmark and 25 of 33 samples from Sweden. No super-*kdr* mutations (e.g. M918T) known to cause resistance to pyrethroids were detected. The implications of these results for resistance management strategies of pollen beetle populations in oilseed rape crops are discussed.

5.1 Introduction

Pollen beetle, *Meligethes aeneus* F. (Coleoptera: Nitidulidae) is one of the major pests in European oilseed rape and can cause significant economic damage without chemical control measures [1]. For almost two decades, pollen beetle populations in Europe have been effectively controlled by synthetic pyrethroid insecticides that act on voltage-gated sodium channels in the insect central nervous system [2,3]. However, the lack of other available insecticide classes with different modes of action and overlapping pyrethroid treatment windows for stem weevil control has resulted in strong pressure for the selection of resistance. As a result, pyrethroid resistance is now widespread among European pollen beetle populations [3-8]. Resistance monitoring initiatives based on adult-vial bioassays using the reference pyrethroid *lambda*-cyhalothrin that were carried out between 2007 and 2010 revealed the presence of pyrethroid resistant populations in almost all European countries sampled, including Germany, France, Poland, UK, Denmark, Sweden and others [7,8]. Fortunately, resistance has not yet been reported for the newer insecticides with different modes of action such as thiacloprid, a recently introduced neonicotinoid insecticide for pollen beetle control in winter/spring oilseed rape, or pymetrozine and indoxacarb, known to act as antifeedant and sodium channel blockers, respectively [9].

Resistance to pyrethroids in pollen beetles is relatively broad spectrum across the whole chemical class, however some pyrethroids such as *tau*-fluvalinate and etofenprox were recently shown to be less affected by cross-resistance issues and have therefore become first choice for control in some countries [10]. Bioassays have also shown moderate levels of

cross-resistance to *tau*-fluvalinate in pollen beetle populations from Denmark and Sweden [10,11], while HEIMBACH *et al.* [12] showed a decrease in the susceptibility of German populations to the non-ester pyrethoid etofenprox over a period of 5 years.

The intense use of pyrethroids against chewing and sucking pest species in many agricultural cropping systems, as well as their use to control disease vectors and urban pests, has resulted in numerous cases of resistance over the past 30 years. Two main types of mechanism are known to be responsible for resistance; one based on modification of the pyrethroid target site and the other caused by enhanced metabolic detoxification from elevated levels of esterases and cytochrome P450 monooxygenases [13].

The biochemical mechanisms underlying pyrethroid resistance in pollen beetles have recently been investigated. The synergistic action of piperonyl butoxide (PBO, a metabolic enzyme inhibitor) in combination with pyrethroids provided initial evidence for the involvement of cytochrome P450 monooxygenases [14,15]. Despite the lack of a correlation between pyrethroid resistance level and elevated cytochrome P450 activity based on biochemical assays with the artificial substrate 7-ethoxycoumarin [14,15], the involvement of monooxygenases in pyrethroid resistance has been clearly demonstrated by the hydroxylation of deltamethrin by pollen beetle microsomes [15]. In this study, the maximum rate of deltamethrin hydroxylation by pollen beetle microsomes correlated well with the level of pyrethroid resistance and was inhibited by both PBO and tebuconazole. Furthermore it was demonstrated that *tau*-fluvalinate and *lambda*-cyhalothrin competitively inhibited the formation of 4-hydroxy deltamethrin, thus confirming pyrethroid cross-resistance based on microsomal oxidation [15].

Target site resistance to pyrethroids is caused by point mutations in the gene for the voltage-gated sodium channel, leading to amino acid substitutions within the channel protein that affect the binding of pyrethroids [16-18]. Two mutations, L1014F and M918T, were originally described in pyrethroid resistant *Musca domestica* and linked to strains phenotypically classified as knock-down resistant (*kdr*) and super-kdr (*s-kdr*), respectively [17]. Since then, the L1014F mutation (or variants such as L1014S) has been identified in a range of different pest species and typically confers moderate (10-20-fold) levels of resistance to all pyrethroids. M918T and other super-kdr like mutations (eg T929I) have also been discovered in a range of pests, and these confer much higher levels of resistance (several 100-fold) [17,18]. These mutations are located in the domain II S4-S5 linker and S5, S6 transmembrane regions of the channel protein and are thought to form part of a hydrophobic binding site for the pyrethroids [18].

The possible role of target site modification in pyrethroid resistance has not yet been investigated in pollen beetles. The objective of this study was to investigate the presence and distribution of *kdr* and *s-kdr* mutations in pyrethroid resistant pollen beetle populations from Europe. To achieve this, a partial sequence of the para-type sodium channel of *M. aeneus* spanning the domain II region containing the *kdr* and *s-kdr* mutation sites was PCR amplified and sequenced. We identified the same nucleotide mutation that causes the *kdr* L1014F substitution in a resistant strain from Denmark and used this to develop a diagnostic assay for the mutation based on SNP-genotyping by pyrosequencing technology. Individuals of several hundred pyrethroid-resistant populations surviving a pyrethroid diagnostic dose (according to [7,8]) in an adult-vial bioassay were subjected to pyrosequencing analysis for *kdr* and the results geographically mapped. The consequence for regional resistance management strategies where target-site resistance was found is discussed.

5.2 Materials and methods

5.2.1 Insect populations

More than 350 European pollen beetle populations were collected in oilseed rape fields between April and June of 2006-2010 and bioassayed for pyrethroid resistance using a recently described adult vial test [7,8]. The test is based on two concentrations of the reference pyrethroid *lambda*-cyhalothrin, 75 ng cm^{-2} and 15 ng cm^{-2} coated onto the inner glass surface and representing 100 % and 20 % of the recommended field rate, respectively (for a detailed description of the method refer to [8]). Many of the populations investigated here were also included in two recently published studies on the status of pyrethroid resistance in pollen beetle in Europe [7,8]. At least 30 populations sampled in 2009 and 2010 from Sweden were also tested with *tau*-fluvalinate due to an increased number of reports of reduced field efficacy with this pyrethroid [11]. Resistance to *tau*-fluvalinate was checked by using the same adult vial test design, based on 100 % and 20 % of the recommended field rates 480 ng cm^{-2} and 96 ng cm^{-2}, respectively. Beetles that survived the bioassay after 24 h at 100 % of the field-recommended rate of either *lambda*-cyhalothrin or *tau*-fluvalinate were stored at -80 °C for subsequent molecular diagnostics. Populations that did not survive the 100 % field-rate of either pyrethroid were discarded.

5.2.2 Amplification and sequencing of *M. aeneus* para-type sodium channel gene fragment and kdr-genotyping using Sanger sequencing

Individual adult pollen beetles were ground in liquid nitrogen and genomic DNA (approx. 1 µg per adult) was extracted using DNAzol purification reagent (Invitrogen) according to the supplier's recommended protocol. Domain II sodium channel gene fragments

were PCR amplified from 100 ng aliquots of gDNA using primers designed against a partial sequence of the pollen beetle para gene deposited in GenBank (sequence accession AF354457, see primer sequences PB1-4 in Table 11). Two rounds of PCR were carried out using various combinations of primers PB1-4 (0.5 µM) in 25 µL reactions containing 1 x Taq enzyme reaction mix (Promega, UK) with standard cycling conditions of 94 °C for 2 min, followed by 30 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min. The ~510 bp sodium channel gene fragments generated in these PCR reactions were ethanol precipitated to remove excess primer and directly sequenced with internal primers (PB5 and PB6) using ABI big-dye terminator reaction kits on a 310 genetic analyser (Applied Biosystems). This was done for a number of individual beetles collected in Denmark in 2007 in order to double-check the (SS/SR/RR) *kdr* results obtained by pyrosequencing. Sequences were analysed using the VectorNTI software package (Invitrogen). Sequence alignment graphics were done by using Geneious v5.5 (www.geneious.com).

Table 11 *Details and sequences of primers used to analyse for* kdr (s-kdr) *mutation.*

Name	**Sequence 5' - 3'**
Py-KDR-Seq1	CACTGTGGTTATCGGTAAT
Py-KDR-Seq2	GCCACTGTGGTTATCGGT
Py-KDR-F	ATGTGTCCTGTATTCCCTTC
Py-KDR-R	[btn]GCTGGATGATCCAAAATTG
Py-s-kdr-Seq	TCTAAATTTACTTATATCCA
Py-s-kdr-F	GGCCGACTCTAAATTTACTTATAT
Py-s-kdr-R	[btn]TCCTTACCCGTATAATTTTTGCC
PB1	TGGCCGACTCTAAATTTACTT
PB2	CTCTAAATTTACTTATATCCAT
PB3	TTGGTGCTGATAAGCTGGATG
PB4	CTGGATGATCCAAAATTGCTC
PB5	GACCACGATCTACCTCGTTG
PB6	ACCAACATACAGTCCCACATC

5.2.3 Pyrosequencing *kdr/s-kdr* diagnostic assay

Pyrosequencing is a DNA sequencing-by-synthesis technique enabling real-time detection of nucleotides forming base pairs in an amplified DNA template strand using an enzyme-cascade finally resulting in bioluminescence signals [19,20]. Individual adult pollen beetles were ground in liquid nitrogen and genomic DNA (approx. 1 µg per adult) was extracted using DNeasy plant kit (Qiagen) or DNAdvance Tissue Kit (Agencourt) according to the supplier's recommended protocol. The domain II sodium channel gene fragment was amplified by PCR from 50 ng aliquots of gDNA using two primers (Table 11, *kdr*: Py-KDR-F &

PyKDR-R; s-kdr: Py-s-kdr-F & Py-s-kdr-R) designed with "Assay Design Software" (PSQ-Biotage AB, now Qiagen) by utilizing the partial sequence of the pollen beetle para gene detailed above (see primer sequences in Table 11). The pyrosequencing protocol comprised 45 PCR cycles with 0.5 µM forward and biotinylated reverse primer in 50 µL reaction mixture containing 1 x Taq enzyme reaction mix (HotstarTaq Master Mix, Qiagen) and cycling conditions of 95 °C for 10 min, followed by 45 cycles of 95 °C for 45 s, 49 °C for 45 s and 72 °C for 1 min, and a final incubation at 72 °C for 5 min. The single strand DNA preparation required for pyrosequencing was carried out using the Vacuum Prep Tool (Biotage AB) in combination with streptavidin coated beads (Streptavidin Sepharose) to separate the biotinylated reverse strand of the PCR products. The pyrosequencing reactions were carried out according to the manufacturer's instructions using one of two different sequence-primers for either *kdr* or *s-kdr* genotyping (Table 11) and the PSQ 96 Gold Reagent Kit (Biotage AB). The genotypes were analysed using the supplied SNP Software (Biotage AB).

5.3 Results

5.3.1 Detection of mutations in para-type sodium channel fragments

A 514 bp fragment of the *M. aeneus para*-type sodium channel gene was PCR amplified from genomic DNA extracted from individual pollen beetles collected in Denmark in 2007 (Fig. 43). This fragment encodes the domain IIS4-IIS6 region of the sodium channel *alpha* subunit which contains five of the putative mutation sites previously associated with *kdr/s-kdr*-type pyrethroid resistance in a range of insect species, i.e. M918, L925, T929, L932 and L1014 [16]. This fragment also contains two short intron sequences (64 bp and 62 bp), the positions of which are also conserved across species [18]. The amino acid sequence of this fragment of the para-type sodium channel of *M. aeneus* shows close homology to that of other insects, with over 90 % direct amino acid identity in this region of the protein (Fig. 44). Based on the comparison of the aligned coleopteran sequences including those from *Leptinotarsa decemlineata* and *Tribolium castaneum* the amino acid identity in this region of the protein is close to 100 %.

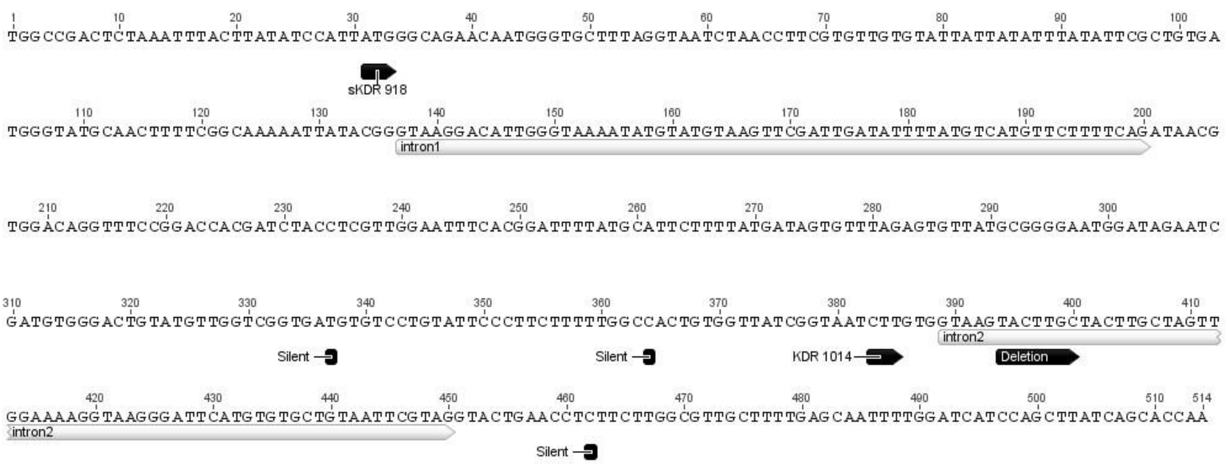

Fig. 43 *Partial nucleotide sequence of a 514 bp genomic fragment of the* para-*type sodium channel gene of* Meligethes aeneus, *spanning the region which includes the* kdr- *and* s-kdr *mutation sites. The* s-kdr *(M918, ATG) and* kdr *(L1014, CTT) mutation sites are marked below the sequence, as well as three silent mutation sites and one 7 bp indel (TACTTGC) in the intron downstream of the* kdr *site.*

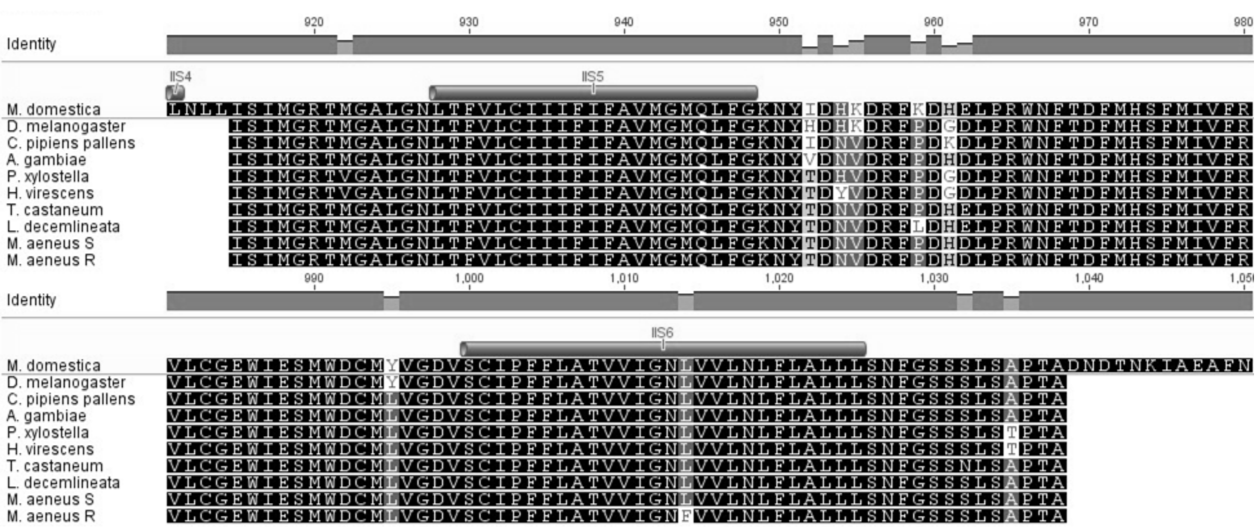

Fig. 44 *Multiple sequence alignment of para-type sodium channel region DII S4-6 sequences from different insect species. Conserved identical amino acid residues are marked in black boxes. The sequence obtained from* M. aeneus *R (bottom) shows the L1014F mutation known to confer knock-down resistance to pyrethroids.*

A total of 14 adult beetles from the obtained Danish sample were sequenced. Those beetles were part of a population which shows only 50 % mortality at 100 % of the field-recommended rate of *tau*-fluvalinate (47.5 g ha^{-1}). The sequencing revealed two distinct

alleles of the sodium channel in the *M. aeneus* population that had been sampled; one corresponding to the 514 bp sequence shown in Fig. 43, and a second allele with a 7 bp indel downstream of the *kdr*-site in the second intron (the deleted bases are shown by an arrow in Fig. 43). Both alleles also carry up to 3 silent nucleotide polymorphisms in the coding sequence 54 bp upstream of the second intron (also marked in Fig. 43). Of the 14 adults tested, 8 were homozygous for the sequence with the 7 bp insertion (allele A), 4 were homozygous for the second allele with the 7 bp deletion (allele B), and the other 2 beetles were heterozygotes carrying both allele types.

Five of the 14 beetles tested were homozygous for the *kdr* mutation (F1014; Fig. 44), 8 were homozygous wild-type (L1014), and the remaining individual was a heterozygote (L/F1014). None of the beetles sequenced contained any of the other mutations (s-*kdr*) mentioned above and known to confer resistance to pyrethroids. Interestingly, the *kdr* mutation was not confined to one of the two allele types described above (A & B), but instead was found in both types. Of the 8 allele A homozygotes, 5 were also homozygous for L1014 (susceptible), 2 were homozygous for the F1014 mutation (*kdr*), and 1 was a heterozygote. Similarly, of the 4 allele B homozygotes, 2 were L1014 homozygotes and 2 were F1014 homozygotes. This result was suprising as it suggests that the *kdr*-like mutation has arisen independently in two different allele types.

5.3.2 Validation of target-site resistance diagnostics by pyrosequencing

The pyrosequencing diagnostic assay identifies all three *kdr* genotypes in individual beetles, designated SS (homozygous L1014), SR (heterozygous L/F1014) and RR (homozygous F1014). The PCR reaction carried out to amplify the template DNA for *kdr*-pyrosequencing produced a ~160 bp fragment of the *M. aeneus* para-type sodium channel (from genomic DNA). For SNP analysis 10 nucleotides starting upstream the putative *kdr*-like polymorphism site (codon CTT at position 1014) were pyrosequenced using the gene specific sequence-primer Py-KDR-Seq1.

Similar to the Sanger sequencing approach described above (5.3.1), pollen beetles of the same Danish sample collected in 2007 were used to validate the pyrosequencing method. In total 16 adult beetles were individually analysed. Four of the 16 beetles tested were homozygous for the *kdr* mutation (F1014; Fig. 45), 7 were homozygous wild-type (L1014), and the remaining 5 individuals were heterozygotes (L/F1014). As shown in the pyrograms in Fig. 45 the assay successfully detects the polymorphism (C/T) at the first coding position of the triplet in position 1014. The nucleotide sequences experimentally obtained for SS, SR, and RR are 5´-<u>CTT</u>GTGGTAA-3´, <u>C/TTT</u>GTGGTAA-3´ and <u>TTT</u>GTGGTAA-3´, respectively, thus based on the sequential reaction of the nucleotides with the template DNA in the order of

which the individual nucleotides were dispensed (Fig. 45). Taking the results of both sequencing approaches together, the Danish sample collected in 2007 contained 15 (50 %), 6 (20 %) and 9 (30 %) individuals of the genotypes SS, SR and RR, respectively.

The primers designed to detect mutations at the M918T *s-kdr* site were used for parallel sequencing with each individual tested, but no mutation was observed at this site.

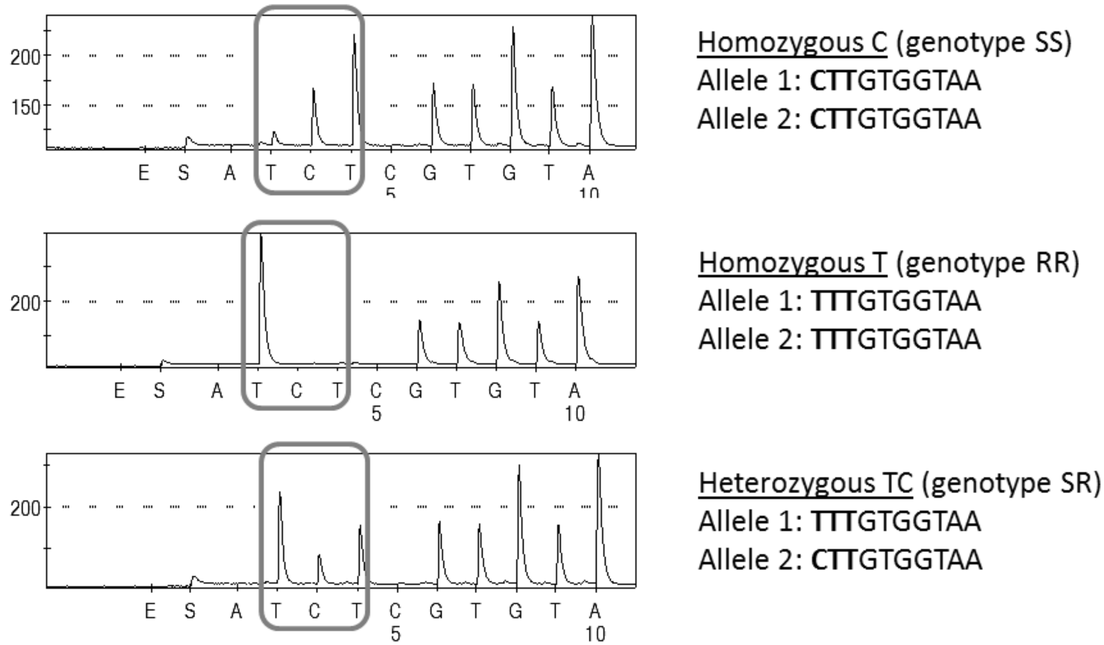

Fig. 45 *Pyrograms displaying homozygous SS, RR, as well as heterozygous SR genotypes of the mutation L1014F found in a Danish population of* Meligethes aeneus *resistant to* tau-*fluvalinate (ca. 50 % mortality at recommended field rate) and collected in 2007.*

5.3.3 Monitoring for target-site resistance and geographical distribution of the L1014F mutation in Europe

In total, more than 350 populations of *M. aeneus* were SNP-genotyped for polymorphisms at both the *kdr*- and *s-kdr* sites by pyrosequencing and mapped according to their geographic location (Fig. 46). We analyzed individuals of 45, 37, 83, 86 and 99 pyrethroid-resistant populations collected in 2006, 2007, 2008, 2009 and 2010, respectively. The populations were collected from oilseed rape in 13 countries and interestingly only samples from two Scandinavian countries, Denmark and Sweden, were found to contain the *kdr* (L1014F) mutation that confers target-site resistance. None of the pyrethroid-resistant samples collected between 2006 and 2010 in Austria, Belgium, Czech Republic, Finland, Lithuania, Latvia, Poland, Ukraine and UK contained this mutation (Table 12). Only one

population collected in 2010 out of 141 sampled in Germany between 2006 and 2010 contained a single heterozygote (SR) individual; all other beetles tested were SS genotypes (Table 12). In 2007 we detected *kdr* for the first time in 3 field-collected samples from Denmark and subsequently in 4 samples collected in 2010, confirming the spread of the mutation in Danish populations. In 2009, numerous populations from different regions of Sweden were collected that showed high levels of resistance in bioassays with *lambda*-cyhalothrin and *tau*-fluvalinate, particularly those from the south of Sweden (Malmö) (Fig. 47a). A lower proportion of resistant individuals were found in the populations collected in the middle of Sweden (Uppsala). All samples from the southern part of Sweden showed a high frequency of the *kdr* allele, but in many cases also contained susceptible genotypes. In contrast, we never found the *kdr* allele in the more northern populations, including those that were resistant to pyrethroids and able to survive 100 % of the recommended field-rate (Fig. 47b).

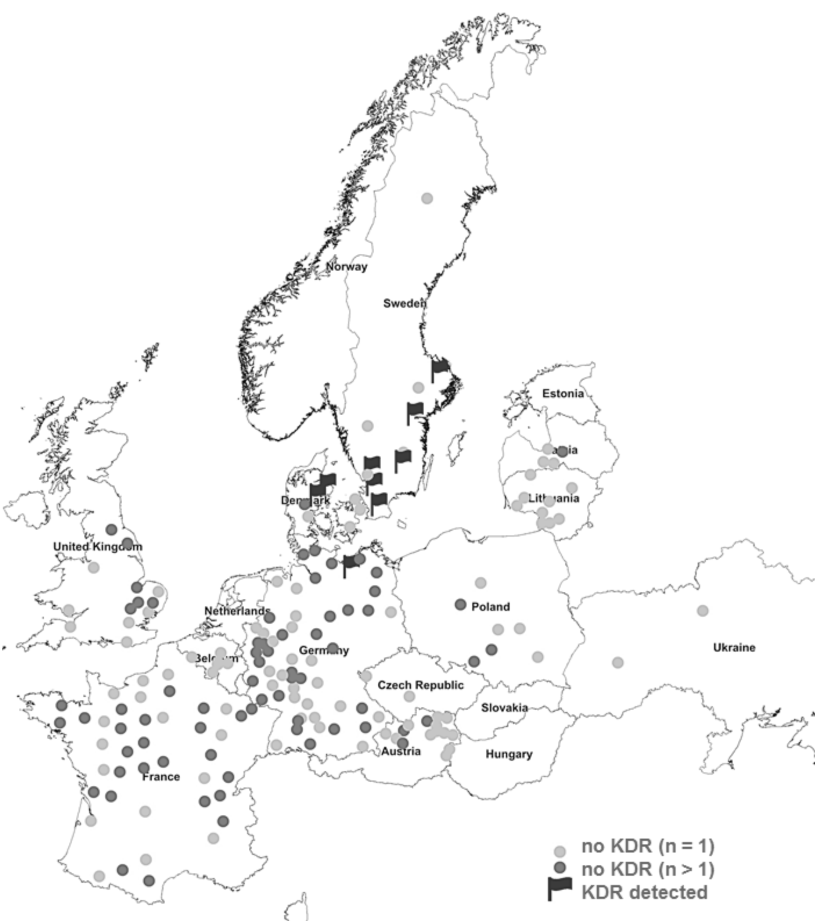

Fig. 46 *Geographical mapping of kdr-based target-site resistance in European populations of pollen beetles. In total 400 collected populations were screened between 2006 and 2010 (Table 12). Individual flags displaying the presence of kdr genotypes may include several positively tested populations. Abbreviations: (n) refers to the number of populations tested per spot.*

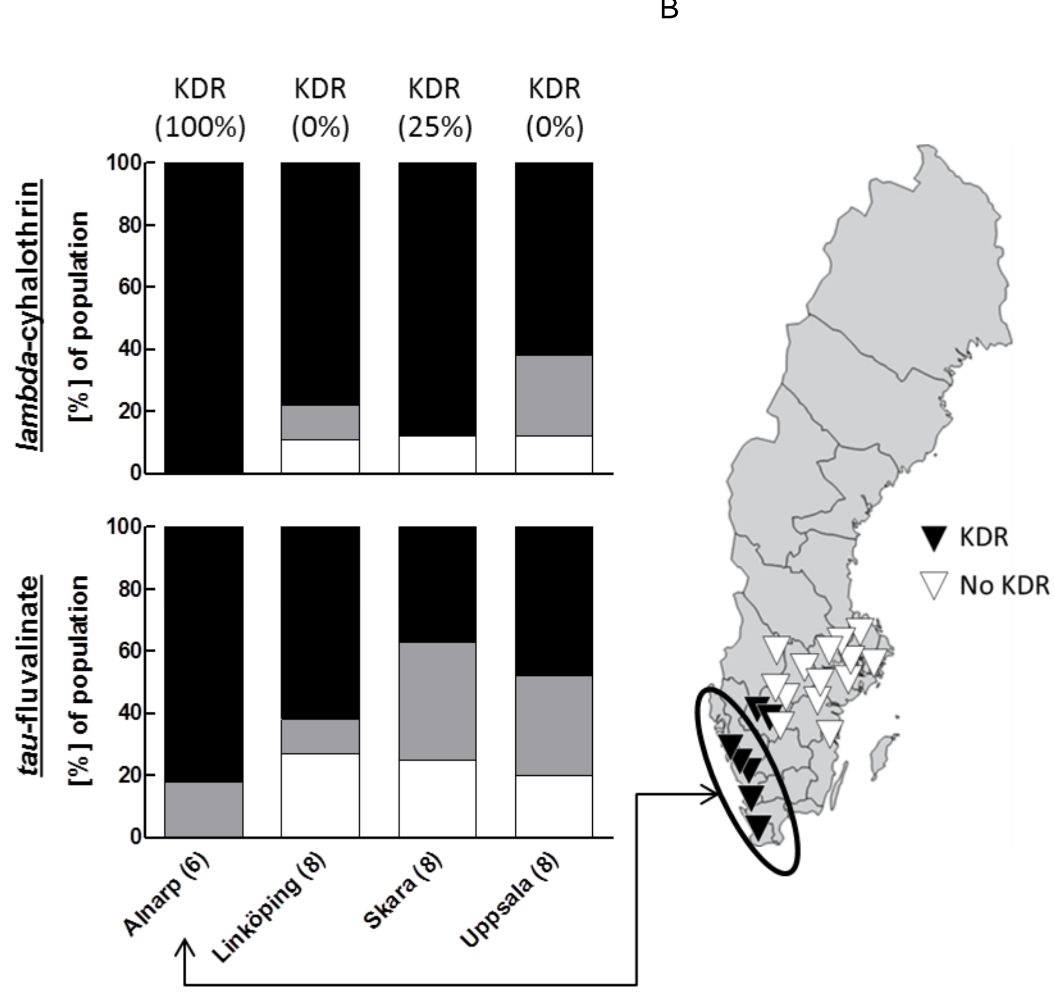

Fig. 47 *(A) Stacked-bar chart showing the proportion of 2009 collections of Swedish pollen beetle populations resistant (black), moderately resistant (grey) and susceptible (white) to* lambda-*cyhalothrin and* tau-*fluvalinate in discriminating dose bioassays. Surviving beetles were genotyped for knock-down resistance (*kdr*) and the proportion homozygous or heterozygous for the L1014F mutation is given in %. (B) Geographical mapping of the populations tested reveals a strong presence of the* kdr *resistance allele in populations sampled in southern regions.*

Table 12 *Number of pyrethroid-resistant populations genotyped for* kdr *resistance (L1014F) and collected in different European countries between 2006 and 2010. Only those beetles which survived 100 % of the field-recommended rate of either* lambda-*cyhalothrin or* tau-*fluvalinate were genotyped. Those populations listed to include SR and RR genotypes always also contained SS individuals, except one from Sweden.*

Genotype	A	B	CZ	DK	F	FIN	G	LT	LV	PL	SE	UA	UK
SS	24	6	2	7	97	3	141	9	5	16	33	2	24
SR	0	0	0	**6**	0	0	**1**	0	0	0	**12**	0	0
RR	0	0	0	**7**	0	0	0	0	0	0	**9**	0	0
Total	24	6	2	7	97	3	141	9	5	16	34	2	24

A=Austria, B=Belgium, CZ=Czech Republic, DK=Denmark, F=France, FIN=Finland, G=Germany, LT=Lithuania, LV=Latvia, PL=Poland, SE=Sweden, UA=Ukraine, UK=United Kingdom

5.4 Discussion

In this study, we demonstrate for the first time the presence of a target-site mutation (L1014F), commonly known as *kdr*, in voltage-gated sodium channels of pyrethroid-resistant pollen beetle collected in oilseed rape in Europe. This mutation has been shown to confer resistance to pyrethroids in a range of insect pests and its effect on the insect sodium channel has been functionally demonstrated [13,16,18]. However, to our knowledge *M. aeneus* is only the second coleopteran species in which this mutation has been detected, following earlier reports in populations of the Colorado potato beetle, *L. decemlineata* [21,22]. An alternative *s-kdr*-like mutation (T929I) has also been described recently in maize weevils, *Sitophilus zeamais* [23]. None of the pyrethroid-resistant pollen beetle populations analyzed in this study carried any of the *s-kdr* mutations at positions M918, L925, T929 and L932 that have been described in other insect pests [16]. Interestingly, we found in some of the amplified fragments a 7 bp deletion (TACTTGC) in the intron downstream of the *kdr*-site, which was not correlated with the presence of the *kdr* mutation. A similar indel (but 5 bp, TCACA) in the intron downstream the *kdr* mutation was recently also described in sodium channel fragments amplified from *Culex quinquefasciatus* [24]. The authors were also unable to link the indel to the presence of the mutation in pyrethroid-resistant mosquitoes.

The pyrosequencing assay developed in this study allows at least 200 beetles to be genotyped per day and is therefore a high-throughput resistance screening methodology for monitoring the spread of *kdr*-like resistance in pollen beetle. Recently a similar approach was described for dieldrin resistance monitoring in the malaria vector *Anopheles funestus* [25].

During the course of the present study we genotyped thousands of individuals but were only successful in detecting the L1014F mutation in populations collected in Denmark and Sweden, with the exception of a single heterozygote beetle from the 141 populations tested from northern Germany. Due to high levels of resistance to pyrethroids such as *lambda*-cyhalothrin, many Danish oilseed rape farmers recently switched to another pyrethroid, *tau*-fluvalinate [10]. The decision was based on the fact that *tau*-fluvalinate seemed to retain better efficacy under field conditions, partly because it is used at 6-7 times higher rates than *lambda*-cyhalothrin and also because it was shown to be less affected by the metabolic resistance mechanism of resistance selected by compounds such as deltamethrin, *lambda*-cyhalothrin and cypermethrin and caused by elevated levels of cytochrome P450 that is already widespread in European pollen beetle populations [15]. However, the continued application of this compound several times per season as described by Hansen [10] may have contributed to the selection for target-site resistance in these populations. The situation in Sweden is even more interesting, because resistant *kdr* genotypes were only detected in the very south, whereas *kdr* was not observed in samples from middle Sweden despite the fact that several populations survived 100 % of the field rate of *tau*-fluvalinate. Why target-site resistance has only evolved in Scandinavian populations remains unclear, but is perhaps related to low economic infestation thresholds (as low as 0.5 beetles per plant [6]) in these areas, triggering more pyrethroid applications and creating high selection pressure in years when high numbers of beetles migrate into winter and spring oilseed rape fields. Another contributing factor is the fact that between 1985 and 2001, i.e. for 15 years exclusively pyrethroids were used for pollen beetle control without any rotation with other compounds.

Although no target site mutations were found in the other European countries, including France, Germany and Poland, high levels of pyrethroid resistance, with ratios between 500-1000-fold, were nevertheless described in hundreds of samples collected from these countries between 2007 and 2010 [7,8]. Many of the samples collected in these countries were shown to have elevated levels of monooxygenases resulting in an enhanced metabolic detoxification of pyrethroids as demonstrated by the formation of 4-OH deltamethrin [15]. It has also been shown in other species that very high resistance ratios to pyrethroids can be explained just by metabolic mechanisms based on the overexpression of cytochrome P450s [13]. Examples include the overexpression of CYP6BQ9 in deltamethrin resistant *Tribolium castaneum* in which confers resistance ratios of up to 4000-fold to deltamethrin [26], and cytochrome P450-based pyrethroid cross-resistance in an isogenic line of *Helicoverpa armigera* confering resistance ratios of > 10,000-fold to certain pyrethroids [27]. Such examples demonstrate the effectiveness of metabolic resistance mechanisms, which expressed at high levels would suffice to render maximum application rates of a pyrethroid

completely useless for control purposes. In such cases any further selection for other mechanisms such as target-site resistance seems unlikely, unless individuals with such a mechanism exhibit strong fitness advantages sufficient to out-compete less fit individuals. However, this additional target-site mechanism would be advantageous since it affects the entire class of pyrethroid chemistry even in the absence of metabolic mechanisms of resistance. For resistance management purposes it may not be advisable to replace pyrethroids that are most affected by metabolic resistance with others that are less affected, as this may provide stronger selection pressure for rare genotypes carrying mutations in the voltage-gated sodium channel, as seen in pollen beetle populations from Denmark and Sweden. Therefore it is strongly recommended to seek for alternative modes of action for pollen beetle control and to follow the resistance management recommendations given by local experts or published annually by the Insecticide Resistance Action Committee (IRAC) [28].

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Chapter 6

A *de novo* transcriptome of European pollen beetle populations and its analysis with special reference to insecticide action and resistance

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Abstract

Pollen beetle, *Meligethes aeneus* is the most important coleopteran pest in European oilseed rape cultivation, annually infesting millions of hectares and responsible for substantial yield losses if not kept under economic damage thresholds. This species is primarily controlled with insecticides but has recently developed high levels of resistance to the pyrethroid class. The aim of this study was to provide a transcriptomic resource to investigate mechanisms of resistance. cDNA was sequenced on both Roche and Illumina platforms, resulting in a total of ~53 m reads which assembled into 43,396 ESTs. Manual annotation revealed good coverage of genes encoding insecticide target-sites and detoxification enzymes. 77 non-redundant cytochrome P450 genes were identified. Mapping of Illumina RNAseq sequences (from susceptible and pyrethroid resistant strains) against the reference transcriptome identified a cytochrome P450 (CYP6BQ23) as highly overexpressed in pyrethroid resistance strains. SNP

analysis confirmed the presence of a target-site resistance mutation (L1014F) in the voltage gated-sodium channel of one resistant strain. Our results provide new insights into the important genes associated with pyrethroid resistance in *M. aeneus*. Furthermore a comprehensive EST resource is provided for future studies on insecticide modes of action and resistance mechanisms in pollen beetle.

6.1 Introduction

The pollen beetle, *Meligethes aeneus* F. (Coleoptera: Nitidulidae) is the major arthropod pest of oilseed rape in Europe [1]. Infestation levels regularly exceed economic damage thresholds and chemical control measures are frequently required to prevent yield losses [2]. The excessive use of pyrethroid insecticides for pollen beetle control throughout Europe for more than 20 years led to wide-scale control failures due to the development of resistance and resulted in serious crop yield losses e.g. in Germany in 2006 [3]. Pyrethroid resistance in pollen beetle was first documented in France in 1999 and has since spread throughout Europe [4,5].

To date, two main resistance mechanisms have been identified in pyrethroid resistant pollen beetle populations: enhanced expression of a cytochrome P450 (CYP6BQ23) [6,7] and an amino acid substitution (L1014F) in the voltage gated sodium channel protein (pyrethroid target-site) leading to knockdown resistance (*kdr*) [8]. Besides these two mechanisms a further study revealed esterases may make a limited contribution to pyrethroid resistance in some pollen beetle populations [9]. Pyrethroid resistance in pollen beetle is not limited to individual members of this insecticide group, but rather affects the whole chemical class [6]. Nevertheless cross-resistance is higher between compounds that are more structurally related e.g. *lambda*-cyhalothrin and deltamethrin, compared to compounds with distinct structural motifs such as bifenthrin, etofenprox and *tau*-fluvalinate [6].

Pollen beetle is a univoltine species and cannot be reared continuously under controlled conditions [10]. For this reason the number of molecular studies on this species is rather limited and available sequence resources are scarce. The state of the sequence information available for this species stands in contrast to the economic importance of this pest. Next-generation sequencing allows high-throughput production of sequence data at a single-nucleotide resolution allowing genome-wide expression analyses [11], and facilitating detailed molecular study of non-model organisms such as the pollen beetle.

The aim of this study was to use next-generation sequencing to produce a *de novo* transcriptome for the pollen beetle as a resource for current and future study of this pest species. This resource was then used as a reference to provide an insight into resistance

related changes in gene expression in pyrethroid resistant pollen beetle populations from different origins in Europe and to identify genes encoding the target-sites of insecticides currently in use for pollen beetle control.

6.2 Results and Discussion

6.2.1 Bioassays

The susceptibility of four pollen beetle populations collected from different locations in Europe towards two different pyrethroid insecticides was assessed using an adult vial test. *Lambda*-cyhalothrin was chosen because it has been routinely used for pollen beetle control for many years and it is the reference pyrethroid for resistance monitoring purposes recommended by the Insecticide Resistance Action Committee (IRAC) [12]. Etofenprox was used because it represents a non-ester pyrethroid. The Ukrainian sample (UA) was used as a reference as the susceptibility of this population to *lambda*-cyhalothrin is comparable to that of susceptible populations tested in 2009 and 2010 [4] (Table 13). The other three pollen beetle populations collected in Germany (D), Poland (PL) and Sweden (SE) were resistant to pyrethroids with resistance ratios for *lambda*-cyhalothrin ranging from ~600 to ~870 based on LC_{50} values and from ~800 to ~2670 based on LC_{95} values. The resistant populations show cross-resistance up-to 140-fold against etofenprox and thus confirm recent findings on pyrethroid cross-resistance patterns in *M. aeneus* [6,8]. The lower resistant ratios of etofenprox are to some extent based on its > 10-fold lower intrinsic activity against pollen beetle which is also reflected by its ~10-times higher field application rate (86 g AI ha^{-1}) compared with *lambda*-cyhalothrin (7.5 g AI ha^{-1}).

6.2.2 High throughput sequencing, assembly and annotation

To develop a transcriptomic resource and gain an insight into genetic adaptions underlying pyrethroid resistance in *M. aeneus* high-throughput RNA/cDNA sequencing was performed by 454 and Illumina sequencing technology at LGC Genomics (Berlin, Germany). Illumina RNA-sequencing of non-normalized libraries of four pollen beetle populations: UA (susceptible), PL, D and SE (all resistant), was performed to obtain insight into gene expression changes and to allow SNP analysis between pyrethroid susceptible and resistant populations. 454 sequencing was carried out on sample PL and only used for *de novo* assembly of the reference transcriptome. 454 sequencing of the normalized cDNA library of sample PL resulted in a total of 595,732 reads with an average length of 505 bp. The assembly of 454 reads by Newbler Assembler 2.6 resulted in 18,307 contigs (9,780 >500 bp, N50 = 1,278 bp) (Table 14). After annotation the dataset was screened for genes of interest (GOI) i.e. P450s and insecticide target sites. The coverage of selected GOI was very low, e.g.

no hit was retrieved for VGSC and only one contig matches to a single alpha subunit of the nAChR.

Table 13 *Log-dose probit-mortality data for two pyrethroid insecticides tested against adult beetles of four strains of* M. aeneus *in an adult vial test (24h).*

Compound	Strain	LC_{50}-value ng cm^{-2}	95% FL[a]	RR[b]	LC_{95}-value ng cm^{-2}	95% FL[a]	RR[b]	Slope (± SD)
Lambda-cyhalothrin	UA	0.2	0.2-0.2	1	1.8	1.5-2.1	1	2.843±0.21
	D	135.8	116.5-158.3	679	2723	1769-4192	1513	1.585±0.10
	PL	174.8	151.3-202	874	4807	3196-7240	2671	1.558±0.09
	SE	120.2	99.1-145.7	601	1465	850.4-2522	814	1.716±0.11
Etofenprox	UA	3.3	2.4-4.5	1	71	28.6-176.6	1	1.834±0.15
	D	158.8	135.9-185.5	48	2979	192.2-4617	42	1.412±0.09
	PL	468.6	380.9-576.6	142	4515	2478-8226	64	1.857±0.14
	SE	127.9	109.7-149	39	4327	2796-6697	61	1.314±0.08

[a] 95% Fiducial limits

[b] RR = resistance ratio obtained by dividing LC-value of strain D, PL or SE by the LC-value of strain UA

[c] Strain abbreviations: UA = Ukraine, D = Germany, PL = Poland, SE = Sweden

Using 454 reads and Illumina reads from sample Poland contigs were assembled with velvetg, clustered with Oases and merged into a combined assembly (Table 14 and additional file 1). Coverages of the contigs ranged from 1 to 928 with a mean coverage of 7.34. CAP3 assembly of Oases loci with more than one assigned transcript resulted in a total of 43,396 sequences (ESTs) representing the final hybrid assembly (N50 = 2,832 bp) (additional file 2). The ESTs were annotated with the descriptions of BLAST hits in Uniprot (72%), SwissProt (56%) and *Tribolium castaneum* entries from UniProt (71%). Additionally, potential protein domains were assigned to 62% of ESTs by screening Pfam-A (E-value cutoff <1).(Table 15). Blast similarity searches between *M. aeneus* ESTs and protein sequences of *T. castaneum* (Order Coleoptera), *D. ponderosae* (Coleoptera), *D. melanogaster* (Diptera) and *A. pisum* (Homoptera) revealed that 86 % (37,409/43,396) of the *M.aeneus* ESTs have significant hits in all five species compared (Fig. 48). This assembly not only improved the numeric indices detailed above but also greatly improved the abundance of GOI. The VGSC was represented in the improved assembly by two contigs encoding 99 % of the coding sequence and nAChR subunits *alpha*1-8 and *beta*1 were represented by 13 contigs including two full length contigs (see section 6.2.4). Cytochrome P450s, carboxylesterases and glutathione S-transferases were represented as 190, 75 and 66 sequences respectively (Table 16). As P450s are of primary relevance to pyrethroid resistance in *M. aeneus* they are described in detail in the

following section, the detailed sequences are provided in additional file 3. The sequence lists of carboxylesterases and glutathione S-transferases are provided in additional files 4 and 5, respectively.

Table 14 *Summary statistics of the transcriptome assemblies of* M. aeneus.

Newbler assembly (454 only)				
Fully assembled reads	419,215			
Partial assembled reads	54,234			
Singletons	82,200			
Repeats	2,161			
Contigs	18,307			
Contigs >500 bp	9,780			
Hybrid assembly (454 and Illumina)				
	K61	K71	K81	K71*
Contigs	341,977	227,156	149,740	145,323
N50	121	141	180	263
Loci	25,908	25,023	23,057	26,733
transcripts	81,655	71,644	60,022	130,323
Coverage of the merging assembly				
Minimum	1			
1stQuartile	2			
Median	4			
Mean	7.34			
3rdQuartile	10			
Maximum	928			
No. of transcripts in oases loci (after CAP3)				
single transcript loci	16,134			
contigs from complex loci	19,305			
singlets from complex loci	7,957			
Sum	43,396			

*Merged assembly

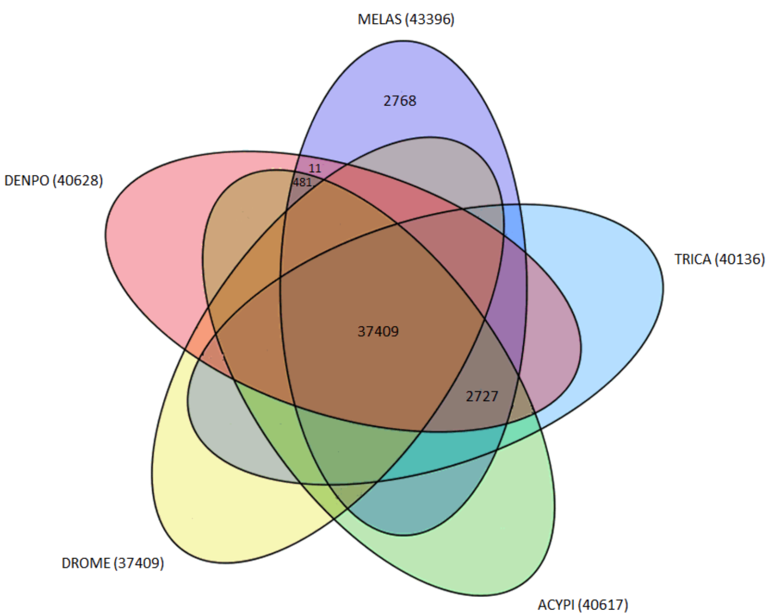

Fig. 48 *Comparison of the assembled sequences of* Meligethes aeneus *(MELAS) with protein sequences of* Tribolium castaneum *(TRICA),* Dendroctonus ponderosae *(DENPO),* Acyrthosiphon pisum *(ACYPI) and* Drosophila melanogaster *(DROME). Values shown in the Venn diagram are the number of MELAS sequences with BLAST hits (E-value ≤$1e^{-3}$) in the species outlined. For 2768 sequences no BLAST hit for any of the above species was obtained.*

Table 15 *Number and percentage of annotated contigs (hybrid assembly)*

Database	No. of sequences	%
SwissProt 1/5	24,460	56
1st UniProt hit	31,073	72
TRICA EST	30,693	71
Pfam	26,941	62
bioproc	25,463	59
molfunc	25,881	60
cellcomp	25,171	58
Total	***43,396***	***100***

Table 16 *Number of annotated sequences of genes known to be related to metabolic insecticide resistance.*

Function	Number of annotated sequences	Mean coverage	Mean length
P450	190	495	1445 bp
CCE	75	1477	1786 bp
GST	66	6298	1310 bp

6.2.3 Cytochrome P450s

P450s are an important enzyme class found in virtually all aerobic organisms that are involved in the metabolism of endogenous substances such as hormones, fatty acids and steroids as well as of xenobiotics such as drugs, pesticides and plant secondary metabolites [13]. The P450 CYP6BQ23 was recently shown to be the main factor causing pyrethroid resistance in *M. aeneus* across Europe through its capability to hydroxylate the alcohol moiety of pyrethroids to a less toxic hydroxy-metabolite [6,7]. Prior to the study described here only this P450 and seven partial P450 sequences have been identified in *M. aeneus,* and as a result very little is known of the size and diversity of the P450 gene family in this species. After manual curation of 190 annotated P450s in the transcriptome of *M. aeneus* we identified 77 non-redundant P450s (including CYP6BQ23) of which 55 contain putative full-length ORFs. The initial P450 count of 190 sequences reduced as many ESTs represented either allelic variants or different fragments of the same P450. All putative full-length P450s contain a transmembrane region (TMHMM predicted, [14]) as well as the I-helix motif A(A,G)X(E,D)T. The identified P450s can be assigned to one of four CYP clans: CYP2 (5 sequences), CYP3 (39 sequences), CYP4 (25 sequences) and the mitochondrial (mito) clan (8 sequences). Out of 77 P450s identified in *M. aeneus* only nine P450s have orthologous genes known in other insect species. One belongs to the CYP4 clan the remaining eight orthologs belong to CYP2 and to the mitochondrial clan. Of these several are thought to be involved in conserved endogenous metabolic pathways [15]. P450s related to ecdysteroid metabolism are: CYP306A1, CYP307A1, CYP18A1, CYP302A1, CYP314A1, CYP315A1 and CYP4AA1 [16–19]. One orthologous gene, CYP303A1, is known to be required for the structure and function of sensory organs in *D. melanogaster* [20]. CYP301A1 is a P450 that can be found in all insect genomes sequence to date and was recently described to be involved in cuticle formation [15].

The small number of P450 genes in pollen beetle showing orthology to other insects fits the known pattern of this gene superfamily within and across taxa [21]. As recently described for the mountain pine beetle, *D. ponderosae* [22], lineage-specific expansions, so called ‘blooms’ in relation to the phylogenetic tree [21], can be found for pollen beetle P450s within the CYP3 and CYP4 clades (Fig. 1). Those blooms reveal gene family-specific expansions, which may have occurred due to species specific adaptation to the environment [22]. Similar to *D. ponderosae*, the greatest expansions can be found within the CYP6 family of the CYP3 clade but the ‘bloomed’ families in *M. aeneus* are distinct from other Coleopteran species i.e. *T. castaneum* and *D. ponderosae*.

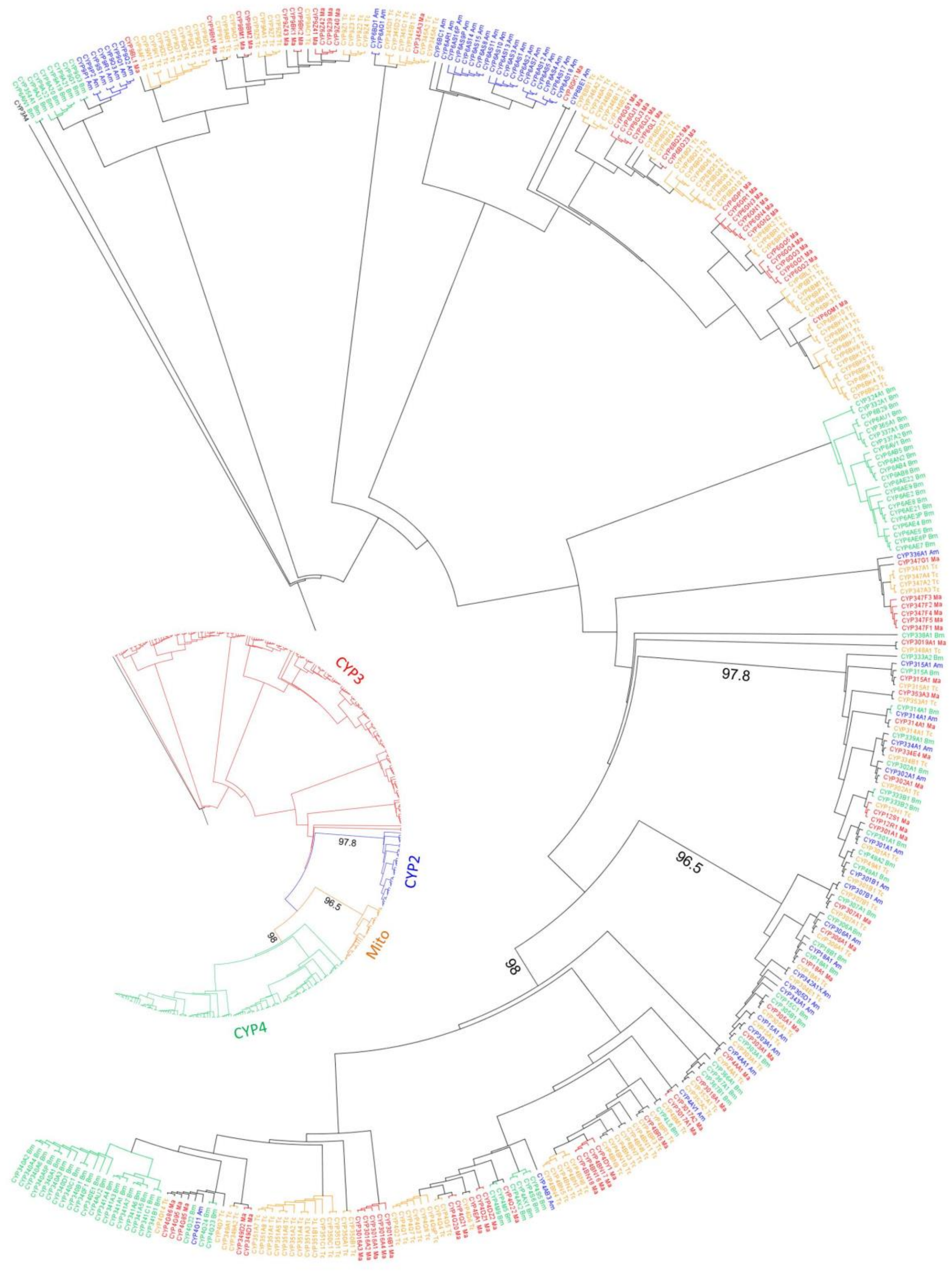

Fig. 49 *Neighbour-joining phylogenetic analysis of cytochrome P450s of* Meligethes aeneus *(Ma, red) along with P450s identified from genome sequences of the red flour beetle (*Tribolium castaneum*) (Ta, orange), western honey bee (*Apis mellifera*) (Am, blue) and silkworm (*Bombyx mori*) (Bm, green), rooted with human CYP3A4 (black). Bootstrap support (%) (1,000 replicates) are shown for important branches only separating CYP clans as indicated by the small embedded tree.*

6.2.4 ESTs encoding insecticide targets used for pollen beetle control

One of the major goals of this study was the identification of ESTs encoding insecticide target sites currently addressed in pollen beetle control throughout Europe. These targets sites are: the voltage gated sodium channel (VGSC) as the target site for pyrethroid insecticides and indoxacarb, the nicotinic acetylcholine receptor (nAChR) as the target of neonicotinoids and acetylcholine esterase (AChE) as the target of organophosphates. Of additional interest was the *RDL* (resistance to dieldrin) locus due to the historical use of organochlorine insecticides, e.g. dieldrin and endosulfan for the control of pollen beetle [23].

The VGSC was represented by two contigs in the transcriptome of *M. aeneus* covering 99 % of the coding sequence; the deduced AA sequence revealed 88.2 % pairwise sequence identity to the VGSC (*paralytic A*) of *T. castaneum* (Table 17). Using the contigs representing the VGSC as a template for mapping Illumina reads conducted by Geneious' "map to reference" function (described below in detail) we were able to close the gap between the two contigs and to obtain the putative full length coding sequence of 6213 bp in a final contig of 8086 bp. This final contig was then used as a template for aligning the Illumina datasets of individual samples to identify polymorphisms. The SNP analysis identified 13, 23, 26 and 31 SNPs (synonymous and non- synonymous polymorphisms) in samples UA, PL, SE and D, respectively, resulting in a polymorphism rate ranging from 0.19 % to 0.42 % (Table 18). Most SNPs identified were synonymous, with only two SNPs leading to amino acid changes one of which caused the L1014F knock down resistance mutation (*kdr*) (Fig. 50) already described for *M. aeneus* [8]. As shown recently the *kdr* mutation was only detected in a Scandinavian population, the other non-synonymous point mutation refers to the AA position 693 in *Musca Vssc1* and causes a tyrosine to histidine (Y/H) change. As *M. domestica* and *T. castaneum* VGSC AA sequences both contain a histidine at the corresponding position we assume this mutation is not linked to pyrethroid resistance. The frequency of the *kdr* mutation in the Swedish sample was 41.3 % (coverage 109), this finding is noteworthy as it shows that *kdr* was not vital to survive the pre-selection with *lambda*-cyhalothrin conducted in this study.

The nAChR was represented in the transcriptome by 13 contigs covering the subunits α1-8 and the β1 subunit. The BLAST search revealed either *T. castaneum* or *L. decemlineata* as the best hit (Table 17). Only contigs of two subunits, i.e. α6 and α8 were found to represent putative full length ORFs. After manual curation of the contigs by short read mapping all other ORFs could be extended but putative full length ORFs were only obtained for α subunits 1-3 and the β1 subunit. SNPs were identified for subunits with a sufficient coverage for all samples (Table 18). The deduced AA sequences of *M. aeneus* nAChR subunits was compared to subunits of insects with known target site resistance affecting neonicotinoid insecticides, i.e. Y151S in *N. lugens* [24] and R81T in *M. persicae* [25]. SNPs causing

variation in deduced AA sequences of nAChR subunits in *M. aeneus* were observed but the mutations reported previously in *M. persicae* and *N. lugens* were not identified in any population (Fig. 51). However, since the first neonicotinoid insecticide to be used in foliar application in oilseed rape in Europe was thiacloprid in 2006 in Germany [4] and to the present day no control failure has been reported [26], we did not expect to find resistance-associated mutations within the nAChR.

Table 17 *BLASTx results for genes of interest coding for insecticide target sites in* M. aeneus.

Gene	Locus transcript	Species	Description	Accession No	% AA Identity	E value
VGSC	10330_Contig1 10221_Transcript_1/1	*T. castaneum*	paralytic A	NP_001159380	88.2	0
ACE	3760_Contig1	*A. diaperinus*	acetylcholinesterase	ABX44668	84	0
nAChR α1	10403_Transcript_1/1 10879_Contig1 6844_Contig1	*L. decemlineata*	nAChR alpha1	ACJ64923	86.5	0
nAChR α2	8750_Transcript_1/2	*T. castaneum*	nAChR alpha2	ACM09847	93.1	0
nAChR α3	13114_Transcript_1/1	*T. castaneum*	nAChR alpha3 (truncated)	ACM09850	94.4	0
nAChR α4	15332_Transcript_1/1	*T. castaneum*	nAChR alpha4 (truncated)	ACM09853	98.7	0
nAChR α5	11795_Contig1	*T. castaneum*	nAChR alpha5	ACM09845	89.2	0
nAChR α6	4346_Contig2	*T. castaneum*	nAChR alpha6 isoform I	ACM09859	94.6	0
nAChR α7	10293_Transcript_1/1 15247_Transcript_1/1	*T. castaneum*	nAChR alpha7	ABV72697	87.3	0
nAChR α8	13563_Contig1	*L. decemlineata*	nAChR alpha8	ACJ64922	90.8	0
nAChR β1	7172_Contig1 19666_Contig1	*T. castaneum*	nAChR beta1	NP_001156000	96.4	0
RDL	6171_contig1	*D. melanogaster*	resistant to dieldrin, isoform E	NP_001261615	93.3	0

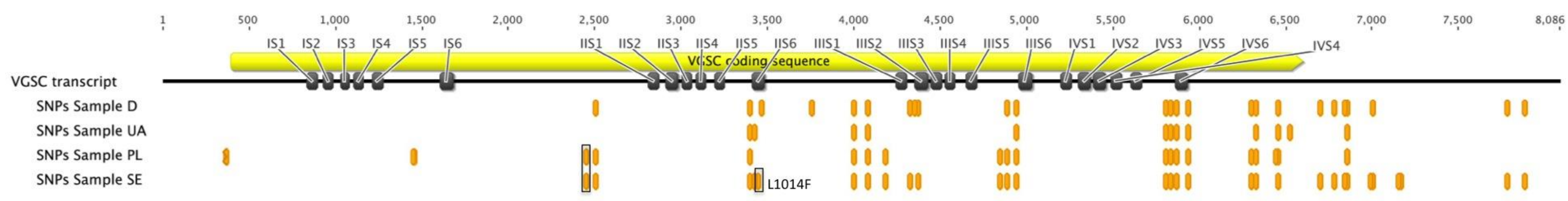

Fig. 50 *SNP distributions in the 8,086nt VGSC sequence of Meligethes aeneus. The coding sequence is indicated by a yellow arrow above the sequence; black boxes indicate subunits 1-6 of the individual transmembrane domains (I-IV); orange ellipses below the sequence indicate the position of SNPs for each strain (boxed ellipses indicate the position of non-synonymous SNPs including L1014F (known as* kdr*).*

Table 18 *Sequence characteristics of manually curated contigs for genes encoding insecticide target sites in for different strains of* M. aeneus.

			Coverage*				**% SNPs (synonymous and non-synonymous)**				**No. of non-synonymous SNPs**			
Gene	**Sequence length (bp)**	**ORF length (bp)**	**UA**	**PL**	**D**	**S**	**UA**	**PL**	**D**	**S**	**UA**	**PL**	**D**	**S**
VGSC	8,086	6,213	21.6	31.3	36.4	45.7	0.16	0.31	0.38	0.32	0	1	0	2
ACE	3,285	1,974	37.2	67.4	67.6	79.4	0.91	1.10	1.25	1.22	5	7	8	6
nAChR α1	2,076	1,632	21.8	30.8	32.1	47	0.92	1.35	1.83	1.97	0	2	0	0
nAChR α2	1,738	1,647	18	26.1	26.3	43.9	0.35	0.69	0.58	0.86	1	1	1	1
nAChR α3	2,000	1,686	9.4	10	12.1	19.1	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
nAChR α4	1,283	1,283	7.9	11.7	10.3	13.2	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
nAChR α5	1,554	1,418	13.4	20.2	17.1	28.3	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
nAChR α6	3,367	1,500	52.6	65.1	72	118.8	1.25	0.95	1.19	1.04	1	1	1	1
nAChR α7	2,101	1,581	8.6	12.8	12.3	15.8	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
nAChR α8	2,162	1,611	13.8	21.1	23.1	36.4	0.42	0.09	0.32	0.74	0	0	0	1
nAChR β1	2,413	1,581	70.8	79.7	89.4	198.1	1.04	1.08	1.08	1.04	1	2	2	2
RDL	1,515	1,419	91.5	120.3	121.5	188.2	6.60	5.94	5.61	5.68	2	2	2	2

*Coverage based on mapping Illumina reads against manually curated contigs

n.c. = not calculated due to insufficient coverage among individual samples

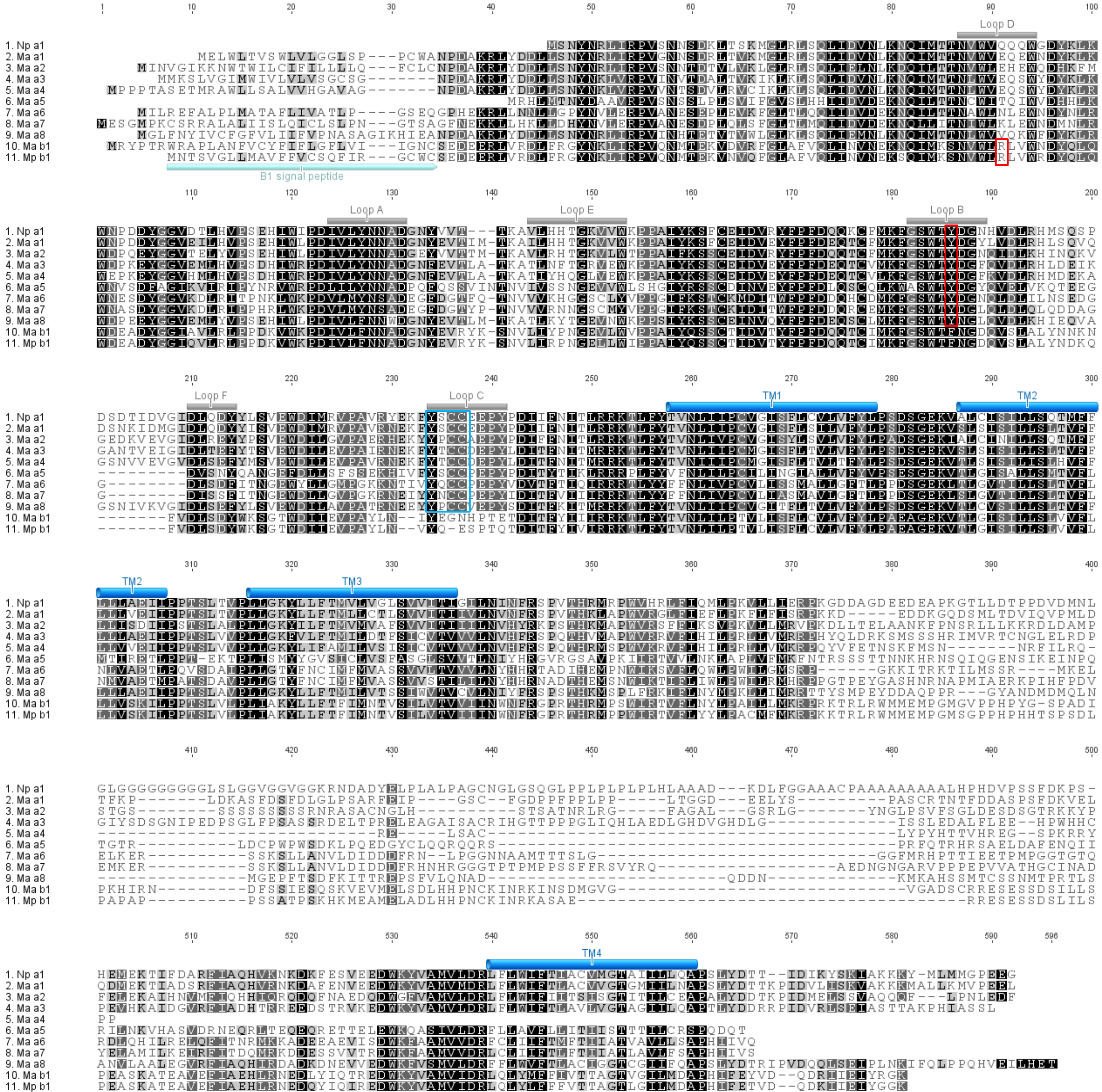

Fig. 51 *Amino acid sequence alignment of* M. aeneus *nAChR subunits (Ma α1-8, β1). Also shown are the sequences of the* Nilaparvata lugens *α1 (Np α1, AAQ75737) and* Myzus persicae *β1 subunit (Mp β1, CAB87995). The predicted signal peptide and transmembrane domains are indicated by an arrow and tubes, respectively. The location of loop domains (loops A-F) involved in ligand binding are indicated by grey bars above the sequence, and the di-cysteine motif characteristic for α subunits is boxed blue. Y151S and R81T mutations known to confer target-site resistance to neonicotinoid insecticides in* N. lugens *and* M. persicae*, respectively, are boxed red. Amino acid changes due to SNPs and deletions identified in* M. aeneus *are: Ma α1 E/D 381 (alignment numbering), D/E 444, Ma α2 L/V 26, Ma α6 G/N 146, Ma α8 G/C 16, Ma β1 R/T 9, deletion-RNDF 405.*

The putative ORF of *ACE* (encoding the AChE) was represented in full length by one contig. The top BLAST hit was from the lesser meal worm, *Alphitobius diaperinus* (AA identity 84 %) and the deduced amino acid sequence shows a high sequence identity with other Coleopteran species such as *T. castaneum* and *L. decemlineata* (Fig. 52). Out of the entire target sites analyzed in this study the *ACE* gene contains the most non-synonymous SNPs (Table 18), but no polymorphism was observed causing an amino acid change at a position previously associated with resistance to organophosphates in other insect species. The ACE sequence was aligned with other insect species and Pacific electric ray, *Torpedo californica* as a reference for numbering (Fig. 52). Pollen beetle's putative *ACE* ORF was screened for the presence of conserved amino acids known to be involved in organophosphate resistance; a valine and leucine were conserved in the pollen beetle sequence at position 129 (*T. californica* 1EA5 numbering) and 150, respectively. Val129 is linked to resistance to organophosphates in *D. melanogaster* [27] whereas Leu150 is associated with resistance in *M. domestica* [28]. However, in both these cases both mutations are known to confer only low levels of resistance on their own but significantly enhance resistance levels when present with other mutations in the *ACE* gene [27–29]. Despite this, to date, the combination of Val129 and Leu150 has not been reported in any study and the impact on the efficacy of organophosphates is unknown. However, the SNP causing a phenylalanine to valine change at position 129 (*T. californica* 1EA5 numbering) should be investigated in further studies as amino acid changes at position 119 (oxyanion hole) are known to confer resistance in *Culex pipiens, Anopheles gambiae* [30] and *Tetranychus urticae* [31].

The *RDL* locus was represented by one contig containing a partial ORF. After manual curation we obtained a contig of 1993bp in length containing a putative ORF of 1416bp. The top BLAST hit was the *RDL* isoform E of *D. melanogaster.* Illumina read mapping to the final contig revealed a high SNP rate above 5.5 % for all samples but only 2 non-synonymous SNPs were found and none of them was at or close to the A302S mutation site known to cause cyclodiene resistance in *D. melanogaster* and a range of other insect species [32,33].

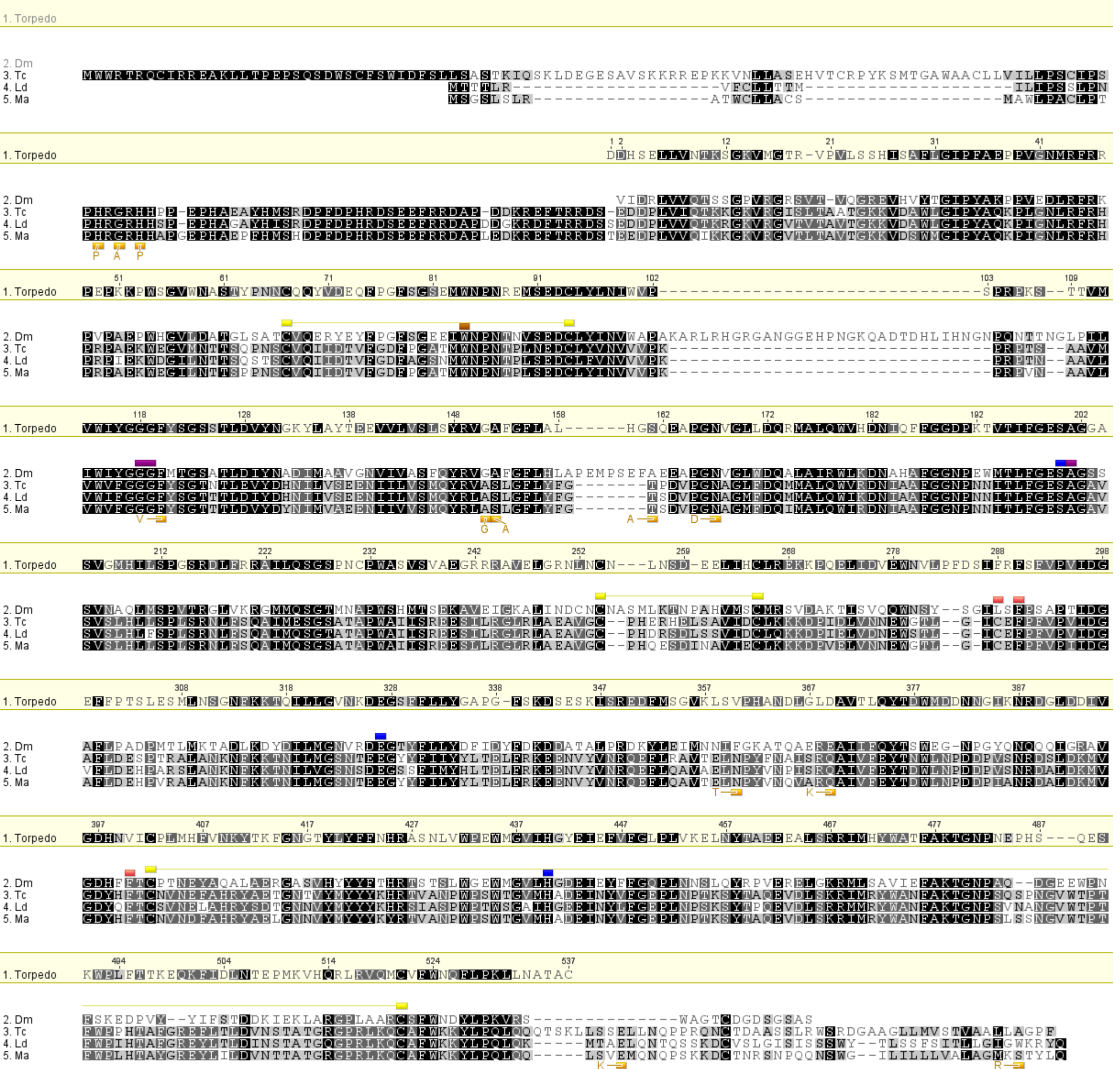

Fig. 52 *Amino acid alignment of* M. aeneus *acetylcholinesterase. Also shown are the sequences of the* Torpedo californica *acetylcholinesterase (1EA5) (reference sequence), Drosophila melanogaster ACE-1 (Dm, 1QO9), Tribolium castaneum ACE-1 (Tc, 662258) and Leptinotarsa decemlineata ACE-1 (Ld, JF343436). Characteristic motifs are indicated as follows (after* HAREL et al.*, 2000 [54]): the intra-molecular disulfide bridges by linked yellow rectangles, the catalytic triad by blue rectangles (S,E,H), the choline binding site by a brown rectangle (W), the acyl pocket residues by red rectangles (L,F,F) and the oxyanion hole residues by purple rectangles (GG,A). Polymorphic sites in M. aeneus ACE are highlighted below the alignment.*

6.2.5 Screening of differentially expressed ESTs, gene ontology analysis and validation by qRT-PCR

Illumina RNA-sequencing of non-normalized libraries of four pollen beetle populations i.e. UA (susceptible), PL, D and SE (all resistant) was performed to obtain a global insight into gene expression changes between pyrethroid susceptible and resistant populations. Individual Illumina datasets were mapped against the above mentioned *de novo* assembled transcriptome of sample PL using bowtie (0.12.9). Approximately 78 % of the Illumina sequences of individual datasets were successfully aligned to the reference transcriptome (additional file 6). Based on FPKM values differentially expressed ESTs were identified among samples. The current study was limited to a single Illumina run covering four individual samples rather than biological replicates as we were interested in identifying common patterns in gene expression among resistant populations collected from different geographies to identify putative resistance genes to be investigated and validated in future studies. For validation of fold-changes calculated based on FPKM values, six of the most interesting candidate genes were analyzed using qRT-PCR. Results of the qRT-PCR runs were correlated with results obtained by RNA-seq (Table 19 & Table 20). Using a cut-off of a 2-fold change in EST expression between all resistant populations and the susceptible population in order to identify putative candidate resistance genes 1,252 and 338 ESTs were up-regulated and down-regulated, respectively (Fig. 53). The complete list of these ESTs along with annotations and calculated fold-change values is provided in additional file 7 and 8. Since all resistant populations but not the susceptible population were pre-treated with *lambda*-cyhalothrin (see material and methods, insect material and bioassays), low expression changes as a result of a common stress response, rather than of a fundamental difference between susceptible and resistant populations, might be anticipated. Focusing on higher expression changes such as ≥ 10-fold, the number of ESTs similarly regulated in resistant samples dropped to 103. The Gene Ontology biological process, cellular component and molecular function categories of ≥ 10-fold over-expressed ESTs were analyzed with Fisher's Exact Test allowing a maximal p-value of 0.01. This analysis revealed a clear pattern of differentially expressed ESTs related to detoxification of xenobiotics as overexpressed in resistant populations. In contrast the differentially expressed ESTs identified in individual resistant populations are not so clearly related to a detoxification category (Table 21, for sequence details refer to additional files 9-12). Only 16 out of 37 commonly over-expressed ESTs (> 10-fold) have annotations from SwissProt. Of these ten out of 16 represent P450s whereas the remaining six ESTs are unrelated to detoxification processes. Nine of the ten P450 ESTs represent CYP6BQ23, the fold-change in expression of these sequences ranged from ~130 to more than 1,800-fold and probably reflects the allelic variation present in the individual populations used in this study (Table 8). This result is consistent with the level of

expression of this P450 in resistant pollen beetle populations reported recently where the role of this P450 in pyrethroid resistance was clearly demonstrated by qPCR screening and functional expression [7]. The fact this former study revealed this particular P450 costitutively overexpressed at a similar level in resistant populations (including in a premature developmental stage) makes induction of expression by the pretreatment with *lambda*-cyhalothrin rather unlikely. Another EST, Locus_15186_Contig1 is more than 15-fold overexpressed among the three resistant strains (Table 19) and represents a partial sequence encoding another P450 (1350nt ORF) that is closely related to CYP6BQ23 (86.1 % amino acid identity) and named CYP6BQ25. This P450 might also contribute to the pyrethroid resistant phenotype as it is overexpressed in all three resistant strains, and based on the high level of amino acid similarity with CYP6BQ23 may also be capable of metabolizing pyrethroid insecticides or their primary metabolites.

Despite the fact the GO analysis revealed a clear pattern of common gene expression among resistant populations other detoxification related ESTs were identified that were not expressed at the same level in all the resistant samples, i.e. another cytochrome P450, some CCEs and GSTs (Table 19). For example, the two ESTs Locus_23909_Transcript_1/1 and Locus_6363_Contig1 represent allelic variants of CYP4Q22 which was > 25-fold, > 5-fold and > 8-fold over-expressed in D, PL and SE, respectively. When compared to the levels of expression of CYP6BQ23 these are fairly modest changes in expression. However it is feasible that this P450 may play a minor role in pyrethroid metabolism. Finally several CCEs and three GSTs were identified. The three GST sequences were commonly over-expressed in all resistant samples ranging from fold changes of 2 to 2.6, 1.3 to 2.3 and 1.1 to 1.9 in D, PL and SE, respectively. Most of the sequences representing differentially expressed CCEs are 2-fold overexpressed in sample D and PL but not in SE except for Locus_6623_Contig1 where the expression pattern is the opposite. Given the low levels of expression of GSTs and CCEs in the resistant population it is likely they play only a minor role in resistance and this is consistent with previous work [9]. Furthermore the cross resistance between etofenprox and *lambda*-cyhalothrin suggests a minor contribution of esterases as the non-ester pyrethroid is affected in the same way in all resistant populations. qRT-PCR on six genes of interest including the highly overexpressed CYP6BQ23, CYP6BQ25, CYP4Q22, a GST and two CCEs confirmed the expression changes of all chosen candidate genes (Table 19 & Table 20). The qRT-PCR carried out with primers based on conserved regions for all contigs of CYP6BQ23 confirmed the massive overexpression of this P450 in all resistant populations ranging from > 400 fold in the Polish sample to ~700 fold in the German and Swedish sample, respectively.

Table 19 *Selected genes coding for detoxification enzymes and differentially expressed between pyrethroid resistant pollen beetle strains (D, PL, SE) and the susceptible reference strain (UA)*

Locus Transcript	SwissProt Acc OA	Gene*	Pfam (score, E-value)	D/UA	PL/UA	SE/UA
16579_Transcript_1/1	Q9V4U7	CYP6BQ23	P450 (89.1, 2.2e-25)	780.37	384.56	747.14
17629_Transcript_1/1	Q27698	CYP6BQ23	P450 (26.8, 1.7e-06)	960.2	268.64	668.28
22383_Transcript_1/1	O61387	CYP6BQ23	P450 (43.9, 1.1e-11)	1,025.73	440.39	1,827.7
23236_Transcript_1/1	Q964Q7	CYP6BQ23	-	613.77	307.14	455.25
23831_Transcript_1/1	Q9VFP1	CYP6BQ23	P450 (50.6, 1e-13)	474.03	132.58	776.37
24713_Transcript_1/1	Q964Q7	CYP6BQ23	P450 (40.3, 1.3e-10)	494.29	202.82	354.78
1499_Transcript_2/220	Q964Q7	CYP6BQ23	-	622.93	281.82	505.9
1499_Transcript_3/220	Q964Q7	CYP6BQ23	-	312.88	140.02	267.25
1499_Transcript_145/220	O61387	CYP6BQ23	P450 (47.9, 6.6e-13)	596.48	295.12	496.14
15186_Contig1	Q9V4U7	CYP6BQ25	P450 (238.0, 1.5e-70)	16.57	28.3	17.36
23909_Transcript_1/1	Q27589	CYP4Q22	P450 (29.2, 3.2e-07)	34.75	7.33	13.61
6363_Contig1	P29981	CYP4Q22	P450 (394.2, 6.4e-118)	28.24	5.91	8.18
16932_Transcript_1/1	O18598	GST sigma class	GST, C-terminal domain (30.7, 2.3e-	2.07	1.3	1.92
7583_Contig1	P46430	GST epsilon class	GST, C-terminal domain (39.6, 3.6e-	2.69	2.39	1.1
9478_Transcript_14/14	Q93112	GST theta class	GST, N-terminal domain (46.3, 3.6e-	2.37	2.14	1.65
12804_Contig1	B2D0J5	CCE clade A	CCE family (314.7, 1.2e-93)	2.28	2.59	0.6
12804_Contig2	B2D0J5	CCE clade A	CCE family (326.1, 4.1e-97)	2.37	2.72	0.59
12804_Transcript_4/6	B2D0J5	CCE clade A	CCE family (270.3, 3.1e-80)	2.34	2.71	0.61
19749_Transcript_1/1	P35502	CCE clade D	CCE family (407.3, 1e-121)	2.43	0.91	1.17
5247_Contig1	B2D0J5	CCE clade E	CCE family (36.8, 1.8e-09)	2.52	2.53	1.93
5247_Contig2	B2D0J5	CCE clade E	CCE family (310.9, 1.6e-92)	2.22	2.16	1.46
6623_Contig1	P35502	CCE clade A	CCE family (363.2, 2.3e-108)	0.66	0.75	3.1
7411_Contig5	P25727	CCE clade A	CCE family (254.9, 1.5e-75)	2.49	2	1.27
7411_Transcript_10/21	P25726	CCE clade A	CCE family (131.9, 2.8e-38)	2.37	1.77	1.15

Locus Transcript	SwissProt Acc OA	Gene*	Pfam (score, E-value)	D/UA	PL/UA	SE/UA
7411_Transcript_14/21	P25727	CCE clade A	CCE family (132.5, 1.9e-38)	2.21	1.59	0.79
7411_Transcript_17/21	O16170	CCE clade A	CCE family (253.2, 5.1e-75)	2.2	1.67	1.22
7411_Transcript_19/21	P25726	CCE clade A	CCE family (349.1, 4.4e-104)	2.05	1.95	1.18
7411_Transcript_8/21	P25726	CCE clade A	CCE family (183.2, 7.8e-54)	2.27	1.75	1.15

<1.5	≥1.5-5	>5-10	>10-100	>100-1000	>1000

*P450 names assigned by P450 nomenclature committee, CCE and GST classes assigned by BLAST search

Table 20 *qRT-PCR validation of candidate-genes.*

Locus	Gene name (gene family)	D	95% FL*	PL	95% FL	SE	95% FL
-	CYP6BQ23 (P450)	690.35	80.93	413.38	44.58	741.78	48.89
15186_Contig1	CYP6BQ25 (P450)	11.82	4.78	23.86	5.23	13.36	5.08
6363_Contig1	CYP4Q22 (P450)	24.63	3.36	7.13	2.18	10.87	3.58
16932_Transcript_1/1	- (GST)	2.24	0.25	1.46	0.53	2.71	1.01
5247_Contig1	- (CCE)	2.98	0.56	1.82	0.53	1.75	0.54
6623_Contig1	- (CCE)	-0.22	0.08	-0.09	0.54	3.45	1.12

*FL = Fiducial limits

Table 21 *GeneOntology categories of at least 10-fold differentially expressed genes.*

Gene Ontology	Common response resistant* versus susceptible strain	SE versus PL**	D versus SE**	D versus PL**
Biological process	Transcription & gene expression Xenobiotic stimulus & metabolic response Response to substances/insecticides/…	Proteolysis Aerobic respiration Viral reproduction GTPase mediated signal Transduction Regulation of cell division	Response to stress GTPase mediated signal Regulation of cell division Aerobic respiration	Response to stress
Cellular component	Mictochondria Endoplasmatic reticulum Microtubuli	Mitochondria Beta galactosidase complex Spindle midzone cytoplasma	Mitochondria Chorion Spindle midzone beta galactosidase complex	
Molecular function	Monooxygenase Activity/oxidoreductase activity Aminopeptidase activity Transition metal binding Electron carrier binding	Cytochrome oxidase activity Endopeptidase activity Oxidoreductase activity	Cytochrome oxidase activity Oxidoreductase activity Hem and tetrapyrrole binding Antioxidant activity Electron carrier binding	
No. of sequences	103	86	102	35
No of annotated seq.	84	40	59	24
No. of seq. with GO term	40	33	45	12

* Includes genes similarly expressed in all resistant samples (D, PL and S) and different from strain UA

** SE vs. PL, D vs. SE and S vs. PL includes genes at least 10 fold differentially expressed between the named two samples.

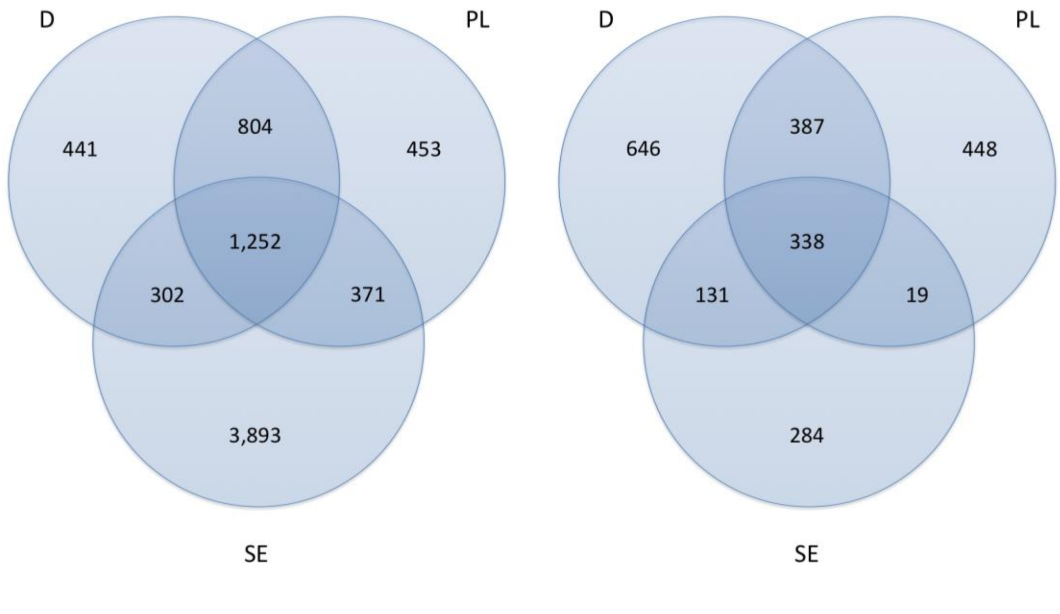

Fig. 53 *Venn diagram of 2-fold over-expressed (left) and 2-fold under-expressed (right) sequences comparing the three resistant samples.*

6.2.6 Conclusions

Despite the economic importance of pollen beetles for European agriculture the lack of genomic/transcriptomic information for this species has hampered molecular research. The overall aim of this study was to provide the first comprehensive transcriptome resource for *M. aeneus* and to use this to investigate the molecular mechanisms underlying pyrethroid resistance in European pollen beetle populations. The transcriptome data obtained in this study has identified two major mechanisms associated with pyrethroid resistance in resistant populations, (1) modification of the target-site in strain SE and (2) enhanced detoxification by one or more cytochrome P450s in strains SE, PL and D. Our findings reported here are consistent with results from previous studies [6–8]. Combinations of target-site and metabolic mechanisms have been described in several other insect species including bed bugs [34,35], houseflies [36] and mosquitos [37] to name just a few. Multiple mechanisms typically often result in significantly higher levels of resistance than individual mechanisms [34,37]. However, in our study the Swedish population SE shows both target-site resistance (*kdr*) and CYP6BQ23 overexpression, but displayed an equally resistant phenotype as the German population D and was significantly more susceptible than the Polish population based on LC_{50} values (the difference was not significant based on LC_{95} values). SCOTT AND GEORGHIOU [38] described a minor contribution of the *kdr* allele for the LPR strain of *M. domestica* in a phenotype showing 5000-fold resistance to permethrin due to overexpression of CYP6D1. However, since only a single pollen beetle strain carried the *kdr* allele at moderate frequency,

the relative contributions of target-site and metabolic mechanisms to pyrethroid resistance remains to be determined and requires more detailed characterization, but it seems highly likely that CYP6BQ23 is the major player in conferring pyrethroid resistance, at least to *lambda*-cyhalothrin. A novel finding of this study was the discovery of a second P450, CYP6BQ25, which is overexpressed in all resistant strains and shows high similarity to CYP6BQ23. Although CYP6BQ25 is expressed at significantly lower levels than CYP6BQ23, it is an interesting candidate for further research as it is linked to pyrethroid resistance in *M. aeneus*.

Unfortunately, pyrethroid resistance is now widespread in pollen beetle populations throughout much of Europe and as a result alternative insecticide classes such as neonicotinoids (e.g. thiacloprid) have been recently introduced as chemical control measures. To facilitate future research efforts on resistance in this species we have characterized numerous genes and gene families encoding the target proteins of the major chemical classes of insecticides. Furthermore the transcriptome offers the possibility to extract genes coding for target-sites not yet addressed for pollen beetle control. Beyond resistance and target-site research this transcriptomic resource will also facilitate molecular studies on fundamental questions such as host plant adaption mechanisms, pollen beetle development and its endocrine regulation for example by transcription factors or neuropeptides.

6.3 Methods

6.3.1 Insect material and bioassays

Adult pollen beetles were collected in winter oilseed rape fields at the pre-flowering stage in Germany (sample D), Poland (sample PL), Ukraine (sample UA) and Sweden (sample SE) in spring 2012. The susceptibility of the collected pollen beetle populations towards two pyrethroid insecticides, i.e. *lambda*-cyhalothrin and the non-ester pyrethroid etofenprox, was assessed by using an adult vial test as described recently [6]. Populations resistant to pyrethroids were further selected with *lambda*-cyhalothrin using a similar bioassay, but at a sub-lethal dose representing 20 % of the field rate (15 ng cm^{-2}) in order to kill remaining susceptible individuals to limit the genetic diversity within the populations in terms of pyrethroid resistance. Survivors of a dose of 15 ng cm^{-2} *lambda*-cyhalothrin were then subsequently transferred to oilseed rape plants in a climate chamber running at 18 °C, 16h:8h day/night and allowed to remain on the plants for one week before they were flash-frozen in liquid nitrogen and transferred to -80 °C. The highly susceptible Ukrainian sample was not pretreated with *lambda*-cyhalothrin as it cannot survive even 100-fold lower doses of pyrethroid insecticides. The Ukrainian population was allowed to feed for one week on oilseed

rape in the climate chamber before it was flash-frozen. All insect samples were shipped on dry ice to LGC Genomics (Berlin, Germany) for further processing.

6.3.2 cDNA library construction, 454 and Illumina sequencing

Sample preparation and sequencing was performed at LGC Genomics (Berlin, Germany). Total RNA was extracted from pools of mature beetles each containing 120 insects using Trizol-GTI-LiCl method [39]. Sample PL (Poland) was sequenced on a 454 FLX Titanium Sequencing platform (Roche) on a half plate single run using cDNA which was normalized using the Trimmer kit (Evrogen). Illumina's TruSeq RNA preparation kit was used to enrich for poly-A RNA and to construct the sequencing libraries for samples PL, SE, UA and D. Poly-A RNA was captured using oligo-T magnetic beads, fragmented chemically and used as template for first strand cDNA synthesis. After the second strand synthesis fragment ends were blunted, an A-overlap was generated and Illumina adapters were annealed. Finally, adapter-ligated library fragments were enriched by PCR. The theoretical insert lengths were between 160 and 260 bp. Sequencing was performed on an Illumina HiSeq2000.

6.3.3 *De novo* assembly

Two assemblies of the *M. aeneus* transcriptome were carried out. (1) 454 reads were assembled by LGC using Newbler Assembler 2.6 (Roche Software) and the following parameters: Seed step 12, seed length 16, min. overlap length 40, minimum overlap identity 90, alignment identity score 2, alignment difference score -3; (2) Illumina reads from the same sample (Poland) were used to improve the 454-assembly: Identical short reads were collapsed keeping the read with the highest quality score sum as the representative of a redundant group. Since the insert lengths of the sequencing library (160-260 bp) led to many overlapping short reads, the collapsed Illumina reads were merged into extended, single-end reads keeping the remaining paired-end reads using Flash 1.2.5 [40] with default settings. This resulted in 3,978,798 extended fragments and 13,023,392 uncombined pairs.

A new assembly was performed using velvet 1.2.08 [41] and oases [42]. K-mer hashes were prepared for k = 61, 71, and 81 with velveth using 454 reads as long fasta, extended Illumina reads as short fastq and uncombined Illumina reads as Short Paired fastq. Contigs were assembled with velvetg for each k-mer size and subsequently clustered with oases. The transcripts of the three sets were then merged into a combined assembly using kmerge = 71. Finally, oases loci with more than one assigned transcript were assembled with cap3 [43] and default settings.

6.3.4 Annotation of predicted proteins

The sequences resulting from the assembly were annotated using SwissProt [44], UniProt [45], *Tribolium castaneum* ESTs from GenBank [46], PFAM [47] and GeneOntology (GO) [48] databases. Sequences were searched via BLASTx against SwissProt, UniProt and *Tribolium castaneum* ESTs. Instead of accepting the best BLAST hit in queries versus SwissProt, the descriptions of the first 5 hits were analysed. Each hit was associated with a score calculated from the E-value, the best HSP score and in accordance to the number of informative and non-informative keywords in the descriptions. The description of the highest scoring hit was accepted. Hmmscan against PfamA was run for the longest translated ORF from the assembly sequences. The GeneOntology annotation includes the complete, non-redundant path through the GeneOntology graph for each top blast hit in the collection of GO representative sequences (seq table of the MySQL incarnation of GO). The GO term annotation was carried out using an in-house script.

6.3.5 Manual curation of genes of interest, phylogenetic analysis and SNP identification

Sequences containing annotations referring to genes of interest (GOI) in particular cytochrome P450s (P450s) and genes encoding insecticide target sites currently addressed in pollen beetle control were manually curated in order to maximize the sequence information using Geneious 6.1 (Biomatters Ltd, Auckland, New Zealand). The largest contigs referring to individual GOI were used as a template for paired short read (Illumina) mapping using up to 5 iterations allowing 2 % mismatches, 10 % gaps, minimum overlap of 25, word length 18 and an index word length of 13. The mapping process was repeated up to three times after manual trimming to conserved regions.

Non-redundant deduced amino acid sequences encoding putative P450s were aligned to available P450 sequences of *Tribolium castaneum, Apis mellifera* and *Bombyx mori* using MUSCLE alignment. A neighbor-joining phylogenetic tree was created with Geneious tree builder using Jukes-Cantor genetics distance model, resampled 1,000 times (Bootstrap, random ssed) and human CYP3A4 as an out-group.

The final set of GOI referring to insecticide target sites were used as a template for single nucleotide polymorphism (SNP) analysis. To identify SNPs short reads of individual populations were mapped against the curated consensus sequences of voltage gated sodium channel (VGSC), acetylcholine esterase (ACE), gamma-amino butyric acid receptor (GABA, *rdl*) and nicotinic acetylcholine receptor subunits (nAChR) allowing 5 % gaps, 10 %

mismatches per read, word length 14 and an index word length of 12. SNPs were called at a minimum coverage of 20, minimum variant frequency of 0.1 and a minimum variant P-value of 10^{-6} while ignoring reads that align at multiple locations.

6.3.6 Mapping of short reads, calculation of expression values and qRT-PCR validation

The Illumina reads from all four samples were mapped separately, i.e. like single end reads, against the PL reference transcriptome using bowtie [49] thus creating a set of technical replicates for each sample. Two mismatches were allowed in a seed of 30 bp and the overall mismatch quality sum threshold was set at 160. No Maq [50] quality score rounding was used. The best 10 alignments were reported excluding reads that aligned more than 20 times (bowtie –n 2 –l 30 –e 160 –nomaground –phred33-quals –k10 –m 20 –best –S MELAS_BCS *input_file*).

Expression values for the reference sequences were calculated using the FPKM algorithm [51] as implemented in Genedata's RefinerGenome [http://www.genedata.com]. Normalization based on length of the contigs and on total count of compatible reads was included. Differentially expressed genes were identified by calculating the arithmetic means of the FPKM values of the two separately mapped reads per paired-end pair and relating them to the averages of the UA sample thus creating fold-change values.

qRT-PCR was performed on a 7900ht real time cycler (Applied Biosystems, CA, USA). Primer pairs were designed using Primer3 [52] to amplify a fragment of 90-150 bp in size for each gene and are listed in additional file 13. Reaction mixtures (15 µl) contained 4 µL cDNA (5 ng), 7.5 µL of SYBR green JumpStart Taq ReadyMix (Sigma Aldrich, MS, USA) and 0.25 µM of each primer. Thermocycling conditions were 2 min at 95°C followed by 40 cycles at 95°C for 10 s, 57°C for 15 s and 72°C for 20 s. A final melt-curve step was included post-PCR (ramping from 65°C-95°C by 0.5°C every 5 s) to check for nonspecific amplification. The efficiency for each primer pair was assessed using a serial dilution of 100 ng to 0.01 ng of cDNA. Only primer pairs were used with a $R^2 \geq 0.99$ and a PCR efficiency > 90 % and < 110 %. Three independent biological replicates containing 15-20 beetles each were run in triplicate in each qRT-PCR experiment. Two reference genes, i.e. α-tubulin (GenBank KC840056.1) and actin (GenBank KC840045.1), were used for normalization according to $_{\Delta\Delta}$Cq method [53].

6.3.7 Database submission

Sequence data generated in this study have been deposited to NCBI as follows:

BioProject (accession no PRJNA223353), project description
http://www.ncbi.nlm.nih.gov/bioproject/PRJNA223353;

BioSample (accession no SAMN02378901), details to the reference sample PL
http://www.ncbi.nlm.nih.gov/biosample/2378901;

Run (accession no SRR1015472), raw data collection
http://www.ncbi.nlm.nih.gov/sra/?term=SRR1015472;

This TSA (Transcriptome Shotgun Assembly) project has been deposited at DDBJ/EMBL/GenBank under the accession GAPE00000000. The version described in this paper is the first version, GAPE01000000.

All ESTs generated in this study incl. the list of P450s genes and genes encoding insecticide target sites are provided in additional file 14.

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Additional files

The additional files mentioned in this chapter can be found in appendix B 9 (.doc files) and/or on http://onlinelibrary.wiley.com/doi/10.1111/imb.12099/suppinfo (.xlsx files).

Additional file 1: Histogram of the coverage of the merging assembly (additional file 1.doc)

Additional file 2: Boxplot of the contig lengths and N50 of the improved assembly (additional file 2.doc)

Additional file 3: P450s nucleotide sequences (additional file 3.xlsx).

Additional file4: CCEs nucleotide sequences (additional file 4.xlsx).

Additional file 5: GSTs nucleotide sequences (additional file 5.xlsx).

Additional file 6:Bowtie results of the alignment of short reads vs. the generated hybrid transcriptome (additional file 6.doc)

Additional file 7: 2-fold up-regulated ESTs between all resistant populations and the susceptible population (additional file 7.xlsx).

Additional file 8: 2-fold down-regulated ESTs between all resistant populations and the susceptible population (additional file 8.xlsx).

Additional file 9: Nucleotide sequences and annotations of >10-fold up-regulated ESTs between all resistant populations and the susceptible population (additional file 9.xlsx).

Additional file 10: Nucleotide sequences and annotations of >10-fold up-regulated ESTs sample SE vs PL (additional file 10.xlsx).

Additional file 11: Nucleotide sequences and annotations of >10-fold up-regulated ESTs sample D vs SE (additional file 11.xlsx).

Additional file 12: Nucleotide sequences and annotations of >10-fold up-regulated ESTs sample D vs PL (additional file 12.xlsx).

Additional file 13: Primer sequences used to validate candidate genes (additional file 13.doc).

Additional file 14: Nucleotide sequences and annotations of ESTs and genes (P450s and insecticide target sites) identified in this study (additional file 14.xlsx).

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Chapter 7

Baseline susceptibility and insecticide resistance monitoring in European populations of *Meligethes aeneus* and *Ceutorhynchus assimilis* collected in winter oilseed rape

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Abstract

Pollen beetle, *Meligethes aeneus* F. (Coleoptera: Nitidulidae) and cabbage seed weevil, *Ceutorhynchus assimilis* PAYK. (Coleoptera: Curculionidae) are important pests in the production of European Winter oilseed rape, *Brassica napus* L. (Brassicaeae), which is grown on several million hectares in Europe. Insecticide treatments are common to control both pests once they exceed economic damage thresholds, however not many chemical classes are available for their control in European oilseed rape. Particularly pollen beetle recently developed high levels of pyrethroid resistance impairing field control at recommended rates in many countries, whereas no resistance is yet reported to another important insecticide, thiacloprid. The major objective of our study was to check the spatio-temporal susceptibility status of pollen beetle against the recently introduced insecticide thiacloprid. From 2009 to 2012 more than 630 populations of pollen beetle collected in 13 different countries were monitored for resistance to thiacloprid by using an adult vial test. No shifting to lower susceptibility of pollen beetle to thiacloprid has been observed between 2009 and 2012. Furthermore we were able to show that pollen beetle larvae are extremely susceptible to thiacloprid whereas larvae of the same strains are significantly more resistant than adults to

pyrethroids such as *lambda*-cyhalothrin. Dose-response data for thiacloprid against cabbage seed weevil populations collected in 2011 in Germany, Sweden and Ukraine showed a ten-fold higher intrinsic sensitivity compared to pollen beetle, and showed only a low variation in response. In addition we also tested 17 cabbage seed weevil populations collected in 5 different countries against *lambda*-cyhalothrin with low variation in response (3-fold), suggesting full baseline susceptibility and no resistance to pyrethroids. The implications of the data presented for resistance management in coleopteran pests in winter oilseed rape will be discussed.

7.1 Introduction

Winter oilseed rape is an economically important crop which is grown on several million hectares and indispensable in many crop rotations in Europe. Its production is threatened by the attack of numerous coleopteran pests such as *Ceutorhynchus* spp. (stem and seed weevils), *Meligethes* spp. (pollen beetles), *Psylliodes chrysocephala* L. and *Phyllotreta* spp. (flea beetles) [1]. Three weevil species are of particular importance as pests in oilseed rape, i.e. rape stem weevil, *Ceutorhynchus napi* GYLL., cabbage stem weevil, *Ceutorhynchus pallidactylus* MARSH., and cabbage seed weevil, *Ceutorhynchus assimilis* PAYK. syn. *C. obstrictus* MARSH.. The latter species is abundant in the crop from early flowering until ripening of the seeds, whereas *C. napi* (similar to *C. pallidactylus*) is abundant in early spring well before flowering. The main damage is caused by *C. assimilis* larval feeding as each developing larvae consumes about five seeds [1]. The damaged pods of the crop then serve as oviposition sites for brassica pod midge, *Dasineura brassicae* WINN. (Diptera: Cecidomyiidae). However, by far the major pest of oilseed rape in Europe is pollen beetle, *Meligethes aeneus* F., which considerably increased in numbers during the last two decades and it was shown that this is linked to expanding oilseed rape acreage in some countries [1,2]. *M. aeneus* destroys the flowering parts through bud feeding resulting in enormous yield losses [1]. Pollen beetle as well as weevil control measures include regular insecticide sprays in most if not all European countries, and depending on country up to five insecticide applications per season are necessary to protect the crop from pollen beetle invasions [3]. After the ban of organophosphates the major insecticide class for pollen beetle and weevil control until 2006 was the pyrethroids [4–6]. Selection pressure by pyrethroids on pollen beetle populations was enormous, so that widespread resistance to this chemical class of insecticides nowadays is a major problem [4,7–12]. Large-scale pyrethroid resistance monitoring campaigns in pollen beetle in as many as 20 countries between 2007 and 2011 revealed high resistance to pyrethroids resulting in limited control and field failure [13,14], whereas resistance in weevils such as cabbage seed weevil, *C. assimilis* is still either absent or limited to just a few regions in Germany [5,15]. In Germany yield losses due to pest pressure and pyrethroid resistance

reached a peak level in 2006 when farmers were unable to fully control pollen beetle infestations on approx. 200,000 ha [16]. The molecular mechanisms involved in pollen beetle resistance to pyrethroids were recently investigated and include cytochrome P450 driven oxidative degradation as well as a target-site mutation leading to a single amino acid change in the voltage-gated sodium channel [17,18]. Since 2007 a few new active ingredients with different modes of action were registered for use in oilseed rape, including thiacloprid, which agonistically binds to insect nicotinic acetylcholine receptors [19]. Other insecticides recently registered for pollen beetle control in some European countries include acetamiprid, indoxacarb, pymetrozine and spinosad [20]. However in Germany by far the most important insecticide after pyrethroids is thiacloprid, which is active against pollen beetle and weevils as well [13]. However, all oilseed rape insect pests share a long history concerning pyrethroid treatments and particularly for pollen beetle, recently introduced resistance management strategies need to be followed to release selection pressure from the pyrethroids which still form a chemical class of utmost importance for the control of other oilseed rape pests [5,13,14]. HEIMBACH & MÜLLER [15] recently reported significant levels of pyrethroid cross-resistance in at least two German populations of *C. assimilis*, however in most regions pyrethroids still work well for cabbage seed weevil as well as stem weevil control.

The objectives of this study were (1) to check the susceptibility status to thiacloprid of *M. aeneus* populations collected all-over Europe in 2011 and 2012, and to compare the obtained data with recently published baseline data [13]; (2) to measure the systemic efficacy of thiacloprid against *M. aeneus* adults, as well as the contact activity to 2nd instar larvae in comparison to *lambda*-cyhalothrin; and (3) to generate baseline susceptibility data for thiacloprid against *C. assimilis* and to compare them with the variation in response to *lambda*-cyhalothrin for populations collected in different European countries to possibly detect susceptibility shifts leading to poor control using manufacturers field recommended rates.

7.2 Materials and methods

7.2.1 Insects

In total 633 *M. aeneus* populations were collected in winter oilseed rape fields from 13 different European countries between 2009 and 2012, including the most important oilseed rape cropping countries France, Germany, Poland and Great Britain (Table 22). A few hundred adult beetles per sample were placed in perforated plastic bags including some oilseed rape buds as food source for shipment. The samples were either shipped to Bayer CropScience, Monheim, Germany or directly tested at the respective collection site by external collaborators. Beetles tested at the authors´ laboratory were stored for 24 h at 4-6 °C upon

arrival in the laboratory. Two hours prior to bioassay the insects were removed from the refrigerator and equilibrated to room temperature (20 ± 2 °C). Those beetles of lower viability and remaining on the bottom of the bag and were not used for the bioassays. Seventeen cabbage seed weevil, *C. assimilis* populations were collected in five European countries in flowering winter oilseed rape fields in 2011 and handled as described above. The sampling sites for cabbage seed weevil populations are shown in Fig. 54.

Table 22 *Number of conducted thiacloprid resistance monitoring tests with* Meligethes aeneus *sorted by year of sampling, testing site and country.*

Year	Site	A	CZ	D	DK	F	FIN	LT	LV	N	PL	S	UA	UK	Sum
2009[3]	BCS[1]	9		19		8								2	33
2010[3]	BCS			32		29					7		2	2	55
2011	BCS	15		32	6	33	2			3	8	7	2	2	110
	External[2]	20	8	117	7			14	18		10	15		3	212
2012	BCS	3	3	7	4	12					23				52
	External		8	112		29	10				8	4			171
Total	BCS	27	3	90	10	82	2	0	0	3	38	7	4	6	250
Total	External	20	16	229	7	29	10	14	18	0	18	19	0	3	383
Total		47	11	319	17	111	2	14	18	3	56	22	4	9	633

A, Austria; CZ, Czech Republic; D, Germany; DK, Denmark; F, France; FIN, Finland; LT, Lithuania; LV, Latvia; N, Norway; PL, Poland; S, Sweden; UA, Ukraine; UK, United Kingdom

[1]Tested at the authors´ laboratory

[2]Tested externally with coated glass vials provided by the authors

[3]The list includes 33 and 55 populations recently taken for thiacloprid baseline susceptibility monitoring and collected in 2009 and 2010, respectively [13] (see Chapter 2)

M. aeneus 2nd instar larvae were collected in May 2011 in winter oilseed rape fields from different regions in Germany. The larvae can be easily collected in the late flowering stage of oilseed rape (BBCH 67-69) by tapping the plants into a close-meshed insect net. The collected larvae were placed with some flowering parts of oilseed rape into a perforated plastic bag and stored for 24 h at 4-6 °C. Two hours before bioassay the larvae were removed from the refrigerator, equilibrated to room temperature (20 ± 2 °C) and separated from flower material by using a sieve (3 mm mesh size).

Fig. 54 *Sampling sites for populations of cabbage seed weevil,* Ceutorhynchus assimilis *in Europe.*

7.2.2 Thiacloprid resistance monitoring bioassay – *M. aeneus*

Thiacloprid resistance monitoring was carried out using an adult vial test as recently described [13]. The method is also available as IRAC (Insecticide Resistance Action Committee) susceptibility method No. 21 "Pollen beetle susceptibility monitoring bioassay – neonicotinoids" [21]. Briefly: Formulated thiacloprid (Biscaya® OD240, Bayer CropScience) was used in three discriminating concentrations coated on the inner glass surface at 1.44 µg cm^{-2}, 0.72 µg cm^{-2} and 0.144 µg cm^{-2} corresponding to 200 % (144 g ha^{-1}), 100 % (72 g ha^{-1}) and 20 % (14.4 g ha^{-1}) of the manufacturers field recommended rate, respectively. Coated vials were dried and kept in the dark at room temperature and used within 4 weeks after preparation [13]. For bioassay purposes 10 *M. aeneus* adults were transferred into each vial using 2-3 replicates per concentration and sample. Beetles in acetone-treated vials served as control. After lidding vials were stored in upright position at 20 ± 2°C for 24 h. Afterwards vials were briefly shaken and assessed for affected beetles. External collaborators conducted the bioassays with material provided by the authors´ laboratory (i.e. coated vials and instructions). Whereas all tests (n = 250) at the authors´ laboratory were conducted by only

two persons between 2009 and 2012, the number of external collaborators doing bioassays (n = 383) exceeded 100 persons (refer to supporting information) that's likely to result in a somewhat higher variation.

7.2.3 Pyrethroid resistance monitoring bioassay – *M. aeneus*

Pyrethroid resistance monitoring bioassays (as described below) were only carried out comparatively with larval bioassays on a few populations of adults collected in 2011 using an adult vial test as recently described [13]. Briefly: Technical grade *lambda*-cyhalothrin was used as a reference pyrethroid at two discriminating concentrations coated on the inner glass surface at 75 ng cm^{-2} and 15 ng cm^{-2} representing 100 % (7.5 g ha^{-1}) and 20 % (1.5 g ha^{-1}) of the manufacturers field recommended rate, respectively. A full description and validation of the methodology for pollen beetle pyrethroid resistance monitoring is given elsewhere [14].

7.2.4 Systemic bioassay

The potential of systemic action of thiacloprid to control *M. aeneus* was examined in a laboratory test system. Inflorescences of untreated oilseed rape plants in the early bud stage (BBCH 51 - 53) were cut and its stems were placed into insecticide solution containing different concentrations of the commercially available 240 g thiacloprid L^{-1} OD formulation (Biscaya® OD 240, Bayer CropScience). Five concentrations were used: 100 % (240 mg AI L^{-1} water, which is equal to manufactures instructions for field application 72 g AI ha^{-1} dissolved in 300 L^{-1}), 20 % (48 mg L^{-1}), 4 % (9.6 mg L^{-1}), 0.8 % (1.92 mg L^{-1}) and 0.16 % (0.38 mg L^{-1}). The inflorescences were placed into insecticide solution 24 h prior to infestation with pollen beetles. Each inflorescence was then infested with 15 *M. aeneus* adults using three replicates per concentration. The mortality was assessed 24 h after infestation with pollen beetles. It is well known that many pyrethroids including *lambda*-cyhalothrin lack any systemic activity due their physicochemical properties [22], however for reasons of completeness we confirmed this lack of systemicity with a similar set-up as described above.

7.2.5 Larval dip bioassay

The susceptibility of *M. aeneus* larvae to *lambda*-cyhalothrin collected in 2011 was compared to thiacloprid using a larval dip bioassay. The larvae were collected in three different regions of Germany with a known presence of pyrethroid resistance in adult pollen beetle. Ten larvae were dipped for 5 s into aqueous insecticide solutions prepared by using the commercially available 100 g *lambda*-cyhalothrin L^{-1} CS formulation (Karate Zeon®, Syngenta) and the commercial available 240 g thiacloprid L^{-1} OD formulation (Biscaya® OD 240, Bayer CropScience) respectively. Five concentrations were tested for each insecticide; for *lambda*-cyhalothrin: 3125 mg AI L^{-1}, 625 mg L^{-1}, 125 mg L^{-1}, 25 mg L^{-1} (field-recommended rate) and 5 mg L^{-1}; and for thiacloprid: 1200 mg AI L^{-1}, 240 mg L^{-1} (field-recommended rate),

48 mg L^{-1}, 9.6 mg L^{-1} and 1.92 mg L^{-1}. For each concentration three replicates were tested. Right after dipping the larvae were placed in a small petri dish (94 mm in diameter) containing a filter disc and ten freshly cut oilseed rape flowers (untreated). Every 8-12 h ten new flowers were placed into each petri dish. Mortality was scored 36 h after dipping using a binocular. Larvae not moving after prodding and showing signs of intoxication were scored as dead.

7.2.6 *C. assimilis* adult vial tests

In order to check for possible variation in *C. assimilis* baseline susceptibility against thiacloprid and *lambda*-cyhalothrin, the adult vial test for *M. aeneus* as described above was used. Dose-response experiments were carried out by using five insecticide concentrations coated on the inner surface of the glass vial and based on the recommended field rate; (1) thiacloprid: 144 ng cm^{-2} (corresponds to 20 % of the field-recommended rate of 72 g ha^{-1}), 28.8 ng cm^{-2} (4 %), 5.76 ng cm^{-2} (0.8 %), 1.5 ng cm^{-2} (0.16 %) and 0.23 ng cm^{-2} (0.03 %) and (2) *lambda*-cyhalothrin: 15 ng cm^{-2} (corresponds to 20 % of the field-recommended rate of 7.5 g ha^{-1}), 3 ng cm^{-2} (4 %), 0.6 ng cm^{-2} (0.8 %), 0.12 ng cm^{-2} (0.16 %) and 0.024 ng cm^{-2} (0.03 %). Mortality was scored after 24 h.

7.2.7 Data analysis

All mortality figures were corrected for control mortality using ABBOTT'S formula [23]. Tests exceeding a control mortality of 20 % were excluded from the analysis. One way Analysis of Variance (ANOVA) with Bonferroni post hoc test was used to determine significant differences of *M. aeneus* thiacloprid susceptibility in 2011/12 compared with the baseline data obtained in 2009/2010 [13]. The data of larval bioassays were compared to those from adult bioassays using ANOVA and mean mortality was compared by Tukey test. According to thiacloprid baseline studies conducted in 2009/10, the expected results for *M. aeneus* mortality in adult vial tests are 98.5 % ± 2.9 at 1,44 µg cm^{-2} (200 % field rate), 95.7 % ± 7.2 at 0.72 µg cm^{-2} (100 %) and 54.8 % ± 12 at 0.144 µg cm^{-2} (20 %) respectively. Lethal concentration (LC) values were calculated by probit regression analysis using Polo Plus software (LeOra Software, Berkeley, CA, USA). The geographical mapping based on pyrethroid resistance monitoring results obtained in the authors´ laboratory between 2004 and 2012 was done with EasyMap Software v10.0 SP17 (Lutum+Tappert, Germany) and is based on postal codes.

7.3 Results

7.3.1 Thiacloprid resistance monitoring in *M. aeneus* populations

The susceptibility of 633 European *M. aeneus* populations against thiacloprid was tested in the years 2009-2012 using an adult vial test (Table 22). The response of those populations collected in 2009 and 2010 served to define the baseline susceptibility of pollen beetle adults to thiacloprid as shown in Fig. 55, where the mean mortality of all European *M. aeneus* populations collected in 2011 and 2012 is plotted in combination with the variation in response obtained in 2009/10 at the chosen discriminating dosages of 200 %, 100 % and 20 % of the recommended field rate (72 g AI ha^{-1}). No statistically significant differences (based on $p < 0.05$) were found between combined data on mean mortality figures for populations tested by external collaborators and in our laboratory, both in 2011 and 2012. Likewise there is no statistical difference in mean mortality at the chosen thiacloprid rates between the years 2011 and 2012 and the baseline shown in Fig. 55, suggesting no shifting towards thiacloprid resistant populations, at least when data of all countries are combined. The externally obtained mean mortality data at 200 % and 100 % of the field rate tend to be slightly lower than the data obtained in our laboratory, albeit the trend is statistically not significant. The comparison of the efficacy of thiacloprid against *M. aeneus* samples collected in individual countries included in the survey also revealed no significant differences between countries and years of sampling (Fig. 56). Thus again suggesting no shifting in thiacloprid susceptibility in *M. aeneus* populations between 2011 and 2012.

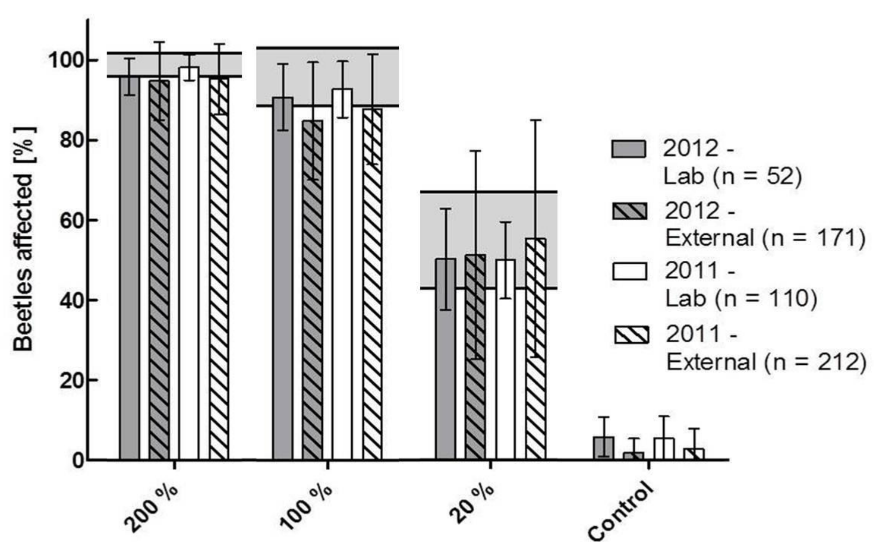

Fig. 55 *Mean mortality ± SD of all* Meligethes aeneus *populations collected in winter oilseed rape in 2011 and 2012 and tested at three field application rates of thiacloprid in an adult vial test (100 % = 72g AI ha^{-1}) (24h). The data obtained in the years 2011 and 2012 are separately plotted for samples tested in the authors´ laboratory (Lab) and tested externally (External) using vials coated in the authors´ laboratory. The grey boxes shown at each rate tested marks the standard deviation of the baseline susceptibility response of pollen beetle populations tested in 2009 and 2010 [13] (see Chapter 2.)*

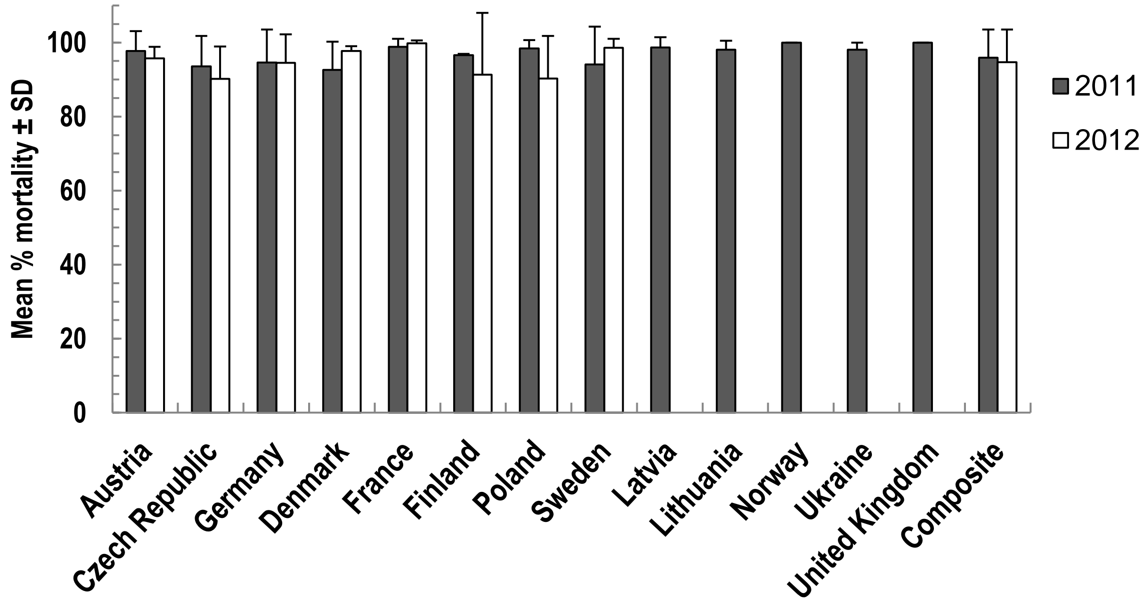

Fig. 56 *Mean % mortality ± SD of* Meligethes aeneus *populations collected in a number of European countries in 2011/12 and tested at 200 % of the field rate of thiacloprid in an adult vial test. Please refer to Table 22 for the number of samples combined for each individual country in the respective years. In 2012 no samples from Latvia, Lithuania, Norway, Ukraine and United Kingdom were tested. The bars named "Composite" refer to the combination of all data sets obtained in 2011 (n = 322) and 2012 (n = 223).*

7.3.2 Systemic action of thiacloprid against *M. aeneus*

This experiment was conducted in order to demonstrate the potential of thiacloprid to control *M. aeneus* by means of its systemic action. Adults feeding on oilseed rape inflorescences immersed with their petioles in thiacloprid solutions show symptoms of poisoning within a few hours and drop off, whereas *lambda*-cyhalothrin in a similar set-up is completely inactive and not systemically transported (data not shown). The systemic bioassay with thiacloprid provided a clear dose response relationship resulting in an LC_{50}-value of 11.3 mg AI L^{-1} (CL95 %: 9.38-13.2 mg L^{-1}) and an LC_{95}-value of 41 mg AI L^{-1} (CL95 %: 21.8-75.0 mg L^{-1}) corresponding to 4.7 % and 17.1 % of the field recommended application rate of thiacloprid (100 %: 72 g AI ha^{-1} and 300 L), respectively (Fig. 57).

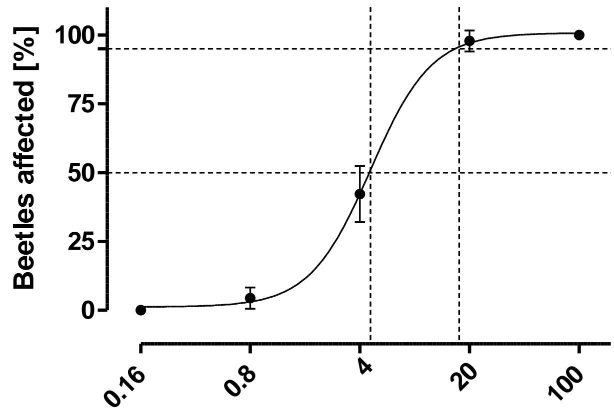

Fig. 57 *Dose response relationship for systemically applied thiacloprid against adults of* Meligethes aeneus *feeding on oilseed rape inflorescences. Data are mean values ± SD (n = 3). Grid lines indicate LC_{50}- and LC_{95}-values (100 % field application rate corresponding to 72 g AI ha^{-1} applied in 300 L water).*

7.3.3 Efficacy of insecticides against *M. aeneus* larvae

Larval dip bioassays revealed a surprisingly low efficacy of *lambda*-cyhalothrin against 2nd instar larvae of *M. aeneus*, when compared to adults collected in the same spot (Table 23). The results obtained at concentrations as high as 100 % of the field-recommended rate (7.5 g ha^{-1}) are significantly different between larvae completely immersed for 5 s in spray liquid and beetles just treated via tarsal contact in adult vial tests. This confirms a significantly lower susceptibility of *M. aeneus* larvae against the pyrethroid *lambda*-cyhalothrin irrespective of the resistance expressed in adult beetles. Extending the dose response relationship by immersing larvae in even higher pyrethroid concentrations such as 25-times the recommended field rate also resulted in mortality figures of just 80 % (Fig. 58). The mean LC_{50}-value for *lambda*-cyhalothrin for three different strains was calculated at 28 mg AI L^{-1} (113 % of the field-recommended rate). In contrast the calculated LC_{50}-value for thiacloprid was 3.1 mg AI L^{-1} against strain M072, i.e. less than 1.5 % of its field-recommended rate. The result indicates high susceptibility of pyrethroid resistant *M. aeneus* larvae against thiacloprid.

Table 23 *Contact efficacy (24 h) of* lambda-*cyhalothrin against adults and 2nd instar larvae of* Meligethes aeneus *at 100 % and 20 % of its recommended field rate, i.e. 7.5 g ha^{-1} and 1.5 g ha^{-1} per 300 L, respectively (Data are mean values ± SD (n=3)). Strain 91-11 collected in Ukraine is pyrethroid susceptible, whereas all others are resistant to pyrethroids.*

Strain	Country	Dose [g ha^{-1}] (Field rate, %)	Adults % Mort ± SD	Larvae % Mort ± SD
40-11	Germany	7.5 (100)	93 ± 12a	50 ± 8.4b
		1.5 (20)	48 ± 7.4a	19 ± 1.0b
59-11	Germany	7.5 (100)	69 ± 13a	43 ± 11b
		1.5 (20)	41 ± 12a	20 ± 8.9b
72-11	Germany	7.5 (100)	79 ± 5.2a	41 ± 16b
		1.5 (20)	15 ± 5.2a	14 ± 12a
91-11	Ukraine	7.5 (100)	100a	nd

Means followed by different letters within a row are significantly different (Tukey test: $p < 0.05$).

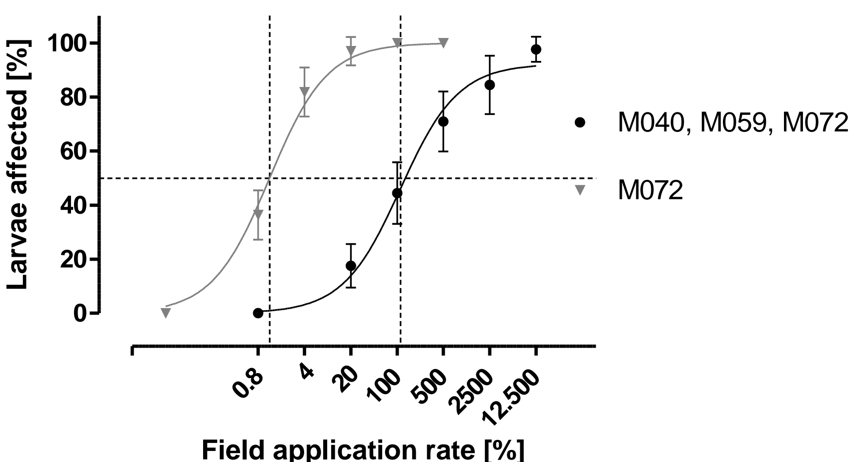

Fig. 58 *Response of larvae of* Meligethes aeneus *collected in 2011 and exposed to different concentrations of commercial formulations of* lambda-*cyhalothrin (Karate Zeon®) (black dots, cumulative for 3 populations) and thiacloprid (Biscaya®) (grey triangles, 1 population) in a larval dip test. Data are mean values ± SD (n = 3).*

7.3.4 Efficacy of insecticides against *C. assimilis* populations

C. assimilis populations were collected in five different countries and eight out of seventeen populations were tested with thiacloprid and all populations showed a high susceptibility to thiacloprid exhibiting less than 3-fold variation based on both LC_{50} and LC_{95} values (Table 24). *C. assimils* populations show a high baseline susceptibility to thiacloprid as

indicated by an LC_{95}-value of 35.3 ng cm^{-2}, corresponding to only 4.9 % of the recommended field rate.

All seventeen populations were tested in *lambda*-cyhalothrin bioassays and all populations were highly susceptible with a calculated composite LC_{95}-value of 0.74 ng cm^{-2}, which correspond to approx. 1 % of the recommended field rate (Table 25). The variation in response of cabbage seed weevil populations to *lambda*-cyhalothrin was similar to thiacloprid, i.e. around 3-fold based on LC_{50} and LC_{95} values.

Table 24 *Log-dose probit-mortality data for thiacloprid obtained from an adult vial test (24 h) against* Ceutorhynchus assimilis *collected in European winter oilseed rape in 2011.*

Strain	Country	LC_{50}-ng cm^{-2} (field rate %)	95 % CL[1] ng cm^{-2}	LC_{95}-ng cm^{-2} (field rate %)	95 % CL ng cm^{-2}	Slope ± SE
60-11	Germany	3.56 (0.5)	2.81-4.47	47.4 (6.6)	32.4-76.3	1.46 ± 0.10
89-11	Germany	2.23 (0.3)	1.46-3.42	31.2 (4.3)	16.4-82.5	1.43 ± 0.10
102-11	Germany	1.96 (0.3)	1.02-3.76	56.1 (7.8)	21.5-290	1.13 ± 0.08
110-11	Germany	2.57 (0.4)	2.06-3.20	26.7 (3.7)	18.8-41.6	1.62 ± 0.11
106-11	Sweden	3.98 (0.6)	2.85-5.53	31.7 (4.4)	19.6-64.3	1.83 ± 0.14
107-11	Sweden	1.76 (0.2)	0.80-3.82	46.3 (6.4)	16.0-351	1.16 ± 0.08
91-11	Ukraine	1.74 (0.2)	1.06-2.87	39.5 (5.5)	18.5-127	1.21 ± 0.08
95-11	Ukraine	4.06 (0.6)	2.72-6.11	75.9 (11)	40.0-190	1.29 ± 0.09
Composite		1.728 (0.2)	1.16-2.58	35.3 (4.9)	18.9-85.4	1.26 ± 0.08

[1]Confidence Limits, 95%

Table 25 *Log-dose probit-mortality data for* lambda-*cyhalothrin obtained from an adult vial test (24h) against* Ceutorhynchus assimilis *collected in European winter oilseed rape in 2011.*

Strain	Country	LC_{50}-ng cm^{-2} (field rate %)	95% CL[1] ng cm^{-2}	LC_{95}-ng cm^{-2} (field rate %)	95% CL ng cm^{-2}	Slope ± SE
60-11	Germany	0.155 (0.2)	0.10-0.24	0.709 (0.9)	0.40-2.10	2.49 ± 0.21
77-11	Germany	0.129 (0.2)	0.10-0.17	0.438 (0.6)	0.31-0.81	3.10 ± 0.32
86-11	Germany	0.072 (0.1)	0.05-0.11	0.422 (0.6)	0.24-1.21	2.14 ± 0.17
89-11	Germany	0.223 (0.3)	0.19-0.26	0.852 (1.1)	0.49-3.30	2.46 ± 0.20
102-11	Germany	0.145 (0.2)	0.08-0.28	0.619 (0.8)	0.31-3.90	2.61 ± 0.23
110-11	Germany	0.167 (0.2)	0.11-0.27	1.120 (1.5)	0.59-3.50	2.00 ± 0.15
106-11	Sweden	0.103 (0.1)	0.06-0.18	0.575 (0.8)	0.29-2.40	2.20 ± 0.18
107-11	Sweden	0.158 (0.2)	0.13-0.19	0.644 (0.9)	0.49-0.93	2.70 ± 0.25
108-11	Sweden	0.110 (0.1)	0.07-0.18	0.767 (1.0)	0.40-2.50	1.95 ± 0.15
91-11	Ukraine	0.239 (0.3)	0.13-0.45	0.962 (1.3)	0.49-5.60	2.71 ± 0.24
95-11	Ukraine	0.238 (0.3)	0.18-0.32	1.200 (1.6)	0.80-2.20	2.34 ± 0.20
117-11	Poland	0.160 (0.2)	0.10-0.26	0.725 (1.0)	0.40-2.54	2.50 ± 0.21
118-11	Poland	0.134 (0.2)	0.10-0.18	0.653 (0.9)	0.42-1.29	2.39 ± 0.20
119-11	Poland	0.135 (0.2)	0.09-0.21	0.713 (1.0)	0.40-2.00	2.28 ± 0.19
120-11	Poland	0.174 (0.2)	0.12-0.26	0.648 (0.9)	0.40-1.73	2.89 ± 0.27
121-11	Poland	0.148 (0.2)	0.07-0.37	0.872 (1.2)	0.38-7.94	2.14 ± 0.17
137-11	Austria	0.125 (0.2)	0.08-0.20	0.734 (1.0)	0.39-2.37	2.14 ± 0.17
Composite		0.140 (0.2)	0.10-0.19	0.740 (1.0)	0.47-1.50	2.28 ± 0.19

[1]Confidence Limits, 95%

7.4 Discussion

The data presented on European populations of *M. aeneus* collected in 2011 and 2012 indicated no shifting in thiacloprid susceptibility when compared with recently published baseline data obtained with populations collected in 2009 and 2010 [13]. In total 162 and 383 samples of different origin were tested by the authors´ laboratory or externally by numerous collaborators (listed in the supplementary section), respectively. So the complete survey is based on 545 *M. aeneus* populations checked for their susceptibility against thiacloprid in 2011 and 2012. Out of 110 pollen beetle populations tested for thiacloprid baseline susceptibility in 2009/10, 51 and 37 populations were only sampled in Germany and France, respectively [13]. However, even though these two countries accounted for more than 50 % of the total oilseed rape acreage in the European Union in 2009 [24], it is good to expand the database by including samples collected in countries not part of the earlier baseline survey. Since 2011 the IRAC susceptibility method No. 21 (www.irac-online.org) is available for thiacloprid resistance monitoring and based on the recently published method by Zimmer and

Nauen [13] (see section 2.3). In order to spatially expand the thiacloprid resistance monitoring efforts, Bayer CropScience (Monheim, Germany) offered in 2011 and 2012 test-kits with Biscaya®-coated glass vials for all stakeholders interested in participating in a European-wide thiacloprid susceptibility monitoring. The supply of test-kits to external stakeholders increased the number of participating countries from six in 2009/10 to thirteen in 2011/12. A comparison of individual data sets obtained externally revealed a slightly higher variation at all discriminating rates tested, most likely due to the fact that more than 100 different persons conducted the bioassays (Fig. 55). The tests conducted in the authors´ laboratory showed less variation most likely due to the fact that all samples received were tested by only two persons (CTZ and HK). Another reason for the observed higher variation in externally conducted tests is possibly linked to declining test kit quality over time as it was recently shown that the formulation used to coat the vials is aging over time when not stored at -20 °C [13]. However in summary the results support the conclusion that thiacloprid susceptibility of European *M. aeneus* populations remained stable in 2011 and 2012. A recent study only covering German *M. aeneus* populations came to a similar conclusion, i.e. stable efficacy of thiacloprid between 2008 and 2011 [15].

This is in contrast to a steady decline in *M. aeneus* pyrethroid susceptibility in many European countries recently reported [14]. Pyrethroids are the most widely used chemical class for the control of a number of coleopteran pests in winter oilseed rape. An example for the spatio-temporal development of pyrethroid resistance is illustrated in Fig. 59, showing that almost all pollen beetle samples collected in 2012 expressed pyrethroid resistance, whereas the problem was less wide-spread when starting the monitoring activities 10 years ago (based on published and unpublished data obtained in the authors´ laboratory [13]). However some European countries such as Lithuania only recently reported a lower susceptibility of *M. aeneus* populations to pyrethroids [25]. The pyrethroid resistance mechanisms yet described include oxidative degradation of pyrethroids by elevated levels of cytochrome P450 monooxygenases and target-site resistance due to an L1014F mutation in the voltage-gated sodium channel [17,18]. Both mechanisms do not affect thiacloprid efficacy as shown by the lack of cross-resistance [13]. However the rapid spread of pyrethroid resistance resulted in the introduction of new insecticide modes of action in order to establish sustainable resistance management strategies to safeguard the use of pyrethroids as important components in such strategies for coleopteran pests other than *M. aeneus* [13,14,26]. Apart from thiacloprid, compounds such as acetamiprid, indoxacarb and pymetrozine were recently tested and introduced as pyrethroid alternatives for *M. aeneus* control in some European countries [20].

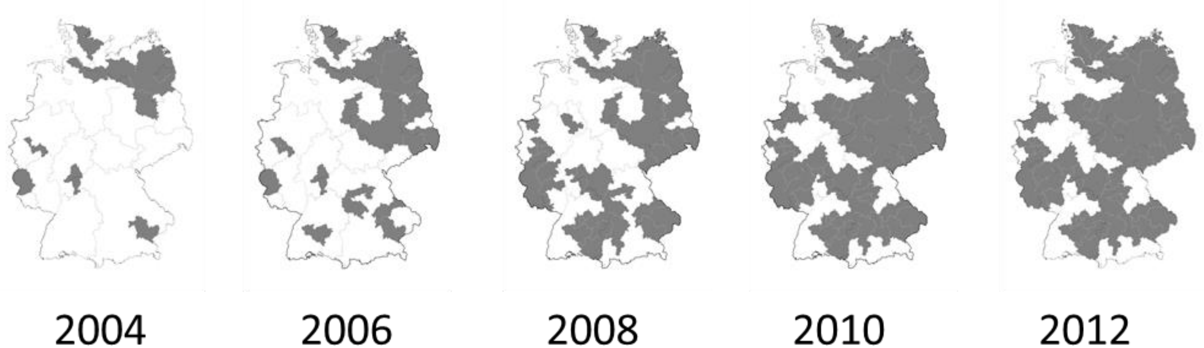

Fig. 59 *Spatio-temporal development (mapped by postal codes) of pyrethroid resistance in* Meligethes aeneus *populations in Germany from 2004 to 2012 based on adult vial tests (24 h). White areas represent regions with either no resistance detected (before 2008) or a lack of test results (2010 and 2012), and grey areas show the presence of pyrethroid resistance in pollen beetles collected.*

An interesting and possibly yet underestimated fact we have shown in this study is the virtual insensitivity of pollen beetle larvae to pyrethroids such as *lambda*-cyhalothrin. Although we were only able to test larvae of a few *M. aeneus* populations a very clear result was obtained by showing that larvae collected in the same fields as adults responded consistently less susceptible, suggesting even stronger selection pressure as pyrethroid-resistant larvae survive field-recommended rates by 50-60 % when directly exposed (Table 23). However, we were not able to test the sensitivity of larvae of pyrethroid susceptible *M. aeneus* populations, so we are not able to draw any conclusion in terms of absolute resistance ratios. In contrast *M. aeneus* larvae are highly sensitive to the neonicotinoid insecticide thiacloprid, suggesting a higher intrinsic efficacy against larvae by providing 100% control at dosages lower than the recommended field rate of 72 g AI ha^{-1}. Such a larvicidal activity is extremely beneficial for population suppression as a whole once thiacloprid is taken up systemically and translocated within the plant as demonstrated for the oil-dispersion formulation, which indeed facilitates the uptake and distribution *in planta* and thus the systemic insecticidal efficacy in several plant species [27]. Furthermore we also confirmed the systemic activity of thiacloprid against adult beetles feeding on winter oilseed rape plants with petioles immersed in thiacloprid solution, whereas expectedly no systemic activity was observed for the pyrethroid *lambda*-cyhalothrin. So it is the combination of effects which best explains the field efficacy of thiacloprid against pollen beetle, i.e. contact and systemic activity against both larvae as well as adults. Whereas the lack of systemicity and larvicidal efficacy of pyrethroids are likely to result in a higher number of survivors and subsequently increased selection pressure and as a consequence a more rapid spread of resistance once the frequency of resistant genotypes is high enough and

alternative modes of action are either not available or not used consequently to eradicate such genotypes.

As mentioned above pyrethroids are also important insecticides for the control of cabbage seed weevil, *C. assimilis*, another important coleopteran late season pest of winter oilseed rape [28]. However, *C. assimilis* resistance to pyrethroids was only recently reported in Germany in a few samples of *C. assimilis* collected in a single spot in northern Germany in 2010 and 2011, whereas the vast majority of the collected strains responded susceptible to *lambda*-cyhalothrin [15]. However the reported LC_{90}-value of 0.76 ng cm^{-2} obtained for a susceptible population of *C. assimilis* is about 1 % of the field-recommended rate, and comparable to the composite baseline LC_{95}-value of 0.74 ng cm^{-2} calculated from seventeen *C. assimilis* populations from 5 European countries analyzed in this study.

The difference in response based on calculated LC_{95}-values for all seventeen *C. assimilis* strains tested is only 3-fold, and considered as natural variation. The fact that HEIMBACH & MÜLLER [15] scored for mortality already after 5 h rather than 24 h explains the somewhat lower lethal concentration values reported in our study. It is worth to mention that all *C. assimilis* populations tested in this study were collected as a by-catch in the same fields where we sampled *M. aeneus* highly resistant to pyrethroids (with the exception of those *M. aeneus* samples collected in the Ukraine). Eight out of seventeen *C. assimilis* populations were also tested for baseline susceptibility towards thiacloprid, and again a very homogenous response was obtained with a natural variation in LC_{95}-values of less than 3-fold. Compared to *M. aeneus*, *C. assimilis* are much more sensitive to thiacloprid as shown by the calculated composite baseline LC_{95}-value of 35.3 ng cm^{-2} (less than 5 % of the recommended field-rate), thus rendering thiacloprid a valuable tool for the control of *C. assimilis* in resistance management strategies in alternation with pyrethroids.

In conclusion, our results demonstrate no general shifting of thiacloprid susceptibility in European *M. aeneus* populations collected in 2011 and 2012, thus suggesting no resistance in those samples analyzed. Furthermore we have shown the systemic action of thiacloprid against pollen beetle adults as well as excellent efficacy against larvae virtually not affected by pyrethroid concentrations well above field recommended rates. All *C. assimilis* populations collected were susceptible to both pyrethroids and thiacloprid, and our composite baseline data form a valuable basis for future resistance monitoring campaigns using discriminating rates of both *lambda*-cyhalothrin and thiacloprid. Resistance management strategies for sustainable control of *M. aeneus* as well as *C. assimilis* populations in winter oilseed rape should imply the rotation of insecticides of different mode of action classes. However particularly thiacloprid - not yet affected by resistance in both *M. aeneus* and *C. assimilis* –

with its added benefits for sustainable pest control such as systemicity and high larvicidal activity is an important tool in future insecticide resistance management tactics in winter oilseed rape cultivation.

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Supplement

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Chapter 8

Target-site resistance to pyrethroid insecticides in German populations of the cabbage stem flea beetle, *Psylliodes chrysocephala* L. (Col. Chrysomelidae)

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Abstract

Cabbage stem flea beetle, *Psylliodes chrysocephala* L. (Coleoptera: Chrysomelidae) is a major pest of winter oilseed rape in several European countries particularly attacking young emerging plants in autumn. Over the last several decades, pyrethroid insecticides have been foliarly applied to control flea beetle outbreaks. Recent control failures in northern Germany suggested pyrethroid resistance development in cabbage stem flea beetles, which were confirmed by resistance monitoring bioassays using *lambda*-cyhalothrin in an adult vial test. The purpose of this study was to investigate the presence of polymorphisms in the *para*-type voltage-gated sodium channel gene of *P. chrysocephala* known to be involved in knock-down resistance (*kdr*). By using a degenerate primer approach we PCR amplified part of the *para*-type sodium channel gene and identified in resistant flea beetles a single nucleotide polymorphism resulting in a L1014F (*kdr*) mutation within domain IIS6 of the channel protein, known as one of the chief pyrethroid target-site resistance mechanisms in several other pest insects. Twenty populations including four archived museum samples collected between 1945 and 1958 were analysed using a newly developed pyrosequencing diagnostic assay. The

assay revealed a *kdr* allele frequency of 90-100 % in those flea beetle populations expressing high-level cross-resistance in discriminating dose bioassays against different pyrethroids such as *lambda*-cyhalothrin, *tau*-fluvalinate, etofenprox and bifenthrin. The presence of target-site resistance to pyrethroids in cabbage stem flea beetle is extremely worrying considering the lack of effective alternative modes of action to control this pest in Germany and other European countries, and is likely to result in major control problems once it expands to other geographies. The striking fact that cabbage stem flea beetle is next to pollen beetle, *Meligethes aeneus* the second coleopteran pest in European winter oilseed rape resisting pyrethroid treatments by expressing a target-site mutation, underpins the importance of diversity in available chemistry for resistance management tactics based on mode of action rotation in order to guarantee sustainable winter oilseed rape cultivation in Europe.

8.1 Introduction

Cabbage stem flea beetle, *Psylliodes chrysocephala* L. (Coleoptera: Chrysomelidae) is a univoltine insect species feeding on cruciferous plants, and it is one of the major pest species in winter oilseed rape (*Brassica napus* L.) in central and northern Europe [1,2]. Adults mainly damage the emerging oilseed rape plants in autumn due to feeding on cotyledons, stems and the first true leaves [3]. After a short feeding period females preferably lay eggs on the stem of newly emerged plants or close to oilseed rape plants in the soil [4–6]. After hatching larvae bore into the petioles and later they move into the main stem where they feed until they reach the final larval instar and pupate in the soil [3,7]. Larval feeding on apical meristems in autumn could result in complete seedling damage as it lost its ability to mount a compensatory response [2]. Flea beetle larvae also cause indirect damage to the plants by making them more susceptible to frost damage and plant pathogens such as *Phoma lingam* [8,9].

Chemical control of cabbage flea beetle infestations these days mainly relies on seed treatments with systemic insecticides such as neonicotinoids to protect the young seedling, and foliar sprays using pyrethroids usually applied later in the autumn season if neonicotinoid seed dressings can no longer protect the plant. Neonicotinoid insecticides act as agonists on insect nicotinic acetylcholine receptors whereas pyrethroids have a different mode of action by modulating voltage-gated sodium channels in the insect central nervous system [10,11]. ALFORD *et al.* [12] recently reported no significant oilseed rape crop damage by *P. chrysocephala* infestations since the use of pyrethroid insecticides in Great Britain. However, the continuous pyrethroid selection pressure due to a lack of alternatives for foliar application created a favorable environment for the emergence of insecticide resistance. This is exaggerated by the fact that pyrethroids are also the major class of insecticides for the control

of other oilseed rape pests throughout the growing season, e.g. pollen beetle (*Meligethes aeneus*), stem and pod weevils (*Ceutorhynchus* ssp.) [13]. First field failures of pyrethroid insecticides in cabbage stem flea beetle control were observed in 2008 in northern Germany and a resistance monitoring initiative confirmed a significant decrease in cabbage flea beetle susceptibility to *lambda*-cyhalothrin tested with discriminating doses in an adult vial bioassay [13]. Resistance to pyrethroids in pest insects is either conferred by elevated levels of detoxification enzymes such as microsomal monooxygenases (cytochrome P450s) or by mutations in the voltage-gated sodium channel protein leading to target-site (knock-down) resistance (*kdr*) [10]. Target-site resistance to pyrethroids was first described in *Musca domestica* and linked to two mutations in the housefly voltage-gated sodium channel gene (*Vssc1*) leading to amino acid changes at positions L1014F (*kdr*) and M918T (*s-kdr*) in domain II of the channel protein [14]. Meanwhile many more mutations in voltage-gated sodium channels conferring pyrethroid target-site resistance have been described, but L1014F remains the most common one described in almost 20 different pest species [15]. These include pollen beetle as another major coleopteran pest in oilseed rape additionally showing a high level of metabolic resistance [16,17]. The emergence and geographic spread of pyrethroid resistance in cabbage flea beetle populations is likely to have a significant impact on the oilseed rape production in Europe, since it is in many regions the only recommended insecticide class for its control [13]. Nothing is yet known on the mechanisms of pyrethroid resistance in cabbage flea beetle however the presence of target-site resistance would be extremely worrying as it is likely to affect the efficacy of the entire chemical class of pyrethroids.

In this study, the possible role of an altered-target site in pyrethroid resistant cabbage flea beetle was investigated. Therefore a fragment of the *para*-type sodium channel gene of *P. chrysocephala* was isolated using a degenerate primer approach and a pyrosequencing based diagnostic was developed allowing the genotyping of *kdr* and super kdr (*s-kdr*) mutations located in the transmembrane spanning domain II. The L1014F *kdr* mutation was identified and the analysis of several samples collected in northern Germany revealed a high allele-frequency of *kdr.* The developed pyrosequencing diagnostic can be used as a tool to monitor the spread of target-site resistance in cabbage flea beetle populations collected in European oilseed rape.

8.2 Materials and Methods

8.2.1 Insects

Adult cabbage stem flea beetles were collected in different parts of Germany between 2009 and 2013 either from freshly harvested winter oilseed rape fields in summer or from oilseed rape fields during emergence of oilseed rape plants in autumn. The sampling was mostly done by using insect nets and exhausters. A simple and efficient way of collecting flea beetles was collecting them directly after crop harvest in stockrooms. Insects were collected in perforated plastic bags with some oilseed rape plant material and tissue paper, transferred to the laboratory and kept at 4°C overnight. Afterwards insects were allowed to equilibrate to room temperature two hours prior to bioassay. Those beetles of lower fitness remaining at the bottom of the bag were excluded from bioassays as well as those obviously affected by transport. Larvae of cabbage stem flea beetles were dissected from infested plants in autumn or early spring and stored in RNA*later* (Ambion®, USA). Historic (museum) samples collected between 1945 and 1958 and taken from the insect archive at the Julius-Kühn-Institute (Braunschweig, Germany) have either been stored in alcohol or formalin.

8.2.2 Bioassay procedure

The test methodology used in this study is based on glass vials coated on the inner surface with different concentrations of pyrethroids dissolved in acetone as recently described for pollen beetle adult vial bioassays [18,19]. Pyrethroids used in bioassays were technical grade and include *lambda*-cyhalothrin, etofenprox, *tau*-fluvalinate and bifenthrin, all obtained from Fluka Chemicals (Buchs, Switzerland). Three doses equivalent to 4 %, 20 % and 100 % of the recommended field application rate of the individual compounds were used (3-5 replicates per concentration, 10 beetles each). The recommended field rates for the tested pyrethroids are given in Table 26. Glass vials treated with acetone only were used as a control. After 24 h, the number of flea beetles severely affected (dead and moribund) were scored and results expressed in percentage mortality. Subsequently the beetles were preserved in RNA*later* (Ambion, USA) for molecular studies. The number of pyrethroids tested per population was dependent on the number of collected beetles per sampling site. However, if the number of beetles was insufficient to test all pyrethroids only *lambda*-cyhalothrin was tested.

8.2.3 Amplification and sequencing of a *para*-type sodium channel fragment of *P. chrysocephala*

To identify polymorphisms in the orthologous *para*-type gene of cabbage flea beetle we used a degenerate primer approach to amplify a DNA fragment encoding domain II of the

voltage-gated sodium channel (VGSC) protein. Nucleic acids were extracted from 15-20 adult beetles according to manufacturer's instructions using Agencourt DNAdvance kit (Beckman Coulter) and TRIzol reagent (Invitrogen, CA, USA) followed by Agencourt RNAdvance Tissue kit (Beckman Coulter) for DNA and RNA extraction, respectively. Degenerate primers were designed based on a multiple alignment of *para*-type VGSC amino acid sequences of *Anopheles gambiae* (GenBank accession no. CAM12801), *Drosophila melanogaster* (AAB59195.1), *Heliothis virescens* (AAC26517), *Leptinotarsa decemlineata* (AAD22957), *Musca domestica* (AAB47605.1), *Plutella xylostella* (BAF37093) and *Tribolium castaneum* (NP_001159380).

Table 26 *Pyrethroid insecticides and their concentrations used in an adult vial test to bioassay cabbage stem flea beetle susceptibility.*

Pyrethroid	ng/cm^2 (g/ha)	Field rate, %
Lambda-cyhalothrin[a]	3 (0.3)	4
	15 (1.5)	20
	75 (7.5)[a]	100
Etofenprox[a]	24 (2.4)	4
	120 (12)	20
	600 (60)[a]	100
Tau-fluvalinate[a]	19.2 (1.92)	4
	96 (9.6)	20
	480 (48)[a]	100
Bifenthrin[b]	4 (0.4)	4
	20 (2.0)	20
	100 (10)[a]	100

[a] Recommended field rate
[b] Currently not registered in Germany

The forward primer was designed on the deduced aa motif NDIIEQ upstream of domain IIS1 (*M. domestica* position 738-743), and the reverse primers were designed to include the deduced aa motifs EMLIKW and MDRIFT located at subunit IIIS2 (*M. domestica* positions 1337-1342 and 1326-1331, respectively). PCR reactions contained 1x RedTaq Readymix (Sigma, USA), 0.5 µM of each primer and 100 ng of cDNA in a total volume of 25 µL. The PCR conditions were 95 °C for 2 min followed by 25 cycles of 95 °C for 30 s, 48 °C for

30 s, 72 °C for 2 min and a final elongation step of 5 min at 72 °C. After a primary PCR using primers “degen F1” and “degen R1” (Table 27), 0.5 µL of this reaction mixture was transferred into new tubes as a template for secondary PCR using primers “degen F1” and “degen R2” with identical PCR conditions. The PCR-amplified fragment was separated with a 1.2 % TAE gel electrophoresis at 120 V for 60 min. The fragment of the expected size of ~1.8 kb was cut out of the gel, purified using Wizard SV gel kit (Promega, USA) and cloned into pBluescript vector using StrataClone PCR cloning kit (Agilent, USA). Minipreps of overnight cultures were purified using GeneJET plasmid kit (Thermo-Fermentas, USA) and Sanger-sequenced using T7 and T3 standard primers. Specific primers were designed based on the obtained sequence to amplify region DIIS1-S6 (VGSC F1 and R1; Table 2). PCR reactions contained 1x RedTaq Readymix (Sigma, USA), 0.5 µM of each primer and 100 ng of cDNA in a total volume of 25 µL. The PCR conditions were 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 57°C for 30 s, 72 °C for 2 min and a final elongation step of 5 min at 72 °C. The PCR products were purified using AMPure Reagent (Beckman, USA) and Sanger-sequenced using VGSC F1, F2 and R1 primers (Table 2). The obtained sequences were assembled and analyzed using Geneious R6.1 (Biomatters Ltd., New Zealand).

8.2.4 Pyrosequencing diagnostic assay for *kdr/s-kdr*

Pyrosequencing is a method of DNA sequencing-by-synthesis allowing real-time detection of nucleotides forming base pairs in an amplified DNA template strand using an enzyme-cascade finally resulting in bioluminescence signals [20]. Genomic DNA (approx. 1 µg per adult) was extracted from individual beetles using DNAdvance Tissue Kit (Beckman, USA) according to the supplier’ recommended protocol; cDNA was prepared as described above. Sodium channel gene fragments were amplified by PCR from 50 ng aliquots of gDNA using two primers per target sequence (Table 27; *kdr*: KDR-F & KDR-R; s-kdr: sKDR-F & sKDR-R) designed with ‘Assay Design Software’ (PSQ-Biotage AB, now Qiagen) by utilizing the partial sequence of the cabbage stem flea beetle *para* gene detailed above (see primer sequences in (Table 27). The pyrosequencing protocol comprised of 45 PCR cycles with 0.5 µM forward and reverse primer (one biotinylated, see Fig. 60) in 50 µL reaction mixture containing 1 x RedTaq Readymix (Sigma, USA) and cycling conditions of 95 °C for 2 min, followed by 45 cycles of 95 °C for 45 s, 57 °C for 45 s and 72 °C for 1 min, and a final incubation at 72 °C for 5 min. The single strand DNA preparation required for pyrosequencing was carried out using the Vacuum Prep Tool (Biotage AB) in combination with streptavidin coated beads (Streptavidin Sepharose) to separate the biotinylated strand of the PCR products. The pyrosequencing reactions were carried out according to the manufacturer’s instructions utilizing the PSQ 96 Gold Reagent Kit (Biotage AB) and using the primers “KDR seq” for *kdr* (L1014) genotyping, “sKDR seq1” and “sKDR seq2” for M918, L925, T929, L932 genotyping,

respectively (Table 27). The genotypes were analyzed using the supplied SNP Software (Biotage AB).

Table 27 *Primer sequences used in this study.*

Name	Sequence 5' to 3'
Degen F1	AAYGAYATHATHGARCARGC
Degen R1	CCAYTTDATNARCATYTC
Degen R2	ACNGTRAADATNCKRTCCAT
VGSC F1	TAGAGCAAGTGAGCACGGAG
VGSC F2	TGCGTATGGGATTGTTGTGC
VGSC R1	TCAGGGAAACAATCTGCCGA
KDR F	GGACTGTATGCTAGTCGGTGATGT
KDR R	[btn]GCTTCGGCTATTTTGTTTGTGTC
KDR seq	CCACTGTTGTCATTGGT
sKDR F	[btn]CTTTTCGATTGCTAAGAGTGTTCA
sKDR R	CCAAATAACTGCATACCCATAACA
sKDR seq1	TAAAGCACCCATAGTTCTA
sKDR seq2	CAAATATAAATATTATAATG

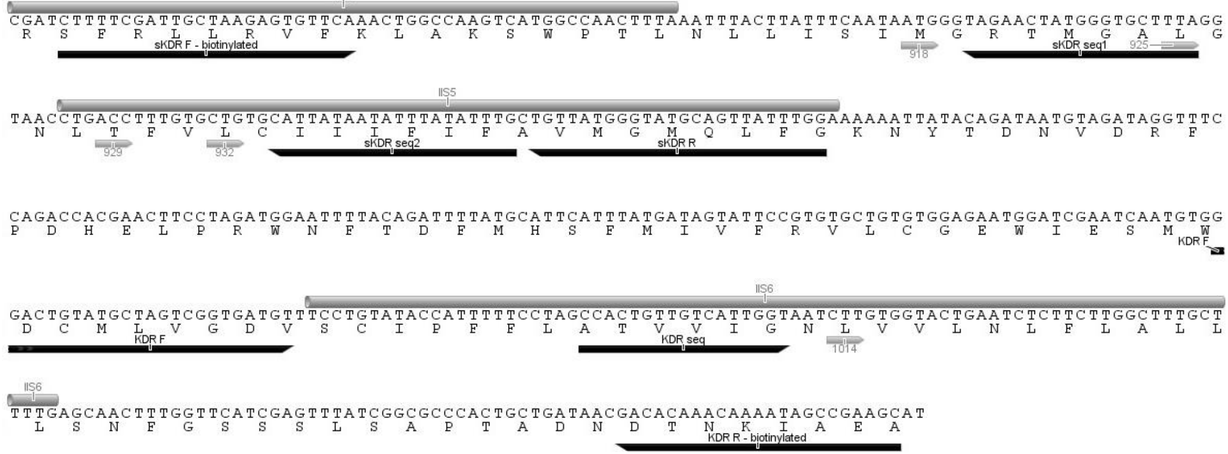

Fig. 60 *Partial nucleotide sequence of a cDNA fragment of the para-type sodium channel of the cabbage flea beetle,* Psylliodes chrysocephala*, spanning the region DII S4-S6. The s-kdr (M918, ATG), kdr (L1014, CTT) and other potential mutation sites (L925, T929 and L932) known to confer resistance to pyrethroids are marked below the sequence. Primers used for pyrosequencing diagnostics are indicated by black arrows below the sequence.*

8.3 Results

8.3.1 Identification of mutations in para-type sodium channel fragments

To identify polymorphisms in the gene sequence encoding domain II of the *para*-type sodium channel of *P. chrysocephala* a PCR approach with degenerate primers successfully resulted in a ~1.8 kb PCR product with the highest similarity to a *para*-type sequence fragment of *L. decemlineata* (87.8 % pairwise identity, E-value = 0), another chrysomelid beetle species. This partial sequence covered the gene region encoding transmembrane domain II which comprises the IIS4-IIS6 region of the voltage-gated sodium channel protein containing five putative *kdr/s-kdr* mutation sites (M918, L925, T929, L932 and L1014) known to be associated with pyrethroid resistance in several insect species (Fig. 60). The amino acid sequence similarity of this particular region of *P. chrysocephala* to other insect species is greater than 90 % and the homology to coleopteran species is close to 100 % (Fig. 60). Based on the cloned *para*-like sequence of *P. chrysocephala*, species-specific PCR/sequencing primers were designed and used for sequencing of several individuals of a number of populations. Analysis of the obtained sequences revealed only a single non-synonymous SNP causing a leucine to phenylalanine substitution at position 1014 (numbering of the *M. domestica* channel protein) (Fig. 60). The beetles containing the L1014F *kdr* mutation were obtained from a region in Mecklenburg-Western Pomerania in Germany with reported pyrethroid field failures. All beetles analyzed by Sanger-sequencing were either homozygous for the wild-type allele L1014 or for the *kdr* allele F1014. No other mutations were found.

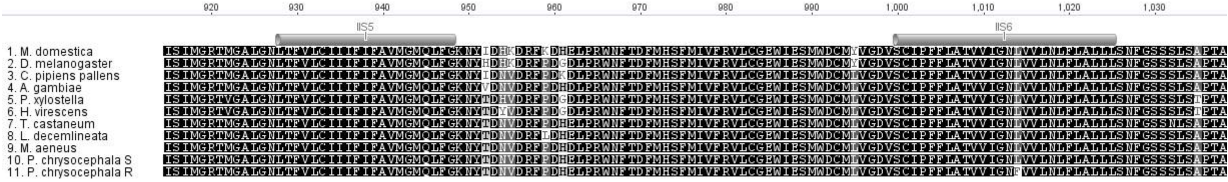

Fig. 61 *Multiple sequence alignment of para-type sodium channel region DII S4/S5-S6 from different insect species (numbering is according to* Musca domestica *Vssc1 sodium channel protein). Conserved identical amino acid residues are marked in black boxes. The sequence obtained from* Psylliodes chrysocephala *R (bottom) shows the L1014F mutation known to confer target-site mediated knock-down resistance to pyrethroids. GenBank Accession numbers of the aligned sequences:* Anopheles gambiae *(CAM12801),* Culex pipiens pallens *(KC977455.1),* Drosophila melanogaster *(AAB59195.1),* Heliothis virescens *(AAC26517),* Leptinotarsa decemlineata *(AAD22957),* Musca domestica *(AAB47605.1),* Plutella xylostella *(BAF37093),* Tribolium castaneum *(NP_001159380) and* Meligethes aeneus *(AF354457).*

8.3.2 Target-site resistance diagnostics by pyrosequencing

For setting up a high-throughput diagnostic system, pyrosequencing assays were developed for *kdr/s-kdr* genotyping. Assays were designed to process both cDNA and gDNA. The *s-kdr* assay covers the region M918 to L932 using two different sequencing primers (Fig. 60). The *s-kdr* assay was limited to the use of reverse sequence primers due to sequence characteristics, whereas the *kdr* assay is limited to use forward sequencing because the assay is designed to suit both cDNA and gDNA. On cDNA it was possible to amplify a region spanning from IIS4 to IIS6 suitable to use for pyrosequencing *s-kdr* and *kdr* region. However the *kdr* sequencing reaction on gDNA failed using the identical setup (data not shown), but re-designing the assay by amplifying two individual PCR products and using a forward sequencing primer for *kdr* (Table 27), the *kdr* diagnostic pyrosequencing assay also worked with gDNA (and cDNA as well).

The pyrosequencing diagnostic assay identifies all three *kdr* genotypes in individual insects, i.e. both larvae and adults: SS (homozygous L1014), SR (heterozygous L/F1014) and RR (homozygous F1014). Insect samples of populations showing significantly decreased susceptibility to pyrethroids and a susceptible reference (showing 100 % mortality at 4 % of the field-recommended rate of *lambda*-cyhalothrin in a vial bioassay) were used to validate the pyrosequencing assay. As shown in Fig. 62 the assay successfully detects the expected nucleotide polymorphism C/T of the first base of the codon translating position 1014. The nucleotide sequences experimentally obtained for SS, SR, and RR are 5'-AATCTTGTGGT-3', 5'-AAC/TTTGTGGT-3' and 5'-AATTTTGTGGT-3', respectively (Fig. 62). The *s-kdr* assay to detect mutations at M918, L925, T929 and L932 was used for parallel sequencing with each individual tested, but no nucleotide polymorphism was observed at the corresponding sites.

In total twenty populations collected primarily in northern Germany were analyzed using pyrosequencing diagnostic. Four of them were taken from a long-standing archive of museum samples and collected between 1945 and 1958, whereas all other samples represented more recent collections with the vast majority collected in 2010 (Table 28). Twenty individuals of each sample collected between 2009 and 2013 in regions of reported field failure were genotyped. Only two populations turned out to be pure wildtype L1014 homozygotes (strains G2 and 41), and another susceptible population (strain 136) contained two beetles heterozygous for the L1014F mutation. The *kdr*-allele was present in most of the samples analyzed and ten populations collected in Mecklenburg-Western Pomerania and Schleswig-Holstein contained only beetles being homozygous for the *kdr*-allele, whereas one sample included 16 homozygotes and 4 heterozygotes (strain 121). One sample collected in

Hesse (PC-05) showed a *kdr*-allele frequency of 0.4 (Table 28). The museum samples collected between 1945 and 1958 were more difficult to analyse and the quality and quantity of gDNA per sample varied widely. However surprisingly the preserved sample collected in 1957 was homozygous for the L1014F mutation, whereas all individuals (larvae and adults) of the three remaining samples from 1945 and 1958 were homozygous for the wild type allele.

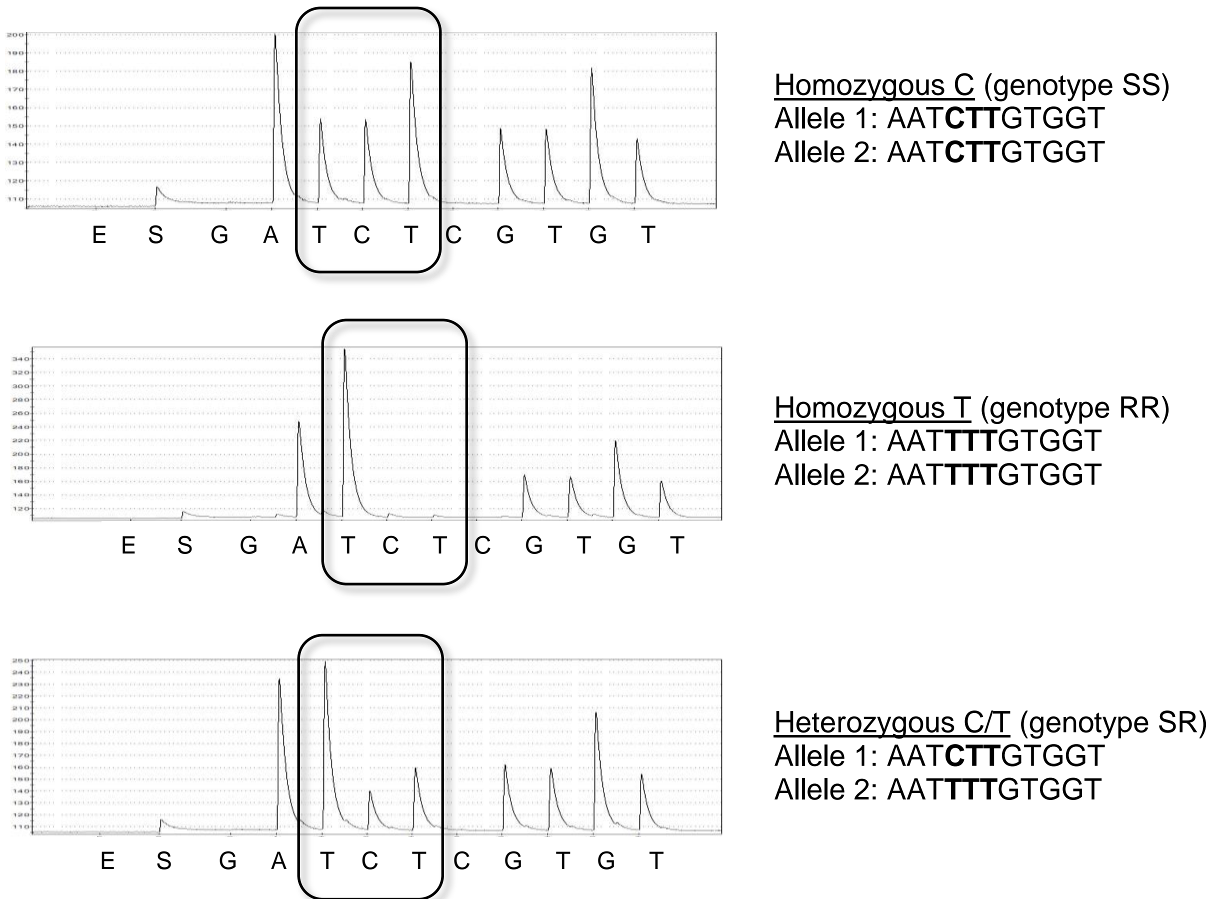

Fig. 62 *Pyrograms displaying homozygous SS, RR as well as heterozygous SR genotypes of the L1014F mutation found in German populations of* Psylliodes chrysocephala *resistant to pyrethroid insecticides and collected in 2010.*

8.3.3 Resistance phenotype conferred by the presence of the L1014F mutation

Depending on the number of beetles collected per sampling site we tested their susceptibility status against up to four different pyrethroid insecticides in adult vial tests (Fig. 4). Based on the pyrosequencing diagnostics reported above we were able to split the obtained bioassay results into populations susceptible and resistant to pyrethroids based on the absence and presence of the L1014F mutation, respectively. The results obtained were similar for all four pyrethroids highlighting the cross-resistance conferred by the detected target-site mutation in those samples showing lower susceptibility. Although the number of data sets for *tau*-fluvalinate, etofenprox and bifenthrin is a little lower compared with the cumulated results obtained for *lambda*-cyhalothrin a clear correlation between the presence of the *kdr*-genotype and pyrethroid cross-resistance is seen (Fig. 63).

Table 28 *Origin and collection year of German cabbage stem flea beetle samples genotyped for kdr resistance (L1014F mutation). All samples were either preserved in alcohol (before 2009) or RNAlater (2009-2013) until pyrosequencing analysis. The dots on the map show the 2010/11 sampling sites with kdr resistance (scale bar = 100 km).*

Sample	Year	Nearest city	Federal State	*KDR* allele frequency
JKI-archive	1945	Lehmkuhlen	Schleswig-Holstein	0
JKI-archive	1957	Kiel-Kitzeberg	Schleswig-Holstein	1
JKI-archive[a]	1958	Kiel Kitzeberg	Schleswig-Holstein	0
JKI-archive[a]	1958	Kiel	Schleswig-Holstein	0
G1[a]	2002	Göttingen	Lower Saxony	0
101	2009	Gadebusch	Mecklenburg-Western Pomerania	1
121	2010	Eldena	Mecklenburg-Western Pomerania	0.9
123	2010	Grombow	Mecklenburg-Western Pomerania	1
119	2010	Lübz	Mecklenburg-Western Pomerania	1
139	2010	Parum	Mecklenburg-Western Pomerania	1
125	2010	Gadebusch	Mecklenburg-Western Pomerania	1
122	2010	Wittenburg	Mecklenburg-Western Pomerania	1
124	2010	Gadebusch	Mecklenburg-Western Pomerania	1
137	2010	Perdöhl	Mecklenburg-Western Pomerania	1
114	2010	Crivitz	Mecklenburg-Western Pomerania	1
PC-02	2010	Hohenhorn	Schleswig-Holstein	1
PC-05	2011	Kassel	Hesse	0.4
136	2011	Neu Eichenberg	Hesse	0.05
G2[a]	2012	Göttingen	Lower Saxony	0
41	2013	Wendhausen	Lower Saxony	0

[a] Larvae only

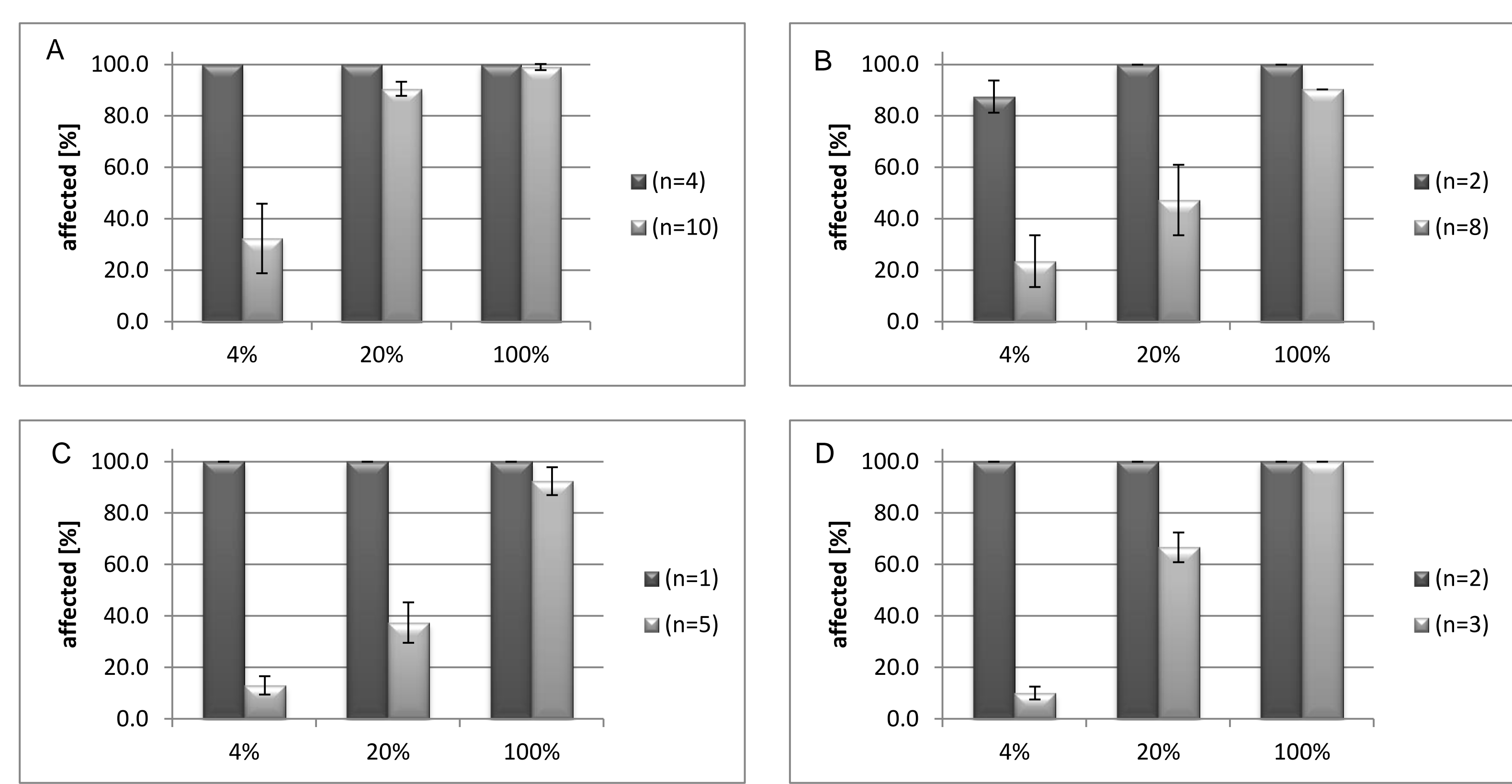

Fig. 63 *Composite percentage mortality figures for susceptible (dark grey; no kdr) and resistant (light grey; kdr L1014F) cabbage stem flea beetle populations after 24 h exposure in an adult vial test to different concentrations of (A)* lambda-*cyhalothrin,(B) Etofenprox, (C)* tau-*fluvalinate and (D) bifenthrin. Test concentrations are given as percentage of the recommended field rate (Table 26); Data are mean values ± SD.*

8.4 Discussion

In this study, we PCR-amplified and sequenced a fragment of the *para*-type voltage-gated sodium channel gene encoding transmembrane domain II from cabbage stem flea beetle, *P. chrysocephala.* We identified a target-site mutation (L1014F) in pyrethroid resistant samples, commonly known as *kdr* and functionally shown to decrease the sensitivity of sodium channels to pyrethroids [21]. This particular mutation is known to confer pyrethroid cross-resistance in at least 20 other pest insects [15], but yet including only two other coleopteran pests, i.e. *M. aeneus* (pollen beetle) and *Leptinotarsa decemlineata* (Colorado potato beetle) [16, 22]. A third coleopteran, the stored product pest *Sitophilus zeamais* was shown to express a *s-kdr*-like T929I mutation in the absence of *kdr* L1014 and weevils surviving pyrethroid treatment were all T929I homozygotes [23]. Recently the same T929I mutation was also found in Bulgarian samples of *L. decemlineata* both in the presence and absence of L1014F, whereas a newly reported T929N mutation in the same study was only found in combination with L1014F [22]. Here we did not find any *s-kdr*-like mutations in several pyrethroid-resistant cabbage flea beetle samples sequenced throughout the study, similar to a recent investigation on pollen beetle, another important pest of oilseed rape, which also failed to find mutations other than L1014F [16]. However, it has been shown by electrophysiological recordings that functionally expressed insect voltage-gated sodium channels (*Vssc1* of *M. domestica*) carrying the L1014F mutation are significantly less sensitive to pyrethroids, which is sufficient to explain the observed *kdr* phenotype [24]. Having said this there is no doubt that the identified target-site mutation in cabbage flea beetle populations collected in German winter oilseed rape contributes to the observed field resistance to pyrethroids (as well as organochlorine insecticides such as DDT which are no longer used) to a similar extent as described in similar studies for other pest species [16,22–24].

We established and validated a pyrosequencing diagnostic method which allows the genotyping of at least 200 cabbage flea beetles per day and is therefore considered to be a high-throughput resistance screening methodology for future monitoring purposes. A similar pyrosequencing method for *kdr* and *s-kdr* diagnostic has recently been described for *M. aeneus* and the disease vector *Culex quinquefasciatus* [16, 25]. The pyrosequencing diagnostic was developed for use of gDNA as well as cDNA and initial difficulties with the reverse *kdr* assay on gDNA is likely due to an intron sequence resulting in a loss of the priming site, as this site was also reported to contain an intron in other insect species [22,25]. The problem was solved by designing a different set of primers (Fig. 1, Table 2). The pyrosequencing *s-kdr* assay additionally spans the region M918 to L932, which is known to contain other mutations such as M918T or T929I [15, 22, 23]. Although we have not detected any *s-kdr* mutation in *P. chrysocephala* pyrosequencing campaigns throughout this study, we

have developed the diagnostics to cover possible additional mutation sites right from the beginning in future monitoring initiatives.

This study on *P. chrysocephala* reveals after *M. aeneus* the second case of a L1014F target-site mutation present in a major coleopteran pest of European winter oilseed rape, and our bioassay findings clearly confirm a high degree of pyrethroid cross-resistance in cabbage flea beetles homozygous for the *kdr* resistance allele. All populations tested and carrying the resistance allele were sampled from recently reported resistance hot spots in Germany [13]. Further evidence for the validity of our conclusions regarding the link between the L1014F mutation and pyrethroid resistance is provided by the fact that structurally diverse pyrethroids such *lambda*-cyhalothrin, *tau*-fluvalinate, etofenprox and bifenthrin (Table 26) are similarly affected in adult vial tests using a range of concentrations up to recommended field rates. In other studies, it has been shown that the presence of metabolic resistance mechanisms sometimes can result in less obvious cross-resistance issues, e.g. in pyrethroid-resistant pollen beetle [17]. However in Swedish populations of pollen beetle, the occurrence of target-site resistance in addition to metabolic resistance resulted in high-levels of pyrethroid cross-resistance completely compromising field efficacy [16]. Many of the cabbage flea beetle samples analyzed in this study revealed a high *kdr*-allele frequency and most of them show a complete lack of wild-type alleles, suggesting a low number of susceptible genotypes due to pyrethroid selection pressure present in those regions sampled.

Interestingly we also detected the L1014F mutation in an archived museum sample of *P. chrysocephala* collected in Northern Germany in 1957 not far away from the current *kdr* hotspot which has a long tradition of winter oilseed rape cultivation in Germany. This may be explained by resistance selection well back in the 20 century by the use of both natural pyrethrins and DDT, which were quite common to control cabbage flea beetle as well as other oilseed rape pests 70 years ago. Natural pyrethrins were used to control oil seed rape pests even before 1920 and DDT was massively used in the 1950s [6, 26]. So there is evidence that in the history of cabbage stem flea beetle control by insecticides this pest was selected twice for the L1014F target-site mutation, i.e. well before the introduction of synthetic pyrethroids by either pyrethrins or DDT or both, and after their introduction. However, for many years other insecticides such as *gamma*-HCH (lindane) and organophosphates were used particularly between 1960 and 1990 [8, 27]. Since no field failures of synthetic pyrethroids in the control of cabbage stem flea beetle were noticed until 2008 [13, 28], it is rather unlikely that the *kdr*-allele detected in the population collected in 1957 was recently present as a conserved mechanism of resistance at high frequency. Otherwise one would have expected a faster emergence of resistance by re-selection of a conserved allelic genotype. Therefore this allele

presumably disappeared over time, as fitness costs may have had an impact in the absence of selection pressure by insecticides targeting voltage-gated sodium channels.

Our results strongly suggest further studies to investigate the spread of pyrethroid resistance in cabbage stem flea beetle, because the emergence of a target-site mutation conferring cross-resistance among pyrethroids is likely to have a strong impact on flea beetle control in Europe in the future. In Germany and many other European countries, only pyrethroids remain available for the control of cabbage stem flea beetles in oil seed rape, because of a recent decision of the EU Commission to suspend neonicotinoid seed treatments in certain crops including oilseed rape [29]. Neonicotinoid seed treatments provide a second mode of action for resistance management purposes and are commonly thought to be indispensable in terms of early season protection of young seedlings from flea beetle attack, so their ban will have strong implications for oilseed rape production and without any doubt increasing pyrethroid selection pressure considering the fact that 67 % of the total area of oilseed rape in the UK is affected by cabbage stem flea beetle [30]. Furthermore the selection pressure on other flea beetle species such as *Phyllotreta* spp. more common in other regions in Europe will increase due to additional (pyrethroid) treatments. Such additional treatments are likely to select for pyrethroid resistance in the absence of another mode of action as it was provided by neonicotinoid seed treatment [31, 32].

For sustainable oilseed rape production and consistent control of flea beetles, it is of utmost importance to follow resistance management principles and to implement alternative modes of action. The recent emergence of pyrethroid resistance in pollen beetle as well as flea beetle is a warning sign that limited mode of action diversity drives rapid resistance development in intensive cropping systems under continuous pest attack such as oilseed rape in Europe. If future flea beetle control measures in oilseed rape have to rely on pyrethroids only due to restricted regulations underestimating the value and urgency of resistance management strategies on mode of action rotation, there is a strong risk of considerable decline in acreage and yield.

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Chapter 9

Concluding discussion

Insecticide resistance in oilseed rape pests is not only a recent phenomenon, as resistance to DDT in pollen beetle was reported in at least two countries, i.e. Poland and in the Czech Republic in the late 1960s and early 1970s [1,2]. However, with the ban of DDT in Europe also resistance slowly decreased over time. Pyrethroid resistance in pollen beetle was first discovered in 1999 [3,4], and is still spreading in Europe [5]. In addition to the pollen beetle resistance problem two more oilseed rape pests, the cabbage stem flea beetle *P. chrysocephala* and the cabbage seed weevil *C. assimilis*, were found to be resistant to pyrethroid insecticides in a region with a very high frequency of oilseed rape cropping in northern Germany [6].

Great efforts were made to monitor the spread of pyrethroid resistance in pollen beetle by authorities such as the Julius-Kühn-Institute in Germany [7], by industry [3] and as a combined approach under the umbrella of IRAC [4]. However, in contrast to the many institutions focusing on resistance monitoring, very little studies have been carried out to elucidate the molecular mechanisms underlying pyrethroid resistance. Therefore this thesis was a combination of bioassays to determine the level of resistance in individual populations of several coleopteran oilseed rape pests followed by biochemical and molecular experiments to elucidate the mechanisms conferring resistance. Understanding the mechanisms of resistance is an important first step in developing resistance management strategies leading to recommendations for the future control of insect pests in oilseed rape.

9.1 Monitoring

The early detection of insecticide resistance development is a prerequisite for resistance management; it starts sometimes with monitoring on the farm level where a farmer may evaluate the efficacy of the control measures taken [8]. In case field performance of insecticides is compromised factors other than resistance also have to be considered such as incorrect application rates, formulation issues or poor application coverage. If these factors can be ruled out laboratory based/manufactured test systems are required to detect significant changes in the susceptibility of a pest towards individual compounds [9,10]. Moreover for the registration of new compounds and the re-registration of established insecticides the EPPO guideline PP 1/213(3) on Resistance Risk Assessment demands baseline susceptibility studies [11]. Establishing baseline susceptibility allows the detection of resistant

populations/strains in monitoring initiatives by calculating the ratio in shift of susceptibility (resistance ratio) based on the benchmark established in a baseline study.

The resistance monitoring of coleopteran oilseed rape pests is based on a simple, robust and reliable adult vial test system which was initially optimized for pyrethroid resistance monitoring in pollen beetle by Bayer CropScience, Syngenta and the Julius-Kühn-Institute (Germany) [4]. The method became part of IRAC's Susceptibility Test Method Series, and is listed as Method No. 011 [12]. It is described in detail in Chapter 2 section 2.2.2 [13]. Although this method is recommended by IRAC for the monitoring of pyrethroid resistance in pollen beetle it can also be used for bioassaying other coleopteran oilseed rape pests as shown in studies of HEIMBACH *et al.* [6,7,14] and in this study (Chapter 7 & Chapter 8 [15,16]). Since all the major coleopteran pests of oilseed rape are univoltine (see section 1.1) and thus preventing mass rearing in the laboratory, the use of a simple, medium throughput system such as the adult vial test is convenient. Test systems involving plants as described by THIEME *et al.* [17] are not commonly used as they are too complicated and don't provide any extra benefits. The adult vial test was therefore adapted for use of several insecticides belonging to different chemical classes. Adult vial tests were subsequently introduced for the neonicotinoid thiacloprid (Chapter 2 [13]), the organophosphate chlorpyrifos and the oxadiazine indoxacarb and can be found in IRAC's Susceptibility Test Method Series under method 021, method 025 and method 027, respectively. The simplicity of the adult vial test allows vials to be prepared in advance to be used directly on site or in the field, and particularly Bayer CropScience provided test kits to numerous testing sites in Europe led for example to > 500 tests in 2012 on pollen beetle populations across Europe [5].

The ongoing monitoring of pyrethroid resistance in pollen beetle allows a detailed insight into the spread of pyrethroid resistance throughout Europe. In 2007 and 2008 resistance mainly affected central Europe i.e. France, Germany, Belgium, the Netherlands, Poland and the Czech Republic [18,19]. Since then it spread rapidly, thus today most if not all European countries may be considered as affected by pyrethroid resistance in pollen beetle except from very eastern countries e.g. the Ukraine [4,5,13,15,20–22].

Although, the simple bioassay system allows high numbers of samples to be tested, the supply of test kits to numerous testing sites involves numerous experimenters, thus care must be taken when interpreting results. In the case of pyrethroids it might not make a big difference if a population is called resistant or highly resistant, both results would lead to a recommendation to use a different chemical class in the field. In contrast for new or re-registered insecticides the situation is more delicate, therefore tests with new insecticides must be carefully assessed in order to detect subtle changes as early as possible to expand their life-span. Today four adult vial test systems are available for monitoring purposes (see

above), covering four distinct modes of action with individual compounds that differ in their physicochemical properties and intrinsic activity, resulting in differences in the expression in symptoms of poisoning. For reliable susceptibility monitoring of new/re-registered compounds test kits should be supplied with detailed information on how to assess the tests and what the result means in terms of susceptibility shifting. Furthermore, each possible shift in susceptibility observed (in laboratory or field) should be confirmed by another laboratory using a full dose-response bioassay, and ideally a re-collected sample from the same field should result in a similar finding.

The massive number of bioassays of pollen beetles conducted throughout Europe was possible because the bioassay is very easy to handle and pollen beetle is fairly easy to collect. The beetle regularly infests oilseed rape in high numbers [23,24] and may be collected using an insect net or by simply shaking the plants into a bucket. Hundreds of beetles can usually be collected in this way in just a few minutes. Compared to pollen beetle other oilseed rape pests are often less abundant in numbers. Thus resistance (baseline) monitoring is more difficult to carry out and therefore a comparable overview regarding the situation is not available. However, the continuous monitoring activities by German authorities even on a smaller number of samples indicated the regionally restricted presence of pyrethroid resistant cabbage stem flea beetles and cabbage seed weevils particularly in Mecklenburg-Western Pomerania [6]. This observation is quite worrying as pyrethroids are also the major chemical class used to control these coleopteran pests and monitoring resources to focus on such problems concerning future pest management in oilseed rape are quite restricted these days. Nevertheless, the efficacy monitoring especially for newly registered chemistry in pollen beetle should be continued to ensure early signs of susceptibility shifts will not be overlooked.

9.2 Mechanisms

To date pyrethroid resistance is present in three different coleopteran species known to be major pests of oilseed rape in Europe [4,6]. Mechanisms commonly involved in pyrethroid resistance are *kdr*-like target site resistance [25,26] and P450-mediated metabolic resistance [27]. Target site resistance is known to confer strong cross-resistance to compounds sharing the same binding site e.g. the L1014F *kdr*-mutation has been shown to confer resistance to pyrethroid insecticides and DDT [25,26,28,29] and point mutations in AChE or nAChR confer resistance to organophosphates/carbamates and neonicotinoids, respectively [30,31]. However the cross-resistance pattern for cytochrome P450 mediated detoxification is less predictable [32]. For instance CYP6P9 from *A. funestus* confers resistance to a broad range of pyrethroids including those containing common structural motifs (Fig. 25, Chapter 3) such as deltamethrin and *lambda*-cyhalothrin but also bifenthrin, which contains a 3-phenylbenzyl instead of a 3-phenyloxybenzyl-alcohol moiety. In contrast CYP6D1

from *M. domestica* confers high levels of resistance (> 5000-fold) to pyrethroids containing a 3-phenyloxybenzyl-alcohol moiety but only low levels to those pyrethroids lacking this moiety [33,34]. P450s may have a broad substrate spectrum [32,35] therefore it is not surprising that they may confer cross-resistance across chemical classes as has been reported several times [36–40]. A very interesting case is CYP6CM1 conferring resistance to many neonicotinoids and the chemically unrelated insecticide pymetrozine in *B. tabaci* [40]. CYP6CM1 is overexpressed in resistant whiteflies up to ~150-fold [41] and resistance ratios ranging from ~400 to > 5000 for neonicotinoids and > 1000 for pymetrozine have been reported [42]. Even though this particular P450 protects the whitefly effectively from pymetrozine and most neonicotinoids [41,42], the neonicotinoid acetamiprid remains unaffected *in vitro* [43]. However, a recently identified P450 in *B. tabaci* named CYP6CX4 was shown to “close the gap” and confers resistance to imidacloprid, thiacloprid and acetamiprid [44]. The complex nature of P450-mediated resistance is not surprising considering the number of P450 genes in several sequenced insect genomes, which is usually around 100, but extremes ranging from 36 genes in *Pediculus humanus* L. up to 180 genes in *Culex pipiens* L. [32,45].

In this study a range of established tools in resistance mechanism research such as synergist trials, biochemical assays, qRT-RCR, pyrosequencing, PCR approaches with degenerate primers and next generation sequencing techniques were combined to elucidate the molecular mechanisms conferring pyrethroid resistance in coleopteran oilseed rape pests with special reference to *M. aeneus*.

9.2.1 Resistance mechanisms in *M. aeneus*

A few studies revealed elevated P450 activity being one major mechanism of pyrethroid resistance in pollen beetle, but all of them failed to functionally link the resistance to P450s [46–48]. Pyrethroid mixtures with PBO suppressing P450 activity in pollen beetle were shown to moderately enhance pyrethroid efficacy under field conditions [49,50]. However a control strategy based on the suppression of metabolic resistance is likely to select for other mechanisms such as *kdr*. Such a tactic is especially risky considering the fact that *kdr* genotypes are already present in some countries such as Denmark and Sweden [51].

In this project, for the first time bioassays on synergist pre-treated pollen beetles from populations collected across Europe confirmed the presence of a P450-based mechanism in all pyrethroid resistant populations (Chapter 3). Native microsomal membrane preparations of resistant pollen beetles incubated with deltamethrin in the presence of NADPH resulted in the formation of the less toxic 4’-OH-metabolite underpinning the involvement of P450s. A single P450, named CYP6BQ23, was identified using a PCR approach with degenerate primers and shown to be highly and significantly overexpressed (up to ~900 fold) in resistant pollen beetle

populations when compared to susceptible populations sampled in Ukraine and Finland. The overexpression of CYP6BQ23 was clearly correlated with the resistant phenotype and the hydroxylation rate of deltamethrin in native microsomal preparations. Recombinant expression of CYP6BQ23 in conjunction with cytochrome P450 reductase in Sf9 insect cells revealed functional evidence of its involvement in pyrethroid resistance, as CYP6BQ23 transfected cells readily detoxified deltamethrin to its 4-OH-derivative [51]. The observed pyrethroid cross-resistance pattern in pollen beetle is comparable to the pattern described by SCOTT & GEORGHIOU [33] for the CYP6D1 mediated pyrethroid resistance in housefly where mainly pyrethroids containing a 3-phenyloxybenzyl-alcohol moiety were affected. The higher efficacy of bifenthrin, etofenprox and *tau*-fluvalinate against pollen beetles observed in laboratory bioassays [6,47,52,53] is also reflected in their slightly better field performance [49,54–56]. Recombinantly expressed CYP6BQ23 was also shown to hydroxylate *tau*-fluvalinate but at a significantly lower level than deltamethrin, thus to some extent explaining the lower cross-resistance observed in both laboratory biossays and field trials. Whereas CYP6BQ23 is shown to provide a high resistance to compounds such as *lambda*-cyhalothrin and deltamethrin, which have been widely used over many years, it is less effective against *tau*-fluvalinate. MOORES *et al.* [57] showed the synergistic action of PBO in combination with *tau*-fluvalinate in pollen beetle populations collected in Poland in 2010, however, the resistance factors based on LC_{50}-values reported were only about 10-fold compared to > 200-fold for *lambda*-cyhalothrin (Chapter 2 [13]). However a combination of *tau*-fluvalinate (and bee-safe neonicotinoids i.e. thiacloprid and acetamiprid) and PBO was shown to result in high honeybee toxicity [58–60], therefore such a mixture has great ecotoxicological disadvantages, particularly in bee attractive crops such as oilseed rape. The nature of the somewhat better field efficacy against pollen beetle of *tau*-fluvalinate is probably a combination of rather limited detoxification rates by CYP6BQ23 and its approx. 7-fold higher application rates.

Tau-fluvalinate which was rarely used in the past [61] was promoted as a resistance management tool to combat resistant pollen beetles based on its better performance in field trials [62]. HANSEN [52] reported an increased usage of *tau*-fluvalinate in Denmark to replace the less effective *lambda*-cyhalothrin and KUDSK [63] later on reported widespread resistance affecting both *lambda*-cyhalothrin and *tau*-fluvalinate in Sweden. Sequencing a fragment of the pollen beetle *para*-type sodium channel gene of *tau*-fluvalinate-resistant populations revealed a target site mutation resulting in a L1014F amino acid substitution, also known as *kdr*. Using a high-throughput pyrosequencing assay beetles of > 400 populations collected throughout Europe were genotyped but *kdr* beetles were interestingly only found in northern Europe. Surprisingly no *kdr* was found in populations obtained from eastern Europe where DDT resistance was reported in the past [1,2] and pollen beetle were under strong pyrethroid selection pressure [64]. Sequence analysis revealed the presence of *kdr* in two allelic variants

with and without a deletion mutation 8 base pairs downstream of the *kdr* mutation located in an intron. This is therefore likely to be an example of a parallel evolution within one species as recently shown in *L. decemlineata* [65], but in a geographical restricted area. The presence of two independent target site alleles in addition to CYP6BQ23 overexpression in Scandinavia is in contrast to the situation in central Europe where only the overexpression of CYP6BQ23 was found. The reasons underlying this phenomena are not well understood, however, factors such as the relatively high cropping area of spring oilseed rape compared to central Europe and the intense use of *tau*-fluvalinate for pollen beetle control may have driven the selection pressure to another level. A close proximity of winter oilseed rape and spring oilseed rape crops provides a prolonged reproduction resource for pollen beetle [66]. Due to its phenology the spring oilseed rape has a lower ability to mount compensation response and is more susceptible to pollen beetle attack compared to winter oilseed rape. As a consequence the economic thresholds are extremely low (0.5-1 beetle/plant in BBCH 51 in Sweden), thus resulting in a higher treatment frequency [61]. Even though it is known pollen beetles may disperse several kilometers a day [67,68] studies on the genetic diversity in pollen beetle populations collected across Europe revealed a high genetic diversity within a population but suggested little long range dispersal [69,70], thus supporting a possibly independent locally restricted resistance development.

In addition to the two major mechanisms i.e. CYP6BQ23 overexpression and *kdr* target-site resistance, carboxylesterases could also play a more minor role in pyrethroid resistance in individual pollen beetle populations [48]. Such a speculation is supported by the transcriptome study carried out in 2012, but no functional evidence for the involvement of esterases in resistance is provided (see Chapter 6 [71]). The global analysis of gene expression in three pyrethroid resistant pollen beetle populations collected in Germany, Poland and Sweden compared to a susceptible reference strain obtained from Ukraine confirmed the presence of *kdr* in Scandinavia and the massive overexpression of CYP6BQ23 in all resistant samples. Interestingly a closely related P450 to CYP6BQ23 named CYP6BQ25 was found to be overexpressed in all resistant samples by approx. 20-fold whereas CYP6BQ23 was overexpressed > 400-fold in all resistant samples analyzed. However, transcript levels do not necessarily precisely correspond to protein levels [72] and are to a greater or lesser extent an indirect measure in terms of ressitance level. Other studies of pyrethroid resistance demonstrated that a P450 does not necessarily has to to be overexpressed beyond 200-fold to be linked to resistance, e.g. CYP6D1 is approx.10-fold [73] and CYP6P3 is approx. 3-fold [74] overexpressed in *M. domestica* and *A. gambiae,* respectively. The high similarity of CYP6BQ25 to CYP6BQ23 and its constitutive overexpression suggests a role in metabolically mediated pyrethroid resistance, perhaps by detoxifying primary metabolites such as 4-OH-deltamethrin, which is principally toxic to pollen

beetles, albeit at a rate 25-fold lower than deltamethrin. However when pharmacokinetically cumulated in concentrations high enough, 4-OH-deltamethrin could principally evoke intoxication symptoms, so its further degradation is toxicodynamically definitely an advantage under constant pyrethroid selection pressure as present in European oilseed rape. Apart from CYP6BQ25 the transcriptomic analysis revealed a third P450 gene, CYP4Q22 also overexpressed in all three resistant populations included in the transcriptome analysis. The role of this P450 also remains to be elucidated. However this is an interesting approach for any future investigations on pollen beetle resistance to pyrethroids. Finally the transcriptomic approach revealed more candidate genes belonging to CCEs and GSTs with fold-changes around 2-fold (see Chapter 6 [71]), which might also be investigated in future projects.

9.2.2 Resistance mechanisms in *P. chrysocephala*

The first case of pyrethroid resistance in the cabbage stem flea beetle was described in 2009 [6]. The resistance was first discovered in Mecklenburg-Western Pomerania, Germany, in an area with a traditionally high cropping frequency of oilseed rape (pyrethroid resistance in pollen beetle was first reported in the same area) [3,6]. Resistance factors based on LC_{50}-values for *lambda*-cyhalothrin where as high as 80-fold among 38 populations tested [6]. The cross-resistance pattern tested with *lambda*-cyhalothrin, *tau*-fluvalinate, bifenthrin and etofenprox revealed that all compounds are similarly affected, although they differ to some extent in their chemical structure. The clear cross-resistance suggested a target-site mutation as the primary resistance mechanism rather than overexpression of detoxification enzymes. A PCR approach with degenerate primers was used to amplify a fragment of the *para*-type sodium channel gene from susceptible and resistant beetles. Subsequent sequencing of the fragments revealed that all resistant beetles contained the L1014F *kdr* mutation. Based on the obtained sequence a high-throughput pyrosequencing assay was designed. The genotyping of resistant and susceptible populations revealed a clear link between the resistance phenotype and the *kdr* trait (see Chapter 8 [16]). A few archived museum samples collected prior to introduction of pyrethroids and obtained from the Julius-Kühn Institute were also analysed, and one sample collected in 1957 also contained the *kdr*-allele. Since the 1950s DDT was massively used in oilseed rape in Germany [75], so the result is not completely unexpected considering the fact that DDT and pyrethroids share the same mode of action by addressing the same binding site in VGSC. As the pyrosequencing diagnostic is only based on a few sequenced base pairs nothing is known about sequence identities (phylogeny) of the 1957s *kdr* allele and those collected more recently. However, based on the fact that no field failures of pyrethroids were reported until 2008 [76] and recent field failures can be correlated with the presence of *kdr* (Chapter 8 [16]) it seems to be unlikely that the allele present already in the 1950s is the origin for today´s problems to control cabbage stem flea beetle in

Germany. The recent discovery of L1014F *kdr* mutations in both cabbage stem flea beetle and pollen beetle is an excellent example of parallel evolution of insecticide resistance in oilseed rape pests, i.e. the presence of *kdr* in a population is likely to be a clear advantage under pyrethroid selection pressure, but fitness costs may affect survival of such genotypes in the absence of pyrethroids. Parallel evolution is a common phenomenon for target-site resistance and reflects the limited options of changes at the target-site level in insects without adversely affecting the natural functionality of the target [30].

9.2.3 Pyrethroid resistance in *C. assimilis*

C. assimilis is the third coleopteran species to be found resistant to pyrethroid insecticides very recently. The resistance arose in the same region in Germany where resistant cabbage stem flea beetles and pollen beetles were first discovered [6]. So far no molecular study has been conducted to elucidate the resistance mechanism(s) in this species. However, likewise *P. chrysocephala* the cross-resistance between *lambda*-cyhalothrin, *tau*-fluvalinate and etofenprox and the similar range of resistance ratios towards *lambda*-cyhalothrin [6] suggests the involvement of a target-site resistance.

9.3 Management

Ideally the development and implementation of insecticide resistance management strategies commence prior to the development of resistance in order to avoid or delay its appearance [77]. Ironically, the starting situation for oilseed rape pest control was favorable for sustainable resistance management, because the inherent resistance risk of all major oilseed rape pests is relatively low compared to pests such as *M. persicae* or *Tetranychus urticae* KOCH, based on their life cycle and host preference for oilseed crops such as *Brassica napus* L., *Brassica rapa* L. and *Sinapis alba* L.. Of course, pollen beetle is also a pest of other Brassicas such as *Brassica oleracea* L., but compared to oilseed crops this is of limited importance and a recent study did not find a host preference of the filial generation to brassicaceous vegetables [78]. However, the recent emergence of probably two independent *kdr* alleles in pollen beetle as well as in cabbage stem flea beetle and a strong metabolic resistance mechanism based on the overexpression of CYP6BQ23 in pollen beetle reflects a serious genetic resistance risk in these pests. The rapid spread of CYP6BQ23 overexpression across Europe prompts questions on the dispersion dynamics of pollen beetles. Pollen beetles may disperse several kilometers a day [67,68], however studies on the genetic diversity in pollen beetle populations collected across Europe suggested little long range dispersal [69,70]. In the past no resistance management strategies were followed prior to the development of pyrethroid resistance in pollen beetle. However, once the extent of the problem became obvious the agrochemical industry started to successfully seek for alternative

modes of action and meanwhile most countries approved registrations of new chemical classes such as neonicotinoids, indoxacarb or pymetrozine, but also older classes such as organophosphates (see Chapter 1). Furthermore some established pyrethroids such as *tau*-fluvalinate became available again because of their higher field efficacy compared to other widely used pyrethroids, e.g. *lambda*-cyhalothrin and deltamethrin (see section 9.2.1).

However, most compounds with an alternative mode of action are registered for pollen beetle control only such as pymetrozine, indoxacarb and organophosphates, so a rotation by mode of action as one of the most effective resistance management strategies at least for pollen beetle control seems possible [79–81]. In addition only two neonicotinoid insecticides (thiacloprid and acetamiprid), are considered to be non-toxic to bees and are allowed to be used in oilseed rape during flowering, all others except some pyrethroids are restricted to pre-flowering use. Although MOORES *et al.* [57] showed the potential of a PBO/*tau*-fluvalinate mixture as a bee-safe control option others reported strong synergism of bee toxicity for such a mixture [58,59]. Furthermore the presence of *kdr* resistance prohibits such a control strategy in order to avoid further selection of target-site resistance.

The early occurrence of pollen beetle in winter oilseed rape is often overlapping with *Ceutorhynchus* spp. [54,61]. Control measures taken against stem weevils currently rely heavily on pyrethroids, thus every spray in the presence of pollen beetle increases the selection pressure. Currently the only way to reduce selection pressure in case co-abundance of stem weevils and pollen beetle is the use of insecticide mixtures. Insecticide mixtures have been shown to be very effective due to synergistic action in various insect species [82–84]. In France a mixture of deltamethrin and thiacloprid (Proteus® OD110) is registered to be used in oilseed rape [85]. However, since thiacloprid is one of the most intensively used non-pyrethroid insecticides in oilseed rape in Europe such a mixture increases the selection pressure on neonicotinoids which provide reliable pollen control as straight products. However in the absence of registered premixed insecticides it is legally possible to use tank mixtures for pest management purposes, e.g. a tank mixture of pymetrozine and pyrethroids as recently recommended by the ECPR-I in Germany [86]. Bee toxicity has to be taken into account while using mixtures of insecticides, since stem weevils emerge early in the spring this pre-flowering application window does not overlap with the presence of bees in oilseed rape.

It is not just pollen beetle adults overlapping with cabbage stem weevils but also pollen beetle larvae overlapping with cabbage seed weevil, which is also controlled by pyrethroid applications, so the selection pressure is also present on the subsequent summer generation of pollen beetle. As demonstrated in Chapter 7 section 7.3.2 [15] pollen beetle 2nd instar larvae are highly resistant to pyrethroids but very susceptible to thiacloprid. Since HEIMBACH & MÜLLER [6] reported *C. assimilis* populations are resistant to a range of pyrethroids, future

control measures should be best based on neonicotinoids which reduces the selection pressure on pyrethroids in both species. However switching from pyrethroids to neonicotinoids for the control of *C. assimilis* is not a sustainable long-term solution, and more bee-safe alternatives of different mode of action classes need to be developed to avoid selection by neonicotinoid insecticides.

The recently discovered *kdr* resistance in the cabbage stem flea beetle is another problem that has to be managed. Presently only a limited number of populations collected in Germany were tested for the presence of this target-site mechanism, and a European wide monitoring will be necessary to check whether the problem is regionally restricted or has already spread. In the case of pyrethroid resistance in pollen beetle only one year after the first evidence of pyrethroid resistance in 1999 in northeastern France it was also reported independently in Denmark and Sweden [52,87,88] and subsequently in many other countries across Europe, such as Germany, Switzerland and Poland [7,89,90]. In contrast to *M. aeneus,* which exceeds economic thresholds almost annually the occurrence of *P. chrysocephala* is irregular. ALFORD *et al.* [91] reported peaks of abundance in a 7-year cycle in central parts of northern Europe. JOHNEN [92] described autumn temperatures as a key factor determining the population dynamics, i.e. in 1989 and 2000 he correlated unusually warm temperatures during autumn month in both years with peak infestations. *P. chrysocephala* is generally highly susceptible to pyrethroid treatments once economic thresholds are exceeded due to decreasing efficacy of seed treatments with neonicotinoids particularly protecting young seedlings [54,91,93,94]. ALFORD *et al.* [91] reported that the importance of *P. chrysocephala* has declined in Great Britain through the foliar use of pyrethroids. A recent decision by the European Union [95] prohibiting the use of neonicotinoid insecticides for seed treatment in several crops including oilseed rape cuts down the control options to pyrethroids only. Considering the resistance development against pyrethroids in *P. chrysocephala* an urgent need for further action is given, i.e. development/registration of alternatives and the avoidance of unnecessary pyrethroid applications by taking into account the economic threshold and phenological models as described by JOHNEN *et al.* [93,96].

It seems naive to try to overcome current problems of insecticide resistance by simply using different insecticides and of course more is needed than just alternating insecticides. Therefore application schemes are developed and provided by industry, authorities and consultants. Examples concerning oilseed rape are management strategies provided by the “Expert Committee on Pesticide Resistance – Insecticides” [86] in Germany and by IRAC [97]. However such strategies can only succeed if enough diversity, i.e. compounds with distinct modes of action are available. As discussed above in theory 4-5 different modes of action are now available in many countries for use in oilseed rape, but in practice the situation is

completely different. The ongoing spread of pyrethroid resistance in oilseed rape pests underlines the requirement for further action. HOKKANEN [23] correlated the increasing problems with *M. aeneus* with the steadily increased acreage of oilseed rape crops. However, oilseed rape has become an indispensable crop in many crop rotations since crop rotations are mainly based on cereals in Europe [24] and sustainable pest management strategies are therefore indispensable, too.

Integrated pest management is the preferred approach for sustainable crop production [98]. The knowledge of naturally-occurring agents of biological control has improved considerably over the past 15 years mainly due to two EU programs (acronyms: BORIS and MASTER [99]). The results of BORIS and MASTER as well as a significant contribution of research not covered by those programs are published in two excellent books about naturally-occurring agents (parasitoids, predators and pathogens) for bio-control of oilseed rape pests [24,100]. Even though the efficacy of natural enemies/pathogens may be good e.g. a parasitism rate of 50 % on pollen beetle larvae [101], so far no basic strategy has been developed to use this potential sufficiently. The use of efficient entomopathogens such as nematodes is too expensive [102,103] and the integration of natural enemies in pest management programmes in oilseed rape is difficult. The complex interaction in a multi-trophic system in different landscapes and unequal cropping practices do not favour integrated pest management approaches [104]. Changes on the landscape level are unlikely to happen in a short period of time; however, the cropping practice may be adapted sooner. As most parasitoids of oilseed rape pests overwinter in the soil a reduced tillage or direct drill of the following crop after the harvest of oilseed rape has a positive impact on the survival rate of parasitoids [105]. The relatively low migration capability of parasitoids suggests positive effects of high cropping frequency within a region for the conservation of parasitoid populations [102]. The impact of pyrethroid insecticides on populations of parasitoids is considered to be serious [106]. The number and the timing of applications as well as the applied dose rates may determine the impact caused by pyrethroid treatments [106]. Studies on other insecticide classes are very limited. However, studies on parasitoids of stem weevils and pollen beetle compared thiacloprid and pyrethroids with similar findings i.e. a reduced parasitism rate after insecticide treatment [107]. The fact that oilseed rape is subsequently attacked by six major pests i.e. *P. chrysocephala*, *C. napi, C. pallidactylus, M. aeneus, C. assimilis* and *D. brassicae* from emergence in early autumn until seed formation in the early summer (Fig. 2, Chapter 1) means there is almost no chance for parasitoids to act in an insecticide free environment. Farmers are taking several control measures i.e. insecticide treatments to protect their crop during the season [61]. Therefore they need to control particularly adult stages, even though the larval stages of some pests have a significant impact on the plants (see section 1.1.1), but systemically acting insecticides to control larvae

are mostly not available. Parasitoids in combination with insecticides may reduce the number of offspring thus lowering the number of adults for the following season. At the end of the day the decision on insecticide treatment in an ideal world should be largely based on economic thresholds rather than protective, thus compromising the potential benefits of natural enemies. This reduces the insecticide selection pressure on insect pests and supports integrated pest management. Furthermore state of the art computer models considering pest and parasitoid phenology should be used to optimize the timing of insecticide treatments [96]. An interesting approach was described by EKBOM [66], since the major pest of oilseed rape are univoltine species an abandonment of oilseed rape cropping (spring and winter crop) on a landscape level for one year should have a massive impact on the reproduction success. However, such a radical approach is unlikely to be followed by farmers.

Another interesting approach regarding sustainable oilseed rape production is to exploit more intensively available options for host plant resistance. Oilseed rape has been genetically modified (GM) to achieve host plant resistance with several traits i.e. protease inhibitors, plant lectins, *Bacillus thuringiensis* (*Bt*) toxins, chitinases and scorpion toxins [108–113]. *Bt* toxins are widely used to confer host plant resistance in a broad range of plants to a variety of insect pests [114]; however, so far no *Bt* toxin was found to be effective against the major oilseed rape pests [24]. No field-tests are published today and the only trait studied for its ability to suppress pollen beetle is pea lectin. Transgenic oilseed rape expressing pea lectin in anthers up to ~0.6 % of total soluble protein was shown to reduce the development of pollen beetle larvae to adulthood by 50 % compared to non-modified control plants [108]. Considering that the mortality occurs during development of the offspring this trait would not protect the plant from damage of the parental generation and probably only cropping on a large scale (geographical region) would have a significant impact on the outbreak of the pest in the following year. A fairly new approach would be the use of RNAi to combat pests in oilseed rape as it was shown to be effective against a coleopteran pest of corn, i.e. *Diabrotica virgifera virgifera* LECONTE [115] and other coleopteran pest species such as *Tribolium castaneum* [116] and *L. decemlineata* [117]. The global acreage of GM crops (mostly cotton and soybean) was 134 million ha in 2009, in contrast only ~100,000 ha were cropped within the EU [118], which is mainly due to regulatory restrictions and limited public acceptance of GM crops in Europe. Even though GM crops are presently not well-established in Europe, it is an option to be considered for future pest management in oilseed rape, particularly if it is for biofuel production rather than human consumption. Thus the food-crop itself has not necessarily to be modified to achieve a long-term effect resulting in a decline in pest populations. LEHRMAN *et al.* [108] hypothesize a long-term impact of the lower fecundity of pollen beetle on GM oilseed rape. Therefore a GM trap crop would be a compromise allowing the use of the technology and keeping the food chain free of GM oilseed rape. The use of

turnip rape (*Brassica rapa*) as a trap crop was shown to be effective in many studies and not just for pollen beetles [119–122]. Host plant resistance must not necessarily involve transgenic events, but could also be facilitated by hybrids obtained in conventional breeding programs. An obvious target to work on brassicaceous crops would be the glucosinolate content and/or their profile [24]. Even after implementing host-plant resistance in combination with trap-crops in a push-pull strategy involving natural enemies or traits as described above, insecticide treatments are likely to be indispensable as a control measure in case of outbreaks and for pests not covered by the alternative approach.

Genetic engineering is not limited to crops to achieve pest control; in fact several strategies to manage pests are achievable using insect transgenesis. Techniques such as the sterile insect technique or the RIDL approach where insects carry a dominant lethal allele [123] are implausible due to the univoltine nature of the major oilseed rape pests, exacerbating the transgenesis and mass rearing. A smart approach seems to be the development of insecticide resistant natural enemies, allowing the natural enemies to survive insecticide treatments, thus stabilizing the population and increasing their numbers/efficiency [124]. However, the most efficient natural enemies of oilseed rape pests are univoltine parasitoids [125], thus the practicability of such an approach is rather limited based on the lifecycle without taking into account any political or public concerns about the release of transgenic insects in Europe.

WILLIAMS [24] describes the integrated pest management approach in oilseed rape as "in its infancy" as the the control of oilseed rape pests mostly relies on insecticides [24,61]. The insecticides used in oilseed rape act all as neurotoxicants, they act rapidly thus preventing crop damage. Spring as well as winter oilseed rape is treated up to 5 times with insecticides to protect the crops mainly from coleopteran pests [61] and a negative footprint on natural enemies was shown in several studies, albeit with old chemistry not showing IPM fitness due to modern insecticide standards [106,107]. The complex insect community on oilseed rape including a diversity of beneficial and pest insects prohibits a simple solution and demands a holistic approach rather than simple one-shot solutions [24]. The management of oilseed rape pests using an integrated approach manages insecticide resistance at the same time, as it would reduce the number of applications and therefore the selection pressure on key pests. Computer based decision support considering climate, the phenology of the pest its natural enemies and of the crop in combination with the economic threshold allows an optimized use of insecticides. Ongoing research to develop biocontrol strategies such as push-pull, screening for biocontrol agents such as entomopathogens, screening for more selective insecticides, conventional plant breeding as well as genetic engineering on crops,

insects and pathogens offers potential to overcome existing problems in the management of oilseed rape pests.

9.4 Future work

The current study supported monitoring activities on coleopteran pests of oilseed rape and focused on the molecular mechanisms of pyrethroid resistance in order to draw conclusions promoting the future management of insecticide resistance as part of integrated management of oilseed rape pests.

M. aeneus

In the present work in Chapter 3 [126] the cross-resistance pattern in pyrethroid resistant pollen beetle populations was examined. Synergist studies suggested a P450-based metabolic resistance mechanism that was confirmed by biochemical assays revealing the hydroxylation of deltamethrin by native microsomal preparations in the presence of NADPH. In the following study (Chapter 4 [127]) CYP6BQ23 was identified, recombinantly expressed and shown to be the major mechanism of pyrethroid resistance in pollen beetle populations collected across Europe. However, except for qRT-PCR no molecular diagnostic tool has been developed. Furthermore the mechanism causing the extreme overexpression of CYP6BQ23 has been elusive yet. It is not based on gene duplication and it should be investigated more deeply in a future study. Understanding the regulation of CYP6BQ23 expression would likely allow the development of molecular diagnostic tools and therefore support studies on the distribution of this mechanism. Another simple pyrethroid resistance diagnostic could be based on antibodies to be used in ELISA detecting the amount of CYP6BQ23 protein in pollen beetle. Today the epidemic outbreak of pyrethroid resistance in pollen beetle is not well understood. Whether the CYP6BQ23-based resistance mechanism evolved once and spread over Europe or the overexpression arose independently in distinct populations across Europe is unclear. The rapid spread of pyrethroid resistance on the mainland of Europe (within two years pyrethroid resistance was reported in north-western France and Scandinavia [52,87,88]) stands in contrast to resistance expansion in the UK. In 2006 and 2007 resistance remained rare in the UK and geographically restricted to costal districts in the south and the east of the country [122], however, from then on resistance spread successively, and in 2012 > 80 % of the populations tested were pyrethroid resistant [5].

The next generation sequencing project in Chapter 6 [71] revealed two more P450s i.e. CYP6BQ25 and CYP4Q22, which are commonly over-expressed in pyrethroid resistant populations of *M. aeneus*. Furthermore candidate genes belonging to carboxylesterases and

glutathione S-transferases were identified. The expression pattern of those genes should be investigated using a bigger sample size. Furthermore functional expression may help to clarify the contribution of individual candidate genes to pyrethroid resistance.

The *kdr* L1014F target-site resistance was shown to be geographically restricted to northern Europe in *M. aeneus* (Chapter 5 [128]). Future studies may investigate the genotype supporting the conservation of the *kdr.* Today it is unclear why *kdr* is geographically restricted whereas metabolic resistance, i.e. the constitutively overexpression of CYP6BQ23, may be found everywhere.

The baseline susceptibility of *M. aeneus* for thiacloprid was established in Chapter 2 [13] and susceptibility monitoring was carried out in the following years (Chapter 7 [15]). Since the neonicotinoids thiacloprid and acetamiprid are the only insecticides other than some pyrethroids that are non-toxic to bees their preservation is very important for future pest management in oilseed rape and therefore susceptibility monitoring should be carried out in future to detect early shifts, which would allow an early response.

Ceutorhynchus spp.

In this study in Chapter 7 [15] the baseline susceptibility of *C. assimilis* was established for *lambda*-cyhalothrin and thiacloprid as reference substances for pyrethroids and neonicotinoids, respectively. Future studies are necessary to establish baselines for *C. napi* and *C. pallidactylus*, the overall goal should be an European wide monitoring of *Ceutorhynchus* spp. since pyrethroid resistance was already reported for *C. assimilis* in Germany (see section 9.2.3) and a high variation in the susceptibility to pyrethroids of the other two weevil species was reported [6,7]. Further studies are required to elucidate the molecular mechanisms conferring pyrethroid resistance in *C. assimilis* as well as the field performance of control agents other than the pyrethroids e.g. synthetic insecticides and entomopathogens, since the only option presently available other than pyrethroids are neonicotinoids and their preservation is thus of key importance for a sustainable pest control in oilseed rape.

P. chrysocephala

In Chapter 8 [16] the molecular mechanism, i.e. the L1014F *kdr* mutation, conferring cross-resistance to pyrethroid insecticides was identified in the *para*-type sodium channel of the cabbage stem flea beetle. Future monitoring is necessary to allow the geographical mapping of resistance and to take the appropriate action to manage this resistance. The developed pyrosequencing assay allows the simple genotyping of larvae and adults on gDNA extractions of single insects and is therefore ideal for future monitoring. Field trials are

necessary to investigate the efficacy of alternative control agents e.g. synthetic insecticides and entomopathogens, since today control relies exclusively on pyrethroid insecticides.

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Appendix A

Table A1 *Supplementary file 1. Primer list.*

Primer name	Purpose	Sequence
5' RACE outer	5' RACE	CATCCTTTTCGATGATCACGTC
5 'RACE inner	5' RACE	ATTCAAAGCCAACTCGTACA
3' RACE outer	3' RACE	AAGGATATGACCTACATGGAGCA
3' RACE inner	3' RACE	AAAACTTACCCTGTACCTGGAACC
CYP6BQ23 F1	CDS verifiying (PCR)	GAATATGCATTGAGCAATGGTGC
CYP6BQ23 R1	CDS verifiying (PCR)	AGTGTCTTATTATGTTTTTCCCTG
CYP6BQ23 R2	CDS verifiying (PCR)	GGCTTAGTCAGTTGAAATCCTC
CYP6BQ23 seq1	Sequencing	ACAATGGCGAGCTAAGTTATGAAGCC
CYP6BQ23 seq2	Sequencing	AACCATGTCATCCTTTTCGAT
CYP6BQ23 seq3	Sequencing	GGAATGTATGTGAATGAGAAGGCGG
CYP6BQ23 seq4	Sequencing	GGCTCAACTTTGCCCTCAAACTCTTCC
Actin F1	Isolation of actin sequences	GGNTWYGCNSRNGANGAYRC
Actin F2	Isolation of actin sequences	ARATHATGTTYGARAMNTWYAA
Actin R1	Isolation of actin sequences	ATCCACATYTGYTGRAANGT
Actin R2	Isolation of actin sequences	CNKKNCKDATRTCNACRTCRCA
Tubulin F1	Isolation of tubulin sequences	TGYTGGGARYTNTAYTGYYTNGARC
Tubulin F2	Isolation of tubulin sequences	GARCAYGGNATHCARCCNGAYGG
Tubulin R1	Isolation of tubulin sequences	ACYTCYTCRTARTCYTTYTC
Tubulin R2	Isolation of tubulin sequences	GGRCACCARTCNACRAAYTGDAT

Table A2 *Supplementary file 2. qRT-PCR primer list.*

Primer name	Sequence	r^{2*}	PCR efficiency	Slope	Y-intercept	Melt °C
CYP6BQ23 qF2	GGAATGTATGTGAATGAGAAGGCGG	0.997	91.1	-3.556	24.703	80.5
CYP6BQ23 qR2	TTCCAAACGATACAGAACCT					
CYP6BQ23 qF1	CAAAACACAATGGCGAGCTA	0.998	92.5	-3.516	25.986	79
CYP6BQ23 qR1	TCTGAGCAGAATCGGAACTG					
CYP6-like 1 qF	CACCGACGACGTCATAGAAA	0.999	92.7	-3.511	27.046	82
CYP6-like 1 qR	TACGCGCATGGATGTATGTT					
CYP6-like 2 qF	ATGACGTACGAGGCCATGAT	1.000	97.3	-3.389	27.714	80
CYP6-like 2 qR	ACGTCGGTTCCAGGTACATT					
CYP4-like 1 qF	AGTGCCGTTTTATGGCAGAG	0.998	99.4	-3.336	24.393	78
CYP4-like 1 qR	CACGTTGGGTTCAGGAAAAT					
CYP4-like 2 qF	CAAAGTTTATGAGGAACTTGTTGAAA	0.993	92.6	-3.514	33.334	78
CYP4-like 2 qR	TCGCATAGCTTCTTTGATCACT					
CYP4-like 3 qF	GGAACGATGCATCAAGGAGT	0.998	99.1	-3.346	24.813	83.5
CYP4-like 3 qR	CCGGAATGCGTTTGTATTTC					
CYP4-like 4 qF	GACTGTATCCTAGCGTTCCCTT	0.999	91.5	-3.544	22.39	80
CYP4-like 4 qR	TGTAAAGGTTTATGTCGCAACC					
CYP4-like 5 qF	AGCAAATAGTTGGCGACGA	0.994	96.8	-3.399	26.884	78
CYP4-like 5 qR	CATTCTTAATCCTTCTTTGATGACA					
CYP4-like 6 qF	CCACAGCTTCTGCCATTTC	0.999	90.8	-3.563	28.743	79
CYP4-like 6 qR	TCCTGAAGATCTCTATGTCCTG					
CYP4-like 7 qF	CCCCGATGTGTTTAATCCTG	0.998	96.7	-3.402	28.999	80
CYP4-like 7 qR	CAATTTCTGGGTCCTGCACT					
Actin qF	CACCACCACCGCTGAAAGGGA	0.999	97.4	-3.386	20.993	82.5
Actin qR	GGGAAGTAGAGGCGGCAGCG					
Tubulin qF	ACCACGAACAACTATCCGTTGCCG	0.998	92.5	-3.515	25.626	82.5
Tubulin qR	ATTTGCCGTGACGGGGGTCG					

*Model fit of standard curve

Table A3 *Supplementary file 3. Amino acid variation of CYP6BQ23*

Strain	Resistance ratio (RR)[1]	Country	Amino acid residue						
			96	156	160	174	251	420	443
70-10	1	Ukraine	E	N	E	K/R	V	C	T
8-10 (1)	140	France	E	N	E	R	V	S	T
8-10 (2)	140	France	E	N	E	R	V	C	A
57-10 (1)		UK	E	N	E	R	V	C/S	A
57-10 (2)		UK	E	N	E	R	V	C/S	A
82-10	459	Germany	E	N	E	R	V	C	A
67-09	10	Austria	E	N	E	K/R	V	C	A
68-10	383	Germany	E	N	E	K/R	V	C	A
79-10		Germany	E	N	E	K/R	V	C	A
96-10	231	Poland	E	N	E	K/R	V	C	A
102-09 (1)		Germany	E	N	E	K/R	V	C	A
102-09 (2)		Germany	E	N	E	K/R	V	C	A
106-10 (1)	369	Czech Republik	D	N	E	K	V/F	C	A
106-10 (2)	369	Czech Republik	D	N	E	K/R	V/F	C	A
107-10	37	Czech Republik	E	N	E	K/R	V	C	A
120-10	162	Sweden	D	N/K	E/D	K/R	V	C	A
127-10	8	Finland	E	N	E	K/R	V	C	A

Appendix B

Fig. A1 *Additional file 1. Histogram of the coverage of the merging assembly*

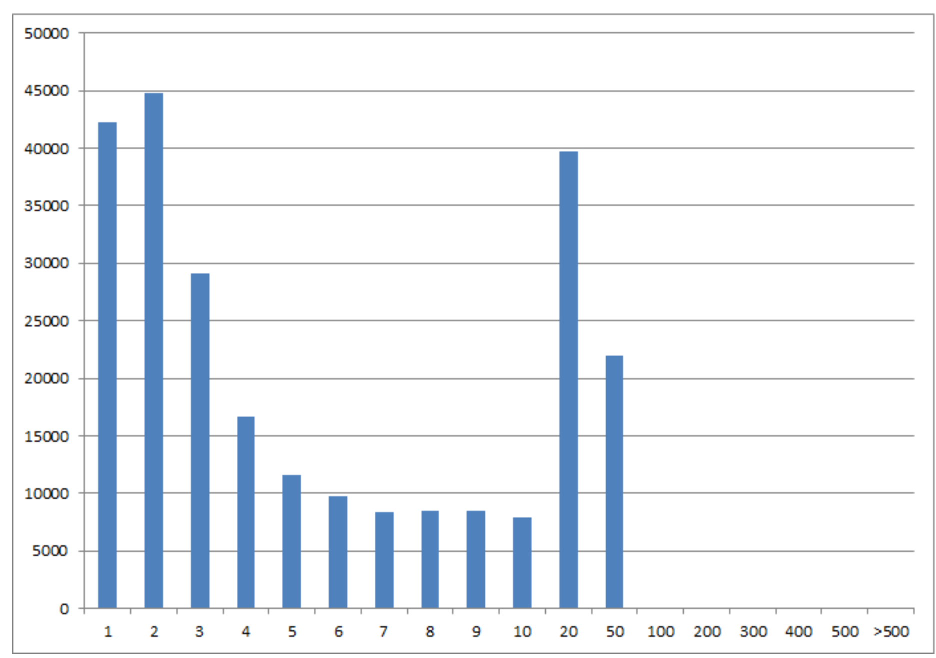

Fig. A2 *Additional file 2. Boxplot of the contig lengths and N50 of the improved assembly*

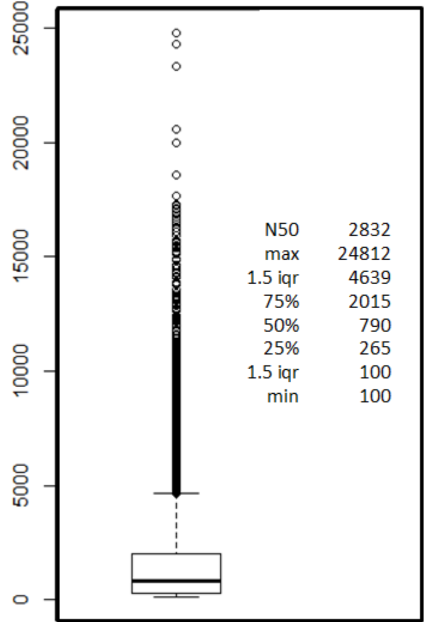

Table A1 *Additional file 3: P450s nucleotide sequences (see CD ROM)*

Table A2 *Additional file4: CCEs nucleotide sequences (see CD ROM)*

Table A3 *Additional file 5: GSTs nucleotide sequences (see CD ROM)*

Table A4 *Additional file 6. Bowtie results of the alignment of short reads vs. the generated hybrid transcriptome*

sample	total reads	Min. 1 alignment	failed	suppressed	reported
UA_1	41,809,924	32,472,457 (77.67%)	9,197,170 (22.00%)	140,297 (0.34%)	131,656,331
UA_2	418,09,924	31,804,070 (76.07%)	9,859,197 (23.58%)	146,657 (0.35%)	129,031,357
SE_1	95,574,799	75,642,802 (79.15%)	19,624,919 (20.53%)	307,078 (0.32%)	306,825,269
SE_2	95,574,799	75,146,539 (78.63%)	20,116,038 (21.05%)	312,222 (0.33%)	305,423,672
D_1	56,000,069	44,383,805 (79.26%)	11,403,380 (20.36%)	212,884 (0.38%)	184,308,254
D_2	56,000,069	43,438,758 (77.57%)	12,339,364 (22.03%)	221,947 (0.40%)	180,501,901
PL_1	52,433,974	41,914,949 (79.94%)	10,321,529 (19.68%)	197,496 (0.38%)	173,146,825
PL_2	52,433,974	40,982,590 (78.16%)	11,243,366 (21.44%)	208,018 (0.40%)	169,525,415

_1 and _2 represents technical replicates

http://onlinelibrary.wiley.com/doi/10.1111/imb.12099/suppinfo

Table A5 *Additional file 7: 2-fold up-regulated ESTs between all resistant populations and the susceptible population (see http://onlinelibrary.wiley.com/doi/10.1111/ imb.12099/suppinfo)*

Table A6 *Additional file 8: 2-fold down-regulated ESTs between all resistant populations and the susceptible population (see http://onlinelibrary.wiley.com/doi /10.1111/ imb.12099/suppinfo)*

Table A7 *Additional file 9: Nucleotide sequences and annotations of >10-fold up-regulated ESTs between all resistant populations and the susceptible population (see http://onlinelibrary.wiley.com/doi/10.1111/ imb.12099/suppinfo)*

Table A8 *Additional file 10: Nucleotide sequences and annotations of >10-fold up-regulated ESTs sample SE vs PL (see http://onlinelibrary.wiley.com/doi/10.1111/ imb.12099/suppinfo)*

Table A9 *Additional file 11: Nucleotide sequences and annotations of >10-fold up-regulated ESTs sample D vs SE (see http://onlinelibrary.wiley.com/doi/10.1111/ imb.12099/suppinfo)*

Table A10 *Additional file 12: Nucleotide sequences and annotations of >10-fold up-regulated ESTs sample D vs PL (see http://onlinelibrary.wiley.com/doi/10.1111/ imb.12099/suppinfo)*

Table A11 *Additional file 13. Primer used for RT qPCR validation*

Primer name	primer sequence 5' to 3'
CYP6BQ23 F	GGAATGTATGTGAATGAGAAGGCGG
CYP6BQ23 R	TTCCAAACGATACAGAACCT
CYP6BQ25 F	GCCCTCAAATTCTTCCACTTTCT
CYP6BQ25 R	AAGGGGTTAAAGCACGGAGG
CYP4Q22 F	ATCGCCAACAACAAAGACGC
CYP4Q22 R	ATTCCCCGATCTGATGTGCC
5247_Contig1 F	ATATGTGCCCAGAGAGCAGC
5247_Contig1 R	ACTGTGCGAAACTCCACGAT
6623_Contig1 F	TCCTCCTCGGAATTTTCGCC
6623_Contig1 R	TGACTTTATTGGGGTCGCCC
16932_Transcript_1/1 F	TCTACGGCGACTTGGATTGG
16932_Transcript_1/1 R	CCCATTCGTTTTCGCCAGTG
Tubulin F	TCGGAGTGTTCTAAGGTGGTGTGA
Tubulin R	TCCGTGGATTACGGTAAGAAGTCGAA
Actin F	CACCACCACCGCTGAAAGGGA
Actin R	GGGAAGTAGAGGCGGCAGCG

Table A12 *Additional file 14: Nucleotide sequences and annotations of ESTs and genes (P450s and insecticide target sites) identified in this study (see http://onlinelibrary .wiley.com/doi/10.1111/ imb.12099/suppinfo).*

Curriculum vitae

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Ich erkläre hiermit, dass ich die vorliegende Arbeit selbstständig gefertigt, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen als solche kenntlich gemacht habe und nicht die Hilfe einer kommerziellen Promotionsvermittlung oder –beratung in Anspruch genommen habe.

Stuttgart, 04. November 2013 Christoph T. Zimmer