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ORIGINAL PAPER

Susceptibility of Danish pollen beetle populations against λ-cyhalothrin and thiacloprid

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Abstract Winter oilseed rape is an important crop in agriculture, where it plays an essential role in cereal-based crop rotations. The main pest in this crop is the pollen beetle which feeds on developing buds. Since 1999, pyrethroid resistance in the pollen beetle has spread throughout Europe. Denmark and Sweden are of interest due to the early detection of pyrethroid-resistant pollen beetle and because of the presence of both target-site and metabolic resistance in the populations. For almost ten years, neonicotinoid insecticides have been used in oilseed rape as well. In this study, the current resistance situation in Danish pollen beetle populations was conducted. The susceptibility of pollen beetle to the synthetic pyrethroid λ-cyhalothrin as well as the neonicotinoid thiacloprid was investigated. Sixty-eight Danish, as well as 6 Swedish and 10 German populations collected between 2014 and 2016 were tested with λ-cyhalothrin. Two-thirds of the Danish populations collected were classified as pyrethroid resistant, whereas Swedish populations were mostly susceptible and all German populations tested were resistant, mostly at higher levels than those detected in Danish strains. Toxicological parameters were calculated by a two-parameter log-logistic model and showed up to 58-fold variation at LC_{50} for λ-cyhalothrin and up to 17-fold variation at LC_{50} for thiacloprid. However, almost all Danish populations were susceptible against thiacloprid and the German populations showed up to sevenfold variation in thiacloprid bioassay. In conclusion, pyrethroid resistance is widespread in Danish pollen beetle populations with regional differences, whereas Danish populations are susceptible to thiacloprid in all regions.

Keywords *Meligethes aeneus* · *Brassicogethes aeneus* · Bioassay · Insecticide resistance · Pyrethroid · Neonicotinoid · Thiacloprid · λ-Cyhalothrin · *Brassica napus*

Key message

- Resistance against pyrethroids is widespread in Danish pollen beetle populations, but the extent of resistance differs regionally.
- Pollen beetle populations are mostly susceptible against thiacloprid in Denmark. There is minor variation between the populations.
- Resistance monitoring is necessary on a regional level to detect changes in susceptibility and to adopt control measures based on the locally available insecticide portfolio.
- Wherever possible rotation of insecticides with different mode of actions is the key to good resistance management.

Introduction

Winter oilseed rape (*Brassica napus*) is an important crop in modern agriculture, where it plays an essential role as a break crop in cereal-based crop rotations (Ahuja et al.

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2010), as well as a source for nutritional and industrial oil-based products (Lamb 1989). In 2014, the European Union produced 24.3 mio t of oilseed rape and turnip rape. The cultivated area of winter and spring oilseed rape in Denmark in 2014 was 192,535 and 1375 ha, respectively (Danmarks Statistik 2016). Since 2000, mostly winter oilseed rape is cultivated in Denmark (Danish EPA 2014), whereas in several other northern countries spring oilseed rape cultivars are still of greater importance (Ekbom 2010).

Oilseed rape is an attractive host plant for many insects, including both pest and beneficial species. Several pests attack winter oilseed rape during its 11-month growing period, especially in autumn after sowing, and in spring where the base for the yield-forming period is established. One of the major pests is the pollen beetle, *Meligethes aeneus* (Coleoptera: Nitidulidae), a univoltine pest adapted to reproduce in winter oilseed rape (Fritzsche 1957). In spring, adults start at temperatures above 11 °C to migrate from their overwintering sites to winter oilseed rape fields (Fritzsche 1957). Adult beetles damage the crop by feeding on developing buds during the green-to-yellow bud stage. In countries where both winter and spring oilseed rape is cultivated, pollen beetles first attack the winter variety for reproduction and later migrate to spring oilseed rape fields (Hansen 2003).

Severe damage due to pollen beetles feeding during early bud stages in winter oilseed rape can cause yield losses up to 100% as seen in 2006 in Germany (Slater et al. 2011). Once exceeding economic damage thresholds, *M. aeneus* is controlled by insecticide treatment such as the pyrethroids. Due to a strong selection pressure, pollen beetle sensitivity to pyrethroids decreased considerably in many European countries (Zimmer and Nauen 2011b). The first case of pyrethroid resistance in *M. aeneus* was reported in Northeastern France in 1999 (Zimmer and Nauen 2011b). Already in 2001, Hansen (2003) detected high levels of resistance in Danish pollen beetle populations. In 2007, pyrethroid target-site resistance based on a L1014F mutation in domain IIS6 of the voltage-gated sodium channel (known as knock-down resistance, kdr) was detected in pollen beetle populations from Denmark and Sweden (Nauen et al. 2012). However, a kdr-mechanism was not found in other parts of Europe yet, despite high levels of resistance (Nauen et al. 2012). In contrary, a metabolic mechanism of resistance based on the over-expression of a cytochrome P450 monooxygenase, CYP6BQ23, was described to confer cross-resistance to pyrethroids all over Europe (Zimmer et al. 2014a; Zimmer and Nauen 2011a). The decreased sensitivity of pollen beetles to pyrethroids prompted industry and regulatory authorities to seek for and register non-cross-resistant alternatives belonging to other chemical classes than pyrethroids. Consequently, in the last decade new modes of action for pollen beetle control have been introduced, such as neonicotinoids (nicotinic acetylcholine receptor competitive modulators), pyridine azomethines (chordotonal organ TRPV channel modulators) and oxadiazines (voltage-dependent sodium channel blockers) classified by the Insecticide Resistance Action Committee (IRAC) in mode of action groups 4A, 9B and 22A, respectively (IRAC 2016; Sparks and Nauen 2015).

The neonicotinoid thiacloprid was introduced to the German market in 2006 as an emergency measure and is since 2007 fully registered in many European countries including Denmark (Middeldatabasen 2016; Zimmer and Nauen 2011b). Cross-resistance was not detected between the pyrethroid λ-cyhalothrin and thiacloprid (Zimmer and Nauen 2011b), and therefore, it is a valuable compound to control pyrethroid-resistant pollen beetles. It has been used for large-scale foliar applications in oilseed rape to control pyrethroid-resistant pollen beetles since 2007.

Even though several chemical control options are available, pollen beetles remain to be a serious pest especially in Northern Europe, where winter oilseed rape as well as spring varieties are challenged by this pest. It is crucial to understand the development and spread of resistance over the years and countries to implement appropriate control strategies based on the best possible options. In 2001 and 2003, a national monitoring of Danish pollen beetle populations was carried out by the Danish Institute of Agricultural Sciences and showed high levels of resistance toward λ-cyhalothrin by using the FAO dip test (Hansen 2008). This testing method is based on dipping individuals into pesticide solutions and an assessment of mortality after 24 h. An adult vial glass method which relies on tarsal contact was established by the pollen beetle working group formed by the IRAC. Since 2007, pollen beetle resistance to pyrethroids is annually monitored in many European countries and reported by IRAC (Slater et al. 2011), by using the adult vial test mentioned above known as IRAC method no. 11 (IRAC 2009). However, only a few populations collected in Denmark were investigated over the years as part of the European-wide IRAC monitoring to investigate pyrethroid resistance development in Europe (Slater et al. 2011).

The objective of the present study was to investigate the current status of pollen beetle resistance across Denmark for both λ-cyhalothrin and thiacloprid susceptibility in an extensive monitoring on the basis of IRAC standardized methods 011 and 021, and the latter is the equivalent of method 011 for neonicotinoids (IRAC 2009, 2011). The main work was performed in 2014, and specific sites were re-visited for sampling in 2015 and 2016. Of particular interest was the distribution of pollen beetle resistance across Denmark toward λ-cyhalothrin (pyrethroid) and to observe possible changes in susceptibility to thiacloprid (neonicotinoid), after more than seven years on the market.

Materials and methods

Pollen beetle populations

Oilseed rape fields were assigned to the monitoring by contacting agricultural consultants and farmers of the various regions in Denmark. The populations were sampled as soon as possible from different locations after the first appearance of pollen beetles to avoid exposure to insecticide application before testing. Approximately 500 pollen beetles were collected per collection site in oilseed rape fields. In total 68 Danish, 6 Swedish and 10 German were tested between 2014 and 2016.

In 2014, 48 populations were sampled across Denmark and tested with extended bioassays (Fig. 1). For comparison, a population from Germany where high resistance was expected and six populations from the southern part of Sweden were collected and sent to the author's laboratory for testing.

After the initial monitoring, the IRAC classification for pyrethroid resistance was used to select pollen beetle locations for further investigations of the involved resistance mechanisms in 2015 and 2016. Especially locations with detected susceptibility, or higher resistance levels to pyrethroids as well as sites with reduced susceptibility to thiacloprid, were resampled as close to the previous field as possible. In 2015, 16 pollen beetle populations were collected, 14 from winter oilseed rape field in selected regions in Denmark, and 2 from spring oilseed rape and five populations were collected in Germany aiming for highly resistant populations. In 2016, four populations were collected from Denmark and four from Germany.

All sampled pollen beetle populations were brought to the author's laboratory for dose–response testing toward pyrethroid (λ-cyhalothrin) and neonicotinoid (thiacloprid) insecticides. All beetles were stored at least 24 h in climatic chambers at 4–6 °C with food and water supply before testing.

Populations were named by a number followed by the year of collection; for example, 11-14 indicates this was population 11 collected in 2014. The location of the pollen beetle collection sites was sorted by regions: DK-NJ = North Jutland, DK-MJ = Central Jutland, DK-SD = South Denmark, DK-NSL = Capital and North Zealand, DK-SL = Zealand. In Sweden, pollen beetle populations from two regions, SE-E = Östergötland county and SE-M = Skåne county, were tested. From Germany, pollen beetle populations were tested from DE-NW = North Rhine-Westphalia, DE-SN = Saxony, DE-MV = Mecklenburg-Western Pomerania, DE-BY = Bavaria.

Pollen beetle bioassays

Extended bioassays were conducted with the standardized methods as described by IRAC (Slater et al. 2011) and elsewhere (Zimmer and Nauen 2011b). These methods are used European-wide which makes it possible to compare the findings with other European countries where λ-cyhalothrin is used as the generic reference compound for pyrethroid resistance testing. The instructions of IRAC's Susceptibility Test Method Series were followed according to No. 11 "Pollen Beetle Susceptibility Monitoring Bioassay—Synthetic Pyrethroids Version 3" and No. 21 "Adult-Vial-Test

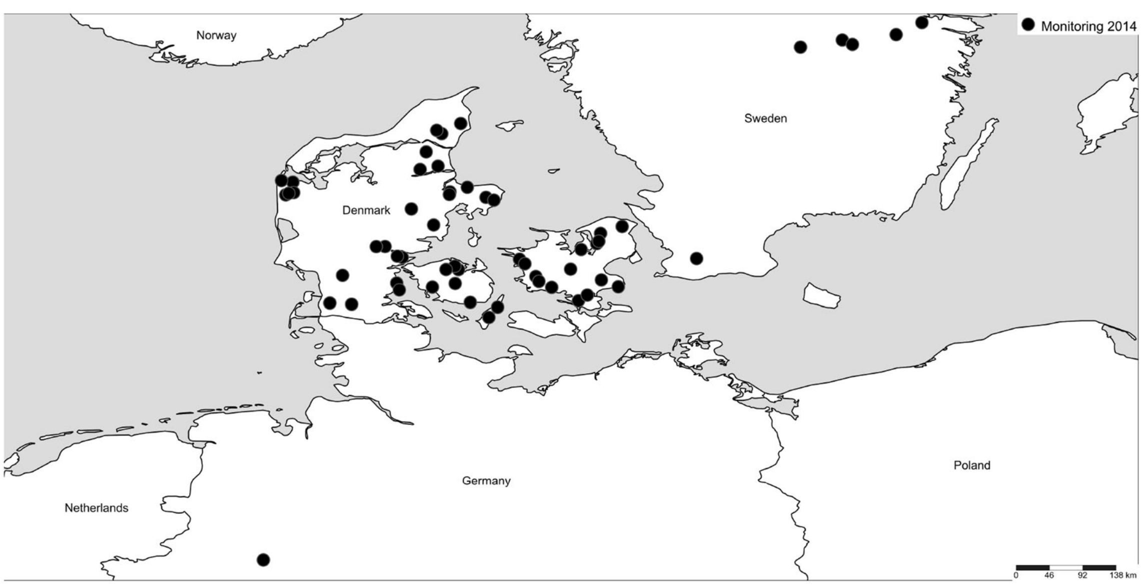


Fig. 1 Monitoring locations marked with *black symbols* were sampled in 2014. Every symbol represents one population, collected in a winter oilseed rape field. This *map* was created with SimpleMappr, http://www.simplemappr.net

for neonicotinoids Version 3.4" (IRAC 2009, 2011). All adult vial glass test kits were produced by Bayer CropSciene in Monheim. As both IRAC methods are based on a limited number of doses due to the aim of a fast testing method, we added a few more doses for generating dose–response curves to achieved comparable LC-values rather than categories. The slope of the dose–response curve indicates homogeneity by a steep slope or heterogeneity by a low slope of the investigated population (Table 1).

Ten active pollen beetles were transferred to each glass vial by using an exhauster connected to a low air flow. In each bioassay, the untreated control glass vial and each concentration had at least two replicates. The assessment was carried out after 24 h in a temperature controlled laboratory at 20 ± 2 °C. Beetles were classified as affected or alive. Affected beetles showed uncoordinated movements, were not able to spread their wings properly or were dead. To minimize variation as much as possible, only one person conducted the assessments because the interpretation of the affected beetles needs some experience (Zimmer et al. 2014b).

The 24-h assessment was performed within the glass vials by adapting the modifications recently described by Zimmer and Nauen (2011b). In a few cases where the sampling conditions were suboptimal due to sudden rainfall, increased control mortality was recorded. In those cases where the control mortality exceeded 20%, the populations were excluded from analyses.

Data analysis

The populations tested were categorized by following the IRAC susceptibility rating scheme for pyrethroids and neonicotinoids. For λ-cyhalothrin, the tested pollen beetle populations were classified into one of five groups as shown in Table 2. In the adult vial test for thiacloprid, the susceptibility was classified based on the observed percentage mortality at the dose rate of 1.44 μg cm^{-2} (200% of field recommended rate), i.e., highly susceptible (>95%), susceptible (94–75%) or reduced susceptibility (<75%) (IRAC 2014).

All maps presented here were created with SimpleMappr (http://www.simplemappr.net).

Dose–response analysis

The lethal concentration (LC) values for the extensive bioassay data for the two tested insecticides λ-cyhalothrin and thiacloprid were calculated by using the two-parameter log-logistic model (Knezevic et al. 2007; Ritz and Streibig 2005). The drc-package from R was utilized for calculation, and multiple dose–response curves were analyzed (Knezevic et al. 2007). Due to the two-parameter log-logistic model, the calculation of the slope parameter results in a negative slope (Ritz et al. 2015). The presented values for the slope were multiplicated with −1 in the analysis presented here. A supplementary analysis was carried out by using the probit model with SAS (version 9.3) program. The probit analysis was used to validate the results to see if they are comparable with other data. No significant differences between the outputs of the two software packages have been found. Therefore, only the results from the drc models are presented here.

Results

Pyrethroid monitoring

For the 3-year period of the pyrethroid resistance monitoring, all populations tested were confined to IRAC susceptibility classes as described above. Based on the adult vial bioassay, 33% of the Danish populations were resistant, 29% moderately resistant and 38% susceptible toward

Table 1 Concentrations of active ingredient coated in the inner surface of glass vials calculated on the recommended label application concentrations for λ-cyhalothrin (7.5 g ha^{-1}) and thiacloprid (72 g ha^{-1}) in 2014–2016 adapted from Zimmer and Nauen (2011b)

Percentage of recommended label application rate	λ-Cyhalothrin Active ingredient (μg cm^{-2})		Thiacloprid Active ingredient (μg cm^{-2})	
	2014	2015–2016	2014	2015–2016
0.8	0.0006	0.0006		0.00576
4	0.003	0.003		0.0288
20	0.015	0.015	0.144	0.144
100	0.075	0.075	0.72	0.72
200			1.44	1.44
500		0.375		

Table 2 IRAC pyrethroid resistance classification scheme for pollen beetles. Table adapted from Zimmer and Nauen (2011a, b)

Concentration of active ingredient (μg cm^{-2})	Affected (%)	Classification	IRAC code
0.075	100	Highly susceptible	1
0.015	100		
0.075	100	Susceptible	2
0.015	<100		
0.075	<100 to ≥90	Moderately Resistant	3
0.075	<90 to ≥50	Resistant	4
0.075	<50	Highly resistant	5

λ-cyhalothrin in 2014 ($N = 48$) (Fig. 2). Denmark can be divided into five regions (see Materials and methods), and the monitoring results for pyrethroid susceptibility for each region in 2014 showed some regional differences (Fig. 2). The regional differences can be seen in relatively more susceptible and moderately resistant populations in Jutland (DK-NJ, DK-MJ, DK-SD), whereas Zealand (DK-SL) mainly showed pyrethroid-resistant pollen beetle populations.

Based on this, selected regions were sampled again in 2015 to follow up on different susceptibility levels. In total, 16 pollen beetle populations were investigated to evaluate whether the obtained sensitivity levels in 2014 remained stable in those regions. In DK-SD, five populations were collected encompassing 60 and 40% moderately resistant and susceptible in 2015, respectively. In DK-NSL, 43% were resistant and 57% moderately resistant ($N = 7$). In DK-SL, ($N = 4$) 75% were resistant and 25% moderately resistant.

To evaluate the results from Danish populations, we included populations from neighboring countries Sweden and Germany. The reference populations ($N = 5$) from Sweden (SE-E) were susceptible, and one (SE-M) was resistant toward λ-cyhalothrin in our tests in 2014. One German population from 2014 was resistant. In 2015, five pollen beetle populations were collected from winter oilseed rape in Germany. Two from DE-NW were resistant and moderately resistant, respectively. Three populations from DE-MV were collected: One was resistant, and two of them were moderately resistant to λ-cyhalothrin. In 2016, three of the four tested populations from Germany were classified as resistant.

Thiacloprid susceptibility monitoring

In 2014, 98% of the 45 Danish pollen beetle populations tested were classified as susceptible and only one population showed a reduced mortality at the tested field rate of 200% (Fig. 3).

The regions with the lowest mortalities at 200% of the recommended field rate of thiacloprid, i.e., 70 ± 20% (DK-MJ 20-14) in 2014, were sampled again in 2015 (DK-MJ 19-15) but then categorized as highly susceptible. In 2015 and 2016 in total, 18 Danish pollen beetle populations were classified as highly susceptible and susceptible (Fig. 3). The lowest measured mortality rate was 83 ± 13% at 200% of the recommended field rate for thiacloprid.

In 2014, six populations collected in Sweden and one in Germany were all highly susceptible to thiacloprid. One German population from 2014 showed slightly reduced susceptibility with 70% mortality at 200% thiacloprid. In 2015–2016, further nine populations were collected in Germany of which 89% were susceptible and 11% showed reduced susceptibility.

Toxicology parameters for λ-cyhalothrin

In 2014, the majority (79%) of tested populations ($N = 48$) had a mortality of 85–100% at 0.075 μg cm^{-2} λ-cyhalothrin (100% field dose). In 21% of the populations ($N = 48$) tested with full dose of λ-cyhalothrin, a reduced mortality (57–84% mortality) was found. The dose–response was calculated for populations which had a response between 0 and 100% mortality to the used doses. Only five Danish populations and the population from Germany had less than three reliable responses which were our criteria for using the model.

In 2014, the LC_{50} values of 48 Danish populations were analyzed and varied from 0.001 μg cm^{-2} in DK-SD (population 33-14), which is equivalent to 2% field rate, to 0.081 μg cm^{-2} (108% field rate) found in a population of DK-SL (population 9-14). The variation for the LC_{50} values within the Danish populations is thus up to 58-fold based on the difference of the most susceptible and most resistant strain in Table 3.

The differences within the regions in Denmark are also reflected in the LC_{50} values. The lowest LC_{50} values ranging from 0.0014 to 0.014 μg cm^{-2} (2–18% field dose) were found in DK-SD where 16 populations were tested (Table 3). DK-SL showed the highest variation within the eleven tested populations with LC_{50} values ranging from 0.013 to 0.081 μg cm^{-2} (17–107% field dose). In 2014, the slope for the Danish populations ranged from 0.6

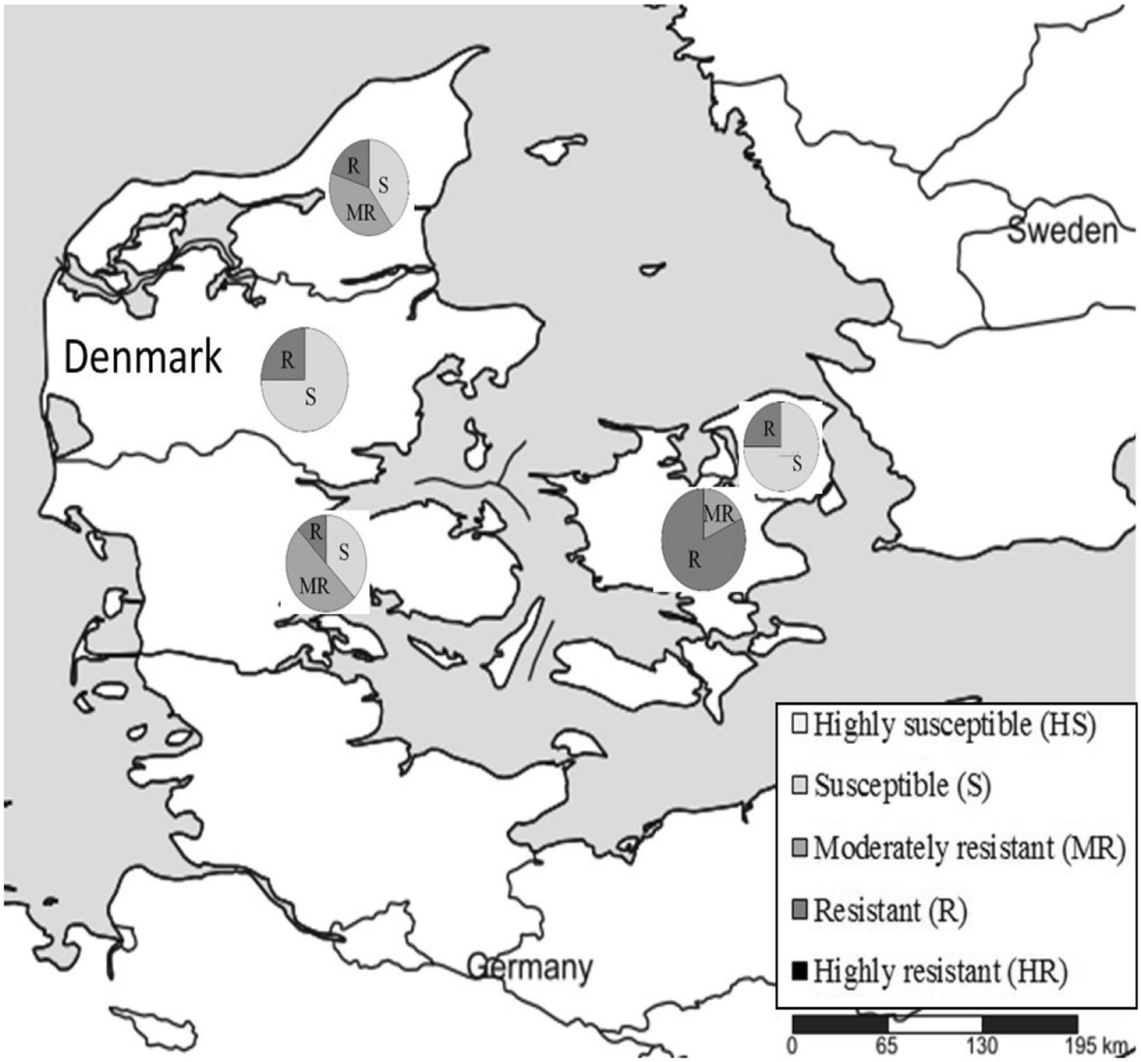
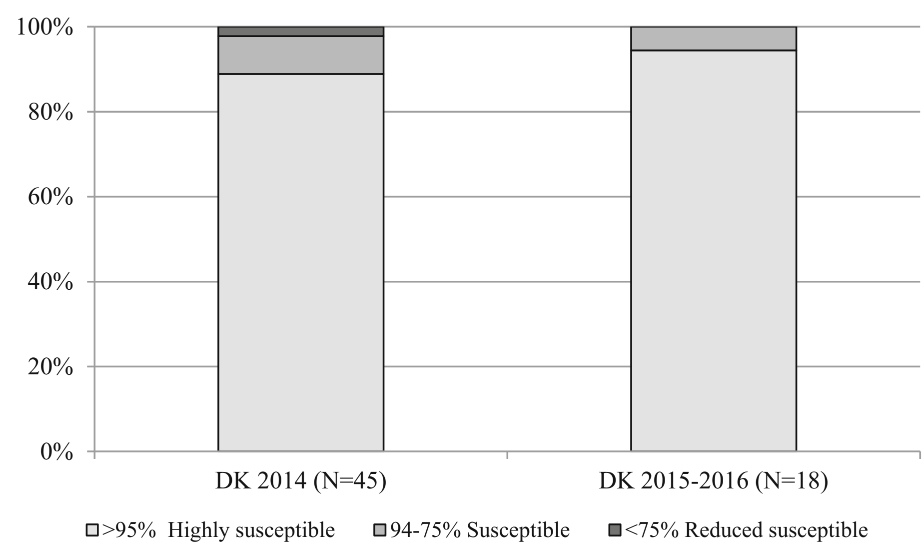


Fig. 2 Initial pyrethroid monitoring of insecticide resistance with Adult-Vial-Tests (11) in 2014. The results grouped into five classes based on IRAC guidelines from highly susceptible to highly resistant. The individual distribution of the 48 Danish populations tested can be found in Fig. 1

Fig. 3 Monitoring of thiacloprid susceptibility level with IRAC No. 21 in 2014. The tested populations with thiacloprid from 2014 and the combined results for 2015 and 2016 are shown. The mortality rate at a thiacloprid dose of 1.44 µg cm^{-2}. In this case, mortality >95% indicated high susceptibility toward thiacloprid



(population 09-14) to 1.9 (population 30-14). A shallow slope like 0.6 with a high LC_{50} value could indicate a heterogeneous population, whereas a steep slope indicates a homogeneous population. The most susceptible Danish population 33-14 had a slope of 1.05, and the most resistant Danish pollen beetle population had a slope of 0.6, indicating heterogeneity.

The data collected in 2015 and 2016 are shown in a combined Table 4, due to the same amount of tested concentrations and replicates. The populations showed a mortality response to at least three and up to five doses. For the tested population with λ-cyhalothrin, the calculated LC_{50} ranged from the most susceptible Danish population (DK-SD 08-15) with 0.004 μg cm^{-2} to the most resistant Danish population (DK-SL 05-15) with 0.039 μg cm^{-2} in 2015 (Table 4). In 2015 and 2016, the slopes ranged from 0.6 (DK-SD 08-15) to 2.7 (DK-NSL 04-15). In this case, strain DK-SD 08-15 had the shallowest slope which likely indicates a heterogeneous population. The highest slope value was achieved by a moderately resistant population (DK-NSL 04-15).

The 2014 reference populations from Sweden had LC_{50} values from 0.003 to 0.006 μg cm^{-2} which are equivalent to 4–8% field dose (Table 3). In 2015 and 2016, a total of nine populations from Germany were tested with λ-cyhalothrin to compare them with Danish pollen beetle populations. The most susceptible DE-NW 02-16 had an LC_{50} of 0.008 μg cm^{-2} and the least susceptible DE-BY 05-16 an LC_{50} of 0.041 μg cm^{-2} which is equivalent to 55% of the recommended field dose (Table 4).

Thiacloprid toxicology

In 2014, three concentrations of thiacloprid were tested (20, 100, 200% of the recommended field dose) as recommended by IRAC (method 021) for testing neonicotinoid susceptibility. The tested populations from 2014 were highly susceptible, and based on the tested doses, it was not possible to calculate the LC_{50} values, because already at a dose of 20% of the application rate of thiacloprid, 93% of the tested populations showed a mortality rate ranging from 55 to 100% (Table 5). Only in 7% of 45 tested Danish populations a mortality of 30–40% at 20% of the recommended dose of thiacloprid was observed. The German population showed a mortality rate of 30 ± 10%, and the six Swedish pollen beetle populations had a mortality rate

Table 3 Results for pyrethroid resistance (λ-cyhalothrin) monitoring for pollen beetle populations from different regions in Denmark 2014

	Population	Region	IRAC class	LC_{50} (CI) (μg cm^{-2})	Slope ± SE	DK fold change LC_{50}	UA RR LC_{50}
AV	$N = 5$	DK-ND	3	0.0179	1.3	13	179
Min	44-14		3	0.0106 (0.0043–0.0169)	1.0 ± 0.2	8	106
Max	32-14		2	0.0239 (0.0110–0.0367)	1.2 ± 0.3	17	239
AV	$N = 12$	DK-MJ	3	0.0140	1.1	10	140
Min	48-14		2	0.0027 (0.0012–0.0041)	1.1 ± 0.2	2	27
Max	37-14		4	0.0430 (0.0204–0.0657)	1.4 ± 0.3	31	430
AV	$N = 16$	DK-SD	3	0.0069	1.1	5	69
Min	33-14		2	0.0014 (0.0005–0.0022)	1.1 ± 0.2	1	14
Max	51-14		4	0.0136 (0.0042–0.0230)	0.8 ± 0.2	10	136
AV	$N = 4$	DK-NSL	3	0.0195	1.3	14	195
Min	13-14		2	0.0129 (0.0073–0.0185)	1.6 ± 0.4	9	129
Max	12-14		4	0.0342 (0.0111–0.0600)	1.0 ± 0.2	24	342
AV	$N = 11$	DK-SL	4	0.0278	1.0	20	278
Min	14-14		3	0.0130 (0.0066–0.0194)	1.3 ± 0.3	9	130
Max	09-14[a]		4	0.0805 (0.0000–0.1950)	0.6 ± 0.2	58	805
AV	$N = 5$	SE-E	1	0.0046	2.1	4	46
Min	55-14		1	0.0030 (0.0017–0.0043)	1.6 ± 0.4	2	30
Max	56-14		2	0.0062 (0.0033–0.0091)	1.5 ± 0.3	4	62
$N = 1$	57-14	SE-M	4	0.0056 (0.0015–0.0097)	0.7 ± 0.2	4	56
$N = 1$	58-14	DE-NW	4	0.0372 (0.0158–0.0586)	1.2 ± 0.4	27	372

Danish (DK) fold change $LC_{50} = LC_{50}/LC_{50}$ (33-14 $LC_{50} = 0.0014$) most susceptible strain; Ukraine (UA) RR $LC_{50} = LC_{50}/LC_{50}$ (Ukraine $LC_{50} = 0.0001$) most susceptible strain known based on Zimmer and Nauen (2011b)

[a] The calculation does not comply with the rules of calculation since the highest dose had a mortality of 57%

Table 4 Danish pollen beetle populations tested with λ-cyhalothrin in 2015 and 2016

	Strains	Region	IRAC class	LC_{50} (CI) (μg cm^{-2})	LC_{95} (CI) (μg cm^{-2})	Slope (SE)	DK fold change LC_{50}	UA RR LC_{50}
AV	$N = 2$	DK-ND	4	0.0196	0.1576	1.5	14	196
Min	08-16		3	0.0185 (0.0120–0.0251)	0.1141 (0.0338–0.1945)	1.6 ± 0.3	14	185
Max	07-16		4	0.0207 (0.0124–0.0291)	0.2010 (0.0389–0.3630)	1.3 ± 0.2	15	207
AV	$N = 5$	DK-SD	3	0.0088	0.2852	0.9	6	88
Min	08-15		2	0.0039 (0.0007–0.0100)	0.4783 (0.0000–1.4000)	0.6 ± 0.1	3	39
Max	09-15		3	0.0158 (0.0064–0.0300)	0.4497 (0.0000–1.0400)	0.9 ± 0.2	12	158
AV	$N = 8$	DK-NSL	4	0.0250	0.2651	1.6	18	250
Min	09-16		3	0.0136 (0.0095–0.0176)	0.0535 (0.0209–0.0862)	2.1 ± 0.4	10	136
Max	05-15		4	0.0391 (0.0180–0.0600)	0.6023 (0.0000–1.3000)	1.1 ± 0.2	29	391
AV	$N = 5$	DK-SL	4	0.0212	0.3652	1.1	15	212
Min	06-16		4	0.0138 (0.0078–0.0198)	0.1980 (0.0206–0.3754)	1.1 ± 0.2	10	138
Max	11-15		4	0.0292 (0.0120–0.0500)	0.6510 (0.0000–1.510)	0.9 ± 0.2	21	292
AV	$N = 3$	DE-MV	3	0.0308	0.1532	2.0	22	308
Min	15-15		3	0.0210 (0.0132–0.0300)	0.1440 (0.0377–0.2500)	1.5 ± 0.3	15	210
Max	17-15		4	0.0375 (0.0242–0.0500)	0.2208 (0.0702–0.3700)	1.7 ± 0.3	27	375
AV	$N = 4$	DE-NW	4	0.0157	0.1829	1.2	11	157
Min	02-16		3	0.0082 (0.0048–0.0116)	0.0859 (0.0164–0.1554)	1.3 ± 0.2	6	82
Max	01-15		4	0.0289 (0.0137–0.0400)	0.3845 (0.0000–0.8100)	1.1 ± 0.2	21	289
N = 1	04-16	DE-SN	4	0.0304 (0.0179–0.0429)	0.3269 (0.0508–0.6030)	1.2 ± 0.2	22	304
N = 1	05-16	DE-BY	4	0.0408 (0.0277–0.0539)	0.1654 (0.0702–0.2606)	2.1 ± 0.4	30	408

Danish (DK) fold change $LC_{50} = LC_{50}/LC_{50}$ (33-14 $LC_{50} = 0.0014$) most susceptible strain; Ukraine (UA) $LC_{50} = LC_{50}/LC_{50}$ (Ukraine $LC_{50} = 0.0001$) most susceptible strain known based on Zimmer and Nauen (2011b)

of 80–100% at 20% of the recommended field dose of thiacloprid, which is considered quite high compared to the recently published baseline data (Zimmer and Nauen 2011b).

In 2015, two lower doses (0.8 and 4%) were added to the bioassay to enable dose–response analysis. The data from 2015 and 2016 are shown in a combined table (Table 6). Thiacloprid LC_{50} ranged for the Danish populations from DK-SL 20-15 with 0.0087 μg cm^{-2} (1% of the field dose) to DK-SL 12-15 with 0.144 μg cm^{-2} (20% of the field dose) which is equivalent to a 17-fold difference. Both, the lowest and the highest LC_{50} values were found in populations from DK-SL. The slope for thiacloprid tested pollen beetle populations ranged from 0.6 (DK-SD 18-15) to 1.4 (DK-NSL 06-15).

The LC_{50} of the tested samples from Germany ranged from DE-MV 17-15 with 0.003 μg cm^{-2} (0.4% of the field dose) to DE-BY 05-15 with 0.614 μg cm^{-2} (85% of the field dose), which were the highest observed LC_{50} values in 2015–2016. The variation within the German populations is thus >200-fold based on the samples tested (Table 6), but less than eightfold compared to the composite European baseline LC_{50} of 0.09 μg cm^{-2} of more than 88 population tested (Zimmer and Nauen 2011b).

Discussion

The main focus of our study was to determine the present resistance situation toward λ-cyhalothrin and to detect possible changes in the susceptibility levels for thiacloprid in Danish pollen beetle populations including some reference populations collected from neighboring countries to serve as a comparison to recent studies conducted by others (Slater et al. 2011; Zimmer and Nauen 2011b). Several populations were collected across Denmark and subjected to an adult vial tests according to IRAC methods, but with an extended concentration range for λ-cyhalothrin and thiacloprid, respectively. As expected pyrethroid resistance is widespread in Danish pollen beetle populations, though none of the tested populations were highly resistant to λ-cyhalothrin according to the IRAC classification (Slater et al. 2011). In contrast to this, Hansen (2003) reported 75–95% survival rate of pollen beetles collected in 2001 and when tested with full recommended rates of pyrethroids employing a FAO dip test, thus indicating the presence of highly resistant populations during this time period. In other European countries, e.g., Germany and France, high resistance levels were found in the majority of pollen beetle populations tested (Slater et al. 2011).

Table 5 Results for thiacloprid bioassay in 2014 with the mortality rate at 0.144 μg cm^{-2} equivalent to 20% of the field application rate of 72 g ha^{-1}

	Population	Region	Mortality (%) ± SD
AV	$N = 6$	DK-ND	61 ± 17
Min	59-14		76 ± 4
Max	30-14		30 ± 0
AV	$N = 13$	DK-MJ	79 ± 11
Min	37-14		100 ± 0
Max	27-14		65 ± 5
AV	$N = 11$	DK-SD	76 ± 17
Min	18-14		100 ± 0
Max	29-14		35 ± 5
AV	$N = 4$	DK-NSL	61 ± 19
Min	11-14		85 ± 5
Max	15-14		40 ± 30
AV	$N = 11$	DK-SL	72 ± 12
Min	06-14		90 ± 10
Max	05-14		60 ± 10
AV	$N = 5$	SE-E	93 ± 8
Min	52-14		100 ± 0
Max	53-14		80 ± 10
$N = 1$	57-14	SE-M	100 ± 0
$N = 1$	58-14	DE-NW	30 ± 10

In 2014, a variation in sensitivity of 58-fold for λ-cyhalothrin among the collected pollen beetle samples collected was seen (Table 3). In the following years, only a tenfold difference between the tested populations was found, which is up to 28-fold to the most susceptible Danish population (DK-SD 33-14) (Table 4). A Danish population collected from DK-MJ in 2010 was tested with λ-cyhalothrin by Zimmer and Nauen (2011b) and showed an LC_{50} of 0.017 μg cm^{-2}, which fits very well within the determined range of the DK-MJ populations (LC_{50} of 0.003–0.043 μg cm^{-2}) from 2014. The most resistant population found by Zimmer and Nauen (2011b) came from Germany and had an LC_{50} of 0.051 μg cm^{-2} toward λ-cyhalothrin. Additionally, Zimmer and Nauen (2011b) sampled a highly susceptible pollen beetle population in Ukraine which served as a reference to calculate resistance ratios. In comparison with this population (UA 70-10) which had an LC_{50} of 0.0001 μg cm^{-2}, the most susceptible population from Denmark in 2014 is 14-fold more tolerant to λ-cyhalothrin. Those truly susceptible populations are difficult to find as the populations are changing over the years due to increased pyrethroid selection pressure even in remote regions. Even the re-sampling in fields, where more susceptible Danish populations were found in 2014, did not result in the same susceptibility level in subsequent years. Pollen beetle populations sampled in consecutive years in regions as close as possible to previously sampled locations revealed similar resistance ratios toward λ-cyhalothrin. Pollen beetles are active flyers and adults hibernate away from the oilseed rape fields (Williams and Cook 2010). The migration of pollen beetles is influenced by climate and proximity to hibernation places to winter oilseed rape fields (Williams and Cook 2010). Denmark can be divided into five parts, i.e., Northern Denmark, Mid-Jutland, South Denmark, Zealand and Capital with Northern Zealand. Each part has specific characteristics in the arable land and climate. The north of Denmark (DK-NJ) is rather cool compared to Zealand (DK-SL). The area for oilseed rape production is 17% in DK-NJ, 27% DK-MJ, 29% DK-SD, 22% DK-SL and 5% in DK-NSL (Danmarks Statistik 2016). The highest intensity of winter oilseed rape cultivation can be found on clay soils, e.g., in Zealand, then Funen (part of DK-SD) and partly in East of Jutland (part of DK-SD and DK-MJ) from 2011 to 2014 (J. E. Ørum pers. comm. 2016). There is a difference in the distribution of resistance populations depending on the geographic location in Denmark. Hansen (2008) revealed that pollen beetles from DK-SL are more resistant to pyrethroids than the ones from Jutland (DK-SD, DK-MJ, DK-NJ), which is also reflected in our findings. However, during the course of our studies in South Jutland (region DK-SD) the most susceptible pollen beetle populations to λ-cyhalothrin were found, located in ca. 50 km distance to each other in 2014 and 2015. The differences in the distribution of resistant populations are probably a reflection of dominating crops, climate and the agricultural practice between regions. Similar differences in pyrethroid resistance levels in pollen beetle population dependent on regional cropping history of oilseed crops have been recently illustrated for Swedish pollen beetle populations (Riggi et al. 2016).

In Denmark, >1–2 insecticide applications against pollen beetles are common (Hansen 2008) which is slightly lower than the European standard of 1–5 applications (Richardson 2008). Pollen beetle is the main pest in winter oilseed rape during spring in Denmark, and fewer applications are done against other pests during this time. In comparison, many different pests during spring are a target for insecticide applications in winter oilseed rape in Germany which can lead to overlapping treatments, especially with pyrethroids (Heimbach and Müller 2013). Frequent insecticide applications can lead to an environment with more selection pressure on the insect pests where susceptible genotypes are eliminated as shown in other cropping systems (Roush and McKenzie 1987).

A major difference to other European countries is that in Denmark and Sweden both winter and spring oilseed is cultivated (Hansen 2003). In 1994 in Denmark the area of winter (96,000 ha) and spring (74,000 ha) oilseed rape was

Table 6 Results for thiacloprid susceptibility testing with Danish pollen beetle populations divided into regions in 2015 ($N = 21$) and 2016 ($N = 6$)

	Strain	Region	LC_{50} (CI) (μg cm^{-2})	LC_{95} (CI) (μg cm^{-2})	Slope (SE)	DK fold change	DE fold change	Baseline fold change
AV	$N = 2$	DK-ND	0.1040	1.570	1.1	12	35	1
Min	07-16		0.0780 (0.0417–0.1100)	1.662 (0.0866–3.240)	1.0 ± 0.1	9	26	0.9
Max	08-16		0.1300 (0.0774–0.1800)	1.478 (0.3222–2.630)	1.2 ± 0.2	15	44	1
AV	$N = 4$	DK-SD	0.0370	1.482	0.8	3	10	0.2
Min	18-15		0.0139 (0.0022–0.0300)	2.201 (0.0000–5.720)	0.6 ± 0.1	2	5	0.2
Max	09-15		0.0439 (0.0232–0.0600)	0.9188 (0.0483–1.790)	1.0 ± 0.1	5	15	0.5
AV	$N = 7$	DK-NSL	0.0751	1.867	1.1	9	25	0.8
Min	20-15		0.0087 (0.0046–0.0100)	0.0890 (0.0129–0.1700)	1.3 ± 0.3	1	3	0.1
Max	12-15		0.1439 (0.0755–0.2100)	4.246 (0.0000–8.880)	0.9 ± 0.1	17	48	2
AV	$N = 5$	DK-SL	0.0531	2.301	0.8	6	18	0.6
Min	11-15		0.0420 (0.0170–0.0700)	2.922 (0.0000–6.750)	0.7 ± 0.1	5	14	0.5
Max	10-15		0.0783 (0.0398–0.1200)	2.287 (0.0000–4.710)	0.9 ± 0.1	9	26	0.9
AV	$N = 3$	DE-MV	0.1520	1.758	1.1	17	51	2
Min	17-15		0.0030 (0.0000–0.0100)	0.0842 (0.0000–0.1800)	0.9 ± 0.2	0	1	0.03
Max	15-15		0.4187 (0.2525–0.5800)	4.777 (0.5750–8.980)	1.2 ± 0.2	17	141	5
AV	$N = 4$	DE-NW	0.1125	1.479	1.1	13	38	1
Min	01-15		0.0545 (0.0230–0.0900)	1.165 (0.0000–2.500)	1.0 ± 0.2	6	18	0.6
Max	02-16		0.2041 (0.1247–0.2800)	2.099 (0.5074–3.690)	1.3 ± 0.2	23	69	2
	04-16	DE-SN	0.3149 (0.1909–0.4400)	3.711 (0.6114–6.810)	1.2 ± 0.2	36	106	3
	05-16	DE-BY	0.6141 (0.4615–0.7700)	1.782 (1.021–2.540)	2.8 ± 0.6	71	207	7

The variation within the Danish samples is calculated on the most susceptible Danish strain 20-15 as a fold change: DK fold change $LC_{50} = LC_{50}/LC_{50}$ (20-15 $LC_{50} = 0.0087$). DE fold change was calculated to the most susceptible strain tested, which was a German pollen beetle population (17-15 $LC_{50} = 0.0030$). Baseline fold change was calculated based on a composite 2010 value of 0.09 μg cm^{-2} (Zimmer and Nauen 2011b)

relatively balanced (EPA 2014). In 2001, Hansen reported about resistant pollen beetles in Denmark (Hansen 2003). Since 2002, the cultivated area on winter oilseed rape has increased which provides rather unlimited feeding and oviposition sites for pollen beetles (Hokkanen 2000; Danmarks Statistik 2016). The parallel decrease in spring oilseed rape cultivation could indicate problems with pest control when winter and spring forms of oilseed rape are cultivated.

The neonicotinoid thiacloprid was introduced in 2007 for pollen beetle control in oilseed rape. An European baseline susceptibility study was conducted and published by Zimmer and Nauen (2011b) which allows to compare data obtained for populations collected in our study. This baseline is also used for registration approval by the European authorities. Now thiacloprid has been on the marked for nearly a decade, and we were interested to check for changes in susceptibility. From 2014 to 2016, several populations were collected across Denmark and analyzed. The existing IRAC classification scheme for thiacloprid is based on percentage mortality scored at 200% of the recommended field dose in a glass vial, and our results span a range from mostly susceptible populations to some showing a reduced susceptibility. The results from the extended bioassay with insecticide-coated vials ranged from LC_{50} of 0.0087–0.144 μg cm^{-2}, which is a 17-fold variation among the tested Danish samples (Table 6). This range for the Danish samples fits within the published thiacloprid baseline for European pollen beetle populations by Zimmer and Nauen 2011b. The generated thiacloprid baseline ranges for the LC_{50} values from 0.04 to 0.144 μg cm^{-2} and LC_{95} from 0.3 to 2.22 μg cm^{-2} in 2010 (Zimmer and Nauen 2011b). Further studies showed no shifting in the generated baseline data for thiacloprid (Zimmer et al. 2014b). In 2015, the tested Danish pollen beetle populations showed a larger range of the LC_{95} values which was 0.34–4.25 μg cm^{-2} in the experiments presented here.

The slope values of the dose–response curves for both λ-cyhalothrin and thiacloprid indicate that in some cases the response to insecticides of pollen beetle populations can be rather heterogeneous. A steep curve likely to represent more homogenous populations was detected for both pyrethroid susceptible as well as more resistant populations. The slopes of the dose–response curves for thiacloprid were rather steep which indicate homogeneous populations although the slopes ranged from 0.6 to 1.4 in 2015 and 2016.

As a reference, some populations from Germany were tested. The LC_{50} values of tested populations from Germany range from LC_{50} values of 0.003–0.614 μg cm^{-2} which are up to 207-fold different, but sevenfold in variation compared to the recently published composite European baseline LC_{50} of 0.09 μg cm^{-2} (Zimmer and Nauen 2011b). It should be noted though that despite the high level of variation in the LC_{50} values, field rates under applied conditions remained active and therefore the variation does not directly convert to field-resistance or product failure (Kupfer and Schröder 2015). The high level of variance in thiacloprid toxicity could be an indicator for the potential of resistance development, so continued monitoring is essential to detect changes in neonicotinoid susceptibility in pollen beetles as early as possible to be able to implement appropriate resistance management strategies based on mode of action rotation (Sparks and Nauen 2015).

This study gives an overview of the current resistance situation in Danish pollen beetle populations toward λ-cyhalothrin as well as their susceptibility to thiacloprid. The high amount of pyrethroid-resistant pollen beetle populations in the Danish region of Zealand (DK-SL) should be considered for the selection of insecticide treatments against pollen beetles in winter oilseed rape. Therefore, alternative mode of actions than pyrethroids should be considered such as neonicotinoids (nicotinic acetylcholine receptor competitive modulators), pyridine azomethines (chordotonal organ TRPV channel modulators) and oxadiazines (voltage-dependent sodium channel blockers) (IRAC 2016; Sparks and Nauen 2015). In Denmark, the main pest in winter oilseed rape during spring is the pollen beetle. In other parts of Europe, several pest species attack the winter oilseed rape in spring where insecticide treatments need to be planned with consideration of changing the mode of actions of the insecticides to avoid the development and insecticide resistances.

Author contributions

CK, RN, KVJ and MK conceived and designed research. CK conducted experiments. CK, RN, KVJ and MK analyzed data. CK, RN, KVJ and MK wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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