Efficacy of alphacypermethrin-treated high density polyethylene mesh applied to jet stalls housing horses against *Culicoides* biting midges in South Africa

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ABSTRACT

The efficacy of alphacypermethrin-treated high density polyethylene (HDPE) mesh applied to jet stalls against *Culicoides* biting midges (Diptera: Ceratopogonidae) was determined by mechanical aspiration of midges from horses and using Onderstepoort 220V downdraught black light traps in four blocks of a 3 x 2 randomised design under South African field conditions. The alphacypermethrin-treated HDPE mesh applied to the stall significantly (P = 0.008) reduced the number of *Culicoides* midges, predominantly *Culicoides* (*Avaritia*) *imicola* Kieffer, mechanically aspirated from horses housed in the stall. The mesh reduced the *Culicoides* midge attack rate in the treated stall compared to the untreated stall and a sentinel horse by 6 times and 14 times, respectively. The number of *Culicoides* midges and *C. imicola* collected in light traps from the untreated and alphacypermethrin HDPE mesh-treated stalls did not differ significantly (P = 0.82). Alphacypermethrin-treated HDPE mesh could be used to reduce exposure of horses in jet stalls to *Culicoides* midges, specifically *C. imicola*, and the risk of midge-borne Orbivirus transmission.

Keywords: *Culicoides imicola*, mechanical aspiration, light trap, pyrethroid, African horse sickness

1. Introduction

Culicoides biting midges (Diptera: Ceratopogonidae) are of importance to health and trade in equids worldwide, primarily due to Orbivirus transmission (Mellor et al., 2000; Meiswinkel et al., 2004). Based on their abundance near livestock *Culicoides* (*Avaritia*) *imicola* Kieffer and *Culicoides* (*Avaritia*) *bolitinos* Meiswinkel are considered the principal vectors of African horse sickness virus (AHSV) and equine encephalosis virus (Nevill et al., 1992; Venter et al., 2002; Meiswinkel et al., 2004; Paweska and Venter, 2004) in South Africa. They are also the dominant species mechanically aspirated from horses at Onderstepoort, South Africa (Scheffer et al., 2012).

Outbreaks in northern Europe of bluetongue virus (Carpenter, 2009), and recently Schmallenberg virus (Hoffmann et al., 2012), have demonstrated the devastating effect of midge-borne viruses on naïve livestock populations. Subsequently, there has been increased concern over the risk of introduction of other midge-borne viruses, such as AHSV, and the need for evidence-based control strategies (Carpenter et al., 2008a, 2009; MacLachlan and Guthrie, 2010; Papadopoulos et al., 2010; Backer and Nodelijk, 2011; MacLachlan and Mayo, 2013; Napp et al., 2013; Robin et al., 2014). Intercontinental trade is a potential mechanism whereby viruses may be introduced into at-risk horse populations, either via movement of infected hosts or vectors (Carpenter et al., 2009; MacLachlan and Guthrie, 2010; Reiter, 2010; de Vos et al., 2012; Napp et al., 2013). Consequently the World Organisation for Animal Health (OIE) have included recommendations in the Terrestrial Animal Health Code on infection with AHSV that insecticide-treated mesh of appropriate gauge be placed over containerised systems transporting horses through regions not free of AHSV (World Organisation for Animal Health, 2013).

The use of mesh to protect stabled horses (Barnard, 1997; Meiswinkel et al., 2000) and the efficacy of insect repellents or insecticides applied to mesh surrounding light traps against *Culicoides* midges have been reported (Braverman and Chizov-Ginzburg, 1998; Page et al., 2009, 2014; Venter et al., 2011, 2014; Del Río et al., 2014a, b). In contrast to the proven efficacy (Lengeler, 2004) and extensive use of pyrethroid-treated mesh against mosquitoes, the use of similar material to protect horses against *Culicoides* midges has received limited attention (Carpenter et al., 2008a). Efficacy of alphacypermethrin-treated high density polyethylene (HDPE) mesh intended for protection of horses against *C. imicola* has recently been demonstrated using light traps and a bioassay (Page et al., 2014).

The objective of this study was to determine if alphacypermethrin-treated HDPE mesh applied to a commercial containerised horse transport system (jet stall) would reduce the number of *Culicoides* midges, particularly *C. imicola* and *C. bolitinos,* mechanically aspirated from horses housed in the stalls in the field. This will support use of the mesh to reduce risk of midge-borne Orbivirus transmission during intercontinental transport of horses.

2. Methods

2.1. Study site and design

The efficacy in reducing the number of *Culicoides* midges aspirated from horses housed in a non-collapsible, 3-compartment jet stall (KLM HMA, European Horse Services, Belgium) of a black, 400 denier, knitted monofilament HDPE mesh with 0.3 mm hole size (RK02 70% Shade Cloth, Alnet, South Africa) treated with alphacypermethrin (Fendona®6, BASF Agro BV Arnhem, Switzerland) was determined in an observer blinded, randomised field study. The study was conducted at the Faculty of Veterinary Science, Onderstepoort (25º38'51.42"S, 28º10'45.96"E, 1 238 m above sea level) between 22 May and 5 June 2014. Comparisons between a mesh-treated and untreated jet stall, located 8.5 m apart to limit interference (Venter et al., 2012) in a grass paddock (58 m x 69 m), were done by mechanical aspiration of midges around sunset from two horses housed in each of the stalls. Treatments were randomised in four blocks of a 3 x 2 design over 12 nights. Each block consisted of three nights where the allocated treatment was maintained for each stall. For the following block treatments were crossed over for the stalls. The same horses were housed in each stall for each block. An additional sentinel horse, in a paddock located 35 m from the jet stalls, was monitored concurrently by mechanical aspiration of midges. Entrance of *Culicoides* midges into the stalls was assessed overnight by 220 V down-draught Onderstepoort suction light traps equipped with 8 W, 23 cm black light tubes (Venter et al., 2009). The study was approved by the Animal Ethics Committee of the University of Pretoria (Study V011-14).

Five healthy adult, female, Thoroughbred horses, mean (range) age 7 (5–13) years, and body mass 529 (463–584) kg were used. Two horses were housed in the outer compartment of each of the stalls overnight from 16h00–06h00 for six nights and thereafter around sunset (17h24-17h27) from 16h00–18h00 for six nights. The middle compartment of each stall was left unoccupied to facilitate access for mechanical aspiration. Between data collection periods the horses were kept with their herd mates in an adjacent grassed paddock. Grass and alfalfa hay was fed *ad libitum* during data collection, and water was provided in buckets every 4 h. Climatic variables (outside temperature, relative humidity, wind speed, rainfall, solar radiation) were recorded hourly using a weather station and data logger (Vantage Pro2 and Weatherlink, Davis, USA) located adjacent to the grass paddock.

2.2. Jet stall treatment

Each 15.5 m^3 jet stall had 1.7 m^2 rectangular openings above the front and rear ramps, permitting entry to *Culicoides* midges. The 35.2 m^2 alphacypermethrin-treated HDPE mesh was custom-made to fit over the treated jet stall in a tent-like fashion, with zip connectors permitting investigator entry located at the end panels. No mesh was applied to the untreated jet stall. The treated mesh was prepared according to the insecticide manufacturer’s instructions for the treatment of bed nets against mosquitoes, for a target dose of 20-40 mg/m^2, on the day prior to each block. The mesh was immersed in 0.28 mg/ml alphacypermethrin suspension for 30 min and air dried overnight at 20 °C and 65% relative humidity. A new mesh was prepared for each treatment block. To counteract depletion of the alphacypermethrin on the mesh due to environmental factors the sides and end panels of the mesh were hand-sprayed at 15h00 on the second and third days of each block with 12.3 mg/m^2 of 0.28 mg/ml alphacypermethrin suspension. Alphacypermethrin uptake was quantified by high performance liquid chromatography (HPLC) analysis of duplicate mesh samples prepared for each block and by duplicate mesh sections (15 x 15 cm) attached to the sides of the stall to monitor the replenishment rate.

2.3. Mechanical aspiration of midges

Midges were aspirated from horses using a customized mechanical aspirator (2820B DC Insect vacuum, BioQuip Products Inc., U.S.A.) mounted on a 12 V hand-held vacuum cleaner (AV1205, Black and Decker, South Africa). One side of each horse inside the stall was aspirated in a systematic manner from cranial to caudal and dorsal to ventral on the neck, back, rump and side by the same investigator. This cycle was repeated until the 2.5 min collection period/horse was completed. Aspiration was performed similarly on both sides of the sentinel horse for 2.5 min per side. The time frame for aspiration lasted from 30 min before to 15 min after sunset, allowing for two 5 min collection periods at each site. Horses were left undisturbed for a 30 min exposure period before the start of aspiration and for a minimum 10 min exposure period between aspiration cycles. The sequence of aspiration for each stall and the sentinel horse was randomised in four replicates of a 3 x 3 Latin square design (Snedecor and Cochran, 1980) to eliminate effects due to site or occasion.

2.4. Light trap collection of midges

Onderstepoort suction light traps were operated inside the rear entrance of each stall, 30 cm from the HDPE mesh enclosing the stall and 2 m above ground level. White polyester netting (hole size 2 mm) was placed around the entrance portal of each light trap to exclude larger insects. The traps were operated from 18h00 (after sunset) to 06h00 (after sunrise); light trap collections and aspiration were therefore not done simultaneously. Catches were made into 500 ml plastic beakers containing 200 ml 0.5% Savlon® (Johnson and Johnson, South Africa) and water solution.

2.5. Culicoides midge identification

Insects collected were stored in 70% ethanol prior to *Culicoides* midge segregation according to species morphology (unpublished wing pattern keys, PVVD, ARC-Onderstepoort Veterinary Institute), sex and parity status (Dyce, 1969) as nulliparous (unpigmented), parous (pigmented), blood fed and gravid females. Large light trap midge collections were sub-sampled (Van Ark and Meiswinkel, 1992).

2.6. Statistical analyses

Statistical analyses were done using SPSS® Statistics version 22 (IBM, USA). Aspirated midge numbers were compared between treatment groups by Kruskal-Wallis one–way ANOVA on ranks. The Mann-Whitney U test was used for pairwise comparisons. Post hoc comparisons were adjusted using the Bonferroni correction of *P* values. When the Bonferroni correction was applied, $P < 0.017$ was considered significant. Attack rate was calculated as the total number of midges aspirated from the horses at each site per minute of collection period. Biting rate was calculated as the total number of freshly blood fed midges aspirated from the horses at each site per minute of collection period.

Midge numbers from the light trap collections were natural logarithm-transformed to achieve normality. Mean numbers of *Culicoides* midges and *C. imicola* were compared between treatment groups using independent samples t-test. Homogeneity of variances was tested with Levene's test. Statistical testing was conducted at the 5% level of significance.

Pearson's Chi-square statistic was used to test the proportion of *C. imicola* in relation to the other *Culicoides* species for independence from treatment. Species diversity for each site was calculated with the Shannon-Wiener index (Al Young Studios, 2012).

3. Results

3.1 Mechanical aspiration of midges

A total of 499 *Culicoides* midges were aspirated from four horses housed in two stalls and one sentinel horse during 36 collections made around sunset for 12 nights. *C. imicola* was the dominant species and comprised 96.6% of midges aspirated, followed by *C. bolitinos* (3.2%) (Table 1). One other species, *C.* (*Culicoides*) *magnus* Colaço, was aspirated. The majority of *C. imicola* aspirated were nulliparous (70.3%), followed by parous, blood fed and gravid females, with no males (Table 2). The proportion of *C. imicola* in relation to the other species collected by aspiration was 98.8% for the stalls combined and 95.6% for the sentinel horse, and was not significantly different between

Table 1. Summary of *Culicoides* species collected by mechanical aspiration from horses and in black light traps operated inside two jet stalls for 12 nights between 22 May and 5 June 2014.

	Mechanical aspiration Total (%)	Light trap Total (%)
C. imicola	482 (96.6)	36075 (99.1)
C. bolitinos	16 (3.2)	173 (0.5)
C. magnus	1 (0.2)	46 (0.1)
C. zuluensis		38 (0.1)
C. gulbenkiani		21 (0.1)
C. pycnostictus		17 (<0.1)
C. nevilli		13 (<0.1)
C. brucei		12 (<0.1)
C. leucostictus		9 (<0.1)
C. enderleini		7 (<0.1)
C. nivosus		1 (<0.1)
Total	499	36412

treatments (X^2 = 3.384, d.f. = 1, P = 0.066). Species diversity was lower in the jet stalls (H' = 0.1) than on the sentinel horse (H' = 0.3) (Table 2).

The alphacypermethrin-treated HDPE mesh applied to the stall significantly (P = 0.008) reduced the number of *Culicoides* midges, predominantly *C. imicola*, mechanically aspirated from horses housed in the stalls, and reduced the *Culicoides* midge attack rate compared to the untreated stall and sentinel horse by 6 times and 14 times, respectively (Table 2). Whilst the number of *Culicoides* midges aspirated in the treated stall was significantly (P < 0.001) lower than the number of midges aspirated from the sentinel horse, the number of midges aspirated in the untreated stall did not differ significantly after Bonferroni correction (P = 0.099) from the sentinel horse (Table 2).

Table 2. *Culicoides* midges, *C. imicola*, and *C. bolitinos* collected during 5 min of mechanical aspiration of two horses housed inside a jet stall with alphacypermethrin-treated (31.8 – 33.7 mg/m^2) HDPE mesh, two horses housed inside an untreated jet stall and a sentinel horse, around sunset for 12 nights between 22 May and 5 June 2014.

		Jet stall				Sentinel horse	
		Alphacypermethrin-treated HDPE mesh		Untreated HDPE mesh			
		n	%	n	%	n	%
C. imicola	Total	26	100	133	98.5	323	95.6
	Median	0 [a]	-	4 [b]	-	9 [b]	-
	Range	0-7	-	0-30	-	0-59	-
	Attack rate	0.2	-	1.1	-	2.7	-
	Biting rate	0	-	0	-	0.1	-
	Nulliparous	17	65.4	90	67.7	232	71.8
	Parous	9	34.6	43	32.3	80	24.8
	Gravid	0	0	0	0	1	0.3
	Blood fed	0	0	0	0	10	3.1
	Male	0	0	0	0	0	0
C. bolitinos	Total	0	0	2	1.5	14	4.1
	Median	0 [a]	-	0 [a,b]	-	0 [b]	-
	Range	0-0	-	0-1	-	0-4	-
	Attack rate	0	-	0.02	-	0.1	-
	Biting rate	0	-	0	-	0	-
	Nulliparous	0	0	1	50.0	10	71.4
	Parous	0	0	1	50.0	4	28.6
	Gravid	0	0	0	0	0	0
	Blood fed	0	0	0	0	0	0
	Male	0	0	0	0	0	0
Culicoides midges	Total	26	-	135	-	338	-
midges	Median	0 [a]	-	4 [b]	-	9 [b]	-
	Range	0-7	-	0-30	-	0-63	-
	Attack rate	0.2	-	1.1	-	2.8	-
	Biting rate	0	-	0	-	0.1	-
	Shannon-Wiener index	-		0.1		0.3	

Median numbers of *Culicoides* midges, *C. imicola*, and *C. bolitinos* within each row with a different superscript differ significantly (*P* < 0.05).

Table 3. *Culicoides* midges, *C. imicola*, and *C. bolitinos* collected by black light traps inside a jet stall fitted with alphacypermethrin-treated (31.8 – 33.7 mg/m^2) HDPE mesh or an untreated jet stall, operated overnight for 12 nights between 22 May and 5 June 2014.

		Jet stall			
		Alphacypermethrin-treated HDPE mesh		Untreated HDPE mesh	
		n	%	n	%
C. imicola	Total	17026	99.0	19049	99.1
	Mean ± S.E.	1419 ± 617	-	1587 ± 646	-
	Nulliparous	13040	76.6	14668	77.0
	Parous	3809	22.4	4212	22.1
	Gravid	11	0.1	23	0.1
	Blood fed	153	0.9	121	0.6
	Male	13	0.1	25	0.1
C. bolitinos	Total	107	0.6	66	0.3
	Mean ± S.E.	9 ± 5	-	6 ± 3	-
	Nulliparous	68	63.6	20	30.3
	Parous	39	36.4	45	68.2
	Gravid	0	0	1	1.5
	Blood fed	0	0	0	0
	Male	0	0	0	0
Other *Culicoides*	Total	60	0.3	104	0.5
Culicoides	Mean ± S.E.	5 ± 2	-	9 ± 7	-
	Nulliparous	34	56.7	60	57.7
	Parous	25	41.7	41	39.4
	Gravid	1	1.7	1	1
	Blood fed	0	0	0	0
	Male	0	0	2	1.9
Total *Culicoides*	Total	17193	-	19219	-
Culicoides	Mean ± S.E.	1433 ± 623	-	1602 ± 653	-
	Shannon Wiener index	0.1	-	0.1	-

3.2 Light trap collection of midges

A total of 36 412 *Culicoides* midges, comprising 11 species, were collected in 24 collections made over 12 nights from two light traps operated simultaneously. *C. imicola* was the most abundant species and comprised 99.1% of midges collected, followed by *C. bolitinos* (0.5%), and *C. magnus* (0.1%) (Table 1). The majority of *C. imicola* collected were nulliparous (76.8%), followed by parous (22.2%), blood fed (0.8%) and gravid (0.1%) females, and males (0.1%) (Table 3).

The mean number of both *Culicoides* midges and *C. imicola* collected in the light traps from the untreated and alphacypermethrin HDPE mesh-treated stalls did not differ significantly (P = 0.82) (Table 3). The proportion of *C. imicola* in relation to the other species collected in the light traps was 99% for both the untreated stall and alphacypermethrin HDPE mesh-treated stall, and was not significantly different between treatments (X^2 = 0.745, d.f. = 1, P = 0.388). Species diversity was similar in the treated (H' = 0.1) and untreated jet stall (H' = 0.1) (Table 3).

The mean ± S.D. alphacypermethrin uptake by the treated HDPE meshes as determined by HPLC analysis on the first, second and third day of each block was 33.7 ± 4.7, 32.3 ± 2.8, 31.8 ± 7.8 mg/m^2, within the target range of 20-40 mg/m^2. The mean ± S.D. outside temperature, relative humidity, wind speed and solar radiation during the data collection were 13.3 ± 0.5 °C, 67 ± 7.1%, 1.1 ± 1.1 km/h and 140.6 ± 13 W/m^2, respectively, with no rain recorded.

4. Discussion

The alphacypermethrin-treated HDPE mesh had a significant effect in reducing the number of *Culicoides* midges, predominantly *C. imicola*, mechanically aspirated around sunset from horses housed in jet stalls under field conditions. A corresponding reduction in the midge attack rate on horses housed in the treated stall compared to the untreated stall and the sentinel horse was shown. In addition, a nil biting rate on horses in both the treated and untreated jet stalls, along with a considerably lower biting rate compared to attack rate for the sentinel horse was determined.

Aspiration of midges from bait animals is considered more reliable for assessment of treatment efficacy (Mullens, et al., 2010) and for gauging midge attack and biting rates (Carpenter et al., 2008b; Gerry et al., 2009; Scheffer et al., 2012; Viennet et al., 2012; Kirkeby et al., 2013; Elbers and Meiswinkel, 2014). The attraction of insects to a light is an artificial response and different cues are involved in the attraction of *Culicoides* midges to hosts than towards light traps. The time frame selected for mechanical aspiration in the present study coincided with the period around sunset when *Culicoides* midges have been shown to attack horses (Braverman, 1988; van der Rijt et al., 2008) and was conducted during the autumn months where peak numbers of midges have been aspirated from horses in the area (Scheffer et al., 2012). Consequently, the attack rate for *C. imicola* on the sentinel horse in the present study (2.7/min) was similar to the attack rate (2.3/min) reported when an AHSV infection rate of 0.43% was detected in aspirated midges (Scheffer et al., 2012). Furthermore, predominantly non-blood fed unpigmented (nulliparous) and parous pigmented (parous) females i.e. females searching for a blood meal, mainly *C. imicola* and *C. bolitinos*, which have been implicated as AHSV vectors (Nevill et al., 1992; Meiswinkel et al., 2004) were aspirated. Similarly, *C. magnus* is also considered as having a high potential vector rating (Nevill et al., 1992) and was previously shown to be susceptible to infection with AHSV (Paweska et al., 2003). The results obtained for the mesh are thus applicable to AHSV control and support the recommended use of insecticide-treated mesh placed over containerised horse transport systems in regions not free of AHSV to reduce the risk of AHSV introduction via intercontinental trade (World Organisation for Animal Health, 2013). Indeed, use of mesh could reduce the risk of AHSV-transmission to naïve horses during outbreaks of AHSV. Likewise, mesh could be used to reduce the risk of naïve midges feeding on AHSV-infected horses, as an alternative to immediate culling of suspected infected horses in non-endemic regions, along with other control measures.

Although fewer midges were collected in the light trap in the treated jet stall the reduction in numbers did not attain significance. This is in contrast to a screening study with the alphacypermethrin-treated HDPE mesh where a significant, 7-fold reduction in midge numbers was reported for a treated light trap (Page et al., 2014). Likewise, Del Río et al. (2014a,b) found no significant reduction in the number of *Culicoides* midges collected in light traps treated with cypermethrin or deltamethrin mesh. Potential reasons for the lack of significant reduction in the number of midges collected in the light trap inside the treated jet stall are related to absence of an immediate knockdown effect of the treated mesh i.e. the midges may have entered the light trap located adjacent to the mesh before being incapacitated. The relatively large mesh hole size, selected so as not to compromise stall ventilation, may not have allowed sufficient midge surface area contact with insecticide (Del Río et al., 2014a), and the midge contact time with the mesh, likely shorter than bioassay contact time (Page et al., 2014), may have been insufficient for immediate knockdown.

Increased mortality rates in midges collected in light traps have been determined after contact with insecticide treated nets (Calvete et al., 2010; Del Río et al., 2014b). Unfortunately the mortality rate of midges collected in light traps in the present study was not investigated, therefore the degree of incapacitation of midges that were able to enter the light trap after passing through the treated mesh is unknown. Nonetheless, during contact bioassays with the alphacypermethrin-treated mesh midge mortality was assessed and observed from 5 min post-exposure (Page et al., 2014), and signs of intoxication and mortality were observed 6 min post-exposure to a deltamethrin-treated mesh (Del Río et al., 2014b). It is considered likely, based on the significant reduction in attack rate determined by aspiration, that the host-seeking ability of the midges (and hence potential for viral transmission) was adversely affected soon after contact with the mesh. The exact interval between contact with alphacypermethrin-treated mesh and onset of midge intoxication having an adverse effect on host-seeking ability is unknown, however. The light trap results highlight the importance of aspiration of midges from bait animals for confirmation of treatment efficacy (Mullens, et al., 2010).

Species richness was greater in the light trap compared to mechanical aspiration, with *C. imicola* predominant for both collection methods. Similar to a previous comparison (Scheffer et al., 2012), a greater proportion of *C. bolitinos*, an endophilic AHSV-vector (Nevill et al., 1992; Meiswinkel et al., 2004; Meiswinkel et al., 2000), was collected by mechanical aspiration than in the light trap. A source of possible variability in the limited species aspirated is the body region sampled, because *Culicoides* species attack different body regions (Braverman, 1988) and the present study focused on the dorsal aspects favoured by *C. imicola*. Species diversity was similar between both light traps, but was greater for aspiration from the sentinel horse compared to inside the jet stalls, likely due to potential exophilic/ endophilic species preferences.

The alphacypermethrin formulation used is recommended by the World Health Organization Pesticide Evaluation Scheme for treatment of mosquito nets, and efficacy and human safety has been demonstrated for malaria control (Ansari and Razdan, 2003; Banek et al., 2010). The alphacypermethrin uptake by the HDPE meshes was within the selected target range (Page et al., 2014), and remained within range with daily replenishment. No rain was recorded during the study period however, so customised replenishment rates may be required under different climatic conditions, particularly if re-use of the mesh is desired.

In conclusion, standardised control measures such as those recommended by the OIE for AHSV (World Organisation for Animal Health, 2013) should be implemented to ensure the safe transportation of equids, especially if these animals are being moved through known AHSV infected areas or areas with unknown risk. Alphacypermethrin-treated HDPE mesh could be used to reduce exposure of horses to *Culicoides* midges, specifically *C. imicola*, and the risk of midge-borne Orbivirus transmission during transport in jet stall containers. Similarly, the alphacypermethrin-treated mesh could also be applied to stable openings (Meiswinkel et al., 2000) to reduce the midge attack rate during AHSV outbreaks. Although a significant reduction in the number of midges attacking horses was demonstrated the alphacypermethrin-treated mesh was not 100% effective, therefore further investigation of use of the mesh in conjunction with additional control measures such as insecticide/ repellent applied to horses (Papadopoulos et al., 2010), or with aerosol insecticide dispensers operated inside jet stalls, is required.

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Conflict of interest statement

The authors declare no conflict of interest.

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