INVESTIGATION OF METHODS FOR PROTECTION OF HORSES IN JET STALLS AGAINST *CULICOIDES* BITING MIDGES

by

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DEDICATION

I dedicate this thesis to Harold, Kate and Connor Page

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ABBREVIATIONS

AHS	African horse sickness
AHSV	African horse sickness virus
ALV	attenuated live virus
ANOVA	analysis of variance
BT	bluetongue
BTV	bluetongue virus
DEET	*N,N*-diethyl-3-methylbenzamide
DEFRA	Department for Environment, Food and Rural Affairs
EEV	equine encephalosis virus
EIA	enzyme immunoassay
EU	European Union
FGM	faecal glucocorticoid metabolite
H'	Shannon-Wiener index
HDPE	high density polyethylene mesh
HPLC	high performance liquid chromatography
HRV	heart rate variability
IATA	International Air Transport Association
IQR	interquartile range
NS	non-structural protein
RH	relative humidity
OIE	World Organisation for Animal Health
OVAH	Onderstepoort Veterinary Academic Hospital
RT-PCR	reverse transcription polymerase chain reaction
ULD	unit load device

UV	ultraviolet
VP	viral protein
WHO	World Health Organisation
WHOPES	WHO Pesticide Evaluation Scheme

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SUMMARY

Culicoides biting midges (Diptera: Ceratopogonidae), specifically *Culicoides* (*Avaritia*) *imicola* Kieffer and *Culicoides* (*Avaritia*) *bolitinos* Meiswinkel have been implicated as vectors of African horse sickness virus (AHSV) and equine encephalosis virus (EEV) in southern Africa. Intercontinental trade is a potential mechanism whereby midge-borne viruses, such as AHSV, may be introduced into immunologically naive horse populations. Horses in containerised air transport systems (jet stalls) may be at risk of exposure to *Culicoides* midges during international export from South Africa. The World Organization for Animal Health (OIE) has recently recommended that during export from, and transit through, African horse sickness (AHS) endemic countries or zones, measures of a physical and chemical nature are applied to protect horses from *Culicoides* midge attack. To contribute to information on effective measures of protection and to generate data on the effect of these on welfare of horses in jet stalls, the efficacy and safety of alphacypermethrin-treated high density polyethylene (HDPE) mesh applied to jet stalls as a method for protection of horses against *Culicoides* midges was investigated at the Faculty of Veterinary Science, Onderstepoort.

Firstly, the repellent and insecticidal efficacy of alphacypermethrin-treated HDPE mesh against *Culicoides* midges was determined using Onderstepoort 220V downdraught black light traps and a contact bioassay. Three traps were operated overnight in four replicates of a 3 x 3 randomised Latin square design near horses. Both an untreated and alphacypermethrin-treated HDPE mesh significantly ($P < 0.05$) reduced the numbers of *Culicoides* midges, predominantly *C. imicola*, collected in the light traps by 4.2 and 7.2 times, respectively. A repellent effect of the alphacypermethrin-treated mesh was not

confirmed because the number of midges collected in the light traps with untreated and alphacypermethrin-treated HDPE mesh were not significantly different (P = 0.656). Bioassay of the insecticidal contact efficacy indicated median *C. imicola* mortality of 100% from 30 and 10 min following exposure to the alphacypermethrin-treated HDPE mesh for 1 or 3 min, respectively. In the bioassay, mortality was significantly higher (P = 0.016) at 5 min post exposure in the midges exposed to the alphacypermethrin-treated mesh for 3 min (74.8%) compared to the 1 min exposure group (59.5%).

Secondly, the efficacy of alphacypermethrin-treated HDPE mesh applied to jet stalls against *Culicoides* midges was determined by mechanical aspiration of midges from horses and using light traps in four blocks of a 3 x 2 randomised design. 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 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).

Finally, the effect of alphacypermethrin insecticide-treated HDPE mesh on ventilation and welfare of horses housed in jet stalls was determined under temperate, climatic conditions. Jet stall microclimate, clinical variables and faecal glucocorticoid metabolites (FGM) of 12 horses were monitored during overnight housing in either a treated or untreated jet stall in two blocks of a 2 x 3 randomized crossover design. Temperature difference between the treated stall and outside differed significantly from the difference between the untreated

stall and outside only at 1/15 time points (P = 0.045, r = 0.70). Relative humidity (RH) difference between the treated stall and outside did not differ from the difference between the untreated stall and outside. Temperature and RH in the treated stall were highly significantly correlated with outside temperature (r = 0.961, P < 0.001) and RH (r = 0.954, P < 0.001), respectively. No significant differences were detected between rectal temperatures, pulse and respiratory rates of horses in the treated stall compared to the untreated stall. Mean FGM concentrations for horses housed in the treated stall peaked earlier (24 h) and at a higher concentration than horses housed in the untreated stall (48 h), but were not significantly different from baseline concentrations. No significant difference was detected in FGM concentrations when the treated and untreated stall groups were compared at individual time points up to 72 h after exiting the jet stall.

These studies determined that alphacypermethrin-treated HDPE mesh served as an effective physical barrier and had a rapid insecticidal effect against *Culicoides* midges, specifically *C. imicola,* under laboratory bioassay and field screening conditions. When practically applied to jet stalls used for air transport of horses alphacypermethrin-treated HDPE mesh significantly reduced the attack rate of *Culicoides* midges on horses, with no adverse effect on jet stall ventilation, clinical variables or stress indicators of horses housed in the stalls. Alphacypermethrin-treated HDPE mesh can be used as an effective physical and chemical method for protection of horses in jet stalls against *Culicoides* midges and the risk of midge-borne Orbivirus transmission under temperate climatic conditions.

CHAPTER 1: GENERAL INTRODUCTION

Culicoides biting midges (Diptera: Ceratopogonidae) are of veterinary significance worldwide, primarily as vectors of arboviruses affecting domestic livestock (Meiswinkel et al., 2004; Mellor et al., 2000; Purse et al., 2015). Based on the numbers collected near livestock *Culicoides imicola* is considered the principal vector of AHSV in South Africa (Meiswinkel et al., 2004; Nevill et al., 1992). Recent outbreaks of a novel orthobunyavirus, Schmallenberg virus (Hoffmann et al., 2012), and the spread of bluetongue virus (BTV) in northern Europe has raised concern of the introduction and spread of other midge-borne viruses, particularly AHSV, and the need for optimised preventative strategies (Backer and Nodelijk, 2011; Carpenter et al., 2008a; Carpenter et al., 2009; MacLachlan and Guthrie, 2010; MacLachlan and Mayo, 2013; Papadopoulos et al., 2010; Robin et al., 2014).

Clear recommendations for pre-export quarantine and testing of horses for AHSV have been in place for many years, however recent amendments to the OIE - Terrestrial Animal Health Code chapter on “Infection with African horse sickness virus” have included recommendations that mesh of appropriate gauge, impregnated with an approved insecticide be placed over containers during transport of horses through regions not free of AHSV (Anon, 2013). During international air transit from South Africa horses may be at short-term risk of exposure to *Culicoides* midges during loading of the jet stalls into an aircraft. Furthermore, there is the possibility that horses in jet stalls may be exposed to AHSV-infected midges during transit through an AHSV-infected country (DEFRA, 2008).

Whilst limited information is available on the effects of microclimate on horses during air transport in insect-proof, climate-controlled enclosed containers (Thornton, 2000), the effects of enclosing solid top HMA-type commercial jet stalls (as used for export of horses

from South Africa) with treated mesh on microclimate, clinical variables and stress of horses are unknown. Monitoring of temperature and relative humidity (RH) inside jet stalls provides an indication of ventilation (Thornton, 2000), while monitoring of clinical variables and faecal glucocorticoid metabolites (FGM) provides a non-invasive means of assessing prolonged stress associated with transport in horses (Schmidt et al., 2010a,b,c).

Investigation of effective and safe physical and chemical methods of protection to mitigate the risk of exposure of equidae to AHSV vectors during transhipment, such as jet stalls enclosed with HDPE mesh treated with an insecticide with proven efficacy against *Culicoides* midges, is required. These measures could ideally also be applied to protect horses from exposure to AHSV during high risk periods in endemic areas in South Africa.

The objectives of the studies conducted were therefore:

- To investigate alphacypermethrin-treated HDPE mesh, applied to Onderstepoort 220V downdraught black light traps, as a physical barrier against *Culicoides* midges.
- To investigate the insecticidal efficacy of alphacypermethrin-treated HDPE mesh, against field-collected, nulliparous *C. imicola*.
- To determine if alphacypermethrin-treated HDPE mesh applied to a jet stall would reduce the attack rate of *Culicoides* midges, particularly *C. imicola* and *C. bolitinos*, on horses housed in the stalls.
- To determine the effect of alphacypermethrin-treated HDPE mesh applied to jet stalls on microclimate, clinical variables and FGM concentrations of horses housed under stationary jet stall, temperate climatic conditions.

CHAPTER 2: LITERATURE REVIEW

2.1. *Culicoides* biting midges

Culicoides midges (Diptera: Ceratopogonidae) (Kettle, 1995) are small insects, measuring 1-3 mm in size (Meiswinkel et al., 2004). Over 1400 species of *Culicoides* midges have been identified worldwide, with approximately 30 species implicated in transmission of over 50 arboviruses (Mellor et al., 2000; Purse et al., 2015; Wilson et al., 2009). Over 120 species of *Culicoides* midges are known to occur in South Africa (Meiswinkel, 1998).

Midges are most abundant during the warm summer months and exhibit predominantly crepuscular or nocturnal behaviour (Kettle, 1995). Dependent on the species, females lay up to 450 eggs (Kettle, 1995) on a moist substrate in which the four larval stages feed and pupate (Meiswinkel et al., 2004; Mellor et al., 2000). The complete life cycle takes three to four weeks under favourable climatic conditions, facilitating development of several generations in a single season. Only female *Culicoides* midges blood-feed, which facilitates egg maturation (Kettle, 1995; Meiswinkel et al., 2004). Nulliparous females have never developed eggs and do not have follicular relics, parous females have laid eggs and have follicular relics, gravid females contain maturing eggs. Age-grading differentiation of midges is of importance as no transovarial transmission of virus has been demonstrated (Osborne et al., 2015), and evaluation of parous or gravid female midges is considered sufficient when aiming to detect Orbiviruses. Nulliparous and parous female midges can be differentiated based on the presence of burgundy-red pigmentation inside the abdominal wall of parous females (Dyce, 1969). This method was recently shown to be inaccurate for *C. imicola* in Israel, whereby 23% of apparently nulliparous females showed

red pigmentation (Braverman and Mumcuoglu, 2009). However, as no practical alternatives are available this method is still a valuable tool for age-grading, with cognisance of the limitations (Braverman and Mumcuoglu, 2009).

Culicoides midges are of importance to global health and international trade in equids, primarily due to Orbivirus transmission (Mellor et al., 2000; Meiswinkel et al., 2004). Based on its wide geographical distribution and preference for bigger mammals, supported by the high numbers collected near livestock, *Culicoides* (*Avaritia*) *imicola* Kieffer is considered the principal biological vector of AHSV, EEV and BTV in South Africa (Nevill et al., 1992; Meiswinkel et al., 2004; Paweska and Venter, 2004), and BTV in southern Europe (Mellor, 1992; Mellor et al., 2000). *Culicoides bolitinos* has also been implicated as a vector of AHSV following an outbreak in the eastern Free State of South Africa (Meiswinkel et al., 2000; Meiswinkel and Paweska, 2003). This endophilic species, whose larval habitat includes cattle dung as well as African buffalo and blue wildebeest dung (Meiswinkel, 1989), is common in cold as well as hot areas and may be more important than the exophilic *C. imicola* as an vector in certain regions (Meiswinkel et al., 2004). In Europe AHSV has been isolated from *C. imicola* as well as from mixed pools of *Culicoides* (*Avaritia*) *obsoletus* Meigen and *Culicoides* (*Culicoides*) *pulicaris* Linnaeus (Mellor et al., 1990).

Culicoides imicola was the most abundant species collected by mechanical aspiration from horses at Onderstepoort, South Africa (Scheffer et al., 2012). Other species collected by mechanical aspiration in order of abundance were: *Culicoides* (*Avaritia*) *bolitinos* Meiswinkel, *Culicoides* (*Avaritia*) *gulbenkiani* Caeiro, *Culicoides* (*Culicoides*) *magnus* Colaço, *Culicoides* (*Hoffmania*) *zuluensis* de Meillon, *Culicoides milnei* Austen, *Culicoides*

#50, *Culicoides* (*Avaritia*) *glabripennis* Goetghebuer, *Culicoides* (*Meijerehelea*) *leucostictus* Kieffer, *Culicoides* (*Remmia*) *enderleini* Cornet & Brunhes, *Culicoides brucei* Austen and *Culicoides tuttifrutti* Meiswinkel (Scheffer et al., 2012).

In South Africa, epidemiological investigations have often employed Onderstepoort suction light traps (Figure 1) to collect *Culicoides* midges. The number of midges collected in light traps are influenced by rainfall during the preceding month, the presence of midge breeding sites, the height of the light trap above ground level, wind speed and relative humidity (Venter et al., 1996). Similar to *Culicoides* (*Avaritia*) *brevitarsis* Kieffer in Australia (Bishop, 2000), light trap catches of *Culicoides* midges and specifically *C. imicola* at Onderstepoort increased significantly with increasing temperature and decreased significantly with increasing wind speed and rainfall, with a tendency for increasing humidity to decrease *C. imicola* catches (Page et al., 2009).

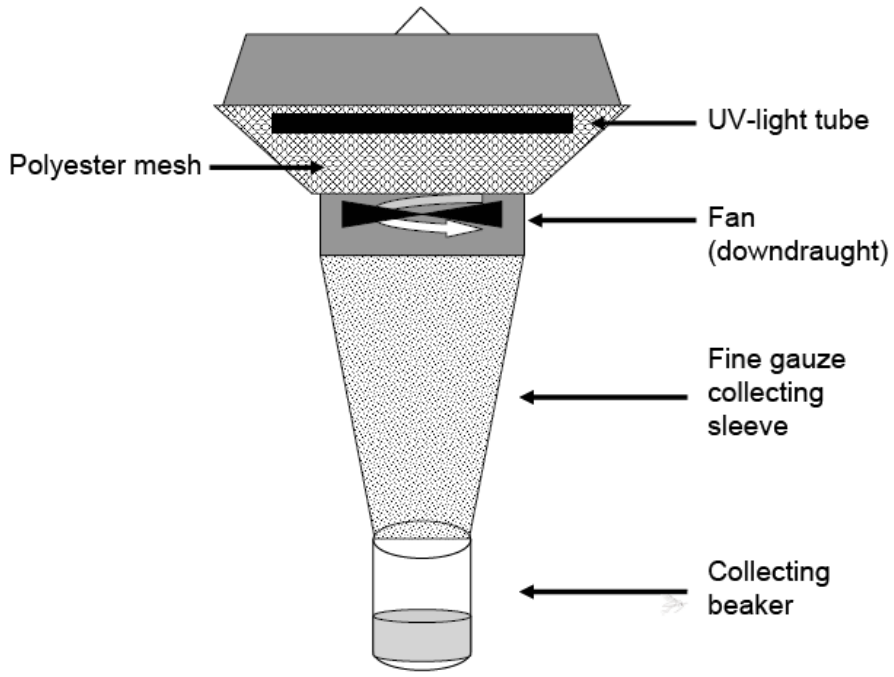

Figure 1. Diagram of the Onderstepoort 220V downdraught black light trap.

While UV-light-baited suction traps are frequently used for collecting midges during epidemiological investigations (Venter et al., 2009), additional sampling methods including CO_2-baited traps and aspiration from sentinel hosts are recommended (Elbers and Meiswinkel, 2014; Scheffer et al., 2012) to better define how midge numbers, species composition and physiological status of light trap catches relate to *Culicoides* species feeding on a natural host (Carpenter et al., 2008b; Viennet et al., 2011). 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; Elbers and Meiswinkel, 2014; Gerry et al., 2009; Kirkeby et al., 2013; Scheffer et al., 2012; Viennet et al., 2012). 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. Besides indicating an over-wintering mechanism for BTV the detection of low numbers of parous female *Culicoides sonorensis* Wirth and Jones during daytime in CDC-type traps baited with dry ice (Mayo et al., 2014) emphasised the importance of use of baited traps without light to investigate midge epidemiology. This was most recently supported by the detection of lower numbers of BTV-infected midges in UV-light traps compared to CO_2-baited traps, suggestive of UV-light aversion in infected midges and indicating underestimation of BTV transmission risk based on using light trap data alone (McDermott et al., 2015).

2.2. *Culicoides* control measures

In addition to the use of vaccines for AHS control, recommended control measures against *Culicoides* midges aimed at reducing the midge attack and biting rate include stabling at night, screening stables with mesh, and the use of repellents or insecticides (Carpenter et al., 2008a; Meiswinkel et al., 2004). Stabling efficacy depends on the exophilic/endophilic behaviour of the vector species (Carpenter et al., 2008a; Viennet et al., 2012), and horses are protected only if the stables are adequately closed (Barnard, 1997; Meiswinkel et al., 2000). Repellents such as DEET (Page et al., 2009), pyrethroids and plant extracts (Braverman and Chizov-Ginzburg, 1998), and organic fatty acids (Venter et al., 2011) have shown efficacy against *Culicoides* midges in light trap studies.

Despite the proven effectiveness (Lengeler, 2004) and widespread use of pyrethroid-treated nets for controlling mosquitoes, the use of pyrethroid-treated mesh for reducing the *Culicoides* biting rate in animals has received limited attention (Calvete et al., 2010; Carpenter et al., 2008a; Del Río et al., 2014a,b.c). In support of further investigation of the insecticidal efficacy of synthetic pyrethroids in horses the susceptibility of colony-reared *Culicoides* (*Monoculicoides*) *nubeculosus* Meigen exposed to hair from horses treated with cypermethrin has been reported (Papadopoulos et al., 2010).

2.2.1. Pyrethrins and synthetic pyrethroid insecticides

Pyrethrins are natural insecticides derived from *Chrysanthemum cinerariaefolium*, while pyrethroid insecticides are synthetic analogues of the pyrethrins. Synthetic compounds were developed to increase insecticidal stability and efficacy (Hansen et al., 1994). Natural pyrethrins and synthetic pyrethroids are lipid-soluble neurotoxicants (Hansen et al., 1994).

Permethrin is a pyrethroid effective as a contact insecticide, causing neurological toxicity resulting in death or knockdown of the target insect (Fradin, 1998).

The mechanism of action of synthetic pyrethroids appears to be interference with sodium channels of parasite nerve axons resulting in delayed repolarisation and paralysis (Taylor, 2001). Type I compounds interfere with the axonal sodium gate and result in delayed repolarisation and repetitive discharge of the nerve whilst Type II compounds also act on the sodium gate, but do so without resulting in repetitive discharge (Taylor, 2001). The insecticidal activity of synthetic pyrethroids may involve both central and peripheral neurons, whilst the knockdown effect is likely due to peripheral neuron effects only (Taylor, 2001). Being highly lipophilic, pyrethroids pass easily through cell membranes and may be absorbed via the skin, inhalation or ingestion (Hansen et al., 1994). Their rapid metabolism, however greatly precludes potential toxicity in humans. Toxicologically these compounds have a useful characteristic, the production of skin paraesthesia, which gives an early indication of accidental exposure (WHOPES, 2003).

Pyrethroids may be applied to mosquito nets or other types of fabric where they act as repellents or insecticides, retain residual activity, are relatively safe to use, are inexpensive and effective at relatively low-concentrations (Barnard, 2000). Pyrethrins and pyrethroid insecticides that have been investigated against *Culicoides* midges in ruminants, include cyfluthrin (Mehlhorn et al., 2008), cypermethrin (Calvete et al., 2010; Papadopoulos et al., 2009; Papadopoulos et al., 2010), deltamethrin (Mullens et al., 2010), fenvalerate (Holbrook, 1986), permethrin (Griffioen et al., 2011; Mullens et al., 2001; Mullens et al., 2000). Permethrin-treated horses with insect bite hypersensitivity showed a 86% positive response in the UK (Stevens et al., 1988) and fewer midges were attracted to horses

treated with topical permethrin in a tent trap study in the Netherlands (de Raat et al., 2008). A recent multicentre investigation of insecticide susceptibility of *Culicoides* midges to synthetic pyrethroids and organophosphates using a modified WHO assay determined that the most toxic insecticides tested were deltamethrin and alphacypermethrin, both synthetic Type II α-cyano pyrethroids (Venail et al., 2015).

Veterinary formulations containing deltamethrin are considered the most effective against *Culicoides* midges by regulatory authorities in the UK at present, however deltamethrin is not currently licensed for use in horses or specifically registered against *Culicoides* midges (DEFRA, 2012). Variable response to deltamethrin was detected in a standardized WHO laboratory assay where susceptibility was higher in colony-reared *C. nubeculosis* than in field populations of *C. obsoletus* or *C. imicola* (Venail et al., 2011). More recently, polyethylene nets impregnated with deltamethrin were evaluated in an adapted WHO laboratory assay and also under field conditions using light traps in Spain, where high (90-100%) mortality was recorded following exposure in the laboratory assay, but no significant reduction in midge numbers was demonstrated under field conditions for a treated trap, despite significantly increased midge mortality observed for the treated trap (Del Río et al., 2014b,c). 'Off-label' topical application of deltamethrin on horses in the UK did not significantly reduce midge biting rates on horses, however biting rates were assessed by Onderstepoort suction light traps operated in the vicinity of the treated horses and not by aspiration which may have confounded the efficacy assessment (Robin et al., 2015).

Cypermethrin is a synthetic pyrethroid insecticide, containing the α-cyano-3-phenoxybenzyl moiety, that has been used as a barrier insect repellent in horses. It is

classified as a moderately toxic material by dermal absorption or ingestion, has no reported adverse effects on reproduction, is not teratogenic, nor mutagenic, is classified as a possible human carcinogen, and may cause adverse effects on the central nervous system (Anon, 1996). Alphacypermethrin (Fendona®) insecticide (APPENDIX B) is recommended by the WHO Pesticide Evaluation Scheme (WHOPES) for treatment of mosquito nets as well as indoor residual spraying for protection against malaria (Banek et al., 2010). Previous human studies have demonstrated the practicality and efficacy of alphacypermethrin when used to treat netting materials (Ansari and Razdan, 2002; 2003). The insecticidal efficacy of alphacypermethrin on hair obtained from treated cattle and wool from treated sheep (Papadopoulos et al., 2009), and cypermethrin on hair from treated horses (Papadopoulos et al., 2010) has been demonstrated *in vitro* against colony-reared *C. nubeculosis* with close to 80% mortality for horse hair 7 days post-treatment.

2.2.2. Insect repellents

Chemical barriers to insects comprise the use of natural and synthetic repellents applied to the skin or to protective screens. The ideal insect repellent would repel multiple species of insects, remain effective for at least eight hours (overnight), cause no irritation, cause no systemic toxicity, be resistant to abrasion and rub-off, and be greaseless and odourless (Fradin, 1998). Direct assessment of efficacy of repellents applied to horses against *Culicoides* species is hampered by the midges' small size and nocturnal behaviour which make observation of repellency difficult. Previous studies have used suction light traps to screen for repellency by comparing numbers of *Culicoides* midges collected in treated and untreated light traps (Braverman and Chizov-Ginzburg, 1998; Braverman et al., 1999; Braverman et al., 2000; Page et al., 2009).

DEET is often used as the “gold standard” in midge repellency trials, however in one study a synthetic pyrethroid, containing a α-cyano-3-phenoxybenzyl compound, was superior to DEET (Braverman and Chizov-Ginzburg, 1998). In a comparable South African field study DEET had a significant repellent effect against *Culicoides* midges and *C. imicola* overnight, while no significant repellent effect was detected for the citronella oil or alphacypermethrin compounds investigated (Page et al., 2009). In a similar repellent study no significant difference was detected between midge counts in light traps treated with a combination of citronella and lemon eucalyptus oils compared to an untreated trap (Venter et al., 2014). Indeed, in this cited study, which was similar to that conducted by Page et al. (2009) a greater number of midges were collected in the citronella-treated light trap, raising the possibility of an attractant effect of citronella on *Culicoides* midges. A similar attractant effect on *Culicoides* midges was reported for a mosquito repellent containing *Eucalyptus* extract in Israel (Braverman et al., 1999). In contrast a mixture of octanoic acid (C8), nonanoic acid (C9) and decanoic acid (Cl0) organic fatty acids applied to polyester mesh resulted in a 1.7 times reduction in the number of *Culicoides* midges collected by the treated Onderstepoort suction light traps with white light compared to untreated control light traps (Venter et al., 2011).

2.2.3. Netting materials or mesh

Whilst no specific guidelines for netting materials for protection against *Culicoides* midges have been published, the following minimum recommendations for mosquito nets are considered relevant: (1) protect against insect/vector entry; (2) be strong and durable; (3) keep its dimensions after washing; and (4) be safe for users. In addition, nets intended for treatment in the field must be able to be treated correctly with an appropriate volume of insecticide formulation diluted in water. The WHO specifications and quality control of netting materials and mosquito nets, includes requirements for mesh count, bursting strength, bursting strength of seams, dimensional stability, durability and storage stability, and other physical characteristics including fabrication method, filament texture and filament count of yarn, linear density, weight of netting material, fire safety, water and insecticide uptake (WHO, 2005).

Adherence to similar requirements is recommended for netting material intended for protection of horses against midges. In some areas increased requirements may be applicable, e.g. a stronger mesh may be required for application to jet stalls to avoid physical damage during the loading and transport process.

In a South African study, closing horse stables with gauze mesh with 1-4 mm hole size resulted in a 14-fold reduction in the numbers of *Culicoides* midges entering (Meiswinkel et al., 2000). In Switzerland, the efficacy of polypropylene netting with hole size of 0.1825 mm^2 was investigated against *Culicoides* midges in three horse housing systems (box, box and paddock and group housing) using light traps for collection of midges (Lincoln et al., 2015). In this study the net protection significantly reduced the midges collected in all three housing systems. Based on the small hole size of the polypropylene netting it was not

considered necessary to apply additional insecticide or repellent to the netting, and no adverse effects related to stall ventilation were reported. One disadvantage of using mesh with a larger hole size is that it may not permit sufficient midge contact with insecticide on the mesh (Del Río et al., 2014a).

2.3. African horse sickness

African horse sickness is an infectious, non-contagious, arthropod vector-borne viral disease of equids caused by infection with AHSV, a non-enveloped RNA virus within the genus *Orbivirus*, family *Reoviridae* (Guthrie and Quan, 2007). The AHSV genome consists of 10 segments of double-stranded RNA that encode seven structural viral proteins (VP1–7) and four non-structural proteins (NS1, NS2, NS3/3A) (Guthrie and Quan, 2007). The outer capsid is composed of VP2 and VP5; the core particle comprises major proteins VP3 and VP7 and minor proteins VP1, VP4 and VP6 (Guthrie and Quan, 2007). Nine serotypes of AHSV have been described (Guthrie and Quan, 2007; Howell, 1962).

Clinical disease is characterised by fever, inappetance, oedema of the subcutaneous and intermuscular tissues, effusion into the body cavities, and haemorrhages particularly affecting the serosal surfaces (Coetzer and Guthrie, 2004). In immunologically naive horses the mortality rate may be over 95%, with donkeys and mules being less susceptible (Coetzer and Guthrie, 2004). The pathogenesis involves endothelial injury and four clinical forms of AHS are described, a peracute pulmonary form, cardiac form, mixed form and horse sickness fever form (Coetzer and Guthrie, 2004). Recently, naturally-infected subclinical cases of AHS were detected in immunised horse by real-time RT-PCR (Weyer

et al., 2013); the role of viraemia detected in these cases in the epidemiology of AHS is unknown.

In South Africa, clinical cases of AHS usually first appear in the north-eastern parts of the country and disease extends southwards with progression depending on the time of year that the first cases occur and the presence of favourable climatic conditions for the breeding of *Culicoides* midges (Coetzer and Guthrie, 2004). The majority of AHS outbreaks occur during the months of March and April, late summer in the southern hemisphere (Coetzer and Guthrie, 2004). Temperatures of 20-22°C and RH between 50-70% has been associated with occurrence of maximum AHS cases in South Africa (Liebenberg et al., 2015).

AHS is a controlled disease in South Africa and is an OIE listed disease (Mellor and Hamblin, 2004). Outside South Africa, AHS has been reported to the OIE since 1993 from Botswana, Burkina Faso, Cape Verde, Cote d'Ivore, Eritrea, Ethiopia, Gambia, Lesotho, Malawi, Mozambique, Namibia, Nigeria, Senegal, Swaziland and Zimbabwe (DEFRA, 2008; Herholz et al., 2008). More recently, outbreaks of AHS have been reported in Senegal in 2007 with AHSV-2 (Diouf et al., 2013; Fall et al., 2015) and in Ethiopia between 2007 and 2010 (Aklilu et al., 2014). The occurrence of AHS in northern Africa (Senegal) and East Africa, including Kenya (Davies et al., 1993), represents a potential risk to horses transhipped by air through these regions, and measures for protection as recommended by the OIE are indicated.

Vaccination is the primary AHS control measure In endemic areas (Coetzer and Guthrie, 2004). A polyvalent, AHS attenuated live virus (ALV) vaccine is commercially available for compulsory vaccination of horses in South Africa. Until 1990 this ALV vaccine comprised two quadrivalent vaccines, bottle one with serotypes -1, -3, -4 and -5 and bottle two with serotypes -2, -6, -7 and -8 (von Teichman et al., 2010). Currently the vaccine consists of a trivalent (serotypes -1, -3, -4) and quadrivalent components serotypes -2, -6, -7 and -8. Serotype 9 was not included due to low regional prevalence in southern Africa. Use of the vaccine strain of AHSV-5 was discontinued in 1990 due to safety concerns (van Dijk, 1998). *In vivo* cross-protection in horses between AHSV6 and AHSV9, and AHSV8 and AHSV5 respectively, has been demonstrated (von Teichman et al., 2010). As future alternatives to ALV vaccines positive preliminary results have been reported in horses with a recombinant canarypox virus vectored vaccine encoding VP2 and VP5 (Guthrie et al., 2009), and a recombinant modified vaccinia Ankaravirus expressing VP2 (Alberca et al., 2014).

2.4. Equine encephalosis

Equine encephalosis is a *Culicoides* vector-borne infectious, non-contagious disease of horses that is caused by EEV, a member of the genus *Orbivirus*, family *Reoviridae* (Howell et al., 2004). As with AHS, disease occurs in late summer and autumn in southern Africa when climatic conditions favour the spread of *Culicoides*-borne viruses (Howell et al., 2004). Cases of ‘‘equine ephemeral fever’’ described by Theiler (1910) are probably the first clinical descriptions of equine encephalosis. Most infected animals are asymptomatic, or only show mild clinical signs characterised by fever, congestion and icterus (Grewar et al., 2015; Howell et al., 2004; Paweska et al., 1999). Other clinical signs include

supraorbital swelling and neurological derangement (Howell et al., 2004). In contrast to AHS, the mortality rate is much lower (Grewar et al., 2015; Howell et al., 2004).

Equine encephalosis virus was first isolated from a mixed pool of *Culicoides* midges (predominantly *C. imicola*) collected between 1968 and 1970 at Onderstepoort (Theodoridis et al., 1979). Serological and virus isolation surveys have indicated that EEV is present throughout South Africa (Paweska et al., 1999; Venter et al., 1999). While both *C. imicola* and *C. bolitinos* can become infected with and permit replication of EEV, oral susceptibility studies indicated that at least four non-*Avaritia* Old World midge species, *C. leucostictus*, *C. magnus*, *C. zuluensis* and *C. onderstepoortensis* may be susceptible to infection and therefore be regarded as potential vectors in the field (Paweska and Venter, 2004; Venter et al., 2002). While EEV was historically thought to be confined to South Africa, an outbreak of febrile disease in horses in Israel in 2008/2009 was linked to a phylogenetically distinct cluster of EEV, with 92% nucleotide identity to the Kaalplaas South African reference strain (Aharonson-Raz et al., 2011). Further evidence suggested that EEV was endemic in Israel, since 2001 at least, and could have originated from Ethiopia, Ghana and the Gambia based on high nucleotide sequence identity (Oura et al., 2012).

As with the risk of introduction of AHSV and BTV, similar climatic and anthropogenic factors may influence the spread of EEV into susceptible equine populations (Purse et al., 2005). In the absence of a vaccine against EEV, vector control measures implemented to limit transmission of AHSV during transhipment of horses will likely also be effective in limiting transmission of other Orbiviruses such as EEV.

2.5. African horse sickness and international export of horses from South Africa

Current OIE conditions for importation of equids from AHS infected countries or zones are specified in the Terrestrial Animal Health Code (World Organisation for Animal Health, 2015) and include the following recommendations relevant to the present studies conducted:

"Recommendations for importation from AHS infected countries or zones

Veterinary Authorities should require the presentation of an international veterinary certificate attesting that the animals:

were held in isolation in a vector-protected establishment

were protected from *Culicoides* attacks at all times during transportation (including transportation to and at the place of shipment)."

"Protecting animals from *Culicoides* attacks

During transportation

When transporting equids through AHS infected countries or *zones*, *Veterinary Authorities* should require strategies to protect *animals* from *Culicoides* attacks during transport, taking into account the local ecology of the *vector*.

a) Transport by road

Potential *risk management* strategies include a combination of:

i) treating *animals* with chemical repellents prior to and during transportation, in sanitized *vehicles* treated with appropriate residual contact insecticide;

ii) *loading*, transporting and *unloading animals* at times of low *vector* activity (i.e. bright sunshine and low temperature);

iii) ensuring *vehicles* do not stop en route during dawn or dusk, or overnight, unless the *animals* are held behind insect proof netting;

iv) darkening the interior of the *vehicle*, for example by covering the roof or sides of *vehicles* with shade cloth;

v) surveillance for *vectors* at common stopping and offloading points to gain information on seasonal variations;

vi) using historical, ongoing or modelling information on AHS to identify low risk ports and transport routes.

b) Transport by air

Prior to *loading* the equids, the crates, *containers* or jet stalls are sprayed with an insecticide approved in the country of dispatch.

Crates, *containers* or jet stalls in which equids are being transported and the cargo hold of the aircraft should be sprayed with an approved insecticide when the doors have been closed and prior to take off. All possible insect harbourage should be treated. The spray containers should be retained for inspection on arrival.

In addition, during any stopover in countries or *zones* not free from AHS, prior to the opening of any aircraft door and until all doors are closed, netting of appropriate gauge impregnated with an approved insecticide should be placed over all crates, *containers* or jet stalls."

A total of 2806 registered horse movements were reported from Africa to Europe between 2004 and June 2007, primarily originating from Egypt and Morocco, with some imports from South Africa (Herholz et al., 2008). To facilitate export of horses to the European Union (EU) from South Africa a protocol was submitted to the European Community

proposing the establishment of an AHS free zone in the Cape Peninsula, an area historically free from AHS (Coetzer and Guthrie, 2004), from horses could be exported under specified conditions. The proposal was accepted by the EU in 1997 (Anon, 1997) and consequently an AHS controlled area was established consisting of the Metropolitan Cape Town AHS free zone within which the vector-protected quarantine facility at Kenilworth Racecourse is located, an AHS surveillance zone (minimum 50 km width) and an AHS protection zone (minimum 100 km width). Vaccination against AHS is compulsory in the protection zone.

Since 1997 periodic outbreaks in the surveillance zone have disrupted trade and currently an alternative approach to facilitate safe export of horses is being explored. This approach includes, amongst other risk reduction measures, horses being pre-loaded into jet stalls inside a vector-protected facility, insecticide-treated mesh protection of the jet stalls, and application of insect repellent to horses.

2.6. Air transportation of horses

The history and veterinary management of horse transport have been reviewed by Leadon (2008) and more recently Padalino (2015) reviewed the effects of transport on equine health and welfare. The risks of equine vector-borne diseases posed by travel to international equine events such as the Olympic games has been reviewed by Herholz et al (2008), including movement data, import and export requirements and vaccination strategies available.

Unit load devices (ULD) are used to transport cargo internationally. Horses are transported by air housed in ULD code HMA-type jet stalls, usually measuring 3.18 x 2.44 x 2.44 m, and capable of transporting three horses per container, on a P6P pallet base. These stalls are suitable for main deck transport on 747F, 767F, 777F, DC-10F, MD-11F aircraft. Certifying authorities for jet stalls include the Federal Aviation Administration and European Aviation Safety Agency. International Air Transport Association (IATA) guidelines for the transportation of horses by air specify container requirements and precautions during loading and transport. These guidelines represent accepted best practice, but are not necessarily based on scientific data (Stewart et al., 2003).

While the frequency of global transport of horses has increased dramatically (Herholz et al., 2008), there is limited published data on jet stall microclimate, stress indicators, or blood parameters related to the welfare of horses during air transport. Leadon et al (1990) reported on environmental, haematological and blood biochemistry changes in 112 horses transported in wooden horse stalls on freight pallets from England to Sydney and the development of respiratory disease (shipping fever) in 6% of the transported horses. The effect of microclimate (temperature and RH) on horses in insect-proof, climate controlled jet stalls during four international flights of 12 to 30 h duration were reported by Thornton (2000) and included assessment of β-endorphin as a stress indicator. In this study highest heart rates were recorded during transitional events e.g. take-off, turbulence and landing, returning to resting rates during level flight, more frequent monitoring of β-endorphin was recommended, and transport in the closed jet stall was not associated with adverse events. Stewart et al (2003) monitored behaviour and heart rate of 16 horses housed in open-topped stalls during seven flights as well as temperature and RH. Similar to

Thornton's (2000) findings, heart rates were higher during transitional events and there were no welfare concerns.

Heart rate, heart rate variability (HRV) and behaviour of horses as indicators of stress during transport were investigated by Munsters et al (2013). Loading into the jet stall was considered the most stressful event with the highest increase in heart rate, and HRV data analysis was not considered useful as a stress indicator in horses transported by air. The relationship between climatic variables at the point of departure on selected blood parameters as a predictor of transport-associated illness of horses travelling between the USA and Amsterdam, and vice versa, was investigated (Maaskant et al., 2013). High ambient temperature and moderate RH at the departure point were associated with elevation of some clinico-pathological variables.

2.7. Faecal glucocorticoid metabolite concentration as a stress indicator in horses

Transport has been deemed a stressful event for horses, based on activation of the hypothalamic-pituitary axis with increased glucocorticoid secretion and changes in heart rate and HRV (Fazio and Ferlazzo, 2003; Fazio et al., 2008, 2013; Schmidt et al., 2010a,b,c). The concentration of glucocorticoid metabolites can be measured in various matrices, and serve as an indicator of physiological stress. Historically, cortisol release in response to transport has been evaluated mainly in blood plasma, however feedback stress reactions associated with repeated venepuncture potentially confound the results and non-invasive alternatives are preferred.

Non-invasive means of assessing transport-associated stress in horses include monitoring of behavioural indices, heart rate and HRV, salivary cortisol and FGM concentrations (Thornton, 2000; Stewart et al., 2003; Schmidt et al., 2010a,b,c; Munsters et al., 2013). Whereas salivary cortisol concentrations reflect acute changes in cortisol release (Peeters et al., 2011; Schmidt et al., 2009), cortisol metabolites in faeces increase approximately 24 h after an increase in blood cortisol concentrations in ponies and mainly reflect prolonged stress (Merl et al., 2000; Schmidt et al., 2010a,b).

Measuring FGM concentrations in horses provides a non-invasive and feedback-free alternative for monitoring adrenocortical function. A group-specific 11-oxoaetiocholanolone enzyme immunoassay (EIA), measuring 11,17-dioxoandrostane (Palme and Möstl, 1997), has been shown to provide valid information in horses (Badenhorst et al., 2015; Merl et al., 2000; Schulman et al., 2014). FGM concentrations have been reported for monitoring adrenocortical response to short, medium, long-distance, and repeated road transport (Schmidt et al., 2010a,b,c), transport and sales consignment (Badenhorst et al., 2015; Schulman et al., 2014).

CHAPTER 3: FIELD AND IN VITRO INSECTICIDAL EFFICACY OF ALPHACYPERMETHRIN-TREATED HIGH DENSITY POLYETHYLENE MESH AGAINST *CULICOIDES* BITING MIDGES IN SOUTH AFRICA[1]

3.1. Summary

The efficacy of untreated and alphacypermethrin-treated HDPE mesh against *Culicoides* biting midges (Diptera: Ceratopogonidae) was determined using Onderstepoort 220V downdraught black light traps and a contact bioassay. Three traps were operated overnight in four replicates of a 3 x 3 randomised Latin square design near horses under South African field conditions. Both the untreated and alphacypermethrin-treated HDPE mesh significantly ($P < 0.05$) reduced the numbers of *Culicoides* midges, predominantly *C. imicola*, collected in the light traps by 4.2 and 7.2 times respectively. A repellent effect of the alphacypermethrin-treated mesh was not confirmed because the number of midges collected in the light traps with untreated and alphacypermethrin-treated HDPE mesh were not significantly different ($P = 0.656$). Bioassay of the insecticidal contact efficacy indicated median *C. imicola* mortality of 100% from 30 and 10 min following exposure to the alphacypermethrin-treated HDPE mesh for 1 or 3 min, respectively. In the bioassay, mortality was significantly higher ($P = 0.016$) at 5 min post exposure in the midges exposed to the alphacypermethrin-treated mesh for 3 min (74.8%) compared to the 1 min exposure group (59.5%). The HDPE mesh could be used to reduce exposure of housed

[1] APPENDIX 3a. Page, P.C., Labuschagne, K., Venter, G.J., Schoeman, J.P., Guthrie, A.J., 2014. Field and *in vitro* insecticidal efficacy of alphacypermethrin-treated high density polyethylene mesh against *Culicoides* biting midges in South Africa. Vet. Parasitol. 203, 184-188.

[2] APPENDIX 3b. Page, P.C., Labuschagne, K., Venter, G.J., Schoeman, J.P., Guthrie, A.J., 2015. Efficacy of

animals to *Culicoides* midges, specifically *C. imicola*, and viruses transmitted by these midges. Mesh treated with alphacypermethrin had the additional benefit of a rapid insecticidal effect on *C. imicola.*

3.2. Introduction

Culicoides biting midges (Diptera: Ceratopogonidae) are of economic and veterinary significance worldwide, primarily due to the Orbiviruses they transmit (Mellor et al., 2000; Meiswinkel et al., 2004). Based on its wide geographical distribution and host preference for bigger mammals, as indicated by the high numbers collected near livestock, *C. imicola* is considered the principal vector of AHSV, EEV and BTV in South Africa (Nevill et al., 1992; Meiswinkel et al., 2004; Paweska and Venter, 2004), and BTV in southern Europe (Mellor, 1992; Mellor et al., 2000).

The spread of BTV in northern Europe and recent outbreaks of a novel orthobunyavirus, Schmallenberg virus (Hoffmann et al., 2012), has demonstrated the devastating effect of these viruses on naïve populations. These outbreaks raised concern over the introduction and spread of other midge-borne viruses, particularly AHSV, and the need for optimised preventative strategies (Backer and Nodelijk, 2011; Carpenter et al., 2008a; Carpenter et al., 2009; MacLachlan and Guthrie, 2010; MacLachlan and Mayo, 2013; Papadopoulos et al., 2010; Robin et al., 2014). Moreover, global expansion of containerised trade, including intercontinental movement of horses (Reiter, 2010), provides potential mechanisms whereby viruses may be introduced (Carpenter et al., 2009; MacLachlan and Guthrie, 2010; de Vos et al., 2012; Napp et al., 2013). Whilst clear recommendations for pre-export quarantine and testing of horses for AHSV have been in place for many years, recent

amendments to the OIE - Terrestrial Animal Health Code have included recommendations that mesh of appropriate gauge, impregnated with an approved insecticide be placed over containers during transport of horses through regions not free of AHSV (Anon, 2013).

In addition to the use of vaccines, recommended control measures against viruses transmitted by *Culicoides* midges include stabling at night, screening of stables with mesh, and the use of effective repellents or insecticides (Meiswinkel et al., 2004; Carpenter et al., 2008a). Stabling efficacy depends on the exophilic/endophilic behaviour of the vector species (Carpenter et al., 2008a; Viennet et al., 2012), and horses are protected only if the stables are adequately closed (Barnard, 1997; Meiswinkel et al., 2000). Repellents such as DEET (Page et al., 2009), pyrethroids and plant extracts (Braverman and Chizov-Ginzburg, 1998), and organic fatty acids (Venter et al., 2011) have shown efficacy against *Culicoides* midges in light trap studies. Recently, insecticidal efficacy against colony-reared *C. nubeculosus* exposed to hair from horses treated with cypermethrin was reported (Papadopoulos et al., 2010). Despite the proven effectiveness (Lengeler, 2004) and widespread use of pyrethroid-treated nets for controlling mosquitoes, the use of pyrethroid-treated mesh for reducing the *Culicoides* biting rate in animals has received limited attention (Carpenter et al., 2008a; Calvete et al., 2010; Del Río et al., 2014a).

The objectives of this study were to determine if alphacypermethrin-treated HDPE mesh applied to light traps will reduce the entry of *Culicoides* midges, particularly *C. imicola*, into the traps. Following on the promising results of Papadopoulos et al. (2010) with cypermethrin and the northern European *C. nubeculosus,* the *in vitro* insecticidal efficacy of alphacypermethrin-treated HDPE mesh against field populations of *C. imicola* was also investigated in a laboratory bioassay. These results may support potential of

alphacypermethrin-treated HDPE nets for protecting livestock against *Culicoides* midges, and be applicable to containerised transport systems of horses.

3.3. Materials and Methods

3.3.1. Light trap assay

The efficacy in reducing numbers of *Culicoides* midges entering a light trap of (1) 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), (2) an identical untreated HDPE mesh and (3) an untreated control black polyester mesh with 2 mm hole size were compared. Comparisons were done overnight for 12 nights in four replicates of a 3 X 3 randomized Latin square design (Snedecor and Cochran, 1980), observer-blinded, field experiment near horses at the Faculty of Veterinary Science, Onderstepoort (25º38'51.42"S, 28º10'45.96"E, 1 238 m above sea level) during late summer between 27 March and 21 April 2011. The meshes replaced the standard white polyester netting around the light source of three 220V down-draught Onderstepoort suction light traps equipped with 8 W, 23 cm black light tubes (Venter et al., 2009) (Figure 1 and 2).

The alphacypermethrin-treated HDPE 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. The mesh was immersed in alphacypermethrin suspension for 30 min, air dried overnight at 20°C and 65% RH, then kept wrapped in tin foil prior to being attached to the light trap with elastic bands. A new mesh was prepared each day for each of the traps. Meshes were removed from the traps 14 h after application.

Alphacypermethrin uptake was quantified by high performance liquid chromatography (HPLC) analysis of a duplicate mesh sample prepared for each replicate. The traps were placed in a row 6.5 m apart to minimise interference between traps (Venter et al., 2012), 2.0 m above the ground, and operated from before sunset (18h00) to after sunrise (06h00). Catches were made into 500 ml plastic beakers containing 200 ml 0.5% Savlon® (Johnson and Johnson, South Africa) and water solution. Overnight insect collections were stored in 70% ethanol prior to the total number of *Culicoides* specimens and the number of *C. imicola* being determined. Climatic variables (outside temperature, RH, wind speed, rainfall) were recorded hourly using a weather station (Vantage Pro2, Davis, USA) and data logger (Weatherlink, Davis, USA).

Figure 2. Onderstepoort suction light trap fitted with alphacypermethrin-treated HDPE mesh.

3.3.2. Contact bioassay

The *in vitro* insecticidal efficacy of meshes against field-collected, nulliparous (unpigmented) female (Dyce, 1969) *C. imicola* was assessed in a contact bioassay conducted between 23 and 27 April 2012. The midges had been collected the previous night in Onderstepoort light traps at the ARC-Onderstepoort Veterinary Institute (25°39'S, 28°11'E; 1 219 m above sea level) and were handled prior to the assay as previously described (Venter et al., 1998). In the absence of laboratory colonies to standardise the physiological age of the midges only nulliparous *C. imicola* were used in the assays. Midges were immobilised for 1 min at -4°C and groups of 10 nulliparous females aspirated into glass Pasteur pipettes on a refrigerated chill table (Figure 3 and 4). At the start of each exposure experiment, the 10 nulliparous females were expelled into one of 60 x 12 mm glass petri dishes (Anumbra, Lasec, South Africa) and exposed to a 19.6 cm^2 alphacypermethrin-treated HDPE mesh (prepared as described for the light trap assay) (Figure 5) for 1 or 3 min, or an untreated control HDPE mesh for 3 min. The meshes were attached to the petri dish by a small disc of adhesive putty (Prestik®, Bostik, South Africa). To maximise contact with the mesh the dishes were gently inverted every 30 sec. Following the exposure period midges were immobilised for 1 min at -4°C and the petri dish cover with the attached mesh was replaced with a clean cover to allow the midges to recover without residual exposure to insecticide (Figure 6). Mortalities were visually assessed at 5, 10, 30, 60 min and 24 h post exposure to the mesh by an entomologist blinded to the treatment group. Midges that were motionless or twitching and incapable of oriented movement were classified as dead. Following the 60 min assessment midges were maintained overnight at 24.5°C and 60% RH until the final efficacy assessment at 24 h. Thirty replicates of each exposure period were conducted.

Figure 3. Chill table for sorting of *Culicoides* midges.

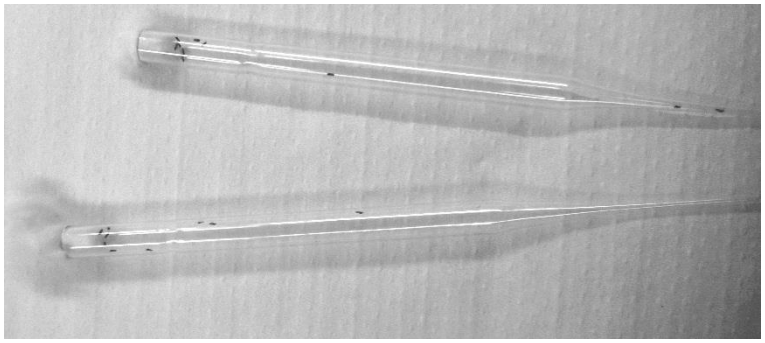

Figure 4. Nulliparous *C. imicola* aspirated into Pasteur pipettes.

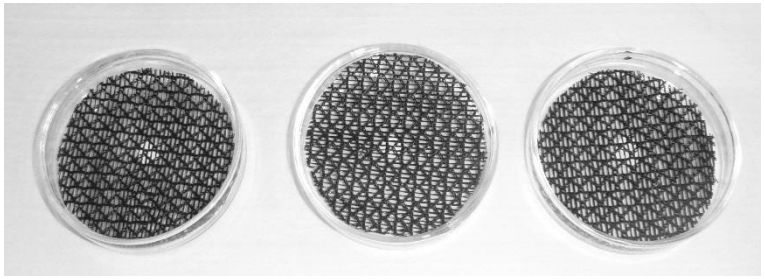

Figure 5. Petri dishes with alphacypermethrin-treated HDPE mesh and untreated control HDPE mesh disc prior to midge exposure.

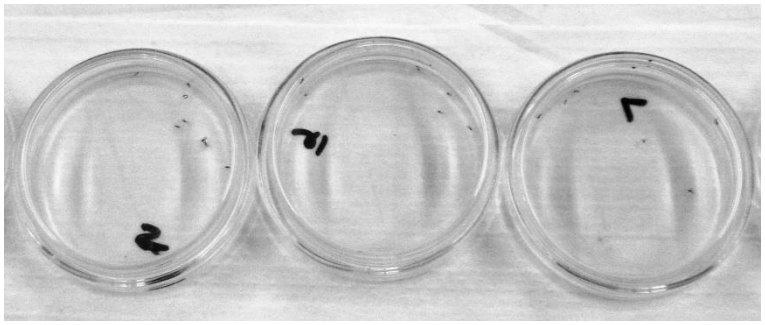

Figure 6. Petri dishes containing *Culicoides* midges for mortality assessment following removal of the HDPE mesh discs.

3.3.3. Statistical analyses

Statistical analyses were done using SPSS® Statistics version 21 (IBM, USA). For the light trap assay midge numbers were natural logarithm-transformed to achieve normality. Mean numbers of *Culicoides* midges and *C. imicola* were compared between treatment groups while controlling for the effects of light trap location and day using ANOVA. Where *F*-ratios were significant, pairwise comparisons were done between treatment groups using Tukey's HSD test. Homogeneity of variances was tested with Levene's test. Statistical testing was conducted at the 5% level of significance.

Contact bioassay midge mortality, corrected for mortality in the untreated control group with Abbott's formula [Mortality % = 100 x (X-Y)/ X; where X = % survival in the untreated control, Y = % survival in the treated sample] (Abbott, 1925), was compared between groups at each time point 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.

3.4. Results

3.4.1. Light trap assay

A total of 37 221 *Culicoides* midges were collected in 36 collections made over 12 nights from three light traps operated simultaneously. *Culicoides imicola* was the most abundant species and comprised 96.1% of midges collected. The mean number of both *Culicoides* midges and *C. imicola* collected with the control trap were significantly higher than the alphacypermethrin-treated HDPE mesh ($P = 0.001$ for both) and the untreated HDPE

mesh light trap ($P < 0.05$ for both) (Table 1) (Figure 7, 8). The smaller total number of *Culicoides* or *C. imicola* collected with the alphacypermethrin-treated HDPE mesh was not significantly different ($P = 0.656$) from that of the untreated HDPE mesh. The proportion of *C. imicola* in relation to the other species collected ranged from 97.1% with the untreated HDPE mesh to 95.9% in the control trap and was not significantly different between treatments ($X^2 = 1.717$, d.f. = 2, $P = 0.424$) (Table 1).

The mean ± s.d. alphacypermethrin uptake by the treated HDPE meshes as determined by HPLC analysis was 36.3 ± 12.3 mg/m^2, within the target range of 20-40 mg/m^2. The mean ± s.d. outside temperature, RH, wind speed, and rain during the light trap collection were 18.9 ± 2.2°C, 79.7 ± 7.2%, 1 ± 0.4 km/h, and 7.2 ± 16.4 mm, respectively.

Table 1. Mean ± s.e. number of *Culicoides* midges and *C. imicola* collected by three black light traps fitted with untreated HDPE mesh, alphacypermethrin-treated (36.3 ± 6.1 mg/m^2) HDPE mesh, or untreated control polyester mesh, operated overnight for 12 nights between 27 March and 21 April 2011.

	Mesh treatment		
	Untreated HDPE mesh	Alphacypermethrin-treated HDPE mesh	Untreated control polyester mesh
Culicoides midges	539 ± 218^a	314 ± 102^a	2 249 ± 763^b
C. imicola	523 ± 214^a	303 ± 99^a	2 156 ± 743^b
% *C. imicola*	97.1	96.3	95.9

Values within each row with a different superscript differ significantly ($P < 0.05$).

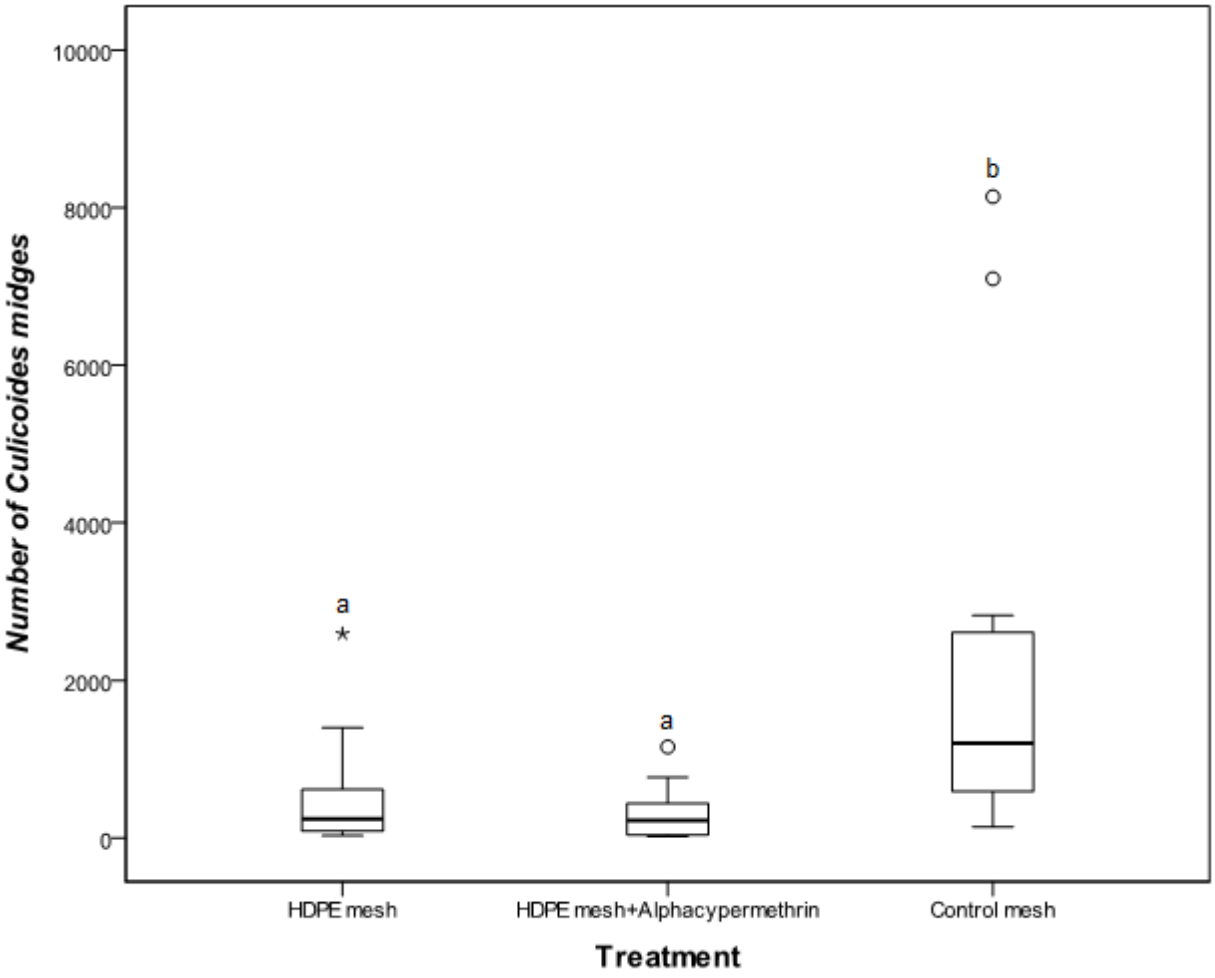

Figure 7. Boxplot of number of *Culicoides* midges collected by three black light traps fitted with untreated HDPE mesh, alphacypermethrin-treated (36.3 ± 6.1 mg/m^2) HDPE mesh, or untreated control polyester mesh, operated overnight for 12 nights between 27 March and 21 April 2011. Horizontal line indicates median, box indicates interquartile range, bars indicate the range, o = outliers; * = extreme outliers. Treatment categories with a different lower case letter differ significantly ($P < 0.05$).

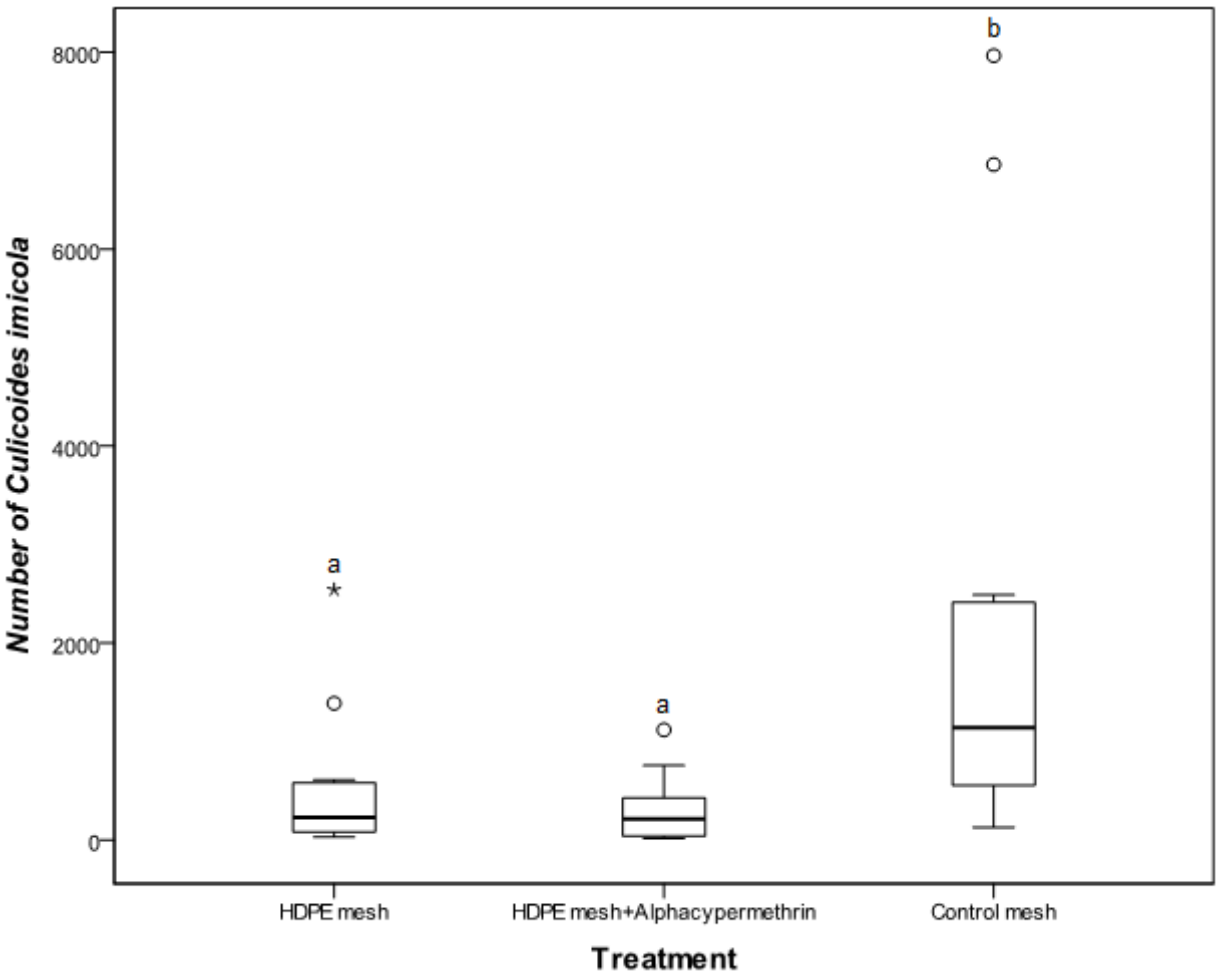

Figure 8. Boxplot of number of *C. imicola* collected by three black light traps fitted with untreated HDPE mesh, alphacypermethrin-treated (36.3 ± 6.1 mg/m^2) HDPE mesh, or untreated control polyester mesh, operated overnight for 12 nights between 27 March and 21 April 2011. Horizontal line indicates median, box indicates interquartile range, bars indicate the range, o = outliers; * = extreme outliers. Treatment categories with a different lower case letter differ significantly (P < 0.05).

3.4.2. Contact bioassay

The median percentage efficacy of the alphacypermethrin-treated HDPE mesh in both the 1 min and 3 min exposure groups was significantly ($P < 0.001$) higher than the untreated control HDPE mesh at all the time points tested (Table 2). Median *C. imicola* mortality was 100% from 30 and 10 min following exposure to the alphacypermethrin-treated HDPE mesh of 1 and 3 min, respectively. The median mortality was significantly higher ($P = 0.016$) in the 3 min alphacypermethrin exposure group (74.8%) compared to the 1 min exposure group (59.5%) at the 5 min time point only.

Table 2. Median (IQR) percentage mortality of field-collected nulliparous (unpigmented), female *Culicoides imicola* exposed to alphacypermethrin-treated HDPE mesh for 1 or 3 min, or to an untreated control HDPE mesh for 3 min in a contact bioassay. Values for the alphacypermethrin-treated HDPE mesh exposure have been corrected with Abbott's formula. Thirty replicates of each exposure period were conducted between 23 and 27 April 2012.

Time post exposure	Exposure		
	Alphacypermethrin-treated HDPE mesh		Untreated control
	1 min	3 min	3 min
5 min	59.5 ^{a*}(35.6-80.3)	74.8 ^{b*} (62.8-100)	11.1 ^{c} (0.0-29.8)
10 min	84.7 ^{a} (72.0-100)	100 ^{a} (82.5-100)	12.5 ^{b} (10.0-29.8)
30 min	100 ^{a} (100-100)	100 ^{a} (100-100)	11.1 ^{b} (0.0-38.1)
60 min	100 ^{a} (100-100)	100 ^{a} (100-100)	20.0 ^{b} (10.0-29.8)
24 h	100 ^{a} (100-100)	100 ^{a} (100-100)	57.1 ^{b} (36.5-77.8)

Values within each row with a different superscript differ significantly ($P < 0.001$).
* indicates $P = 0.016$.

3.5. Discussion

The HDPE mesh had a significant effect in reducing the numbers of *Culicoides* midges, predominantly *C. imicola*, collected by the light trap. The magnitude of reduction for the untreated HDPE mesh and alphacypermethrin-treated HDPE mesh was 4.2 and 7.2 times, respectively, with efficacy likely related to the smaller hole size of the HDPE mesh, compared to the untreated control polyester mesh. Whilst the light trap with the alphacypermethrin-treated mesh consistently collected fewer *Culicoides* midges than the untreated HDPE mesh, a significant repellent effect was not demonstrated. This is in agreement with a previous study at Onderstepoort where no repellent effect against *Culicoides* midges was demonstrated for a different alphacypermethrin formulation tested at a lower concentration (0.3%) (Page et al., 2009). However, a limitation of using light traps alone to screen mesh for repellent and insecticidal efficacy against *Culicoides* midges is that the relative strong attractant effect of the light may override any repellent effect (Page et al., 2009; Venter et al., 2009) and a large mesh hole size may not allow sufficient contact with the insecticide (Del Río et al., 2014a). Paradoxically, a potential benefit of the lack of a repellent effect of alphacypermethrin against *Culicoides* midges is that it could increase (or at least not decrease) the numbers of midges coming into direct contact with treated mesh, and thereby promote insecticidal efficacy.

Exposure of *C. imicola* to the alphacypermethrin-treated HDPE mesh in the contact bioassay resulted in a rapid insecticidal effect. The initial magnitude and rate of inducing mortality was greater after 3 min exposure compared to 1 min exposure of midges to the mesh. Subsequently, at 30 min post exposure both groups had reached maximal effect which was maintained up to the final assessment at 24 h. A similar contact assay and

exposure period to that reported by Papadopoulas et al. (2010) was used. The aim of the present study was, however, to assess the potential of alphacypermethrin-treated meshes for protecting containerised transport systems of horses, and not to screen the potential for direct treatment of horses with alphacypermethrin. Therefore it was elected to use a treated mesh, instead of hair clippings, for exposure of midges. Although the contact bioassay was done with nulliparous females, the results would not be expected to be different for parous females or males, however this was not investigated. As male midges are not generally abundant in light trap catches near hosts (Venter et al., 2009) the light trap results could vary for males.

Although mortality in the bioassay treated groups was consistently significantly higher than that of the control group, a study limitation was the relatively high mortality (up to 57% after 24 h) recorded in the control group. This was likely related to the use of field-collected midges and experimental handling procedures. Dehydration during the holding period and the effect of chilling (Nunamaker et al., 1996) as an immobilising method may have contributed to the high mortality in the control group. Improved holding conditions and alternate immobilisation methods such as CO_2 (Meyer and Schmidtmann, 1979) need to be evaluated for use in future insecticide bioassays against *Culicoides* midges.

The alphacypermethrin formulation used is recommended by WHOPES for treatment of mosquito nets as well as indoor residual spraying (Banek et al., 2010) and efficacy and safety has been demonstrated for malaria control (Ansari and Razdan, 2002; 2003). However, welfare concerns associated with reduction of airflow and animal confinement, as well as practicality restricts the use of completely enclosed structures for housing livestock species and combinations of protective measures against *Culicoides* midges may

therefore be required (Calvete et al., 2010). On the other hand, in horses (which are generally more accustomed to stabling) the use of complete barriers may be more feasible, providing that ventilation is not compromised. For example, a South African field study found that closing stables by meshing with gauze cloth resulted in a 14-fold reduction in the numbers of *C. imicola* and *C. bolitinos* entering the stables (Meiswinkel et al., 2000).

In conclusion, the worldwide increase in the transportation of animals and products in freight containers may increase the risk of viruses being transported (Reiter, 2010). Whilst the available data on *Culicoides* midge involvement in vector-borne pathogen importation via transport networks is limited (Carpenter et al., 2009; Napp et al., 2013), standardised measures such as those recommended by the OIE for AHSV (Anon, 2013) should be in place to ensure the safe transportation of animals, especially if these animals are being moved through known infected areas or areas with unknown risk. Based on the positive results of the present study, HDPE mesh has potential to reduce exposure of housed horses to *Culicoides* midges, specifically *C. imicola*, during high risk periods for AHSV transmission, or during containerised transport. Additionally, treating this mesh with alphacypermethrin may increase the overall field efficacy thereof in reducing *Culicoides* midge biting rates. Meanwhile, further investigation of the specific mesh in reducing midge biting rate under field conditions and the effect of the mesh on ventilation of housing is required.

CHAPTER 4: EFFICACY OF ALPHACYPERMETHRIN-TREATED HIGH DENSITY POLYETHYLENE MESH APPLIED TO JET STALLS HOUSING HORSES AGAINST *CULICOIDES* BITING MIDGES IN SOUTH AFRICA[2]

4.1. Summary

The efficacy of alphacypermethrin-treated 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 *C. imicola*, 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.

[2] APPENDIX 3b. Page, P.C., Labuschagne, K., Venter, G.J., Schoeman, J.P., Guthrie, A.J., 2015. Efficacy of alphacypermethrin-treated high density polyethylene mesh applied to jet stalls housing horses against *Culicoides* biting midges in South Africa. Vet. Parasitol. 210, 84-90.

4.2. Introduction

Culicoides imicola and *C. bolitinos* are considered the principal vectors of AHSV and EEV in South Africa (Nevill et al., 1992; Venter et al., 2002; Meiswinkel et al., 2004; Paweska and Venter, 2004). They are the dominant species mechanically aspirated from horses at Onderstepoort, South Africa (Scheffer et al., 2012).

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 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 (Anon, 2013). The efficacy of alphacypermethrin-treated HDPE mesh as a physical barrier against *Culicoides* midges, and the insecticidal efficacy against field-collected, nulliparous *C. imicola* was demonstrated using light traps and a bioassay (Chapter 3, APPENDIX 3a). These positive study findings supported further investigation of the mesh for protecting horses against *Culicoides* midges under field conditions

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.

4.3. Materials and Methods

4.3.1. 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 (Figure 9), located 8.5 m apart to limit interference 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 Onderstepoort 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 (Figure 10 and 11). 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, RH, 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.

4.3.2. 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% RH. 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 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.

Figure 9. Jet stall protected with alphacypermethrin-treated HDPE mesh (a) and untreated jet stall (b).

Figure 10. Horses in outer compartments of jet stall prior to stall closure.

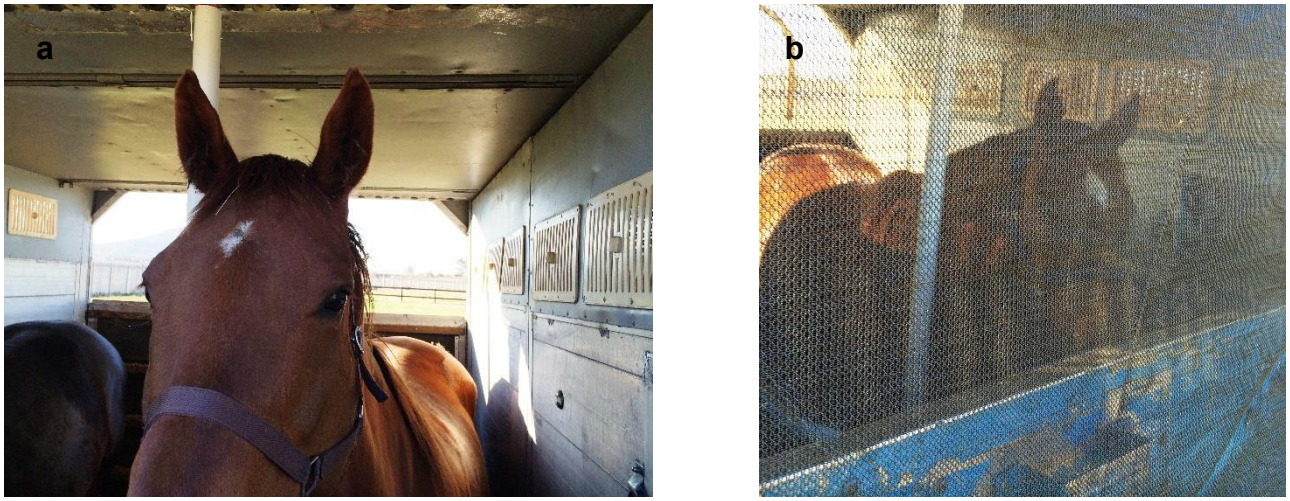

Figure 11. Horse inside an untreated (a) and a treated jet stall (b).

4.3.3. 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) (Figure 12). 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.

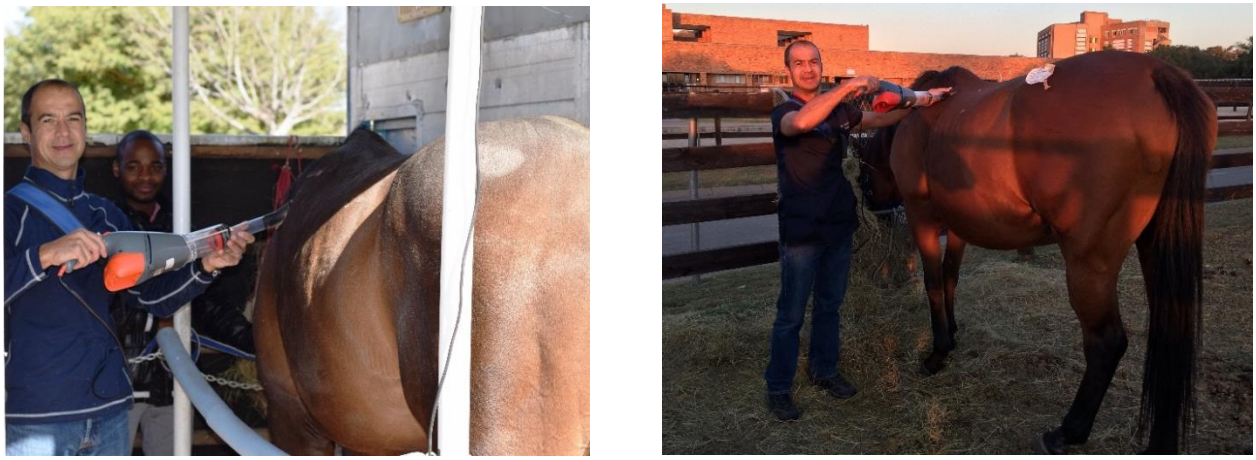

Figure 12. Mechanical aspiration of midges from a horse inside a jet stall (a) and sentinel horse (b).

4.3.4. Light trap collection of midges

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.

4.3.5. *Culicoides* midge identification

Insects collected were stored in 70% ethanol prior to *Culicoides* midge segregation according to species morphology (unpublished wing pattern keys, Parasites, Vectors, and Vector Borne Diseases, Agricultural Research Council-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).

4.3.6. 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 (*H'*) (Al Young Studios, 2012).

4.4. Results

4.4.1. 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. *Culicoides. imicola* was the dominant species and comprised 96.6% of midges aspirated, followed by *C. bolitinos* (3.2%) (Table 3). One other species, *C. magnus,* was aspirated. The majority of *C. imicola* aspirated were nulliparous (70.3%), followed by parous, blood fed (Figure 13) and gravid females, with no males (Table 4). 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 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 4).

Figure 13. Blood-fed *Culicoides* biting midge.

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 4) (Figure 14). The *Culicoides* midge biting rate on horses housed in both the treated and untreated stalls was zero, compared to a 0.1/ min biting rate on the sentinel horse (Table 4). 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 4).

Table 3. Summary of *Culicoides* species collected by mechanical aspiration from horses and in 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)	36,075 (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	36,412

Table 4. *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	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	-
	H'	-		0.1		0.3	

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

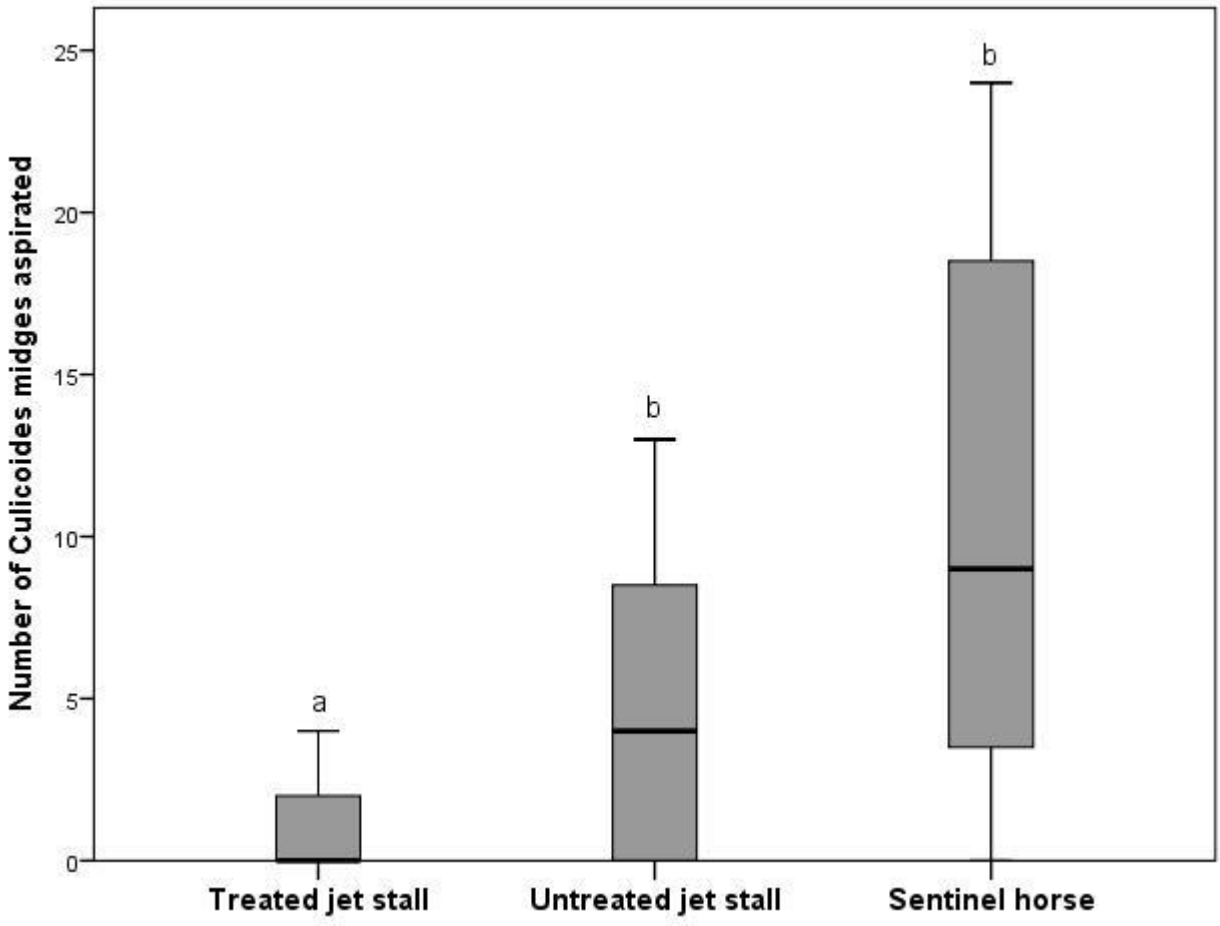

Figure 14. Boxplot of number of *Culicoides* midges collected during 5 min of mechanical aspiration of two horses housed inside a jet stall with alphacypermethrin-treated (31.8 – 33.7 mg/m^2) HDPE mesh, two horses housed inside an untreated jet stall and a sentinel horse, around sunset for 12 nights between 22 May and 5 June 2014. Median numbers of *Culicoides* midges within each group with a different superscript differ significantly ($P < 0.05$).

4.4.2. 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. *Culicoides 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 3). 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 5).

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 5). 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 5).

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, RH, 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.

Table 5. *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
	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	-
	Mean ± S.E.	1433 ± 623	-	1602 ± 653	-
	H'	0.1	-	0.1	-

4.5. 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 zero 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; Elbers and Meiswinkel, 2014; Gerry et al., 2009; Kirkeby et al., 2013; Scheffer et al., 2012; Viennet et al., 2012). 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 (Anon, 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 (Chapter 3, APPENDIX 3a). 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 (Chapter 3, APPENDIX 3a), 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 (Chapter 3, APPENDIX 3a), 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 WHOPES 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, 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 (Anon, 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.

CHAPTER 5: THE EFFECT OF ALPHACYPERMETHRIN-TREATED HIGH DENSITY POLYETHYLENE MESH PROTECTION ON JET STALL MICROCLIMATE, CLINICAL VARIABLES AND FAECAL GLUCOCORTICOID METABOLITES OF HORSES

5.1. Summary

African horse sickness is of importance to health and international trade in horses worldwide. During export from and transit through AHS endemic countries or zones, physical and chemical measures to protect horses from AHS vectors are recommended by the OIE. Protection of containerized air transport systems for horses (jet stalls) with alphacypermethrin insecticide-treated high density polyethylene mesh is effective in reducing the *Culicoides* midge vector attack rate. In order to determine the effect of this mesh on jet stall ventilation and horse welfare under temperate climatic conditions, jet stall microclimate, clinical variables and FGM of 12 horses were monitored during overnight housing in either a treated or untreated stall in two blocks of a 2 x 3 randomized crossover design. Temperature difference between the treated stall and outside differed significantly from the difference between the untreated stall and outside at 1/15 time points ($p = 0.045$, $r = 0.70$). Relative humidity difference between the treated stall and outside did not differ from the difference between the untreated stall and outside. Temperature and RH in the treated stall were highly and significantly correlated with outside temperature ($r = 0.961$, $p < 0.001$) and RH ($r = 0.954$, $p < 0.001$), respectively. No significant differences were detected between rectal temperatures, pulse and respiratory rates of horses in the treated stall compared to the untreated stall. Mean FGM concentrations for horses housed in the treated stall peaked earlier (24 h) and at a higher concentration than horses housed in the

untreated stall (48 h), but were not significantly different from baseline. No significant difference was detected in FGM concentrations when the treated and untreated stall groups were compared at individual time points up to 72 h after exiting the jet stall. The results of this study show that alphacypermethrin-treated HDPE mesh can be used under temperate climatic conditions to protect horses in jet stalls against AHS vectors without compromising jet stall microclimate and horse welfare.

5.2. Introduction

African horse sickness is an infectious, non-contagious, arthropod-borne viral disease that is of importance to health and international trade in equids (Coetzer and Guthrie, 2004; MacLachlan and Guthrie, 2010). Intercontinental trade is a potential route whereby viruses such as AHSV may be introduced into equid populations, either via movement of infected hosts or *Culicoides* midge vectors (Carpenter et al., 2009; MacLachlan and Guthrie, 2010; Reiter, 2010; de Vos et al., 2012; Napp et al., 2013).

In order to reduce risk of AHSV transmission during export from or transit through AHS endemic countries or zones, measures of a physical and chemical nature have been recommended by the OIE to protect horses against AHS vectors (Anon, 2013). Alphacypermethrin insecticide-treated HDPE mesh had a rapid insecticidal effect against *Culicoides* midges (Chapter 3, APPENDIX 3a) and was shown to significantly reduce the midge attack rate on horses housed in jet stalls (Chapter 4, APPENDIX 3b).

While horses are frequently transported by air in jet stalls, there is limited published data on jet stall microclimate, stress indicators, or blood parameters related to the welfare of horses during air transport (Leadon et al., 1990; Maaskant et al., 2013; Munsters et al.,

2013; Stewart et al., 2003; Thornton, 2000). Stall environment and microclimate affects equine respiratory health in particular during air transport (Leadon et al., 2008). There are currently no reports on the influence of effective physical or chemical vector protection measures, such as alphacypermethrin insecticide-treated HDPE mesh or polypropylene mesh (Lincoln et al., 2015), applied to jet stalls on stall microclimate or stress indicators of horses.

Non-invasive means of assessing transport-associated stress in horses include monitoring of behavioural indices, heart rate and heart rate variability, salivary cortisol and FGM concentrations (Thornton, 2000; Stewart et al., 2003; Schmidt et al., 2010a,b,c; Munsters et al., 2013). Whereas salivary cortisol concentrations reflect acute changes in cortisol release (Schmidt et al., 2009; Peeters et al., 2011), cortisol metabolites in faeces increase approximately 24 h after an increase in blood cortisol concentrations in ponies (Palme et al., 1996) and mainly reflect prolonged stress (Merl et al., 2000; Schmidt et al., 2010a,b). FGM concentrations have been reported for monitoring adrenocortical response to short, medium, long-distance, and repeated road transport (Schmidt et al., 2010a,b,c), transport and sales consignment (Badenhorst et al., 2015; Schulman et al., 2014), but not in response to jet stall housing or air transport of horses.

The aims of the study were to determine the effect of alphacypermethrin insecticide-treated HDPE mesh applied to commercial, HMA-type jet stalls on jet stall microclimate and FGM concentrations of horses housed under stationary jet stall, temperate climatic conditions.

5.3. Materials and Methods

5.3.1. Animals

Twelve clinically healthy, adult horses (six geldings and six mares) of mixed breed (eight Thoroughbred, two Basuto, two Boerperd) with mean (range) age 11 (5–20) years, and body mass 482 (404–584) kg were included in the study (Table 6). The horses were resident at the study site as part of the research herd. Other than being led into the stalls for subjective behaviour assessment one week prior to the start of the study, horses were not acclimatised to the stalls. Grass and alfalfa hay was fed *ad libitum* during data collection, and water was provided in buckets every 4 h. Wood shavings were used as bedding in the stalls. During overnight data collection herd mates were kept in adjacent paddocks in direct view of the front of the jet stalls to reduce group separation stress. The study horses were kept grouped with their herd mates in an adjacent grassed paddock during the day. No non-routine activities were conducted with the horses from 1 week before until study completion. The study was approved by the Animal Ethics Committee of the University of Pretoria (Study V011-14).

Table 6. Signalment of horses enrolled in the study.

Gender	Breed	Age (years)	Mass (kg)
Female	Thoroughbred	5	463
Female	Thoroughbred	7	491
Female	Thoroughbred	20	500
Female	Thoroughbred	5	550
Female	Thoroughbred	6	558
Female	Thoroughbred	13	584
Male	Basotho	13	404
Male	Basotho	14	404
Male	Thoroughbred	6	421
Male	Boerperd	9	440
Male	Thoroughbred	20	444
Male	Boerperd	13	520

5.3.2. Study design

A randomised, crossover experimental study was performed from 28 to 30 April 2014 and from 5 to 7 May 2014 (late summer in the southern hemisphere). Horses were housed between 16h00 and 06h00 in two jet stalls, located 8.5 m apart in a grass paddock (58 m x 69 m), at the Faculty of Veterinary Science, Onderstepoort (25º38'51.42"S, 28º10'45.96"E, 1 238 m above sea level).

The horses were grouped according to gender and ranked according to body weight, then assigned to same gender pairs to ensure comparable horse body weight housed in each stall each night. One of each gender pair of horses was randomly assigned to either a treated or untreated stall and monitored overnight in two experimental blocks of a 2 x 3 design. The first block consisted of three consecutive nights followed by a five day rest period. Treatments were then crossed over for the stalls and three consecutive nights of data collection for the second block conducted in the same sequence. Horses were therefore housed in the same gender pair and stall compartment for each block, but with the treatment crossed over.

5.3.3. Jet stalls

Two commercial, non-collapsible, Federal Aviation Administration approved (15.5 m^3, length 3.18 m, width 2.44 m, height 2.44 m, tare weight 900 kg, maximum gross weight 6 804 kg) HMA-type jet stalls (KLM, European Horse Services, Belgium) were used. The inside of the stall had 3 adjacent compartments for housing horses, with a separate grooms' compartment in the front for monitoring, feeding and providing water. During data collection horses were housed in the outer compartment of each stall with the middle

compartment left unoccupied to facilitate access for clinical monitoring. When closed each stall had a 1.7 m^2 rectangular opening above the front and rear ramps for ventilation.

5.3.4. Jet stall treatment

One stall was treated with a black, 400 denier, knitted monofilament HDPE mesh (RK02 70% Shade Cloth, Alnet, South Africa) with 0.3 mm hole size, impregnated with 20-40 mg/m^2 alphacypermethrin (Fendona®6, BASF Agro BV Arnhem, Switzerland) for protection against *Culicoides* midges, as previously described (Chapter 4, APPENDIX 3b). The mesh was custom-made to fit over the stall in a tent-like fashion, with zip connectors for access located at the end panels. When fitted, the mesh covered the ventilation openings of the stall. No mesh was applied to the untreated stall.

5.3.5. Clinical variables

The horses' habitus, appetite and vital signs (rectal temperature, pulse and respiratory rates) were recorded before entering the jet stall (16h00), and every 4 h while housed inside the stall.

5.3.6. Climatic variables

Microclimate (temperature and RH) inside each stall was monitored hourly with data loggers (iButton® DS1923, Fairbridge Technologies CC, Wendywood, South Africa) secured 1 m above the floor in the grooms' compartment. Outside temperature and RH were monitored on a logger located 10 m away from the stalls. Data were downloaded with commercial software (Coldchain Thermo Dynamics Software, Fairbridge Technologies CC, Wendywood, South Africa) at the end of each treatment block. Additional outside climatic

variables (wind speed, rainfall) were recorded hourly using a weather station and data logger (Vantage Pro2 and Weatherlink, Davis, USA) located in an adjacent paddock.

5.3.7. Faecal sample collection and analysis

Faecal samples were collected before entry into the jet stall (Baseline), 24, 48 and 72 h after exiting the jet stall. Faecal samples were collected by manual extraction from the rectum using a lubricated, plastic rectal glove. The faecal material was transferred into a 25 ml plastic specimen container, frozen within 4 h of collection, and kept at -20˚C until hormone extraction and assay.

Faecal samples were lyophilized, pulverized, and sifted (Ganswindt et al., 2010). Thereafter, faecal extracts were prepared and FGM hormone analysis performed as previously described using a group-specific 11-oxoaetiocholanolone enzyme EIA, measuring 11,17-dioxoandrostane (Palme and Möstl, 1997), shown to provide valid information on adrenocortical function in horses (Badenhorst et al., 2015; Merl et al., 2000; Schulman et al., 2014). Serial dilutions of faecal extracts produced displacement curves parallel to the standard curve of the assay. Intra- and inter-assay coefficients of variation, determined by repeated measurement of high- and low-concentration quality controls, ranged between 1.9 - 6.6% and 6.2 - 10.2%, respectively. Cross-reactivity of the antibody and the assay was as previously reported (Ganswindt et al., 2002; Palme and Möstl, 1997).

5.3.8. Statistical analyses

Statistical analyses were performed with SPSS® Statistics (SPSS® version 23, IBM, USA). Data were assessed for normality by evaluating descriptive statistics, plotting histograms and performing the Kolmogorov-Smirnov test. All statistical tests were two-tailed at the 5% level of significance.

Differences between treated and untreated jet stall and outside climatic variables were computed and compared at hourly time points by Kruskal Wallis test. Where the test statistic was significant pairwise comparisons were conducted with p-values adjusted for multiple comparisons. Effect sizes ($r = z/\sqrt{n}$) were calculated for pairwise comparisons. Pearson's correlation coefficient was calculated for the relationship between jet stall microclimate and outside climatic variables. Clinical variables (rectal temperature, pulse, respiration) were compared within each treatment group with Friedman's ANOVA. Where the test statistic was significant pairwise comparisons were conducted with p-values adjusted for multiple comparisons. Wilcoxon signed-rank tests were used to compare clinical variables between horses housed in the treated stall versus the untreated stall.

Natural-log-transformed FGM concentrations were used for data analysis. FGM data within each treatment group were compared with one-way repeated measures ANOVA with *post hoc* Bonferroni adjustment of p-values for pairwise comparisons. The assumption of sphericity was confirmed with Mauchly's test. Comparisons of FGM data between treatment groups were performed using paired samples t-tests.

5.4. Results

5.4.1. Climatic variables

Temperature and RH (mean ± s.e.) recorded in the treated stall, untreated stall and outside were 11.5 ± 1.5°C, 9.2 ± 1.5°C, 6.0 ± 1.8°C and 77.6 ± 2.6%, 85.8 ± 2.1% and 96.1 ± 3.4%, respectively. Mean ± s.e. temperature and RH at hourly time points are shown in Figure 15 and 16, respectively. Mean (range) outside wind speed during the data collection period was 0.2 km/h (0-0.3 km/h) and no rain was recorded.

Temperature difference (median [IQR]) between the treated stall and outside (6.1°C [4.1-7.1°C]) differed significantly from the difference between the untreated stall and outside (3.6°C [2.0-4.1°C]) at 1/15 time points (P = 0.045, r = 0.70), while temperature difference between the treated stall and outside differed significantly from the difference between the treated stall and untreated stall (2.5°C [1.5-3.0°C]) at 13/15 time points (P = 0.001 – 0.041, r = 0.71 – 1.06) (Figure 17).

Relative humidity difference between the treated stall and outside (12.5% [5.1-19.3%]) did not differ from the difference between the untreated stall and outside (8.0% [4.1-13.5%]) at any time point, while RH difference between the treated stall and outside differed significantly from the difference between the treated stall and untreated stall (4.8% [2.3-7.7%]) at 4/15 time points (P = 0.005 – 0.033, r = 0.73 – 0.91), and RH difference between the untreated stall and outside differed significantly from the difference between the treated stall and untreated stall at 1/15 time points (P = 0.033, r = 0.73) (Figure 18).

Temperature in the treated stall (r = 0.961, n = 90, $P < 0.001$) and untreated stall (r = 0.988, n = 90, $P < 0.001$) were highly and significantly correlated with outside temperature (Figure 19 A, B). RH in the treated stall (r = 0.954, n = 90, $P < 0.001$) and untreated stall (r = 0.969, n = 90, $P < 0.001$) were highly and significantly correlated with outside RH(Figure 19 C, D).

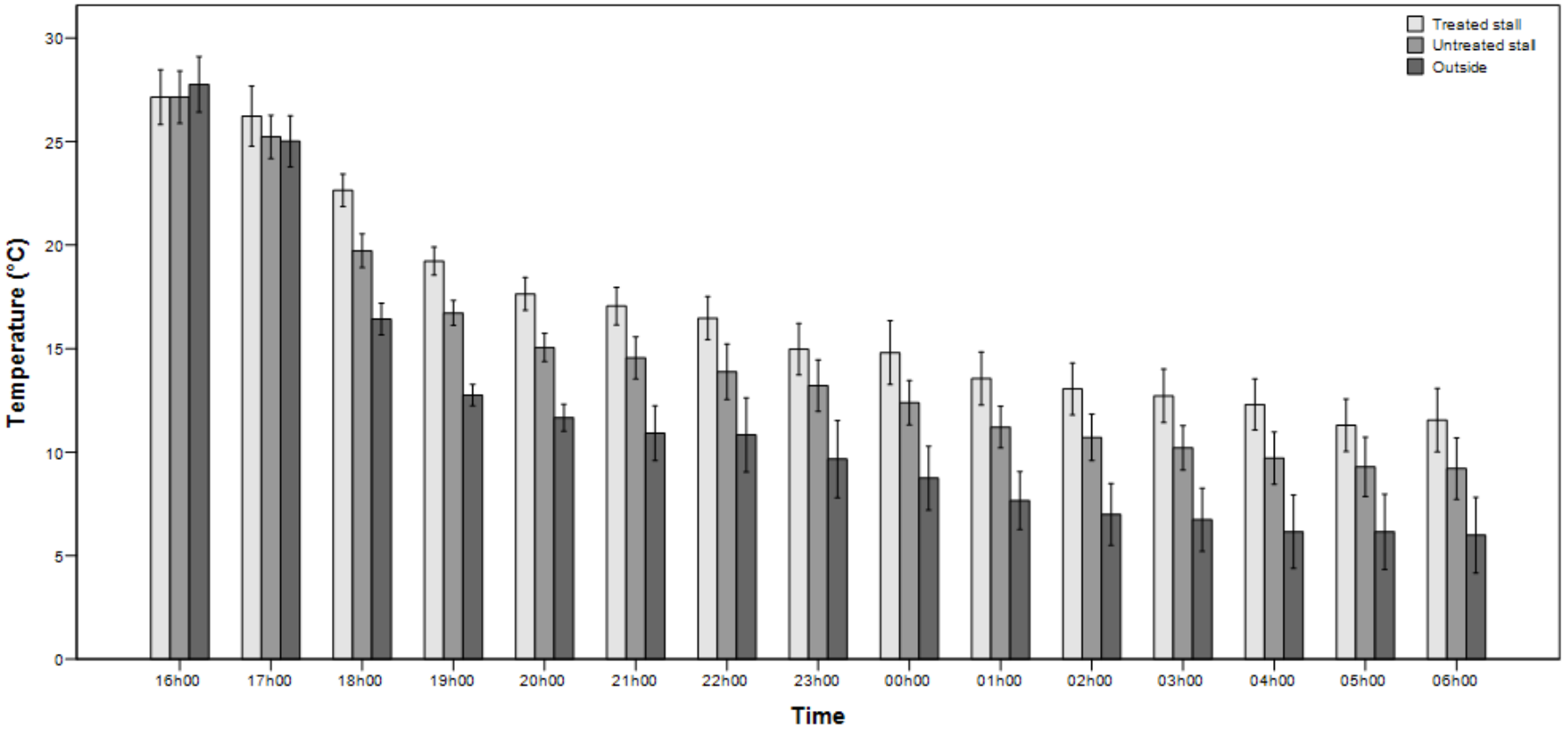

Figure 15. Temperature (°C) (mean ± s.e.) recorded at hourly time points in a treated jet stall, an untreated jet stall and outside over 6 nights under temperate climatic conditions.

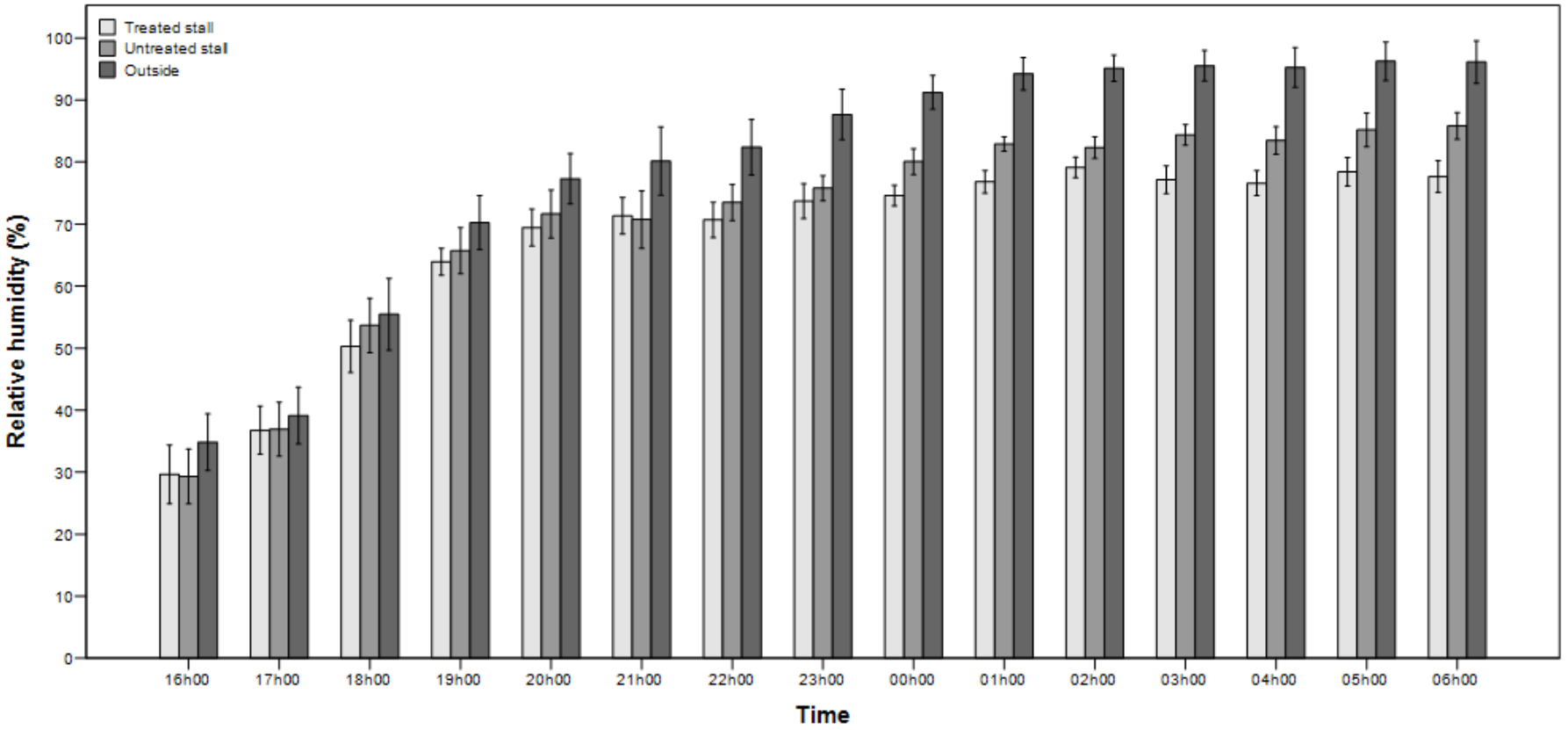

Figure 16. Relative humidity (%) (mean ± s.e.) recorded at hourly time points in a treated jet stall, an untreated jet stall and outside over 6 nights under temperate climatic conditions.

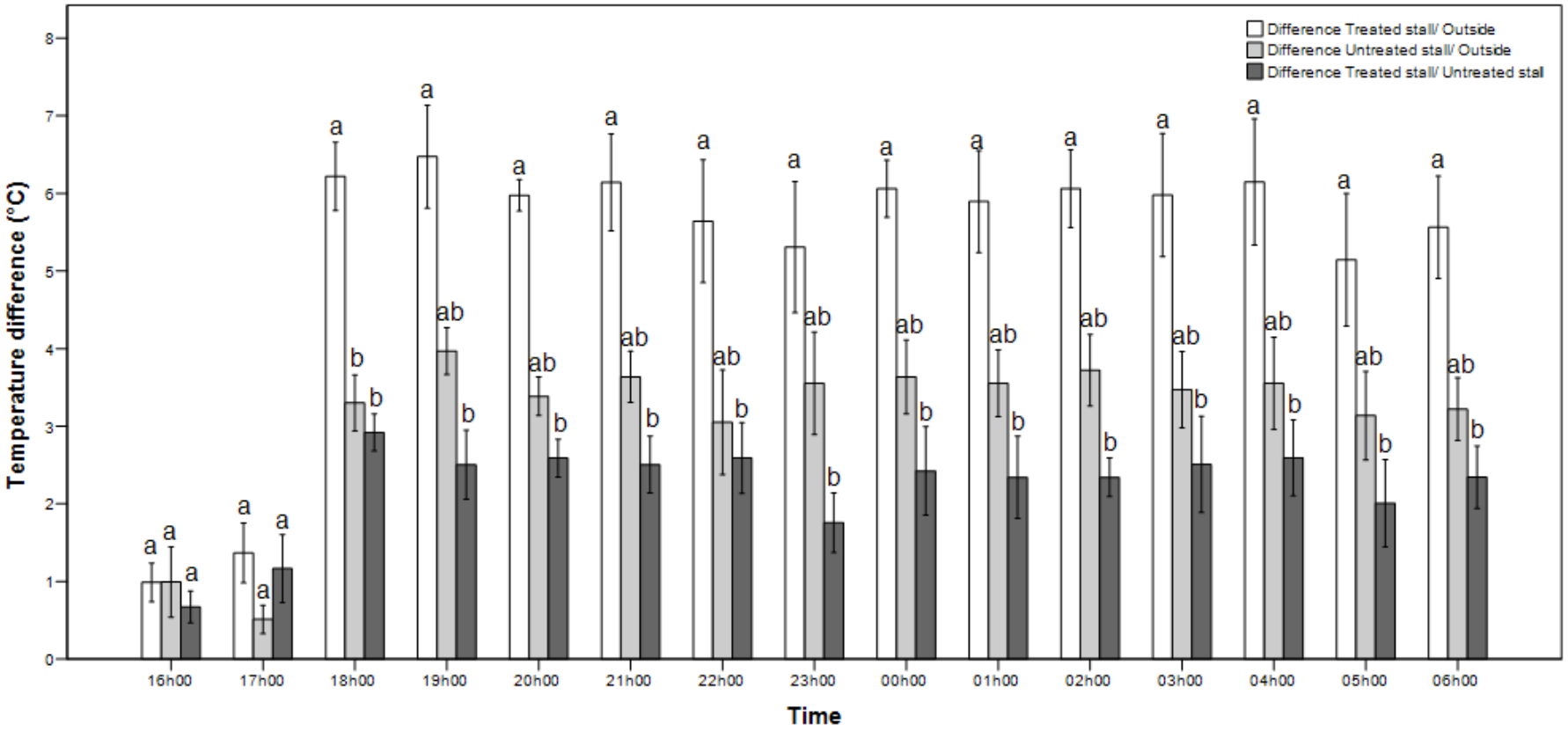

Figure 17. Hourly differences in temperature (°C) (mean ± s.e.) between a treated jet stall and outside, an untreated jet stall and outside, and a treated jet stall and an untreated jet stall recorded over 6 nights under temperate climatic conditions. Within each time point bars with a different lower case letter differ significantly ($P < 0.05$).

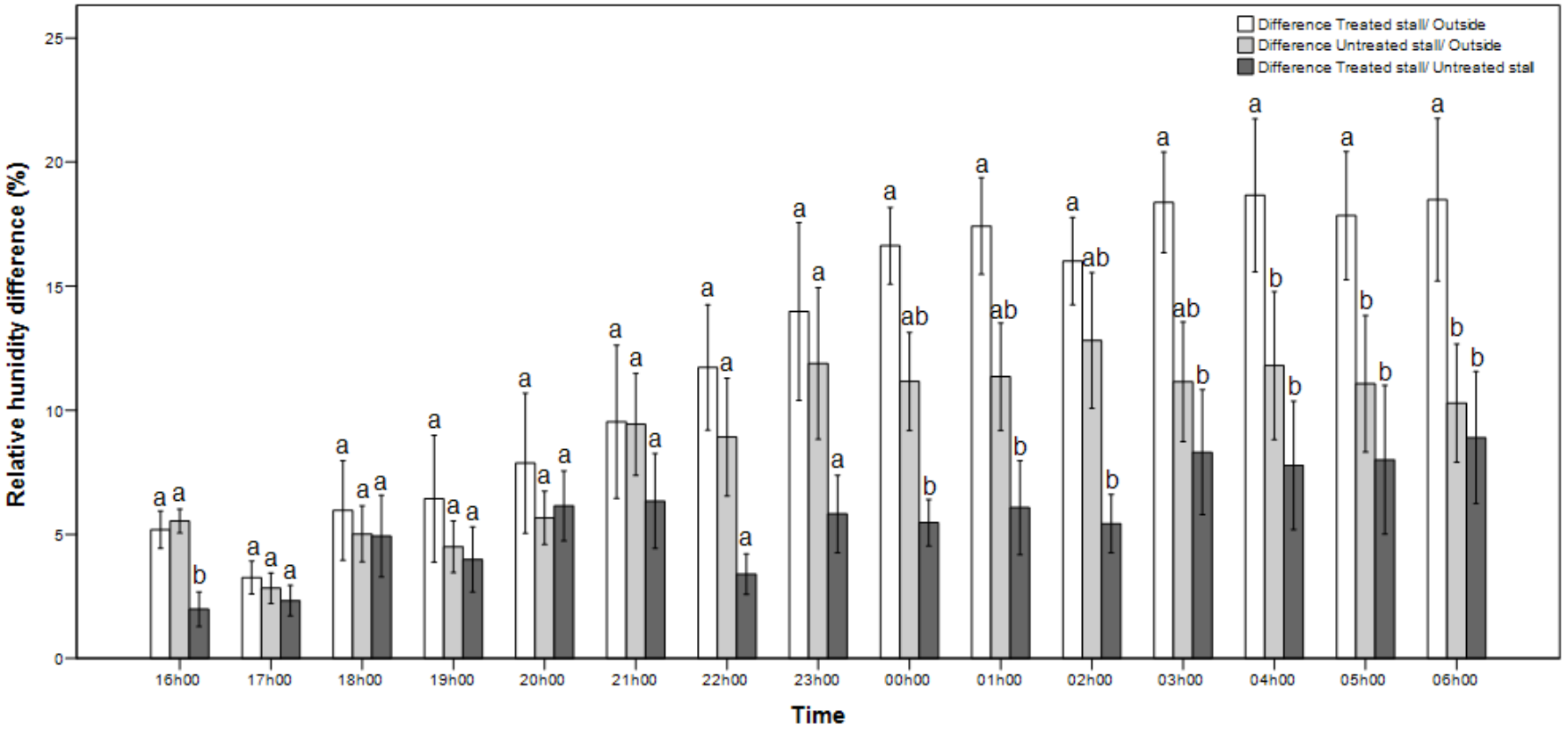

Figure 18. Hourly differences in relative humidity (%) (mean ± s.e.) between a treated jet stall and outside, an untreated jet stall and outside, and a treated jet stall and an untreated jet stall recorded over 6 nights under temperate climatic conditions. Within each time point bars with a different lower case letter differ significantly ($P < 0.05$).

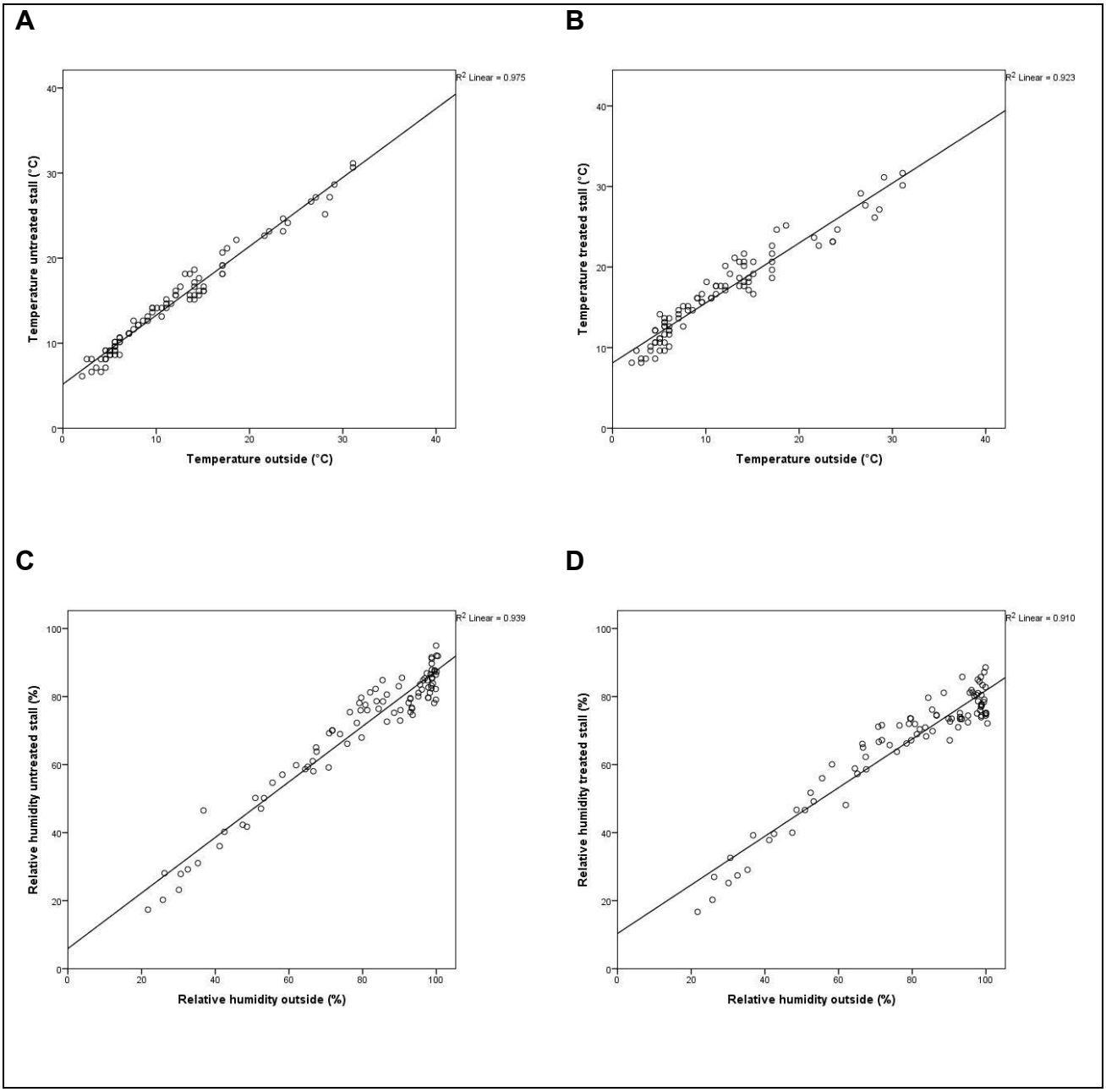

Figure 19. Relationship between temperature in an untreated jet stall and outside (A), temperature in a treated jet stall and outside (B), relative humidity in an untreated jet stall and outside (C), and relative humidity in a treated jet stall and outside (D), determined at 90 data points over 6 nights under temperate climatic conditions.

5.4.2. Clinical variables

No adverse effects of housing in the treated or untreated stalls were recorded in any of the horses. All horses remained alert and responsive with subjectively normal appetite, water intake, regular faecal output and urination. Vital signs recorded in the treated and untreated groups before entering the stall and during overnight housing are summarised in Table 7.

No significant differences were detected between the 4-hourly rectal temperatures (X^2_F = 6.824, df = 3, *P* = 0.078), pulse (X^2_F = 2.520, df = 3, *P* = 0.472) and respiratory rates (X^2_F = 2.806, df = 3, *P* = 0.422) for the horses in the untreated stall. For the horses in the treated stall rectal temperature differed significantly for the 20h00 compared to the 04h00 time point only (X^2_F = 11.283, df = 3, *P* = 0.01), while pulse (X^2_F = 1.031, df = 3, *P* = 0.794) and respiratory rates (X^2_F = 5.346, df = 3, *P* = 0.148) did not differ significantly between time points. There was no significant difference between rectal temperature (*P* = 0.246 – 0.918), pulse (*P* = 0.261 – 0.812) and respiratory rates (*P* = 0.065 – 1.0) of horses in the treated stall compared to the untreated stall at individual time points (Figures 20 – 22).

Table 7. Clinical variables (mean ± s.d.) of horses in the treated and untreated jet stall groups before entering the stall (16h00) and during overnight housing (20h00, 0h00 and 04h00).

	Group	Time point			
		16h00	20h00	0h00	04h00
Rectal temperature (°C)	Treated stall	37.8 ± 0.7	38.1 ± 0.7	37.8 ± 0.6	37.5 ± 0.7
	Untreated stall	37.7 ± 0.6	37.8 ± 0.6	37.6 ± 0.3	37.5 ± 0.3
Pulse (beats/min)	Treated stall	37 ± 9	40 ± 11	37 ± 7	37 ± 4
	Untreated stall	44 ± 11	38 ± 7	39 ± 9	35 ± 11
Respiration (breaths/min)	Treated stall	16 ± 7	21 ± 12	16 ± 5	14 ± 4
	Untreated stall	14 ± 3	15 ± 5	16 ± 6	14 ± 3

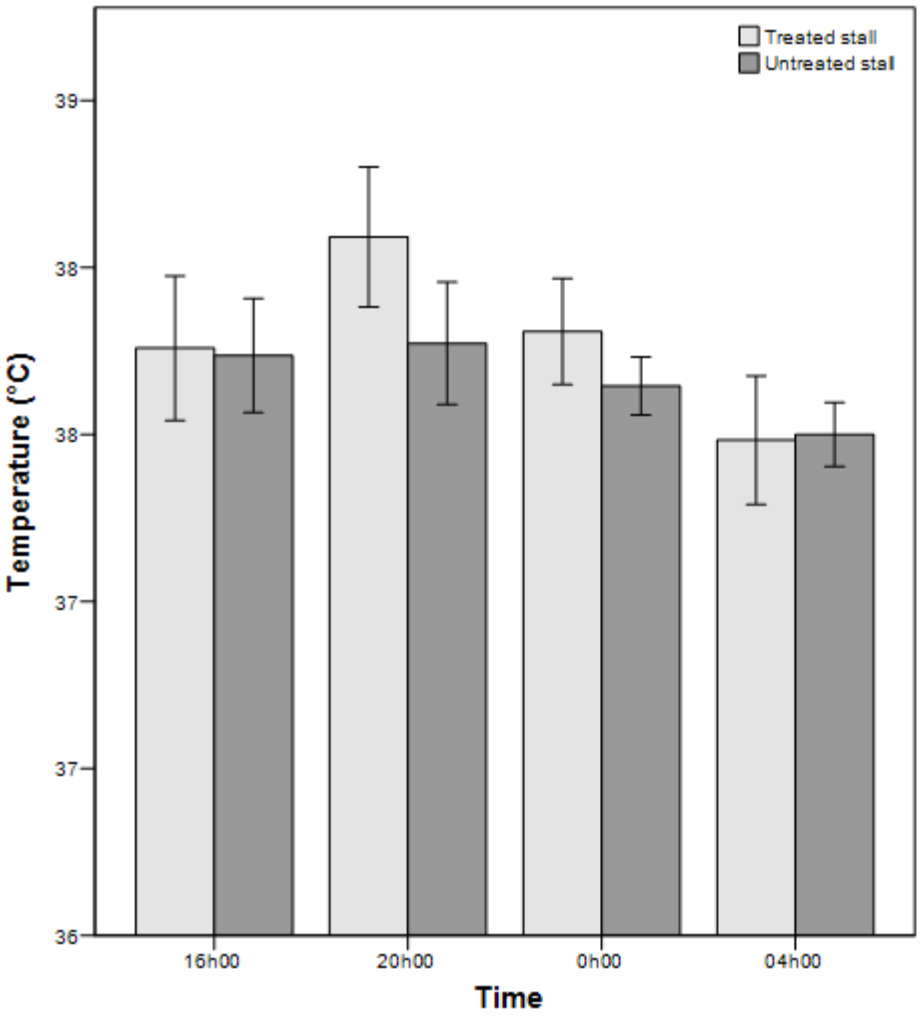

Figure 20. Rectal temperature (°C) (mean ± s.e.) of horses in the treated and untreated jet stall groups before entering the stall (16h00) and during overnight housing (20h00, 0h00 and 04h00).

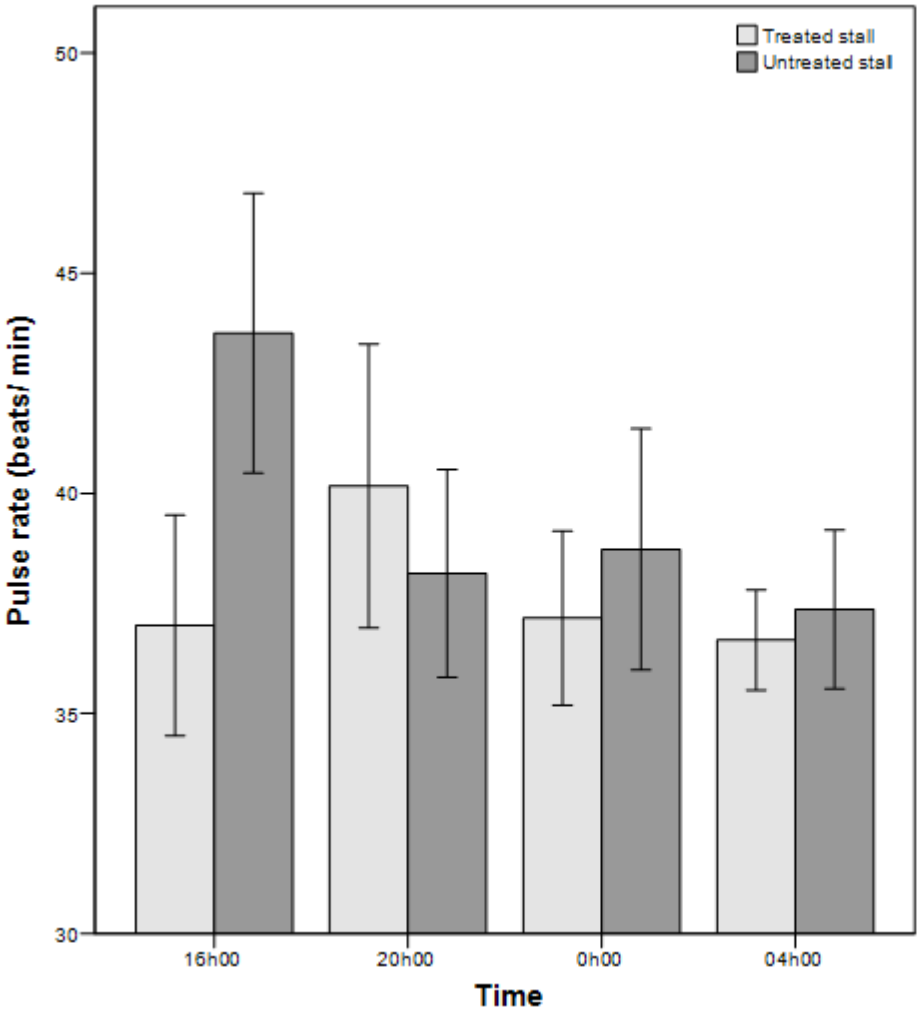

Figure 21. Pulse rate (beats/ min) (mean ± s.e.) of horses in the treated and untreated jet stall groups before entering the stall (16h00) and during overnight housing (20h00, 0h00 and 04h00).

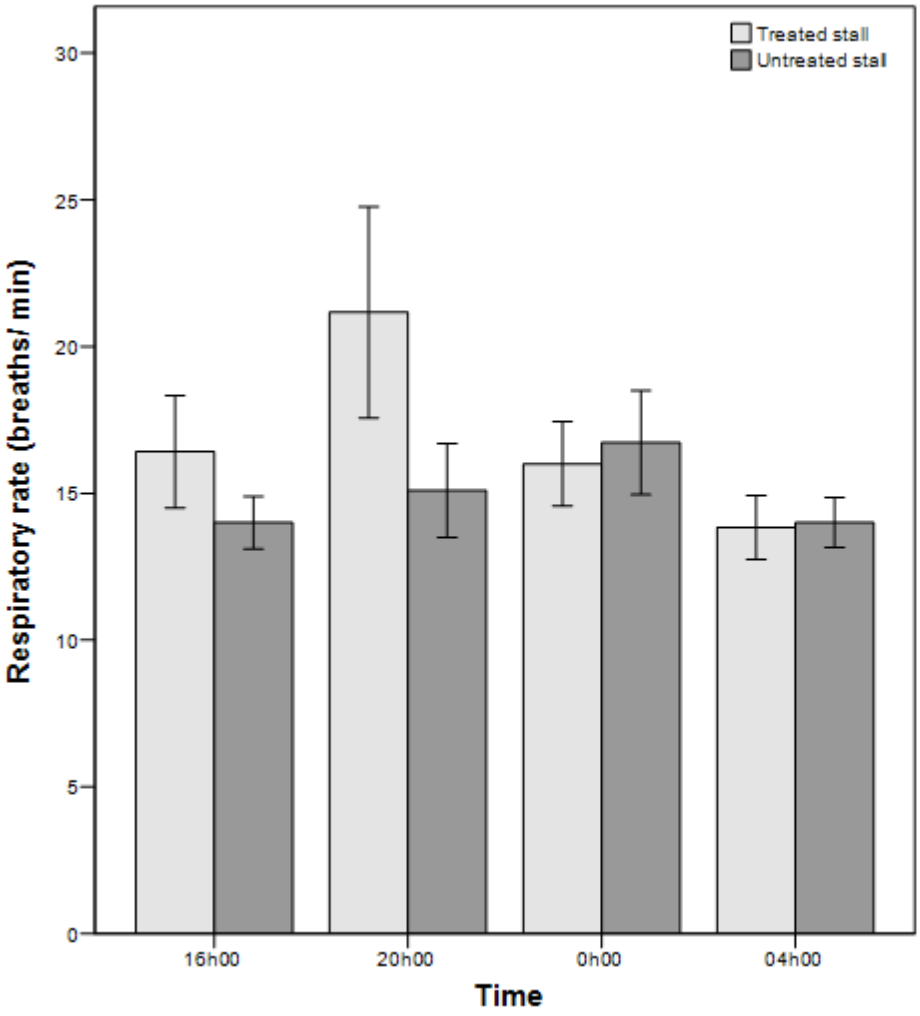

Figure 22. Respiratory rate (breaths/ min) (mean ± s.e.) of horses in the treated and untreated jet stall groups before entering the stall (16h00) and during overnight housing (20h00, 0h00 and 04h00).

5.4.3. FGM concentrations

An increase in mean FGM concentrations from baseline levels was detected in both treatment groups after overnight housing in the jet stalls (Figure 23). Mean FGM concentrations for horses housed in the treated stall peaked earlier (24 h) and at a higher concentration than horses housed in the untreated stall (48 h).

No significant difference was detected in FGM concentrations between baseline samples and samples obtained up to 72 h after exiting the stall for both the treated ($F = 2.001$; $df = 3, 33$; $P = 0.133$) and untreated stall ($F = 0.777$; $df = 3, 27$; $P = 0.517$) groups. No significant difference was detected in mean FGM concentrations when the treated and untreated stall groups were compared at individual time points ($t = -1.712$ - 0.190, $P = 0.121 – 0.893$).

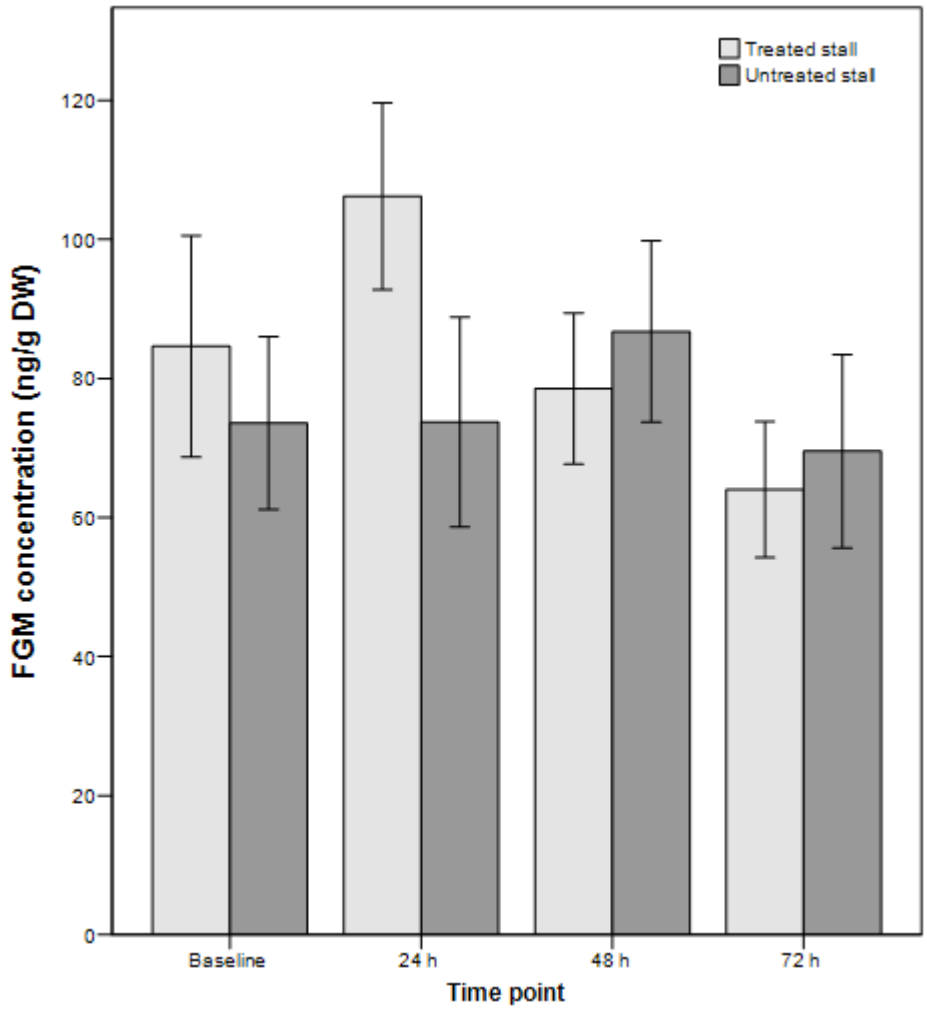

Figure 23. FGM concentrations (mean ± s.e.) from horses housed in a treated and untreated jet stall, between 16h00 and 06h00 for 6 nights, before entrance into the stall (Baseline), 24, 48, and 72 h after exiting the stall.

5.5. Discussion

Alphacypermethrin-treated HDPE mesh applied to commercial jet stalls as a physical and chemical protection measure against AHS vectors did not compromise jet stall microclimate, clinical variables or indicators of physiological stress in horses housed under stationary stall, temperate climatic conditions.

Closed top HMA-type jet stalls are commonly used to transport horses internationally. These stalls avoid potential fire alarm concerns associated with air conditioning units of climate-controlled stalls, however ventilation and microclimate could be affected by application of mesh around the stalls for vector protection. The outside temperatures in the present study were within or lower than a guideline range for transport of horses (10-21°C), while the RH was higher than the recommended range (45-50%) (IATA, 1998), as expected for the overnight study conditions. Similarly, the outside and jet stall temperatures in the present study were within or lower than corresponding cargo hold and jet stall temperature ranges reported during air transit of horses, and the outside and jet stall RH was similar or higher than reported (Leadon et al., 1990; Maaskant et al., 2013; Munsters et al., 2013; Stewart et al., 2003; Thornton, 2000). Marked differences in study conditions and the type of jet stall utilised limit direct comparison of microclimate between the present and the cited studies, however.

In order to better assess the impact of the mesh on microclimate the differences computed between treated and untreated jet stall microclimate and outside climatic variables were analysed as outside conditions, which were highly correlated with those inside the jet stalls, varied daily making comparisons of differences more appropriate than actual values (Purswell et al., 2010). In the present study, while the temperature in the treated stall was

consistently higher than the outside temperature, likely associated with retention of metabolic heat produced by the horses (McCutcheon and Geor, 2008) and low overnight outside temperatures, the treated stall/ outside temperature difference was only significantly higher than untreated stall/ outside difference at one (18h00) time point. The difference between the treated stall/ outside RH also did not differ significantly from the untreated stall/ outside RH, in fact the treated stall RH was consistently lower than the outside RH despite anticipated evaporative heat loss which would be expected to increase RH (McCutcheon and Geor, 2008). These findings support no increased risk to thermoregulation with application of the HDPE mesh to a jet stall as compared to an untreated stall under similar climatic conditions.

While a thermal index limit of 28°C is specified for equine competition there is currently no defined value to prevent equine thermal stress during equine transport (Purswell et al., 2010) and further research has been recommended to improve thermal environment and ventilation of equine transport systems (Padalino, 2015). In the absence of data under more extreme environmental conditions for similar jet stalls protected with HDPE mesh, opening of the mesh flaps adjacent to stall ventilation points (e.g. above the front and back ramps) to promote ventilation is recommended as a precautionary measure if hot or humid environmental conditions arise during the transport process, such as during stopovers with exposure to ambient air (Thornton, 2000). Opening the mesh of stalls located at the rear of an aircraft first is recommended based on the temperature and RH gradient in the cargo hold whereby the stalls at the rear experience warmer, moister conditions, especially when the aircraft is stationary (Leadon et al., 1990). Furthermore, it is recommended that jet stall microclimate be monitored regularly during the transport process, particularly under more extreme environmental conditions when mesh protection is used. As emergency opening

of the mesh to promote ventilation could increase risk of exposure to AHS vectors, additional methods of protection such as insecticides or repellents applied to horses in the stalls or insecticidal aerosols sprayed in the cargo hold is advised.

Overnight housing in the treated jet stall had no significant effect on clinical variables compared to housing in an untreated stall. Horses were assessed under stationary jet stall field conditions in the present study which precluded confounding factors associated with air transport, such as loading into the aircraft, take off, turbulence, landing and unloading. Increased heart rates have been reported during these transitional events with return to resting values during level flight (Munsters et al., 2013; Stewart et al., 2003; Thornton, 2000). In the present study, respiratory rate also did not differ significantly between time points monitored in the treated stall, further supporting a lack of thermal stress as respiratory rate would be expected to increase (McCutcheon and Geor, 2008) as a heat loss mechanism if the treated mesh compromised ventilation.

While the effects of road transport (Schmidt et al., 2010a; Schmidt et al., 2010b; Schmidt et al., 2010c) on cortisol release, heart rate and HRV have been reported for horses there is limited information on the effect of air transport on stress indicators (Munsters et al., 2013; Stewart et al., 2003; Thornton, 2000), and none have reported on the effect of jet stall transport on FGM. In contrast to monitoring of adrenocortical function by measurement of plasma and salivary cortisol, which reflect acute changes in cortisol release (Schmidt et al., 2009), FGM concentrations reflect only marked or prolonged increases in cortisol release with a lag time of 24 h related to species-specific intestinal passage time in horses (Merl et al., 2000; Schmidt et al., 2010c). In the present study, which was limited to investigating the effects of stationary housing in a jet stall, no

significant difference from baseline was detected in FGM concentrations, neither was there a significant difference between treatment groups. These findings, which are supported by the clinical variable data, indicate that stationary housing in an untreated jet stall or one protected with alphacypermethrin-treated HDPE mesh under temperate climate conditions is not associated with a significant stress response in horses. The earlier and higher peak in FGM concentrations for horses housed in the treated stall warrants further investigation in a larger cohort, however, but may be associated with other factors, e.g. mesh-associated impaired visual contact with herd mates compounding a herd group disruption effect (Schulman et al., 2014).

While caution is advised in practical application of the study findings based on the recognised study limitations including a stationary jet stall investigated under temperate field conditions, relatively low number of horses of varying breed, age and gender enrolled, and lack of a control group not housed in a jet stall, the findings support the safety of the mesh applied to comparable jet stalls under similar climatic conditions.

In conclusion, alphacypermethrin-treated HDPE mesh applied to jet stalls housing horses had no adverse effect on jet stall microclimate, clinical variables or stress indicators of horses housed in the stalls under temperate climatic conditions. While this mesh could be used as a safe and effective physical and chemical method for protection of horses in jet stalls against *Culicoides* midges under similar temperate climatic conditions, further investigation is required under more extreme climatic conditions as well as during air transport.

CHAPTER 6: GENERAL CONCLUSIONS

1. Alphacypermethrin-treated HDPE mesh served as an effective physical barrier against *Culicoides* biting midges under field screening conditions using Onderstepoort 220V downdraught black light traps.
2. Alphacypermethrin-treated HDPE mesh had a rapid insecticidal effect against field-collected, nulliparous (unpigmented) female *C. imicola* in a laboratory bioassay.
3. Alphacypermethrin-treated HDPE mesh applied to jet stalls significantly reduced the attack rate of *Culicoides* midges on horses housed in the jet stalls.
4. Alphacypermethrin-treated HDPE mesh applied to jet stalls housing horses had no adverse effect on jet stall microclimate under temperate climatic conditions.
5. Alphacypermethrin-treated HDPE mesh applied to jet stalls had no adverse effect on clinical variables or stress indicators of horses housed in the stalls under temperate climatic conditions.
6. Alphacypermethrin-treated HDPE mesh could be used as a safe and effective physical and chemical method for protection of horses in jet stalls against *Culicoides* midges under similar temperate climatic conditions.
7. Investigation of additional methods of protection used in conjunction with alphacypermethrin-treated HDPE mesh, e.g. DEET insect repellent applied to horses in jet stalls, may be warranted to further improve protection of horses against *Culicoides* midges.

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APPENDICES

APPENDIX 1. Specification of high density polyethylene mesh

ALNET
A Trusted Name A Tested Reputation
Registration No 1953/002549/07

Alnet (Pty) Ltd
Moorsom Ave, Epping 2, 7460, Cape Town
Private Bag X306, Epphdust, 7475, South-Africa
Tel: +27 21 530 2400 • Fax: +27 21 534 4003 or +27 21 534 8338
e-mail: sales@alnet.co.za • Website: www.alnet.co.za

70% Shade Cloth Monofilament Black
Specification RK02

WEIGHT G/M²	= ± 201.5g/m²
SHADE %	= ± 62.0 %
BURST Kpa	= ± 250.0Kpa
Stitches per inch	= 20
Warp Breaking Strength	= 9.0Kn/mm
Elongation	= 79.0%.
Weft Breaking Strength	= 19.0 Kn/mm
Elongation	= 83.0%
Hole size	=0.3mm

General Information

Material	= 400 Den monofilament
Material Usage	= 100% High Density Polyethylene
Fabric Construction	= Raschel Knitted Monofilament.
Additives	= UV Stabilizer – ADBOO4 & Colorants
Durability Testing	= Test sources SABS, Stellenbosch University

Legend

Warp	It is the direction in which the cloth is knitted
Weft	It is the direction 90 from the knitted direction
UV	Ultra Violet

Durban
No 4 Larsen Park
40 Ebonyfield Ave
Springfield Park
PO Box 74319
Rochdale Park 4034
Tel: +27 31 579 1480
Fax: +27 31 579 1014

Johannesburg
Summit Square II – Unit 1
397 Roan Crescent
Corporate Park North
Randjespark Ext 121, Midrand
PO Box 50708
Randjiesfontein 1683
Tel: +27 11 314 3493
Fax: +27 11 314 3703

Port Elizabeth
5 Cowie Street
Sidwell 6001
PO Box 3255
North End
6056
Tel: +27 41 453 6051
Fax: +27 41 453 6052

St Helena Bay
Sandy Point
Harbour
St Helena Bay 7390
PO Box 13
St Helena Bay 7390
Tel: +27 22 736 1025
Fax: +27 22 736 1422

Directors: M Appelo (Chairman)*, DJ Du Rand (Alternate)*, AJ Kok, Prof J Van Zyl Smit
Executive*

APPENDIX 2. Fendona® 6 Directions for use

BASF
The Chemical Company

FENDONA® 6

Reg. No. L 5678; Act No 36 of 1947 **N-AR 0729**
W 130 053
For full particulars, see enclosed leaflet.
A long lasting suspension concentrate contact and stomach insecticide for the control of malaria vectors, nuisance and biting flies, cockroaches (american and german), bedbugs, fleas, mosquitoes, hide beetle larva, fishmoths, stableflies, ants and Alphitobius larva and -beetle.
Active Ingredient: Alpha-cypermethrin (Pyrethroid) ..... 60 g / ℓ

Reg. Nr. L5678 Wet Nr 36 van 1947
'n Suspensiekonsentraat kontak- en maaginsekdoder met 'n lang nawerkende aksie vir die beheer van malariavektore, hinderlike- en steekvlieë, kakkerlakke (amerikaanse en duitse), weeluise, vlooie, vismotte, muskiete, velkewerlarwes, perdevlieë, miere en Alphitobius-kewer en -larwe.
Aktiewe bestanddeel: Alfa-sipermetrien (Piretroïed) ..... 60 g / ℓ

Registered by / Geregistreer deur:
BASF South Africa (Pty) Ltd.
(Co. Reg. No. / Mpy. Reg. Nr. 66/10235/07)
P.O. Box / Posbus 2801, Halfway House, 1685

Manufactured and formulated by /
Vervaardig en geformuleer deur:
BASF Agro BV Arnhem (NL), Waedenswil Branch
Moosacherstraße 2, CH-8820 Waedenswil, Switzerland

EMERGENCY NUMBER / NOODNOMMER 082-893-3643
UN / VN No: 3082 **App. code 22 / 01 / 01**

1 ℓ

Batch Number /
Lotnommer:

Date manufactured /
Datum vervaardig:

4 014348 441803

® Registered Trademark of BASF /
Geregistreerde Handelsmerk van BASF 81037384 ZA 0118

~xmam1228205530.indd 1 02.12.2008 09:12:29

WARNINGS

- Concentrate is poisonous when swallowed and moderately toxic by skin contact.
- Not significantly hazardous to bees, various other beneficial pest parasites and predators and fish under field conditions, provided the product is used as directed.
- Do not spray over or allow drift to contaminate drinking water and water bodies such as dams, ponds, rivers, streams or fish hatcheries.
- Moderately toxic to wildlife.
- Store in a cool place away from food and feedstuffs.
- Keep concentrate out of reach of children, uninformed persons and animals.
- Do not apply directly onto animals.
- Do not apply to surfaces where food is handled.

Although this remedy has been extensively tested under a large variety of conditions the registration holder does not warrant that it will be efficacious under all conditions because the action and effect thereof may be affected by factors such as abnormal climatic and storage conditions, quality of dilution water, compatibility with other substances not indicated on the label and the occurrence of resistance of a pest against the remedy concerned as well as by the method, time and accuracy of application. The registration holder furthermore does not accept responsibility for damage to the environment or harm to man or animal or lack of performance of the remedy concerned due to failure of the user to follow the label instructions or due to the occurrence of conditions which could not have been foreseen in terms of the registration. Consult the supplier in the event of any uncertainty.

PRECAUTIONS

- Avoid eye splashes, skin contact by and inhalation of spray mist.
- Wear protective clothing: Overall, gloves and facemask.
- Wash with soap and water after use or after accidental skin contact.
- Do not eat, drink or smoke while mixing or spraying or before washing hands and face.
- Prevent contamination of food, feedstuffs, eating utensils and drinking water.
- Remove pets and aquaria or cover aquaria.
- Thoroughly wash and rinse the spray equipment / containers / basins after use and dispose of wash water where it will not contaminate crops, grazing, rivers and dams.
- TRIPLE RINSE empty container in the following manner: Invert the empty container over the spray or mixing tank and allow to drain for at least 30 seconds after the flow has slowed down to a drip. Thereafter, rinse the container three times with a volume of water equal to a minimum of 10 % of that of the container. Add these rinsings to the contents of the spray tank.
- Destroy empty container and do not re-use for any other purpose.

DIRECTIONS FOR USE

USE ONLY AS DIRECTED

Compatibility: Do not mix **FENDONA 6** with any other chemical.

Mixing instructions for spraying:
Half fill the sprayer with clean water. Measure the required volume of **FENDONA 6** and pre-mix this with some water. Add this mixture to the water in the sprayer. Fill the sprayer with water to the required level and shake the sprayer well to ensure proper mixing or ensure continual mechanical or hydraulic agitation before spraying commences. Prepared spray mixture must not be left in the spray tank for any length of time e.g. overnight.

General directions:

- **FENDONA 6** is a long lasting suspension concentrate contact and stomach insecticide for the control of malaria vectors, flies, cockroaches, bedbugs, fleas, fish moths, mosquitoes, ants, hide and skin beetles and larvae and litter beetle and larva. The length of control depends on:
  - the insect species being treated,
  - the level of infestation,
  - the type of surface (or bed-net fabric),
  - whether the treatment is applied indoors or outdoors,
  - exposure to sunlight and weather conditions and,
  - depending on dosage rate, months of control can be expected indoors and on bed-nets, and several weeks outdoors under favourable conditions.
- **FENDONA 6** spray mixture is colourless and odourless and can be used indoors or outdoors, in and around homes, factories, hotels, shops, food handling and processing establishments, hospitals, schools, dairy parlours, pigeries, stables, poultry houses, dog kennels, refuse dumps and similar places where high levels of hygiene are required.

Poultry uses:

- **Poultry sheds:** All cracks, crevices and potential hiding places where the litter beetle may seek shelter must receive special attention. Ensure thorough wetting of all surfaces including vertical surfaces up to at least one metre above the ground. **FENDONA 6** will control but will NOT eliminate existing populations of litter beetles that have invaded and colonised the insulation of cavity walls or other protected places.
  - **Broiler sheds:** Should be treated before restocking with each new batch of chicks, i.e. after the clearing, cleaning and disinfecting operations have been carried out.
  - **Laying sheds:** Should be treated on a regular basis. Frequency will depend on the type of surface to be treated. Dropping and litter heaps may require treatment more often. New and empty cages can be treated BEFORE stocking with hens. DO NOT SPRAY THE BIRDS DIRECTLY OR ALLOW THE SPRAYMIST TO DRIFT OVER THEM.

Malaria vector control:

Surface sprays

- Use a sprayer fitted with a nozzle that will deliver coarse droplets at a low pressure. Do not spray at high pressure that will result in a spray mist.
- Ensure total coverage of all surfaces. All cracks, crevices and potential hiding places where mosquitoes may seek shelter must receive special attention.
- Allow spray to dry on treated surfaces before re-entering.

Bed-nets

- All nets need to be washed and dried before treatment. Some new nets are sometimes starched, and must therefore be washed first to remove the starch. Ensure that all traces of soap are rinsed out after washing and before treating with **FENDONA 6**, as alkaline soap residues could influence the persistence of the treatment.
- The volume of **FENDONA 6** to be used per bed-net is dependent on the length of time that control is required, as well as the size of the net.

Months of control required (dose active ingredient)	Single bed-net (±12 m²)	Double bed-net (±16 m²)	Queen size bed-net (±20 m²)	King size bed-net (±24 m²)
4 – 6 months (20 mg a.i. / m²)	4 mℓ	5.5 mℓ	6.5 mℓ	8 mℓ
more than 9 months (40 mg a.i. / m²)	8 mℓ	10.5 mℓ	13.5 mℓ	16 mℓ

- Bed-net fabric, mesh size and accessories such as a roof, border or string ties, affect the volume of water needed to treat and thoroughly wet a net. Generally 30 mℓ mixture / m² is needed for synthetic materials (e.g. polyester) and 130 – 150 mℓ / m² for cotton.
- Rough guidelines for the amount of water needed to wet different size bed-nets are given below (it is recommended however that the exact absorption volume for each type and size of net be determined before treatment).

Fabric	Single bed-net (±12 m²)	Double bed-net (±16 m²)	Queen size bed-net (±20 m²)	King size bed-net (±24 m²)
Synthetic material (e.g. Polyester)	360 mℓ	480 mℓ	600 mℓ	720 mℓ
Cotton	1700 mℓ	2250 mℓ	2800 mℓ	3400 mℓ

Mixing Instructions for treating bed-nets.

- Use a suitable container / bucket / bowl / bag, that is large enough to hold the bed-net as well as the required amount of water.
- Treat nets outside or in a well-ventilated area and use gloves.
- Fill the container with the predetermined volume of water to fully wet the bed-net to be treated. Add the correct volume of **FENDONA 6** (see above) to the water and mix well.
- Soak the net in the mixture until it is thoroughly wet.
- Remove the net and allow excess to drip back into the container. (If the correct amount of mixture was used, there should be very little left).
- Allow the net to dry evenly by laying it on a bed or bedding (which helps to kill bedbugs) or on a plastic sheet outside in the shade.
- When net is dry, it may be hung over the bed.
- After treating nets, wash hands, arms, and container and dispose of wash water where it will not contaminate crops, grazing, rivers and dams.
- The effect of the treatment will not be the same if the net is washed after treatment, and a re-treatment is advisable.
- Bed-nets treated with **FENDONA 6** will not be stained or have an odour after treatment and will not be more flammable.
- To treat several identical nets, a larger volume of mixture can be prepared by multiplying the volume of **FENDONA 6** as well as the volume water needed per net, by the total number of nets to be treated.

All spray applications must be made with suitable equipment that is in good working order and correctly calibrated to ensure the desired coverage for that particular method of application.

PEST	DOSAGE	DIRECTIONS FOR APPLICATION
HIDES & SKINS Hide beetle larvae	35 mℓ / 10 ℓ water	**Hides and skins:** Thoroughly wet all parts. Repeat treatment every 3 months or when necessary.
Hide beetle adults	35 mℓ / 10 ℓ water	**Warehouse floors, walls and other surfaces:** Apply spray mixture to the point of run-off. Repeat treatment every 3 months or if necessary.
MOSQUITO ADULTS (including malaria vectors)		Several months of control can be expected indoors at the highest dosage rates (160 mℓ / 10 ℓ water), but at the lower dosage rate only 4 – 6 weeks' control can be expected. The length of control is determined by the dosage rate and the type of treated surface. (See also **General Directions**, under **DIRECTIONS FOR USE** above)
	40 – 85 mℓ / 10 ℓ water (12 – 25.5 mg a.i. / m²)	Apply as a coarse spray to surfaces where insects settle in areas where re-sprays can be done every 4 – 6 weeks. Apply to point of run-off. Use higher dose rate for longer lasting control or where infestation is severe. Repeat if necessary.
		MALARIA VECTOR - SURFACE TREATMENTS
	160 mℓ / 10 ℓ water (50 mg a.i. / m²)	Recommended for use as a surface spray on coarse surfaces (such as clay and brick internal and external walls, roofs and ceilings, e.g. thatched). Use this rate where applications can be made only **once or twice** during the malaria infection period of October to May. Apply as a coarse spray to the point of run-off by using 50 mℓ spray mixture / m². If the applications will be made on smooth surfaces (such as glass and wood inside and outside the house) apply as a coarse spray to the point of run-off by using 25 mℓ spray mixture / m² by mixing 330 ml of **FENDONA 6** with 10 ℓ water. This will also result in applying 50 mg a.i. / m².
		BED-NET TREATMENT
	20 mg a.i. / m² OR 40 mg a.i. / m²	Mix the required volume **FENDONA 6** with enough water to wet a bed-net. Soak the net in the mixture until it is thoroughly wet. Use the higher rate for longer period of control. For amount of **FENDONA 6** refer to **Bed-nets** under **MALARIA CONTROL** above. See further information under **General directions** under **DIRECTIONS FOR USE** above as well.
POULTRY SHEDS Lesser Mealworm / Litter beetle (Alphitobius diaperinus)	40 - 85 mℓ / 10 ℓ water	Apply as a coarse spray to the interior surfaces of the sheds. Ensure thorough wetting of the floors, walls and other surfaces. Repeat if necessary. Use higher dose rate for longer lasting control or where infestation is severe. (See under **General Directions above**). DO NOT SPRAY THE BIRDS DIRECTLY OR ALLOW THE SPRAYMIST TO DRIFT OVER THEM.
PUBLIC HEALTH: Adult house flies Adult stable flies Ants, Bedbugs Cockroaches (american & german) Fish moths, Fleas	40 - 85 mℓ / 10 ℓ water	Apply as a coarse spray or with a paintbrush to cracks, crevices and any place where these insects may hide and on surfaces over which they may crawl or settle. Apply to point of run-off. Use higher dose rate for longer lasting control or where infestation is severe. Repeat if necessary.

APPENDIX 3. Publications

Veterinary Parasitology 203 (2014) 184–188

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Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Field and *in vitro* insecticidal efficacy of alphacypermethrin-treated high density polyethylene mesh against *Culicoides* biting midges in South Africa

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ABSTRACT

The efficacy of untreated and alphacypermethrin-treated high density polyethylene (HDPE) mesh against *Culicoides* biting midges (Diptera: Ceratopogonidae) was determined using Onderstepoort downdraught black light traps and a contact bioassay. Three traps were operated overnight in four replicates of a 3 × 3 randomised Latin square design near horses under South African field conditions. Both the untreated and alphacypermethrin-treated HDPE mesh significantly ($P < 0.05$) reduced the numbers of *Culicoides* midges, predominantly *Culicoides* (*Avaritia*) *imicola* Kieffer, collected in the light traps by 4.2 and 7.2 times, respectively. A repellent effect of the alphacypermethrin-treated mesh was not confirmed because the number of midges collected in the light traps with untreated and alphacypermethrin-treated HDPE mesh was not significantly different ($P = 0.656$). Bioassay of the insecticidal contact efficacy indicated median *C. imicola* mortality of 100% from 30 and 10 min following exposure to the alphacypermethrin-treated HDPE mesh for 1 or 3 min, respectively. In the bioassay, mortality was significantly higher ($P = 0.016$) at 5 min post exposure in the midges exposed to the alphacypermethrin-treated mesh for 3 min (74.8%) compared to the 1 min exposure group (59.5%). The HDPE mesh could be used to reduce exposure of housed animals to *Culicoides* midges, specifically *C. imicola*, and viruses transmitted by these midges. Mesh treated with alphacypermethrin had the additional benefit of a rapid insecticidal effect on *C. imicola.*

1. Introduction

Culicoides biting midges (Diptera: Ceratopogonidae) are of economic and veterinary significance worldwide, primarily due to the Orbiviruses they transmit (Mellor et al., 2000; Meiswinkel et al., 2004). Based on its wide geographical distribution and host preference for bigger mammals, as indicated by the high numbers collected near livestock, *Culicoides* (*Avaritia*) *imicola* Kieffer is considered the principal vector of African horse sickness virus (AHSV), equine encephalosis virus and bluetongue virus (BTV) in South Africa (Nevill et al., 1992; Meiswinkel et al., 2004; Paweska and Venter, 2004), and BTV in southern Europe (Mellor, 1992; Mellor et al., 2000). *Culicoides imicola* is predominant on livestock throughout South Africa and is also the most abundant species collected

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on horses at Onderstepoort, South Africa (Scheffer et al., 2012).

The spread of BTV in northern Europe and recent outbreaks of a novel orthobunyavirus, Schmallenberg virus (Hoffmann et al., 2012), has demonstrated the devastating effect of these viruses on naïve populations. These outbreaks raised concern over the introduction and spread of other midge-borne viruses, particularly AHSV, and the need for optimised preventative strategies (Carpenter et al., 2008, 2009; MacLachlan and Guthrie, 2010; Papadopoulos et al., 2010; Backer and Nodelijk, 2011; Tarlinton et al., 2012; MacLachlan and Mayo, 2013; Napp et al., 2013). Moreover, global expansion of containerised trade, including intercontinental movement of horses (Reiter, 2010), provides potential mechanisms whereby viruses may be introduced (Carpenter et al., 2009; MacLachlan and Guthrie, 2010; de Vos et al., 2012; Napp et al., 2013). Whilst clear recommendations for pre-export quarantine and testing of horses for AHSV have been in place for many years, recent amendments to the World Organisation for Animal Health (OIE) – Terrestrial Animal Health Code have included recommendations that mesh of appropriate gauge, impregnated with an approved insecticide be placed over containers during transport of horses through regions not free of AHSV (World Organisation for Animal Health, 2013).

In addition to the use of vaccines, recommended control measures against viruses transmitted by *Culicoides* midges include stabling at night, screening of stables with mesh, and the use of effective repellents or insecticides (Meiswinkel et al., 2004; Carpenter et al., 2008). Stabling efficacy depends on the exophilic/endophilic behaviour of the vector species (Carpenter et al., 2008; Viennet et al., 2012), and horses are protected only if the stables are adequately closed (Barnard, 1997; Meiswinkel et al., 2000). Repellents such as *N,N*-diethyl-3-methylbenzamide (Page et al., 2009), pyrethroids and plant extracts (Braverman and Chizov-Ginzburg, 1998), and organic fatty acids (Venter et al., 2011) have shown efficacy against *Culicoides* midges in light trap studies. Recently, insecticidal efficacy against colony-reared *Culicoides* (*Monoculicoides*) *nubeculosus* Meigen exposed to hair from horses treated with cypermethrin was reported (Papadopoulos et al., 2010). Despite the proven effectiveness (Lengeler, 2004) and widespread use of pyrethroid-treated nets for controlling mosquitoes, the use of pyrethroid-treated mesh for reducing the *Culicoides* biting rate in animals has received limited attention (Carpenter et al., 2008; Calvete et al., 2010; Del Río et al., 2014).

The objectives of this study were to determine if alphacypermethrin-treated high density polyethylene (HDPE) mesh applied to light traps will reduce the entry of *Culicoides* midges, particularly *C. imicola*, into the traps. Following on the promising results of Papadopoulos et al. (2010) with cypermethrin and the northern European *C. nubeculosus*, the *in vitro* insecticidal efficacy of alphacypermethrin-treated HDPE mesh against field populations of *C. imicola* was investigated. These results may support potential of alphacypermethrin-treated HDPE nets for protecting livestock against *Culicoides* midges, and be applicable to containerised transport systems of horses.

2. Methods

2.1. Light trap assay

The efficacy in reducing numbers of *Culicoides* midges entering a light trap of (1) 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), (2) an identical untreated HDPE mesh and (3) an untreated control black polyester mesh with 2 mm hole size were compared. Comparisons were done overnight for 12 nights in four replicates of a 3×3 randomised Latin square design (Snedecor and Cochran, 1980), observer-blinded, field experiment near horses at the Faculty of Veterinary Science, Onderstepoort (25°38′51.42″ S, 28°10′45.96″ E, 1 238 m above sea level) during late summer between 27 March and 21 April 2011. The meshes replaced the standard white polyester netting around the light source of three 220 V down-draught Onderstepoort suction light traps equipped with 8 W, 23 cm black light tubes (Venter et al., 2009).

The alphacypermethrin-treated HDPE 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. The mesh was immersed in alphacypermethrin suspension for 30 min, air dried overnight at 20 °C and 65% relative humidity (RH), then kept wrapped in tin foil prior to being attached to the light trap with elastic bands. A new mesh was prepared each day for each of the traps. Meshes were removed from the traps 14 h after application. Alphacypermethrin uptake was quantified by high performance liquid chromatography (HPLC) analysis of a duplicate mesh sample prepared for each replicate. The traps were placed in a row 6.5 m apart to minimise interference between traps (Venter et al., 2012), 2.0 m above the ground, and operated from before sunset (18h00) to after sunrise (06h00). Catches were made into 500 ml plastic beakers containing 200 ml 0.5% Savlon® (Johnson and Johnson, South Africa) and water solution. Overnight insect collections were stored in 70% ethanol prior to the total number of *Culicoides* specimens and the number of *C. imicola* being determined. Climatic variables (outside temperature, RH, wind speed, rainfall) were recorded hourly using a weather station (Vantage Pro2, Davis, USA) and data logger (Weatherlink, Davis, USA).

2.2. Contact bioassay

The *in vitro* insecticidal efficacy of meshes against field-collected, nulliparous (unpigmented) female (Dyce, 1969) *C. imicola* was assessed in a contact bioassay conducted between 23 and 27 April 2012. The midges had been collected the previous night in Onderstepoort light traps at the ARC-Onderstepoort Veterinary Institute (25°39′ S, 28°11′ E; 1 219 m above sea level) and were handled prior to the assay as previously described (Venter et al., 1998).

In the absence of laboratory colonies to standardise the physiological age of the midges only nulliparous *C. imicola* were used in the assays. Midges were immobilised for 1 min at −4 °C and groups of 10 nulliparous females aspirated

into glass Pasteur pipettes on a refrigerated chill table. At the start of each exposure experiment, the 10 nulliparous females were expelled into one of 60 mm × 12 mm glass Petri dishes (Anumbra, Lasec, South Africa) and exposed to a 19.6 cm^2 alphacypermethrin-treated HDPE mesh (prepared as described for the light trap assay) for 1 or 3 min, or an untreated control HDPE mesh for 3 min. The meshes were attached to the petri dish by a small disc of adhesive putty (Prestik®, Bostik, South Africa). To maximise contact with the mesh the dishes were gently inverted every 30 s. Following the exposure period midges were immobilised for 1 min at −4 °C and the petri dish cover with the attached mesh was replaced with a clean cover to allow the midges to recover without residual exposure to insecticide. Mortalities were visually assessed at 5, 10, 30, 60 min and 24 h post exposure to the mesh by an entomologist blinded to the treatment group. Midges that were motionless or twitching and incapable of oriented movement were classified as dead. Following the 60 min assessment midges were maintained overnight at 24.5 °C and 60% RH until the final efficacy assessment at 24 h. Thirty replicates of each exposure period were conducted.

2.3. Statistical analyses

Statistical analyses were done using SPSS® Statistics version 21 (IBM, USA). For the light trap assay midge numbers were natural logarithm-transformed to achieve normality. Mean numbers of *Culicoides* midges and *C. imicola* were compared between treatment groups while controlling for the effects of light trap location and day using analysis of variance (ANOVA). Where *F*-ratios were significant, pairwise comparisons were done between treatment groups using Tukey's HSD test. Homogeneity of variances was tested with Levene's test. Statistical testing was conducted at the 5% level of significance.

Contact bioassay midge mortality, corrected for mortality in the untreated control group with Abbott's formula [Mortality % = $100 \times (X - Y)/Y$; where X = % survival in the untreated control, Y = % survival in the treated sample] (Abbott, 1925), was compared between groups at each time point 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.

3. Results

3.1. Light trap assay

A total of 37 221 *Culicoides* midges were collected in 36 collections made over 12 nights from three light traps operated simultaneously. *Culicoides imicola* was the most abundant species and comprised 96.1% of midges collected. The mean number of both *Culicoides* midges and *C. imicola* collected with the control trap were significantly higher than the alphacypermethrin-treated HDPE mesh ($P = 0.001$ for both) and the untreated HDPE mesh light trap ($P < 0.05$ for both) (Table 1). The smaller total number of *Culicoides* or *C. imicola* collected with the alphacypermethrin-treated HDPE mesh was not significantly different ($P = 0.656$) from that of the untreated HDPE mesh. The proportion of *C. imicola* in relation to the other species collected ranged from 97.1% with the untreated HDPE mesh to 95.9% in the control trap and was not significantly different between treatments ($\chi^2 = 1.717$, $df = 2$, $P = 0.424$) (Table 1).

The mean ± S.D. alphacypermethrin uptake by the treated HDPE meshes as determined by HPLC analysis was 36.3 ± 12.3 mg/m^2, within the target range of 20–40 mg/m^2. The mean ± s.d. outside temperature, RH, wind speed, and rain during the light trap collection were 18.9 ± 2.2 °C, 79.7 ± 7.2%, 1 ± 0.4 km/h, and 7.2 ± 16.4 mm, respectively.

3.2. Contact bioassay

The median percentage efficacy of the alphacypermethrin-treated HDPE mesh in both the 1 min and 3 min exposure groups was significantly ($P < 0.001$) higher than the untreated control HDPE mesh at all the time points tested (Table 2). Median *C. imicola* mortality was 100% from 30 and 10 min following exposure to the alphacypermethrin-treated HDPE mesh of 1 and 3 min, respectively. The median mortality was significantly higher ($P = 0.016$) in the 3 min alphacypermethrin exposure group (74.8%) compared to the 1 min exposure group (59.5%) at the 5 min time point only.

4. Discussion

The HDPE mesh had a significant effect in reducing the numbers of *Culicoides* midges, predominantly *C. imicola*, collected by the light trap. The magnitude of reduction for the untreated HDPE mesh and alphacypermethrin-treated HDPE mesh was 4.2 and 7.2 times, respectively, with efficacy likely related to the smaller hole size of the HDPE mesh, compared to the untreated control polyester mesh. Whilst the light trap with the alphacypermethrin-treated mesh consistently collected fewer *Culicoides* midges than the untreated HDPE mesh, a significant repellent effect was not demonstrated. This is in agreement with a previous study at Onderstepoort where no repellent effect against *Culicoides* midges was demonstrated for a different alphacypermethrin formulation tested at a lower concentration (0.3%) (Page et al., 2009). However, a limitation of using light traps alone to screen mesh for repellent and insecticidal efficacy against *Culicoides* midges is that the relative strong attractant effect of the light may override any repellent effect (Page et al., 2009; Venter et al., 2009) and a large mesh hole size may not allow sufficient contact with the insecticide (Del Río et al., 2014). Paradoxically, a potential benefit of the lack of a repellent effect of alphacypermethrin against *Culicoides* midges is that it could increase (or at least not decrease) the numbers of midges coming into direct contact with treated mesh, and thereby promote insecticidal efficacy.

Exposure of *C. imicola* to the alphacypermethrin-treated HDPE mesh in the contact bioassay resulted in a rapid insecticidal effect. The initial magnitude and rate of inducing mortality was greater after 3 min exposure compared to 1 min exposure of midges to the mesh. Subsequently, at

Table 1
Mean ± S.E. number of *Culicoides* midges and *C. imicola* collected by three black light traps fitted with untreated HDPE mesh, alphacypermethrin-treated (36.3 ± 6.1 mg/m^2) HDPE mesh, or untreated control polyester mesh, operated overnight for 12 nights between 27 March and 21 April 2011.

	Mesh treatment		
	Untreated HDPE mesh	Alphacypermethrin-treated HDPE mesh	Untreated control polyester mesh
Culicoides midges	539 ± 218[a]	314 ± 102[a]	2 249 ± 763[b]
C. imicola	523 ± 214[a]	303 ± 99[a]	2 156 ± 743[b]
% *C. imicola*	97.1	96.3	95.9

Values within each row with a different superscript differ significantly ($P < 0.05$).

30 min post exposure both groups had reached maximal effect which was maintained up to the final assessment at 24 h. A similar contact assay and exposure period to that reported by Papadopoulos et al., 2010 was used. The aim of the present study was, however, to assess the potential of alphacypermethrin-treated meshes for protecting containerised transport systems of horses, and not to screen the potential for direct treatment of horses with alphacypermethrin. Therefore it was elected to use a treated mesh, instead of hair clippings, for exposure of midges. Although the contact bioassay was done with nulliparous females, the results would not be expected to be different for parous females or males, however this was not investigated. As male midges are not generally abundant in light trap catches near hosts (Venter et al., 2009) the light trap results could vary for males.

Although mortality in the bioassay treated groups was consistently significantly higher than that of the control group, a study limitation was the relatively high mortality (up to 57% after 24 h) recorded in the control group. This was likely related to the use of field-collected midges and experimental handling procedures. Dehydration during the holding period and the effect of chilling (Nunamaker et al., 1996) as an immobilising method may have contributed to the high mortality in the control group. Improved holding conditions and alternate immobilisation methods such as CO_2 (Meyer and Schmidtmann, 1979) need to be evaluated for use in future insecticide bioassays against *Culicoides* midges.

The alphacypermethrin formulation used is recommended by the World Health Organization Pesticide Evaluation Scheme for treatment of mosquito nets as well as indoor residual spraying (Banek et al., 2010) and efficacy and safety has been demonstrated for malaria control (Ansari and Razdan, 2002, 2003). However, welfare concerns associated with reduction of airflow and animal confinement, as well as practicality restricts the use of completely enclosed structures for housing livestock species and combinations of protective measures against *Culicoides* midges may therefore be required (Calvete et al., 2010). On the other hand, in horses (which are generally more accustomed to stabling) the use of complete barriers may be more feasible, providing that ventilation is not compromised. For example, a South African field study found that closing stables by meshing with gauze cloth resulted in a 14-fold reduction in the numbers of *C. imicola* and *Culicoides* (*Avaritia*) *bolitinos* Meiswinkel entering the stables (Meiswinkel et al., 2000).

In conclusion, the worldwide increase in the transportation of animals and products in freight containers may increase the risk of viruses being transported (Reiter, 2010). Whilst the available data on *Culicoides* midge involvement in vector-borne pathogen importation *via* transport networks is limited (Carpenter et al., 2009; Napp et al., 2013), standardised measures such as those recommended by the OIE for AHSV (World Organisation for Animal Health, 2013) should be in place to ensure the safe transportation of animals, especially if these animals are being moved through known infected areas or areas with unknown risk. Based on the positive results of the present study, HDPE mesh has potential to reduce exposure of housed horses to *Culicoides* midges, specifically *C. imicola*, during high risk periods for AHSV transmission, or during containerised transport. Additionally, treating this mesh with alphacypermethrin may increase the overall field efficacy thereof in reducing *Culicoides* midge biting rates. Meanwhile, further investigation of the specific mesh in reducing midge biting rate under field conditions and the effect of the mesh on ventilation of housing is required.

Table 2
Median (IQR) percentage mortality of field-collected nulliparous (unpigmented), female *Culicoides imicola* exposed to alphacypermethrin-treated HDPE mesh for 1 or 3 min, or to an untreated control HDPE mesh for 3 min in a contact bioassay. Values for the alphacypermethrin-treated HDPE mesh exposure have been corrected with Abbott's formula. Thirty replicates of each exposure period were conducted between 23 and 27 April 2012.

Time post exposure	Exposure		
	Alphacypermethrin-treated HDPE mesh		Untreated control
	1 min	3 min	3 min
5 min	59.5[a*] (35.6–80.3)	74.8[b*] (62.8–100)	11.1[c] (0.0–29.8)
10 min	84.7[a] (72.0–100)	100[a] (82.5–100)	12.5[b] (10.0–29.8)
30 min	100[a] (100–100)	100[a] (100–100)	11.1[b] (0.0–38.1)
60 min	100[a] (100–100)	100[a] (100–100)	20.0[b] (10.0–29.8)
24 h	100[a] (100–100)	100[a] (100–100)	57.1[b] (36.5–77.8)

Values within each row with a different superscript differ significantly ($P < 0.001$).
[*] $P = 0.016$.

Conflict of interest statement

The authors declare no conflict of interest.

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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 220 V downdraught black light traps in four blocks of a 3×2 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.

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 et al., 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

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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 (25°38′51.42″ S, 28°10′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 × 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 × 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 to 06h00 for six nights and thereafter around sunset (17h24–17h27) from 16h00 to 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 × 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 customised 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 3×3 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, 2014).

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)	36,075 (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	36,412

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 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).

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

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	–
	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 ($P < 0.05$).

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., 2011; 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

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	17,026	99.0	19,049	99.1
	Mean ± S.E.	1,419 ± 617	–	1,587 ± 646	–
	Nulliparous	13,040	76.6	14,668	77.0
	Parous	3,809	22.4	4,212	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
	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	17,193	–	19,219	–
	Mean ± S.E.	1,433 ± 623	–	1,602 ± 653	–
	Shannon Wiener index	0.1	–	0.1	–

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., 2000, 2004), 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 Organisation 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.

Conflict of interest statement

The authors declare no conflict of interest.

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Animal Ethics Committee

PROJECT TITLE	**Protection of horses against *Culicoides* biting midgets during international air transit**
PROJECT NUMBER	**V011-14**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. P Page**

STUDENT NUMBER (where applicable)	**882 219 04**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Equine**	
NUMBER OF ANIMALS	**6**	
Approval period to use animals for research/testing purposes		**17 March – 30 May 2014**
SUPERVISOR	**Prof. AJ Guthrie**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 24 February 2014
CHAIRMAN: UP Animal Ethics Committee	Signature