

Target-site and metabolic resistance against λ -cyhalothrin in cabbage stem flea beetles in Denmark

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Abstract

The cabbage stem flea beetle, *Psylliodes chrysocephala* (L.) (Coleoptera Chrysomelidae) is a major pest of oilseed rape throughout Europe. Pyrethroids has been widely used for control of *P. chrysocephala*, but in recent years control failure has occurred, possibly due to resistance. Thirteen out of 15 populations collected in Denmark were susceptible to λ -cyhalothrin. Just two populations, both from the south of Denmark had decreased susceptibility. The target site resistance allele, *kdr*, was found in a frequency from 0.00 to 0.76. There seemed to be a good relationship of the frequency of the target site mutation and resistance levels with some regional differences. Based on the data presented here target site mutation is a good indicator of decreased λ -cyhalothrin susceptibility. However, it is not the sole contributor to pyrethroid resistance in Danish *P. chrysocephala*, and the potential involvement of metabolic resistance should be investigated.

Key words: *Psylliodes chrysocephala*, pyrethroid, insecticide resistance, *kdr*, knockdown resistance.

Introduction

The Brassicaceae family is a broad family of approximately 3,200 different species. These are important in agriculture worldwide due to their nutritional, medical and crop rotation potential. This means Brassicaceae crops and the growth of these are of significant economic value (Ahuja *et al.*, 2010). Oilseed rape (*Brassica napus* L.) has many functions in agriculture both as a source of oil and protein. Oilseed rape has been cultivated for many years in Europe, with more than 175,000 ha in Denmark alone, a number, which is likely to increase in the future.

The large area used for oilseed rape production in Europe makes this crop very important and control of cruciferous plant pests is an essential part of the production. Unfortunately, many pests have evolved resistance to the most commonly used pesticides. Insecticide resistance is the results of selection pressure on the genotypes available in the field. This is partly due to inconsiderate use of pesticides as well as the ban on neonicotinoid seed treatment, where the same mode of action is used without alteration, leading to high selective pressure causing development of resistance.

Oilseed rape has a range of pests, which destroy the pods or the stem, causing reduced yields. Some of the most important pests of this crop include the pollen beetle *Brassicogethes aeneus* (F.) (Coleoptera Nitidulidae), cabbage stem flea beetle *Psylliodes chrysocephala* (L.) (Coleoptera Chrysomelidae) and *Ceutorhynchus* spp. (Coleoptera Curculionidae) weevil species, *Delia brassicae* (Wiedemann) (Diptera Anthomyiidae) and *Myzus persicae* Sulzer (Rhynchota Aphididae). The cabbage stem flea beetle is the first insect pest to infest emerging winter oilseed rape in the autumn after sowing (Ferguson *et al.*, 2003) and has been an issue for many years both in the UK (Graham and Alford, 1981) and Germany (Zimmer *et al.*, 2014a). Adult *P. chrysocephala* emerge from summer diapause in mid- to late August

where they migrate to winter rape crops and mate, feed and lay their eggs in the soil close to host plants. The main damage is caused by the tunneling of feeding larva (Williams, 2004) which weakens the lower part of the stem and upper part of the roots making infested plants more susceptible to damage by frost or hard winds and fungal infections.

Control of flea beetles has relied on seed treatment with systemic insecticides such as neonicotinoids as well as spraying with pyrethroids. Both groups of insecticides work on the insect nerve system, causing paralysis by affecting the nicotinic acetylcholine receptor or the voltage-gated sodium channel, respectively. A temporary European regulatory restriction on systemic neonicotinoids was reinforced in December 2013 due to possibly lethal effects on honeybees (EU Commission, 2013). Knowledge about the resistance of the cabbage stem flea beetle towards different insecticides is scarce, but resistance in the pollen beetle is well documented. Both pests can be found in the field simultaneously, so resistance issues in pollen beetles might indicate similar issues with resistance in cabbage stem flea beetles. Pyrethroid resistance for pollen beetles is a known problem and resistant populations have been found in Denmark (Hansen, 2003; Kaiser *et al.*, 2018).

For *P. chrysocephala* and many other insect species, a mutation known as knockdown resistance (*kdr*) has been found to cause target-site resistance towards pyrethroids (Williamson *et al.*, 1993). The mutation is an amino acid substitution (L1014F) in the sodium channel, which causes 10 to 20-fold resistance depending on insect species, while an additional *super-kdr* mutation (M918T) can cause up to 100-fold resistance towards pyrethroids (Hemingway and Ranson, 2000; Rinkevich *et al.*, 2013). These two are the most common alterations, but several mutations related to pyrethroid resistance have been found (Davies, 2008; Soderlund, 2005). Pyrethroid resistance has been found in *P. chrysocephala* in Germany, but seems only to be present in

certain areas (Heimbach and Müller, 2013). Furthermore, the presence of *kdr* has been shown in German flea beetle populations (Zimmer *et al.*, 2014b) from the northern part of Germany, with no other possible responsible mutation for target-site resistance observed. Resistance and *kdr* in the northern part of Germany could indicate similar issues in the southern part of Denmark, since some of the locations in Zimmer's study are not far from the Danish border.

Recently, Danish, German and English *P. chrysocephala* populations were tested for pyrethroid resistance and presence of the *kdr* mutation in order to determine whether the mutation has spread from Germany to Denmark (Højland *et al.*, 2015). Here, a correlation between frequency of the *kdr* allele and resistance level was found for the German samples. Metabolic pyrethroid resistance has been observed in England (Højland *et al.*, 2015). The Danish samples tested in the study showed high mortality at relatively low doses of λ -cyhalothrin, indicating no resistance or at low levels.

A key element in maintaining effective control of insect pests is keeping resistance levels low. Understanding the molecular biology behind insecticide resistance can prove essential in development of new insecticides as well as maintain continued effect of the pesticides presently available. Here, we further investigate λ -cyhalothrin resistance levels in Danish *P. chrysocephala* populations and test whether the observed resistance levels are related with the frequency of the target site mutation.

Materials and methods

Insect collection

Live *P. chrysocephala* adults were collected from oilseed rape fields in 2014 and from storage facilities in 2015. Collecting from storage facilities just as the harvest is brought in allows for collection of vast numbers in a short time period. In Denmark samples were collected at:

Dalmose, DK-4261 (N55.292250, E11.422656),
Korsør, DK-4220 (N55.332991, E11.140449),
Flakkebjerg, DK-4200 (N55.325056, E11.390832),
Kalundborg, DK-4400 (N55.686731, E11.163374),
Egense, DK5450 (N55.545003, E10.398003),
Holstebro, DK-7600 (N56.527179, E8.567961),
Thyholm, DK-7790 (N56.652657, E8.511435),
Århus, DK-8472 (N56.228209, E9.825486),
Bramming, DK-6740 (N55.441006, E8.761026),
Sommersted, DK-6560 (N55.323670, E9.350937),
Rødekro, DK-6230 (N55.073298, E9.298419),
Sønderborg, DK-6400 (N54.968547, E9.668529),
Haderslev, DK6100-1 (N55.319438, E9.561694),
Haderslev, DK6100-2 (N55.180041, E9.483832),
Toftlund, DK-6520 (N55.192269, E9.085182).

Insects were collected in plastic containers with some oilseed rape plant material and tissue paper, transferred to the laboratory and kept at temperatures ranging from 4-12 °C until bioassays were performed. The beetles were allowed to equilibrate to room temperature prior to bioassay. Only live, mobile beetles were used for bioassay.

In addition to the Danish samples, we received *P. chrysocephala* samples collected in the Southern part of Sweden from fields, where pyrethroids have been sprayed. In Sweden samples were collected at:

Häljarp (N55.861345, E12.937775),
Helleberga (N55.875659, E12.998172),
Kattarp (N56.140548, E12.781438),
Åstorp (N56.134197, E12.944760),
Tygelsjö (N55.514454, E12.997785),
Maglarp (N55.387175, E13.081839),
Hemmesdyngge (N55.369641, E13.356871),
Forsa (N57.075239, E16.438502),
Boberg (N56.868596, E12.606057),
Vreta Kloster (N58.489585, E15.497995),
Kölby (N56.642763, E16.188410),
S. Möckleby (N56.355798, E16.419627),
Christinelund (N57.949419, E16.445400),
Källingemöre (N56.873992, E16.668879),
Mörbylånga (N56.523149, E16.388134),
Viken (N56.15884, E12.635822),
Skurup (N55.404752, E13.481255),
Bjursnäs (N56.542808, E16.180473).

These samples contained dead individuals and were tested for *kdr* frequency, but not for resistance levels.

Bioassay

The test method used is based on IRAC (Insecticide Resistance Action Committee) method 031, with additional insecticide concentrations added (www.irac-online.org/methods/weevils-and-flea-beetles/ 2014). Glass vials were coated on the inner surface with different concentrations of λ -cyhalothrin dissolved in acetone. Different doses, ranging from 0.12 ng cm⁻² to 75 ng λ -cyhalothrin cm⁻², equivalent to 0.16-100% of the recommended field application rate, were used with 2-4 replicates per concentration. Glass vials treated with acetone alone served as controls. After 24 h, the number of cabbage stem flea beetles severely affected (dead or moribund) were scored and results expressed in percentage mortality. Beetles that were capable of coordinated movement were scored as 'mobile/unaffected'. The dose of 37.5 ng cm⁻² λ -cyhalothrin, equivalent to 50% of the field rate, was not included in the bioassays of 2014, since IRAC method 031 was not available before November 2014. The experimental method and resistance patterns of some of the populations have been included in an earlier study (Højland *et al.*, 2015).

DNA purification

When possible, beetles used for DNA purification were not used in the bioassays. This is to ensure a randomization of tested individuals and avoiding bias towards a specific genotype. Extraction of gDNA from beetles was performed by firstly homogenizing individual beetles followed by extraction with the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol (Qiagen, Ballerup, Denmark). Gel electrophoresis and spectrophotometry (Nanodrop) were performed to assess the integrity and the concentration of each DNA sample.

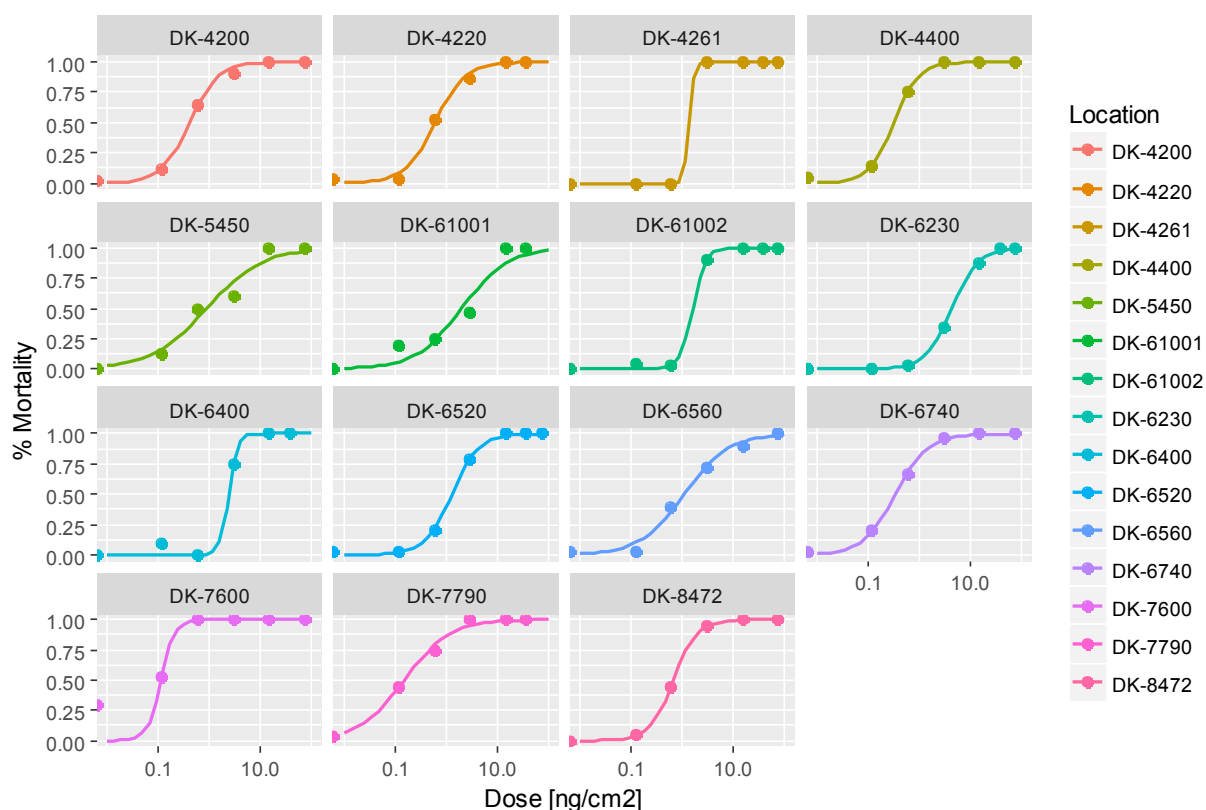


Figure 1. Dose-response curves for the Danish CSFB populations.

DNA purification

The presence of the L1014F *kdr* mutation in the beetles were tested by a TaqMan assay (Livak, 1999), also described in Højland *et al.* (2015). For each population, 19-36 individual beetles were tested for the *kdr* allele. Primer and probe sequences for the assay were designed as described previously (Højland *et al.*, 2015). A total of 392 beetles were tested, 13% of which was included in Højland *et al.* (2015).

Statistical analysis

Statistical analysis was performed by using the R statistical software. The drc-package from the R-project was used for analysis of dose response curves and LC₅₀ calculation (Knezevic *et al.*, 2007).

Results

Bioassay

A total of 15 *P. chrysocephala* samples were obtained from different regions in Denmark, eight of which (collected in 2014) has also been described in Højland *et al.* (2015). The remaining seven were primarily collected in the southern part of Jutland. The dose-response curves for each of the Danish populations are given in figure 1. The field dose 75 λ -cyhalothrin ng cm⁻² caused full mortality in all samples. Likewise, for most of the populations, a rate of 15 ng λ -cyhalothrin cm⁻² did also cause full mortality, except in DK-6560 and DK-6230, where the mortality was 90 and 87%, respectively. Only two samples (DK-61001 and DK-6230) had more than 50%

survival at 3 ng cm⁻² λ -cyhalothrin, both were collected from the southern part of Jutland. The resistance factor (RF) ranged from 1 to 27 compared to the susceptible DK-7790 sample.

Distribution of the L1014F target site mutation

Fifteen Danish and 21 Swedish populations (N = 392) were analyzed for the *kdr* allele (13% were included in Højland *et al.*, 2015). The L1014F mutation was found in most of the Danish populations (table 1). In populations DK-7790 and DK-6740 the *kdr* allele were absent, while the remaining samples had a *kdr* frequency of 0.03-0.76. Two of the four samples collected in east Denmark had low *kdr* frequencies and in two samples no *kdr* alleles were present. For the Swedish population, the mutation was detected in two samples with *kdr* frequencies of 0.1 and 0.25. In the remaining 19 samples, the *kdr* mutation was not observed.

Relationship of LC₅₀ values and *kdr* mutation distribution

The relationship between the *kdr* frequency and LC₅₀ were assessed by plotting the LC₅₀ values for each population with the corresponding *kdr* frequency (figure 2). There was no significant correlation, which was tested by linear regression. Samples from East Denmark had a *kdr* frequency < 0.10 and LC₅₀ values < 0.62 ng cm⁻² λ -cyhalothrin corresponding to a RI of 5. One exception was the DK-4261 sample, which had a LC₅₀ value of 1.3 ng cm⁻² and a RI of 7, but still a *kdr* frequency below 0.10. The same pattern was observed for the samples from North Denmark. Samples from South Denmark

Table 1. Toxicological analysis of Danish flea beetle populations with λ -cyhalothrin vial test. LC_{50} values in $ng\ cm^{-2}$ with confidence intervals, slope with standard error, resistance factor (RF) at LC_{50} and *kdr* frequency for each population.

Location	n	λ -cyhalothrin			<i>kdr</i>		
		LC_{50} (90% C.I.)	Slope \pm SE	RF	n	Frequency	
East Denmark	DK-4200	276	0.42 (0.34-0.50)	1.6 ± 0.41	3	20	0.08
	DK-4220	159	0.62 ± 0.07 (0.43-0.81)	1.5 ± 0.41	4	20	0.00
	DK-4261	157	1.3 ± 0.04 (1.24-1.45)	56*	9	19	0.00
	DK-4400	240	0.32 ± 0.02 (0.26-0.38)	2.0 ± 0.48	2	20	0.03
South Denmark	DK-5450	237	0.91 ± 0.30 (0.09-1.7)	0.67 ± 0.12	6	36	0.46
	DK-61001	116	2.1 ± 0.84 (0-4.4)	0.81 ± 0.38	13	20	0.50
	DK-61002	207	1.6 ± 0.12 (1.3-1.9)	4.2 ± 3.7	10	20	0.56
	DK-6230	180	4.4 ± 0.16 (4.0-4.8)	1.5 ± 0.35	28	20	0.76
	DK-6400	114	2.4 ± 0.35 (0-12)	19*	16	20	0.10
	DK-6520	200	1.3 ± 0.06 (1.2-1.5)	1.7 ± 0.31	9	20	0.48
	DK-6560	178	1.1 ± 0.16 (0.6-1.6)	1.0 ± 0.20	7	30	0.34
	DK-6740	180	0.34 ± 0.02 (0.29-0.40)	1.4 ± 0.29	2	19	0.00
North Denmark	DK-7600	180	0.12 ± 0.03 (0.03-0.20)	7.6*	1	20	0.03
	DK-7790	240	0.16 ± 0.02 (0.10-0.21)	0.91 ± 0.20	1	20	0.00
	DK-8472	120	0.66 ± 0.01 (0.64-0.69)	1.9 ± 0.62	4	21	0.05

*The slope of the bioassay was steep, and calculations of fiducial limits were not possible.

showed a more diverse pattern with most samples having frequencies of *kdr* > 0.30 and LC_{50} values > 0.9 $ng\ cm^{-2}$ λ -cyhalothrin.

Discussion

The toxicological analysis of Danish cabbage stem flea beetle populations categorized most of the populations as susceptible. Two of the populations, DK-6560 and DK-6230, showed 90% and 87% mortality, respectively at 15 $ng\ cm^{-2}$ λ -cyhalothrin.

Before initiating the monitoring, we hypothesized that populations from south of Jutland to harbor resistant populations, due to its proximity to Germany, where flea beetle control failure has been reported from northern Germany (Heimbach and Müller, 2013). The DK-6560 population was collected in the southern part of Jutland, which increased our focus on this area in the following collections. Another population with decreased susceptibility was identified, but the remaining six samples from this region were susceptible. The DK-6230 sample originated from one of three fields and another collection (DK-61002) was made on the early seedlings on one of these fields where oil-seed rape was grown, but no sign of resistance was observed in this sample.

The L1014F mutation is known from several different insect species and correlation between pyrethroid resistance levels and frequency of the mutation is well-documented (Rinkevich *et al.*, 2013). Earlier investigations have showed *kdr* to be responsible for resistance in German cabbage stem flea beetles, while in the UK, resistant beetles without *kdr* have been observed (Højland *et al.*, 2015). The *kdr* mutation was found in most of the Danish populations, with a few exceptions. The *kdr* frequency was highest in samples from the south of Denmark, where *kdr* frequencies was up to

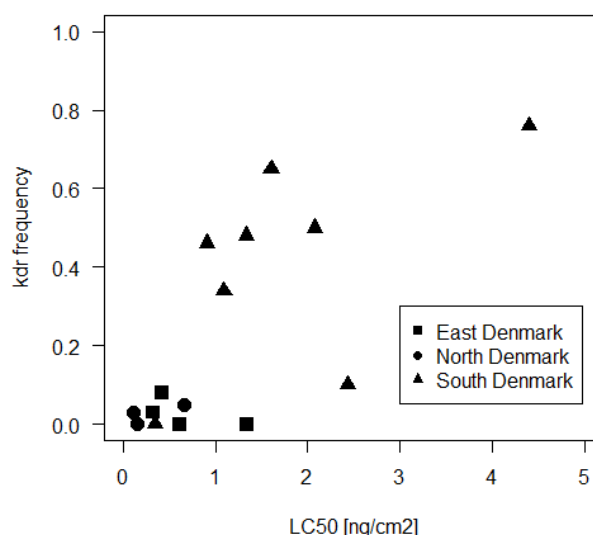


Figure 2. Relationship of the *kdr* mutation and λ -cyhalothrin LC_{50} values. Each region of samples is marked by a different symbol.

0.76. This indicates that the pattern observed in Northern Germany and Southern Denmark is similar. The opposite pattern was observed for samples collected in East Denmark. Here, *kdr* was found in two of four samples, but at frequencies below 0.08. The pattern of East Denmark corresponds with the Swedish samples, where only two of 21 samples contained the *kdr* mutation due to one and two individuals being homozygote resistant, respectively. The fact that *kdr* was absent in most Swedish populations does not necessarily mean it is absent in all populations, but rather that it is present at a very low frequency in Sweden. Our data suggest that *kdr* is found throughout Denmark, but with a relatively high frequency closer to Germany, but lower when moving eastwards to Sweden.

The *kdr* mutation commonly cause 10-20 fold resistance towards pyrethroids in other insects, so to test whether resistance ratios and *kdr* frequencies are connected a plot of these two factors was made. For all regions in this study, there was a good relationship between frequency of the *kdr* mutation and resistance levels. Samples from North Denmark as well as from East Denmark were low on both parameters. However, samples from South Denmark had a more diverse pattern with some locations scored both high of *kdr* frequency and resistance index, while a few samples did not follow the trend. Based on the data analyzed here, samples from South Denmark are more interesting to pursue in search for resistant populations than those from the other areas. A sample from South Denmark with a RI of 16 was observed, despite a low *kdr* frequency of the sample. This could indicate that *kdr* is not the only factor involved in pyrethroid resistance in Danish *P. chrysocephala*.

In conclusion, most *P. chrysocephala* populations collected in Denmark were susceptible to λ -cyhalothrin according to the IRAC classification method 031. Two populations, both from the south of Denmark were found to have decreased susceptibility. The target site mutation was widely distributed throughout Denmark with a regional gradient showing high *kdr* frequencies in South Denmark and low or no *kdr* frequencies in North Denmark, East Denmark and Sweden. There are some degree of correlation between the λ -cyhalothrin resistance levels and the frequency of the *kdr* allele. However, a few of the samples did not fit the correlation model, which could indicate metabolic resistance or other target site mutations to also play a role in λ -cyhalothrin resistance in Denmark.

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