The influence of carbon dioxide on the numbers of *Culicoides* midges collected with suction light traps in South Africa

G.J. Venter[1,2*], S.N.B. Boikanyo[1], D.M. Majatladi[1], and L. Morey[3]

[1]ARC-Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort, 0110 South Africa

[2]Department of Veterinary Tropical Diseases, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa

[3]ARC-Biometry Unit, P.O. Box 8783, Hatfield, 0001 South Africa

*Corresponding author: Tel.: +27-12-5299181; Fax: +27-12-5299180. E-mail address: Venterg@arc.agric.za (G.J. Venter)

Running head: Influence of CO_2 on light trap collections

Abstract. To implement risk management against diseases transmitted by *Culicoides* species Latreille, 1809 (Diptera: Ceratopogonidae) it will be essential to identify all potential vectors. Light traps are the most commonly used tool for the collection of *Culicoides* midges. Taking into account the indiscriminative artificial attraction of light, these traps will collect all night flying insects and not only livestock associated *Culicoides* midges. Factors that could increase the efficacy of these traps for especially livestock associated *Culicoides* midges need to be investigated. In the present study, results obtained with CDC- and Onderstepoort light traps baited with CO_2 were compared to those of un-baited traps. Comparisons were done in two replicates of a 4 x 4 randomized Latin square design. With both traps, the mean numbers of *Culicoides* midges collected in 16 baited collections were higher than those in 16 un-baited collections. Despite exceptionally low numbers collected with the CDC traps, the increase in the numbers and frequency of collection of *Culicoides imicola* Kieffer, 1913 was more pronounced in the CDC compared to that in the Onderstepoort trap. These results indicate that the addition of CO_2 could increase the efficiency of these traps for the collection of *C. imicola*, and other livestock associated *Culicoides* species.

Key words: Insect Collection, CO_2, *Culicoides imicola*

Introduction

Worldwide more than 33 species of protozoa and 20 species of filarial nematodes are reportedly transmitted by various species of *Culicoides* midges Latreille, 1809 (Diptera: Ceratopogonidae) (Linley, 1985). In addition, over 75 different viruses have been isolated from in excess of 50 species of *Culicoides* midges (Meiswinkel *et al*., 2004). Amongst these arboviruses causing important veterinary diseases are some almost exclusively vectored by *Culicoides* midges e.g. bluetongue virus, epizootic haemorrhagic disease virus and African horse sickness virus. The expansion and persistence of bluetongue virus over the last decade together with the detection of Schmallenberg virus in Northern Europe have indicated the potential involvement of several novel species of *Culicoides* in the transmission of livestock viruses (Carpenter *et al*., 2009). Virus detection in field collected specimens coupled to oral susceptibility results in the laboratory infers that susceptibility to virus infection is not restricted to a few individual species but that it is potentially widespread in the genus *Culicoides* (Carpenter *et al*., 2009; Venter *et al*., 2011a).

The involvement of a wide range of *Culicoides* species, each with a unique and often unknown biology, complicates the epidemiology and integrated control of these diseases. For the implementation of integrated risk management, it will be essential to know the abundance and species composition of the *Culicoides* populations in an area. To determine the vector capacity of these populations the feeding frequency on the relevant host must be known. Since development of the first suction light traps for mosquitoes in 1930 (Mulhern, 1942), various suction light trap models are today still the most extensively used tool for collecting and monitoring of *Culicoides* species (Venter *et al*., 2009). Due to their artificial stimuli, light traps will unavoidably attract large numbers of other insects, e.g. beetles and moths and the results does not inevitably reflect the biting rate on the livestock involved (Gerry *et al*., 2009; Viennet *et al*., 2011; Scheffer *et al*., 2012). Light traps furthermore sample only a negligibly small percentage of the night active adult population (Meiswinkel *et al*., 2004).

It is well established that the presence of livestock near light traps increases the numbers of mammophilic species of *Culicoides* collected (Garcia-Saenz *et al*., 2011; Viennet *et al*., 2013).

Semiochemicals that act as olfactory cues, as an additional attractant to light, may therefore increase the specificity and efficiency of traps. Odour baited traps have the potential to more accurately characterise host vector relationships than light trapping alone and would be less labour intensive than animal-bait trapping (Harrup *et al*., 2012). Several studies have indicated carbon dioxide (CO_2) to be an attractant for a number of blood feeding insects including *Culicoides* midges e.g. *Culicoides furens* (Poey), 1853, *C. hollensis* (Melander and Brues), 1903 and *C. melleus* (Coquillett), 1901, in North America (Kline *et al*., 1994) and *C. impunctatus* Goetghebuer, 1920 in Scotland (Bhasin *et al*., 2001). Studies in Australia and Scotland have shown that CO_2 can act synergistically with specific enantiomers of octenol to increase the numbers of species of *Culicoides* collected (Ritchie *et al*., 1994; Harrup *et al*., 2012). Conversely, some abundant livestock associated species e.g. the Palaearctic *C. obsoletus* (Meigen), 1818 are reported to respond poorly to CO_2 (Mullens *et al*., 2005; Gerry *et al*., 2009).

Although *C. imicola* Kieffer 1913, an abundant proven orbivirus vector in South Africa, is attracted to and can be collected in incredibly large numbers near livestock (Meiswinkel *et al*., 2004), nothing is known about additional factors that would attract this and other Afrotropical *Culicoides* species to livestock. It was shown that the addition of a mixture of octenol and methylphenol to the Onderstepoort 220 V trap did not influence species richness, numbers collected, sex ratios or age-grading results (Venter *et al*., 2011b).

The aim of the present study was to determine if the addition of CO_2 to a light trap might increase the range and numbers of *Culicoides* species collected and if this could affect the age-grading and sex ratio results of the population sampled. The artificial attraction of light, and especially that of the strong 8 W black light of the Onderstepoort trap, for *Culicoides* species coupled with the strong downdraught of the fan, could counteract any potential attractant effect of additional products. Previous studies indicated the Centres for Disease Control miniature light trap (CDC trap), equipped with a less powerful fan and light source, to be less attractive than the Onderstepoort trap for livestock associated *Culicoides* species in South Africa (Venter *et al*., 2009). This less attractive light trap may therefore be more suitable to determine if the addition of CO_2 to the trap might increase the efficacy thereof.

Because of its proven efficiency the Onderstepoort 220 V black light traps are routinely used in many countries for the collection of *Culicoides* midges (Venter *et al*., 2009). In order to collect especially livestock associated *Culicoides* these traps are usually deployed near livestock. To determine if the additional use of CO_2 will increase the efficiency of this trap, evaluations were done near livestock. These results may provide an insight into factors that will attract *Culicoides* midges to livestock and contribute to the development of integrated control strategies.

Material and Methods

All evaluations with CO_2 were done at the Agricultural Research Council-Onderstepoort Veterinary Institute (25°39'S, 28°11'E, 1 219 m above sea level) during the rainy season in summer.

To determine if the addition of CO_2 to light traps will influence the numbers and species composition of *Culicoides* midges collected four CDC traps fitted with 6 V incandescent light sources were used from 4 to 20 December 2013. Two of these traps were baited with CO_2 and two were un-baited control traps. To minimise the effect of background CO_2 all four traps were deployed at least 500 m from livestock or bigger mammals. Some large trees sheltering wild birds and vervet monkeys (*Chlorocebus pygerythrus*) at night were present in the area.

To determine if the addition of CO_2 would increase their efficiency, two Onderstepoort traps baited with CO_2 were compared to two un-baited traps. Comparisons in this second study were done from 21 to 31 January 2014 near cattle. The traps were hung underneath the eaves of an open-sided stable housing 15 to 20 cattle at night.

To ensure that treatment means were independent of any effects due to site or occasion, comparisons with each trap design and treatment were done in two replicates of a 4 (sites) × 4 (days) randomized Latin square design (Perry *et al*., 1980). In each of the two replicates, two baited traps were compare with two un-baited traps over a period of four nights. To minimise interference between treatments, trap sites were at least 15 m apart (Venter *et al*., 2012). Traps were baited with 1.5 kg of dry ice wrapped in absorbent paper and placed in a plastic bucket. The bucket was sealed with a lid through which a plastic pipe of 10 mm diameter protruded and it was placed in an isolated polystyrene box above the trap with the pipe's outlet fitted just above the trap light source. In the morning, after collection, any remaining dry ice was weighed. On average 1.1 kg of dry ice was used overnight (12 hours) at each baited trap.

In both studies, traps were hung 1.4 m above ground level. Insects were collected into water to which 0.5% Savlon® antiseptic had been added. Traps were operated from two hours before dusk to two hours after dawn. In the morning, insects were transferred to 80% ethanol and stored in the dark until analysed. Based on abdominal pigmentation (Dyce, 1969), females of all species were age-graded into un-pigmented (nulliparous), pigmented (parous), gravid or freshly blood-fed. Captured males were also counted.

Analysis of variance (ANOVA) was used to differentiate between trap treatment effects. The data was normally distributed and treatment means were separated using Fisher's protected t-test of least significant difference (LSD) (Snedecor & Cochran, 1980). Data was analysed using the statistical program GenStat® (Payne *et al*., 2007). Depending on the sample size proportions of insect counts between treatments were compared using either Fisher's exact or Chi-squared ($\chi 2$) tests. All statistical testing was done at the 5% significance level.

Results

CDC trap comparisons

In this comparison, 32 collections were made on eight nights between 4 and 20 December 2013 with the CDC traps fitted with 6 V incandescent light source. The average minimum night temperature for this period was 15°C. Of the 188 *Culicoides* midges found, 136 (72.3%) were collected in the 16 collections made with the two baited CO_2 traps and 52 (27.7%) in the 16 collections made with the two un-baited traps (Table 1). Although only relatively small numbers were collected, the mean number (8.5) of *Culicoides* midges in 16 collections made with the baited traps was significantly higher ($P = 0.003$) than that of 3.3 in the un-baited traps (Table 1).

Table 1. Summary of *Culicoides* midges collected at the Agricultural Research Council-Onderstepoort Veterinary Institute to determine the effect of the addition of CO_2 to a CDC light trap (incandescent light) on *Culicoides* numbers, species composition and age grading results of the population. Collections were made from 4 to 20 December 2013 in the absence livestock. (P values ≤ 0.05 = a statistical significant difference)

Light trap treatment	CO_2 present	CO_2 absent	Statistical significance (P value) *Fisher's protected t-test $^{+}$Fisher's exact test
Number of collections made	16	16	
Number of species present	5	5	
Total *Culicoides* collected	136	52	
Mean collection size	8.5	3.3	0.003^{*}
Range in collection size	1-25	0-23	
Non blood feeding insects	828	713	0.312^{*}
Culicoides : other insects	1 : 6.1	1 : 13.7	
C. bedfordi			
Frequency of collection	14	9	
Total collected	56	30	
Mean collection size (%)	3.5 (41.2)	1.9 (57.6)	
Range in collection size	0-16	0-13	
Age grading results			
Un-pigmented females (%)	49 (87.5)	28 (93.3)	0.486^{+}
Pigmented females (%)	2 (3.6)	1 (3.3)	
Gravid females (%)	1 (1.8)	0	1.000^{+}
Males (%)	4 (7.1)	1 (3.3)	0.654^{+}
C. leucostictus			
Frequency of collection	10	5	
Total collected	58	16	
Mean collection size (%)	3.6 (42.4)	1.00 (30.3)	
Range in collection size	0-19	0-8	
Age grading results:			
Un-pigmented (%)	34 (58.6)	10 (62.5)	1.000^{+}
Pigmented (%)	17 (29.3)	3 (18.8)	0.532^{+}
Males (%)	7 (12.1)	3 (18.8)	0.443^{+}

Culicoides midges belonging to at least six species were collected. The most abundant in the baited traps were two ornithophilic species, i.e. *C. leucostictus* Kieffer, 1911 (42.4%) and *C. bedfordi* Ingram and Macfie, 1923 (41.2%) (Table 1). In the un-baited traps *C. bedfordi* (57.6%) was dominant, with *C. leucostictus* representing 30.3% of the collected *Culicoides* midges (Table 1). Despite being the most abundant, *C. bedfordi* was present in only 14 and nine of the 16 collections made with the baited and un-baited traps respectively (Table 1). Similarly, *C. leucostictus* was present in 10 and five of the collections made with baited and un-baited traps (Table 1). A single specimen of *C. pycnostictus* Ingram and Macfie, 1925 was collected with the baited traps and two specimens of *C. nivosus* de Meillon, 1937 were only collected in the un-baited traps.

Culicoides imicola, considered the most abundant livestock associated *Culicoides* species in the area (Venter et al., 2012; 2014), represented 14.1% (n = 19; mean = 1.2) and 3.0% (n = 2; mean = 0.1) of the *Culicoides* midges in the baited and un-baited traps respectively. It was collected seven times in the baited and only twice in the un-baited traps.

Un-pigmented females were the dominant grouping for both *C. bedfordi* and *C. leucostictus* (Table 1). No statistical differences were found in the age grading results for the two collection methods (Table 1). No freshly blood engorged females were found.

The number of other insects collected exceeded the number of *Culicoides* midges by a factor of 6.1 and 13.7 in the baited and control traps respectively (Table 1) but the proportional representation of *Culicoides* midges was significantly ($P < 0.001$) higher in the baited traps.

Onderstepoort trap comparisons

Similar to the comparisons made with the CDC traps in 32 collections made on eight nights between 21 and 31 January 2014 with the 220 V Onderstepoort trap (Table 2). The average minimum night temperature for this period was 16°C. Of the 8 537 *Culicoides* midges collected, 6 547 (76.7%) were collected in the 16 collections made with the two baited traps and 1 990 (23.3%) in the 16 collections made with the two un-baited traps (Table 2). The mean number of *Culicoides* midges in the 16 collections with the two baited traps (409.2) was significantly (P = 0.042) higher than those in the un-baited traps (124.4) (Table 2).

Culicoides midges belonging to at least 14 species were collected. While all were present in the baited traps, only seven of these were seen in the un-baited traps. In both trapping regimes, *C. imicola*, accounting for 98.1% and 97.9% of the *Culicoides* midges in the baited and un-baited traps

Table 2. Summary of *Culicoides* midges collected at the Agricultural Research Council-Onderstepoort Veterinary Institute to determine the effect of the addition of CO_2 to an Onderstepoort 220 V black light trap on *Culicoides* numbers, species composition and age grading results of the population. Collections were made from 21 to 31 January 2014 near livestock. (P values ≤ 0.05 = a statistical significant difference)

Light trap treatment	CO_2 present	CO_2 absent	Statistical significance (P value) *Fisher's protected t-test $^{+}$Fisher's exact / $^{++}$Chi-squared test
Number of collections made	16	16	
Number of species collected	14	7	
Total *Culicoide*s collected	6547	1990	
Mean collection size	409.2	124.4	0.042*
Range in collection size	16-2639	13-663	
Non blood feeding insects	1562	1 073	0.049*
Culicoides : other insects	1 : 0.24	1 : 0.55	
C. imicola			
Frequency of collection	16	16	
Total collected	6422	1948	
Mean collection size (%)	401.4 (98.1)	121.8 (97.9)	0.043^{+}
Range in collection size	16-2603	11-654	
Age grading results:			
Un-pigmented females (%)	2780 (43.3)	1047 (53.7)	$< 0.001^{++}$
Pigmented females (%)	3314 (51.6)	804 (41.3)	$< 0.001^{++}$
Freshly blood-fed (%)	41 (0.6)	4 (0.2)	0.035^{++}
Gravid females (%)	269 (4.2)	83 (4.3)	0.941^{++}
Males (%)	18 (0.3)	10 (0.5)	0.181^{++}
C. enderleini			
Number of collection present	8	6	
Total collected	52	23	
Mean collection size (%)	3.3 (0.8)	1.4 (1.1)	
Range collection size	0-18	0-8	
Age grading results:			
Un-pigmented females (%)	14 (26.9)	11 (47.8)	0.111^{+}
Pigmented females (%)	31 (59.6)	5 (21.7)	0.003^{+}
Freshly blood-fed females (%)	1 (1.9)	0 (0)	1.000^{+}
Gravid females (%)	1 (1.9)	1 (4.4)	0.522^{+}
Males (%)	5 (9.6)	6 (26.1)	0.082^{+}

respectively, was the most abundant (Table 2). The mean number of *C. imicola* was significantly higher (*P* = 0.043) in the baited (401.4) than the un-baited traps (121.8) (Table 2). The second most abundant species *C. enderleini* Cornet and Brunhes, 1994 in both trap treatments represented 0.8% and 1.1% of the *Culicoides* midges collected (Table 2). It was present in eight and six of the 16 collections made with the baited and un-baited traps (Table 2).

Culicoides leucostictus, an abundant ornitophilic species in the first study, represented a mere 0.5% (*n* = 31) and 0.05% (*n* = 1) of the *Culicoides* collected. Only three specimens of *C. bedfordi* were collected in each trapping regime.

In the baited traps, pigmented *C. imicola* females represented 51.6% of the total collected (Table 2). In the control traps, un-pigmented females, representing 53.7% of the *C. imicola* individuals, were the most abundant (Table 2). While the mean number of un-pigmented *C. imicola* females was not significantly different ($P = 0.088$) between the baited (173.8) and un-baited traps (65.4), a significantly higher ($P = 0.024$) mean number of pigmented females were collected in the baited (207.1) compared to the un-baited traps (50.3). As a result the proportional representation of un-pigmented ($P < 0.001$), pigmented ($P < 0.001$) and freshly blood fed ($P = 0.035$) females was significantly different between treatments (Table 2). No significant differences were found in the proportional representation of gravid females ($P = 0.941$) and males ($P = 0.082$) (Table 2).

Similar to *C. imicola,* the proportional representation of pigmented older *C. enderleini* females was significantly ($P = 003$) higher in the baited traps compare to that in the un-baited traps (Table 2). No significant differences were found in proportional representation of the other age groups or males collected (Table 2).

In the collections made in the immediate vicinity of the cattle the mean number of *Culicoides* midges exceeded the number of other insects collected by a factor of 0.24 and 0.55 in the baited and un-baited traps respectively (Table 2). The proportional representation of *Culicoides* midges in the baited traps was significantly ($P < 0.001$) higher.

Discussion

With both the CDC and the Onderstepoort traps, significantly higher mean numbers of *Culicoides* midges were collected with the baited CO_2 traps. On average, the addition of CO_2 increased the mean numbers collected by a factor of 2.6 and 3.3 for the CDC and Onderstepoort traps respectively. The addition of CO_2 to CDC traps, in the absence of livestock, not only increased the frequency but also the mean numbers of *C. imicola*, a mammophilic species, by a factor of 12. In the Onderstepoort traps the numbers of *C. imicola* were increased by a factor of only 3.3. It must, however, be taken into account that, due to the presence of cattle and consequently higher background levels of CO_2, at the sites *C. imicola* may already have been abundant. Despite the relative inefficiency of the CDC traps for the collection of South African *Culicoides* species, the present results indicate that the addition of CO_2 may give a more reliable representation of livestock host-vector relationships than the suction light trap on its own. . These results are in agreement with those on some North American (Kline *et al*., 1994) and Scottish *Culicoides* species (Bhasin *et al*., 2001). In North America, the CDC trap, most often baited with CO_2, seems to be the preferred method for the collection of *Culicoides* midges (Smith & Mullens, 2003). Taking into consideration the relative low numbers collected in the present trials, the attraction of *C. imicola* and other South African *Culicoides* species to CO_2 needs further evaluation.

The significantly higher proportions of pigmented older females collected in the baited traps suggest that CO_2 may be differentially attractive to various age groups. (Table 1 & 2). Since transovarial transmission of orbiviruses is not known to occur in the genus *Culicoides* (Osborne *et al*., 2015), the number of parous females is of importance in evaluating the vector potential of populations.

. Host location behaviour involves multiple and complex visual, thermal and olfactory stimuli, which will be difficult to replicate (Logan *et al*., 2010). Carbon dioxide mimics animal exhalation and thus renders these traps more specific for capturing blood-feeding insects looking for a blood meal. While trapping using CO_2 as bait reflects biting populations of *C. sonorensis* Wirth and Jones, 1957 in the USA (Mullens & Gerry, 1998), these and other major vector *Culicoides* species appear to utilise more complex attraction cues (Bishop *et al*., 2008; Harrup *et al*., 2012; Mullens & Gerry, 1998). Previous studies indicated that octenol did not increase the number of South African *Culicoides* collected with the 220 V Onderstepoort trap (Venter *et al*., 2011b). It still needs to be determined if the simultaneous use of CO_2 and octenol might have a synergic effect on the numbers of *C. imicola*, as is the case for some North American species (Cilek & Kline, 2002). Since CO_2 act as a non-visual olfactory stimulus it may be more effective in collecting day-active *Culicoides* midges.

Carbon dioxide is relatively expensive and is not always practical for use in routine big scale surveillance programmes. The optimum release rate of the CO_2 still needs to be determined. In the evaluation of the Triple Trap no apparent effect of the CO_2 released by this trap could be found (Venter *et al*., 2013). Although it was shown that the addition of CO_2 might increase the effectivity of light traps for the collection of livestock associated *Culicoides* midges the prevailing wind, ambient temperatures and background levels of CO_2 and other stimuli may influence the dispersal and effectivity of the CO_2. These variables, in combination with other factors that may influence the number of *Culicoides* midges collected with light traps, might explain the relatively great night-to-night variations found.

Due to automatic trapping, independent of the operator, light traps will remain a practical and an economic trapping systems to determine species presence and abundance in an area. It should be realised that light is an artificial attraction stimulus and does not mimic or reflect any host response. Light traps will collect all phototropic insects present but will not replace the host. Compared to the potential dispersal capacity of olfactory cues, the range of attraction of light traps can be relatively short (Venter *et al*., 2012). The great number of factors (known and unknown) that influence midge behaviour as well as trap efficiency renders it difficult to compare light trap results meaningfully. These deficiencies restrict the use of light traps to broad-scale surveillance rather than local scale assessments of distribution and abundance (Bishop *et al*., 1994).

It is already known, since 1934, that CO_2 can be an effective attractant for a number or of haematophagous Diptera (McNelly, 1989; Logan *et al*., 2010). Further studies involving *Culicoides* should concentrate on alternative kairomones either as individual components or in synergistic mixtures. This should be considered, as part of a wider understanding of host-selection with factors such as visual and thermal cues taken into account. This may contribute to more accurate estimates of activity that can in turn be used to produce more accurate risk assessment tools and eventually methods of control.

Acknowledgments

We thank the ARC-OVI and the NRF (South Africa) for supporting this work. The authors wish to thank Martin Geier (Biogents, Germany) and Brad Mullens (University of California, USA) for fruitful discussions relating to CO_2 and the insect collection. We thank Dr Truuke Gerdes and Dr Kerstin Junker for constructive comments on earlier drafts of this manuscript. We thank the anonymous referees for their valuable input for improving the quality of this manuscript.

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