THE THREAT OF AFRICAN HORSE SICKNESS VIRUS IN THE UK: FURTHERING OUR UNDERSTANDING OF VECTOR BIOLOGY AND HOW BEST TO PROTECT HORSES IN THE EVENT OF AN OUTBREAK

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By

Matthew George Robin

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ABSTRACT

Recent changes in the global distribution of several vector-borne viral diseases have been linked to climate change and globalisation, leading to concerns that they will increasingly threaten northern Europe and the UK. African horse sickness (AHS) is a vector-borne viral disease of equids that is spread by *Culicoides* midges. The disease is associated with up to 95% mortality in naïve populations of horses and an outbreak in the UK would therefore have devastating consequences for both animal welfare and the equine industry. AHS has never been reported to occur further north than Spain, however it has been suggested that appropriate midge species and climatic conditions are now present in northern Europe to support an outbreak.

Given the integral role of the vector in AHS epidemiology, data describing the distribution of *Culicoides* species throughout the UK is key to predicting the risk and potential spread of a disease outbreak. It is suspected that *Culicoides* species of the Obsoletus and Pulicaris groups would be most likely to act as vectors; however, only very limited data exist regarding the *Culicoides* species present on UK equine properties. Chapter 2 of this thesis aimed to describe the species of *Culicoides* collected by light-suction trapping on equine properties in the UK. Fourteen equine properties were identified and collection took place at each overnight for 3 sessions. The study identified the presence of potential AHS virus (AHSV) vector *Culicoides* species on both urban and rural equine properties within the southeast UK. PCR analysis revealed that engorged members of these species contained equine DNA, proving a direct vector-host interaction.

Current recommendations for preventing the spread of AHS are limited. DEFRA currently suggest using topical deltamethrin for AHS control in the face of an outbreak; however, no data is available regarding its efficacy in the horse. The aims of Chapter 3 of this thesis were to investigate the effect of topical deltamethrin on the blood-feeding of *Culicoides* on horses and to investigate which species appear to preferentially blood-feed on horses. Three pairs of horses were placed in partially enclosed cages, which allowed samples representing the *Culicoides* interacting with individual horses to be collected. Four collection sessions were run before 1 horse from each pair was topically treated with 1% deltamethrin and another 4 sessions were completed. Collected *Culicoides* were identified and each midge examined to see if it had blood-fed. There was no

significant effect of treatment on blood-feeding by *Culicoides*. The most abundant species collected were from the Obsoletus (44.3%) and Pulicaris (34.7%) groups. These species were also more likely to have blood fed than other species, supporting their potential role as AHSV vectors if the virus were to reach the UK.

LIST OF ABBREVIATIONS

AHS	African horse sickness
AHSV	African horse sickness virus
BT	Bluetongue
BTV	Bluetongue virus
BF	Blood-fed
CDC	Centre for Disease Control
IBH	Insect bite hypersensitivity
NBF	Non blood-fed
OVI	Onderstepoort Veterinary Institute
PCR	Polymerase chain reaction

CHAPTER ONE

LITERATURE REVIEW

INTRODUCTION

African horse sickness is an infectious, non-contagious, insect-borne viral disease of equids that is endemic in the central tropical region of Africa and is associated with mortality rates up to 95% in naïve populations [1]. Due to the combination of high mortality and the ability of the virus to expand out of its endemic area without warning, the World Organisation for Animal Health classifies AHS as a listed disease. Possible references to AHS have been found from ancient times; however, the first recorded outbreak occurred in imported European horses in Africa in 1719 [2]. AHS virus (AHSV) is a member of the genus Orbivirus (family Reoviridae) and consists of nine different serotypes [3]. The principal vectors for transmission of AHSV are almost certainly *Culicoides* species biting midges, which are ubiquitous on farms throughout most of the inhabited world [4, 5]. The geographical distribution and seasonal occurrence of AHS is entirely dependent on those of the vector; therefore understanding the dynamics and behaviour of *Culicoides* is essential to understanding and predicting possible outbreaks in the UK [6].

It has been suggested that recent changes in the global distribution of several vector-borne viral diseases, including AHS and bluetongue (BT), may be associated with climate change and globalisation [7]; particularly the transportation of animals and animal products. This had led to concerns that these diseases will increasingly threaten northern Europe and the UK [8, 9]. The occurrence of the first BT outbreak in the UK in 2007 was of particular concern as this disease is closely related to AHS.

LIFE CYCLE AND PATHOGENESIS OF AFRICAN HORSE SICKNESS

In the field, AHSV is transmitted almost exclusively via the bites of *Culicoides*, although disease associated with AHSV has also been described in humans following nasal exposure to virus from broken vaccine vials and in dogs following ingestion of virus infected meat [10, 11]. More recently, disease has been reported in a dog with no history of eating infected meat [12]. Following viral inoculation, there is a period of viral replication within the regional lymph nodes of the bite area, before haematogenous dissemination throughout the body to multiple target tissues occurs (in particular the lungs, spleen, other lymphoid tissue and endothelial cells). Virus multiplication in these tissues then gives rise to a secondary viraemia (intrinsic incubation) of varying duration and titre, depending upon a number of host and serotype factors [13]. The underlying

pathology of AHS in the target organs is vascular endothelial damage and subsequent effusion, cardiovascular compromise and haemorrhage. Four different forms of disease are recognised, depending on the target organs and severity of disease: the peracute pulmonary form (Dunkop), which is characterised by rapidly progressive respiratory failure and death within hours (*figure 1.1)*; the cardiac form (Dikkop), which is associated with abdominal pain, oedema of the head and neck and heart failure (*figure 1.2)*; the mixed form, where both cardiac and pulmonary signs occur to some degree; and the final form, horsesickness fever, which is associated with a mild fever that may be subclinical and is seen only in reservoir species (discussed later) and partially immune horses [13]. Mortality rates of 70-95% have been reported, with the highest rates seen in naïve populations, therefore an outbreak in the UK could be devastating [14].

Figure 1.1: A case of the pulmonary form of AHS showing frothy fluid at the nostrils associated with acute respiratory failure and death.

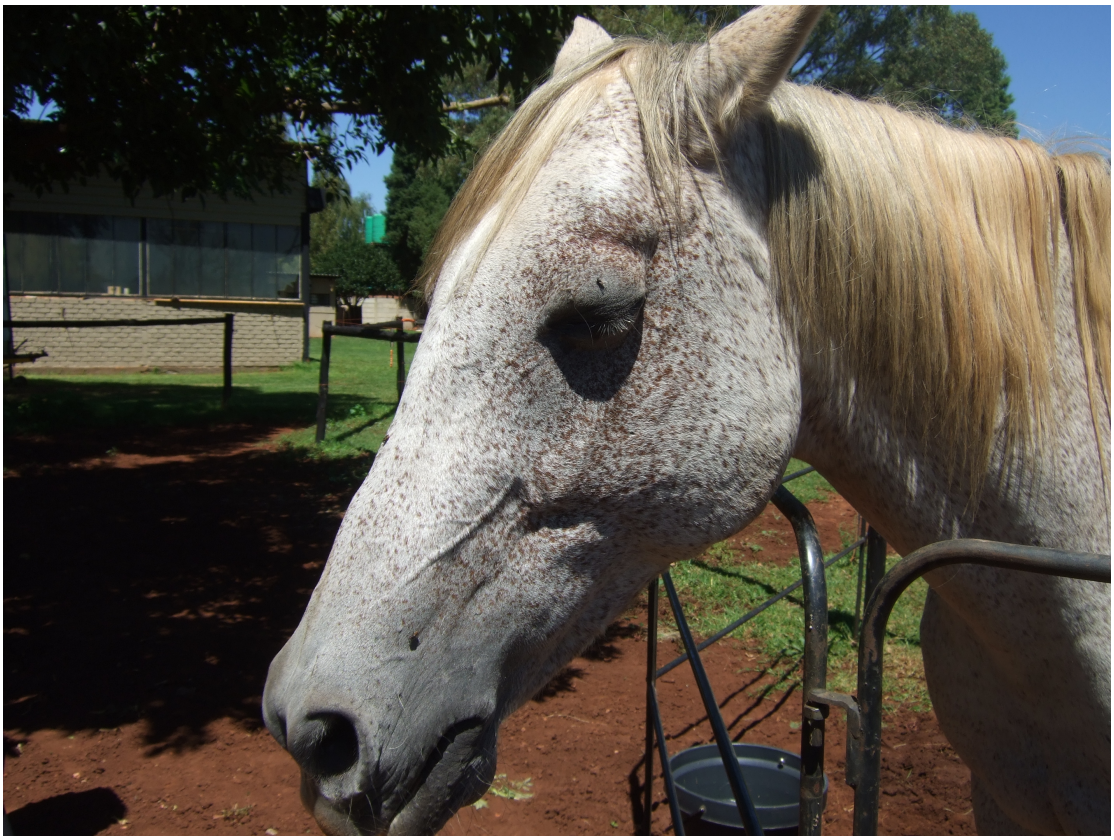

Figure 1.2: A case of the cardiac form of AHS showing oedema of the supraorbital space and head.

Vector infection occurs when a midge feeds on a viraemic vertebrate host. In horses, the viraemic phase typically lasts only 2-8 days; however, reservoir mammalian host species may have a more prolonged period of infectivity [15]. Following ingestion by the midge, the virus must translocate from the insect gut to the salivary glands and replicate in sufficient numbers to allow successful infection of the next mammalian host [5]. The stages within the midge make up the extrinsic incubation period.

GLOBAL DISTRIBUTION AND EPIDEMIOLOGY OF AFRICAN HORSE SICKNESS

There are nine antigenically distinct serotypes of AHSV and all are endemic in sub-Saharan Africa. However, outbreaks of various serotypes have occurred outside of this region [13]. Major epizootics associated with AHSV-9 have been reported in the Middle East, India and Asia [16, 17]. Epizootics have also occurred in European countries (Spain and Portugal), both in 1966 and more recently in 1987-91 when an outbreak of AHSV-4 occurred in Spain, Portugal and Morocco [18]. Prior to this, all outbreaks outside the endemic region had been associated with AHSV-9 only, and there have now also been epizootics caused by serotypes 2, 4, 6, 7, 8 and 9 in more northern parts of Africa [19, 20].

Outbreaks outside the endemic region are often short-lived; however this was not the case during the most recent outbreak in the Iberian Peninsula. In this case, the diagnosis of AHS was first made in September 1987 and official eradication was declared by December, as it was assumed that the virus was incapable of overwintering in Europe. However, a series of more severe outbreaks then occurred elsewhere in Spain, commencing in October 1988, demonstrating that this assumption was incorrect [18]. During the course of the 4-year AHSV-4 outbreak in the region, there was no evidence of any other AHSV serotype within 2000km that may have acted as a different virus source (an outbreak of AHSV-9 did occur in Saudi Arabia in 1989) and it therefore appears that the virus was able to overwinter at least 4 times [13, 21]. Theoretically, AHSV can only survive either within the vector or within a vertebrate host and potential mechanisms are outlined below.

Vertical transmission within *Culicoides* is a possible overwintering mechanism. Until recently there was no evidence that transovarial or venereal transmission of arboviruses occurred in any *Culicoides* species, meaning that the transmission and persistence of AHSV must rely entirely on a cycle between susceptible vertebrate hosts and vectors [5, 22]. The demonstration of BT virus (BTV) RNA within *Culicoides* larvae and pupae supports a theory that the virus could survive in overwintering larvae and become active the following year [23]. However, this is only weak evidence and there is no evidence at all to support this occurring with AHSV [24]. Another theory relates to the increased lifespan of adult *Culicoides* at lower temperatures (discussed later). It has been suggested that if winters are relatively mild and short, it may be the case that infected midges could survive from one season to the next [25].

Given the rapid progression and high mortality rate of AHS in horses, it is highly unlikely that the virus is able to overwinter in this species, certainly in naïve populations. Reservoir species provide a more plausible mechanism, as AHSV infection in these species is usually associated with lower mortality and prolonged viraemia, increasing the likelihood of viral survival and transmission to vectors [13]. Historically, zebra were considered the reservoir host for AHSV and their role in the epidemiology of the disease in South Africa has been well documented [26]. The abilities of AHSV-9 and AHSV-4 to persist in West Africa and Spain, where there are no zebra herds, suggests that other species may be able to act as reservoir hosts. Donkeys have been suggested as potential reservoir hosts and have been shown to become viraemic following inoculation with

virulent AHSV-4 in the absence of clinical signs [27]. The longest reported viraemia in vertebrate hosts appears to be in the zebra, at around 6 weeks [26]. In order to maintain AHSV in an area, there must be enough reservoir hosts to provide susceptible foals to become infected and the climatic conditions must support the required numbers of competent vectors [26, 28]. While the minimum size of a reservoir herd is unknown, the incidence of AHSV is much lower in areas of South Africa where zebra herd sizes are less than 100 [26]. In 2009 there were around 300 zebra and 10,000 donkeys in the UK, with over half of the donkeys housed at 8 sites belonging to a single charity [29]. It therefore seems possible, though unlikely, that an adequately sized donkey herd exists in the UK to allow maintenance of a continuous AHSV presence if vector conditions were appropriate.

THE ROLE OF *CULICOIDES* BITING MIDGES

When considering the potential risk of AHS to the UK, the dynamics and behaviour of the *Culicoides* species present and the possible effects of climate change on these species must be considered. When discussing the risk of AHSV to the UK, comparisons are often made with BTV, with is closely related. Both viruses are endemic in tropical regions of Africa, where they share the same *Culicoides* vector species, and both have made incursions north into Europe [4, 7, 30]. Bluetongue is vastly more widespread than AHS and this is likely to reflect differences in the size of the susceptible host population, particularly reservoir species. Interestingly the steady decline in the number of AHS cases in South Africa over the last century, while most likely due to increased vaccination, also coincides with a reduction in zebra numbers [13].

Culicoides midges (*figure 1.3*) are among the world's smallest and most widespread insects. They are considered a biting nuisance to humans and livestock, transmit viral and parasitic diseases and are the major cause of insect bite hypersensitivity (IBH) in horses, which is thought to be triggered by *Culicoides* salivary antigens [31]. There are currently over 1400 different species of *Culicoides* identified, with around 30 of these thought to be capable of virus transmission and over 50 different viruses isolated from midges worldwide [5, 24, 32]. The taxonomy of the Western Palaeartic region *Culicoides* is based on several morphological traits. The wing pattern in particular is very important, with variations in venation, colour, marking pattern and covering by short hairs used for differentiation (see *figure 1.4*). Other features, including thoracic

Figure 1.3: A single Culicoides midge shown blood feeding. The Pirbright Institute

colouring, antennae and abdominal spermathecae, are also used [33, 34]. Unfortunately, identification can require a highly specialised knowledge of insect morphology that is no longer readily available [35, 36]. The members of the Obsoletus group are only readily distinguishable using the morphological traits (terminal genitalia) present in adult males (which play no part in arboviral disease transmission) and the wing patterns of members of the Pulicaris group overlap considerably [37]. It is possible to achieve some separation of female members of both groups as shown in *table 1.1*. Given the potential importance of several of these species in arboviral transmission (discussed below), polymerase chain reaction (PCR) assays have recently been developed to provide rapid and accurate identification [36].

Figure 1.4: Wing patterns of members of the Palaeartic Obsoletus (top) and Pulicaris (bottom) groups. Images not to scale. www.iikculicoides.net

Subgenus	Species group and female morphology	Species	Comments on morphological identification of females
Avaritia	**Obsoletus** Readily identified from Pulicaris group based on wing pattern and smaller size of Obsoletus group individuals	***C. chiopterus***	Smaller size than other Obsoletus group species and paler wings.
		C. dewulfi	Very similar morphology to other Obsoletus group species and variable size and wing pattern but can be identified
		C. obsoletus	Cannot readily be distinguished from *C. scoticus.* Can be distinguished from *C. chiopterus* and *C. dewulfi.*
		C. scoticus	Cannot readily be distinguished from *C. obsoletus.* Can be distinguished from *C. chiopterus* and *C. dewulfi.*
Monoculicoides	**Pulicaris** Readily identified from Obsoletus group based on wing pattern and larger size of Pulicaris group individuals	***C. pulicaris***	Can be separated from *C. punctatus* by wing vein tip appearance, although a clear distinction can be difficult.
		C. punctatus	Can be separated from *C. pulicaris* by wing vein tip appearance, although a clear distinction can be difficult.

Table 1.1: classification of important potential AHSV vector species of Culicoides in the UK [37]

The life-cycle of *Culicoides* includes the egg, 4 larval stages, the pupa and the adult. The egg and immature stages are water dependent and a wide range of possible breeding sites have been described, including ponds, streams, marshes, bogs, tree holes, leaking water pipes, animal dung, and rotting vegetation [38]. Importantly many of these are present around areas where horses are commonly housed. The duration of the larval stages varies from 4 days to several weeks depending on climatic conditions and many species overwinter as 4th stage larvae [39]. Only female adults blood feed and therefore only these are of interest when considering virus transmission. The ability of a vector to transmit a pathogen under field conditions is termed vectorial capacity. In the case of AHSV transmission, this is determined by the *Culicoides* density in relation to the vertebrate host, the amount of *Culicoides* actually blood-feeding, the duration of the extrinsic incubation period, the vector competence and the life expectancy of the insect [40]. All of these factors are significantly affected by environmental and climatic conditions and the effects of changing global climate must be considered for each.

ENVIRONMENTAL EFFECTS ON THE VECTORIAL CAPACITY OF *CULICOIDES* SPECIES FOR AHSV AND POSSIBLE EFFECTS OF CLIMATE CHANGE

Female *Culicoides* will typically feed once for every batch of eggs that they develop (although females of a small number of species can lay their first batch without a blood

meal). Egg development occurs more rapidly at higher temperatures and the blood-feeding rate therefore increases at higher temperatures [41, 42]. For viral transmission to occur the midge must then survive for enough time to allow extrinsic viral replication to occur and another blood feed must then take place. The extrinsic incubation period of AHSV in *Culicoides* species is temperature dependent, with reduced times occurring at higher temperatures (around 25°C) and no viral replication occurring below 15° [43, 44]. The adult lifespan of *Culicoides* appears to be inversely related to temperature [43]. At low temperatures, survival times of up to 3 months have been recorded and at temperatures above 20 °C, high rates of mortality lead to a shorter average lifespan [43]. Overall, the reduced extrinsic incubation period seen at higher temperatures (27-30°C) more than makes up for the associated reduction in lifespan, increasing the vector capacity [45].

The ability of many *Culicoides* species to act as effective vectors appears to be primarily related to abundance, with the probability of any individual vector species midge transmitting BTV predicted to be as low as 1 in 35 000, although this was specifically calculated for *C. brevitarsis* in Australia and it is thought that the probability may be even lower for many other species [46]. It was suggested that during the 1999 BT outbreak in Bulgaria, the ability of Obsoletus group *Culicoides* to transmit disease was increased by high abundance and survival rates [47]. Rainfall increases adult abundance by providing suitable breeding grounds [5]. The annual precipitation rate in the UK is expected to increase and this might therefore be expected to increase midge abundance, although the relationship may well be more complicated, particularly as only winter rainfall might actually increase [48].

It has been predicted that the effects of climate change will result in UK temperatures continuing to rise by at least 0.2°C per decade for the foreseeable future [49]. It has been demonstrated that the proportion of *C. sonorensis* that is capable of transmitting AHSV increases with temperature and that *Culicoides* species traditionally considered non-vectors of AHSV have increased susceptibility to infection if raised under warmer conditions [44]. It has been hypothesised that this is due to changes in the gut of the midge that allow the virus to pass more easily to the salivary glands prior to transmission: the so called 'leaky gut phenomena' [44, 50]. This effect is very important when considering which species may be able to act as vectors of AHSV in the UK.

The effects of temperature on midge abundance and disease do appear to vary by species. In Spain, the abundance of *C. imicola* is positively correlated with temperature and the abundance of *C. imicola* correlates with outbreaks of AHS and BT in this region [30]. Conversely, modelling has predicted that the abundance of Obsoletus group *Culicoides* will decrease towards 2050 due to the increase in temperature as it appears more abundant in cooler locations [9]. Additionally, higher temperatures have been shown to increase mortality rate and decrease life-span in *Culicoides* [51].

POSSIBLE SCENARIOS FOR AN AHSV OUTBREAK IN THE UK

There are 2 different scenarios that must be considered when examining the possibility of an AHS outbreak in the UK:

1 - A CHANGE IN DISTRIBUTION OF TRADITIONAL AHSV VECTOR SPECIES

It is possible that the effects of climate change may alter the distribution of the traditional vectors of AHSV, allowing them to become established further north and effectively taking the virus with them. In Africa, *C. imicola* is considered the principal vector of AHSV and makes up over 90% of species caught during light-trapping in AHS endemic areas, with *C. bolitinos* also recognised as a secondary vector in some regions [24, 52]. AHS is not known to have occurred in any parts of the world where *C. imicola* is not present, supporting the theory that an outbreak is unlikely in its absence. It has been suggested that the current northern spread of *C. imicola* observed in the Iberian Peninsula could extend the distribution of *C. imicola* into central Europe by the early part of the 21st century [5, 53]. Interestingly a single *C. imicola* midge was caught in Switzerland in 2006 [54]; however, it was assumed that it's presence was a result of wind transportation from further south as no *C. imicola* were caught in a 2009 study in the same area [55]. Therefore, while this movement of the traditional AHS vectors to northern Europe and the UK is a possibility, it appears that it is not an immediate one [56].

2 - INDIGENOUS *CULICOIDES* SPECIES ACTING AS VECTORS

The second scenario is that the northern spread of AHS may not depend entirely on *C. imicola* and indigenous *Culicoides* species in northern Europe might be able to act as vectors for AHSV [25]. This could be due to inherent ability to transmit the virus or

climate change mediated effects on vector capacity similar to those previously described [7].

It is first important to consider the identification of *C.imicola* and *C. bolitinos* as the principal AHS vectors, as their role has never been definitively proven. Biting insects have long been suspected to be the cause of the spread of AHS and the disease was first induced in horses following inoculation with *Culicoides* extract in 1944 [4]. The ability of *Culicoides* to actually transmit AHSV was more convincingly demonstrated when the North American BTV vector, *C. variipennis*, was shown to be an efficient lab vector for AHSV [57]; however, transmission between live hosts has still not been demonstrated. Epidemiological studies have since demonstrated spatial and temporal associations between the abundance of *C.imicola* (as caught by light traps) and the incidence of AHS in Spain, Portugal, Morocco and South Africa [53, 58-60]. However, recent studies have demonstrated that catches obtained from light traps do not appear to correlate with either the overall biting rate or the species composition of midges found feeding vertebrate hosts [61, 62]. Therefore, it may be the case that the studies previously described underestimate the potential vector roles of other species. This theory is supported by the fact that during the 1987-1991 outbreak in Spain, AHSV-4 was isolated from non-*C. imicola* mixed pools (containing *C. cataneii, C. lailae* and the Obsoletus and Pulicaris groups) [63]. As the range of Obsoletus and Pulicaris groups currently extend much further north than *C. imicola*, which is not found in the UK, it is possible that they may be able to act as AHSV vectors in this region [63].

During the BT outbreaks in Europe, disease occurred in regions where the traditional vector could not be found [47]. A vector role of Obsoletus and Pulicaris group midges for BTV has been demonstrated, based on abundance, spatial and temporal correlation with disease outbreaks, and viral isolation from midges caught during outbreaks [64, 65]. During these outbreaks, temperatures were among the warmest since records began and this may well have significantly increased the ability of indigenous species to act as BTV vectors [66]. It should be noted that in Spain, outbreaks of AHS and BT have been correlated with the abundance of *C. imicola*, but not Obsoletus and Pulicaris groups, suggesting that the latter two are still relatively unimportant vectors in areas where the traditional vector is present [30].

Overall, the evidence suggests that members of the Obsoletus and Pulicaris groups can be considered potential vectors of AHSV in the UK and northern Europe (see *table 1.1*).

POSSIBLE ENTRY ROUTES OF AHSV TO THE UK

1 - AHSV ENTRY TO THE UK IN AN INFECTED VERTEBRATE

It is highly unlikely that the virus could enter the UK within a legally transported viraemic horse due to the stringent regulations in place and the rapid progression of the disease [67]. The UK is officially listed as AHS free by the World Organisation for Animal Health, which publishes updated regulations required for membership and import of horses from suspect areas [68]. Briefly, horses imported into the UK from countries subject to AHS restrictions must be subject to quarantine in vector proof housing for at least 40 days prior to transport and during this time AHS status is assessed by paired antibody titres and polymerase chain reaction. Post-import sampling is not routinely carried out in the UK unless deemed necessary by veterinary risk assessment; however a small percentage of randomly selected animals imported are sampled upon their arrival in the UK to monitor the certification standards of the exporting country. These regulations appear appropriate based on the disease seen in horses; however, the movement of AHSV within a reservoir species not showing clinical signs presents a more difficult problem. The importation of infected zebra from Namibia to a safari park near Madrid was reported to be the most plausible explanation for the 1987-91 outbreak of AHS in the Iberian Peninsula [18]. The longest reported viraemia in zebra is 6 weeks, thus it may be possible for an infected animal to remain clinically undetected during the 40-day quarantine [26]. Failure of the paired serology testing would also have to occur; however this may be more likely at the lower viraemic levels that occur in reservoir hosts. The risk from illegal transport of reservoir hosts is impossible to quantify.

Another role for reservoir hosts, particularly the donkey, would be in the northern spread of the virus around Europe before an outbreak was diagnosed. It is also worth remembering that in endemic regions of Africa, AHSV appears to circulate amongst native horse breeds with a far lower mortality rate than usual, with up to 96% of sampled animals seropositive in parts of West Africa [69]. Although animals such as these are rarely transported internationally, their presence reminds us that species other than zebra and donkeys should be considered when assessing reservoir hosts and sub-clinical carriers. The climatic conditions would also have to be appropriate for an outbreak to occur at the exact time of introduction. A recent risk assessment in The Netherlands concluded that equine movements between October and March posed a

negligible risk of introducing AHSV as the *Culicoides* population would be inadequate to transmit disease [70].

2 - AHSV ENTRY TO THE UK IN AN INFECTED MIDGE

There are two possible ways that a virus-infected midge could reach the UK. The first is by becoming trapped within a plane or other cargo vessel, especially those transporting vegetative materials (for example packaged flowers) from North Africa to Europe. This risk is impossible to quantify, as available data are not suitable for estimating the chance of introduction via transportation [71]. Again, the climatic conditions would also have to be appropriate for an outbreak to occur at the exact time of introduction.

The second method of virus introduction via the midge is wind dispersal. Adult *Culicoides* rarely fly further than a few hundred metres from their breeding grounds; however they may be passively dispersed over much greater distances if wind patterns are appropriate [5]. The wind dispersal of infected *Culicoides* has been implicated as the cause of the overseas spread of AHSV from Morocco to Spain in 1966 and BTV from mainland Europe to the UK in 2007 [72, 73]. For this to occur in the UK, AHSV would already have to be circulating within northern Europe (by methods previously described). Wind-based modelling to predict *Culicoides* flight activity has been demonstrated to accurately describe outbreaks of BTV within Europe and could be used to predict the likelihood of AHS outbreaks in the future [74].

3 - VACCINE COMPLICATIONS

The final way that AHSV could enter the UK is by reversion to virulence of attenuated vaccine strains. AHSV vaccinated horses can be imported into the UK and recently an AHSV strain circulating in The Gambia was thought highly likely to have been derived from a live-attenuated AHSV-9 vaccine strain [69]. The illegal importation of unregulated vaccines during an outbreak may also contribute to this. In addition, both the field transmission and re-assortment of live attenuated vaccine strains of BTV have been demonstrated in Europe, raising concerns about their use [75, 76].

CONCLUSIONS - THE RISK OF AHSV TO THE UK

Overall, it appears that climate change and globilisation have resulted in a myriad of

factors that increase the risk of AHS to the UK equine population. Changes in vector capacity mean that the potential distribution of AHSV within Europe now includes the same locations as BTV and the introduction of AHSV-infected equines or *Culicoides* could produce extensive and persistent epidemics throughout Europe [7].

CULICOIDES IN THE UK – WHAT DO WE KNOW?

Overall, very little is known about the ecological characteristics of the various *Culicoides* species that directly influence their efficiency as vectors [77]. As *C. imicola* has never been detected in the UK, the potential roles of the species making up the Obsoletus and Pulicaris groups in the spread of AHSV must be investigated, given their abundance and potential vector role [7, 47, 65, 77].

Within the UK, *Culicoides* species have traditionally received attention for 3 main reasons: their trigger role in insect bite hypersensitivity (IBH) in horses, their ability to act as a biting nuisance to man and, most recently, their role as BTV vectors.

The role of *Culicoides* species in IBH, the most common skin disease of horses in the UK, has been well researched and documented at the immunological level since their discovery as a trigger for the disease [78, 79]. In Scotland, *C. impunctatus* (a member of the Pulicaris group) has long been identified as the species responsible for human irritation and much data has been generated regarding the ecological characteristics of this species and methods of eradication [80]. Importantly *C. impunctatus* has been demonstrated to support BTV multiplication following virus ingestion, although it does not appear to have played a major role in disease transmission thus far [81]. In the Netherlands and France, members of the Obsoletus and Pulicaris groups have been shown to be the *Culicoides* species most attracted to horses and this is very concerning given their potential vector role [82, 83]. In the UK, members of these groups have been shown to comprise 93.5-97% of *Culicoides* caught on farms using light-vacuum traps [77, 84]. These investigations were carried out on rural properties housing mostly cattle and sheep. The preferential *Culicoides* landing sites on the horse and peak activity times of several species has been described, with Obsoletus group midges noted to be the most numerous [85, 86]. A more recent study on the experimental infection of Obsoletus group *Culicoides* with BT virus used *Culicoides* collected from a stable, however the exact species composition of the catches were not described [87]. Currently there appears to

be no dedicated light-suction trap based survey of *Culicoides* on equine premises in the UK.

CONTROL OF DISEASE: VACCINATION

Annual vaccination of horses is the mainstay of controlling AHS in endemic regions, with the first highly effective live attenuated vaccine produced in 1936 [15]. Vaccine administration is typically timed to occur before peak midge numbers and is given in late autumn or early spring. The vaccine contains live-attenuated forms of seven of the nine AHSV serotypes, with serotypes five and nine omitted due to the generation of cross-protection by serotypes eight and six respectively [88]. Horses that have received three or more courses of immunisation are usually well protected, although the vaccine cannot be relied upon to fully protect all horses [15]. A recent study showed that 16% of immunised horses in an AHS endemic area were infected with AHSV over a two year period [89]. As half of these cases were sub-clinically infected, they would likely have a significant impact on disease epidemiology, particularly with regard to illegal equid transportation and disease introduction. It is important to note that the methodology of the study meant that the authors could not determine if the level of viraemia seen in the sub-clinically infected horses was enough to infect *Culicoides*.

Outside of the endemic regions, vaccination has been successfully used to control outbreaks of AHS, with the 1966 and 1987-91 outbreaks in the Iberian Peninsula probably the most relevant examples to the UK [18]. Hundreds of thousands of horses were vaccinated during these outbreaks, using either a polyvalent, live-attenuated or monovalent (serotype four) vaccine [18]. The administration of AHS vaccine is currently prohibited in the UK and would result in loss of OIE disease-free status. However, vaccination would be essential to control a disease outbreak among naïve horses and the African horse sickness (England) regulations include the use of vaccination where the presence of virus has been confirmed and initial control measures (primarily movement restrictions and vector protection) have been unsuccessful [90]. According to the regulations, vaccination would cease after elimination of the virus in order to regain disease-free status.

As previously discussed, there have long been concerns about reversion to virulence of attenuated vaccine strains and recently an AHSV strain circulating in The Gambia was thought highly likely to have been derived from a live-attenuated AHSV-9 vaccine strain

[69]. Given that field transmission of live-attenuated vaccine strains of BTV has been demonstrated in Europe, this raises serious questions about the use of live AHS vaccines in the UK [76]. Alternative vaccine types, including inactivated virus and recombinant structural protein vaccines have been developed, with a recent study demonstrating that multi-serotype VP2 subunit vaccines are potentially feasible [91-93]. While not yet commercially available, these vaccines are a potentially safer alternative to the live-attenuated types, particularly for use in non-endemic countries. The availability of vaccines to use in the face of a UK outbreak is a cause for concern as the vaccine banks suggested in the African horse sickness (England) regulations have yet to be approved [90].

CONTROL OF DISEASE: PREVENTION OF VECTOR-HOST INTERACTION

The prevention of *Culicoides* blood-feeding on horses is an essential part of controlling an AHS outbreak and there are several control methods that have been recommended. These include the eradication of breeding grounds using insecticides or environmental intervention, the killing of adult midges using insecticide treatment of either host animals or the environment, the use of vector protected housing, the use of midge repellents on hosts and the use of attractants to lure and kill adult midges [94].

In the case of the Obsoletus and Pulicaris groups, there is very little data on breeding habits and host-oriented responses, making the development of control strategies difficult [94]. There are very few studies that assess methods used to prevent *Culicoides* from biting horses, making it almost impossible to determine their potential use during an AHS outbreak [95]. Much of the UK based research has focused on *Culicoides* as a biting nuisance in humans and not specifically on prevention of transmission of disease. Despite IBH being the most common skin disease of horses, the only truly effective control method for IBH is complete allergen avoidance, which has been known for over 50 years [96, 97]. While moving horses to areas without midges would be effective for preventing AHSV transfer, it is often highly impractical and potentially inappropriate during a disease outbreak. Stabling of horses has been shown to reduce the signs of IBH and has long been used to prevent AHS in Africa [85, 98]. Insect blankets with both neck and hood covers have been shown to limit the feeding rate of Culicoides on horses in The Netherlands, and the authors of this study suggested that this might be helpful to protect horses from bites of AHS-infected *Culicoides* [99]. Glucocorticoids and antihistamines have been shown to reduce some of the clinical signs associated with IBH

[100, 101]; however, they clearly have no role in preventing the spread of vector borne disease. As *Culicoides* are crepuscular, with peak activity at dawn and dusk, it is recommended that any protective effects are focused at this time, with many horses housed mid-afternoon to mid-morning in order to reduce the clinical signs of IBH [96, 102]. Unfortunately, many *Culicoides* species, including *C. obsoletus, C. scoticus, C. chiopterus* and *C. dewulfi,* have been shown to feed on sheep during the day, potentially making this method unsuitable for effective disease control [5, 103].

The effect of topical application of insecticides on *Culicoides* biting rates has been investigated and insecticidal pyrethroids are commonly used to control arthropod-borne diseases in cattle and sheep [94]. Currently DEFRA advises that the pyrethroid, deltamethrin, is the most effective product against midges, although they emphasise that it is not licensed in the horse nor specifically against midges in any species [90]. The insecticidal efficacy of deltamethrin against various *Culicoides* species appears well established in livestock. The application of 0.75% deltamethrin to shorn sheep has been shown to prevent biting by *Culicoides* [104]. A recent double-blinded field trial showed that the application of topical 0.75% solution of deltamethrin to sheep produced a significant reduction in Culicoides biting rate for up to 35 days, albeit with only small numbers caught in the study [105]. However, there is no specific information available concerning the use of deltamethrin in horses and no literature is currently available regarding the effect of topical deltamethrin on *Culicoides* biting rate in horses.

There is *in vitro* evidence that following the topical application of cypermethrin to horses, a high proportion of *C. nubeculosus* die within an hour of contact with treated horsehair [95]. The disease-control implications of this result are unclear: it appears that although the product should help to reduce the spread of the virus from an infected host, infected midges may still be able to transmit virus to a treated horse. The effect of another synthetic pyrethroid, permethrin, on the amount of *Culicoides* midges caught by tent-trapping around horses has been studied *in vivo* [106]. This study showed no significant effect of permethrin on the total midge catches or percentage of blood fed midges caught near treated horses after 24 hours. As topical pyrethroid use in UK equids would be off-licence, it is important to note that no adverse effects were reported. Interestingly, there were also data in this study to suggest that the effect of the permethrin on the midge catch may have been greater after 48 hours, and this should be taken into account in future studies. Certainly it has been shown that 4-7 days after the topical application of permethrin to sheep, there was a significant reduction in the total midge count and percentage of blood-fed midges caught using similar methodology

[107].

Assessment of the *Culicoides* blood-feeding rate was used in the in-vivo studies discussed previously as a measure of the effects of the treatment on vector-host interaction (and therefore indirectly on disease transmission). However, other methods have also been used. It has been shown that the application of 4% high cis-permethrin to horses with IBH significantly reduced the clinical signs of IBH in 86% of 43 horses [108]. This suggests that the biting rate of the midges was definitely reduced by the product; unfortunately adverse effects including itching and swollen skin were noted in this study. Possibly the most direct indication of the effects of the permethrins on the transmission of arboviral disease is a field study conducted in cattle. This demonstrated that 2-weekly application of permethrin did not reduce exposure to BTV as measured by serology [109]. However, the viral challenge was considered to be very high in this study, with many more untreated cattle nearby, and the regime used may simply have been overwhelmed [109].

Injectable avermectins are also used to control ectoparasites in many species, including the horse. Unfortunately their efficacy against different *Culicoides* species varies significantly, with toxic doses required in some cases and there is no data available on their efficacy against European *Culicoides* species [94].

The use of repellents has been recommended to control AHS and there is a vast amount of products marketed for horses that remain entirely untested. N,N-diethyl-3-methylbenzamide (DEET) has been shown to reduce the biting rate of *C. impunctatus* in humans [110]. The application of 15% DEET impregnated mesh to vacuum light traps has been shown to significantly reduce *Culicoides* catches when compared to untreated mesh [111]. Interestingly this study also showed that treating the mesh with alpha cypermethrin had no effect on the *Culicoides* catch. Therefore, the use of a repellent may be more effective than an insecticide at reducing disease transmission. Unfortunately there is *in vivo* evidence of adverse effects, including hypersteatosis and dermatosis, occurring in horses when DEET is applied topically at concentrations greater than 15% [112].

Organic fatty acid impregnated mesh has been shown to reduce the *Culicoides* catches by light-traps in South Africa; however the ability to reduce the biting rate or reduce the spread of disease has not been demonstrated [113]. Alpha-cypermethrin treatment of mesh did not appear to have a significant repellent effect on trap catches, when

compared to untreated mesh [114].

Overall, there is a paucity of *in vivo* evidence regarding the effects of both insecticides and repellents on the biting rate of *Culicoides* midges in horses. Although it is unlikely that any product would achieve complete protection from biting, their use in an outbreak situation would probably bring some protection and be achievable for horse owners. Further investigation is therefore warranted.

As previously mentioned, one of the most effective protection methods appears to be vector-proofed housing. In South Africa, the stabling of horses at night has long been identified as a way of reducing the risk of AHS [98]. However, the housing must be constructed to clearly defined specifications and there are various levels of vector proofing attainable. The behaviour of the different midge species is also important, depending on whether they display endophilic or exophilic activity [94]. It has been demonstrated that while catches of exophilic *C. imicola* are higher outside open stables than they are inside, catches of endophilic *C. bolitinos* are greater inside than out [115]. This suggests that the biting risk from endophilic species may actually be increased by housing horses in normal stables with open windows and top-doors. On UK cattle farms, catches of Obsoletus group *Culicoides* were 6.5 times greater outdoors compared to indoors [116]. This was in the absence of any specific vector proofing of the housing; however, the greatest difference was seen in those buildings where doors were closed. These results suggest primarily exophilic behaviour of the Obsoletus group; however there were still significant catches indoors. When vector proofing (closed doors and gauzed windows) was applied to equine housing in South Africa, there was a 14-fold reduction in the catch of both endophilic and exophilic species [115]. This study used only cheap and simple methods of vector proofing; however it is unclear if the 14-fold reduction would be enough to prevent disease spread in an outbreak situation in areas of high midge abundance. The use of impregnated mesh is also likely to reduce the entry of midges into animal housing as previously discussed [113, 114].

Regarding other control methods, the use of chemo-attractants to bait traps has been trialled in Scotland based on knowledge of host-location in *C. impunctatus* [117]. The host kairomones carbon dioxide and 1-octen-3-ol have been shown to attract members of the Obsoletus group in the UK, although effective use as a control method is not yet possible [118]. In Scotland it is thought to be impractical to apply insecticides or undertake habitat manipulation on sufficient scale to effectively control midges [119]. Certainly it appears unlikely that the large-scale coordinated effort required could take

place in time to reduce an outbreak. There are also concerns over the environmental effects of treatments. The covering of muck heaps on farms, which has been suggested as a smaller scale method of habitat manipulation, has been shown not to affect *Culicoides* abundance and is therefore unlikely to be an effective method of controlling arboviral disease [120].

Overall the most effective and feasible method of preventing *Culicoides* blood-feeding on horses during an outbreak of AHS appears to be vector-proofed housing. It is not currently known how many equine properties have any form of vector proofed housing available; however, it is likely to be uncommon. As it is impossible that completely *Culicoides*-proof housing could be made available for all horses, the use of other methods, including topical insecticides and repellents, would be indicated. Knowledge of the efficacy of the various products available is limited and studies are required before any can be specifically recommended based on scientific evidence.

CHAPTER TWO

INVESTIGATION OF *CULICOIDES* SPECIES ON UK EQUINE PREMISES

Work presented in this chapter has been accepted for publication during the writing of this thesis:

Robin M, Archer D, Garros C, Gardès L, Baylis M. The threat of midge-borne equine disease: investigation of Culicoides species on UK equine premises. *Veterinary Record*. 2014;174(12):301

INTRODUCTION

It has been suggested that recent changes in the global distribution of several vector-borne viral diseases may be associated with climate change and globalisation (particularly the transportation of animals and animal products) leading to concerns that these diseases will increasingly threaten northern Europe and the UK [8, 9]. It is suspected, as previously discussed, that the appropriate midge species and climatic conditions are present in northern Europe to support an outbreak of AHS, even if the traditional vector species (*C. imicola, C. bolitinos*) are not present. In the UK, members of the Obsoletus and Pulicaris groups have been shown to comprise the vast majority of midges caught on farms using light-vacuum traps [77, 84]. These investigations were carried out on rural properties housing mostly cattle and sheep. The preferential *Culicoides* landing sites on the horse and peak activity times of several species has been described, with Obsoletus group midges noted to be the most numerous [85, 86]. There has been no dedicated light-suction trap based survey of *Culicoides* on equine premises in the UK. This information is essential for modelling the potential spread of disease and in formulating evidence-based disease control strategies.

AIMS

The primary aims of the investigation were to identify the *Culicoides* species present on urban and rural equine properties in the UK and to confirm which of these had fed on horses and could therefore potentially act as disease vectors.

The variation in the *Culicoides* catches at different sites within selected properties was also investigated to assess how representative a single trap site per property was, particularly as this may be a method of assessing the risk of disease spread during an outbreak. The available *Culicoides* control methods at each property was recorded to determine the available methods of preventing vector-host interaction in the case of an AHSV outbreak. Finally, any international equine transportation on or off each property was recorded, as this represents a possible route of disease introduction, particularly if AHSV were to reach Europe.

MATERIALS AND METHODS

Trapping was carried out in and around the Newmarket area in South-East UK in May/June 2012. Newmarket was selected as the most appropriate location for several reasons. The town is estimated to be home to around 2500-3000 horses, with a similar number in studs in the surrounding countryside. This provides a unique mix of both urban and rural equine properties with important potential implications for the spread of equine disease. The stud yards and racecourses, which host some of the world's most important flat races, generate a vast amount of national and international equine movement. Newmarket's location in East Anglia is also of importance, as the first cases of BT were seen in this area in 2007 and wind dispersal of infected midges from mainland Europe was hypothesised as a possible source of the virus [73, 121]. Thus if AHSV were to enter in a similar way, Newmarket would likely be one of the first areas affected.

Two online databases [122, 123] were used to identify equine stud farms and racing yards with a CB8 postcode and these were all marked onto an Ordnance Survey map. A 12 x 12km-sized area was then divided into 25 squares and a single property within each square was randomly selected. Property owners were contacted to gain permission to carry out insect trapping on the premises. If permission was not granted then another property within the square was randomly selected until a suitable property was selected. The properties were allocated as either urban (within Newmarket and surrounded by buildings) or rural (outside the main town and surrounded by vegetation). The properties were identified by a code, based on their location in the grid map (A-E, followed by number 1-5, see *figure 2.1*)

Ethical approval was granted by the Veterinary Research Ethics Committee, University of Liverpool. Informed consent was obtained prior to data collection.

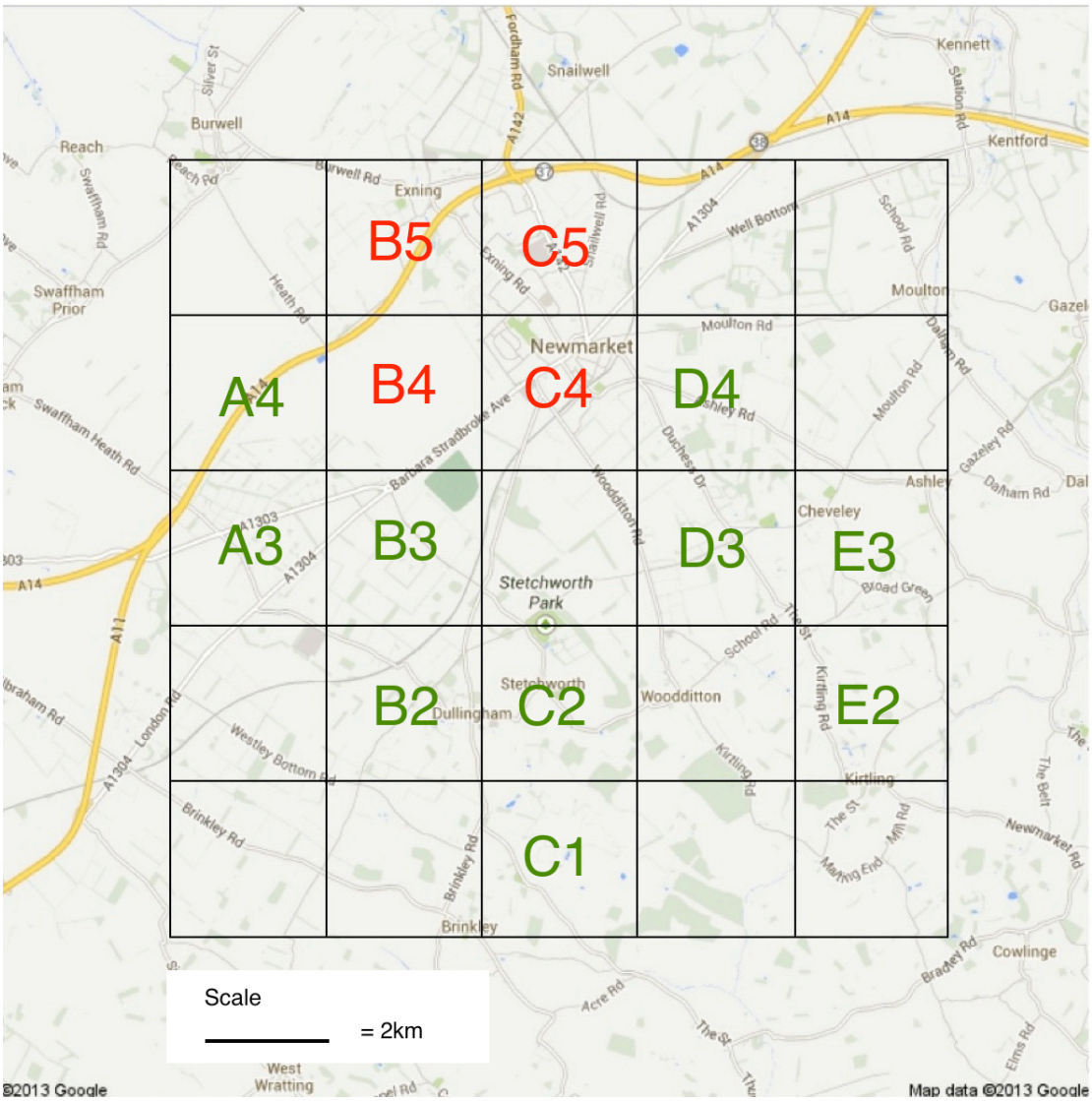

Figure 2.1. Approximate locations of selected properties. Property names in green are rural locations and those in red are urban locations. Map data © 2013 Google.

To investigate the *Culicoides* species present on the selected properties, a single trap site was selected at each. The trap site had to be less than 10m from a horse and at a height of 2m. To investigate the variation in *Culicoides* catches within a single property, three properties were randomly selected (B3, C2, C5) and five trap sites chosen at each. Again, these sites all had to be less than 10m from a horse at a height of 2m and, additionally, they could not be directly visible from one-another.

A single trap site was selected at each property situated as close to horses as possible. Ultraviolet light-emitting-diode Centre for Disease Control and Protection (UV LED CDC) traps (Bioquip[1]) were used for *Culicoides* collection (*Figure 2.2*). These traps used LED lights to attract insects, before sucking them into plastic beakers situated beneath the lights using fan-generated suction. The beakers contained 100ml of water, to which a

Figure 2.2: A CDC LED light-trap hanging in position. The LED lights act as an attractant to the vacuum above the fan, which collects them in the beaker below.

drop of detergent was added to break the surface tension and submerge the insects. The traps were each powered by a 6V battery. Overnight maximum/minimum temperature and humidity were recorded at all locations on each night using a hygrothermometer (Brannan[2]) and wind speed was also measured at the time that each trap was set.

All trapping took place during May/June 2012. Traps were set up and activated between 16.00 and 18.30 and collections were retrieved the following morning between 07.00 and 09.30. Trapping at a single site on each of the properties took place on consecutive nights until three successful (more than 10 *Culicoides* in total) catches were obtained. Trapping took place at all properties on each of these nights. The additional trapping at multiple sites on the properties B3, C2, and C5 took place over two consecutive evenings, with trapping taking place on all three properties on each night.

The collected insects were transferred to a preservative solution of 70% ethanol, before being transported to the laboratory for analysis. Individual *Culicoides* species were identified using a dissecting microscope and standard wing-pattern identification keys (*figure 2.3*)[33-35]. Male and female *Culicoides* were separated based on the increased size of males and the presence of long, feathery paired antennae on the head. As male *Culicoides* do not feed and are therefore unable to act as AHSV vectors, they were not recorded. The females making up the Obsoletus group (*C. obsoletus*, *C. scoticus*, *C. chiopterus* and *C. dewulfi*) cannot readily be individually identified using microscopic techniques. Therefore, 100 Obsoletus group *Culicoides* were randomly selected and

identified using a species-specific polymerase chain reaction (PCR) assay previously described [36]. Females were graded into pigmented, non-pigmented and engorged (*figure 2.4)*[124]. In order to identify the species origin of the blood within engorged *Culicoides*, all blood-fed Obsoletus group *Culicoides* were analysed following the PCR assays previously described [125]. Blood meal source was molecularly identified using host-species primers, including horse as a simplex PCR and cattle/sheep/goat as a multiplex PCR. Universal primers for vertebrates were also used as a control of host DNA quality and quantity.

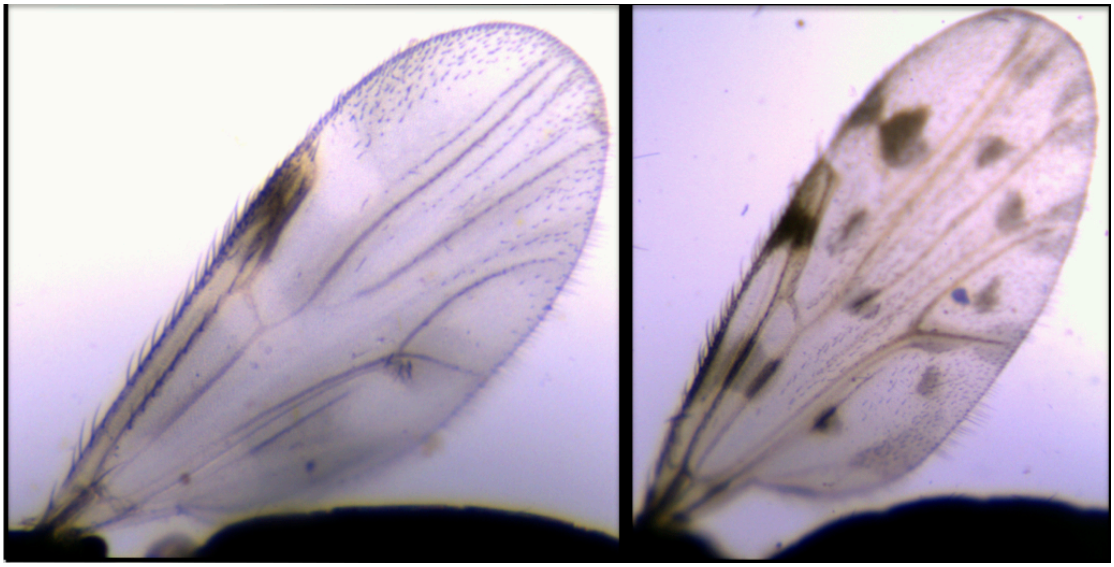

Figure 2.3: Photographs demonstrating wing patterns of Obsoletus (left) and Pulicaris (right) group Culicoides collected during the study.

Figure 2.4: Photographs demonstrating non blood-fed (left) and blood-fed (right) Obsoletus group Culicoides. The blood meal is clearly visible within the abdomen of the midge on the right.

A brief questionnaire (see appendix) was designed to record methods of vector protection utilised at each of the properties, other species housed nearby and equine movements on and off the premises.

RESULTS

The trapping initially took place at all 14 properties; however, collection was discontinued at property B2 at the owner's request. When collecting data from all 13 properties, the traps had to be run overnight on 14 nights in order to obtain the three sets of sufficient catches as planned. On the 11 nights where catches were not used the data was not considered suitable; either because there were no *Culicoides* caught at all or less than 10 in total. The data collection using multiple traps on properties B2, C3 and C5 took place over two consecutive nights as planned.

TRAPPING DATA

Culicoides were caught at least once at each of the 13 locations. A total of 1467 female *Culicoides* species were caught at the 13 sites over three nights (*table 2.1*). Of these, 99% (1452) were from the Obsoletus group. The total Pulicaris group catch was nine midges (eight *C.pulicaris* and one *C. punctatus*). Taken together with the Obsoletus group, this gave a total vector species catch of 99.6% (1461/1467). The mean individual trap catch per night over three nights was 37.6 *Culicoides*; however there was marked variation in the mean catch per trap between the three nights (2.2, 97.2 and 13.6 *Culicoides* per trap per night respectively). Meteorological data and average catch per trap are shown in *Table 2.2.* The mean individual trap catch per night over three nights was 91.0 at urban properties (or 120.3 when excluding the indoor location) and 122.4 at rural properties.

Species/group	**Female *Culicoides* catch over 3 nights**													
	A3	**A4**	**B3**	**B4**	**B5**	**C1**	**C2**	**C4**	**C5**	**D3**	**D4**	**E2**	**E3**	**Total**
Obsoletus group	400	200	102	2	63	149	13	153	143	5	37	166	18	**1451**
C. pulicaris				1				1				5	1	**8**
C. punctatus								1						**1**
C. circumscriptus	1											2		**3**
C. festivipennis											1			**1**
C. pictipennis											2	1		**3**
Total *Culicoides*	401	200	102	3	63	149	13	155	143	5	40	174	19	**1467**

Table 2.1: Total numbers of Culicoides females of each species caught at each of 13 equine properties over three nights.

	Night 1	Night 2	Night 3
***Culicoides* per property**	2.2	97.2	13.6
Wind speed (m/s)	1.48	0.04	0.09
Max. temperature (°C)	18.7	21.3	20.6
Min. temperature (°C)	11.2	12.2	10.2
Max. humidity (%)	85.5	83.4	83.1
Min. humidity(%)	41.2	49.8	49.7

Table 2.2 : Average numbers of Culicoides and average meteorological data (mean of all 13 sites) obtained during Culicoides collection

On the three properties where multiple trap locations were used there were marked variations in the *Culicoides* counts at the different locations, this was particularly evident at property B3 (*table 2.3)*.

Properties and trap number	**Total *Culicoides* species**		
	Night 1	**Night 2**	**Total**
B3-1	27	2	**29**
B3-2	23	6	**29**
B3-3	63	2	**65**
B3-4	33	1	**34**
B3-5	2	0	**2**
C2-1	2	0	**2**
C2-2	4	0	**11**
C2-3	1	0	**1**
C2-4	0	0	**0**
C2-5	0	0	**0**
C5-1	2	1	**3**
C5-2	0	0	**0**
C5-3	1	0	**1**
C5-4	0	0	**10**
C5-5	0	0	**0**

Table 2.3: Total numbers of Culicoides females caught in five traps at each of three properties over two consecutive nights.

Premises	Female *Culicoides* catches									
	Obsoletus group					Other species				
	Non-pigmented	Pigmented	Gravid	Blood-fed	Total	Non-pigmented	Pigmented	Gravid	Blood-fed	Total
A3	261	137	0	2	400	0	1	0	0	1
A4	90	107	0	3	200	0	0	0	0	0
B3	59	43	1	0	103	0	1	0	0	1
B4	1	1	0	0	2	0	0	0	0	0
B5	37	24	0	2	63	0	0	0	0	0
C1	83	64	0	2	149	0	0	0	0	0
C2	8	5	0	0	13	0	0	0	0	0
C4	92	61	0	0	153	1	1	0	0	2
C5	90	53	0	0	143	0	0	0	0	0
D3	3	2	0	0	5	0	0	0	0	0
D4	20	17	0	0	37	2	0	0	0	2
E2	78	87	0	1	166	3	5	0	0	8
E3	6	12	0	0	18	1	0	0	0	1
Total	828`	613	1	10	1452	8	8	0	0	15

Table 2.4: Culicoides females following sorting into non-pigmented, pigmented, gravid or blood-fed categories.

PCR DATA

OBSOLETUS GROUP SPECIES PCR RESULTS

Species of *Culicoides*	Amount identified	Percentage
C. obsoletus	47	**52.3**
C. scoticus	35	**38.9**
C. dewulfi	8	**8.9**
C. chiopterus	0	**0**
Total	90	**-**

Table 2.5: Results of PCR assay to identify members of Obsoletus group

90% of the selected Obsoletus group *Culicoides* were successfully amplified using PCR (*table 2.5*). *C. obsoletus* (52.3%) and *C. scoticus* (38.9%) were the most commonly identified members of the Obsoletus group, with *C. dewulfi* (8.9%) detected far less frequently. No *C. chiopterus* were identified.

SOURCE OF BLOOD MEALS

Sample	Simplex PCR Horse	Simplex PCR Birds	Simplex PCR All vertebrates	Multiplex PCR Cattle, sheep goats	Multiplex PCR: Human, goat, dog, cattle
1	Slight signal	Negative	Negative	Negative	Negative
2	Negative	Negative	Negative	Negative	Negative
3	Negative	Negative	Negative	Negative	Negative
4	Positive	Negative	Negative	Positive for sheep	Negative
5	Positive	Negative	Positive	Negative	Negative
6	Negative	Negative	Negative	Negative	Negative
7	Negative	Negative	Negative	Negative	Negative
8	Negative	Negative	Positive	Negative	Negative
9	Positive	Negative	Negative	Negative	Negative
10	Negative	Negative	Negative	Negative	Negative

Table 2.6: PCR assay identification of origin of blood meals in 10 engorged Culicoides.

A total of 10 blood-fed *Culicoides* females were collected and all were from the Obsoletus group. Species-specific PCR analysis identified the blood meal sources of three as equine, with an additional blood meal also producing a weak positive equine signal (*Table 2.6*). The blood meal of one *Culicoides* (sample four) contained both equine and ovine DNA.

QUESTIONNAIRE DATA

8/13 (62%) of the properties used some form of housing or management strategy to reduce insect bites. Rugs (5/8) and reduced turnout at dawn/dusk (4/8) were the most commonly utilised methods. None of the properties had any vector proofed housing available. International horse movement had occurred on or off of 54% (7/13) of the properties within the month prior to trapping taking place.

DISCUSSION

A total of 1467 *Culicoides* were collected at 13 equine properties over three nights in the Newmarket area in June 2012. The majority (99.5%) were species considered to be potential vector species of AHSV. Vector species were present on all properties with no apparent difference between urban and rural locations. The presence of equine blood within the potential vector species confirms that not only are these species present on equine properties, but they are also blood-feeding on the horses. These findings are of significance when considering the AHS threat to the UK, as vector–host interaction is a key predictor of a vector's capacity to transmit viral disease.

The AHS Control Strategy for Great Britain states that *'where a control notice has been served, the veterinary officer will then, as far as is reasonably possible assess places likely to facilitate the survival of the vectors, or to accommodate them, and the practicality of using appropriate vector control measures in such places … and begin an epidemiological inquiry to try to establish at least the presence and distribution of vectors'* [126]. Such an investigation would most likely be conducted using light traps in a similar way to our study.

Light traps are the standard sampling method for *Culicoides* midges when conducting epidemiological investigations and have long been used in surveillance programmes throughout the world. They provide an indication of the *Culicoides* population and abundance in an area, although it has always been unclear how the numbers, species composition and physiological status of catches relate to the *Culicoides* actually feeding on a natural host. [94]. Information obtained from light traps does not always appear to correlate well with either the overall biting rate or the species composition found feeding on sheep [61, 62]. This has not been similarly investigated for the horse and further work into the association between light-suction traps and animal-baited methods is required.

The type of light trap used also has an effect on the catch [127]. The type used in this study was battery powered and utilised LEDs, making it different to the mains-powered Onderstepoorte-style trap (OVI) more commonly used. The battery-powered trap was chosen for convenience as the traps could be quickly set up and no mains cables were required on the busy equine yards, which improved the health and safety of persons and animals on the properties. No specific information is available regarding their

performance when compared to the OVI; however there have been comparisons between the OVI and similar, smaller traps (although these utilised different types of light). In South Africa the OVI collected an average 2.5 times as many *Culicoides* as the smaller trap [127]; however there was no significant difference between the two in Europe [128]. The larger OVI trap is more sensitive at collecting less abundant *Culicoides* species and this provides important information about species variety, as the most abundant species are not necessarily the most competent vectors [127]. The importance of all of these factors is very difficult to determine, particularly as much of the evidence supporting the BTV and AHSV vector roles of certain *Culicoides* species is based on associations between disease occurrence and species abundance as measured by light trapping [53, 58-60]. Given that the ability of many *Culicoides* species to act as effective vectors appears primarily related to abundance, it seems appropriate to consider the 99.5% potential vector catch as significant [47].

There were marked differences in both the night-to-night *Culicoides* catches at each property and also the catch within individual properties on the same night. *Culicoides* counts are known to vary significantly according to climate and weather changes and higher wind speeds and lower ambient temperatures have been associated with lower Obsoletus group catches [5, 116] Over the three nights there were variations in the temperature, wind speed and humidity at each of the sites and this may well account for some of the differences in catch numbers. Night two had by far the highest mean *Culicoides* count per property and also the highest mean max/min temperatures and lowest mean wind speed. Additionally, the nights when no *Culicoides* were caught were subjectively all associated with rainfall and increased wind-speeds. The low total catch at property B4 is likely explained by the indoor location of the trap, if we assume that the majority of the *Culicoides* species caught are exophilic, although there is limited data describing the behaviour of Palaeartic *Culicoides* species. A study from Canada suggests that *C. obsoletus* are exophilic [129]; however, more recently, *C. obsoletus* have been shown to enter stables to feed [83]. Local scale modelling of *Culicoides* on non-equine properties has demonstrated significant variation in the abundance and species counts between neighbouring farms and this also appeared to be the case in Newmarket [84]. These variations in catches would have to be taken into account when conducting an epidemiological enquiry in the face of a disease outbreak.

The ability of the *Culicoides* species identified in this study to transmit AHSV is currently unknown. Members of the Obsoletus and Pulicaris groups have been identified as

potential vectors of arboviral disease in northern Europe and our findings show that these are the most abundant species on equine properties in the UK [130]. The use of the species specific PCR allowed accurate identification of members of the Obsoletus group that was previously based on highly specialised microscopic examination of morphological features of males only, very few of which were caught in the present study. Individual species identification is essential due to the variations in vector capacity that occur in individual species. Accurate epidemiological investigation and risk assessment both require accurate knowledge of the geographic distributions of individual members of the groups. Ninety percent of the midges tested were successfully identified using the PCR technique and this is similar to a previously published value of 93% [61]. *C. obsoletus* and *C. scoticus* were the most common members of the Obsoletus group identified, making up 52.3 and 38.9% respectively, and future studies should focus on the individual roles of *C. obsoletus* and *C. scoticus.* No *C. chiopterus* were identified and other studies using light traps in Northern Europe have also found relatively low numbers of this species when compared to the other Obsoletus group members [61, 84]. It has been shown that light-traps significantly underestimate the biting rate of *C. chiopterus* in sheep and the results of the present study should therefore be interpreted with caution [61].

The findings of the PCR analyses of the blood meals provides definitive proof that potential vector species are blood feeding on horses in the Newmarket area; however some of the results require further discussion. Samples four and nine produced a positive result for the horse PCR, but not for the vertebrate PCR as would be expected. The most likely reason for this is related to degeneration or digestion of the host DNA within the engorged *Culicoides*. The length of the DNA sequence amplified in the vertebrate PCR is longer than that amplified in the equine specific PCR. Therefore, it is possible that sufficient DNA degeneration may have occurred to prevent detection of the vertebrate sequence, without affecting the equine specific sequence. Degeneration is also likely to be responsible for the negative results of all PCR tests on samples two, three, six, seven and ten, as well as the reduced signal on sample one. The samples were preserved in 70% ethanol for up to six weeks prior to PCR analysis and in future this time frame should be reduced in order to minimise degeneration of host DNA. Interestingly, the blood meal origin from sample four was identified as both equine and ovine, suggesting that the midge had fed on both species. The host preference of northern European *Culicoides* has been investigated. One study showed that cattle were the preferred host of Obsoletus and Pulicaris group *Culicoides,* even if other species

(including horses) were present [131]. The blood meal origin in the majority of engorged Obsoletus group midges caught in Sweden was equine; however the midges appeared to feed opportunistically, with host selection reflecting host availability in the vicinity of the traps [132]. Another study investigating the *Culicoides* catch on sticky boards attached to a number of different host species also concluded that the horse was the most attractive host [83]. Although the total numbers were too low for accurate comparison, the results of catching in Newmarket appear to reflect this finding, with equine being both the most common blood-meal origin, despite several other species, particularly sheep, were housed on many of the properties.

The mean pigmented rate for the Obsoletus group was 57%, compared to previously published rates of 84 % in the UK [84]. The significance of this finding is difficult to determine as many different factors, including the type of light trap have been shown to affect the ratio of pigmented:non-pigmented midges collected [127]. A high pigmented rate indicates a high survival rate within the population and this is likely to enhance the transmission potential in a vector species [47]. The total percentage of blood fed females (0.7%) is similar to those found in other studies, including 2.9% [131] and 0.27% [132]. The cues that attract *Culicoides* to their hosts are essentially unknown and it is therefore difficult to predict why light traps appear not to attract large numbers of blood-fed individuals [94].

The reasons for selecting the geographical location of the trapping area (400km^2 in Newmarket, southeast UK) have been described. Relatively, this is a very small area and the accuracy of using this data to represent *Culicoides* distribution in other parts of the UK is a limitation of the study. There is known to be significant local-scale variation in Obsoletus group abundance in the UK and, although satellite data can be used to explain much of this variation, it is unclear if this would be accurate enough to model disease vector abundance on a national scale [84]. Catching on several more equine premises throughout the UK would be required to determine if this is the case.

CHAPTER TWO – MAIN CONCLUSIONS

Potential AHSV vector species were present on all investigated properties housing equines in the Newmarket area of the UK. Members of the Obsoletus group were the most abundant midges caught in light-traps (99% of total), with *C. obsoletus* and *C. scoticus* making up 52.3 and 38.9% respectively of the Obsoletus group total. Engorged Obsoletus group members contained equine derived DNA, proving a direct vector-host interaction. There was no apparent difference in the *Culicoides* catches on rural vs urban premises. No vector-proofed housing was available on any investigated property. There is potential for an AHS outbreak to occur in the UK if the virus were imported.

CHAPTER THREE

THE EFFECT OF TOPICAL APPLICATION OF DELTAMETHRIN TO HORSES ON BLOOD-FEEDING BY *CULICOIDES* MIDGES

Work presented in this chapter has been accepted for publication during the writing of this thesis:

Robin M, Archer D, Garros C, Gardès L, Baylis M. Repellent effect of topical deltamethrin on blood feeding by *Culicoides* on horses. *Veterinary Record*. 2015

INTRODUCTION

Vector-host interaction is a key factor in the spread of vector-borne diseases and the prevention of *Culicoides* blood-feeding on horses is an important part of controlling an AHS outbreak. There are several methods described to prevent midges from biting livestock, including the use of repellents and insecticides. One of the control methods for AHS that has been proposed by DEFRA is the topical application of deltamethrin to horses, although they emphasise that the drug is not licensed in the horse, nor specifically against *Culicoides* in any species [90]. The insecticidal susceptibility of *C. obsoletus* to deltamethrin has been investigated *in vitro*, with 24hr LD_{90} values between 0.00105 and 0.00203% demonstrated, consistent with high susceptibility [133, 134]. One of these studies went on to investigate the efficacy of topically applying 0.75% deltamethrin to the top-line of sheep on the mortality rate of *C. nubeculosus*, another Palearctic species of *Culicoides* [134]. In this case, despite the theoretical applied dose being around 180 times the *in vitro* LD_{50}, the maximum mortality rate of *Culicoides* feeding on the thigh of the sheep was only 45% and results were highly variable. In contrast, the topical application of 0.75% deltamethrin over all exposed skin of shorn sheep completely prevented biting by *C. obsoletus*, another Palaearctic species, although sample sizes in this study were low [104]. In a study in horses using another pyrethroid (permethrin) there appeared to be a small reduction in the number of blood-fed *Culicoides* caught near horses, however the result was not significant [106]. An extensive search of the literature failed to find any data investigating the efficacy of deltamethrin in preventing *Culicoides* from blood-feeding on horses.

As discussed in Chapter One, there is substantial evidence that Palaearctic *Culicoides* species of the Obsoletus and Pulicaris groups acted as vectors of BT virus in northern Europe from 2006 [65, 130]. Given their potential involvement during the 1987-91 AHS outbreak in the Iberian Peninsula, these species are therefore considered potential vectors for AHS in the UK [63]. Studies carried out in mainland Europe have demonstrated that members of the Obsoletus and Pulicaris groups are caught in large numbers around horses [82, 106]. However, data on the interaction of the individual species with horses in the UK are lacking. Additionally, very little is known about the feeding preferences of the individual members of the Obsoletus group (*C. obsoletus*, *C. scoticus*, *C. chiopterus* and *C. dewulfi*) for horses. A host-baited study carried out in France using a single horse showed that *C. scoticus*, *C. dewulfi* and, to a lesser extent, *C. obsoletus* were the species most commonly caught [83]. No study specifically

investigating the blood-feeding rate of different members of the Obsoletus group on horses was found during a literature search and this data is essential when considering AHSV transmission.

AIMS AND HYPOTHESES

This study had two main aims. The first was to investigate the effect of topical deltamethrin on the total number and blood-feeding of *Culicoides* caught around horses. Our hypothesis was that the application of topical deltamethrin would reduce both the total amount of potential vector species of *Culicoides* caught around horses and the percentage of these that had blood-fed.

The second aim was to investigate which *Culicoides* species preferentially blood-feed on horses to provide evidence for their potential roles as vector species. Our hypothesis was that previously identified potential vector species (members of the Obsoletus and Pulicaris groups) caught around horses would be more likely to have blood-fed than other species.

METHODOLOGY: PRELIMINARY STUDIES

The study utilised a modification of a design first used by van der Rijt *et al.* (2008) *Culicoides* were collected by a light-trap that was hung within a midge-proof netting that partially enclosed a horse. The horse and netting were both enclosed within a cage (see *figures 3.2-3.3)* that provided a safe structure from which to hang the nets and enclose the horse. The collected midges were analysed, both microscopically and using PCR, to identify species, evidence of engorgement (blood-feeding) and blood-meal source. Data from each collection of midges was used as an indication of the *Culicoides*-horse interaction during that session.

Prior to commencement of data collection, three preliminary studies were carried out, in order to develop the most effective and practicable method of undertaking the sampling. The three preliminary studies are each described in full, prior to a description of the main study.

Ethical approval was granted by the Veterinary Research Ethics Committee, University of Liverpool.

PRELIMINARY STUDY ONE: IDENTIFICATION OF LOCATION TO UNDERTAKE MAIN STUDY

AIM

To determine the optimum location for *Culicoides* sampling, based on the abundance of *Culicoides* caught by overnight light-suction trapping

METHOD

Potential locations were chosen based on their practicability for positioning of the cages and safe handling of the horses around the main study site. The abundance of *Culicoides* at each of the sites was estimated based on overnight light-suction trapping at each location.

Trapping was carried out using UV LED CDC traps (Bioquip[1]) as described in Chapter Two. The traps were set up and activated between 19:00 and 20:00 and insects caught in the traps were retrieved the following morning between 07.00 and 08:00. The collected insects were transferred to the laboratory for identification and counting. Only total female *Culicoides* counts were undertaken, as males do not blood feed.

RESULTS

A total of eight different trapping locations were selected. Five of these were on the main University of Liverpool Leahurst campus, two were at an adjacent farm as shown in *figure 3.1* and an additional trap was positioned at a nearby riding school located approximately 3km away (location eight).

Initial trapping was carried out for four nights at sites one to five on the main campus. This was followed by a further two nights of trapping that included sites six and seven and then a final two nights of trapping that included site eight. Sites two to four were not included in the final two nights as additional livestock were present at this time and it was felt that this might affect the results.

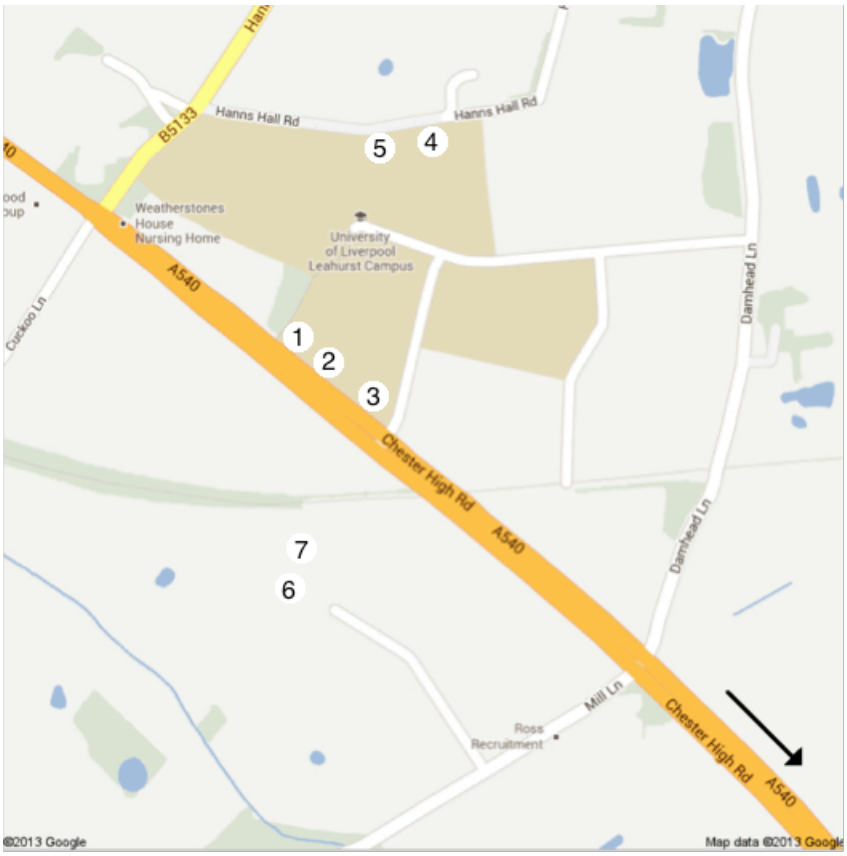

Figure 3.1 – locations (numbered 1-7) for overnight trapping using light-suction traps in preliminary study one. The arrow points in the direction of site eight, located 3km away on a riding school. Map data © 2013 Google.

Trap location	**Total female *Culicoides* caught overnight on each date**							
	Night 1	**Night 2**	**Night 3**	**Night 4**	**Night 5**	**Night 6**	**Night 7**	**Night 8**
1	77	39	34	45	54	36	10	54
2	44	9	8	23	34	3	-	-
3	38	36	32	12	23	56	-	-
4	53	6	14	2	4	12	-	-
5	**68**	**103**	**98**	**160**	**108**	**84**	**257**	**92**
6	-	-	-	-	48	38	67	76
7	-	-	-	-	58	38	21	56
8	-	-	-	-	-	-	60	45

Table 3.1 – Total female Culicoides collected overnight over eight nights during sampling at each of eight sites in preliminary study one

CONCLUSION

The *Culicoides* counts were consistently highest at site five (see *table 3.1*) and this location was therefore chosen to undertake the main study.

STUDIES UTILISING CAGES

- **PRELIMINARY STUDY TWO**
- **PRELIMINARY STUDY THREE**
- **MAIN STUDY – DELTAMETHRIN TRIAL**

ANIMALS

Horses were recruited in pairs for the investigation and were matched as closely as possible for size and colour. The selection criteria used for recruitment were that the horses must be healthy (with no history of any dermatological condition), have a temperament suitable for placement in the cages and not have been treated with any topical insecticide or repellent for at least six months. Six horses were used in total (three pairs) and these were recruited from three sources: The University of Liverpool teaching herd and two private horse owners who provided informed consent for their horses to be used.

Cage design

Two galvanised steel cages were designed and commissioned for the study (*figures 3.2-3.3*). Each cage measured 2.4m long x 1.2m wide x 2.4m high. Each cage was made out of 3mm iron grille and measured 2.4m long x 1.2m wide x 2.4m high. The diameter of the holes was approximately 5cm. This size was chosen as it allowed adequate enclosure of a horse, without the animal having to touch the sides of the cage. This was felt to be important as contact with the cage could reduce the available biting area on the horse and might also be associated with rubbing off of the insecticide. The two longest sides, rear and top of the cage were solid and fixed in position. A hinged door was present at the front of each cage; however these were never closed during the investigation. (The doors were present so that the cages could be utilised in future investigations using sheep instead of horses). The cages were situated on flat ground at location five on The Leahurst Campus, approximately 2m apart [106]. Both were securely fixed to the ground using 10mm diameter nylon rope and grounded fittings. The sides and rear of each cage were lined internally with netting of an aperture size < 1.5mm^2, which was assumed to act as a barrier to *Culicoides* [102].

PRELIMINARY STUDY TWO: METHOD PRACTICALITY AND EQUIPMENT TESTING

AIMS

To assess the safety of the method and ensure that horses could be trained to stand in the cages with the traps in place. To determine the best method for collecting *Culicoides* within the cages.

Method

A single horse was trained to back into the cage on several occasions prior to trapping (*figures 3.2 and 3.3*). Once the horse was considered relaxed in the cage, initial trapping was carried out. The horse was placed in the cage for one hour at sunset. A comparable study in The Netherlands using similar *Culicoides* species showed that this was the time when most midges were caught [106]. A haynet was provided to ensure that the horse was relaxed and the handler was able to remain at a distance of 15m. This ensured that a human standing nearby had negligible effect on trapping of midges in close vicinity to the horses under investigation.

The horse remained in the cage for one hour, during which time the amount of insects in the netting was visually checked by torchlight, to determine if insects could then be aspirated for assessment. As no trapped midges were visualised it was felt that an alternative trapping method within the netting would be more appropriate. Initially, a single UV-LED CDC[1] light-trap was hung from the centre of the cage roof. The light trap was activated as soon as the horse entered the cage and the horse remained in the cage for one hour. The collected insects were transferred to a preservative solution of 70% ethanol, before being transported to the laboratory for analysis. As male *Culicoides* do not feed, they are unable to act as AHSV vectors and were not recorded. Engorged females were identified based on abdominal pigmentation [124]. Sessions took place using a single CDC trap (*figures 3.2 and 3.3)*, multiple CDC traps or a single Onderstepoort Veterinary Institute (OVI)[3] trap (*figure 3.4*), to determine which collected the most *Culicoides*. This was done based on evidence of varying efficacy of trap types as discussed in Chapter Two.


Figures 3.2 and 3.3: Experimental setup used in preliminary study two, showing a single horse within the cages. A single CDC light-trap is positioned above the horse. Netting covers the two sides, roof and part of the rear of the cage.

RESULTS

All types and combinations of traps were well tolerated by the horse. The results of sampling (*table 3.2*) showed that the OVI trap caught the greatest number of *Culicoides* females.

Session number	**Traps used**	**Total number of *Culicoides* females**	**Number of blood-fed *Culicoides* females**
1	1 CDC trap	2	2
2	2 CDC traps	15	12
3	1 CDC trap	8	6
4	1 OVI trap	42	29
5	2 CDC traps	16	16
6	1 OVI trap	36	21
7	1 CDC trap	5	4

Table 3.2: Results of preliminary catching with 1 horse in cage trap and varying light traps used. CDC = Centre for Disease Control trap, OVI = Onderstepoort Veterinary Institute trap.

CONCLUSIONS

No adverse or dangerous events occurred during the trapping and it was therefore felt appropriate to continue with the cage-based investigations. A single OVI trap per cage

caught the most *Culicoides* females per session and was therefore used for the main investigation.

PRELIMINARY STUDY THREE – SUITABILITY OF METHOD

INTRODUCTION AND AIMS

Previous comparable studies utilising drop traps have relied on aspiration of insects from within the netting as previously described [106, 107]. Our method used light traps within the cages to catch the insects instead of aspiration. It was therefore important to establish that the *Culicoides* caught within each cage were representative of *Culicoides* interaction with the horse within the cage and not simply the *Culicoides* population in the environment.

METHOD

The study was set up in the same way as described in preliminary study one; however three catches were obtained during each one hour trapping session. One catch was from a single OVI light trap situated within a cage that contained a horse (as in preliminary study 1); a second was from a single OVI light trap situated in the adjacent, empty cage; and a third catch was obtained from a single OVI trap hung nearby, but out of direct sight (to act as a control). Trapping took place over four nights, with one horse used on nights one and two, and a different horse used on nights three and four.

RESULTS

Location of trap	***Culicoides* catches**											
	Night 1			**Night 2**			**Night 3**			**Night 4**		
	Total	**BF**	**%BF**	**Total**	**BF**	**%BF**	**Total**	**BF**	**%BF**	**Total**	**BF**	**%BF**
Cage, with horse	26	3	10	10	4	29	40	28	41	70	40	36
Cage, no horse	9	0	0	0	0	0	2	0	0	25	0	0
Control	5	0	0	1	0	0	5	1	17	16	0	0

Table 3.3: Culicoides female catches from light-suction traps in cages (that were either empty or contained a horse) and hung nearby as a control. BF = blood fed.

CONCLUSIONS

The results of the showed that the number of *Culicoides* caught in the trap situated in the cage containing the horse was much greater than that from the trap in the empty adjacent cage and the control trap. From these data, the trap situated in the cage

containing the horse appears to represent the *Culicoides* interacting with the horse, rather than a sample of the *Culicoides* present in the environment. The decision was therefore made to continue using this method for the main study, with the percentage of the potential vector species of *Culicoides* within the light-trap catch that had blood-fed being used as an indication of the rate of *Culicoides* feeding on the horse within the cage.

MAIN STUDY – DELTAMETHRIN TRIAL

AIMS

To investigate the effect of topical deltamethrin on the total number and blood-feeding of *Culicoides* caught around horses. Our hypothesis was that the application of topical deltamethrin would significantly reduce both the total amount of potential vector species of *Culicoides* caught around horses and the percentage of these that had blood-fed.

To investigate which *Culicoides* species preferentially blood-feed on horses. Our hypothesis was that previously identified potential vector species (members of the Obsoletus and Pulicaris groups) caught around horses would be more likely to have blood-fed than other species.

METHOD

The cages were designed and set up as previously described, with each containing a single OVI light-trap. Horses participated in the study in pairs and each pair completed the full trial prior to starting with the next pair. All horses were housed under the same conditions and were kept at pasture for the duration of the study. Trapping of insects took place on consecutive evenings at sunset as previously described. Both horses were positioned in the cages (as shown in *figure 3.4*), prior to activation of both light-traps. Following activation of the traps, the handler (one person for all sessions) withdrew to a distance of 15m. All horses had a hay-net available and none required additional handler interaction during trapping. Trapping took place for one hour, whereupon the traps were deactivated and the horses removed from the cages and returned to the paddocks.

Trapping took place on consecutive evenings. If no *Culicoides* were caught, an additional session took place the following evening until a catch was obtained. After four sessions (pre-treatment sessions), one of the horses in each group was randomly selected and treated with topical 1% deltamethrin solution (*Coopers Spot On ready-to-use*[4]). As the product is not licensed for use in horses, the manufacturer's instructions on dosage and application to cattle were used (10ml of solution applied to a single location on top of

spine just behind the withers). At this time, the horses were moved to separate paddocks situated to prevent any direct physical contact between the two horses. Trapping continued each evening until four more catches (post-treatment sessions) were obtained.

Figure 3.4: set up of equipment for main investigation showing a pair of horses within the cages. A single OVI light-trap is positioned above each horse. Netting covers two sides and part of the rear of the cage.

CULICOIDES IDENTIFICATION AND ANALYSIS

Individual *Culicoides* species were identified using a dissecting microscope and standard wing-pattern identification keys, as described in Chapter Two [35]. As male *Culicoides* do not feed and are therefore unable to act as AHSV vectors, data about these were not recorded. Members of the Obsoletus and Pulicaris groups were classified as potential vector species, as described previously. All other species were considered non-vectors. Blood-fed individuals were identified based on the presence of blood within the abdomen. To prove that the blood-fed *Culicoides* had fed on horses, 20 engorged vector species *Culicoides* were analysed to identify the origin of their blood meal, using PCR assays as described in Chapter Two [125]. Blood meal source was molecularly identified using host-species primers, including horse as a simplex PCR and cattle/sheep/goat as a multiplex PCR. Universal primers for vertebrates were also used as a control of host DNA quality and quantity.

A sample of 100 (50 blood-fed and 50 non blood-fed) of the Obsoletus group *Culicoides* collected during the deltamethrin study was randomly selected for species identification. As the females making up the Obsoletus group cannot be accurately individually identified using microscopic techniques, the same species-specific polymerase chain reaction (PCR) assay described in Chapter Two was used to confirm numbers of each species [36].

STATISTICAL ANALYSIS

Data were analysed using the SPSS software package (version 21.0.0.0).

The treatment effect of topical deltamethrin on both blood-fed and total *Culicoides* was investigated using paired-samples T-tests. Initially, a contrast value (C) was calculated for each pair to represent the treatment effect on variables of interest (percentage of collected vectors that had blood-fed and total number of *Culicoides* collected) using data generated as shown in *table 3.4*, where C = (AT-BT)-(AU-BU).

	Pair 1		**Pair 2**		**Pair 3**	
	T1	**U1**	**T2**	**U2**	**T3**	**U3**
Mean value of variable of interest before treatment (B)	**BT1**	**BU1**	**BT2**	**BU2**	**BT3**	**BU3**
Mean value of variable of interest after treatment (A)	**AT1**	**AU1**	**AT2**	**BU2**	**AT3**	**AU3**

Table 3.4: T = treated horse in each pair (1-3), U = untreated/control horse in each pair (1-3). BT (1-3) = mean value over first four sessions (before treatment) for treated horses 1-3, BU(1-3) = mean value over first four sessions for untreated horses 1-3, AT (1-3) = mean value over last four sessions (after treatment) for treated horses 1-3, AU (1-3) = mean value over last four sessions (after treatment) for untreated horses 1-3.

A paired sample T-test was then used to investigate the significance of the C values generated for each of the variables of interest. Significance was set at $p < 0.05$.

The relative reduction in the percentage of vectors BF caught around each horse (D) was also calculated for each pair, as it was though that this represented a more clinically relevant effect of treatment. Again, using data generated as shown in *table 3.*4, D = (AT/BT) – (AU/BU). The data was log transformed prior to analysis using a paired sample T-test. Significance was set at $p < 0.05$.

The relationship between blood-feeding and *Culicoides* species was investigated using Fisher's Exact Test. The relationship between vector species and blood-feeding was investigated using a chi-squared test. Significance was set at $p < 0.05$.

RESULTS

A total of 2,534 *Culicoides* were caught over the 24 sessions, giving a mean *Culicoides* catch per session of 105.6 (range 10 – 587) and a mean catch per cage of 52.8 (range 1 – 321). The overall blood-feeding rate of vector species was 16.0%. The most abundant species were members of the Obsoletus (44.3%) and Pulicaris groups (34.2%), giving a total potential vector species percentage of 78.3% (*table 3.5)*.

***Culicoides* species**	**Total**	**Percentage of total *Culicoides***
Total *Culicoides*	2534	100
Total Vectors	1984	78.3
Obsoletus group BF	174	6.9
Obsoletus group NBF	945	37.3
Pulicaris group BF	146	5.7
Pulicaris group NBF	721	28.5
***C. festivipennis* BF**	11	0.4
***C. festivipennis* NBF**	499	19 .7
***C. circumscriptus* BF**	1	0
***C. circumscriptus* NBF**	39	1.5

Table 3.5: Collated data on female Culicoides species collected from all three pairs of horses over all 24 catching sessions. BF = blood-fed. NBF = non blood-fed

VECTOR SPECIES VS NON-VECTOR SPECIES

Species	**Number of *Culicoides* (%)**	
	BF	**NBF**
Vectors	318 (16.0)	1666 (84.0)
Non-vectors	12 (2.2)	538 (97.8)

Table 3.6: total amount of blood-fed (BF) and non blood-fed (NBF) vectors (Obsoletus and Pulicaris groups) and non-vectors (other species) caught over all 24 trapping sessions.

Sixteen percent of vector species and 2.2% of non-vector species caught had blood fed (*table 3.6).* Vector species were significantly more likely to have blood-fed than non-vector species ($p < 0.001$)

OBSOLETUS GROUP ANALYSIS

Ninety-eight percent of the selected Obsoletus group *Culicoides* were successfully amplified using the PCR (*table 3.7*). *Culicoides dewulfi* (46.9%) and *C. obsoletus* (44.9%) were the most abundant species, with *C. scoticus* (7.1%) and *C. chiopterus* (1.0%) both caught in much lower numbers.

Species	**Number of *Culicoides* (%)**	
	BF	**NBF**
C. dewulfi	18 (36)	28 (58.4)
C. obsoletus	29 (58)	15 (31.3)
C.scoticus	3 (6)	4 (8.4)
C.chiopterus	0 (0)	1 (2.1)

Table 3.7: results of PCR analysis to identify species of 50 randomly selected BF (blood-fed) and 50 NBF (non blood-fed) Obsoletus group Culicoides

There was a significant ($p = 0.034$) effect of species (within the Obsoletus group) on blood-feeding, with more *C. obsoletus* and less *C. dewulfi* blood-fed than expected (see appendix for cross-tabulation data).

SPECIES ORIGIN OF BLOOD-MEALS

All 20 of the Obsoletus group and Pulicaris group samples analysed using blood-meal species origin specific PCR were successfully amplified using the equine specific primers.

EFFECT OF TOPICAL DELTAMETHRIN TREATMENT ON BLOOD-FED VECTOR SPECIES

1) ANALYSIS OF PERCENTAGE BF VECTORS (C VALUE)

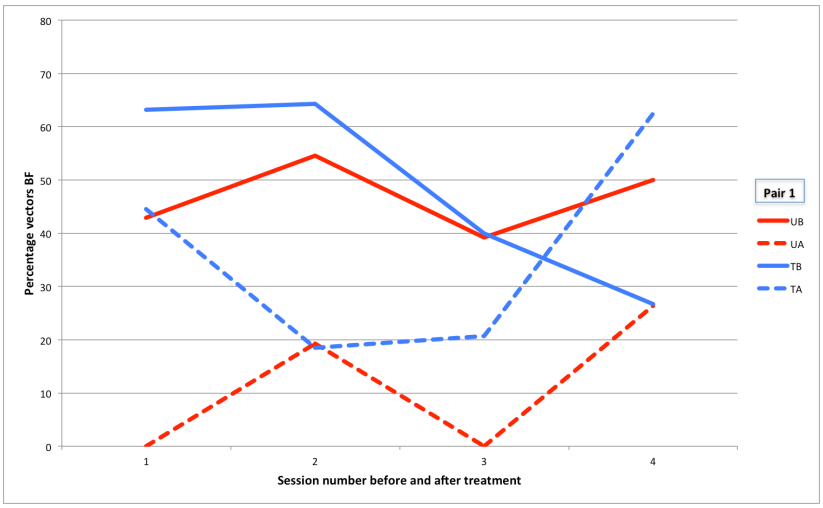

Figure 3.5a: pair 1

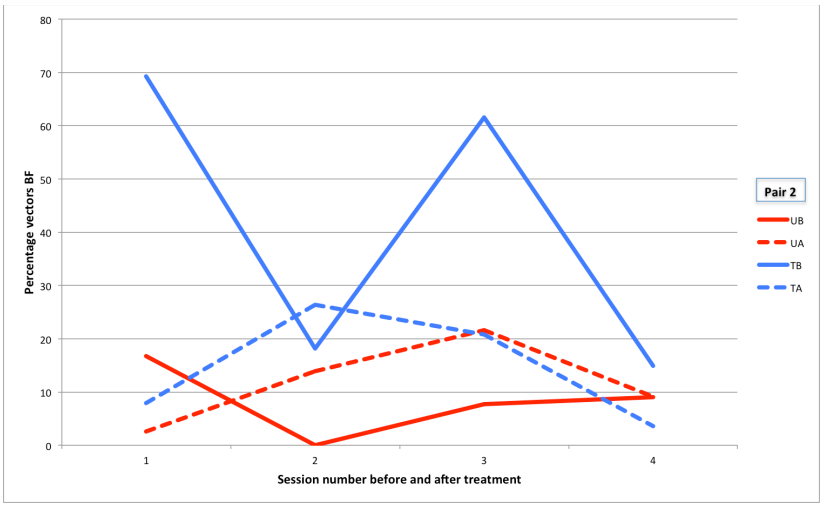

Figure 3.5b: pair 2

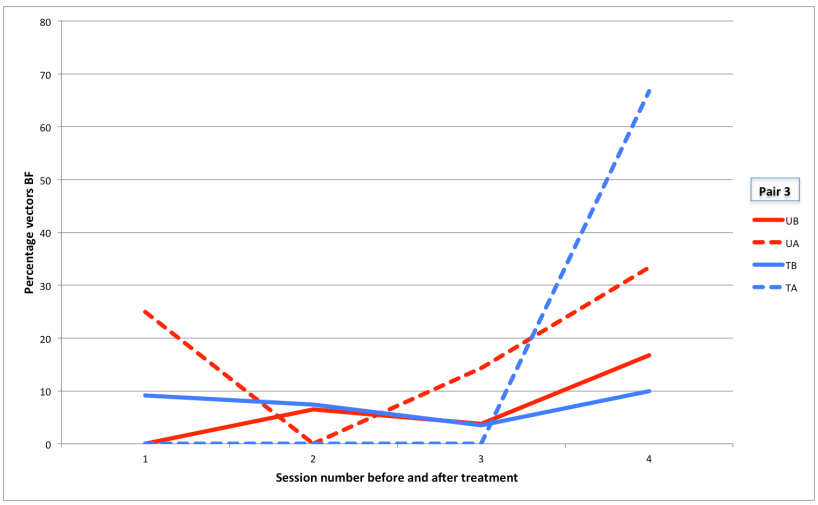

Figure 3.5c: pair 3

Figures 3.5a-c: Line graphs to show percentage vectors blood-fed (BF) for each pair of horses during four sessions before treatment and four sessions after treatment with deltamethrin. Red lines represent the untreated horse in each pair and blue lines represent the treated horse in each pair. Solid line represents data during first four session and dotted line during last four sessions.

A paired sample students T-test was used to investigate the effect of treatment on the percentage of collected vectors that had blood-fed (*table 3.8*).

Mean	SD	SE	95% CI		Sig. (2-tailed)
			Lower	Upper	
2.95	26.5	15.31	-62.95	68.80	0.866

Table 8: Results of students T-test for C. SD = standard deviation of mean, SE = standard error of the mean, CI = confidence interval of the mean

The data does not support an association between treatment and percentage of blood-fed vectors caught (P=0.866).

2) ANALYSIS OF RELATIVE CHANGE IN PERCENTAGE BF (D VALUE)

	Mean	SD	SE	95% CI		Sig
				Lower	Upper	
Ln(D)	-0.06	0.54	0.31	-1.40	1.28	0.862
Exp(D)	0.94	1.72	1.36	0.24	3.60	-

Table 3.9: Results of students T test for D value

The data does not support a significant effect of treatment on the change in percentage of blood-fed vectors caught (p = 0.862) (*table 3.9*). Based on the 95% CI, the treatment effect on percentage vectors BF lies between a reduction to 24% of the original value and an increase to 360% of the original value.

EFFECT OF TREATMENT ON TOTAL *CULICOIDES* COLLECTED (C VALUE)

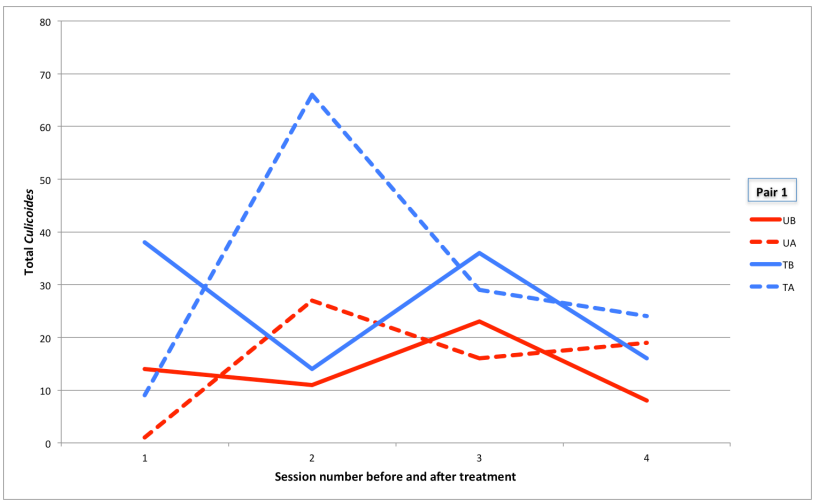

Figure 3.6a: pair 1

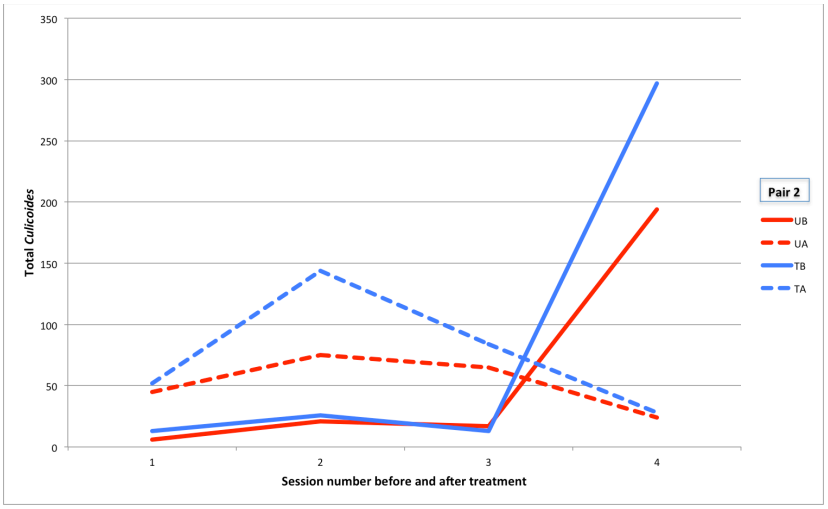

Figure 3.6b: pair 2

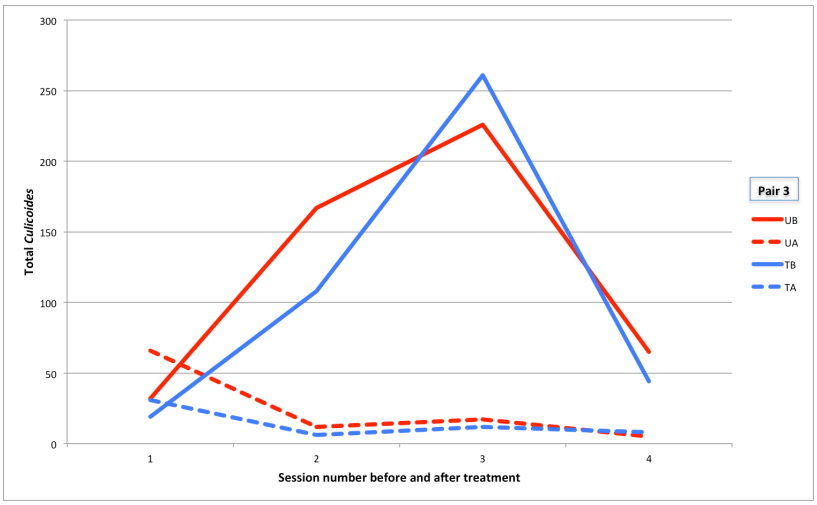

Figure 3.6c: pair 3

Figures 3.6a-c: Line graphs to show total Culicoides collected for each pair of horses during four sessions before treatment and four sessions after treatment with deltamethrin. Red lines represent the untreated horse in each pair and blue lines represent the treated horse in each pair. Solid line represents data during first four session and dotted line during last four sessions.

Figures 3.6a-c show the total *Culicoides* caught during each session for each pair. Again there is marked variation from session to session in the total catch for each horse.

Using the same method for calculating C as previously described, the following results were obtained for the effect of treatment on the total number of female *Culicoides* collected:

Mean	**SD**	**SE**	**95% CI**		**Sig. (2-tailed)**
-2.25	4.68	2.70	-13.89	9.39	0.493

Table 3.10: Results of paired T-test for total *Culicoides* collected

The data also does not support a significant treatment effect of topical deltamethrin on the total number of *Culicoides* caught (p=0.493).

DISCUSSION

The data does not show a significant treatment effect of topical deltamethrin on the blood-feeding of *Culicoides* on horses, and does not support its use as a repellent during an outbreak of AHS. The most abundant species collected during the study were members of the Obsoletus and Pulicaris groups and there was a significant relationship between blood-feeding and species, with *Culicoides* from these two groups apparently more likely to have blood fed. All engorged members of these species assessed by PCR had blood-fed on horses. These data provide important evidence of vector-host interaction and supports the theory that these species have the potential to act as AHS vectors.

The implications of the results of the present study for the control of vector-borne disease must be carefully considered. Deltamethrin is a synthetic pyrethroid, whose mechanism of action involves interference with sodium channels of *Culicoides* nerve axons that results in delayed repolarization and paralysis [135]. The application of topical deltamethrin to horses could be expected to have 3 possible effects on *Culicoides*-horse interaction: a repellent effect that prevents blood-feeding; *Culicoides* death after blood-feeding that prevents onward spread of virus from a viraemic horse and/or reduces the population of available vectors; and, finally, no effect at all. The results of the present study do not support a repellent effect of deltamethrin to protect an individual horse from virus inoculation; however, no conclusions can be drawn on the onward spread of the virus or the vector population.

Studies in other species suggest that, while the application of topical insecticides does not protect the treated animal directly from vector-borne diseases, it instead reduces the abundance and life expectancy of the vector population [136]. Non-AHS susceptible hosts (mostly sheep and cattle) have a significant effect on the amount of *Culicoides* within an area and effective use of insecticides to minimise *Culicoides* numbers would rely on the continuous and effective treatment of all animal hosts within an area [137]. Unfortunately, the efficacy of such use has not yet been definitively demonstrated and the 2-weekly application of topical permethrin to cattle did not reduce seroconversion to BT virus [109]. However, the viral challenge was very high in this study, with many more untreated cattle nearby, and the regime used may simply have been overwhelmed.

Up to 80% *Culicoides* mortality has been demonstrated within an hour following

exposure to the plucked hair of horses treated seven days previously with another pyrethroid, cypermethrin [95]. As the extrinsic incubation period (time for viral amplification and migration within *Culicoides*) of AHSV is far greater than one hour, 80% of *Culicoides* contacting the hair would be unable to act as onward virus vectors. Regarding the UK equine population and AHS transmission it would theoretically be the case that treatment of every single horse and donkey could break the cycle of transmission by preventing onward spread from viraemic individuals, although a similar study using deltamethrin instead of cypermethrin is required to support its use.

Possible explanations for the deltamethrin having no effect include resistance and failure to achieve required concentrations for toxicity at biting locations. Insecticide resistance among vectors of many arboviral diseases (particularly malaria) is well established. The susceptibility of *Culicoides* species to insecticides is relatively poorly documented, although a standardised susceptibility test has been described [134]. Given the high susceptibility of *Culicoides* generally reported to deltamethrin, it seems unlikely that resistance would have been a significant problem in the present study [133, 134]. Our results are in agreement with a previous study showing a lack of efficacy of another pyrethroid, permethrin, in horses [106]. In that study, post-treatment samples were taken at a single time point 24 hours after application of insecticide and the authors recommended repeated measures on subsequent days to see if any effect was observed. Despite the repeated sampling for four days after application of the deltamethrin used in this study, no significant effect of the treatment was detected. In sheep, the reported efficacy of topical deltamethrin varies greatly, with effective *Culicoides* mortality occurring for between 10 days to five weeks [104, 105, 134]. The peak mortality rate has been reported to occur between zero and four days after application of deltamethrin, depending on the method of application and the present study should therefore have detected an effect [104, 134]. The product used was selected based on 'the cascade' and is a one percent solution of deltamethrin licensed for livestock use in the UK. The dose used was the equivalent of the licensed cattle dose and the product was applied to a single area behind the withers as per the manufacturer's instructions for usage on cattle. There are concerns that lack of spread of compounds applied in this way results in lack of efficacy in distant parts of the body [136]. Following the application of deltamethrin to the backs of sheep, *Culicoides* exposed to the hair collected from primary biting regions (belly, face, legs) showed lower mortality than those exposed to hair from the backline, although results were variable [94, 136]. These results were not significantly improved by applying the products to the flanks of the

animals, rather than just along the back line [136]. In cattle, deltamethrin has been shown to reach the feet following topical treatment along the spine [138]. *Culicoides* appear to feed on all parts of the horse, with some species showing apparent predilection sites. In one study involving a single horse, the majority of *C. scoticus* and *C. dewulfi* were collected from the back (main site) and head, whereas *C. obsoletus* had no preferential sites [83]. Another study showed that the hindquarters and mane were *Culicoides* predilection sites [139]. Following the application of cypermethrin to the face, legs, back and hindquarters of horses, the toxicity of collected hair to *Culicoides* was consistently lower from the legs and highest from the back [140]. A further study using an *in vitro* assay to assess the efficacy of deltamethrin applied in a single area of the back would help to determine whether or not lack of compound spread contributed to the results of the present study. In particular this may explain the higher blood-feeding rate of *C. obsoletus* observed as these *Culicoides* would have been more likely to feed elsewhere if the insecticide was only present at required concentrations on the back of the horse.

Other studies carried out around horses in Europe have also demonstrated the importance of members of the Obsoletus and Pulicaris groups, although it must be remembered that their distribution is environmental, as well as host, dependent [82, 106, 139, 141]. In the present study, *C. dewulfi* (46.9%) and *C. obsoletus* (44.9%) were the most abundant members of the Obsoletus group, with *C. scoticus* (7.1%) and *C. chiopterus* (1.0%) caught far less frequently. The specific members of the Obsoletus group were significantly associated with blood-feeding, with *C. obsoletus* apparently more likely to be blood-fed. Taken together with the relatively high numbers of this species, the data highlights the importance of *C. obsoletus* as a potential vector of AHS.

In the present study, the treatment effect was expressed as the relative change in the percentage of blood-fed vectors after treatment, compared to before treatment. The 95% CI tells us that the treatment effect of deltamethrin on the pre-treatment BF rate lies between a reduction of 76% and an increase of 260%. This 95% CI is very wide and additional pairs of horses could be used to give a more accurate estimate of the treatment effect. A cross-over design could not be used, due to the unknown residual effect of the deltamethrin. When considering future study refinements, it is important to bear in mind what treatment effect would be required for the treatment to be recommended clinically as a repellent. This is particularly difficult to define as there appears to be no accepted value and the variation in methodology between studies

makes comparisons difficult. In human trials investigating the efficacy of repellents, the dosages that repel 50, 90 and 95% of mosquitos are quoted; however, most seem to provide at least 90% protection from biting for a period of time [142].

Several studies investigating the interaction between horses and *Culicoides* have examined catches obtained from aspiration of *Culicoides* caught within a drop-trap around the horse [82, 99, 106]. Our decision to use light-traps to sample *Culicoides* within a similar space was made to increase the number of *Culicoides* sampled, and therefore the analytical power of the study. It has been stated that UV light-traps cannot be used directly to assess *Culicoides* biting rates and the collection of a species in a light trap placed near an animal does not prove that it was feeding on it [62]. The information obtained from light traps does not always appear to correlate well with either the overall biting rate or the species composition found feeding on sheep when sampled by direct aspiration from the host [61, 62]. Indeed, a study that compared *Culicoides* catches from sticky covers/blankets on a single horse with those in a nearby light-trap showed that *C. scoticus*, *C. dewulfi* and, to a lesser extent, *C. obsoletus* were the species most commonly attracted to the host, with the importance of *C. obsoletus* overestimated by UV light trapping [83]. The results of the current study appear to be in agreement with this, although the methodologies (Viennet *et* al used a trap hung out of direct sight of the host at a distance of around 50m, compared to trap hung directly above the host within a semi-closed environment) are substantially different. This might also explain the lower blood-feeding rate seen in the previous study (0.5%), compared to the average of 16% in the present study.

It has been shown that proximity to cattle increases the total numbers of *Culicoides* and blood-fed females caught using light-traps [116, 143]. In a study where light-traps were positioned directly above sheep (but not within a midge-proofed enclosure), the female *C. obsoletus* catch increased linearly with host number [144]. These results help support the assumption made in our study that the *Culicoides* caught in the light-traps were attracted by the horse directly below each trap in the cages and therefore represent the specific population of *Culicoides* feeding on them. The addition of partial enclosure of the trap and host by the midge-proof netting should have helped to further isolate the relevant population of *Culicoides*. This is supported by the PCR results that showed 100% of the engorged *Culicoides* testes contained equine DNA. This could be further improved by the use of individual horse specific primers to establish if the *Culicoides* had fed on the specific horse in the same cage, rather than the adjacent one.

The effect that the light attractant on the light-trap might have on the host seeking behaviour of *Culicoides* sampled is difficult to predict as their ecology is poorly described. During preliminary work undertaken as part of this study, the population of midges caught in the light-trap situated within an empty cage (no horse) was very different to that obtained by a trap within the adjacent cage that contained a horse. In particular, no engorged *Culicoides* were ever caught in the empty cage. This suggests that the *Culicoides* were interacting with the host prior to being attracted to the light-trap. The use of the netting also has limitations, as the front and part of the back was open to allow *Culicoides* to enter the cage and interact with the horses. This setup allows some *Culicoides* that have bitten the horse to leave the cage without entering the trap. Unfortunately this is difficult to completely overcome, and the number of *Culicoides* entering a confined space is proportional to the size of the entrance [145].

The wide variation in the total *Culicoides* catch per cage (1-321) is similar to that reported in a similar study that used aspiration within netting instead of the light traps to collect samples [99]. This was most likely due to meteorological factors, which were not recorded during this study but are known to affect *Culicoides* counts [5, 116].

CHAPTER THREE – MAIN CONCLUSIONS

The results of the present study do not support the use of topical deltamethrin solution as a method for preventing *Culicoides* from blood-feeding on individual horses and transmitting AHSV to those individuals. Further studies are required to identify a more suitable repellent product to prevent *Culicoides* blood-feeding on horses, as well as to determine the role that deltamethrin might play in preventing onward viral transmission from a viraemic horse.

Obsoletus and Pulicaris group *Culicoides* were the most likely to have blood-fed and all engorged members of these groups analysed had blood-fed on horses, supporting their potential role as vectors if AHS virus were to reach the UK. Within the Obsoletus group, *C. obsoletus* was the most likely species to have blood-fed.

CHAPTER 4

FINAL CONCLUSIONS

The data presented in this thesis arise from two different studies, both of which provide information that improves current knowledge of AHSV vector biology and methods of protecting horses in the face of an outbreak.

Potential AHSV vector *Culicoides* species of the Obsoletus and Pulicaris groups were present on both urban and rural equine properties within the southeast UK, where they made up 99.6% of all *Culicoides* collected. These species also made up 78.3% of *Culicoides* collected during the cage-trapping studies, which were assumed to sample those *Culicoides* that were interacting directly with horses. All engorged members of the Obsoletus and Pulicaris groups collected in the cage-trapping study and analysed by PCR were found to contain equine DNA. Additionally, several engorged members of the Obsoletus group collected on properties in the southeast UK also contained equine DNA. These data provide evidence of direct interaction between these species and horses, supporting their possible role as AHSV vectors.

The data provides information on the relative importance of members of the Obsoletus group. When examining light-trap catches on equine properties, *C. obsoletus* (52.3%) and *C. scoticus* (38.9%) were the most commonly identified members of the Obsoletus group. *Culicoides dewulfi* was detected far less frequently (8.9%) and no *C. chiopterus* were identified. In the cage-trapping study the results were different, with *C. dewulfi* (46.9%) and *C. obsoletus* (44.9%) making up almost all of the Obsoletus group *Culicoides* sampled. In addition, *C. obsoletus* was statistically more likely to have blood-fed than *C. dewulfi.* As previously discussed, this variation in the apparent importance of members of the Obsoletus group is most likely due to environmental factors controlling the *Culicoides* species present in the different areas. In addition, there is debate over the use of light-traps to represent vector-host interaction and further studies are required to compare *Culicoides* sampled by direct aspiration from horses with those caught in light-suction traps [94]. Despite these limitations when comparing individual species, the data from this thesis showed that the collected Obsoletus and Pulicaris group *Culicoides* were statistically more likely to be blood-fed than other species, supporting the theory that these species have the potential to act as AHS vectors.

The data obtained from the present study does not support the use of topical one percent deltamethrin solution as a method for preventing *Culicoides* from blood-feeding on individual horses and transmitting AHSV to those individuals during an outbreak. Further work investigating the repellent effect of other products, particularly DEET, in a

similar way is recommended. It is important that the other potential roles of deltamethrin discussed are not discounted, and further work is required to determine the role that it may have in preventing onward viral transmission from a viraemic horse.

None of the properties investigated in the southeast UK contained any form of vector-proofed housing. Given the evidence that even basic vector proofing of stables significantly reduces access by *Culicoides*, it would seem most appropriate to improve owner awareness of the importance of preparing for novel vector-borne diseases and investigate simple modifications to equine housing that could be quickly applied in the face of an AHS outbreak [115].

Finally, there were marked variations in the total *Culicoides* catch between different nights on different properties and within properties on the same night during collection in the southeast UK and also on different nights during cage-trapping. As discussed, this was most likely due to established geographical and meteorological factors, including wind speed, rainfall and vegetation, which are know to affect *Culicoides* abundance. However, it would still present a significant problem to veterinary officers attempting to establish the local distribution of *Culicoides* in an outbreak situation (an important part of the UK Government control strategy for AHS) and these difficulties would have to be taken into account.

APPENDIX

Manufacturers details

[1] Bioquip Products Inc., 2321 Gladwick Street, Rancho Dominguez, CA 90220, USA

[2] S. Brannan and Sons Ltd, Leconfield Industrial Estate, Cleator Moor, Cumbria, CA25 5QE

[3] Onderstepoort Veterinary Institute, Onderstepoort, South Africa

[4] Zoetis UK Limited. 5th Floor, 6 St. Andrew Street, London EC4A 3AE

Databases

[a] www.directoryoftheturf.com and www.newmarketracehorsetrainers.co.uk. Both accessed in March 2012.

Chapter Two

Questionnaire

Site Name

Date

1) Do you use any of the following methods to prevent insect bites? *Please tick as appropriate.*

	All horses	Sweet itch horses
Application of repellent or insecticide to horses. *If yes please list products used*		
Use of blankets/masks		
Reduced turnout at dawn/dusk/overnight		
Feed supplements		
Covers on muck-heap		N/A
Application of insecticide to environment *If yes please list where applied, product used and when last applied*		N/A
Protected housing *If yes please answer question 10*		

2) Which of the following protected housing measures do you use to prevent insect bites? *Please tick as appropriate.*

Mesh over openings (<1.6mm^2)	
Application of insecticide to mesh	
Indoor fans	
Insect traps (electrical)	
Insect traps (non-electrical)	
Controlled environment buildings	
Other. *Please describe below*	

3) Was there any international movement of horses on or off this site within the last month?

Yes	*Please go to question 14*
No	*Please go to question 16*

4) Were any of these movements…? *Please tick as appropriate.*

Tripartite (UK/ROI/France)	
To/from other EU states	
To/from non-EU states	

5) During which months of the year does international movement of horses on and off this site occur? *Please circle as appropriate.*

Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

6) If possible, please describe the specific destinations and origins of each horse movement on and off this property within the last 7 days. *PTO if necessary*

Chapter Three

Raw species data for all three pairs:

Pair 1

	Session number															
	1		2		3		4		5		6		7		8	
Pair 1	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**
Total Culicoides	38	14	14	11	36	23	16	8	9	1	66	27	29	16	24	19
Total vectors	38	14	14	11	30	23	15	8	9	1	65	26	29	16	24	19
Total BF vectors	24	6	9	6	12	9	4	4	4	0	12	5	6	0	15	5
% BF vectors	63.2	42.9	64.3	54.6	40	39.2	26.7	50	44.5	0	18.5	19.3	20.7	0	62.5	26.4
Obsoletus gp BF	17	4	4	4	11	8	3	1	3	0	8	3	6	0	14	4
Obsoletus gp NBF	9	5	5	3	17	14	8	4	5	1	49	21	21	14	8	12
***C. punctatus* BF**	7	1	5	2	1	1	1	3	1	0	4	2	0	0	0	0
***C. punctatus* NBF**	4	3	0	1	0	0	2	0	0	0	4	0	1	0	0	1
***C. pulicaris* BF**	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1
***C. pulicaris* NBF**	1	0	0	1	1	0	1	0	0	0	0	0	1	2	1	1
***C. festivipennis* BF**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***C. festivipennis* NBF**	0	0	0	0	6	0	1	0	0	0	1	1	0	0	0	0
***C. circumscriptus* BF**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***C. circumscriptus* NBF**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Pair 2

	Session number															
	1		2		3		4		5		6		7		8	
Pair 2	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**
Total Culicoides	13	6	26	21	13	17	297	194	52	45	144	75	84	65	28	24
Total vectors	13	6	22	13	13	13	297	191	51	39	129	65	82	65	28	22
Total BF vectors	9	1	4	0	8	1	44	17	4	1	34	9	17	14	1	2
% BF vectors	69.3	16.7	18.2	0	61.6	7.7	14.9	9	7.9	2.6	26.4	13.9	20.8	21.6	3.6	9.1
Obsoletus gp BF	8	1	0	0	4	0	8	12	2	1	15	2	2	0	1	2
Obsoletus gp NBF	4	4	13	6	2	6	37	112	36	28	32	25	5	4	11	9
***C. punctatus* BF**	0	0	4	0	4	0	34	4	2	0	19	6	12	13	0	0
***C. punctatus* NBF**	0	1	5	7	3	6	212	56	11	10	63	30	60	35	12	9
***C. pulicaris* BF**	1	0	0	0	0	1	2	1	0	0	0	1	3	1	0	0
***C. pulicaris* NBF**	0	0	0	0	0	0	4	6	0	0	0	1	0	12	4	2
***C. festivipennis* BF**	0	0	4	8	0	4	0	3	1	6	15	10	2	0	0	0
***C. festivipennis* NBF**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
***C. circumscriptus* BF**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***C. circumscriptus* NBF**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Pair 3

	Session number															
	1		2		3		4		5		6		7		8	
Pair 3	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**	**T1**	**U1**
Total Culicoides	19	32	108	167	261	226	44	65	31	66	6	12	12	17	8	5
Total vectors	11	18	41	78	201	186	10	6	6	8	2	8	5	7	3	3
Total BF vectors	1	0	3	5	7	7	1	1	0	2	0	0	0	1	2	1
% BF vectors	9.1	0	7.4	6.5	3.5	3.8	10	16.7	0	25	0	0	0	14.3	66.7	33.4
Obsoletus gp BF	1	0	2	4	7	5	1	1	0	2	0	0	0	0	2	1
Obsoletus gp NBF	6	12	26	57	144	128	7	4	4	5	2	8	4	5	1	2
***C. punctatus* BF**	0	0	1	1	0	2	0	0	0	0	0	0	0	1	0	0
***C. punctatus* NBF**	4	6	12	16	42	39	2	1	2	1	0	0	1	1	0	0
***C. pulicaris* BF**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***C. pulicaris* NBF**	0	0	0	0	8	12	0	0	0	0	0	0	0	0	0	0
***C. festivipennis* BF**	0	0	0	3	2	0	0	0	1	1	1	0	0	0	1	0
***C. festivipennis* NBF**	8	14	63	77	54	36	30	56	22	54	3	3	4	7	4	2
***C. circumscriptus* BF**	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
***C. circumscriptus* NBF**	0	0	4	8	4	4	4	3	2	3	0	1	3	3	0	0

Collated data

***Culicoides* species**	**Pair 1**	**Pair 2**	**Pair 3**	**Total**	**%**
Total *Culicoides*	352	1106	1084	2534	-
Total Vectors	342	1051	593	1984	78.3
Obsoletus group BF	90	58	26	174	6.9
Obsoletus group NBF	196	334	415	945	37.3
***C. punctatus* BF**	28	98	5	131	5.2
***C. punctatus* NBF**	16	520	127	663	26.2
C. *pulicaris* BF	3	10	0	13	0.5
C. *pulicaris* NBF	9	29	20	58	2.3
***C. festivipennis* BF**	0	53	9	62	2.4
***C. festivipennis* NBF**	9	2	437	448	17.7
***C. circumscriptus* BF**	0	0	1	1	0.0
***C. circumscriptus* NBF**	0	0	39	39	1.5

Cross-tabulation data for chi-squared analysis

1. **All species**

Species/group	**Females**	**Engorged**		**Total**
		BF	**NBF**	
Obsoletus gp	Count	174	945	1119
	Expected count	146.4	972.6	1119
C. punctatus	Count	131	677	808
	Expected count	105.7	702.3	808
C. pulicaris	Count	15	44	59
	Expected count	7.7	51.3	59
C. festivipennis	Count	11	500	511
	Expected count	66.9	444.1	511
C. circumscriptus	Count	1	39	40
	Expected count	5.2	34.8	40
Total	Count	332	2205	2537
	Expected count	332	2205	2537

2. **PCR results of random sample of 100 Obsoletus group *Culicoides***

Species	**Females**	**Engorged**		**Total**
		BF	**NBF**	
C.obsoletus	Count	29	15	44
	Expected Count	22.4	21.6	44
C.dewulfi	Count	18	28	46
	Expected Count	23.5	22.5	46
C.scoticus	Count	3	4	7
	Expected Count	3.6	3.4	7
C.chiopterus	Count	0	1	1
	Expected Count	0.5	0.5	1
Total	Count	50	48	98
	Expected Count	50	48	98

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