Determining and Modelling the Bluetongue Vector Landscape

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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This thesis is based on research carried out in the Department of Epidemiology and Population Health at the University of Liverpool. Except where indicated, the content of this thesis is my own work.

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ABSTRACT

Bluetongue (BT) is a seasonal vector-borne, viral, disease that causes significant economic and welfare problems in ruminants. It is transmitted by species of *Culicoides* midges (Diptera: Ceratopogonidae), and as such, the distribution of the disease is restricted to regions where the vectors are present. Once restricted to tropical and subtropical regions of the world, serotypes of BT have been causing outbreaks in southern Europe, following its introduction in 1998, and in 2006, BT serotype-8 emerged in northern Europe, causing devastating economic, welfare and production consequences. The northwards expansion of BT has been attributed to a shift in the geographic limit of the *Culicoide*s *imicola* Meigen vector, and the involvement of the newly implicated Palaearctic vectors, the Obsoletus and Pulicaris Groups. Little is known about the ecological characteristics of the newly implicated vectors, or indeed those believed to be non-vectors, including their distribution and abundance, making disease risk assessment and management difficult.

Within this thesis, a series of field experiments were initiated on a group of farms to gain insight into the distribution and abundance of *Culicoides* species. The results highlighted that a very high level of variation is seen when trapping *Culicoides* at the local-scale, yet it is possible to build a strong model explaining this variation using a mixture of host and environmental variables, with satellite-derived ecological correlates. This high level of variation in midge catches present between farms undermines attempts to record their nationwide distribution in larger scale models. The results uniquely model Obsoletus Group abundance, and highlight a difference in host involvement between vector and non-vector models. Further field studies which showed a lack of significant variation both between years and at the within-farm level highlight the robustness of this model in predicting the distribution of the BT vectors species, such that it could prove useful for exploring targeted surveillance and control methods.

Culicoides distributions do not remain static, therefore an understanding of their flight behaviour is critical to determining the distance over which an insect may transmit a disease agent and the size of the area over which control should be applied. Laboratory studies were undertaken to validate the use of commercial fluorescent dusts as a quick and effective method of marking *Culicoides* for both field and laboratory studies, and a 'self-marking' technique was conceived. Dispersal studies, using the dusts, determined the distances that Obsoletus Group females and males, as well as *C. pulicaris* females, are able to disperse over a set period of time. This knowledge of flight speed and distance is of utmost value as a critical component in the modelling of BT disease and other *Culicoides*-borne diseases.

The Obsoletus Group contains four members (*C. obsoletus*, *C. scoticus*, *C. chiopterus* and *C. dewulfi*) which are difficult to differentiate down a microscope. Using morphometric analyses, female *C. obsoletus* and *C. scoticus* individuals could be separated under a stereomicroscope based on abdominal measurements.

Studies such as those contained in this thesis, therefore, are of utmost value in providing information on critical components in the modelling of BT disease and other *Culicoides*-borne diseases.

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"An investment in knowledge pays the best interest." -Benjamin Franklin

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"If a cluttered desk is a sign of a cluttered mind, of what, then, is an empty desk a sign?" – Albert Einstein

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I might stick to writing a fiction novel next time round...

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Common abbreviations are defined below, whilst specialist abbreviations are defined below and on their first use within the text.

a0	Overall Mean Amplitude
a1	Annual Amplitude
a2	Biannual Amplitude
a3	Triannual Amplitude
AHS	African Horse Sickness
AHSV	AHS (see above) Virus
AIC	Akaike's Information Criterion
ANOVA	Analysis of Variance
APRS	Automatic Packet Recording System
BBSRC	Biotechnology and Biological Sciences Research Council
BGP	Brilliant General Purpose
BP	Base Pairs
BT	Bluetongue
BTV	BT (see above) Virus
BTV-x	BTV (see above) Serotype-x e.g. BTV-8
°C	Degrees Celsius (Centigrade)
C.	Culicoides
cm	Centimetres
COI	Cytochrome Oxidase Subunit 1
CD	Coefficient of Difference
CV	Coefficient of Variation
d1	Proportion of Variance explained by the Annual Cycle
d2	Proportion of Variance explained by the biannual Cycle
d3	Proportion of Variance explained by the triannual Cycle
da	Proportion of Variance explained by three cycles combined
DEFRA	Department for Environment, Farming and Rural Affairs
d.f	Degrees of Freedom
dLST	Day-time Land Surface Temperature
DNA	Deoxyribonucleic Acid
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
EVI	Enhanced Vegetation Index
FMD	Foot and Mouth Disease

Continued Overleaf…

g	Gram
GB	Great Britain
GIS	Geographical Information System
GLM	General Linear Model
GPS	Global Positioning System
h	hour
IAH	Institute for Animal Health
IAH-P	IAH (see above) Pirbright Site
ITS-1	Internal Transcribed Spacer 1
ITS-2	Internal Transcribed Spacer 2
kg	Kilogram
LandIS	Land Information System
LRZ	Lower Risk Zone
LST	Land Surface Temperature
NDVI	Normalised-Difference Vegetation Index
µm	Micrometer
m	Metres
m^2	Metres squared
MET	Meteorological
MIR	Middle Infra-Red Reflectance
ml	Milliliters
MLV	Modified Live Vaccine
mm	Millimeters
mn	Minimum
MODIS	Moderate Resolution Imaging Spectroradiometer
MRR	Mark-Release-Recapture
mx	Maximum
N	Sample Size
NAME	Numerical Atmospheric Dispersion Modelling Environment
NDVI	Normalized Difference Vegetation Index
nLST	Night-time LST (see above)
OIE	World Organisation for Animal Health
OVI	Onderstepoort Veterinary Institute
p1	Phase (Peak Timing) of the Annual Cycle
p2	Phase (Peak Timing) of the Biannual Cycle
p3	Phase (Peak Timing) of the Triannual Cycle
PC1	Principal Component Axis 1
PC2	Principal Component Axis 2
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction

Continued Overleaf…

r^2	Variance Explained
SD	Standard Deviation
SE	Standard Error
sp.	Species
TAE	Tris-Acetate-EDTA Buffer
TOV	Toggenburg Orbivirus
UK	United Kingdom
US(A)	United States (of America)
UV	Ultra Violet
V	Variance
VBD	Vector-Borne Disease
vr	Variance
WAHID	World Animal Health Information Database

Common taxonomic abbreviations are defined below.

Obsoletus Complex	*C. obsoletus* and *C. scoticus*
Obsoletus Group	*C. obsoletus*, *C. scoticus*, *C. chiopterus* and *C. dewulfi*
Pulicaris Group	*C. pulicaris*, *C. punctatus* and *C. impunctatus*

CHAPTER ONE

GENERAL INTRODUCTION

This Chapter gives an overview of vector-borne diseases (VBDs), more specifically bluetongue (BT) and its recent developments within Europe. I will then go on to review current knowledge on the biology of *Culicoides* biting midges, the vectors of BT, as well as their capacity to transit viruses. The effects of climate and weather on *Culicoides* populations will then be considered before, finally, describing the chapters of this thesis.

1.1 Vector-borne Diseases

In the past 130 years since the scientific basis behind insects as potential vectors of disease was established (Pearn, 2004), hundreds of infectious agents have been found to require a haematophagous arthropod for transmission between vertebrate hosts (Gubler, 1998). Prevention and control of such diseases was historically based on controlling arthropod vectors, with yellow fever in Cuba being the first VBD successfully controlled in this way.

VBD control and elimination programs only had temporary benefits however and re-emergence of a number of diseases has greatly intensified since the 1970s (Hammon, 1973; Bruce-Chwatt, 1979; Gubler, 1996; Krogstad, 1996). Many new, or previously unseen, infectious diseases have also emerged, many of which are of growing public health concern (Gratz, 1999) and other diseases have spread to geographical areas where they were not previously found. This is the case with bluetongue disease.

1.1.1 Factors Affecting VBD Spread

Factors responsible for the emergence or resurgence of VBDs are complex. They include insecticide and drug resistance, changes in public health policy, emphasis on emergency response, lessening emphasis on prevention programs, demographic and societal changes, and genetic changes in pathogens (Lederberg *et al.*, 1992). Reversing the trend of emergent and resurgent VBDs is a major challenge with little funding for vaccine research (with the exception of malaria) and a narrow use of

those that are available for diseases such as yellow fever and tick-borne encephalitis (Gubler, 1998).

Along with BT, several other VBDs have been emerging into new areas for the first time, such as malaria, dengue fever, chikungunya and the plague (Watson *et al*., 2005). This has been attributed to a range of climate-driven factors, as well as ones associated to changes in industry and urbanisation. These affect the spread of VBDs by increasing the ability of the vector to transmit a disease, or in creating suitable habitats for it to establish. In some cases, whether accidental or induced by human activity, diseases which had been related to one type of vector species have also been seen in new competent vectors which may have different hosts and habitats, as is the case for West Nile (Gubler, 2007) and BT. Human travel, deforestation, irrigation projects and the upsizing of farm animal holdings are examples of anthropogenic causes of VBD spread, whilst climate change and El Niño events are examples of climate driven factors responsible for this occurrence (Gratz, 1999).

1.2 Bluetongue Virus

One of the prime examples of a disease that has spread to a new area is BT, a notifiable vector-borne viral disease of ruminants which, until recently, was restricted to tropical and subtropical areas of the world (Mellor *et al*., 2008). BTV, of which 26 serotypes are known (Maan *et al*., 2012), is a double-stranded RNA virus (Mertens *et al.*, 2008) and member of the genus *Orbivirus* (family Reoviridae). The virion, about 70 nm in diameter, consists of a core containing 10 segments of double stranded RNA and composed of five proteins and an outer capsid made up of two other proteins (Grimes *et al*., 1997).

BTV is transmitted by midges of the genus *Culicoides* (Mellor *et al.*, 2000), and until recently *C. imicola* was believed to be the only important vector in southern Europe, but other, newly recognised vector species are also involved (Mellor *et al.*, 2008). BTV is transmitted between ruminants almost exclusively through the bite of adult female vectors, and global distribution of the disease is therefore restricted to regions

where these vectors occur, and transmission to the season of vector activity (Mellor *et al.*, 2000).

BTV infects all species of ruminants, but severe disease is normally restricted to certain breeds of sheep (MacLachlan, 1994) and some species of deer (Robinson *et al.*, 1967; Stair *et al.*, 1968, Taylor, 1986). In sheep, BTV causes an acute disease with high morbidity and mortality, with clinical symptoms including high fever, excessive salivation and erosion of the mouth mucosae, swelling of the face and tongue and rarely, cyanosis of the tongue (Erasmus, 1990; Verwoerd & Erasmus 1994; Geering *et al*., 1995). Congestion may be prominent and foot lesions are noted in some animals, initiating with coronitis and subsequent lameness (OIE, 2008a). Mortality rates in sheep are usually between 2-30% and infection in pregnant ewes can cause abortions or foetal abnormalities. Finally, during the long convalescent period, many sheep shed their fleece (Geering *et al.*, 1995).

Not all animals develop clinical symptoms and cattle and goats usually exhibit subclinical infections, therefore serving as viral reservoirs (MacLachlan, 1994). BTV serotype 8 (BTV-8) however, which caused an unprecedented epidemic in northern Europe between 2006 and 2008, is associated with distinct clinical signs in some cattle in infected herds (Backx, 2008).

There is no treatment for bluetongue. Prevention may be possible by vaccination and by controlling midge populations (with insecticides or, where practical, by control of breeding sites), but neither is totally successful (IAH, 2005).

1.2.1 Historical Perspective

Although distributed worldwide, many BT serotypes are linked to specific vectors and therefore tend to be restricted to certain geographic regions. For example, BTV-1, 3, 9, 15, 16, 20, 21 and 23 occur in Northern Australia, BTV-1, 3, 4, 6, 8, 12 and 17 circulate in the Central American- Caribbean Basin, and BTV-1 to 16, 18, 19 and 24 tend to be responsible for African outbreaks (IAH, 2010).

Previous to 2006, BT had been circulating regularly in the Mediterranean and Balkan regions since the late 1990s (Purse *et al.*, 2006), with the exception of sporadic outbreaks in the 50s which caused great economic losses, mostly to the sheep industry. It spread to these areas via two main pathways. The first of these was the eastern and western movement of BTV-1, 4, 9 and 16 originally affecting the Greek islands and moving into the Balkan regions and the Mediterranean islands of Corsica, Sicily and Sardinia. The second was the northerly expansion of BTV-2 and recently BTV-1 from Algeria to Tunisia, into mainland Italy, the Spanish Balearic islands and finally mainland Spain and Portugal (Purse *et al.*, 2005).

BT has historically made only brief, sporadic incursions into the southern fringes of Europe (Campano Lopez & Sanchez Botija, 1958; Manso Ribeiro *et al.*, 1958; Mellor & Pitzolis, 1979; Jennings *et al*., 1983; Mellor & Boorman, 1995; Baylis *et al*., 1997). Since 1998 however, an unprecedented epidemic of bluetongue has occurred involving six strains of BTV (BTV-1, 2, 4, 8, 9 and 16) spreading across 12 countries and 800 km further north in Europe than previously reported (Mellor & Wittmann, 2002; Purse *et al*., 2005). These strains entered Europe within eight years of each other and their emergence appears to be due to recent changes in the European climate. These changes have influenced virus persistence during winter, the northward expansion of the major Old World BTV vector *Culicoides imicola* and, beyond this vector's range, transmission by indigenous European *Culicoides* species, thereby expanding the risk of transmission over much larger geographical regions (Purse *et al*., 2005).

1.2.2 European BTV-8 Epidemic

The largest epidemic of BTV-8 ever known began in 2006 and affected the Benelux, France and Germany and, between September 2007 and January 2008, spread to Denmark, Switzerland, Spain, the United Kingdom and the Czech Republic (OIE, 2008b; Hofmann *et al*., 2008). On 14 August 2006, a private veterinary practitioner in Limburg, in the south of the Netherlands, notified the veterinary authorities of BT-suspect cases on four different holdings in the country. By the 22nd August, 40 outbreaks were reported and by the 1st September 138 had occurred. These were the

first indications of a rapidly spreading BTV-epidemic in north-western Europe, which subsequently affected cattle and sheep holdings in the Netherlands, Belgium, Germany, France, and Luxembourg. On 28 August 2006, the Central Reference Laboratory at Pirbright, UK, reported that the outbreaks were caused by BTV-serotype 8 (BTV-8) (EFSA, 2007).

This outbreak occurred some 5^{o} of latitude further north than had ever been previously recorded in Europe, and was caused by a serotype that was not circulating in the Mediterranean countries (EFSA, 2007). BTV-8 was also not known to be active in any of the regions bordering the Mediterranean Basin, although surveillance was limited. Phylogenetic analysis has since identified the strain as genetically distinct from American, Asian or vaccine strains of BTV-8 and most closely resembles an isolate from sub-Saharan Africa (Maan *et al*., 2008).

The virus survived the winter and the epidemic flared up even more strongly in 2007, and by November, over 30,000 outbreaks had been reported in a total of nine Central European countries. Since its introduction in 2006, BTV-8 has been recorded in 19 European countries (Table 1.1).

This serotype had previously only been detected in sub-Saharan Africa, the Caribbean, India and Pakistan and the epidemiological mechanisms underlying this BT epidemic are still unclear, including its origin, route and mode of entry and the contributing factors that influenced the establishment and the spread of this disease (EFSA, 2007; Mintiens *et al*., 2008). The European Food Safety Authority (EFSA) outbreak report however, indicated that the place of introduction was likely to be located in a 20km circular area in the Maastricht region in the Netherlands. This circle includes the geographical location of the first officially reported outbreaks in Belgium, Germany and the Netherlands including the Belgian farm where the earliest clinical symptoms were reported.

The possible methods of introduction most commonly cited are plant imports from North Africa and an international equestrian event - in both cases, infected midges were said to have been imported. The potential for infected *Culicoides* to be imported along with or independently of the import of animals, plants or other

'materials', and the effectiveness of measures to reduce such a possibility, merit further study.

Table 1.1. Year of introduction of BTV-8 into European countries (data from: OIE, 2013).

Year of Introduction	Country
2006	Netherlands Belgium Germany France (including Corsica) Luxembourg
2007	Czech Republic Switzerland Denmark United Kingdom
2008	Austria Hungary Italy (including Sardinia) Sweden Norway Slovakia Spain
2009	Greece (Island of Lesbos only)
2010	Turkey Cyprus

1.2.3 BTV in the UK

The first case of BTV-8 in the UK was detected on the 15th September 2007 near Ipswich, Suffolk (ANON, 2007d). The most likely entry route was identified as wind-borne movement of infected vectors from the continent (Gloster *et al*., 2007a, b, 2008), with overnight winds from the Ostend area of Belgium on the 4th and 5th of August, providing suitable conditions for entry of the vectors responsible for the first outbreaks (Gloster *et al*., 2008). The UK outbreak was restricted primarily to the east and south-east of England (DEFRA, 2008b). Altogether, 137 outbreaks (in

indigenous ruminants) were detected from the start of the event to the end of June 2008, but all of them were as a result of infection from virus circulation in 2007. Two of these were on the same location, but occurred at a different time (DEFRA, 2009; OIE, 2009). The 85 seropositive cases detected in 2008 were identified through statutory testing for movement out of a restricted zone.

Of the countries where transmission has been recorded, only the UK did not reported further cases of transmission in the year following incursion. The UK situation remained static since the last confirmed holding for BTV-8 (an imported case) was detected on 14th November 2008. There was no evidence of circulating disease in 2008 or 2009 and no positive cases were found as a result of post import tests during 2009. It was agreed with the European Commission that as of 12th June 2010 the whole of Great Britain changed its status from a BTV 8 Protection Zone to a BTV-8 Lower Risk Zone (LRZ). A LRZ is an option within the bluetongue regulations which enables Member States that do not have circulating disease to allow their livestock holders to vaccinate against bluetongue. It also imposes tighter controls on animals brought into the country from 'confluent zones' (zones of the same BTV serotype) to help keep disease out.

As of 26th November 2010 measures to reduce the risk of virus entry into the UK changed from single post import testing for all bluetongue virus (BTV) susceptible animals entering Great Britain at 5-7 days to a risk-based regime whereby imports are tested depending on the risk assessment of each susceptible consignment. On 5th July 2011 Great Britain was officially declared free from bluetongue and the LRZs across England and Wales were lifted (DEFRA, 2013).

1.2.3.1 Other BTV Threats to the UK

Since the start of bluetongue's invasion into Europe, a total of 10 serotypes have been recorded. Between 1998 and 2007, six different serotypes (BTV-1, 2, 4, 8, 9 and 16) have been isolated and identified from epizootics occurring across Europe. In 2008 a novel orbivirus was detected in goats from Switzerland and was initially termed Toggenburg orbivirus, before becoming BTV-25. Since 2008, two additional serotypes, BTV-6 and 11 were detected in northern Europe.

1.2.3.1.1 Southern Europe

In 2012, the European Commission showed that the incidence of BTV has declined to just 34 outbreaks for all serotypes within southern Europe. The breakdown by serotype and member state for last year can be seen in Table 1.2.

Table 1.2 Number and serotype of BTV cases recorded by European member states from 1st January to 26th November 2012.

Member State	Outbreaks in 2012	Serotypes
Cyprus	9	BTV-4
Greece	7	BTV-4, -16
Italy	12	BTV-1, -9
Portugal	1	BTV-1
Spain	5	BTV-1

BTV-1 and BTV-4

BTV serotypes active in southern Europe, that could continue to threaten the UK, include a BTV-1 strain first identified in July 2007 in Andalusia, Spain (ANON, 2007c). It is likely that this outbreak spread from the major epizootic of BTV-1 that was ongoing in Morocco (ANON, 2006c), Algeria (ANON, 2006a) and Tunisia (ANON, 2007e) in 2006 and 2007. The BTV-1 strain has subsequently expanded into Portugal (ANON, 2007b), Sardinia (ANON, 2006b) and France (ANON, 2007a), and into areas outside the range of *C. imicola*, with cases recorded as far north as Brittany (DEFRA, 2008a). Although vaccination and other control methods were ongoing the World Organisation for Animal Health (OIE) considered BTV-1 endemic in Spain and Portugal.

BTV-1 imports were also identified in the UK and the Netherlands in 2008. The disease in the UK was found in 5 cattle imported from south-west France to a farm near Blackpool, but there was no evidence of disease circulation. In the Netherlands an imported bull, also from south-west France, was found to be positive for that serotype, but investigations revealed that no spread of BTV-1 had occurred.

In 2009, eight French holdings had outbreaks of BTV-1, while in 2010 only one outbreak was reported. In Spain, 377 outbreaks of BTV-1 were seen in 2009, with no outbreaks of BTV-4 since 2006. However in 2010, although the number of BTV-1 outbreaks dropped to only 4, there was a re-incursion of BTV-4 with 2 outbreaks occurring in the south, despite ongoing compulsory vaccination for BTV-1,-4 and -8 in that year. The last BTV outbreak in continental France was on 10 June 2010.

Although France now appears free of BTV, both BTV-1 and BTV-4 serotypes continue to be a problem further south, with BTV-1 causing outbreaks in Spain, Portugal and Italy, and BTV-4 occurring in Cyprus and Greece, in 2012.

BTV-2, BTV-9 and BTV-16

Isolates of BTV-2 outbreaks in Corsica, Sardinia and Sicily between 2001 and 2002 were identical to previous isolates from outbreaks recorded in Tunisia in 2000, indicating a movement of the serotype from Africa into Italy. Outbreaks of BTV-16, and BTV-2 and -6, occurred in mainland Italy in 2006 and 2009 respectively, and although BTV-2 appears to have disappeared from Europe, BTV-9 and -16 and have continued causing outbreaks in Italy in June 2012 and Greece.

1.2.3.1.2 Western Europe

BTV-6 and BTV-11

Infections, but not transmission, of two additional serotypes were recorded in northern Europe in 2008, BTV-6 in the Netherlands (ANON, 2008; International Society for Infectious Diseases, 2008a), Germany (ANON, 2009; International Society for Infectious Diseases, 2008d) and Belgium, and BTV-11 in Belgium (De Clercq *et al*., 2009; FAVV, 2009). Phylogenetic analysis has shown that both these strains are genetically almost identical to the seed viruses used for the development of the Modified Live Vaccines (MLVs) produced by Onderstepoort Biological Products, South Africa (International Society for Infectious Diseases, 2008b, 2008c, 2009). BTV-6 and BTV-11 have not previously been reported in Europe and MLVs against them have not been used in Europe. Investigations suggest that no virulent BTV-6 virus strains circulated in the Netherlands (van Rijn *et al.*, 2012), Germany or

Belgium (IAH, 2005). No further reports of these serotypes were made in Europe from 2009 onwards.

BTV-25

Swiss authorities also detected an entirely new type of bluetongue-like virus in goats in 2008. The virus was initially called Toggenburg Orbivirus (TOV), but based on genetic analysis, it was proposed that it should be classified as a new serotype of bluetongue, serotype 25. Serological and virological test results from archived Swiss goat samples collected in 1998 have since indicated the presence of BTV-25 in the Swiss goat population at that time, prior to any BT disease outbreak in this part of Europe (Hofmann *et al.*, 2008b).

1.2.3.1.3 Northern and Eastern Europe

In northern Europe, national bluetongue monitoring programmes and pre-movement testing in late 2012 led to the findings of bluetongue positive animals in Lithuania, Latvia, Poland and Estonia. The Pirbright Institute, identified the virus as BTV-14, which was known to have been circulating in the western part of Russia for at least two years. Clinical signs were not observed and further sequencing data suggested the circulation of the BTV-14 reference or vaccine strain from South Africa, possibly indicating the use of a live attenuated BTV-14 vaccine in the field (European Commission, 2013).

1.2.4 Clinical Signs and Surveillance of BTV-8

Morbidity, mortality and case fatality were much higher in sheep flocks compared to cattle herds and there was a long interval between first clinical signs observed by the animal owners and reporting of a clinically suspect situation to the competent authorities (≥2 weeks). This is partly due to a lack of familiarity amongst farmers and veterinary practitioners in this part of Europe with BT signs during the early phase of the BTV-8 epidemic (Backx, 2008).

In contrast to previous experience that BTVs does not produce more than transient and mild, if any, clinical signs in cattle, the BTV-8 incursion indicated that a small number of cattle within a herd can show distinct clinical signs. These signs, including lesions of the nasal mucosa, salivation, fever, teat lesions and coronitis, were expressed differently to those in sheep flocks, the most prominent of which included erosions of the oral cavity, facial oedema, dysphagia (difficulty swallowing), congestion, redness of oral mucosa, and lameness (Backx, 2008).

Whereas infected sheep tended to show clear clinical signs, often only a few sheep within a flock were PCR or sero-positive. These findings therefore suggested that a monitoring system based on clinical signs could be considered for sheep flocks. In contrast, since in infected cattle herds only a small proportion (if any) of animals tended to show clinical signs but a large proportion of cattle were PCR or sero-positive (even when no clinical signs were seen), a monitoring system based on serological screening of cattle seemed to be the more effective option for surveillance in cattle herds (Backx, 2008).

1.2.5 Vector Biology

For its dissemination, bluetongue virus is reliant on various species of biting midges of the order Diptera (2-winged flies), genus *Culicoides*, which are the only known biological vectors. To date four species of *Culicoides* have been incriminated as vectors in southern Europe; the most significant of these is the Afro-asiatic *C. imicola* responsible for at least 90% of BTV transmission in the Mediterranean Basin. It is regarded by some researchers to be a recent invader from Africa and to be spreading northwards. The three remaining vectors are endemic to the Palaearctic region but have until now played only a minor role in the spread of BTV; however, their importance appears to be increasing as BTV is moving northwards at a pace that is outstripping the slow advance of *C. imicola*.

Surveys were implemented in most of the BTV-8 countries affected in 2006 in parallel with occurrence of outbreaks. Not a single specimen of *C. imicola* was detected amongst a total of approximately 100,000 *Culicoides* collected in northern France, Belgium, Luxembourg, Germany and The Netherlands (Meiswinkel *et al*.,

2007). This demonstrates that other species endemic to the Palaearctic region are quite capable of transmitting BTV and, judging from the rapid spread of the virus, it required no 'pre-adaptive' phase in indigenous *Culicoides*. In the Netherlands *C. dewulfi*, *C. obsoletus* and *C. scoticus* were found to be real-time (RT)-PCR positive to BTV-8 - evidence that more than one species of *Culicoides* was involved in the outbreak of BT across north-western Europe. All except one (*C. imicola*) of the previously identified vectors of BTV in southern Europe are known to occur widely across northern Europe.

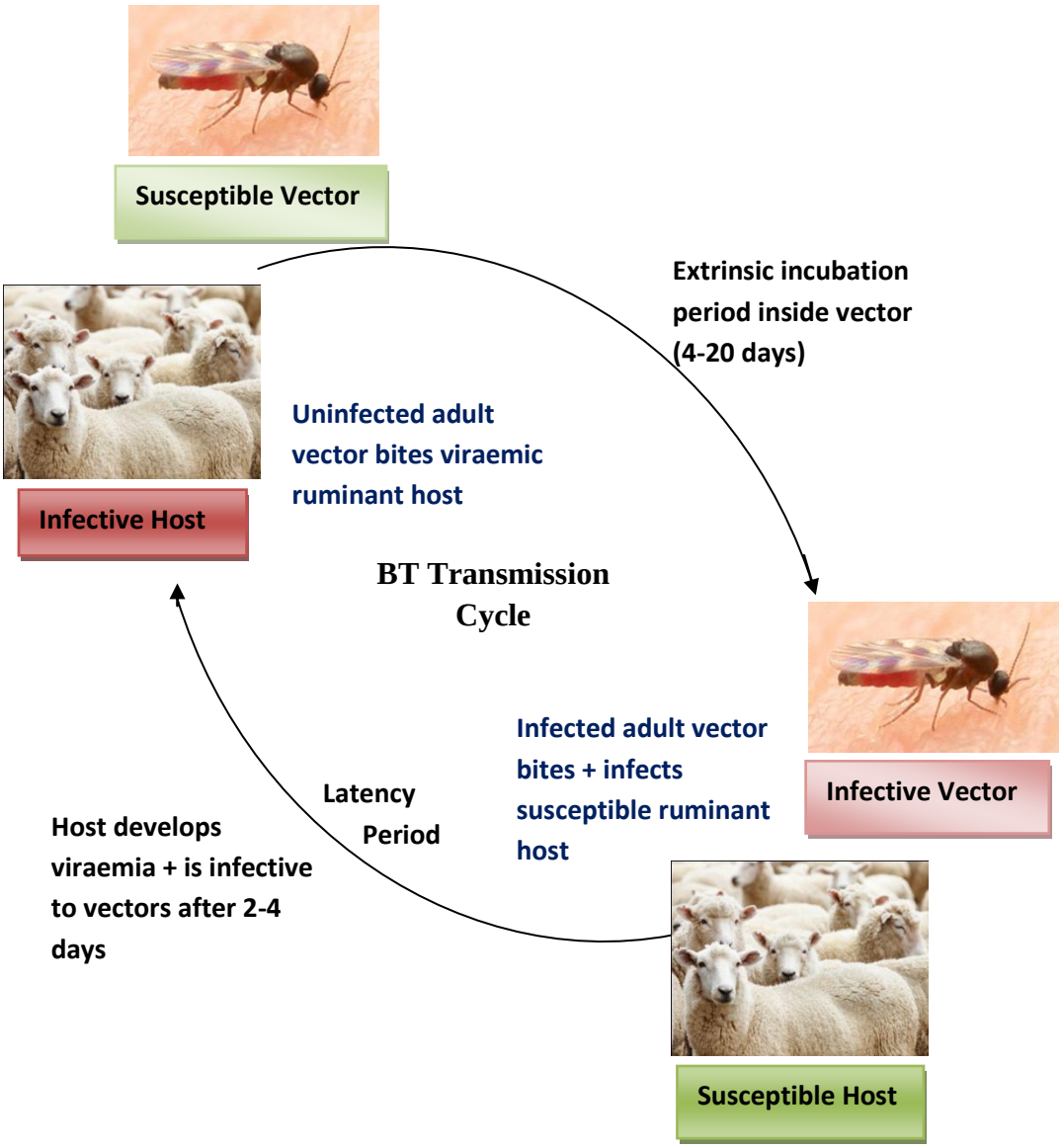

Figure 1.1 The Transmission Cycle for BTV (reproduced from Purse *et al*., 2005**).**

The BT transmission cycle can be seen in Figure 1.1. The female midges take a blood meal before laying their eggs. In doing so, they take up BTV from viraemic vertebrate hosts. The virus multiplies in the midge and can infect other animals at the next blood meal. Temperatures over 25°C are optimal for the virus to multiply. If they fall below 12°C for a prolonged period, the virus cannot multiply. Animal-to-animal transmission is not possible. Since biting midges winter largely in the form of eggs or in the larval stage, there are very few adult females around during the cold season to transmit the virus. For this reason, the disease shows marked seasonality.

1.2.5.1 Oral Infection

When an uninfected female *Culicoides* feeds on an infected host the blood and virus taken up during a blood meal is deposited in the hind part of the mid-gut (Megahed, 1956). The virus particles attach themselves to the surface of the gut cells so that they then enter by either direct plasma membrane penetration or by receptor-mediated endocytosis; here they replicate. Progeny virons are released from the gut cells into the haemocoel where they infect various secondary organs, including the salivary glands. Here the virons replicate and the virus particles accumulate in the salivary ducts ready for transmission during subsequent biting (Chandler *et al*., 1985; Fu e*t al*., 1999). Individuals that have the ability to become infected after ingesting the virus and then to transmit it are known as 'vector-competent'.

Even within known *Culicoides* vector species, only a proportion of individuals will actually be vector competent (Jones and Foster, 1978; Jennings and Mellor, 1987). Individuals that are not competent have a series of barriers that limit the dissemination of the virus, thus preventing transmission. These obstructions include a mid-gut escape barrier which allows virus replication in the mid-gut cells but prevents the virus from exiting into the haemocoel; a mid-gut infection barrier that prevents the virus from even replicating in the mid-gut cells (Jenning and Mellor, 1987); and a dissemination barrier whereby the virus in the haemocoel is unable to infect the secondary target organs (Fu *et al*., 1999). These barriers seem to be hereditary traits and populations of *C. variipennis* have been selectively bred for either high or low incidence of expression of these barriers (Jones and Foster, 1974;

Fu *et al*., 1999). The genetic traits are apparently controlled by a single gene with a major locus and a modifier. In the major locus the maternal genotype determines the progeny phenotype and the paternal gene is always dominant in the offspring. In addition, other external factors may also affect vector competence, including temperature (Mullens *et al*., 1995; Mellor *et al*., 1998; Wittmann, 2000).

1.2.6 Vector-free Period

BT is a seasonal disease generally observed in late summer and early autumn. Virus transmission begins in the early spring with the onset of insect flight activity and continues until the first hard frosts. Therefore a vector-free period has been proposed.

The discovery that larger numbers of *Culicoides* may be found indoors compared with outdoors, especially towards the end of the season, has discredited earlier attempts to declare the vector-free period to be when "…<10 *Culicoides* are found in a light trap suspended outdoors for one night" (Meiswinkel, 2007). Consequently, it will be necessary to amend this criterion possibly by using light traps suspended both inside and outside animal housing. Because of a lack of reliable data on vector competence rates, transmission rates and vector ecology in the northern European context, it is not yet possible to define the vector-free season with an acceptable degree of certainty. The most recent entomological data to emerge is that low numbers of adult *Culicoides,* including freshly blood fed individuals, have on occasion been captured in light traps operated throughout the winter (January, February and March 2007) in various Member States in north-western Europe (EFSA, 2007). In all likelihood this persistent activity of adult *Culicoides* owes much to the mild temperatures that have continued to prevail across northern Europe.

1.2.7 Overwintering of BTV

In temperate regions with cold winters, the vectors survive severe weather as larvae and the transmission cycle is interrupted, whereas BTV may be maintained in year-round transmission cycles in temperate regions with mild winters (Gerry & Mullens,

2000), as well as in the tropics. However, even in these regions, the cycles may be interrupted by dry seasons or other adverse environmental conditions (Gibbs *et al*., 1992). Clearly a mechanism(s) is required for the viruses to survive periods of inclement climatic conditions.

A number of hypotheses have been proposed for the seasonal recurrence of BTV (Griot, 2000; Nevill, 1971; Tabachnick *et al*., 1996).

1. Overwintering in vertebrate host (currently unproved)
    a. Transovarial transmission
    b. Transplacental infection
2. Local overwintering of BTV in their vectors.
3. Persistent infection
4. Re-introduction of BTV by infected vertebrate hosts or vectors.
5. Low-level cycling throughout winter.

In terms of the re-introduction of BTV by infected vertebrate hosts or vectors, this is unlikely. You would expect that, with random introduction, a greater diversity of serotypes then observed would be expected in an endemic area, such as is the case with BTV-11 in northern Colorado (White *et al*., 2004).

The OIE specifies that the maximum infective period for BTV infected animals is 60 days. In northern Europe there is a vector-free period of around 90 days (January-March) and a transmission-free period of around 120 days (mid December-mid April). Consequently BTV should not be able to overwinter in northern Europe. During the 2006-2007 BTV-8 outbreak however, overwintering was seen in Germany, Holland, Belgium and northern France, and particularly around areas where intense transmission had occurred in the previous year. This suggests that the virus overwintered in either ruminant or insect hosts, or both, in 2006.

1.2.8 Prevention of BTV

In an effort to reduce the impact of outbreaks of BTV-8, the competent authorities recommended that livestock be housed at night in the belief that this would reduce significantly the *Culicoides* attack rate (and thereby lower the BTV transmission

rate). All farmers within the 20 km infection zone were therefore required to keep their animals indoors each night and to treat the animals monthly with a pour-on insecticide. But in France and in The Netherlands it has been discovered that *Culicoides* enter animal housing quite freely (Meiswinkel, 2007). Of particular concern, is that >95% of these comprised the vector species *C. obsoletus* and *C. dewulfi*. Work in these two Member States showed that early in the season when night-time temperatures remained high larger numbers of *Culicoides* were captured in light traps operated outside stables. However, later in the season, when the temperatures began to drop to single digits, a reversal occurred and more *Culicoides* were captured both earlier in the evening, and inside, rather than outside stables (Meiswinkel, 2007). These data suggest that during the cooler times of the year *Culicoides* emerge from their resting places sooner in the day (when it is still reasonably warm) probably to attack livestock while still at pasture. It is well known that species such as *C. obsoletus* will intensify their attacks on overcast days when low-light conditions prevail. In such situations it is possible that attacking *Culicoides* may follow the cattle returning to their milking sheds and accompany them indoors. Once inside the biting midges would then be able to complete their blood feeding activities, only to be captured subsequently in the light traps operated nearby. This sequence of events would explain why increased numbers of midges were captured inside animal houses late in the season and why a high percentage of them were freshly blood fed. In order to protect housed animals from attack by *Culicoides*, it may be required that such housing be sealed to a level where the lack of circulation of air might become a welfare problem or which is economically not viable. But even if such well-sealed buildings were to be ventilated, perhaps by screening with insect-proof mesh, this would do little to prevent *Culicoides* from entering the housing along with cattle in the late afternoon (as described above). To control this possible influx by *Culicoides* would require the installation of walk-through insecticidal sprayers (Meiswinkel, 2007).

Overall, there is a paucity of information on the behavioural activities of vector species of *Culicoides*, especially in relation to their interactions with host animals and their biting activities. The discovery of significant numbers of *Culicoides* in buildings towards the end of the season has raised two additional points of concern as to whether some species of *Culicoides* breed indoors and if BTV-infected late-

season adult *Culicoides* can overwinter inside cattle sheds to emerge months later in spring, initiating a recrudescence of the BTV transmission cycle. These questions have yet to be answered.

Naturally infected animals produce antibodies that are detectable for life, in contrast to animals vaccinated with inactivated vaccine which produce antibodies for about one year. In experimentally infected animals, PCR positive results are observed up to 200 days post infection in ruminants (Bonneau *et al.*, 2002).

1.3 *Culicoides* Biting Midges

Culicoides biting midges (Diptera: Ceratopogonidae) are economically important nematocerous Diptera. Nematocerous flies are considered the oldest 'suborder' within the Diptera, and include Culicidae (mosquitoes), Tipulidae (crane flies), Chironomidae (non-biting midges), Bibionidae (march flies), in addition to economically important disease vectors (black flies, biting midges and sand flies) or crop pests (gall midges). They occupy various habitats (fully aquatic, semi-aquatic, wood-boring, desert dwelling, etc.) and trophic levels (predators, herbivores, fungivores, detritivores and parasites).

More than 1,400 *Culicoides* species have been identified and they occur on all major landmasses excluding Antarctica, New Zealand and the Hawaiian Islands (Boorman, 1993; Meiswinkel *et al*., 1994; Mellor *et al*., 2000). *Culicoides* species cause economic damage in terms of biting nuisance and as vectors of disease-causing pathogens. In the first case they limit tourism and outdoor activities in many parts of the world (e.g. Scotland, USA, Caribbean, Australia; Kettle 1995) as well as causing severe allergic dermatitis (sweet itch) in horses (Mellor and McCaig 1974). In the second case they have the ability to transmit a whole range of pathogens including 53 viruses (Table 1.3; Meiswinkel *et al.,* 1994), 12 species of protozoa and 18 species of filarial nematode (Linley, 1985).

Table 1.3 Summary of viruses isolated worldwide from *Culicoides* species (Meiswinkel *et al*., 1994; Wittmann, 2000).

Virus	Family	Genus	*Culicoides* spp.	Continents
Rift Valley fever	Bunyaviridae	Phlebovirus	mixed pool	Africa
Lokern	"	Bunyavirus	*variipennis*	North America
Main Drain	"	"	"	"
Belmont	"	"	*marksi, bundyensis*	Australia
Aino	"	"	*Brevitarsis*	Asia, Australia
Akabane	"	"	*imicola, milnei* *brevitarsis,* *wadai, oxystoma*	Africa, Asia Australasia "
Buttonwillow	"	"	mixed pool	North America
Douglas	"	"	*Brevitarsis*	Australasia
Oropouche	"	"	*Paraensis*	South America
Peaton	"	?	*Brevitarsis*	Australasia
Sabo	"	"	mixed pool	Africa
Sango	"	"	"	"
Sathuperi	"	"	"	Africa, Asia
Schmallenberg	"	"	*obsoletus group*	Europe
Shamonda	"	"	*Imicola*	Africa
Shuni	"	"	mixed pool	"
Thimiri	"	"	*histrio* *nivosus*	Australasia, Asia Africa
Tinaroo	"	"	*Brevitarsis*	Australasia
Congo	"	Nairovirus	mixed pool	Africa, Asia
Dugbe	"	"	"	Africa
Nairobi sheep disease	"	"	*tororoensis*	"
Issyk-kul	"	-	*schultzei* s.l.	Asia
African Horse Sickness	Reoviridae	Orbivirus	*imicola,* *tororoensis* *bolitinos* *obsoletus group* *pulicaris*	Africa, South Europe Africa, Asia Africa South Europe
Bluetongue	"	"	*actoni, brevitarsis* *orientalis, wadai* *fulvus* *obsoletus* *exspectator*	Australasia " Australia South Europe, Africa

			imicola bolitinos, tororoensis, milnei, pycnostictus, insignis variipennis, stellifer, pusillus	Africa, South Europe Africa, Asia North America " Central America
Epizootic haemorrhagic disease	Reoviridae	Orbivirus	mixed pool, *kingi schultzei brevitarsis variipennis*	Africa " Australasia North America
Equine encephalosis	"	"	mixed pool	Africa
Eubenangee	"	"	*marksi*	Australasia
Bunyip Creek	"	"	*brevitarsis oxystoma*	" "
CSIRO Village	"	"	*brevitarsis*	"
D'Anguilar	"	"	"	"
Marrakai	"	"	mixed pool	"
Nyabira	"	"	*imicola*	Africa
Cul. 1/69	"	"	mixed pool	"
Cul. 2/69	"	"	"	"
Chuzan	"	"	*oxystoma*	Eastern Paleartic
Gweru	"	"	mixed pool	Africa
Wallal	"	"	*dycei, marksi*	Australasia
Mudjinbbarry	"	"	*marksi*	"
Mitchell River	"	"	mixed pool	"
Warrego	"	"	*dycei, marksi actoni*	" "
Letsitele	"	"	*bolitinos, imicola zuluensis, magnus*	Africa "
Kotonkan	Rhabdoviridae	Lyssavirus	mixed pool	"
Vesicular stomatitis – New Jersey	"	Vesiculo-virus	"	North and South America
Bovine ephemeral fever	"	?	*imicola, coarctatus brevitarsis*	Africa, Asia Australasia
Kimberley	"	?	"	"
Kununurra	"	?	*austropalpalis*	"
Tibrogargan	Rhabdoviridae	?	*brevitarsis*	Australasia

Bivens Arm	"	?	*Insignis*	North America
Sweetwater Branch	"	?	"	"
Wongabel	"	?	*austropalpalis*	Australia
Ngaingan	"	?	mixed pool	"
Wongorr	"	?	*pallidothorax*	"
Israel Turkey meningitis	Flaviviridae	Flavivirus	mixed pool	Africa, Asia
Eastern equine encephalo-myelitis	Togaviridae	Alphavirus	"	Asia, Australasia, North and South America

1.3.1 Biology

1.3.1.1 Life Cycle

The lifecycle of *Culicoides* varies between species in terms of duration at different stages and location of breeding sites (Uslu & Dik, 2007), but the overall process is fairly similar. The complete lifecycle can occur in two to six weeks, dependent on the species involved as well as the environmental conditions.

It is the reproduction of *Culicoides* that is responsible for their biting behaviour. Once fertilised, the eggs cannot properly develop without the female taking a blood meal. The blood-meal provides protein for egg development. Male midges do not take blood, and some species mature a first batch of eggs autogenously but then take a blood meal for subsequent egg batches (Boorman and Goddard 1970). Autogeny is not a trait of species like *C. imicola*, *C. brevitarsis*, *C. variipennis* or *C. obsoletus*, but many species, including *C. reithi, C. circumscriptus* (Becker, 1960) and *C. impunctatus* (Boorman and Goddard 1970), have been recorded as such.

Eggs are usually laid, singly or in batches (IAH, 2006) that can range from 25-300 eggs, in a variety of semi-aquatic habitats, as the larvae need a certain amount of free water to complete their lifecycle (Meiswinkel *et al*., 1994; Mellor, 1996). These environments can include, but are not limited to, damp or saturated soils, bogs,

marshes, animal holes, tree holes, irrigation pipe leaks and even rotting fruit (Meiswinkel *et al*., 1994, Mellor, 1996). The eggs are about 400µm x 50µm and are initially white, but become rapidly darker and tend to hatch within 2-7 days (Meiswinkel *et al*., 1994). Jobling (1953) found that the egg stage in *C. vexans* may last over four months, while the larval stage may last for six.

As *Culicoides* are members of the Diptera suborder Nematocera, the eggs hatch and go through four larval instars before pupating and emerging as adults. The duration of instar stages is dependent on temperature and other climate variables and is also dependent upon species. It can last for as little as 4 days in the tropics but, in temperate areas, the fourth instar larvae diapauses over the winter prolonging this phase to many months (Braverman, 1994; Kettle, 1995).

Pupae are formed amongst litter or float on the surface of free water in the breeding site (IAH, 2006). This stage is very brief, with adult *Culicoides* emerging after 2 or 3 days. *Culicoides* biting midges are among the smallest haematophagous insects and range between 1 and 3mm in size. The adults themselves are usually short-lived, with individuals rarely surviving longer than 20 days. In exceptional conditions, adults have been kept alive for as long as 90 days (Mellor *et al*., 2000).

The gonotrophic cycle usually occupies between one and three weeks, depending upon the ambient temperature and the majority of species are univoltine, but some have several generations through the year, depending on local conditions.

1.3.1.2 Feeding Preferences and Flight

Culicoides are mostly crepuscular with peak activity at dawn and dusk, particularly when conditions are warm, still and humid (Boorman, 1993). Females usually fly to find a blood-meal, a mate or an oviposition site, but as males do not blood feed they remain closer to breeding sites than females.

The adult flight range, on the one hand, is usually short, with an active dispersal range of a few hundred meters to 3 km (Lillie *et al.*, 1981; Kettle, 1995). American studies indicate a mean distance travelled of between 2 and 2.2 km over 4 days (range 0.5-3.5 km) (Lillie *et al.*, 1985), with 1.2 km covered in 12 hours.

Topographic, environmental and climatic factors have not been considered in terms of their influence on the variation in flight distance or direction. On the other hand, being so small, *Culicoides* are easily dispersed by wind and this allows them to travel great distances, even across the sea (Hayashi *et al*., 1979; Sellers, 1992).

Short-range dispersal studies however, remain inconclusive regarding the effect of wind speed and direction on *Culicoides* dispersal; some studies report recaptures most marked in the direction of prevailing wind (Breeland & Smith, 1962; Brenner *et al*., 1984; Williams, 1962), others state that dispersal was not aided by wind (Lillie *et al*., 1985). A number of studies even note some midges dispersed further against the wind (Brenner *et al*., 1984).

Most *Culicoides* species will feed on a range of hosts, for example cattle, sheep, horses, birds, and humans, however others have more specific host preferences or aversions, for example *C. imicola* does not feed on humans (Braverman & Phelps, 1981; Kettle, 1995).

1.4 *Culicoides* as Vectors of Disease

Up to now 24 species of *Culicoides* from around the world have been associated with BTV (Table 1.4). These include species that have been proven to biologically transmit the viruses; species from which the viruses have been isolated in the field and species that became infected after experimentally ingesting virus infected blood-meals. There are also 5 other *Culicoides* species (*C. arakawae, C. circumscriptus, C. gemellus, C. shultzei* and *C. nudipalpis*) associated with BTV, however there is no published evidence to date that proves they are involved in transmission (Dyce, 1989; Meiswinkel and Baylis 1998).

Culicoides imicola was initially the only proven field vector for African horse sickness virus (AHSV) and BTV in Europe. It is an Afro-Asiatic species that was recorded for the first time in Europe from Spain, in 1982 (Mellor *et al*., 1983). The species has now also been confirmed throughout the year in adult form in parts of Spain and Portugal (Rawlings *et al*., 1997), as well as on some Greek islands

including Lesbos (Mellor *et al*., 1984; Mellor, 1998) and Rhodes (Boorman, 1986), and also France (Biteau-Coroller *et al*., 2006). As a result of the year round presence of the vector all of these areas are potential endemic zones for both BT and AHS.

BTV and AHSV have also been isolated from *Culicoides* species that were not initially considered to be important vectors. For example BTV was isolated from *C. obsoletus* in Cyprus (Mellor & Pitzolis, 1979) and AHSV was recovered from mixed pools of *C. obsoletus* and *C. pulicaris* in Spain (Mellor *et al*., 1990). Both species have a wide distribution across Europe. During the 1999 BTV outbreak in Bulgaria, the role of major vector was suggested for *C. obsoletus*. Its involvement in the spread of BTV has been suggested because *C. imicola* appears to be totally absent from this area, whereas *C. obsoletus* was the most numerous species trapped at infected sites (Baylis *et al*., 2001; Mellor & Wittmann, 2002).

Epidemiological & virological investigations at the Institute for Animal Health, Pirbright, (IAH-P) have confirmed that members of the Obsoletus and Pulicaris Group midges are the BTV transmitters in non-imicola outbreak areas, as they:

1. Comprise >90% of the *Culicoides* populations in non-imicola outbreak areas;
2. Show fine-scale spatial & temporal correlation with BTV activity;
3. BTV has been regularly isolated from them in the field; and
4. They support BTV replication to levels at which transmission occurs.

(Mellor, unknown)

It is believed that changes in the climatic drivers of BTV infection in Europe have:

1. Caused an extension in the range of some vectors (*C. imicola*), so that transmission occurs more extensively;
2. Caused faster virus development in the insect vector, so transmission occurs earlier;
3. Increased the proportion of an insect population able to transmit, so transmission occurs more frequently;
4. Extended the ability to transmit to additional spp. of *Culicoides*, e.g. *C. obsoletus* & *C. pulicaris*.

In the New World, *C. imicola* and AHS are absent. Nevertheless, BTV is common throughout much of North, Central and South America and Australia, with the main vector species being *C. variipennis* for North America and C. *brevitarsis* for Australia. The vectors in Central and South America are less well studied, but include *C. insignis* and *C. pusillus* (Mellor, 1990). Recently, new evidence has suggested that *C. variipennis* is in fact a complex of three genetically defined sub-species (*C. v. occidentalis*, *C. v. sonorensis* and *C. v. variipennis*) and it is believed, on the basis of field isolations, that *C. v. sonorensis* is the primary vector species for BTV in North America (Tabachnick, 1992; Tabachnick & Holbrook, 1992).

Table 1.4 *Culicoides* species associated with BTV (Wittmann, 2000).

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Wittmann, E. J. (2000) Temperature and the Transmission of Arboviruses by *Culicoides* Biting Midges. PhD Thesis. University of Bristol, UK.

1.4.1 Species found in UK

Forty seven species of *Culicoides* have been noted in Britain, but others undoubtedly remain to be discovered, either having been overlooked, or may yet appear here as a result of global warming and consequent immigration from continental Europe (IAH, 2009). The subgenera and their individual species known to be represented in Britain are shown in Table 1.5.

1.4.2 Species that transmit BT

As previously mentioned, the vectors of BT suggested to be involved in transmission in Europe outside the range of *C. imicola*, are two species groups: the Obsoletus Group and Pulicaris Group. The Obsoletus Group are members of the *Avaritia* (Fox) sub-genus and consist of four species in the UK: *C. obsoletus* Meigen, *C. dewulfi* Goetghebuer, *C. chiopterus* Meigen and *C. scoticus* Downes and Kettle. The females of *C. obsoletus and C. scoticus* are morphologically cryptic. The Pulicaris Group are members of the sub-genus *Culicoides* Latreille and consists of two species: *C. pulicaris* Linnaeus and *C. punctatus* Meigen, although additional species and rearrangement of this group has been suggested (Gomulski *et al*., 2006; Pages *et al*., 2009). All of these species are present in the UK.

The role of the Obsoletus and Pulicaris Groups in BTV transmission in different areas has been inferred by fine scale temporal and spatial overlap of their distributions with outbreaks (Purse *et al*., 2007). Virus isolations have also been made from the wild-caught, parous, non-engorged adults of both species groups (Savini *et al*., 2005, 2003; Caracappa *et al*., 2003) and from laboratory-based infection studies (Carpenter *et al*., 2006, 2008; Jennings and Mellor, 1988).

Table 1.5 *Culicoides* subgenera and species present in Britain (IAH, 2009).

Subgenera	Species
Avaritia	***Culicoides chiopterus***
	Culicoides dewulfi
	Culicoides obsoletus
	Culicoides scoticus
Beltranmyia	***Culicoides circumscriptus***
	Culicoides manchuriensis
	Culicoides salinarius
Culicoides	***Culicoides deltus (delta + lupicaris)***
	Culicoides fagineus
	Culicoides grisescens
	Culicoides impunctatus
	Culicoides newsteadi (+halophilus)
	Culicoides pulicaris
	Culicoides punctatus
MonoCulicoides	***Culicoides nubeculosus***
	Culicoides parroti
	Culicoides puncticollis
	Culicoides riethi
	Culicoides stigma
Oecacta	*Culicoides alazanicus*
	Culicoides albicans
	Culicoides brunnicans
	Culicoides cataneii
	Culicoides clastrieri
	Culicoides clintoni
	Culicoides dendriticus
	Culicoides duddingstoni
	Culicoides festivipennis
	Culicoides furcillatus
	Culicoides griseidorsum
	Culicoides heliophilus
	Culicoides kibunensis
	Culicoides maritimus
	Culicoides pictipennis
	Culicoides poperinghensis
	Culicoides simulator
	Culicoides truncorum
	Culicoides vexans
SilvatiCulicoides	***Culicoides achrayi***
	Culicoides fascipennis
	Culicoides pallidicornis
	Culicoides picturatus
	Culicoides subfasciipennis
Wirthomyia	*Culicoides minutissimus*
	Culicoides reconditus
	Culicoides segnis

1.4.3 Species found in Wales

There are no reported studies investigating the species of *Culicoides* present in Wales, and few that report species collected for other means. One such study however (McCall & Trees, 1993), investigating the transmission of *Onchocerca* sp. in North Wales, reports that 87% of their total ceratopogonid catch was made up of three species groups: The Pulicaris Group, Obsoletus Group, Fascipennis Group, with the Pulicaris Group exhibiting the highest prevalence of all species caught (Table 1.6) and a 2-fold higher abundance than that of the second most abundant group, the Obsoletus Group.

Table 1.6 *Culicoides* caught in two areas of north Wales (McCall & Trees, 1993).

Ceratopogonidae	Site 1	Site 2
***C. pulicaris* Group**	1,287	553
***C. obsoletus* Group**	386	584
***C. fascipennis* Group**	234	166
***C. vexans* Group**	50	118
***C. heliophilus* Edwards**	167	109
***C. stigma* Mg.**	9	16
C. nubeculosus	1	0
Total	**2,134**	**1,546**

The above study was conducted on two farms within 10 miles of the town of Bala, which is located in the Snowdonia National park, north Wales. Both farms were mixed cattle and sheep farms. Another study, investigating the use of housing as a means of protecting animals against *Culicoides*, (Baylis *et al*., 2010) reported *Culicoides* catches as seen in Table 1.7. In this study an abundance of the Obsoletus Group was observed, with this group present in 27-fold higher numbers compared to the Pulicaris Group.

Table 1.7 *Culicoides* caught at two time-periods in Bala, north Wales, (Baylis *et al*., 2010).

Species Trapped	May-June	October
Obsoletus Group	66,159	3,967
Pulicaris Group	2,586	45
Other *Culicoides*	1,092	3
Total *Culicoides*	**71,345**	**4,625**

Both studies highlight that both of the newly implicated vector groups are highly abundant in the Bala region of north Wales, especially when compared to other *Culicoides* species present. The observed difference in relative numbers of Obsoletus Group members caught in each of the studies is unclear, but may be due to the timing of trappings (daytime for McCall & Trees, 1993; over night for Baylis *et al*., 2010); the time of year trappings were made (fortnightly between April-October for McCall & Trees, 1993; 12 nights in May-June and October for Baylis *et al*., 2010), as Berisha *et al.* (2010) found that Pulicaris Group members were trapped in greatest numbers in May, June and July, but Obsoletus Group members were trapped for longer period throughout the year; or possibly due to climatic or environmental affects making the region more favourable for the Obsoletus Group in 2007 than in 1983-1984.

1.5 Climate Variables and *Culicoides*

BTV occurrence may be delineated spatially by landscape features (Guis *et al*., 2007), however, outbreaks will only arise where climatic conditions are permissive to both virus replication (Mullens *et al*., 1995; Wittmann & Baylis, 2000) and vector activity (Mellor *et al*., 2000). Quantification of these constraints would allow accurate predictions of vector abundance and /or distribution to be made, providing critical support for decision making, including the development of a control strategy to minimise exposure risk.

Climate and weather variables have a profound effect on the biology and behaviour of *Culicoides* individuals and populations. As the midges are so small, they are vulnerable to even small changes in the atmosphere including temperature, wind speeds and light intensity (Mellor & Leake, 2000). This in turn affects the distribution of BTV, making weather variables and climate change an important aspect of arboviral disease epidemiology.

1.5.1 Effects on Distribution

The geographic distribution of a vector is strongly influenced by temperature, with lower temperatures having a larger impact than higher temperatures (Gates, 1993). For example, it is known that in Iberia the northern limit of *C. imicola* is determined by low temperatures (Baylis & Rawlings, 1998). From this it is apparent that other parts of Europe that have similarly suitable temperatures as those of southern Iberia are at risk of a *C. imicola* introduction. In addition, should climate change occur as predicted, and mean annual temperatures rise, then larger areas of Europe will become vulnerable to the establishment of populations of *C. imicola*. In relation to this is one of the key aspects of arboviral epidemiology: the ability of the adult vectors to overwinter. This is particularly relevant in Mediterranean regions where winters are often too cold for adult vectors to persist throughout the year, which in turn prevents diseases like BT and AHS from becoming endemic in these areas. However, climate change could alter this situation in many areas and the predicted higher winter temperatures could allow adult vector *Culicoides* to persist, as may have occurred in the 1987-1991 outbreak in Iberia (Rawlings *et al*., 1998).

1.5.2 Effects on Dispersal

Dispersal is a major factor controlling *Culicoides* distribution and is mostly affected by wind. Due to their small size, adult *Culicoides* are easily picked up by the wind. Sellers (1992) identified optimal conditions of wind speeds of 10-40 km/h, at a height of up to 1.5 km and at temperatures between 12 and 35 °C, whereby *Culicoides* could theoretically be dispersed up to 700 km. Similarly many studies

have put forward circumstantial evidence suggesting that the spread of *Culicoides*-borne disease may be caused by wind dispersal (Murray, 1987; Homan *et al*., 1990; Sellers & Maarouf, 1991; Braverman, 1992; Braverman & Chechik, 1996). Unfortunately the concept is difficult to prove as other methods of introduction cannot be entirely ruled out. Significantly though, most of the major outbreaks of BTV and AHS in Europe have been attributed to wind dispersal of *C. imicola* from infected areas to uninfected ones (Sellers *et al*., 1977; Boorman & Wilkinson, 1983; Mellor, 1987).

1.5.3 Effects on Activity Rates

Activity rates in *Culicoides* are affected by a number of weather variables. Studies have shown a positive correlation between activity and temperatures. There appear to be lower and upper temperature limits which suppress activity in different *Culicoides* species. For example, *C. variipennis* has been shown to have suppressed activity rates when temperatures are lower than 10^{o}C and higher than 32^{o}C (Nelson & Bellamy, 1971). Other species are affected by either low or high temperature thresholds, like *C. brevitarsis*, which is inactive at temperatures lower than 18^{o}C (Murray, 1987), or *C. furens* and *C. barbosai* which have reduced activity at temperatures higher than 24^{o}C (Kettle, 1969).

Similarly, light intensity has been shown to affect midge activity. Most *Culicoides* are nocturnal and decreasing light intensity is a trigger for activity. Interestingly, complete darkness can also suppress activity, as shown in *C. variipennis* which was more active during moonlight than during dark periods (Nelson & Bellamy, 1971).

Recently, wind speed has also been shown to affect activity rates. These two elements are negatively correlated for *C. impunctatus* (Blackwell, 1997) and *C. brevitarsis* (Kettle *et al*., 1998), and virtually all activity of *C. imicola* and *C. brevitarsis* is suppressed at wind speeds greater than 3 m/s and 2.2 m/s respectively (Walker, 1977; Murray, 1987). Finally, rainfall has been recorded as an activity inhibitor, whereas high humidity levels, promoted by rainfall, increase *Culicoides* activity rates (Murray, 1986).

1.5.4 Effects on Adult Survival

There have been many laboratory studies on the effect of climate variables on survival rates of adult *Culicoides* (Hunt *et al*., 1989; Wellby *et al*., 1996). Wittmann (2000) confirmed that *C. variipennis*' daily survival rate decreased with increasing temperatures, so much so that on average midges lived three times longer at 15^{o}C than at 30^{o}C. In terms of vector transmission these findings on their own suggest that at high temperatures fewer adults will survive, implying that fewer individuals are able to transmit the virus. Relative humidity, however, also may have an impact on survival rates and can moderate the deleterious effects of high temperatures. In fact it has been shown for both *C. variipennis* and *C. brevitarsis* that there is an increase in survival rates at high humidity levels and high temperatures, whereas low humidity and low temperatures have a detrimental effect on adult survival rates (Murray, 1991; Wittmann, 2000).

Rainfall can also affect adult survival. Murray (1991) recorded how *C. brevitarsis*' survival rates appeared to increase after rainfall. This was detected by the fact that before rainfall many midges fed twice and only a few fed three times, but after rainfall the age structure shifted to one of a few midges feeding twice and many feeding three times. This change to a longer lived population could increase the chances of an arboviral outbreak.

In Morocco it appears that wind speed has a significant impact on adult survival (Baylis *et al*., 1998). In a comparison across catch sites it was found that there was a significant correlation between wind speed and mortality rates in *C. imicola*. At windier sites fewer *C. imicola* seemed to survive. It is not, however, clear whether this was because the midges were actually being killed by high wind speeds, or whether they were just being dispersed. Whatever the reason though, it was clear that wind speed affected the age structure of a *C. imicola* population thus affecting the ability of that population to cause and sustain a BT or AHS outbreak.

1.5.5 Effects on Seasonality

Understanding the factors that affect the seasonal distribution of *Culicoides* species is essential to the understanding of arboviral epidemiology. The seasonality of disease outbreaks is associated with the timing of the annual peak in vector numbers (Baylis *et al*., 1997; Mohammed & Mellor, 1990). Furthermore, the proportion of adult vectors surviving through winter months underpins the ability of viruses becoming endemic in an area (Mellor, 1996). With most *Culicoides* species, and certainly with *C. imicola,* the effects of temperature and rainfall are the most important.

In areas where winters are colder, it is temperature that has the biggest impact. In such areas adult *C. imicola* disappear or the population is greatly reduced. However, in the spring the population builds up, through multiple generations, and peaks just a few weeks after the hottest time of the year (Mellor *et al*., 2000). In tropical areas, where winters are warm enough for adult *Culicoides* survival, it appears that the annual cycle of a population is more affected by the timing of the wet season. In fact it is quite clear that in Nigeria and Sudan *C. imicola* populations peak at the end of the seasonal rains (Mohammed & Mellor, 1990; Mellor *et al*., 2000). Similar observations have been made for *C. brevitarsis* in tropical areas of Australia (Murray, 1991).

1.5.6 Effects on Abundance

Abundance in *Culicoides* populations is increased greatly when the previously discussed factors are at optimal levels: low dispersal, high activity, high survival and year-round breeding. However abundance is also affected by climate variables.

Temperature correlates strongly with abundance, particularly at the limits of a species' distribution, for example with *C. imicola* in Spain cool temperatures inhibit abundance. But high temperatures favour it (Rawlings *et al*., 1998). In other areas rainfall has more of an impact, whereby the greater the rainfall, the greater the abundance. This has been recorded in South Africa (Nevill, 1971) and Israel (Braverman & Galun, 1973) for *C. imicola*. Similarly in the Caribbean, rainfall has

been correlated with both the incidence of BTV and the abundance of its primary vector, *C. insignis* (Homan *et al*., 1990). Rainfall influences abundance through its effect on the availability of breeding sites. In the case of *C. imicola*, it is known that this species breeds in wet, organically enriched soil or mud (Braverman *et al*., 1974; Walker, 1977; Braverman & Boorman, 1978) so it is likely that rain provides ideal breeding conditions, particularly after dry periods.

Other climate variables also affect abundance: a study in Morocco showed negative correlations between wind speeds and relative abundance of *C. imicola* (Baylis *et al*., 1998). This is most probably because winds speeds increase adult mortality and dispersal, as mentioned above, this affecting abundance. Similar patterns were also found in Spain and Portugal, again for *C. imicola* (Baylis & Rawlings, 1998).

1.6 Summary

While considerable progress has been made in understanding the epidemiology of BT virus, several significant areas remain poorly understood, particularly in understanding the role of *Culicoides* vectors in transmission, and the subsequent use of *Culicoides* surveillance data in modelling.

One of the main limiting factors of the models predicting *Culicoides* abundance and distribution is the quality of entomological data available. No amount of statistical manipulation or ecological descriptors can overcome sampling bias or inadequate scale created by poor data collection (Reisen, 2010). At present, all available *Culicoides* abundance and distribution data in Europe is derived from collections which are often based on just one or two trap nights at single location, widely distributed across a country (a 45 km by 45 km sampling grid was recommended by the European Union for surveillance (European Commission, 2000a, 2007)).

Within the climatic range of virus replication, the distribution of BTV epidemics is dependent almost entirely upon the distribution and abundance of vectors, and in particular upon local-scale variation in vector abundance that produces variation in vector-host ratios (Gubbins *et al*., 2008). Identification and quantification of the wide

spatial variation in abundance of *Culicoides* vectors at both the local and within-farm scale, in relation to measurable ecological factors is therefore essential for developing targeted control strategies and for assessing risk of BTV transmission.

While *Culicoides* modelling has yet to be undertaken at a high resolution, the best method for sampling *Culicoides* themselves has also yet to be determined. Within Europe, the Onderstepoort Veterinary Institute (OVI) 8w Ultra-Violet (UV) black-light suction trap (Agricultural Research Council, South Africa) has been recommended as the 'gold standard' for *Culicoides* surveillance (EFSA, 2007; Mellor & Hamblin, 2004) and although successful in capturing large numbers of adult *Culicoides* (Meiswinkel, 1998), these traps may provide a biased estimate of the total flying population or of host-seeking activity of potential BTV vector species (Carpenter *et al*., 2008). Conditions of operation, such as trap height (Venter *et al*., 2009) may also significantly affect the accuracy with which *Culicoides* populations are reflected in trap catches and in turn have implications for the accuracy of model predictions based on these population estimates.

Following the sampling of *Culicoides* in the field, the identification of trapped species, undertaken in order for the data to be used for modelling purposes, also proves problematic. BTV transmission in northern Europe is thought to be carried out primarily via the Palaearctic vector groups, the Obsoletus Group and the Pulicaris Group. The cryptic nature of these species groups has hampered the acquisition of detailed ecological knowledge required for parameterisation of BTV risk models. Augot *et al.* (2010), Nielsen & Kristensen (2011), and Pages & Monteys (2005) all describe morphometric techniques which can be used in the identification of the cryptic species, but geographic and seasonal variation in *Culicoides* species may mean these techniques cannot be generalised for identifications throughout Europe.

To investigate several of these important questions highlighted within this literature review, this thesis will investigate the relationship between a range of ecological correlates (both climatic and non-climatic) and the distribution and abundance of *Culicoides* populations on a group of farms in north Wales. The data collected during sampling at these farms will also be used to investigate the variation in abundance at different resolutions, the dispersal of these species between the farms, and whether

morphometric identification can be used to differentiate between the cryptic species involved in BT transmission.

1.7 Aims of the Thesis

The aim of the work discussed in this thesis was to inform future BT modelling by investigating key areas in the understanding of *Culicoides* life history characteristics, sampling, distribution, dispersal and identification which, as of yet, remain poorly understood.

- *Chapter 2* details the materials and methods replicated throughout this thesis;
- *Chapter 3* investigates the variation in *Culicoides* abundance at the local scale, on a set of farms in north Wales;
- *Chapter 4* determines the on-farm variation in *Culicoides* density and distribution, and how this compares to the between-farm variation identified in Chapter 1;
- *Chapter 5* investigates methods for marking *Culicoides* midges in order for mark-release-recapture methods to be undertaken in the field;
- Chapter 6 determines the dispersal of *Culicoides* midges on a group of farms in order to determine the distance travelled over a set period of time;
- *Chapter 7* determines whether morphometric identification can be used to differentiate between the morphologically cryptic Obsoletus Group species members;
- *Chapter 8* reviews the information gained from these studies in order to assess their impact on the future of BT modelling.

CHAPTER TWO

MATERIALS AND METHODS

The following methods describe techniques that are common to more than one investigation within this thesis. Additional materials and methods are presented in the individual chapters to which they are relevant.

2.1 Study Region

Fieldwork was undertaken on 35 study sites in the Bala region of north Wales. Bala is situated in Snowdonia National Park (Figure 2.1)**,** which covers 2,140 km^2 of countryside in North Wales. Snowdonia is the second largest National Park in England and Wales and the second oldest, acquiring National Park status in 1951 (Bala & Penllyn Tourism Association, 2013).

The Park contains a variety of landscapes, and habitats for animals, birds and plants; from 23 miles (37 km) of coastline with sand dunes and estuaries, to glacial valleys, the remnants of broad-leaved woodlands of oak, ash, rowan and hazel, lakes, streams and rugged mountains (Bala & Penllyn Tourism Association, 2013).

The Park's entire coastline is a Special Area of Conservation, which runs from the Llŷn Peninsula down to the mid-Wales coast. A large proportion of the Park is today under designation (or under consideration for designation) as Sites of Special Scientific Interest, National Nature Reserves, Special Areas of Conservation, Special Protection Areas, Biosphere and Ramsar sites, such as Bala Lake (Bala & Penllyn Tourism Association, 2013).

Bala itself has dramatic scenery with the surrounding mountain ranges reaching almost 3,000 ft high, deep valleys, fast flowing streams, rivers, waterfalls, forests and many lakes. The town of Bala lies on the north of Bala Lake (Llyn Tegid), the largest natural lake in Wales, which is over 5.6 km long, 1.2 km wide and over 43.5 m deep in places (Bala & Penllyn Tourism, 2013).

The Bala region is an area with an ubiquitous population of *Culicoides* and a high density of livestock farms, primarily focusing on sheep production and beef cattle farming. As this region also contains a wide diversity of altitude and landscape

features, it is of particular interest when looking to understand what ecological drivers are important for determining vector suitability and abundance.

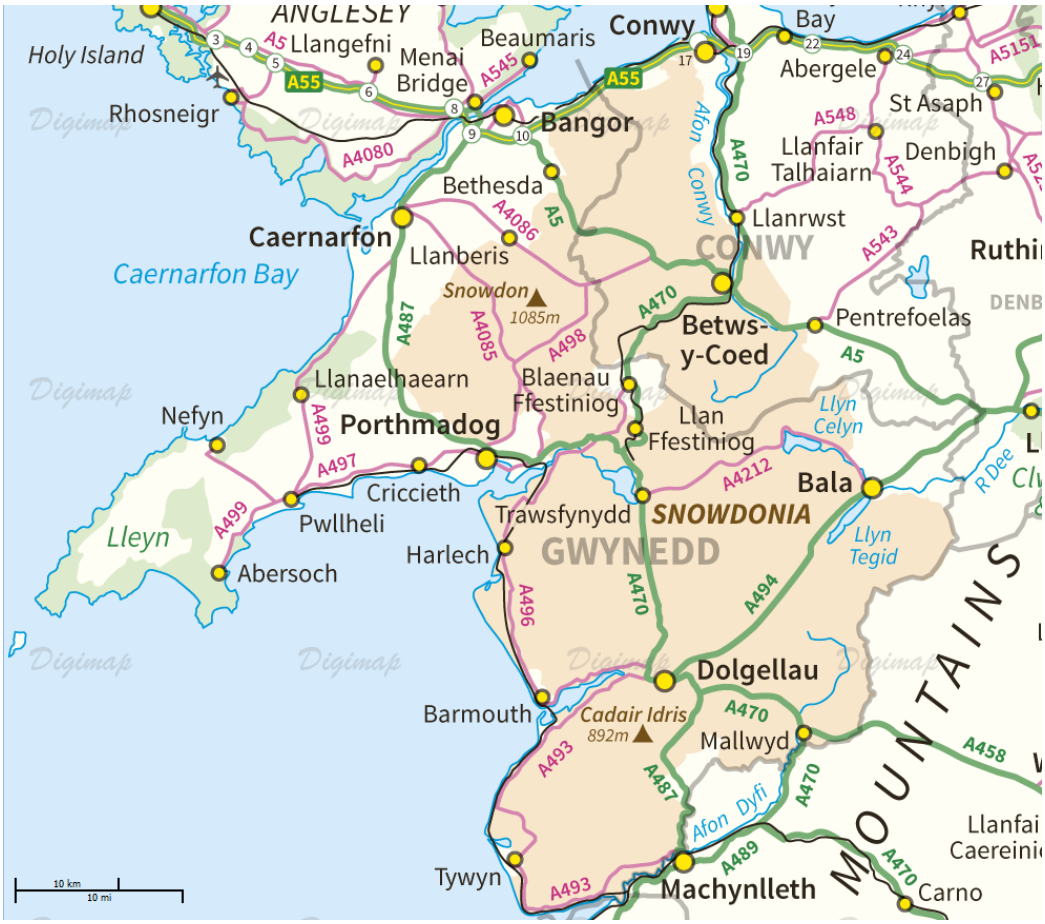

Figure 2.1 The location of Snowdonia National Park and the town of Bala within the park. (© Crown Copyright/database right 2014. An Ordnance Survey/EDINA supplied service)

Sites were selected by overlaying a 6 x 6 km grid on top of a 1: 25,000 ordinance survey map of the area just north of Bala Lake (the town and surrounding areas). In each grid square (36 in total) one farm or smallholding was then selected to participate in the series of studies, although due to the terrain, there were 2 squares containing no properties, as shown in Figure 2.2. One further control farm, outside the gridded region, was also selected to participate (35 farms participated in total). All farms were recruited via personal contact and agreed to take part in the studies following a short meeting held with the farmers to discuss the project.

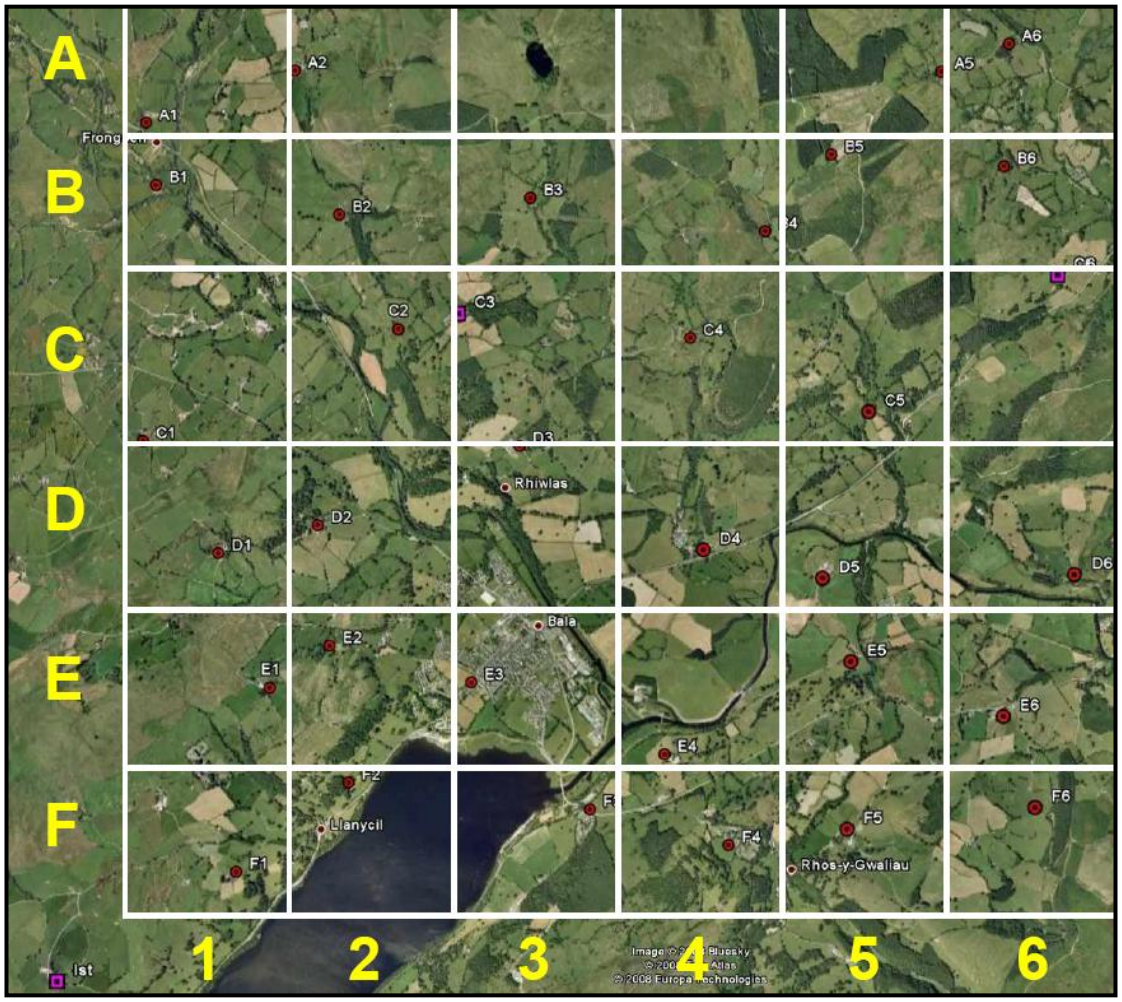

Figure 2.2 Location of 35 trapping farms within a 6 x 6 km grid covering Bala, north Wales.

All sites were used in Chapter 3, sites A1, A6, B1, B2, C1, C4, C5, C6, D2 D3, D5 and F4 were used in Chapter 4, and 19 of the sites were used in Chapter 6 (see Table 2.1).

Table 2.1 Farm characteristics (location, livestock type and number) of 35 study sites in the Bala region of North Wales. The Chapters the sites are used in are also given. Unknown denotes that those animals were present on the site, but the number of them was unknown. For anonymity, the coordinates given are for the centre of the grid square that each site belongs to.

Farm ID	British National Grid Coordinates		Livestock				Chapter (s)
	x	y	Beef	Dairy	Sheep	Other	
A1	290850	339847	150	0	2600	4 (Dogs)	3, 4, 6
A2	291850	339847	Unknown	0	Unknown	0	3
A5	294850	339847	0	0	140	0	3
A6	295850	339847	0	0	30	18 (Dogs)	3, 4, 6
B1	290850	338847	0	0	0	0	3, 4, 6
B2	291850	338847	0	0	150	0	3, 4, 6
B3	292850	338847	0	0	160	0	3
B4	293850	338847	0	0	900	0	3
B5	294850	338847	0	0	0	0	3, 6
B6	295850	338847	0	0	8	0	3
C1	290850	337847	96	0	180	0	3, 4, 6
C2	291850	337847	0	0	0	0	3
C3	292850	337847	30	0	550	3 (Dogs)	3, 6
C4	293850	337847	40	0	28	2 (Horses)	3, 4, 6
C5	294850	337847	Unknown	0	230	0	3, 4, 6
C6	295850	337847	90	0	500	0	3, 4, 6
D1	290850	336847	0	0	0	0	3
D2	291850	336847	90	0	1200	40 (Pigs)	3, 4, 6
D3	292850	336847	350	0	2000	4 (Horses)	3, 4, 6
D4	293850	336847	0	0	0	0	3, 6
D5	294850	336847	177	0	14	0	3, 4, 6
D6	295850	336847	25	0	200	0	3
E1	290850	335847	0	0	Unknown	0	3, 6
E2	291850	335847	0	0	0	0	3
E3	292850	335847	0	0	0	0	3, 6
E4	293850	335847	0	0	0	0	3
E5	294850	335847	30	0	900	0	3
E6	295850	335847	0	70	80	0	3
F1	290850	334847	11	0	200	2 (Dogs)	3, 6
F2	291850	334847	0	0	0	0	3
F3	292850	334847	2	0	0	0	3
F4	293850	334847	0	0	41	0	3, 4, 6
F5	294850	334847	60	0	1200	0	3
F6	295850	334847	0	0	Unknown	0	3, 6
Ist	289850	333847	117	0	600	7 (Dogs)	3

2.2 Trapping *Culicoides* Midges

A number of protocols can be undertaken to trap *Culicoides*. For the collection of adult *Culicoides*, light-baited traps and semiochemical-baited traps (such as CO_2 traps) can be used to trap both live individuals and to kill insects on capture. Drop-trapping techniques can also be employed to trap live individuals, by suspending a fabric netting above a cage surrounding a host and lowering the netting around the cage to trap C*ulicoides* attracted towards the host.

For larval collection, substrates can be collected and examined directly for the presence of *Culicoides* larvae using the combined sieving and sugar floatation technique described by Kettle and Lawson (1952). Substrate samples can also be transferred into an emergence chamber, so that adults can be identified on emergence. Such emergence traps can also be used directly in the field at larval development sites (Pajor, 1987).

2.2.1 Collection of Adult *Culicoides*

Onderstepoort Veterinary Institute (OVI) 220v downdraft black light traps (Agricultural Research Council, South Africa) fitted with an 8W 23cm black UV bulb are now the international standard for entomological surveillance of bluetongue vectors.

The trap itself is comprises a number of parts, as seen in Figure 2.3. The blue casing of the trap (a), houses an 8W back UV bulb (b), which attracts *Culicoides* towards it. A fine mesh surrounding the casing (c) allows larger insects to be filtered out before they reach the light source. A fan (d) housed directly below the light source sucks the insects down and through a column of netting (e) before depositing them in the collecting vessel (f) at the bottom.

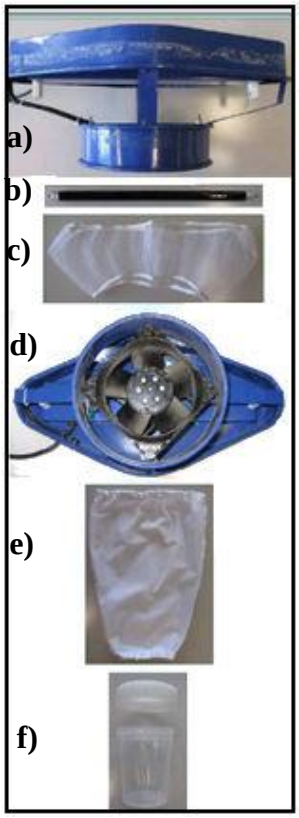

Figure 2.3 The components of an Onderstepoort Veterinary Institute black light trap. a) casing; b) 8W black UV bulb; c) mesh netting; d) fan; e) netting column; and f) collecting vessel.

Collections of adult *Culicoides* in Chapters 3, 4 and 6 were made using OVI traps suspended at a height of 1.5m to 2.0 m above ground level and hung from a tree or building on the site, or from a tripod if no suitable hanging places were available (Figure 2.4). Insects were collected into a 500 ml beaker containing approximately 200 ml of water with a drop of detergent to reduce surface tension.

All traps were set between 1600 h and 1800 h and samples collected between 0800 h and 1000 h the next day, except in Chapter 6 where traps were left to continue running during the day time. The contents of each collecting pot was stored in 70% ethanol prior to identification.

In order to trap live adult *Culicoides* the 500 ml beaker was substituted for a plastic pot with mesh bottom.

Figure 2.4 Onderstepoort Veterinary Institute (OVI) 8W UV light trap, hung from a tree (left) and a tripod (right).

2.3 Keeping Field-Caught *Culicoides* Alive in a Laboratory

Field-caught *Culicoides* were kept alive in the laboratory for use in Chapter 5. Insects were trapped using the OVI traps as described previously and a lid placed on the trapping container prior to transport to the laboratory. Once in the laboratory, the trapping container was place up-side-down so that the gauze bottom was on the top of the container (Figure 2.5).

A piece of flattened cotton wool was soaked in a 10% sucrose solution, before being partly squeezed so that it did not drip liquid, and placed on top of the gauze for sustenance. More sucrose solution was added to the cotton wool every 3 hours. The trapping pots were stored in the laboratory at 17˚C.

Figure 2.5 The maintenance of *Culicoides* in plastic trapping containing incorporating gauze lids. Cotton wool embedded with a 10% sucrose solution was placed on top of the gauze lids for sustenance.

2.4 Morphological Identification of *Culicoides*

Culicoides collected in Chapters 3, 4, 5, 6 and 7 were, following the removal of non-*Culicoides* genera, identified to species level where possible based on wing morphology and using the keys of Campbell and Pelham-Clinton (1960) and Delecolle (1985).

Due to the large numbers of adult insects collected in Chapters 3 and 4, these collections were sorted under a stereomicroscope to remove individuals that were non-*Culicoides*, before being further sorted to species level by Karien Labuschagne at the Onderstepoort Veterinary Institute, South Africa.

Morphologically, adult *Culicoides* can be distinguished from all other Diptera by the following morphological features: a well developed radial cell (Figure 2.6), but lacking an R4 and R5 region, a 15 segmented antenna, 13 antennal flagellomeres, a short anepisternal suture (Figure 2.7), and short cerci (Kondratieff, 2005).

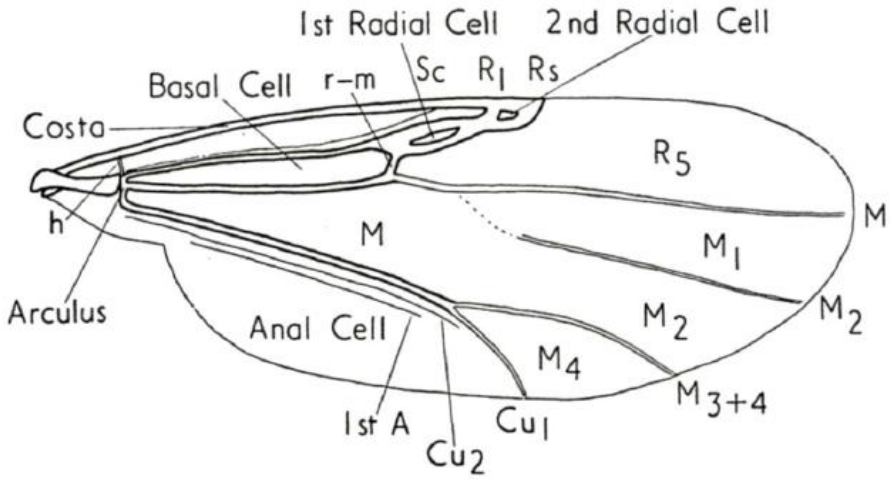

Figure 2.6 Standard notation for *Culicoides* wing venation (taken from Campbell and Pelham-Clinton, 1960)

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Downes, J. A. & Wirth, W. W. (1981) Ceratopogonidae. Pp. *In Manual of Nearctic Diptera, Vol 1. Agriculture Canads, 27*, 393-421.

Figure 2.7 *Culicoides*: A) female adult; B) male head; C) larvae. (From Downes & Wirth, 1981).

For the identification of *Culicoides* to species level, many species have wing patterns of light and dark areas that aid in identification (Borkent, 2005; Campbell & Pelham-Clinton, 1960). Additionally, details of the patterns on the dorsal side of the thorax (mesonotum), size and shape of the antennal segments, distribution of sensory pits along the antennae, relative position of the eyes, number of spermatheca in females and shape of the genital organs in males may also be required to make a full identification (Figure 2.8).

Figure 2.8 Standard notation for *Culicoides* morphology of the head (left); and male genitalia (right). [drawings provided by Dr. John Boorman]

The Palaearctic vector groups, the Obsoletus Group and Pulicaris Groups, were identified morphologically to differing degrees. The Pulicaris Group members, comprising *C. pulicaris* and *C. punctatus*, were identified to species level for both males and females. Female Pulicaris Group species were identified from their wing patterns, with *C. pulicaris* differentiated from *C. punctatus* by the dark tips to wing veins M1, M2 and CU1 (Lane, 1981), while the tips of the wing veins in *C. punctatus* typically end in small pale spots (Figure 2.9).

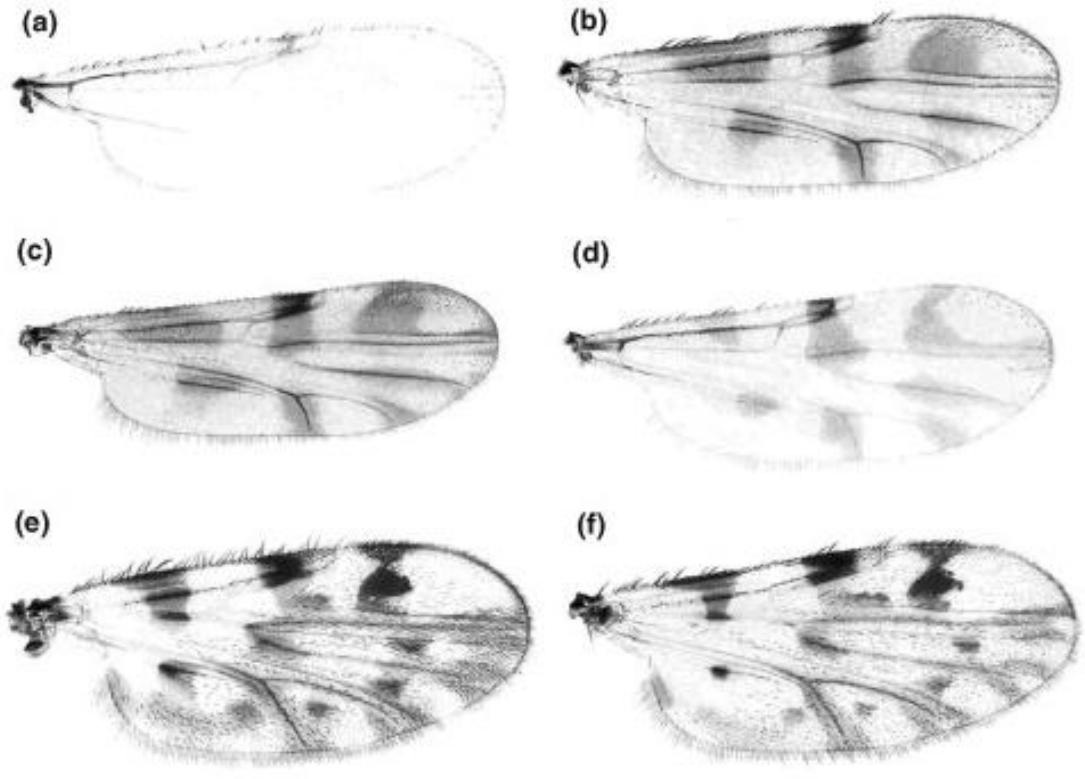


Figure 2.9 Photographs of wings of *Culicoides* species implicated as BT vectors in northern Europe. The Obsoletus Group members include, a) *C. chiopterus*, b) *C. obsoletus*, c) *C. scoticus*, and d) *C. dewulfi*. The Pulicaris Group members are e) *C. pulicaris*, and f) *C. punctatus*.

For the Obsoletus Group members, *C. obsoletus, C. chiopterus, C. dewulfi* and *C. scoticus*, only the females of *C. chiopterus* and *C. dewulfi* were able to be reliably differentiated from the other species. *C. chiopterus* is a smaller species than the others in the group, and the wings of both it and *C. dewulfi* are paler in their markings than for *C. obsoletus* and *C. scoticus*, with *C. chiopterus* in particular almost devoid of colour (Figure 2.9). A pale spot can be seen in the distal part of the wing in R5 and this has an apical edge obliquely from the costa to m1 (appearing almost triangular in shape) in *C. dewulfi*, whereas for *C. obsoletus* and *C. scoticus* this pale spot is rounded. This pale spot is often absent in *C. chiopterus*.

On examination of the spermatheca (Figure 2.10), *C. chiopterus* and *C. dewulfi* were able to be further confirmed as one or other of those species, with a pronounced

difference in size between the two spermatheca present in *C. dewulfi*, but equal sized spermatheca in *C. chiopterus* (as well as *C. obsoletus* and *C. scoticus*).

Figure 2.10 The relative size of spermatheca in female *C. dewulfi* (left – taken by X Allene, CIRAD, France) and *C. chiopterus*.

The wings of the females are more profoundly marked than males of the same species, and are in general slightly shorter and broader. However, the males of all species of both the Obsoletus and Pulicaris Groups were identified to species level based on the morphology of their genitalia, as described in the key of Campbell and Pelham-Clinton (1960) and Delecolle (1985) [see Figure 2.11].

Within their species, or species groups, the parity stage of females was identified as either nulliparous, parous, blood-fed or gravid based on abdominal pigmentation (Dyce, 1969).

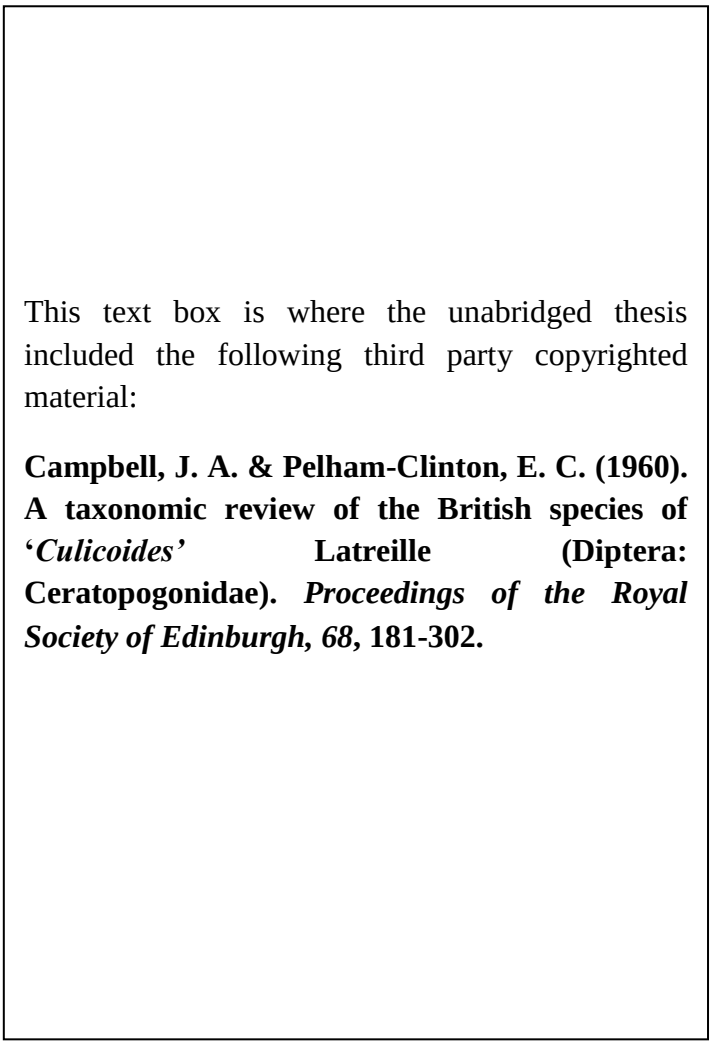


Figure 2.11 Morphological characteristics of male Obsoletus Group (a, b, c, d) and Pulicaris Group (e, f) species [reproduced from Campbell and Pelham-Clinton (1960)]

2.5 Molecular Identification of *Culicoides*

Molecular identification of the Obsoletus Group members was undertaken for Chapter 7. This work was undertaken at CIRAD, in Montpellier, France, where I took part in a collaborative project sponsored by the EDENext (European Commission) project. The molecular identification was carried out based on the work of Nolan *et al*. (2007).

Prior to DNA extraction, the *Culicoides* were individually removed from their ethanol storage vials and placed on absorbent paper, to remove any remaining ethanol. *Culicoides* were added to a Macherey Nagel round well block with 500 µL of 5% Chelex® 100 resin (Bio- Rad Laboratories, Inc., Hercules, CA, U.S.A.) [Figure 2.12].

Figure 2.12 Machery Nagel round well block containing 500 µL of Chelex resin and one *Culicoides* Obsoletus Group member per well.

For tissue lysis to occur, 3 mm Qiagen tungsten carbide beads were added to each well in the round well block. The blocks were agitated twice for 30 seconds, with 30 agitations per cycle in a Qiagen Tissuelyser II. The beads were removed, and extraction of DNA was achieved by incubating the *Culicoides* at 56°C for 1 hour (700 rpm), then 30 minutes at 96°C (650 rpm) in the 500µL Chelex resin suspension, using an Eppendorf Thermomixer.

Primers and PCR amplifications conditions were as described by Nolan *et al*. (2007), with 4 forward primers for:
C. obsoletus: UOAobsF (5'-TGCAGGAGCTTCTGTAGATTTG-3'),
C. scoticus: UOAscoF (5'-ACCGGCATAACTTTTGATCG-3'),
C. chiopterus: UOAchiF (5'-TACCGCCCTCTATCACCCTA-3'), and
C. dewulfi: UOAdewF (5'-ATACTAGGAGCGCCCGACAT-3')

and 1 reverse primer:
C1-N-2191 (5'-CAGGTAAAATTAAAATATAAACTTCTGG-3'). (Dallas *et al*., 2003)

The polymerase chain reactions (PCR) of the mitochondrial Cytochrome C oxidase I (COI) gene were performed in a total volume of 25 µL, containing 2.5 µL buffer, 0.2 µm of 25 µM dNTPs, 0.5 µL of each 10 µM forward primer, 0.5 µm of the 10 µM reverse primer, 18.5 µL H_2O and 0.25 µL 5 u/µL Taq polymerase. The PCR reaction was performed in a PTC-100 Cycler (MJ Research, Inc., Montreal, QC, Canada) under the following conditions: an initial denaturation step at 92 °C for 2 min 15 s, followed by 30 cycles of 92 °C for 15 s, 61°C for 15 s, 72 °C for 30 s, and ending with a final elongation step at 72 °C for 1 min. Results were visualized on a 1% agarose gel after 50 min electrophoresis at 110 V in 0.5x TAE buffer, using gel red staining at 1:20,000.

The length of amplified products was used to determine the species of each sample. *C. obsoletus* exhibited products at 335 base pairs (bp), *C. scoticus* at 229 bp, *C. chiopterus* at 435 bp, and *C. dewulfi* at 493 bp (Figure 2.13).

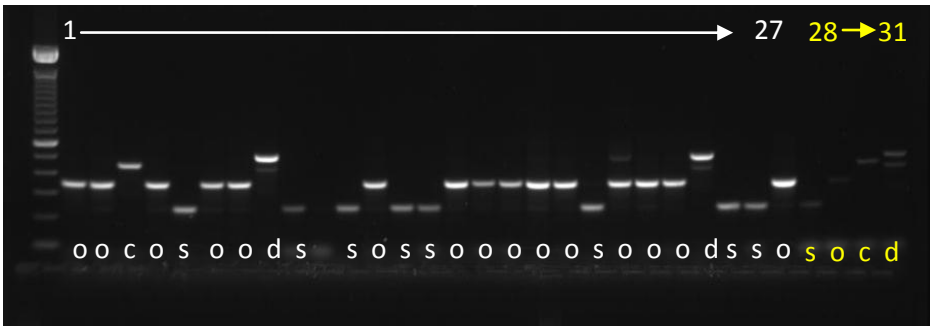


Figure 2.13 Gel image of 27 *Culicoides* Obsoletus Group members. Lanes 1 to 7 contain samples with the species indicated by o= *C. obsoletus*, s= *C. scoticus*, c=*C. chiopterus*, and d= *C. dewulfi*. Lanes 28 to 31 contain the positive controls for each of the species and are labeled using the same key as the sample lanes.

2.6 Slide mounting *Culicoides*

Slide-mounting of *Culicoides* species was undertaken in Chapter 7 and, as with the molecular identification of *Culicoides*, this work was undertaken at CIRAD in Montpellier. Individual females belonging to the Obsoletus Group were dissected on a slide under a drop of Canada Balsam. The slide was visualized down a stereomicroscope and the body was dissected using sterile needles, which were re-sterilised before the next sample. The head (dorsal side up), wings and posterior abdominal segment (ventral side up) of each of these specimens were subsequently mounted on the slide under 3 separate cover slips in Canada Balsam (Figure 2.14). The remaining thorax, legs and anterior abdomen were stored in 75% ethanol for DNA analysis at a later stage.

Each slide contained a label where details on the date of collection, the geographic location and the ID of the farm the sample was collected from, the sample code used for molecular analysis, whether the specimen was male or female and, following molecular identification of the stored remains of the dissected midge, the species.

Mounted specimens were placed flat on plastic slide-holder trays which were then transferred into a warming oven set at 34°C for 4 days. The slide mounts were then removed and stored vertically in slide boxes.

Figure 2.14 Location and orientation of slide-mounted segments of *Culicoides*. a) wing; b) head positioned dorsal-side upmost with antennae extended to the right; and c) posterior end of the abdomen situated ventral-side upmost.

CHAPTER THREE

BETWEEN FARM VARIATION IN *CULICOIDES* DENSITY

This chapter was accepted for publication (see Appendix A):

Kluiters G, Sugden D, Guis H, McIntyre KM, Labuschagne K, Vilar MJ and Baylis M (2013) Modelling the spatial distribution of *Culicoides* biting midges at the local scale. Journal of Applied Ecology, 50: 232-242

MB conceived and designed the study. DS, HG, KMM & MJV undertook field samplings. KL identified the trapped *Culicoides* to species level. GK undertook statistical analyses and model building on the trapping data. GK wrote the first draft of the manuscript and all authors contributed to approving the final version of the manuscript.

3.1 Abstract

Culicoides midges (Diptera: Ceratopogonidae) are ubiquitous on farms in the United Kingdom (UK), but little research has explored their spatial abundance, an important determinant of disease risk. Models to explain and predict variation in their abundance are needed for effective targeting of control methods against BTV and other *Culicoides*-borne diseases. Although models have been attempted at the national scale (e.g. Scotland), no investigations have taken place at a finer spatial scale.

Midge abundances were estimated using light traps on 35 farms in the Bala region of north Wales. *Culicoides* catches were combined with remotely-sensed ecological correlates, and on-farm host and environmental data, within a general linear model (GLM). Drivers of local scale variation were determined at the 1 km resolution.

Local-scale variation in abundance exhibited an almost 500-fold difference (74 to 33,720) between farms in maximum Obsoletus Group catches. The Obsoletus Group model explained 81% of this variance and was dominated by normalised difference vegetation index (NDVI). This is consistent with previous studies suggesting strong impacts of forest cover and vegetation activity on distribution, as well as shaded breeding site requirements.

The variance explained was consistently high for the Pulicaris Group, *C. pulicaris* and *C. punctatus* (80%, 73%, and 74%), the other probable BTV vector species in the UK. The abundance of all vector species increased with the number of sheep on farms, but this relationship was missing from any of the non-vector models. This is particularly interesting given that none of the species concerned are known to utilise sheep-associated larval development sites. Performance of the non-vector models was also high (65–87% variance explained), but species differed in their associations with satellite variables.

At a large spatial scale, there is significant variation in *Culicoides* Obsoletus Group abundance, which undermines attempts to record their nationwide distribution in larger scale models. Satellite data can be used to explain a high proportion of this

variation and, if shown to be generalizable, they may produce effective predictive models of disease vector abundance. We recommend undertaking a prior survey for farms with high *Culicoides* catches within the sampling area and checking stability in catch size between seasons and years.

3.2 Introduction

BT has undergone an unprecedented emergence following the entry of BTV-8 into northern Europe in 2006. The Palaearctic Obsoletus and Pulicaris Groups of *Culicoides* biting midges are implicated in virus transmission (Carpenter *et al*., 2009). Although BT has long been notifiable to the World Organization for Animal Health (OIE), the recent introduction and establishment of BTV-8 into northern Europe has intensified interest in understanding the drivers of *Orbivirus* outbreaks.

Recent advances have been made in understanding larval development sites, taxonomy and molecular recognition of *Culicoides,* but little is currently known regarding certain ecological characteristics of the vector and non-vector species (Conte *et al.* 2007a), including their distribution and abundance, making disease risk assessment and management difficult. As BT and other *Culicoides*-borne diseases are transmitted almost entirely by the bites of their vector species (Mellor *et al.*, 2000), their distribution and infection intensity are dependent on the distribution and abundance of *Culicoides*.

The spatial distribution of *Culicoides* between farms is influenced to varying degrees by the abundance and proximity of a range of resources, namely hosts, oviposition and resting sites. Environmental and climatic conditions will influence the distribution of these resources within the landscape. Species distribution models are numerical models that combine observations of species occurrence and abundance with environmental predictors. By matching species distributions with spatial and/or temporal patterns in environmental factors, suitability maps can then be produced for vectors and diseases across unsampled areas (Rogers & Randolph 2003).

3.2.1 The Current State of *Culicoides* Modelling

Statistical models facilitate informing opinions where ecological knowledge is lacking, by identifying relationships between climatic and environmental factors, and the known distribution of vectors (Baylis *et al.*, 1999; Baylis & Rawlings, 1998). Suitability maps can then be produced for vector occurrence across regions where vector distribution is unknown, on the basis of their climate and environment

(Rogers & Randolph, 2003). Analysing patterns in *Culicoides* abundance with different ecological characteristics in the same model framework (Calvete *et al.*, 2008; Conte *et al.*, 2007a) can inform an understanding of biological mechanisms underlying the sensitivities of midge species to particular environmental factors, despite limited ecological knowledge of these species.

Models of haematophagous insect distribution and abundance are, however complicated by trap catch data only providing a relative rather than an absolute measure of abundance for an area [for a review see Southwood & Henderson, 2000]. The accuracy of this estimate will vary with both sampling effort and trap efficiency, species sampled and trap type. Variations in seasonality of species sampled and the timing of collections will also influence estimates of relative abundance. In haematophagous insects, it is unknown how the attraction to and collection at light traps is related to the numbers and species composition attracted to hosts (Carpenter *et al.*, 2008). Discrepancies in the abundance or occurrence of a species in trap catches compared to those observed on susceptible hosts has implications for models describing the spatial and temporal distribution of vector-borne diseases.

A wide range of statistical techniques is available for modeling species distribution and abundance, allowing for the incorporation of both linear and non-linear responses to predictors. Some approaches also allow for effects such as spatial autocorrelation to be explicitly included within the models (Legendre, 1993; Rangel *et al.*, 2006).

3.2.1.1 Spatial Scale

The spatial scale at which these techniques have been used varies greatly but larger-scale studies have primarily been the focus, while local scale, high-resolution, models are less common. Country-wide risk mapping, as was undertaken in Switzerland prior to the BTV-8 incursion, highlights the use of regression analyses together with GIS techniques in determining vector suitability maps for the major European vector group *C. obsoletus* (Racloz *et al.*, 2007), whilst discriminant analysis has been used in terms of continent-wide mapping in Europe and Africa (Tatem *et al.*, 2003). Discriminant analysis and regression have also been used in

terms of country wide vector-distribution and risk mapping in Sicily (Purse *et al.*, 2004b), various regions in the Mediterranean (Baylis *et al.,* 2001) and Morocco (Baylis *et al.,* 1998), Iberia (Baylis & Rawlings, 1998; Wittmann *et al*., 2001) and Italy (Conte *et al.,* 2003) respectively.

The spatial scale and intensity of a study impacts on the temporal frequency of trapping, because of the limited resources, mostly time, available for processing catches. At the extreme ends of the scales, national-level surveys have undertaken single nights of catches at a large number of sites (Meiswinkel *et al*., 2008; Hartemink *et al*., 2009), while other studies report daily catches over several months at a single site (Birley & Boorman, 1982; Gerry & Mullens, 2000). Many studies adopt protocols in between these extremes: in Morocco, 22 trap sites were sampled twice weekly for two years (Baylis *et al.,* 1997). The trade-off between spatial and temporal resolution employed by such studies has rarely been overcome, except in recent years where national-level BT concerns led, in some instances, to country-wide surveillance using government resources (Calvete *et al.,* 2006; Conte *et al*,, 2007a).

The lack of local-scale modelling is likely to be due to climate-driven models performing poorly when validated at the local scale with independent data (Capela *et al.,* 2003), since non-climatic factors such as landscape configuration, farm husbandry, host availability and microclimate also influence abundance. Local scale distribution of *Culicoides* and BTV are best explained by models that incorporate landscape (Guis *et al.,* 2007), topographical (Conte *et al.,* 2007b), or host factors (Calvete *et al.,* 2008) alongside climate. Purse *et al*. (2012) found that although local-scale abundance patterns of *Culicoides* in Scotland were explained by models combining host, landscape and climate factors, predicted abundances of species varied widely among farms even over short distances (less than a few km), highlighting the need for higher-resolution models to be explored.

3.2.1.2 Climatic, Environmental and Host Variables

Seasonal climatic variables derived by temporal Fourier processing are good predictors of vectors and vector-borne disease patterns, including tsetse flies and

trypanosomiasis (Rogers, 2000), malaria (Rogers *et al.*, 2002), tick-borne diseases (Randolph *et al*., 2000) and bluetongue and its vectors (Purse *et al.*, 2007; Tatem *et al.*, 2003). Previous models indicate that climatic determinants of distribution differ between *Culicoides* species, with Purse *et al*. (2004b) finding the distributions of *C. obsoletus* and *C. newsteadi* were primarily related to remotely-sensed temperature variables (land surface temperature (LST), air temperature (TAIR)), while normalised difference vegetation index (NDVI) was the most important for *C. pulicaris*, and *C. imicola* was modelled using a combination of LST, NDVI and middle infra-red reflectance (MIR) in Sicily. Within Switzerland, the Pulicaris Group has been found to become more prevalent in trap catches with increasing altitude (1,200 m to 2,000 m above sea level) (Tschuor *et al*., 2009).

The usefulness of climatic variables as predictors for BTV outbreak occurrence has been confirmed repeatedly (Baylis *et al*., 2001; Purse *et al*., 2004a, b; Tatem *et al*., 2003). Purse *et al.* (2012) highlighted that the best performing models based on a single set of predictors were climate models, followed by landscape and then host models, for *Culicoides* distribution across Scotland. Within climatically suitable areas, it is probable that non-climatic factors such as soil type (Baylis *et al*., 1999), host abundance (Calvete *et al*., 2008; Guis *et al*., 2007), landscape structure (Guis *et al*., 2007), terrain (Conte *et al*., 2007a; Guis *et al*., 2007) and farm husbandry (Meiswinkel *et al*., 2000) determine the distribution and abundance of vector population at a finer spatial scale. The inclusion of such factors in addition to climatic correlates had therefore been shown to give models of the highest explanatory power (Purse *et al.*, 2012; Calvete *et al*., 2008; Conte *et al*., 2007a; Mellor *et al*., 2000).

Currently, only five European based investigations into *Culicoides* abundance or BTV transmission have incorporated non-climatic ecological variables (Calvete *et al*., 2009; Conte *et al*., 2007a, b; Guis *et al*., 2007; Purse *et al*., 2012) and only two have done so for the Palaearctic group species (Conte *et al*., 2007a; Purse *et al*., 2012). These models, however, indicate the potential for non-climatic variables to explain variation in *Culicoides* abundance in addition to that currently explainable by purely climate based models (Pili *et al*., 2006; Calistri *et al*., 2003).

3.2.1.3 Modelling in the UK

As understanding of the role of the Palaearctic vector groups in transmission within Europe increased, the importance of developing habitat suitability models for these groups, in addition to *C. imicola*, has been realized (Calvete *et al*., 2008, 2009; Conte *et al*., 2007a; Purse *et al*., 2006). However, few attempts have been made to model the relationship between climate, host and environmental factors and the distribution of current and potential bluetongue vectors within the United Kingdom.

To date, models concerning the Palaearctic BT vectors (Conte *et al*., 2007a, b; Purse *et al*., 2004a) have focused on links to climatic conditions within areas towards the southern limit of the vector's range. In contrast to *C. imicola*, the Palaearctic vector groups are widely distributed across the temperate regions of Europe. Thus, the links identified within the Mediterranean Basin to climate correlates between the Obsoletus Group and distribution and temperature (Calvete *et al*., 2008; Purse *et al*., 2004a, 2007) and indicators of moisture availability (NDVI and seasonality in precipitation) (Calvete *et al*., 2008; Conte *et al*., 2007a; Purse *et al*., 2004a, 2007) may not accurately portray these species' preferences within the temperate regions of their distribution.

Purse *et al*. (2012) investigated the effects of landscape, host and climate on *Culicoides* in Scotland, producing abundance models that performed well for the non-vector species *C. impunctatus* and *C. deltus* as well as the vector *C. punctatus*, yet failed to produce a strong model for the major BT vectors the Obsoletus Group, and *C. pulicaris*. Although all *Culicoides* species share the same basic habitat requirements, i.e. presence of host for bloodmeals and breeding sites (Mellor *et al*., 2000), they differ in their life history characteristics and therefore, the extent to which their distribution and abundance is affected by environmental factors. This highlights that there is still a need for strong models of the BT vector species groups to be produced.

3.2.2 Research Justification

The *Culicoides* Obsoletus complex is ubiquitous on farms in Great Britain but its density varies greatly. We need to determine the pattern of variation, in particular the spatial scale over which density varies; and to identify its causes so that predictive models can be developed.

Little is known about the ecological characteristics of the newly implicated *Culicoides* vector species (Conte *et al*., 2007a), or indeed those believed to be non-vectors, including their distribution and abundance, making disease risk assessment and management difficult. As BT and other *Culicoides*-borne diseases are transmitted almost entirely by the bites of their vector species (Mellor *et al*., 2000), their distribution and intensity of infection are dependent on the distribution and abundance of *Culicoides*.

To date, few models aim to explain the high level of local-scale spatial variation observed in BTV-vector abundance (Calistri *et al*., 2003; Pili *et al.*, 2006). In addition, the differing relative importance of climatic variables as determinants of species occurrence at the presence-absence level, indicate that the Palaearctic vector groups have differing environmental requirements. There have been few attempts to model the relationship between climate, host and environmental factors and the distribution of current and potential bluetongue vectors within the United Kingdom at a high spatial resolution. The lack of work on these newly implicated vector species compared to *C. imicola*, especially when considering their differing life-history characteristics and species distribution limits, highlights a need for the production of strong distribution models for the Palaearctic group species.

By analysing patterns in BT vector abundance in relation to both climatic and non-climatic ecological correlates in the same region, we hope to gain some understanding of the biological mechanisms underlying the differential sensitivity of the different BTV vector species groups, as well as those currently believed to be non-vectors, to particular environmental factors. Examining the importance of climatic and non-climatic ecological correlates within these species will potentially lead to improvements in BTV risk assessments through the explanation of a greater

degree of the variation observed in *Culicoides* abundance and vector-to-host ratios within modelling frameworks. Such knowledge will also determine whether predictor sets can be generalised across midge species with particular ecological characteristics (groups).

This study aimed to measure the light trap catch of *Culicoides* at a local, farm-level, scale and identify determinants of the abundance. It aimed to address the hypothesis that predictive mapping techniques for Palaearctic *Culicoides* abundance can be improved by integrating remotely-sensed correlates with climatic, environmental, and farm-level host and management correlates at the 1 km scale. It also aimed to test the hypothesis that it is possible to determine significant differences between BTV vector models compared to non-vector models.

Specific objectives included building a model for the Obsoletus Group (*C. chiopterus, C. dewulfi, C. obsoletus, and C. scoticus*), determining whether predictors could be generalised across midge species with particular ecological characteristics (groups), and investigating the use of satellite imagery at the local spatial scale. The use of freely available satellite-derived variables (Moderate Resolution Imaging Spectroradiometer, MODIS, imagery) in predicting vector distribution was also investigated, testing the hypothesis that, even at a high spatial scale, local variation in the satellite data allows accurate predictions of *Culicoides* abundance and that this freely accessible source is a useful tool in abundance prediction.

The fieldwork for this study was carried out within the University of Liverpool's Lucinda Group in July 2008, and the raw data recorded during the fieldwork was provided to me at the start of my PhD.

3.3 Materials and Methods

3.3.1 Study Region: The Bala Area of North Wales

BTV-8 emerged in northern Europe in 2006 and, following the first outbreaks in the country in August 2007, England and Wales initiated voluntary vaccination campaigns, of which Scotland's was compulsory. With Wales accounting for 15% of all sheep within the EU community (DEFRA, 2006), as well as exhibiting the highest sheep density in the UK (Figure 3.1), incursions of BT could cause devastating production losses.

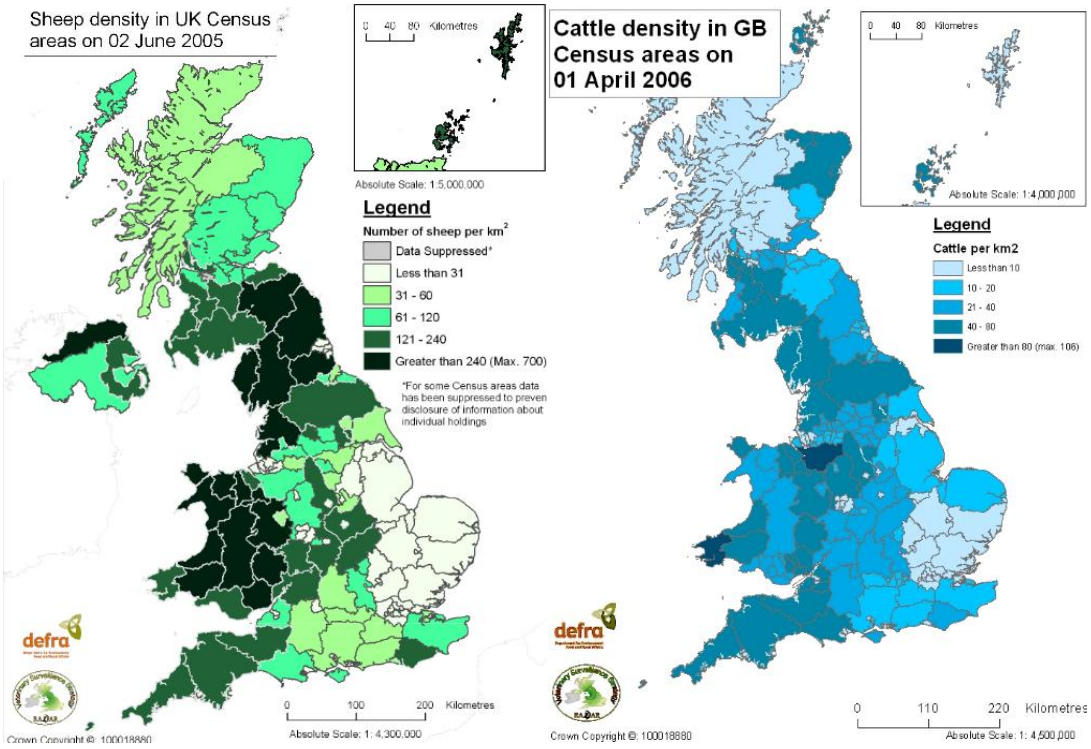


Figure 3.1 The density of cattle and sheep in UK or Great Britain (GB) Census areas, where a) Sheep density in the UK on 2nd June 2005 (Defra, 2005); and, b) Cattle density in GB on 1st April 2006 (Defra, 2006) (© Crown Copyright, 2014).

To test the research hypotheses, this study focuses on the prediction of *Culicoides* abundance in the Bala region of Gwynedd, North Wales. The town of Bala is situated among the Arenig and Berwyn mountains on the north eastern end of Bala

Lake, or Llyn Tegid, which is Wales' largest natural lake (Figures 3.2 and 3.3). The glacially formed lake is four miles long by a mile wide and lies in Snowdonia National Park (Information Britain, 1998).

The Bala region (Figure 3.2) is an area with an ubiquitous population of *Culicoides* and a high density of livestock farms, primarily focusing on sheep production and beef cattle farming. This region also contains a wide diversity of altitude and landscape features which make the region of particular interest when looking to understand what ecological drivers are important for determining vector suitability and abundance, and indeed, whether combining host, environment, climate and remotely-sensed correlates can improve vector abundance modelling.

Figure 3.2 The Bala Region of North Wales (© Crown Copyright/database right 2014. An Ordnance Survey/EDINA supplied service.)

3.3.2 Farm selection

A 6 x 6 km grid was overlaid on top of a 1: 25,000 ordinance survey map of the area just north of Bala Lake (the town and surrounding areas). In each 1 km grid square (36 in total) one farm or smallholding was then selected to participate in the study, although due to the terrain, there were 2 squares containing no properties, as shown in Figure 3.3. One further control farm, outside the gridded region, was also selected to participate (35 farms participated in total). All farms were recruited via personal contact and agreed to take part in the study following a short meeting held with the farmers to discuss the project.

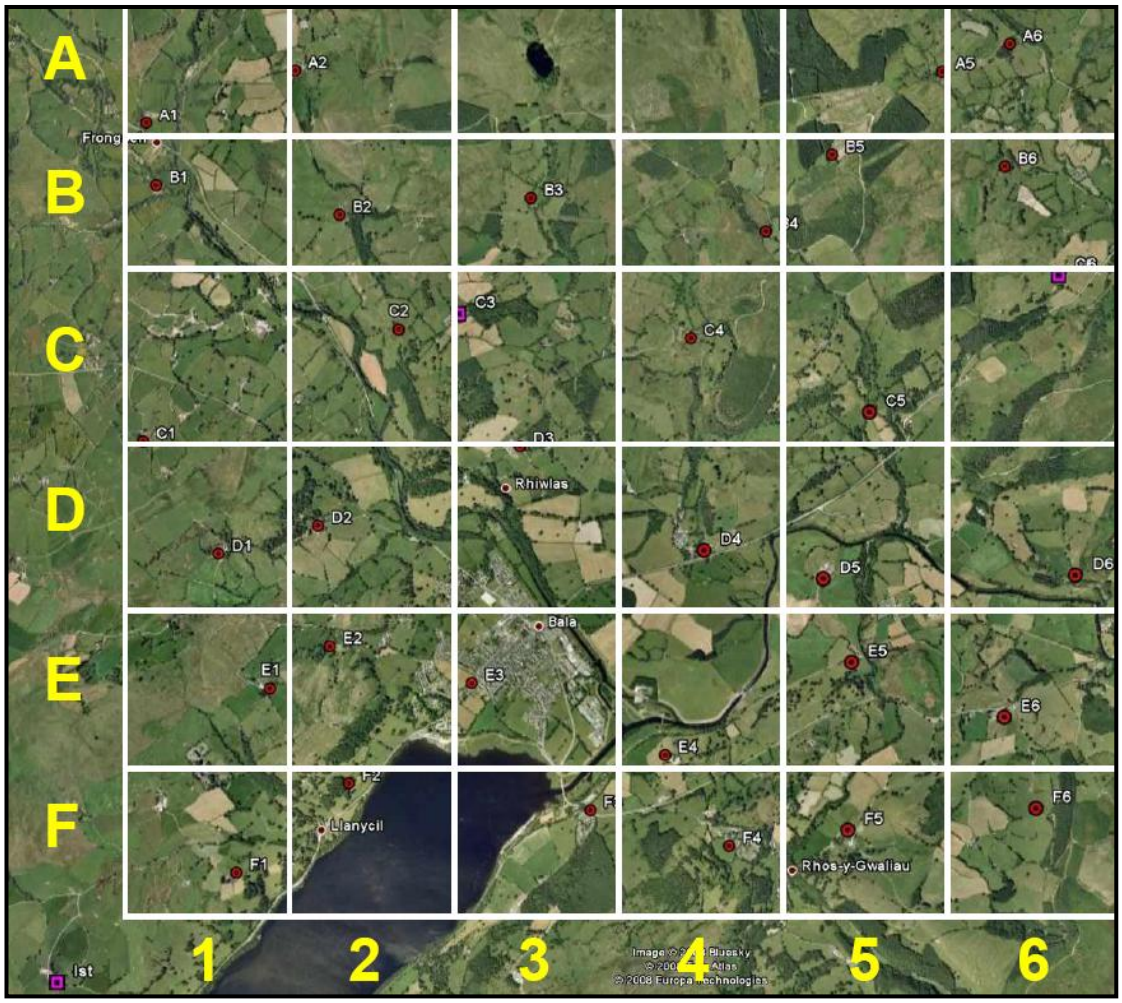


Figure 3.3 Location of 35 Trapping Farms within a 6 x 6 km Grid Covering Bala, North Wales.

A triplicated randomised trapping grid allocated the selected trapping sites to a 12-night trapping schedule (Table 3.1) between 7th July and 18th July 2008. Each farm was therefore sampled for a total of 3 trapping nights spread out over 12 nights.

3.3.3 Questionnaire Design

A short 2 page questionnaire was designed in order to determine characteristics of the farm and environment surrounding the traps (see Appendix B).

The questionnaire aimed to capture data on:

(i) Host characteristics
   a. number and distance of dairy, beef, sheep, horses and other animals from the trap;
   b. use and frequency of insecticide administration on animals, buildings, dung heaps;

(ii) Farm surroundings/environment
   a. presence of breeding sites (dung or manure heaps, leaf litter and food heaps) within 250 m radius of traps;
   b. water sources (standing and running water, waterlogged ground, man-made sources such as troughs) within 250 m of traps.

The altitude of each trapping site was measured using a Garmin eTrex® H GPS receiver. A copy of the questionnaire can be seen in Appendix B.

3.3.4 Experimental Methodology

Trapping was undertaken using 15 Onderstepoort-type down draught black light traps, fitted with an 8W black bulb, and connected to either a mains power source or a car battery. Such traps measure a mixture of *Culicoides* abundance in an area as

well as their activity and attraction to light. Traps on three of the farms were run on each consecutive night throughout the trapping period to act as controls.

Traps were positioned as close to livestock as possible and the number of livestock within 50 m was recorded each night. Midges were collected in 500-ml beakers containing approximately 200 ml of water, and a small amount of washing-up liquid to break the surface tension of the water. Traps were set between 1600–1800 h and collected between 0800–1000 h the following morning when collections were transferred to 70% ethanol for storage.

Culicoides sorting and counting was undertaken by Karien Labuschagne from the Onderstepoort Veterinary Institute Agricultural Research Council, South Africa. Large collections were sub-sampled (Van Ark & Meiswinkel, 1992) and females were age-graded into nulliparous, parous, gravid or blood-fed based on abdominal pigmentation (Dyce, 1969). Males were also counted, but all other insects were stored uncounted. For the Obsoletus Group, the females of four constituent species (*C. chiopterus*, *C. dewulfi*, *C. obsoletus* and *C. scoticus*) were counted together, while males were counted separately. Only females were considered in the analyses as males do not take blood meals and consequently do not act as vectors of disease between vertebrates. For the Pulicaris Group, *C. pulicaris*, *C. punctatus* and *C. impunctatus* catches were modelled together, as well as separately. In Europe, members of the Obsoletus Group, as well as the *C. pulicaris* and *C. punctatus* members of the Pulicaris group are considered the most important vectors (Carpenter *et al.*, 2009; Mellor & Pitzolis, 1979); the other *Culicoides* species trapped were considered to be non-vectors of BT.

Table 3.1 Trapping Schedule, in the form of a thrice-replicated randomised trapping grid, for 35 farms in the Bala region of Wales. Yellow/Green/Blue = first/second/third nights of trapping. Purple = control farms

Farm ID	Night											
	1	2	3	4	5	6	7	8	9	10	11	12
A1			X		X						X	
A2	X				X					X		
A5		X						X				X
A6		X				X					X	
B1		X				X				X		
B2				X	X							X
B3		X				X				X		
B4			X					X	X			
B5			X					X				X
B6				X			X		X			
C1		X				X					X	
C2			X					X	X			
C3	X	X	X	X	X	X	X	X	X	X	X	X
C4	X					X				X		
C5	X							X	X			
C6	X	X	X	X	X	X	X	X	X	X	X	X
D1				X	X						X	
D2	X						X		X			
D3			X				X					X
D4		X						X	X			
D5			X		X							X
D6		X			X						X	
E1	X				X				X			
E2				X				X		X		
E3				X			X			X		
E4				X			X		X			
E5		X			X						X	
E6	X				X							X
F1		X					X				X	
F2			X				X			X		
F3	X					X						X
F4	X					X					X	
F5			X			X				X		
F6				X			X			X		
Ist	X	X	X	X	X	X	X	X	X	X	X	X

3.3.5 Satellite-derived Climate Data

Seventy remotely-sensed variables were derived from MODerate-resolution Imaging Spectroradiometer (MODIS) imagery from the NASA Terra satellite (Scharlemann *et al*., 2008). Five variables with environmental significance were extracted at 1 km grid resolution between 2001– 2005: NDVI, MIR, day and night land surface temperature (dLST and nLST) and enhanced vegetation index (EVI). NDVI is a measure of chlorophyll abundance, correlated with soil moisture, rainfall and vegetation biomass, coverage and productivity (Campbell, 1996). MIR is correlated with water content, surface temperature and vegetation canopy structure (Boyd & Curran, 1998). EVI is similar to NDVI, measuring vegetation activity correlated with levels of soil moisture (Chen *et al.,* 2006; Waring *et al.,* 2006), but has improved sensitivity in wet zones with high biomass. For each of these factors, 14 temporal Fourier processed (Rogers, 2000) predictors were produced (Table 3.2).

Table 3.2. Temporal Fourier processed predictors of five environmentally significant variables derived from MODIS imagery

MODIS Variable	Explanation
a0	Overall mean amplitude
a1	Amplitude of the annual cycle
a2	Amplitude of the biannual cycle
a3	Amplitude of the triannual cycle
p1	Phase (peak timing) of the annual cycle
p2	Phase (peak timing) of the biannual cycle
p3	Phase (peak timing) of the triannual cycle
d1	Proportion of variance explained by the annual cycle
d2	Proportion of variance explained by the biannual cycle
d3	Proportion of variance explained by the triannual cycle
da	Proportion of variance explained by the annual, biannual and triannual cycles combined
mn	Minimum of the seasonal cycle
mx	Maximum of the seasonal cycle
vr	Variance

3.3.6 Data Analysis

For Pearson Product-Moment correlations between trap catches of different *Culicoides* spp., the critical value for significance was adjusted to a lower threshold using the Bonferroni correction to take account of multiplicity of *P* values.

Nightly species, or group, catches were $\log_{10}(n+1)$ transformed and the maximum of the three catches per farm was used in model building (hereafter, log-max catch). The maximum catch was preferred to the mean because *Culicoides* catches are readily reduced by weather conditions and, arguably, the maximum provides a better measure of abundance over a short time period (Baylis *et al.,* 1997). Abundance models were not parameterised for seven of the 19 species due to low catches. For the 12 other species, none of the distributions differed significantly from normality (Anderson-Darling Test for Normality, $P \geq 0.4$ for all species). Log-max catches for these species were related to satellite-derived ecological correlates, host, and environmental variables using General Linear Models (McCullagh, 1989) in R version 2.8.1 (R Development Core Team, 2008).

Predictor sets were:

(i) host factors – on farm sheep, dairy, beef, horse, other animal density; insecticide use – on animals, buildings, dung heaps;

(ii) environmental factors – presence of breeding sites composed of leaves, dung heaps, food piles, number of breeding sites within 250 m; presence of running, standing, poor draining, artificial or other water sources, number of water sources within 250m;

(iii) 70 remotely-sensed variables – 14 NDVI, 14 EVI, 14 MIR, 14 dLST and 14 nLST variables derived from MODIS satellite data (as described by Scharlemann *et al*. 2008).

The number of explanatory variables available for multivariable modelling was reduced, in order to minimise the risk of overfitting the model. Explanatory variables were examined for univariable Pearson Product-Moment correlations with log-max catch. Only variables with a probability of correlation < 0.2 were retained. Collinear variables least correlated with log-max catch were removed.

For model development, a best subsets approach was first used to select a subset of variables (within each of the three predictor sets) that best explained the variation in abundance of each species, where the R^2 and adj-R^2 were the highest possible, while each variable was significant at the $P \leq 0.05$ level individually. Finally, the subsets of variables were combined across predictor sets into a global model and the selection procedure, based on R^2 (adj-R^2) values and individual variable significance, repeated to produce a final model for each species. When multiple models fulfilled these criteria, the final model was determined on the basis of having a lower Akaike's Information Criterion value (AIC, Akaike 1973).

The small number of sampled sites precluded partitioning of the species dataset into a calibration and evaluation dataset. Therefore, to evaluate likely generalisation errors of the final models (i.e. overfitting), leave-one-out regression analysis allowed cross-validation to occur, whereby each data-point in turn is left out of the analysis and the final model refitted. The stability of variance explained, coefficients and fitted abundance values were evaluated across leave-one-out models.

Variograms were computed from the models' residuals to identify the presence of residual spatial autocorrelation, or second-order (local) effects. Second-order effects describe small-scale variation due to interactions between neighbours (Pfeiffer *et al*., 2008). The Moran's I correlation coefficient (Moran, 1950) was employed, with fixed neighbourhood sizes of 1.5, 2 and 3km, to evaluate the spatial pattern and examine the existence of the residual spatial autocorrelation between neighbouring farms. Moran's I is one of the most established indicators of spatial autocorrelation and, like the correlation coefficient, the values of Moran's I range from 1 (strong positive spatial autocorrelation), through 0 (a random pattern), to -1 (strong negative spatial autocorrelation).

To examine the assumption of independency, the Moran's I formula used is given in Equation 3.1.

$$I = \frac{N \sum_i \sum_j W_{ij}(X_i - \bar{X})(X_j - \bar{X})}{(\sum_i \sum_j W_{ij}) \sum_i (X_i - \bar{X})^2}$$

Where:

N	=	The number of cases
Xi	=	The variable at location i
Xj	=	The variable at location j
X	=	The mean
Wij	=	A weight applied between locations i and j
W = {Wij}	=	A contiguity matrix and the weight assigned in this study are 1 if zone i is adjacent to zone j and zero if other wise

Equation 3.1 Formula for Moran's I, an indicator of spatial autocorrelation

3.4 Results

3.4.1 Trapping

The 175 trap catches produced a total of 357,233 *Culicoides* of 19 species in the Bala region. The single largest catch was 65,763 midges in one trap over one night, while the mean of the maximum catches was 2,706 midges per trap per night. One catch contained zero *Culicoides* due to trap malfunction. A total of 61.9% of the *Culicoides* trapped across sites belonged to the Obsoletus Group and 31.6% to the Pulicaris Group. Of the latter, *C. punctatus* and *C. impunctatus* were the most abundant species, making up 15.4 and 14.4% of the total *Culicoides* sampled, whilst *C. pulicaris* comprised 1.8%. Of the other species, *C. achrayi* contributed 5.5%, while the others made up less than 1% each (Table 3.3). Due to the low catches of *C. brunnicans*, *C. circumscriptus*, *C. kibunensis*, *C. minimus*, *C. nubeculosus*, *C. pictipennis* and *C. stigma* models of abundance were not parameterised.

Spatial variation in maximum abundance of the Obsoletus and Pulicaris Groups, along with the three Pulicaris Group species individually can be seen in Figure 3.4. The Obsoletus Group exhibited an almost 500-fold difference in maximum catches (74–33,720) between farms, but *C. punctatus* displayed the highest variation with an almost 4,000-fold difference (6–23,656) across sites, while the Pulicaris Group exhibited the lowest, 330-fold difference (85–28,423). Similar spatial patterns are seen between the male Obsoletus Group members in Figure 3.5.

High correlation emerged between trap catches of females of certain *Culicoides* spp. on farms (Table 3.4). Obsoletus Group abundance was highly correlated to that of the vectors *C. pulicaris* and *C. punctatus*, but also the non-vector *C. achrayi*, which was associated with five of the eight species examined. The vector species, *C. pulicaris* and *C. punctatus*, were significantly associated. The non-vector species *C. impunctatus*, although a member of the same sub-genus as *C. pulicaris* and *C. punctatus*, was most strongly associated to the other non-vector species *C. achrayi*. Males of the individual species were highly correlated to the females (except *C. pulicaris* where P=0.08), apart from *C. fascipennis* and *C. festivipennis* which were not correlated. Males of the Obsoletus Group constituent species (*C. chiopterus*, *C.*

dewulfi, *C. obsoletus*, *C. scoticus*) were also highly correlated to each other (*P*=0.001) except for *C. chiopterus* with only 11 males in the total maximum catch.

Mean parous rate and range for the eight species modelled were, for the Obsoletus Group 0.84 (0.4–0.94), *C. achrayi* 0.83 (0.36–0.94), *C. albicans* 0.64 (0–1), *C. fascipennis* 0.79 (0–1), *C. festivipennis* 0.31 (0–1), *C. impunctatus* 0.83 (0.49–0.99), *C. pulicaris* 0.34 (0–0.72) and *C. punctatus* 0.78 (0.31–0.99). The parous rates of four of these species were significantly correlated to their abundance (*C. achrayi*: r=0.46, *P*=0.008; *C. albicans*: r=0.35, *P*=0.06; *C. impunctatus* r=0.39, *P*=0.03; *C. pulicaris* r=0.35, *P*=0.05).

Table 3.3 *Culicoides* species caught in Bala

Species Trapped	Female (% of total catch)	Male (% of total catch)
Obsoletus Group Total	**211927 (64.92)**	**9246 (30.02)**
By Species		
C. chiopterus	-	14
C. dewulfi	-	1694
C. obsoletus	-	6576
C. scoticus	-	962
Pulicaris Group Total	**97262 (29.80)**	**15615 (50.71)**
By Species		
C. impunctatus	37229	14225
C. pulicaris	5992	574
C. punctatus	54041	816
Other *Culicoides*	**17250 (5.28)**	**5933 (19.27)**
By Species		
C. achrayi	14111	5458
C. albicans	1135	346
C. brunnicans	23	0
C. circumscriptus	5	0
C. delta	402	1
C. fascipennis	1249	56
C. festivipennis	168	26
C. kibunensis	45	41
C. minimus	1	0
C. nubeculosus	101	5
C. pictipennis	9	0
C. stigma	1	0
Total	**326439 (100)**	**30794 (100)**

Table 3.4 Pearson Product-Moment correlation coefficients of the abundance of females of different species of *Culicoides* spp. on farms around Bala

Significance (determined using the Bonferroni correction) is given where * = $P \leq 0.001$

	C. achrayi	*C. albicans*	*C. festivipennis*	*C. fascipennis*	*C. delta*	*C. impunctatus*	*C. punctatus*	*C. pulicaris*
Obsoletus Group	0.637 *	0.337	0.395	0.336	0.372	0.401	0.745 *	0.828 *
C. pulicaris	0.605 *	0.348	0.278	0.393	0.312	0.408	0.838 *	
C. punctatus	0.593 *	0.482	0.268	0.606 *	0.326	0.414		
C. impunctatus	0.703 *	0.531	0.139	0.293	-0.288			
C. delta	0.104	0.124	0.503	0.306				
C. fascipennis	0.407	0.385	0.261					
C. festivipennis	0.342	0.369						
C. albicans	0.582 *							

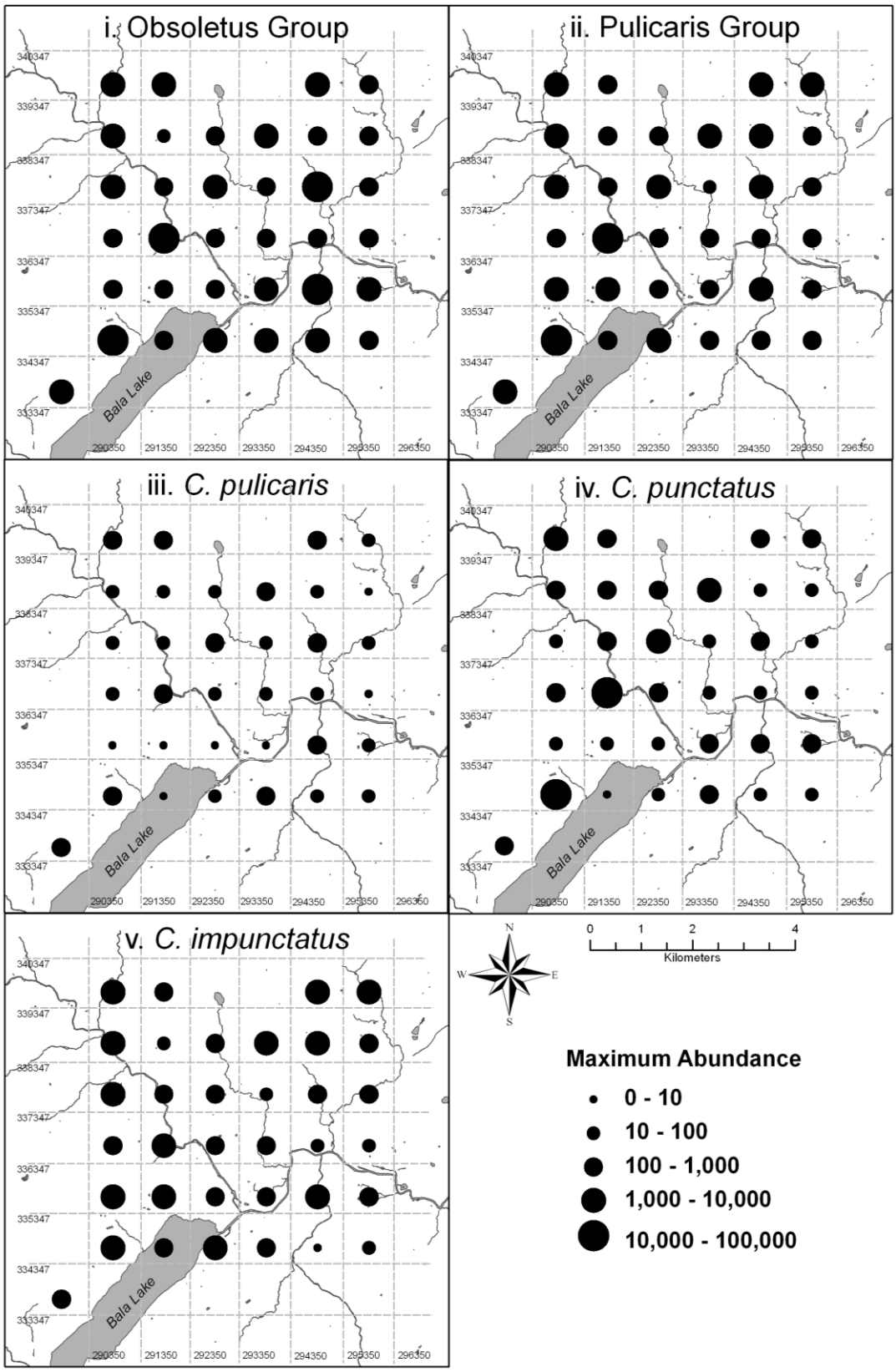


Figure 3.4. Spatial variation in maximum $\log_{10}(n+1)$ abundances of the i. Obsoletus Group, ii. Pulicaris Group, and the Pulicaris Group constituent species: iii. *C. pulicaris*, iv. *C. punctatus*, v. *C. impunctatus*, across trapping sites. Abundances have been centred in each grid square for anonymity

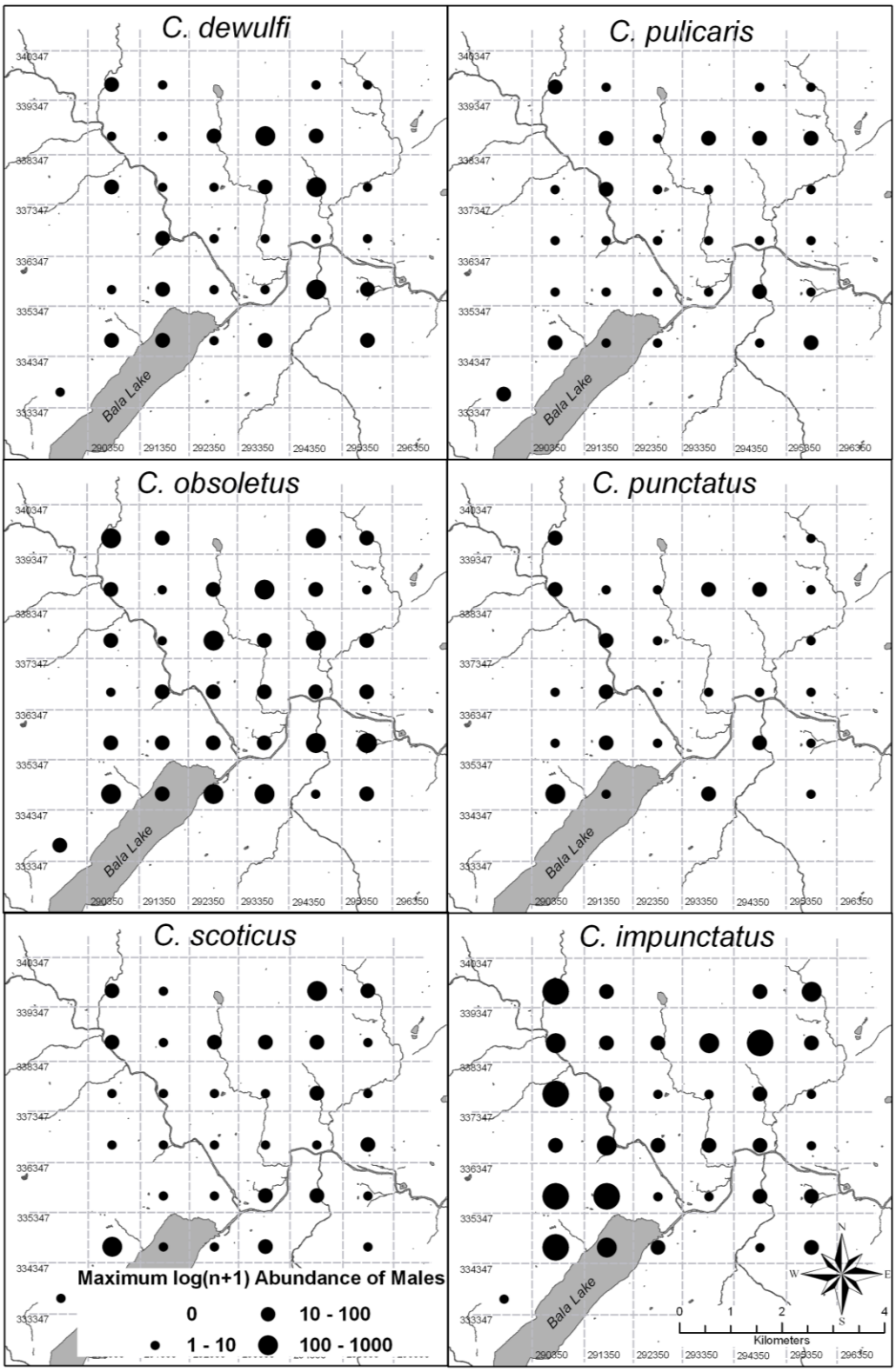


Figure 3.5. Spatial variation in maximum log(n+1) abundances of the male Obsoletus Group species i. *C. dewulfi*, ii. *C. obsoletus*, iii. *C. scoticus;* and the Pulicaris Group constituent species: iv. *C. pulicaris*, v. *C. punctatus*, vi. *C. impunctatus*, across trapping sites. Abundances have been centred in each grid square for anonymity. No map was drawn for the *C. chiopterus* member of the Obsoletus Group as there were only 11 males trapped across 4 sites in the maximum catches.

3.4.2 Questionnaire Data

Of the 35 farms, 22 (62.9%) kept sheep, 14 (40%) beef cattle and two (5.7%) horses, while only one kept dairy cattle (Table 3.5). Other animals included pigs, dogs and chickens. Three farms had sheep present on premises during the study, but did not own them and were unaware of the exact numbers. As 'number of sheep' kept on a farm was determined to be an important variable early on, it was decided that data from those three farms would be omitted from model building, leaving 32 observations.

Table 3.5. Host animals on farms around Bala

Host Variable	Number of Farms (%)	Mean[a] Number of Animals	SD	Range
Sheep	22 (62.86)	541.41	688.92	8-2600
Beef Cattle	14 (40)	90.57	91.29	2-350
Horses	2 (5.71)	3	1.41	2-4
Dairy Cattle	1 (2.86)	70	NA	NA
Pigs	1 (2.86)	40	NA	NA

SD, standard deviation; NA, not applicable

[a] Mean of those farms with animals

Table 3.6. Breeding sites and water sources on farms around Bala

Predictor Variable	Number of Farms (%)	Mean Distance (m)[a]	SD	Distance Range (m)
Breeding Sites[b]				
Dung	21 (60)	18.29	25.85	1–100
Leaf Litter	24 (68.57)	21.83	21.80	1–75
Food Heaps	8 (22.86)	54.29	44.01	20–150
Water Sources[b]				
Running	24 (68.57)	104.22	91.12	2–250
Standing	9 (25.71)	197.22	104.79	5–250
Wet Ground	18 (51.43)	70.15	86.06	2–250
Artificial	22 (62.86)	12	16.77	1–50

SD, standard deviation

[a] From light trap; [b] Within 250m of the trapping site

Nineteen farms (54.3%) used insecticides regularly on animals, but none on buildings or for dung management, although 21 (60%) farms had dung piles within 250 m of the trapping site (mean 18.29 m, range 1–100 m). In terms of other breeding sites, 24 (68.57%) farms had leaf litter between 1 and 75 m (mean 22 m), and eight (22.86%) had food heaps within 20–150 m (mean 54 m) of the traps (Table 3.6). The mean altitude of the farms was 237.9 m, but varied between 169.1 and 335.1m.

3.4.3 MODIS Satellite Data

In the 2001–2005 MODIS data, daytime temperatures peaked in Bala in early to mid-June, and night-time temperatures in late June. Site temperatures ranged from -0.8 to 20.8°C (mean 10.9 °C) in the daytime and -6 to 9.2°C (mean 0.9 °C) at night time. The peak of NDVI occurred between late June and early August and the seasonal range in this index varied from 1.617 to 1.871. The peak of EVI occurred between early and late June and the seasonal range at sites varied from 1.140 to 1.739. For MIR, a peak occurred across most sites between early March and May, although the peak on one farm occurred in late February. The seasonal range varied from 0.036 to 0.129.

3.4.4 Overall Performance of Model Variables

The variables included in each of the final group or species-specific models are shown in Table 3.7. Overall, NDVI was the only explanatory variable selected in all models, while MIR was only seen in six of the models. The only host variable within the final models was the ‘number of sheep’ on a farm, while for environmental variables the number of water sources, food heaps and dung heaps were included. The vector group models (Obsoletus and Pulicaris Groups) both contained a host variable (sheep), as did the two individual vector species models (*C. pulicaris* and *C. punctatus*), while this remained absent in the non-vector models.

Table 3.7. Abundance models for each species or group, including percentage of variance explained (R^2 and adjusted R^2) and model AIC (Akaike's Information Criterion). After each variable, ( ) indicate the sign of the correlation coefficient.

Species/ Group	NDVI	EVI	MIR	LST		Other	AIC (Null Model AIC)	Mean R^2(%) (Adjusted R^2(%))
				dLST	nLST			
Obsoletus Group	p3 (+) d1 (+) da (-) mx (+)	-	a3 (+) d3 (-)	d2 (-)	p3 (+)	Sheep (+) Water (-)	40.035 (73.587)	81.23 (72.29)
Pulicaris Group	p3 (+) d1 (+) da (-)	a3 (+) d1 (+) d2 (+) da (-)	p2 (+)	-	-	Sheep (+)	28.462 (62.533)	80.41 (72.39)
C. impunctatus	p2 (-)	a1 (-) a3 (+) d3 (-) mx (-)	-	a0 (+) p1 (+)	p3 (-) d2 (+) d3 (-)	-	35.565 (68.759)	80.93 (71.85)
C. pulicaris	p3 (+) d3 (-) mx (+)	p1 (-)	-	p3 (-)	p3 (+)	Sheep (+)	48.876 (76.84)	73.10 (65.25)
C. punctatus	p3 (+) d1 (+) mx (+)	p1 (-) d1 (-)	-	-	-	Sheep (+)	44.518 (75.851)	74.17 (67.97)
C. achrayi	a1 (+) a3 (-) d1 (-) d3 (+) da (+) vr (-)	a3 (+) p2 (-) d1 (+) d2 (+) da (-) mx (-)	-	-	p1 (+) p2 (+) p3 (-)	Breeding Environments (-)	31.712 (58.435)	84.10 (67.15)
C. albicans	a1 (-) p3 (+) d1 (+) d3 (-) vr (+) mn (+)	a0 (-) mx (+)	p1 (+) da (-) mx (+)	-	p3 (+)	Water (+)	41.030 (50.362)	65.78 (41.07)
C. delta	vr (+) mx (+)	-	a1 (-) p2 (-) mn (-) mx (+)	a0 (+) a1 (+) mx (-)	a3 (+) p3 (-) mn (-)	-	28.469 (63.01)	84 (73.90)
C. fascipennis	a1 (+)	a1 (-) d1 (+) da (-) vr (+) mx (+)	-	a1 (-)	a1 (+) d1 (-) d2 (+) vr (-)	-	15.671 (61.5)	87.97 (81.35)
C. festivipennis	a1 (-) p1 (+) d1 (+) da (-) vr (+) mn (+)	a0 (+) da (+)	p1 (-)	a1 (+) a2 (+) a3 (-) vr (-)	p3 (+)	-	23.014 (44.433)	78.57 (60.93)

Remotely-sensed variables: NDVI=normalised difference vegetation index; EVI=enhanced vegetation index; MIR=middle infra-red reflectance; dLST=day land surface temperature; nLST=night land surface temperature.

MODIS variables: a=amplitude; p=phase (peak timing); d=proportion of variance explained by the: 1=annual cycle, 2=biannual cycle, and 3=triannual cycle. da=proportion of variance explained by all three cycles combines; mn=minimum of the seasonal cycle; mx=maximum of the seasonal cycle; vr=variance.

3.4.5 Model Evaluation

Moran's I tests for spatial autocorrelation were insignificant at all neighbourhood sizes for all species and groups, except for *C. festivipennis* which exhibited negative spatial autocorrelation at the 1.5 and 2 km neighbourhood sizes (in a 1.5 km neighbourhood I = -0.39, p = 0.003). The Moran's I for the Obsoletus group model verged on, but did not reach significance at the 1.5 km neighbourhood size (I = -0.2596, E[I] = -0.0323, p = 0.06). The insignificant Moran's I values suggest that spatial autocorrelation has no, or little, influence on patterns of midge trap catches at these scales.

Leave-one-out regression analysis was used for cross-validation due to the small number of sampled sites. Fig. 3.6 shows the fitted abundance values from the leave-one-out regressions for the six most abundant *Culicoides* species. The stability of the fitted abundance values and variance suggest there would be low generalisation errors if our predictions were extended to another region of north Wales.

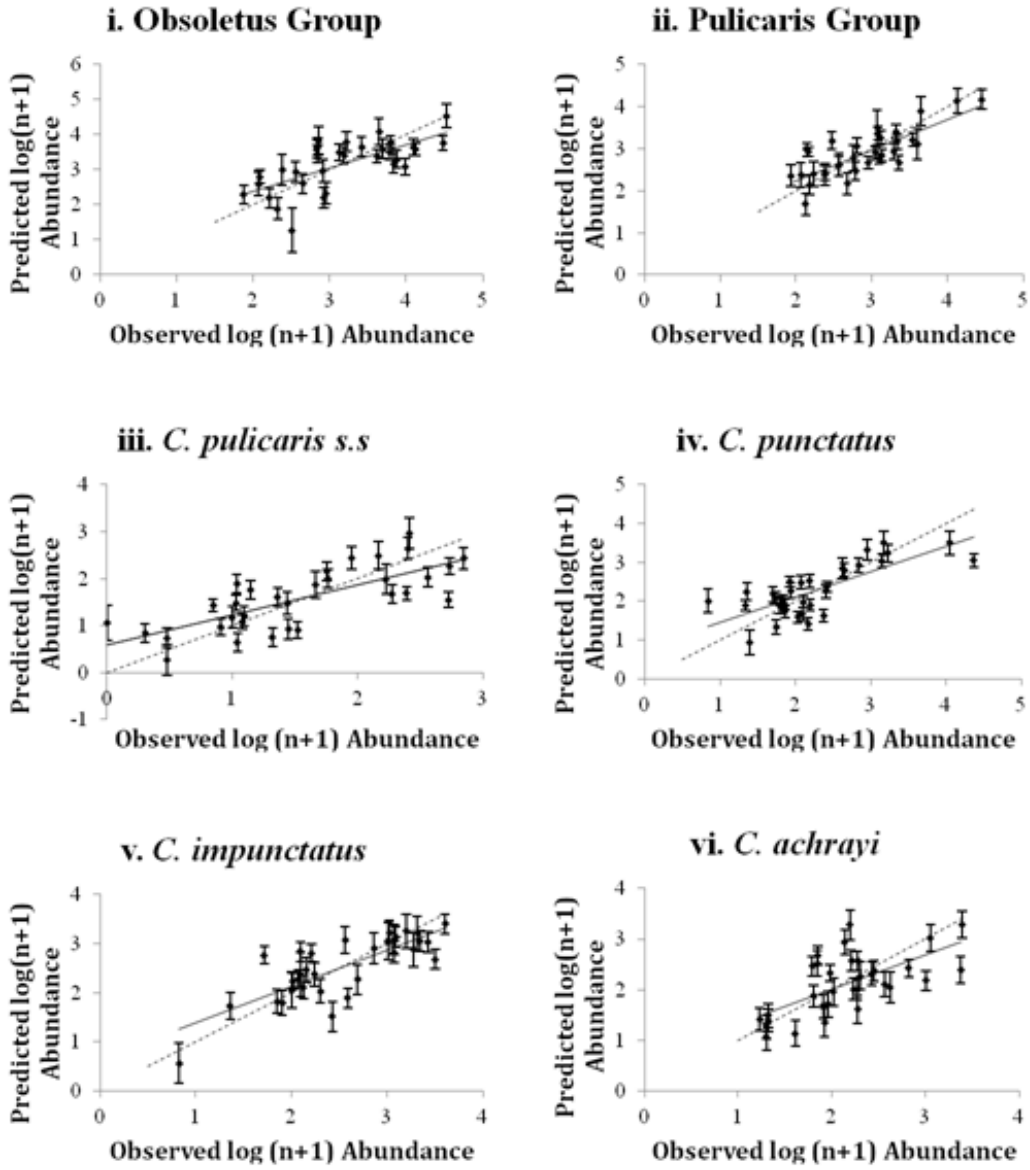


Figure. 3.6. Predicted versus observed log(n+1) abundance for the Obsoletus and Pulicaris Groups, and for *C. pulicaris*, *C. punctatus*, *C. impunctatus*. Error bars indicate the magnitude of standard errors, solid black line indicates line of best fit and dashed black line indicates line of equality

3.5 Discussion

This study represents the first attempt to explain and predict trap catch patterns of the Obsoletus Group, Pulicaris Group species, and other potential UK vectors at a high resolution (1 km) in relation to a range of ecologically relevant satellite, environmental and host factors. *Culicoides* inhabit a wide range of moist microhabitats in agricultural and natural ecosystems (Mellor *et al*., 2000). As such, mapping potential areas of disease risk and targeting disease control measures requires models that can explain and predict local scale variation in abundance, rather than simply occurrence, of species.

The Obsoletus and Pulicaris groups were the most abundant *Culicoides* caught in the Bala area, in agreement with previous studies in north Wales (McCall & Trees, 1993; Baylis *et al*., 2010). *C. achrayi* and *C. impunctatus* were the most abundant non-vector species. *C. achrayi* is also highly prevalent on Belgian farms (Haubruge, 2008). In contrast, relatively few *C. achrayi* were trapped at Chester Zoo (Vilar *et al*., 2011) during the same year and instead a high proportion *C. kibunensis*. The reasoning behind this is unclear, but apparent opposing preferences for a farm or zoo environment are likely due to differences in these species' life-history characteristics.

Large differences between *Culicoides* catches on neighbouring farms highlights the fact that catches on one farm should not be deemed representative of a region. This raises questions about the validity of nationwide entomological surveillance schemes which, inevitably, trap at coarse resolutions. Our results suggest that large-scale surveillance should consist of multiple trapping sites in each sampling area, or undertaking a prior survey of multiple farms in each region and proceeding with those that yield the highest catches.

A trade-off exists between temporal and spatial aspects of most surveys. In our study, the intense spatial detail (one trap per km^2) and high frequency nightly trapping required a degree of effort, and generated a volume of midges, that precluded undertaking trapping longer than three nights per site and 12 nights in total. This may limit our ability to generalise findings in both space and in time. We cannot be sure that the same farm-to-farm heterogeneity occurs elsewhere in the UK

or at Bala at other times of year or in other years. However, a study near Bala in May-June, 2007 reported significant heterogeneity in *Culicoides* catches between just four farms over 12 nights trapping on each farm, suggesting that our findings are robust, at least for Bala. If verified, the small-scale variability observed in this study should highlight an area of concern for those interpreting large-scale studies with scarce sampling points.

High local spatial variation may also explain the difficulty of building strong large-scale models for the Obsoletus Group (Purse *et al*., 2012). We have successfully modelled high spatial variation at the small spatial scale for several species, including the Obsoletus Group. One reason for the success of high-resolution models is that nearby farms may differ in the levels of important explanatory variables while distant sites spread across large areas may differ in the nature of those explanatory variables.

Abundances of several vector species (Obsoletus Group, *C. pulicaris* and *C. punctatus*) were correlated, suggesting common predictors of abundance due to similar life history characteristics, such as the presence of live hosts. Similarly, male abundances of three of the Obsoletus Group species were correlated, again suggesting similar ecological factors favour abundance. It is of interest to note that *C. impunctatus*, believed to be a non-vector species, is closely related to the vectors *C. pulicaris* and *C. punctatus*, yet shows strongest correlation to the distribution of other non-vector species.

Calvete *et al*. (2008) found that incorporating host variables into bioclimatic models greatly increased the variance explained in BTV-4 occurrence in Spain. In Italy, both biotic (forest and vegetation activity) and abiotic (topography, temperature and aridity index) axes were found to govern the occurrence of the *C. obsoletus* group (Conte *et al.*, 2007a). Half of our models contained satellite variables only, and there was no significant difference between the explanatory power of these or the mixed predictor models. This concurs with Calvete *et al*. (2008) who, from the superior performance of climate only models, inferred that bioclimatic variables were the main ecological factors driving BTV occurrence across Spain. Satellite-derived ecological correlates dominated in number in the final models for all species in Bala, highlighting that the importance of these ecological drivers extends to the local scale.

3.5.1 The Obsoletus Group Model

For the Obsoletus Group, four species (*C. chiopterus*, *C. dewulfi*, C. *obsoletus* and *C. scoticus*) with a mix of host and breeding habitat preferences are modelled together. Even so, the model explains a high amount of variance (81%) and is consistent with other studies that have detected large impacts of landscape factors, such as forest cover and vegetation activity on the distribution patterns of the Obsoletus Group or Complex (Calvete *et al*., 2008; Conte *et al*., 2007a; Purse *et al*., 2004a). The goodness of fit of the group model could either indicate that one species is highly dominant (and thus the model most influenced by its requirements) or that all species have similar habitat requirements. Further work should be undertaken to explore whether these species' habitats differ.

The distributions of the Obsoletus Group were dominated by remotely-sensed NDVI variables. Most NDVI variable coefficients were positive, indicating a preference for microclimates with high levels of moisture, favouring vegetation growth. This is supported by previous observations that *C. obsoletus* breeds preferentially in forest litter (Amosova, 1956; Dzhafarov,1964). Conte *et al*. (2007a) also found significant correlation between areas of deciduous and mixed broadleaved/coniferous forests and the Obsoletus Complex. The inclusion of two MIR variables, also correlated with vegetation levels (Boyd & Curran, 1998), further supports this theory. The correlation of 'number of water sources' with the Obsoletus Group indicates that moist habitats are favourable, likely due to their semi-aquatic larval stage, and, indeed, *C. obsoletus* has been reared from wet areas of Scotland (Kettle & Lawson, 1952).

Obsoletus Group trap catches also increased with the number of sheep on farms. This is in agreement with Garcia-Saenz *et al*. (2010) who found a linear increase between *C. obsoletus* trap catches and sheep number.

Molecular identification was deemed unnecessary as there is currently insufficient information to determine vector competence of the Obsoletus Group species individually. If it becomes clear that there are important differences between vector competences, molecular identification of the Obsoletus Group species would provide further evidence of their ecological differences. Whilst sufficient numbers of males

were caught and could be used to model the Obsoletus Group species, there is little evidence their relative abundance is proportional to the females of each species.

3.5.2 The Pulicaris Group Vector Species

Like the Obsoletus Group, *C. pulicaris* and *C. punctatus* abundances were positively correlated with NDVI variables and sheep number. Catches of *C. pulicaris,* a wet-soil and bog species, have been associated with high, stable, levels of moisture (high, less variable NDVI) elsewhere in Europe (Purse *et al.*, 2004b, 2005). The *C. punctatus* model is similar to that of *C. pulicaris,* only lacking in LST, highlighting the close relationship of the two species. The inclusion of LST variables in the *C. pulicaris* model may highlight the species' need for more stable temperatures, as highlighted by Parker (1950) who found that *C. punctatus* eggs are less adversely affected by above-normal temperatures than *C. pulicaris* eggs.

The Pulicaris Group model appears more closely related to its two constituent vector species (*C. punctatus* and *C. pulicaris*) than to that of the third, non-vector, species *C. impunctatus*. The inclusion of 'number of sheep' on a farm suggests that, like with the Obsoletus group, Pulicaris group numbers also increase as sheep numbers increase.

3.5.3 Non-Vector Models

The *C. impunctatus* model, unlike the other Pulicaris Group species models, was dominated by LST variables. The annual mean of the dLST indicates a preference for warmer temperatures and a later peak in daytime temperatures (positive coefficient for p1). EVI featured heavily in this model, with an increased trap catch in locations with low variation in vegetation activity throughout the year and, in contrast to the Obsoletus Group model, less densely covered areas with more access to sunlight. This is likely to represent this species' preference for organically enriched, soil breeding sites with high water content (Blackwell *et al*., 1999; Blackwell *et al*., 1994b). Kettle & Lawson (1952) describe that immature stages of *C. impunctatus* are commonly found in bogland sites in wetter areas of moorland

where *Sphagnum* and *Polytrichum* moss growth is thin enough to permit penetration by *Juncus articulates*, a species of rush that thrives in hot overhead sunlight.

The exclusion of 'number of sheep' in the *C. impunctatus* model may be attributable to its autogenous nature (Boorman & Goddard, 1970) making the species less reliant on host blood-meals than anautogenous vector species. In turn, the only models incorporating host factors are those that have, currently, been implicated as BT vector species.

As I did not design, or take part in, the fieldwork for this study there are a number of changes I would make if the study were to be repeated. Firstly, the size of the trapping grid should be doubled in order to recruit twice the number of farmers and enable the data to be split into a calibration and evaluation dataset. Ideally, the holding recruited in each grid square would be a farm with known numbers of livestock, whereas in this study two grid squares lacked any premises, and 3 premises had sheep belonging to another farmer present and were unaware of numbers – precluding them from the analysis. It was not possible to analyse the section of the questionnaire covering 'degree of openness' and this information would be more informative on a scale of 0-4, where 0 is a fully open landscape (e.g. no buildings, hedges surrounding the trap), 1 is enclosed on one side (e.g. by the wall of a building), 2 is enclosed on 2 sides, 3 is enclosed on three sides, and 4 is fully enclosed.

The MODIS data used was averaged over a five year period, the latest year of which was 3 years prior to the fieldwork. Ideally the temporal resolution of this data would be the year the fieldwork took place and, as the sampling employed a snapshot approach, it should also be at the same time of year as the trapping, Finally the use of weather stations monitoring, temperature, humidity, wind speed and rainfall at each of the trapping farms would have enabled weather data to be included in the models (other than purely the dLST and nLST provided by the MODIS dataset). This may have increased the variance explained by the models further and would have allowed us to assess the impact of weather data on top of environmental and host factors.

3.5.4 Conclusions

The high explanatory power of all models built highlights the success of using freely available satellite data to model *Culicoides* distribution. Although the leave-one-out regression analyses provide strong evidence that these vector models would have strong predictive power when applied to another area of north Wales, external validation involving sampling in another region will be the ultimate test. With no strong Obsoletus group abundance models published for the UK and no previous work highlighting a difference in host involvement between vector and non-vector models, the vector species models arising from this work are of utmost value.

Four of the species had parous rates that were significantly correlated to their abundance. A high parous rate is an indication of survivorship and the correlation for these four species suggest that survivorship, as opposed to proximity to breeding sites, is the reason for the high population sizes, reinforcing the reliability of this data.

Analysing a large number of explanatory variables relative to the number of data points creates a danger of overfitting. Leave-one-out regression analysis is a useful technique for examining overfitting, as overfitted models often show poor predictive ability. The leave-one-out regressions of our models show good predictive ability and therefore, while we accept there was a risk of overfitting, our models show no evidence of it.

Our study highlights the high variation in *Culicoides* abundance that can occur between neighbouring farms, but work still needs to be undertaken to determine the level of variation present at the smaller, within-farm scale, and the factors driving that variation. Given the remarkable heterogeneity detected, we recommend that large-scale surveillance includes multiple sites per region or that a prior survey of each region is undertaken to determine those farms with the highest *Culicoides* catches.

CHAPTER FOUR

BETWEEN YEAR, AND ON FARM VARIATION IN *CULICOIDES* DENSITY

The study was conceived and designed by Georgette Kluiters (GK). GK & Matthew Baylis were awarded a Wellcome Trust Vets Vacation Scholarship which enabled Kevin Jones (KJ) to support the field trapping. GK & KJ undertook the field sampling. Karien Labuschagne sorted the *Culicoides* to species level. GK undertook analyses on the data.

4.1 Abstract

High levels of variation in midge catches have been identified between farms as close as 1 km from each other. Risk managers for disease incursions may rely on models of disease, or the distribution of their vectors, to effectively target control methods against diseases, such at BTV. Little work has explored the annual variation in *Culicoides* distributions or abundances on farms, which may undermine attempts to base control methods on the distribution of vectors from previous years of trapping, or indeed how catches may vary in different environments within farms.

Midge abundances were estimated using light traps on 12 farms in the Bala region of north Wales. Traps were located in three differing environments on each farm (in a yard or concreted area, in an open field, amongst high vegetation and trees). Trap catches on the 12 farms were compared between the years 2008 and 2010, while the abundance of *Culicoides* species trapped in each of the farm environments was investigated using a one-way ANOVA or Kruskal-Wallis test.

Maximum catches for all species were, on average, 11-fold greater in 2010, than 2008. The 500-fold local-scale variation in abundance identified for the Obsoletus Group in 2008 (74 to 33,720) was reduced to a 50-fold difference in 2010. The most prevalent species were members of the Obsoletus Group in 2008, while in 2010 the Pulicaris Group was the most prevalent, with *C. impunctatus* totaling 67% of all *Culicoides* trapped in that year.

For both the 2008 and 2010 trapping results, the abundance of the Obsoletus Group was highly correlated to that of the vector species *C. pulicaris* and *C. impunctatus*, while the non-vector species *C. impunctatus* only showed weak positive correlation to the other members of its subgenus, *C. pulicaris* and *C. punctatus*. This highlights that although overall numbers of *Culicoides* have increased between the years, the underlying distribution of these species is likely to be similar.

The mean number of sheep, beef and horses kept on the 12 farms decreased over the two year period, yet the one farm keeping pigs had increased the number of them. The number of farms using insecticides on their animals decreased from 10 to eight, while none of them used insecticides on buildings or for dung management in either year. The proximity of breeding sites (dung and leaf litter) and water sources to the

trapping sites increased over the two year period, suggesting a change in farm management practices over this time.

Culicoides catches were consistently higher from catches located in areas of high vegetation in comparison to catches from traps set up on concrete or in an open area, for all but one species (*C. fascipennis*). A significant difference between environments was only observed for *C. pulicaris*, *C. achrayi*, *C. festivipennis* and *C. reconditus* however, and not for any of the other vector species.

Our study highlights that the level of variation present at the within farm scale is smaller than that between farms and that the life-history characteristics of the individual species do not seem to play as big a role at this smaller scale, as they do at the local scale. The results confirm that the annual variation in catches was not significant and that the distribution of midges between farms stayed relatively consistent between years. Greater consistency in the spatial distribution of *Culicoides* was seen for the vector species and those species trapped in greater abundance.

Given the higher catches of each species trapped in the areas of farms with high-density vegetation, we recommend that while traps should be placed as close to animals as possible, they should be placed within a vegetation-rich area to maximise the numbers of each species caught, especially when sampling for rare or uncommon species. The results from this study, in combination with the high-variation captured in the model produced in Chapter 3, highlight the robustness of the model in predicting the distribution of the BT vectors species, highlighting that it could prove useful for exploring targeted surveillance and control methods.

4.2 Introduction

The previous chapter highlighted that there is significant variation in *Culicoides* density (almost 500-fold for the Obsoletus Group) between farms that are situated close together, undermining previous unsuccessful attempts to record the Obsoletus Group's nationwide distribution in large-scale models. It was possible to capture this variation in a local-scale GLM model built from satellite-derived proxies for climate data and a host of environmental variables. The variance explained was consistently high for both the Obsoletus Group and the Pulicaris Group, and its constituent species, suggesting the model itself is robust. If the model is robust, it could prove useful for targeted surveillance and control.

This chapter attempts to assess the model's robustness in a number of ways:

(i) To see if the variation in 2008 was still present in 2010; and whether farms classified as having high/low catches in 2008 have the same in 2010;

(ii) To see if there was equal high variation within a farm, such that the choice of trapping site on a farm would be critical, and to try to explain this within-farm variation.

4.2.1 Annual Variation in *Culicoides* Density

The 2008 Bala study identified a high level of variation in *Culicoides* catches between farms, with an almost 500-fold difference in maximum catches (74 – 33,720) for the Obsoletus Group, and an almost 4,000-fold difference (6 – 23,656) for *C. punctatus*. As yet, little is known regarding whether this high level of variation is seen every year or, whether the spatial distribution of the species trapped are consistent between years. Knowledge of this is important in terms of determining whether this model, and others produced using a snap-shot sampling approach, need to account for annual changes in density and distribution of *Culicoides* or whether such changes are insignificant.

Suitability maps of *Culicoides* derived from trapping data for use in terms of disease control need to be applicable to the situation at the farm level for a number of years post sampling. As trapping data, in terms of obtaining maximum catches, is usually obtained in the summer months, and the identification of trapped *Culicoides* can take several months to complete, models produced using such means tend to use data that has been collected from at least the previous year's surveillance. This effectively means that the data is already a year out of date when the model is first employed. It is therefore important for the variation in such data to remain constant over time in order for the model to provide meaningful results at the time of use.

Kaufmann *et al.* (2012) monitored biting midges on farms in 12 locations in Switzerland for a period of three years and found that annual variations in midge numbers at the sampled locations were low. They proposed that this indicated that the monitoring of midges should preferably be done by investigating a large number of sites for one season instead of few locations for extended periods of time.

4.2.2 Variation in Midge Catches Between Different Farm Environments

It is important to consider what may influence trap catches at the farm-scale, even when aiming to produce a model at the regional or national scale. As trapping data is initially collected at the farm-scale it is necessary for those undertaking entomological surveys to assess the best place to locate a trap on a farm in order to obtain the data they are after.

A number of studies have investigated the positioning of traps in terms of the height at which they should be set (Braverman & Linley, 1993; Venter *et al*., 2009a), the type of trap that should be used in different circumstances (Scheffer *et al*., 2012; Venter *et al*., 2009b), and the colour light bulb that is best for attracting *Culicoides* (Venter & Hermanides, 2006). As well as trap-specific adjustments however, the environment immediately surrounding the trap may also have an influence on what the trap is catching. Currently the only guidelines for *Culicoides* surveys are to set the traps as close to livestock as possible in order to increase the chances of catching the maximum numbers of the active population at the time of trapping.

Climatic variables, which may be important determinants of *Culicoides* distribution on a larger-scale probably becomes less important at the local on-farm scale, as the variation is reduced. Instead, environmental features may become a more important determinant of Culicoides density at the local scale. As each species of *Culicoides* has varying life history characteristics, it is feasible to hypothesise that the numbers of each species trapped will vary depending on the environment the trap is placed in, and whether that environment corresponds to the characteristics of a certain species.

A number of studies have detected large effects of landscape features, such as forest cover and vegetation, on the distribution patterns of the Obsoletus Group or Complex (Calvete *et al*., 2008; Conte *et al*., 2007a; Purse *et al*., 2004a). This suggests that a trap positioned in a region of high vegetation would catch a higher proportion of the population of these species than a trap positioned elsewhere. *C. pulicaris*, on the other hand, a wet-soil and bog species, has been associated with high, stable, levels of moisture (Purse *et al.*, 2004b, 2005) and may be expected to have maximum catch potential nearby to wet ground and water-sources. An example of a non-vector for BT would be *C. impunctatus* which, according to Kettle & Lawson (1952) is found in less densely covered areas with more access to sunlight, suggesting that perhaps trapping away from over-head vegetation is preferable.

If the location of a trap has an effect on the *Culicoides* species or numbers that it collects, a high level of variation may well be seen within a farm, such that the choice of site on a farm is critical. It is therefore important to determine whether the within farm variation is equally high in comparison to the between farm variation in *Culicoides* catches.

4.2.3 Research Justification

This study aimed to provide insight into the variation in light trap catches of *Culicoides* on a set of farms between 2008 and 2010. It aimed to address the hypothesis that, as with the annual variation in year-round trapping of *Culicoides* on farms, the annual variation in snapshot sampling of *Culicoides* is not significant. It also tested the hypothesis that the number and proportion of *Culicoides* species

trapped by an OVI trap is dependent upon the surrounding environment that the trap is placed in within a farm, and that it is possible to identify significant differences in catches by trapping in different environments.

To address the above issues trapping was undertaken on a sample of the farms from 2008, in the summer of 2010. This smaller cohort of farms was selected as each farm was to have 3 OVI traps running simultaneously in three differing environments on the site.

Specific objectives included identifying where best to locate a trap on a farm in order to maximise catches of the BT vector species (Obsoletus Group, *C. pulicaris* and *C. punctatus*) based on the environment that trap was set in; to compare host and farm-management factors between 2008 and 2010; and to evaluate whether the 2010 catches agreed with the 2008 ranking of the farms based on the size of the *Culicoides* catches.

The award of a Wellcome Trust Vets Vacation Scholarship enabled Kevin Jones to support the field trapping undertaken in this project.

4.3 Materials and Methods

4.3.1 Study Region

To test the research hypotheses, this study was undertaken within the Bala region of North Wales during 2010, two years after the 'Between Farm Variation' study was undertaken (Chapter 3).

This study was carried out on 12 farms from the original 34 farms used during the 2008 study in order to perform a more targeted sampling approach on farms with a known catch potential. The data collected from the catches on these 12 farms was used to identify both the on-farm variation between three differing trapping environments on farms, as well as to investigate the variation in midge density on these farms between the years 2008 and 2010.

4.3.2 Farm Selection and Trapping Environments

The 34 farms used during the 2008 study were categorised into three groups: high, medium and low, according to their maximum midge catches recorded in that year. A sub-set of 4 farms were randomly selected from each of the categories for further study in 2010 (Table 4.1).

Table 4.1 Density categories of the 12 Bala farms for the 2010 *Culicoides* on-farm variation study. The density category is determined from the *Culicoides* numbers caught in the 2008 Bala study.

Farm Grid Square	Log(n+1) Max Catch	Max Catch	Density
C6	2.62	416	Low
B2	2.64	433	Low
D5	2.91	809	Low
C4	2.95	898	Low
D3	3.18	1,525	Medium
A6	3.45	2,832	Medium
C1	3.62	4,142	Medium
B1	3.62	4,211	Medium
F4	3.86	7,230	High
A1	4.14	13,864	High
C5	4.55	35,433	High
D2	4.82	65,763	High

Three trapping locations were selected on each of the 12 farms, set within three different environments. The environments selected were: an area dominated by concrete, buildings or walls; an open landscape with little shelter (not enclosed by buildings or trees); and, an area of high vegetation (trees, hedges) (see Figure 4.1).

To control for nightly variation in midge activity, it was decided that the three traps on each farm should operate simultaneously. In choosing locations, care was therefore taken to ensure that the selected sites were far enough away from one another so that they did not interfere with other traps' catches.

Figure 4.1 Three differing environments selected on 12 farms in Bala in order to determine whether the environment surrounding the trap influences the number of species of *Culicoides* trapped on those farms, where a) concrete/stone environment; b) open environment; and c) trees and high vegetation.

4.3.3 Questionnaire Design

Two short questionnaires were used to determine characteristics of the farms and environment surrounding the traps (see Appendix C).

The questionnaires captured data on:

1. Farm Details:
    a. Size of farm, type of farm, number of animals

2. Insecticide Use:
    a. Which animals are treated, whether buildings or dung was treated

3. Trap Details:
    a. Latitude and longitude, altitude, trap and anemometer height, time set and collected
    b. Environment around trap (leaf litter, dung heaps, water sources)

4.3.4 Experimental Methodology

Culicoides field trapping was done over 12 nights in July 2010 using Onderstepoort light traps. The trapping schedule was randomly assigned to the 12 Bala farms, with all three trap sites on an individual farm run simultaneously. The trapping schedule can be seen in Table 4.2.

Table 4.2 2010 Bala Farm Trapping Schedule.

Yellow= 1st night, Green= 2nd night, Blue= 3rd night, on each farm. Farm Ids in white indicate low catches in 2008; those in light grey indicate medium catches; and, those shaded dark grey indicate high catches in 2008.

Farm ID	**Night**											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
C6	X			X					X			
B2					X					X		X
D5		X				X		X				
C4			X				X				X	
D3									X	X	X	
A6	X	X	X									
C1					X	X	X					
B1				X				X				X
F4		X									X	X
A1			X	X	X							
C5	X					X				X		
D2							X	X	X			

The traps were operated from 1600-1700 h to 0900-1000 h from 21st July 2010 to 1st August 2010. Each nightly catch was collected in a 500 ml plastic beaker containing approximately 200 ml of water plus a small amount of washing-up liquid, to act as a surfactant. Every morning the beaker was removed, replaced with a new one, and the catch stored in 70% ethanol. The trapping on each farm occurred at three sites over three nights (e.g. nine trappings per farm).

Culicoides sorting and counting was undertaken by Karien Labuschagne from the Onderstepoort Veterinary Institute Agricultural Research Council, South Africa. Large collections were sub-sampled (Van Ark & Meiswinkel, 1992) and females were age-graded into nulliparous, parous, gravid or blood-fed based on abdominal pigmentation (Dyce, 1969). Males were also counted, but all other insects were stored uncounted. For the Obsoletus Group, the females of four constituent species (*C. chiopterus*, *C. dewulfi*, *C. obsoletus* and *C. scoticus*) were counted together, while males were counted separately.

4.3.5 Analyses

Before analyses were undertaken, nightly species, or Group, catches were $\log_{10}(n+1)$ transformed so as to try to normalise the distributions. The Obsoletus Group members were categorised together for the purposes of analysis as the females of the species could not be reliably differentiated using a light microscope. Members of the Pulicaris Group (*C. impunctatus*, *C. pulicaris* and *C. punctatus*) were analysed both at Group level and at species level. The heterogeneity in catch size and composition of all trapped *Culicoides* on farms was compared for the 2008 and 2010 trapping results, and spatial density patterns were mapped for the Obsoletus and Pulicaris Groups, and the constituent members of the latter group, using ArcMap 10.0.

Further analyses were not undertaken for eight of the remaining 12 trapped species due to low catches. For the 9 species, and species Groups, that were analysed further, five of the distributions did not differ significantly from normality (Anderson-Darling Test for Normality, $P \geq 0.4$) following the $\log_{10}(n+1)$ transformation, while

C. achrayi, *C. fascipennis*, *C. festivipennis* and *C. reconditus* were significantly non-normal.

Pearson Product-Moment correlations were undertaken between trap catches of these 9 different *Culicoides* spp. or groups trapped in 2010, whereby the critical value for significance was adjusted to a lower threshold using the Bonferroni correction to take account of multiplicity of *P* -values.

4.3.5.1 Variation in Trap Catches between 2008 and 2010

Depending on whether the species data were normally distributed or not, a paired t-test (Obsoletus and Pulicaris Group, *C. pulicaris*, *C. punctatus* and *C. impunctatus*) or Kruskal-Wallis test (*C. fascipennis*, *C. festivipennis* and *C. kibunensis*) was used to compare of the mean number of *Culicoides* collected on the 12 farms throughout Bala in the years 2008 and 2010.

The maximum catch of each species was determined for each of the three trapping sites per farm, for the year 2010. These 36 data points were reduced to 12 data points by averaging the 3 maximum catches per farm. The maximum catches of each species were compared per farm between 2008 and 2010 using Pearsons Correlation Coefficient. As these separate significance tests were testing the same hypothesis (whether the pattern of *Culicoides* on farms in 2008 remained consistent in 2010), the probabilities from these separate tests were combined using the methods of Fisher (1954, section 21.1). The computation is based on the fact that ln *P* is distributed as $-\frac{1}{2}\chi^2_{[2]}$, or -2 ln *P* is distributed as $\chi^2_{[2]}$. This method has been used repeatedly by Sokol and Rohlf (1981), and by evaluating twice the negative natural logarithm of the individual probabilities, considering each to be a $\chi^2_{[2]}$, and summing these values, we obtain a total that can be determined under 2*k* degrees of freedom (where *k* = the number of separate tests and probabilities).

4.3.5.2 Within Farm Variation in Trap Catches

A one-way ANOVA was used to investigate differences in mean *Culicoides* densities among the three trapping environments on farms (concrete, open space, high vegetation) for the normally distributed species (Obsoletus and Pulicaris Groups, as well as the later Group's constituent species). Where a difference was found, Fisher's Least Significant Difference was used post-hoc to highlight where significant differences existed between these environments. For the non-normally distributed catches (*C. achrayi*, *C. fascipennis*, *C. festivipennis* and *C. reconditus*), a Kruskal-Wallis test corrected for tied ranks, was conducted to evaluate the differences among the three trapping environments on the median catch densities of the species. Here, any differences observed were identified between the environments using the post-hoc Mann Whitney U Test with Bonferroni adjustments.

4.4 Results

4.4.1 Trapping

Eighteen different species of *Culicoides* were trapped in the Bala region. The single largest catch was 97,983 midges caught in one trap over one night, with a mean of 7,945 midges per trap per night. Around 76% of the total number of *Culicoides* trapped across sites belonged to the Pulicaris Group and 20% to the Obsoletus Group. Of the Pulicaris Group, *C. impunctatus* was the most abundant species, (97%), whilst *C. pulicaris* and *C. punctatus* made up 2% and 1% respectively. Of the other species caught, *C. achrayi* contributed 3%, while *C. circumscriptus*, *C. comosioculatus*, *C. fasciepennis*, *C. festivipennis*, *C. grisescens*, *C. jurensis*, *C. minutissimus*, *C. parotti*, *C. reconditus* and *C. stigma* made up less than 1% each (Table 4.3).

Spatial variation in the maximum abundance of the Obsoletus and Pulicaris Groups, as well as for the three Pulicaris Group species individually can be seen in Fig. 4.2. The Obsoletus and Pulicaris Groups both exhibited a 50-fold difference in maximum catches between farms, but as with the 2008 trapping data *C. punctatus* displayed the highest variation, with an almost 150-fold difference across sites, while *C. pulicaris* exhibited the lowest with less than a 40-fold difference.

Due to the low catches of *C. circumscriptus*, *C. comosioculatus*, *C. grisescens*, *C. jurensis*, *C. kibunensis*, *C. minutissimus*, *C. parotti* and *C. stigma*, these species were not normally distributed and further analyses were not undertaken on these species.

There was high correlation between abundance in *Culicoides* spp. on farms (Table 4.4). As with the 2008 trapping results, the Obsoletus group abundance was highly correlated to that of the vector species *C. pulicaris* and *C. punctatus*, but also that of *C. achrayi*. The group also had a weak positive correlation to *C. impunctatus*, *C. festivipennis* and *C. delta*. *C. pulicaris* was most strongly associated with the vector species *C. punctatus* and the Obsoletus group, and *C. punctatus* showed similar strong associations to the Obsoletus group and *C. punctatus*, as well as the non-vector *C. achrayi*. The non-vector species *C. impunctatus*, showed weak positive

correlation to the other members of the same sub-genus, *C. pulicaris* and *C. punctatus*, as well as the other non-vector species *C. achrayi*.

Table 4.3 *Culicoides* Species Trapped in Bala during 2010

Species Trapped	Female (% of total catch)	Male (% of total catch)
Obsoletus Group Total	**150888 (26.08)**	**33766 (9.45)**
By Species		
C. chiopterus	-	0
C. dewulfi	-	1751
C. obsoletus	-	18444
C. scoticus	-	13571
Pulicaris Group Total	**404220 (69.87)**	**311545 (87.15)**
By Species		
C. impunctatus	385174	309424
C. pulicaris	12244	1592
C. punctatus	6802	529
Other *Culicoides*	**23404 (4.05)**	**12158 (3.40)**
By Species		
C. achrayi	16469	8998
C. circumscriptus	77	8
C. comosioculatus	1	1
C. fasciipennis	1257	491
C. festivipennis	661	370
C. grisescens	1081	479
C. jurensis	0	1
C. kibunensis	79	60
C. minutissimus	0	1
C. parotti	4	0
C. reconditus	3775	1741
C. stigma	0	8
Total	**578512**	**357469**

Table 4.4. Correlation between abundance of *Culicoides* spp. on farms around Bala in 2010

Significance is given where * = p≤0.1; ** = p≤0.05; *** = p≤0.01

	C. reconditus	***C. festivipennis***	***C. fascipennis***	***C. achrayi***	***C. impunctatus***	***C. punctatus***	***C. pulicaris***
Obsoletus Group	0.429	0.348	0.422	0.735 ***	0.348	0.670 **	0.800 ***
C. pulicaris	0.194	0.033	0.292	0.654 **	0.658 **	0.658 **	
C. punctatus	0.452	0.607 **	0.225	0.843 ***	0.566 *		
C. impunctatus	0.018	0.156	0.355	0.533 *			
C. achrayi	0.760 ***	0.733 ***	0.248				
C. fascipennis	0.096	0.235					
C. festivipennis	0.807 ***						

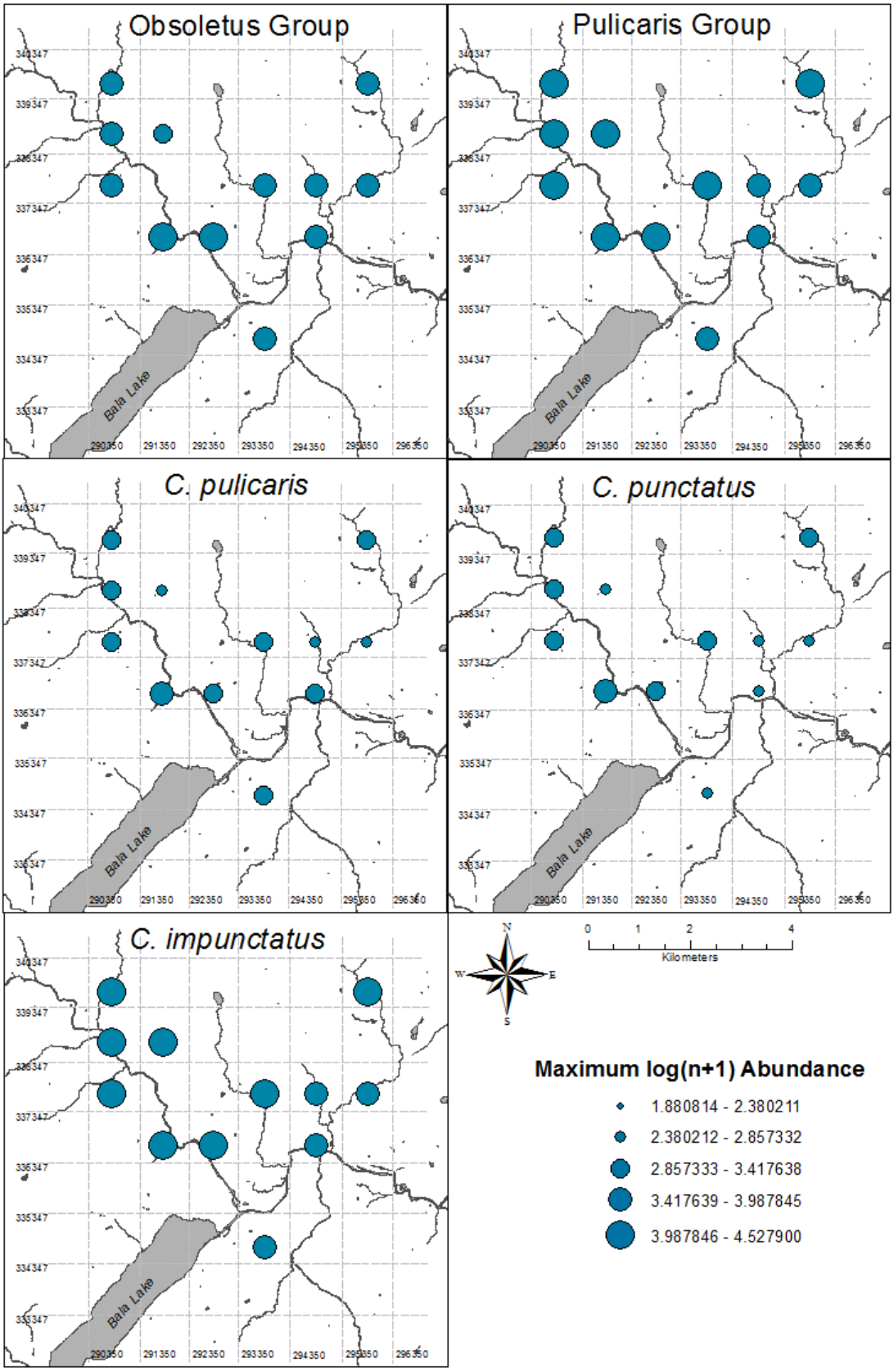


Fig. 4.2 Spatial variation in maximum log (n+1) abundances of the Obsoletus group, Pulicaris group and the Pulicaris group constituent species across trapping sites

4.4.2 Questionnaire Data

Of the 12 farms, the number keeping sheep decreased from 12 (100%) in 2008 to 11 (92%) in 2010. The converse was seen for farms keeping beef cattle where the number increased from 9 (75%) farms in 2008 to 10 (83%) in 2010. Only one farm kept pigs during both years (Tables 4.5 and 4.6). Other animals included horses, dogs and chickens, although the numbers of the latter two were not collected in 2008. None of the farms kept dairy cattle. The mean numbers of each species of animal seen on the farms decreased over the two years, except for pigs.

Table 4.5 Host animals on farms around Bala in 2008

Host Variable	Number of Farms (%)	Mean[a] Number of Animals	SD	Range
Sheep	12 (100)	856.6	1250.18	10 - 2600
Beef Cattle	9 (75)	127.9	102.42	30 - 350
Dogs	-	-	-	-
Chickens	-	-	-	-
Horses	2 (17)	3	1.41	2 - 4
Pigs	1 (8)	40	NA[b]	NA[b]

SD, standard deviation; [a] Mean of those farms with animals; [b] Not applicable

Data on dogs and chickens were not collected in 2008.

Table 4.6 Host animals on farms around Bala in 2010

Host Variable	Number of Farms (%)	Mean[a] Number of Animals	SD	Range
Sheep	11 (92)	837.2	761.52	144 - 2200
Beef Cattle	10 (83)	79.2	80.13	20 - 294
Dogs	6 (50)	4	4.36	1 - 9
Chickens	2 (17)	15.5	6.36	11 - 20
Horses	2 (17)	2	NA[b]	NA[b]
Pigs	1 (8)	75	NA[b]	NA[b]

SD, standard deviation; [a] Mean of those farms with animals; [b] Not applicable

Eight farms (66.7%) used insecticides regularly on animals in 2010, while 10 (83.3%) had done so previously in 2008, but in neither year had any of the farms used insecticides on buildings or for dung management. In 2008 only 3 farms had dung piles within 10 m of the trapping site, but in 2010 this increased to 10 (83.3%) farms. In terms of other breeding sites, only 2 (17%) farms had leaf litter within 5 m in 2008, increasing to 11 (91.7%) farms in 2010, and the seven farms that had water sources within 10 m of the traps in 2008 increased to eleven (91.7%) farms in 2010. The mean altitude on the farms was 240.2 m, but varied between 169 and 344 m.

4.4.3 Variation in Trap Catches between 2008 and 2010

Maximum catches for all species on each farm were, on average, 11-fold greater in 2010 than in 2008. On classifying the farms into those with a low, medium or high density of *Culicoides*, none of the farms exhibited a maximum catch of <1000 midges, a cut-off which would have classified them in the low density category in 2008. In 2010, the maximum catches ranged between 3,562 and 97,983 on farms with 8 (67%) of the farms falling into the high density category (≥10,000 midges), while the range seen in 2008 was 416 – 65,763.

On comparing the proportion of the total *Culicoides* catch achieved by each farm, 50% of the farms remained within the same density category as in 2008. Only 2 farms achieved lower maximum catches in 2010, moving them from the high catch density category to the low density category.

Comparing the numbers of the Obsoletus Group caught on each farm, 8 (67%) of the farms remained within the high, medium or low density group they were classed in during 2008. The range of maximum catches in 2010 was 326 to 14,767, while in 2008 the range was far broader, being 77 to 33,722. Those farms with smaller catches (<1000 midges) in 2008, increased their maximum catches by 6-fold on average, while those with larger catches (>2000 midges) exhibited a 2-fold decrease on average.

For the Pulicaris Group, 7 (58%) of the farms remained within the same density category as for the 2008 catches, but the maximum numbers of *Culicoides* trapped

were significantly higher in 2010. The range for 2008 was 87 to 28,425 while for 2010 it was significantly greater at 1,891 to 95,500 midges per farm. The non-vector member of this group, *C. impunctatus* had 8 farms remaining in the same density categories as in 2008, but the range of *Culicoides* trapped significantly increased from 24 to 4,062 in 2008 to 1,762 to 95,500 in 2010, an average increase of 80-fold in the catches. This increase was less marked for *C. pulicaris* with an average increase in catch numbers of 9-fold, while for *C. punctatus* 7 (58%) of the farms showed a decrease in numbers trapped in 2010.

Two farms, D2 and A1, exhibited consistently high numbers of the Obsoletus Group and Pulicaris Group members, and their constituent species, during both trapping years. On the converse, four farms, B2, C4, C6 and D5, exhibited consistently low numbers of those species during both years.

For the other non-vector species, *C. kibunensis*, *C. fasciipennis* and *C. festivipennis*, less than 50% of farms had trappings of these species in the same density category as in 2008. A number of species were trapped in one year and not the other; *C. brunnicans*, *C. delta*, *C. minimus*, *C. nubeculosus* and *C. pictipennis* were trapped during 2008 only, while *C. comosioculatus*, *C. jurensis*, *C. minutissimus*, *C. parotti* and *C. reconditus* were only found in 2010. *C. comosioculatus* had not previously been recorded in the UK.

Statistically significant annual variation in midge abundance was neither found for the mean number of all midges trapped, nor for the majority of analyses of the midges belonging to the species or species Groups trapped in both years (Table 4.7). Statistically significant variability between the years was determined in three instances. *C. impunctatus* showed the greatest level of significance, while significant results for *C. fascipennis* and *C. festivipennis* are likely relating to low and highly variable abundances of these species during both years.

Table 4.7 Statistical ([1]paired T-test or [2]Kruskal-Wallis) comparison of the mean number of *Culicoides* collected on 12 farms throughout Bala in the years 2008 and 2010. The category 'All' also includes species that were not trapped during both years. * indicates a significant difference.

Species or Species Group	**Year**		***P*-value**
	2008	**2010**	
Obsoletus Group[1]	7,030	4,705	0.468
Pulicaris Group[1]	3,401	20,016	0.044
C. pulicaris[1]	156	426	0.022
C. punctatus[1]	2,265	298	0.307
C. impunctatus[1]	984	19,641	0.028*
C. fascipennis[2]	11.50	38.10	0.047*
C. festivipennis[2]	2.50	26.37	0.044*
C. kibunensis[2]	2	6.48	0.65
All[1]	11,463	26,240	0.149

Table 4.8 Correlation between the abundance of individual *Culicoides* species and Groups observed on farms in 2008 and their abundance on the same farm in 2010. * Indicates a significant *P*-Value. The natural logarithm of each *P*-Value (ln(*P-value*)) is also given.

Species or Species Group	**Correlation**	***P*-Value**	**ln*(P*-value)**
Obsoletus Group[1]	0.492	0.104	-2.263
Pulicaris Group[1]	0.344	0.273	-1.298
C. pulicaris[1]	0.53	0.076	-2.577
C. punctatus[1]	0.672	0.017*	-4.075
C. impunctatus[1]	0.686	0.014*	-4.269
C. fascipennis[2]	-0.454	0.138	-1.981
C. festivipennis[2]	0.583	0.047*	-3.058
C. kibunensis[2]	-0.046	0.887	-0.120
T**otal**			**-19.640**

Although the abundance of the majority of species showed strong positive correlation (Figure 4.3) between the years 2008 and 2010, this correlation was only statistically significant for *C. punctatus*, *C. impunctatus* and *C. festivipennis* ($P \leq 0.05$) while verging on significance for three other species ($P \leq 0.14$ for Obsoletus Group, *C. pulicaris* and *C. fascipennis*) (Table 4.8). After combining the *P*-values in

order to determine an overall significance test of the hypothesis, $-2\Sigma \ln P$ was greater than $\chi^2_{.001[16]}$ (39.280 and 29.252 respectively), which, if the data can be treated as independent, highlights that there was significant positive correlation between the catches (rankings) on the farms ($P < 0.001$) between both years.

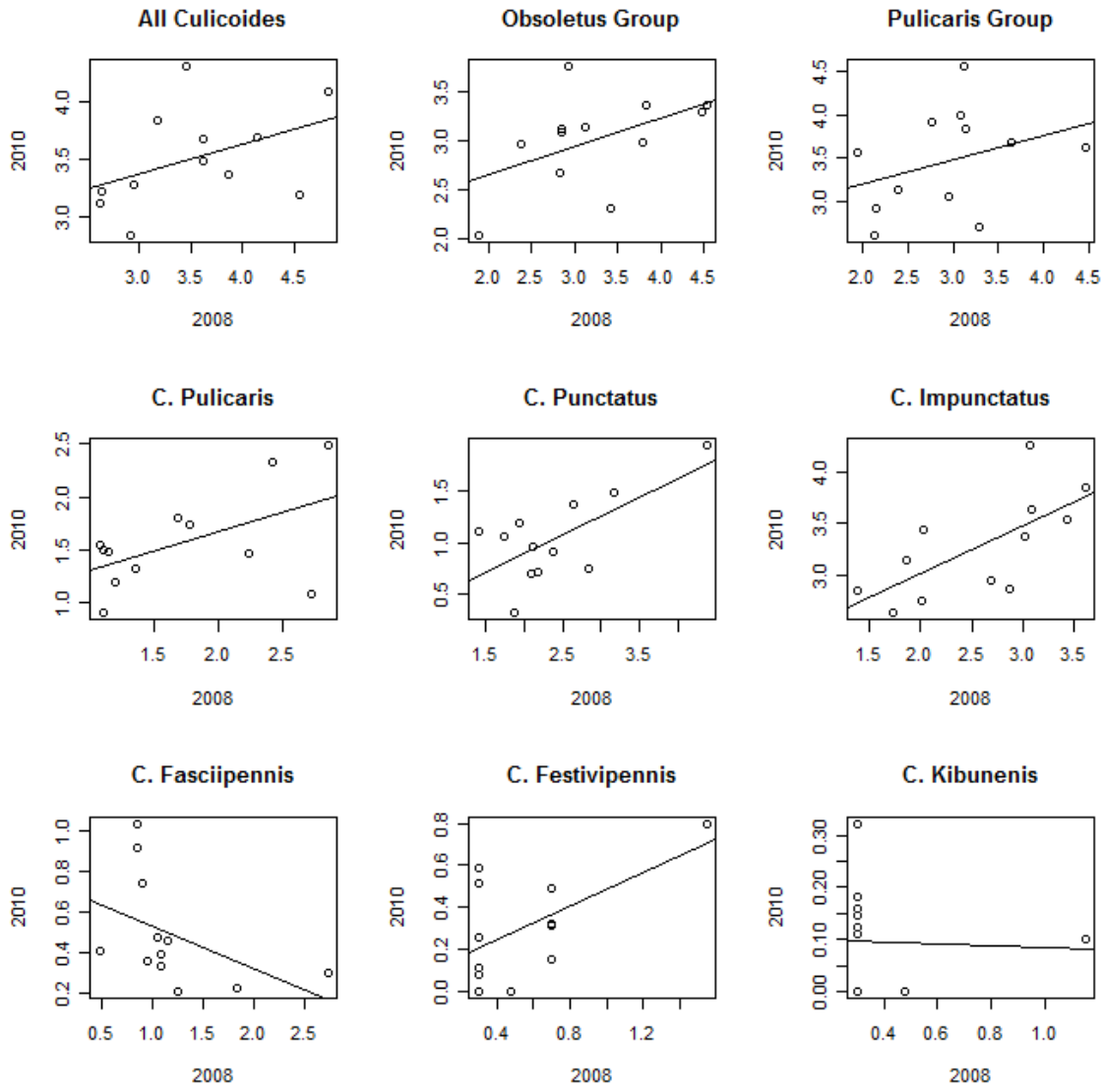


Figure 4.3. Correlation between the $\log_{10}(n+1)$ abundance of *Culicoides* species trapped on farms in Bala in 2008 and 2010

4.4.4 Within Farm Variation in Trap Catches

Mean *Culicoides* densities on farms were consistently higher from catches in traps located near trees, in comparison to catches from the traps set up in a built environment or in an open area, except for *C. fascipennis* where the highest mean density was trapped in a built-up environment (Tables 4.9 and 4.10).

For *C. pulicaris*, there was a significant difference in the mean catch between the three environments (One-way, ANOVA, P=0.05), with the highest catches near trees, followed by open areas, and the lowest catches were in the built environment (Table 4.9). The difference between trees and the built environment was significant by the Fishers Least Significant Difference post-hoc test. No significant differences were apparent for the Obsoletus Group or the other Pulicaris group species, although it is worth noting that in all cases the highest catches were among trees.

There was a significant difference in the median catch for *C. achrayi* and *C. festivipennis* between the three environments, and the *P*-value was verging on significance for *C. reconditus*, (Kruskal-Wallis, P=0.009, P=0.02 and P=0.08 respectively). Post-hoc tests were conducted to evaluate pairwise differences among the three groups using the Mann Whitney U Test with Bonferroni adjustments (Table 4.10). The results of these tests indicate significant differences between the catches obtained near to trees, and those on concrete or in open areas. Higher numbers of *Culicoides* were trapped near trees than on concrete or in open areas.

Table 4.9 Results of One –way ANOVA and Fishers Least Significant Difference to investigate differences among three trapping environments (concrete, open area, high vegetation) on the mean catch densities of the normally distributed *Culicoides* species trapped in Bala in 2010.

Species	**Mean log_{10}(n+1) *Culicoides* Catch by Environment**			**Difference obtained from Fishers**
	Concrete	**Open**	**Tree**	
Obsoletus Group	2.742	2.652	2.912	-
Pulicaris Group	3.240	3.188	3.470	-
C. pulicaris	1.398	1.552	1.761	C-T**
C. punctatus	0.904	1.067	1.154	-
C. impunctatus	3.217	3.163	3.447	-

Significance is given where * = p≤0.1; ** = p≤0.05; *** = p≤0.01
C-T indicates significant difference between concrete and tree environments.

Table 4.10 Results of Kruskal-Wallis and Mann Whitney U Test, with Bonferroni correction, to investigate differences among three trapping environments (concrete, open area, high vegetation) on the median catch densities of the non-normally distributed *Culicoides* species trapped in Bala in 2010.

Species	**Mean log_{10}(n+1) *Culicoides* Catch by Environment**			**Kruskal-Wallis Statistic**	**Difference obtained from Mann Whitney U**
	Concrete	**Open**	**Tree**		
C. achrayi	1.425	1.393	1.968	9.33***	C-T***; O-T***
C. fascipennis	0.554	0.371	0.539	1.32	
C. festivipennis	0.155	0.201	0.554	7.54**	C-T***; O-T**
C. reconditus	0.959	0.862	1.315	5.06*	C-T*; O-T**

Significance is given where * = p≤0.1; ** = p≤0.05; *** = p≤0.01
C-T indicates significant difference between concrete and tree environments; while O-T indicates significant difference between open and tree environments.

Although greater numbers of almost all *Culicoides* species were trapped in areas of high-vegetation compared to the other two environments, there was little difference in the proportions of the different species caught (Figure 4.4). *C. pulicaris*, *C. punctatus*, *C. fascipennis*, *C. festivipennis* and *C. reconditus* each comprised of less

than 2% of the total catch in any environment, while *C. achrayi* numbers increased to 4% of the total catch near high vegetation. *C. impunctatus* dominated all environments with catches comprising 72-73% on concrete and near high vegetation, with this proportion rising to 81% of all species trapped in open areas on farms. Conversely, the proportions of the Obsoletus Group trapped were slightly higher on concrete and near to vegetation (20 and 23% respectively) compared to open areas where they were 14% of the total *Culicoides* catch.

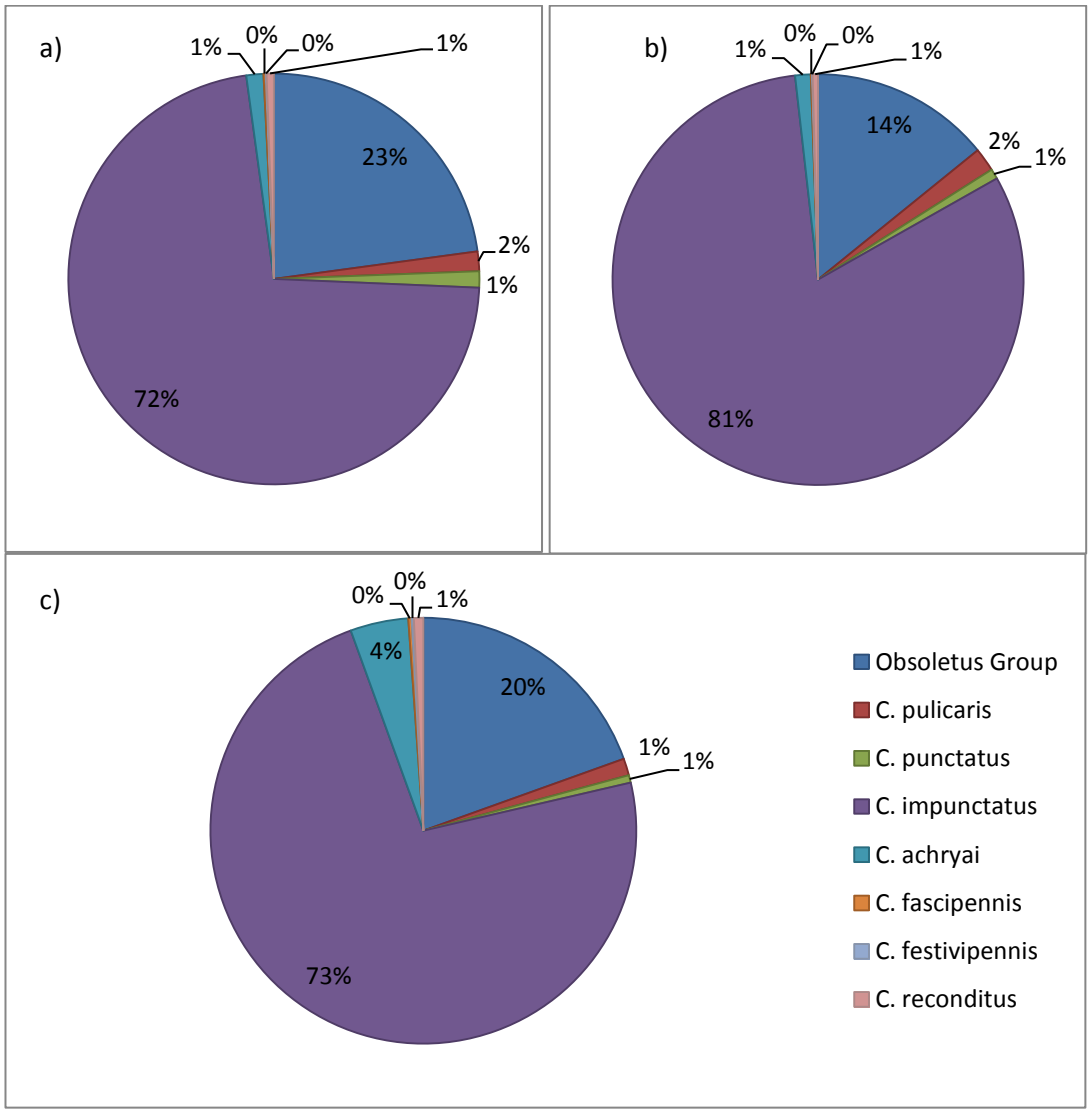


Figure 4.4. The proportions of *Culicoides* species in three different environments in Bala, where a) trapping on concrete; b) trapping in an open area; and, c) trapping in high vegetation.

4.5 Discussion

4.5.1 Annual Variation in *Culicoides* Density

This study represents the first attempt to determine whether significant annual variation exists between snapshot samplings undertaken at the peak of the *Culicoides* season in two different years. Models of vector abundance may be employed to investigate disease control scenarios a number of years after being produced, therefore it is important for the variation in trapping data to remain constant over time in order for the model to provide meaningful results at the time of use. As such it is important to determine whether there is significant annual variation in *Culicoides* density.

In agreement with the 2008 trapping data, the Pulicaris and Obsoletus Groups were the most abundant *Culicoides* caught in the Bala area in 2010. However, while the Obsoletus Group members were most prevalent, and *C. punctatus* accounted for the highest proportion of the Pulicaris Group members in 2008, the most prevalent species in 2010 was *C. impunctatus*, which was even more numerous than the three Obsoletus Group members combined. The reason for this is unclear, with Zimmer *et al*. (2013) finding similar results when trapping in the bogs of a Belgium nature reserve, with the species generally being found in wet soil with good access to sunlight (Kettle & Lawson, 1952).

Of the eight species, and species Groups, that were trapped in both 2008 and 2010, *C. impunctatus*, *C. fascipennis* and *C. festivipennis* were the only species to show significant variation in the mean numbers collected between the years. Of these, the significant results for the latter two species are likely related to the low, and highly variable, abundances of these species during both years. A number of less prevalent species were not present in both years' catches, with *C. albicans*, *C. brunnicans*, *C. delta*, *C. minimus*, *C. nubeculosus* and *C. pictipennis* only caught in 2008, while *C. comosioculatus*, *C. grisescens*, *C. jurensis*, *C. kibunensis*, *C. minutissimus*, *C. parotti* and *C. reconditus* were only trapped in 2010. *C. comosioculatus* is of particular interest as it has not previously been identified in the UK, having only recently been discovered in Belgium (Zimmer *et al*., 2013).

Although trap catches during 2010 contained considerably larger numbers of individuals of each species than those from 2008, spatial variation in the maximum abundance of each species between sites was far lower than observed in 2008 and the ranges were generally lower. Less variability in catches was observed between neighbouring farms than previously seen in 2008. This may be expected due to fewer farms being sampled in 2010, as well as more sites selected per farm in this year. As such, the high variation observed in 2008 may be partly due to differences in the selected trapping sites on the farms themselves, which in turn may be the reason for some farms moving from low to high catch groupings in 2010 when the selected sites were consistent between farms.

Although the majority of the Obsoletus Group and Pulicaris Group members were categorized in the same density categories as they were in the 2008 study, many of the non-vector species changed density categories. This was to be expected, as these less prevalent species also showed very high variation between farms, and may not have been present on all farms in both years.

Correlations between the abundance of *Culicoides* spp. on farms concurred with the results from 2008 whereby the vector species were positively correlated, suggesting common predictors of abundance due to similar life history characteristics, such as the presence of live hosts. Once again, *C. impunctatus*, believed to be a non-vector species, is closely related to the vectors *C. pulicaris* and *C. punctatus*, yet also shows positive correlation to the distribution of the non-vector *C. achrayi*.

On combining the abundance data for the species that were trapped during both 2008 and 2010, the overall abundance of *Culicoides* showed significant positive correlation between the two years, highlighting that those farms with higher trap catches in 2008 also had higher trap catches in 2010. If the catches of each species can be assumed to be independent, our results suggest a low annual variation in both the abundance and spatial distribution of *Culicoides* between the sampled farms.

Previous studies have also identified similar low annual variation in midge numbers on farms when undertaking sampling at weekly intervals throughout the year (Kaufmann *et al.*, 2012). Our study confirms that this low annual variation in midge numbers remains is also present when sampling is undertaken using a snapshot

approach at just one time-point in the year. The low annual variation observed confirms that models of BT vector distribution, with data originating from a snapshot sampling approach, remain relatively stable when predicting distributions between years.

4.5.2 Variation in Midge Catches between Different Farm Environments

For both nation-wide and smaller-scale entomological surveillance activities, it is necessary to determine a trapping protocol whereby the positioning of traps on farms is standardised in order to reduce the inclusion of bias in the sample design. As different *Culicoides* species have different life-history characteristics, it can be hypothesised that the environment surrounding the trap will have an impact on the number and species of *Culicoides* sampled by that trap.

In our study, the mean *Culicoides* densities trapped on the 12 farms were consistently higher from catches in traps located in areas of high vegetation, in comparison to catches from the traps set up on concrete or in an open area. Although not all species showed significant differences in catch numbers between the three environments, significant differences were observed for *C. pulicaris*, *C. achrayi*, *C. festivipennis* and *C. reconditus*. These results highlight that greater numbers of most species of *Culicoides* can be sampled when trapping in an area of high vegetation, away from areas of concrete, or open spaces. This may be due to such environments being used as both breeding sites, as well as resting areas for *Culicoides*.

Little difference was observed however, in the proportions of each species caught in the three environments, suggesting that trapping in areas of high vegetation is not better for one individual species, but equally beneficial for increasing the numbers of all species. Trapping in this environment may well, therefore, be beneficial when trying to capture rare species that generally have a low prevalence. The reasoning behind this remains unclear; on the one hand there may truly be a larger population of midges in this location, but on the other hand the location is likely to be more sheltered, with less wind, increasing levels of activity and also trapping efficacy.

The level of variation present at the within- farm scale is, therefore, far less than at the between-farm scale. This suggests that although it is important to standardise the location of traps on sampling farms, the choice of trapping site is not critical to obtaining unbiased estimates of *Culicoides* density on farms.

If the study was to be repeated it would ideally be split into two separate pieces of fieldwork. One would use all 34 farms that were sampled in the 2008 study (rather than only 12) and would have traps placed in the same locations on these farms as they were in that year in order to compare the variance in catches between those years. Increasing the number of farms may aid in providing a more convincing relationship in the catch correlation between 2008 and 2010. This correlation contains only 12 data points and therefore may currently be heavily influenced by outliers.

The second study would compare the within-farm and between farm variance as undertaken here by placing traps in the three differing environments on the farms. In order to analyse this data, multilevel modelling could be used to estimate separately the variance between farm and the variance between sites on the same farm.

The trapping schedule has three consecutive nights' worth of sampling on five of the farms. This is not ideal as, had similar weather e.g. rain occurred on these consecutive nights, the catches from these farms may have been adversely affected. Although this study highlights a consistency in the spatial variation of catches and farm ranking between years, the study should ideally be replicated annually to determine whether differences occur over longer periods of time. Finally the study would have benefitted from the Obsoletus Group members being identified to species level using PCR methods, but as this was not undertaken for the 2008 study, it was deemed unnecessary in this case.

4.5.3 Conclusions

Overall, our results successfully confirm the robustness of the local-scale model produced in Chapter 3, by highlighting both a consistency in the spatial distribution of trapped *Culicoides* and the ranking of farm catches between years, as well as confirming a lack of significant variation in catches from differing locations within a farm.

The lack of significant annual variation observed in midge abundances, with a correlation in trap catches observed on farms between 2008 and 2010, highlights that trapping data collected from one year can be assumed to be fairly consistent between years, even when using a snapshot sampling approach. The remarkably high level of variation in *Culicoides* catches observed in 2008 was not as great in 2010, although the numbers of each species trapped were significantly higher. This may be due to more stable weather conditions during the trapping period in 2010 compared to those in 2008, therefore providing a greater level of consistency in results between farms. This variation in catches that may be accountable to the weather conditions during trapping was partly accounted for by ensuring that maximum, rather than mean, trap catches were analysed. Using the maximum catch increases the likelihood of farm estimates being the best possible approximation of the population of each species present, rather than an approximation that may have been influenced by reduced trap catches due to adverse weather (had we used the mean instead).

Correlations remained between the BT vectors, as well as for the non-vectors in terms of spatial distribution suggesting, once again, that individuals within these groups share similar life-history characteristics. This once again supports the validity of our model in using ecological correlates for defining the landscape of the vectors and non-vectors.

The number of *Culicoides* trapped in areas of high vegetation was consistently greater than compared to trapping in an area of predominantly concrete or an open area. Although only statistically significant for four of the species, this highlights that trapping in this location has the potential to attract larger numbers of many species of *Culicoides.* While this was the case, because the proportions of each species trapped in the different locations remained roughly equal, differences in the life-history characteristics of these species were unlikely to be the cause of this. Instead, overarching influences on trap catches, such as the degree of shelter around the trap providing security from high winds or rain, as well as the proximity to resting sites may highlight why greater numbers of all species could be trapped near to trees, rather than in open, or built-up environments.

Although a number of other studies have already investigated the role that trap type (Scheffer *et al*., 2012; Venter *et al*., 2009b), height (Braverman & Linley, 1993;

Venter *et al*., 2009a) and bulb colour (Venter & Hermanides., 2006) have in trapping *Culicoides*, our results highlight that more work is needed in order to understand the role of trap location in increasing the numbers of midges caught, which may be particularly useful for studies aiming to trap novel species.

Multi-level modelling would provide a good follow-up method to the results we have produced here. Such an approach could be used to estimate separately the variance between trapping sites within the same farm, and the variance between farms.

When comparing the trapping data between the 2008 and 2010 studies, it is important to recognise a number of crucial differences between the studies. The earlier study took place during the first half of July, while the later study took place during the second half of July and into the beginning of August. Although we assume that both trappings sampled the *Culicoides* population in Bala at the peak time-point during the year, the slight variation in start dates may have meant we missed the peak for some species, but caught others during the alternate year. Similarly, differences in weather variables, both during each trapping period and for the months previous to trapping may have impacted on species availability in either study.

In conclusion, our study highlights that the level of variation present at the within-farm scale is smaller than that between farms. The reasoning for this remains unclear, but the life-history characteristics of the individual species do not seem to play as big a role at this smaller scale, as they do at the farm scale. Given the higher catches of each species trapped in the areas of farms with high-density vegetation, we recommend that while traps should be placed as close to animals as possible, they should ideally be placed within a vegetation-rich area to maximise the numbers of each species caught, especially when sampling for rare or uncommon species. The high level of local-scale variation captured in the model from Chapter 3, in combination with the lack of significant variation both between years and at the within-farm level highlights the robustness of this model in predicting the distribution of the BT vectors species, and could prove useful for exploring targeted surveillance and control methods.

CHAPTER FIVE

LABORATORY INVESTIGATION OF FLUORESCENT DUSTS AS A MEANS OF MARKING *CULICOIDES* BITING MIDGES

The study was conceived and designed by Georgette Kluiters (GK). GK and Matthew Baylis were awarded a BBSRC-funded Summer Studentship which enabled Kristina Hunter (KH) to support the laboratory studies. GK & KH collected the *Culicoides* and implemented the laboratory investigations. KH undertook preliminary analyses on the data for her report to the BBSRC and GK undertook full analyses on the data at the end of the study.

5.1 Abstract

Investigation of insect flight patterns frequently involves the use of dispersal studies. Different approaches have been attempted to study the dispersal of *Culicoides* spp. midges: long-distance dispersal studies have utilised evidence from disease outbreaks, whereas short-distance studies have involved direct capture of adult midges near breeding sites. An optimal approach would be a mark-release-recapture study (MRR) using wild-caught midges in their natural environment, however without the availability of a suitable marker this would be impossible. At present, no studies have been performed to identify markers that are suitable for use in midges within the Obsoletus Group, and visible by eye or down a light microscope.

A series of 11 experiments were undertaken to determine the effectiveness of three colours of Brilliant General Purpose (BGP) fluorescent dusts (Brilliant Group, San Francisco, California) in marking *Culicoides* midges. Three particular areas of interest were focused on, namely dust properties, the effect on *Culicoides*, and means of application in the field.

All three dusts were insoluble in water, 10% washing up liquid and 70% ethanol. They were visible down a microscope, with and without the use of a black light, and the pink and green dusts were highly visible without the need for a microscope. The dusts remained adherent to the marked *Culicoides* for the duration of the experiments, did not transfer between marked and unmarked individuals or the environment, and remained adherent when the *Culicoides* were stored in an ethanol or water-based solution.

The dusts had no effect on the mortality rate of the insects over the 48 hrs of the experiment. There were no significant differences between the recorded behaviours undertaken by un-dusted control *Culicoides* and the BGP fluorescent dusted *Culicoides*.

Field-based marking of *Culicoides* can be achieved using a ‘self-marking’ technique, whereby the trapping vessel is pre-dusted with fluorescent dust prior to trapping the individuals to be marked. Not only does this reduce the mortality rate involved in marking individuals, but it reduces the time needed to handle specimens prior to release.

BGP fluorescent dusts provide a quick and effective method of marking *Culicoides* for both field and laboratory studies.

5.2 Introduction

Culicoides flight behaviour is believed to drive the dispersal of midge-borne diseases, like bluetongue, from farm-to-farm; and modelling studies suggest that BT outbreaks cannot occur in the absence of local spread by midges (Turner et al, 2012). Culicoides flight behaviour is therefore a critical aspect of BT epidemiology, but it remains poorly understood (Carpenter *et al*., 2009). To improve our understanding of midge flight behaviour, and therefore how BT spreads from farm to farm, we need to determine dispersal patterns, in particular the distance over which midges fly during a set period; and to identify factors that contribute to the direction and flight distance.

Investigation of insect flight patterns frequently involves the use of dispersal studies (Danthanarayana, 1986; Lillie *et al*., 1985; Valerio *et al*., 2012). Different approaches have been attempted to study the dispersal of *Culicoides* spp. midges: long-distance dispersal studies have utilised evidence from disease outbreaks (Burgin *et al*., 2013; Ducheyne *et al*., 2007; Eagles *et al*., 2012; Hendrickx *et al*., 2008; Sedda *et al*., 2012), whereas short-distance studies have involved direct capture of adult midges near breeding sites. An optimal approach would be a mark-release-recapture study (MRR) using wild-caught midges in their natural environment, however without the availability of a suitable marker this would be impossible. Any selected marker must not have a detrimental effect on the survival or behaviour of marked specimens or the environment, but must also remain adherent under adverse conditions, be highly visible and make a distinct differentiation between marked and unmarked individuals (Hagler & Jackson, 2001). At present, no studies have been performed to identify markers that are suitable for use in midges within the Obsoletus Group, as has been performed in other studies for larger insects such as mosquitoes.

5.2.1 Characteristics of Effective Markers

A wide variety of materials and methods have been used to mark animals for biological research (see Hagler & Jackson, 2001 for a review of these techniques). Vertebrate biologists often mark their test subjects with bands, brands, tattoos, tags,

notches, paints, and radiolabels (Basavaraju *et al*., 1998; Stock, 1979). Unfortunately, most vertebrate-marking techniques are not practical for marking insects because they are cumbersome, heavy, and/or costly (Southwood, 1978). As a result, entomologists are often challenged to develop unique methods for marking insects.

An ideal marking material is durable, inexpensive, nontoxic (to the insect and the environment), easily applied, and clearly identifiable (Hagler & Jackson, 2001). Furthermore, the marker should not hinder or irritate the insect or affect its normal behavior, growth, reproduction, or life span (Hagler & Jackson, 2001). The marking method employed will depend on the insect being marked, the environment that the insect will encounter, and the nature of the experiment.

Insects can be marked individually or in large groups. Individual marks, usually in the form of a painted label or a physical tag, permit the identification of a specific individual in a population. Mass marking, usually in the form of an application of dust, paint, or dye, permits the identification of a group of insects within a larger population. In addition to individual-marking and group-marking techniques, self-marking techniques have also been developed in which insects mark themselves by contacting marking materials that are natural to their environment (e.g. pollen) or materials that have been strategically placed in their environment by researchers (e.g. bait formulations or substances at the entrances of nests or hives) (Hagler *et al*., 2011; Niebylski & Meek, 1989).

5.2.2 Methods for Marking Insects

5.2.2.1 Tags

Tags can be used to mark insects inexpensively, and allow insects to be identified on an individual level for MRR studies. They are of most use for long-term studies for which other markers could not be retained, but the size, shape, and placement of tags must not restrict the insect's movement or interfere with behavior (Hagler & Jackson, 2001). The application of individual tags however is time-consuming and

makes tagging procedures impractical for mass marking insects. Moreover, the physical nature of tags limits their application to relatively large insect species (Hagler & Jackson, 2001).

5.2.2.2 Mutilation

Marking by mutilation ensures that the marks are usually persistent and can readily and accurately be recognized in the field without the aid of any specialized equipment. However, like tagging, mutilation can also and time-consuming, and is only applicable to a small number of insect species (Hagler & Jackson, 2001).

5.2.2.3 Paint and Ink

Paints and inks can be applied to large batches of insects for MRR studies using various spraying devices (e.g. hand atomizers and spray guns), so is easy, rapid, and inexpensive (Hagler & Jackson, 2001). The marker is also usually recognizable on recaptured insects without microscopic examination. Researchers must be sure the paint and solvent (if diluted) are nontoxic and do not alter insect behavior (Walker & Wineriter, 1981). However, this method isusually reserved for larger insects as these sprays can be destructive to small and delicate insects.

5.2.2.4 Dyes

In the 1960s and 1970s, most of the progress toward marking insects with dyes was made with the concurrent development of sterile insect release and area-wide pest management programmes (Gast & Landin, 1966; Hendricks, 1971; Schroeder *et al*., 1974; Steiner, 1965). Certain oil-soluble dyes can accumulate in insect body fluids or tissues after insects have eaten them, with adult insects emerging marked when larvae are fed on those dyes (Gast&Landin, 1966), and dyes can also be transferred to the eggs in some species (Gast & Landin, 1966).

Advantages include the use of different coloured dyes to mark different cohorts of individuals (Jones, 1990), that insects can occasionally be self-marked (Lloyd *et al*.,

1968; Wilson *et al*., 1971), and many dyes can be rapidly and nondestructively detected visually in recaptured insects (Graham & Mangum, 1971). Disadvantages include some dyes not being visible by direct inspection, with insects needing to be crushed on filter paper (Coppedge *et al*., 1979; Showers *et al*., 1989) or ground in solvent (such as acetone) (Argauer & Cantelo, 1972). Very few dyes examined as potential insect markers have proven to be effective, with most dyes harmful to insects or exhibiting too short a retention interval (Gast & Landin, 1966; Naranjo, 1990; Ostlie *et al*., 1984; Su *et al*., 1991).

5.2.2.5 Dust or Powder

Dusts have been used to mark insects for over 75 years (Darling, 1925). To date, they are probably the most commonly used materials for externally marking a variety of insects (Service, 1993). Although various kinds of dusts have been used to label insects (Polivka, 1949; Service, 1993; Southwood, 1978; Taft & Agee, 1962), the most common commercial dust used to mark insects is Day-Glo (Day-Glo Color Corp., Cleveland, OH). This dust is available in a wide variety of colours, affordable, and visible to the naked eye, with its detection on insects enhanced under UV light (Beier *et al*., 1982; Stern & Muller, 1968).

Sometimes adjuvants (e.g. flour, sand, or gum Arabic) are mixed with dusts to provide better adhesion of the dust particles (Beier *et al*, 1982; Reinecke, 1990), but this is unnecessary on insects with hairy surfaces. Dusts are usually applied to insects by putting them in a container with a given amount of dust and shaking or tumbling the container (Shroeder & Mitchell, 1981). These procedures however are not practical for dusting small and delicate insect species because of the immediate high mortality and application of too much dust on the insect (Meyerdirk *et al*., 1979). Not only can too much dust cause mortality, but also decreased mobility, and interference with sensory organs (Cook & Hain, 1992). Therefore, innovative devices and self-marking techniques are available for applying very small quantities of dusts to insects (Sheppard *et al*., 1973). The use of an insufflator, for example, may be used to ‘puff’ dust onto mosquitoes (Service, 1993), or a dust storm can be created in an enclosed cage with a vacuum duster (Dunn & Mechalas, 1963).

Many mark-capture studies have employed self-marking techniques in which dusts were placed strategically near insect nest and hive entrances (De Grandi-Hoffman & Martin, 1995), on floral-visitation sites (Musgrave, 1950), and insect traps (Gentry & Blythe, 1978; Hogsette, 1983). Price & Slosser (1983) placed fluorescent dust in pheromone-baited traps modified to allow for the escape of trapped boll weevils exposed to the dust. The self-marking techniques used on insects have the advantage of eliminating the damage associated with artificially handling the insects (Hagler & Jackson, 2001).

Dusts are most frequently used for marking insects in MRR studies. Field-collected or laboratory-reared insects are dusted *en masse* and released into the field for dispersal studies (Schroeder & Mitchell, 1981; Stern & Mueller, 1968). As dusts come in a variety of colours, are inexpensive, readily available, environmentally safe, and easily applied and detected, they make excellent markers for most insects.

There are disadvantages to using dusts however - if too much dust is applied, it can produce adverse behavioral effects or kill insects, so preliminary tests should be undertaken to determine the optimal amount of dust to apply. Dusts are often not persistent enough for long-term studies, and some dust particles have the potential to be transferred to unmarked insects in the field or in traps (Miller, 1993).

5.2.2.6 Other Marking Options

Other marking options include pollen, biochemicals, radioactive isotopes, trace elements, protein marking and genetic engineering.

The use of pollen has received limited attention in the study of insect activity (Courtney *et* al., 1982), as several factors limit its use for wide-scale application. An effective pollen marker must be geographically remote from the areas in which the pollen-bearing insects are caught, pollen analysis is costly, time-consuming, tedious, and requires expertise in pollen taxonomy (Jones *et al*., 1995), and the marker can be

influenced by the time of year of study. The use of genetic and biochemical markers, has not been well documented as these types of markers are rare, and usually associated only with insects that have been reared in the laboratory for many generations (Hagler & Jackson, 2001). Induced mutations, using radiation or mutagenic chemicals, may include other non-visible but detrimental mutations affecting physiology or behaviour (Bartlett, 1982).

Labelling insects with radioactive isotopes was popular from the 1950s to the 1970s (Davey, 1965), until stricter environmental protection laws and the development of simpler, less expensive, and more reliable methods have reduced the usefulness of these isotopes as insect markers (Hagler & Jackson, 2001). A review of radioactive-isotope marking is provided by Service (1993). As an alternative to radioactive isotopes, rare- or trace-element-marking techniques were developed in the 1970s and were reviewed by Akey *et al*. (1991). The detection of these elements can be difficult, expensive and time-consuming (Akey & Burns, 1991), may not be retained well in certain insect species (Guillebeau *et al*., 1993), and can adversely affect development, survival and fecundity of certain insects (Van Steenwyk *et al*., 1992).

5.2.3 Methods used to Mark *Culicoides*

Davies (1965) explored the use of radioactive substances for labeling *Culicoides* larvae under both laboratory and field conditions. Direct application of P-32 to larvae, or to a tray of mud collected from the field, enabled the larvae present to metabolise enough radioactive phosphorus to be detectable in the adult 9 weeks later. Holbrook *et al*. (1991) undertook a similar procedure where they reared *C. variipennis* adults from media with different concentrations of rubidium. Concentrations of 500 parts per million and above reduced pupal production, adult emergence and adult longevity, but they found that lower concentrations had no noticeable effects and one marked fly was readily detected in a pool of five.

Although Davies mentions that these techniques may be useful for adult dispersal studies, they rely on the ability to rear or colonize your species of interest in a laboratory setting which, for the Obsoletus Group species, has yet to be achieved.

Similarly, as the radioactive labels were applied to larvae rather than adults, it is unlikely that such a technique could be used successfully on wild-caught adult *Culicoides* to be marked and released immediately.

Campbell & Kettle (1975) used dyes to mark adult *Culicoides* immediately after emergence, in order to easily classify flies by age. Dissection of adult *C. brevitarsis* showed that ingested sucrose solution (10%) was stored in the crop, which is easily visible as an enlarged, semi-translucent zone in the anterior half of the abdomen in intact insects. Previous experiments (Campbell & Kettle, 1975) had already shown that newly emerged individuals of both sexes fed readily on sucrose solution, hence colouration of that solution could provide an easy method for marking. Of the 23 dyes tested, 6 proved suitable for marking purposes (not lethal, produced consistent staining). As they were unable to induce *C. brevitarsis* to take a blood meal in captivity however, the influence of blood feeding on the retention of the mark was not assessed. As this method relies on the *Culicoides* feeding on a sucrose solution, it is only really applicable to mark-release-recapture studies when releasing laboratory colonies, and not for capturing and releasing field-caught *Culicoides*.

The use of micronized fluorescent dusts for marking *C. variipennis* was evaluated by Lillie *et al.* (1981). Dusts were obtained from United States Radium Corporation and Day-Glo Colour Corporation, and applied to CO_2 anaesthetized *Culicoides* using an insufflator. Although none of the Day-Glo dusts were satisfactory for marking as they did not adhere to the cuticle of the flies, three of the six US Radium dusts were believed to be suitable as marking agents. Lillie *et al*. (1981) successfully used this technique to study the dispersal of field-collected larvae that were reared in a laboratory, before being marked with micronized fluorescent dust and released in Colorado. Later in 1985, Lillie *et al*. undertook the same marking technique on wild caught *Culicoides*. Here a needle of a 5ml syringe was inserted through the screen adaptor on the lower portion of a CDC trap and injected approximately 0.4ml of micronized fluorescent dust, while leaving the fan running to circulate the dust. Their preliminary tests suggested that 100% of the specimens in the collection bag could be marked using this technique.

Brenner *et al* (1984) also successfully used fluorescent dusts, produced by Radiant Pigment Corporation, to study the dispersal of *C. mohave* in southern California.

They employed a different method again to those described above. Once wild-caught midges were caught, they were anaesthetized briefly with CO_2, passed through a course sieve to eliminate larger insects and placed in a cardboard container whose internal surfaces had been coated with 0.2 g of fluorescent powder. The carton was revolved slowly to mark the adults, before they were released.

Although the fluorescent dust marking methods mentioned above have proved successful for marking *Culicoides* in the field, neither the United States Radium Corporation nor the Radiant Pigment Corporation exist today, and none of the methods have been trialed on the *Culicoides* Obsoletus Group members.

A recent study by Kirkeby *et al*. (2013) used fluorescein isothiocyanate to dust the Palaearctic vector Groups, but although the staining could be detected by ELISA plate scanning, it could not readily be seen by the eye, and may also be removed from *Culicoides* by the addition of ethanol to samples, so storage of these samples over time is unfeasible.

5.2.4 Research Justification

The main means of spread of BT from farm to farm is thought to be via the dispersal of *Culicoides*. While this dispersal is undoubtedly a vitally important aspect of BT epidemiology, it has not yet been recorded in terms of the newly implicated vector species.

Until now, dispersal studies have only been undertaken on *C. brevitarsis*, *C. mohave* and *C. variipennis,* as well as the Pulicaris Group in Denmark. As such, investigation of marking agents has mainly been undertaken on these species. The use of dyes and radioactive substances as markers has been shown to be effective in a laboratory setting. Their use in mark-release-recapture experiments in the field however, requires either the use of laboratory reared or colonised individuals marked when they were larvae (Davies, 1965; Holbrook *et* al., 1991) or newly emerged adults (Campbell & Kettle, 1975); or for dispersal to be investigated by dosing larval field sites with the marker which is then metabolised and found in the emerging adults (Davies, 1961). Both methods are time consuming and limitations include the need

to colonise or feed *Culicoides* in a laboratory, knowledge of where breeding sites are situated, and not having the ability to select the location of 'release'.

Although the use of micronized fluorescent dusts has produced good results in a laboratory setting (Lillie *et al*., 1981), as well as proved successful in field trials (Brenner *et al*., 1984; Lillie *et al*., 1981, 1985), a number of problems still remain. Firstly the use of easily identifiable fluorescent dusts that can be seen by eye has not been tested on Obsoletus Group members. Secondly, the manufacturers of such dusts used in previous studies, on other *Culicoides* spp., no longer exist.

This study therefore aimed to address the hypothesis that Obsoletus Group members can be marked effectively using micronized fluorescent dusts under both a laboratory and field settings. It also tested the hypothesis that the fluorescent dusts have no adverse effects on *Culicoides* behaviour, life-span or flight compared to unmarked controls. The ability to detect marked individuals trapped in both water and ethanol was also investigated, as well as whether there was any transfer of dust to other individuals or the environment. As no self-marking method currently exists for marking *Culicoides* in the field the final aim of the study was to explore the possibility of self-marking.

The aim of this study was to determine whether three commercial Brilliant General Purpose (BGP) fluorescent dusts (BPG Brilliant Green, BPG Brilliant Pink and BPG Brilliant Yellow), manufactured by Brilliant Group Inc., US, are suitable markers for use when studying C*ulicoides* spp. and to devise a suitable marking protocol for both field and laboratory use. The award of a BBSRC-funded Summer Studentship enabled Kristina Hunter to support the laboratory studies undertaken in this chapter.

5.3 Materials and Methods

5.3.1 Selection of Fluorescent Dusts

Brilliant General Purpose (BGP) Fluorescent Pigments manufactured by Brilliant Group (San Francisco, USA) were selected as the fluorescent dusts to be tested due to their small particle size (3-5 microns), wide range of colours which would be useful for repetitions of MRR experiments, non-toxic nature, and availability. The BGP series of fluorescent pigments are principally used in the coloration of paints, coatings, inks and plastics. Three colours were selected to be tested as marking agents – pink (BGP-PK111), green (BGP-GR118) and yellow (BGP-YE117). See Appendix D for the material safety data sheet.

5.3.2 *Culicoides* Collection and Identification

All Obsoletus Group midges used in this set of studies were wild-caught specimens captured using an Onderstepoort-type down-draught black light trap placed at 2 m in height at the University of Liverpool's Leahurst Veterinary School, Wirral, UK. *Culicoides* were trapped in bags in order to catch live specimens. The *Culicoides* were maintained in plastic containers at 17°C with a 10% sucrose solution embedded in cotton wool and placed on gauze lids for sustenance (Figure 5.1).

Separation of the Obsoletus Group from other species was performed following incapacitation by cooling on a coldplate set to -15˚C, midges were maintained at this temperature for 15 seconds until all activity had ceased before separation could occur. Midges were identified as Obsoletus Group members via stereomicroscope examination of specimen wing patterns using the Institute of Animal Health wing pattern analysis key (IAH, 2009).

Live-trapped *Culicoides* were killed in the laboratory by being placed in a -80˚C freezer for 20 minutes.

Figure 5.1. The maintenance of *Culicoides* in plastic trapping containing incorporating gauze lids. Cotton wool embedded with a 10% sucrose solution was placed on top of the gauze lids for sustenance.

5.3.3 Experimental Methodology

A series of experiments were undertaken to determine the effectiveness of the BGP fluorescent dusts in marking *Culicoides* species. Here, three particular areas of interest were focused on:

1. Dust properties;
2. Effect of dust on *Culicoides*; and
3. Dust application.

Within these themes a series of 11 experiments were undertaken.

5.3.4 Investigation of Dust Properties

5.3.4.1 Dust Solubility

100 ml of three test solutions (water, 10% washing up liquid in water, and 70% ethanol) were prepared in triplicate in 500 ml plastic beakers. These solutions were held at three different temperatures (10°C, 20°C or 30°C) using a hotplate (Stuart

Heat-stir SB162, Bibby Scientific Limited, UK) or coldplate (Leica EG1130, Leica Microsystems, Germany). 1 g of each dust (green, pink and yellow) was added individually to the test solutions, and the solutions were agitated using a magnetic stirrer (Stuart Heat-stir SB162, Bibby Scientific Limited, UK) for 30 minutes. Dust solubility was assessed via filtration of beaker contents using 5 µm filtration-paper. The tests were repeated using 0.01 g of each coloured dust.

5.3.4.2 Dust Visibility

0.01 g of each dust was gently spread using a spatula onto a variety of surfaces (clean white paper towels, opaque and transparent plastic) and the excess removed by shaking. Surfaces were inspected directly using a stereomicroscope under both natural and blacklight. The procedure was repeated by dusting 30 killed *Culicoides* stored in a conical flask with dusts, before the live-dusted *Culicoides* were visualised from the experiments that followed.

5.3.4.3 Dust Adherence

0.01 g of test markers were prepared in 15 ml tubes. Thirty killed *Culicoides* were added to test tubes ranging in number and agitated using an electronic shaker (IKA MS2 Minishaker, IKA Laboratory Technology, Germany) or gently rolled for 10 seconds. Specimens were removed from the tubes, examined under a stereomicroscope to determine if dusts were adherent, before being stored in clean 15 ml tubes at 10°C and re-checked at 24 and 48 hours. Areas marked by each of the coloured dusts were noted.

10 ml of test solutions (pure water, 10% washing up liquid in water or 70% ethanol) were prepared in 15 ml plastic conical tubes held at one of three specified temperatures (10°C, 20°C or 30°C) using a hotplate or coldplate. Individual marked midges were added to the test solutions and maintained at the specified temperature for 30 minutes. Midges were individually removed from the solutions and examined under a stereomicroscope to determine adherence. They were further stored at the

specified temperatures using an incubator and re-checked at 24 and 48 hours. *Culicoides*, and the solutions they were contained in, were fluoresced under a blacklight to observe the presence of fluorescent dust.

5.3.4.4 Dust Transfer to the Environment

Thirty live *Culicoides* were marked using the rolling method mentioned above. Marked midges were placed into clear plastic containers with the base lined with paper towel. *Culicoides* were maintained in the containers for 48 hours before being killed and removed. The gauze, paper towel and plastic container surfaces were inspected using a portable black-light for evidence of dust transfer.

5.3.5 Investigation of Effects of Dust on Culicoides

5.3.5.1 Dust Toxicity

Live-caught *Culicoides* were separated by cooling and marked using the tube rolling method discussed above. Thirty marked midges and 30 un-marked controls were transferred to separate plastic containers. The mortality rate of the marked and un-marked individuals were observed and recorded every 30 minutes for 4 hours. *Culicoides* were re-checked at 24, 48 and 72 hours.

5.3.5.2 Impact on Behaviour

Thirty marked *Culicoides* and 30 un-marked controls were placed into separate clear plastic containers. Scan sampling was used to determine the proportion of midges exhibiting the listed behaviours of flying, climbing, walking, eating, cleaning and resting at the following intervals: every 5 minutes during the first hour, every 15 minutes for the second hour and every 30 minutes for the third hour.

5.3.5.3 Dust Transfer between Culicoides

Thirty marked and thirty control midges were placed into the same clear plastic container. The *Culicoides* remained in the container for 24 hours before being killed and inspected under a stereomicroscope using both natural and black-light conditions to observe evidence of any dust transfer.

5.3.6 Investigation of Optimal Dust Application

5.3.6.1 Laboratory Marking

Three approaches to marking *Culicoides* were compared within the laboratory, and information recorded included the ease of application, evenness of coverage, mortality, injury to midges and waste residue.

Thirty live *Culicoides* were placed in plastic containers secured with a clean gauze-lid. 0.01g of dust was measured and placed on the surface of the gauze, a fine brush was used to gently sieve the dust through the gauze. Midges were observed for responses and killed and examined under natural light and black-light via a stereomicroscope.

Thirty live *Culicoides* were placed in a glass vacuum flask and secured with a bung. 0.01g dust was injected into the hose barb at the neck. Gentle traction was applied to the syringe to gradually introduce dust to the flask. Midges were observed for responses and killed and examined under natural and black-light via a stereomicroscope.

Plastic trapping pots were pre-dusted with 0.01g of dust to create a fine layer of dust on all surfaces. Thirty live *Culicoides* were placed in the pots and allowed to walk through the dust layer independently for 15 minutes. Midges were observed for responses and killed and examined under natural or black-light via stereomicroscope.

5.3.6.2 Field-based Marking

Due to the success of the pre-dusted trapping pot method in marking *Culicoides*, and the possibility of it being employed as a self-marking method, the use of pre-dusted trapping pots was studied in terms of field-based marking. Plastic collection pots were pre-dusted with 0.1 g of dust. Care was made to ensure even coverage of the sides and base of the pot. Pots were transported to the Leahurst field-site, attached to the OVI trap and run over night. Trapping pots containing live marked C*ulicoides* were collected the following morning and all caught midges were killed and inspected under natural and black-light via a stereomicroscope.

The pre-dusted collection pots, used in the field-based marking experiment, were replicated by varying the weights of dust used. Five weight intervals of dust were used, ranging from 0.1 g to 1 g, with pots run overnight in traps and the proportion of dead and live midges determined the following day. All remaining live midges were then killed by rapid cooling before inspection.

5.4 Results

5.4.1 Investigation of Dust Properties

Both 0.01 g and 1g of the three fluorescent dusts were insoluble in water, 10% washing-up liquid solution and ethanol at 10, 20 and 30^{o}C. The dusts were visible, both directly and under a stereomicroscope in natural or black-light conditions, with no variation in visibility detected. They were visible on all surfaces dusted, with no difference in visibility identified.

The three dust markers adhered to all midges for the duration of the experiment (48 hours). BGP yellow and green dusts demonstrated greatest attachment to the wings, wing base and legs, while BGP Brilliant Pink adhered to the thorax. Five of the killed C*ulicoides* marked by agitating the pre-dusted tubes on an electronic shaker showed damage to their wings and antennae, while *Culicoides* marked via rotation of the tube showed no such damage.

Following storage of the killed and marked *Culicoides* in liquid for 48 hrs, the dusts remained adherent on the individuals and no extraneous particles were found either on the container or liquid they were stored in. The dusts remained adherent for the 48 hour duration in water, 10% washing up liquid solution, and ethanol at 10, 20 or 30^{o}C. No transfer occurred with any dust from marked midges to any of the test surfaces over the 48 hours of the experiment.

5.4.2 Effect of Dust on Culicoides

An increase in the rate of mortality was observed for the control and dust marked *Culicoides* (Figure 5.2) over time. The BGP green dust-marked *Culicoides* had a mortality rate that seemed to exceed that of the Yellow or Pink dusts, as well as the un-dusted control *Culicoides*, but this was insignificant statistically (one way ANOVA, P=0.846).

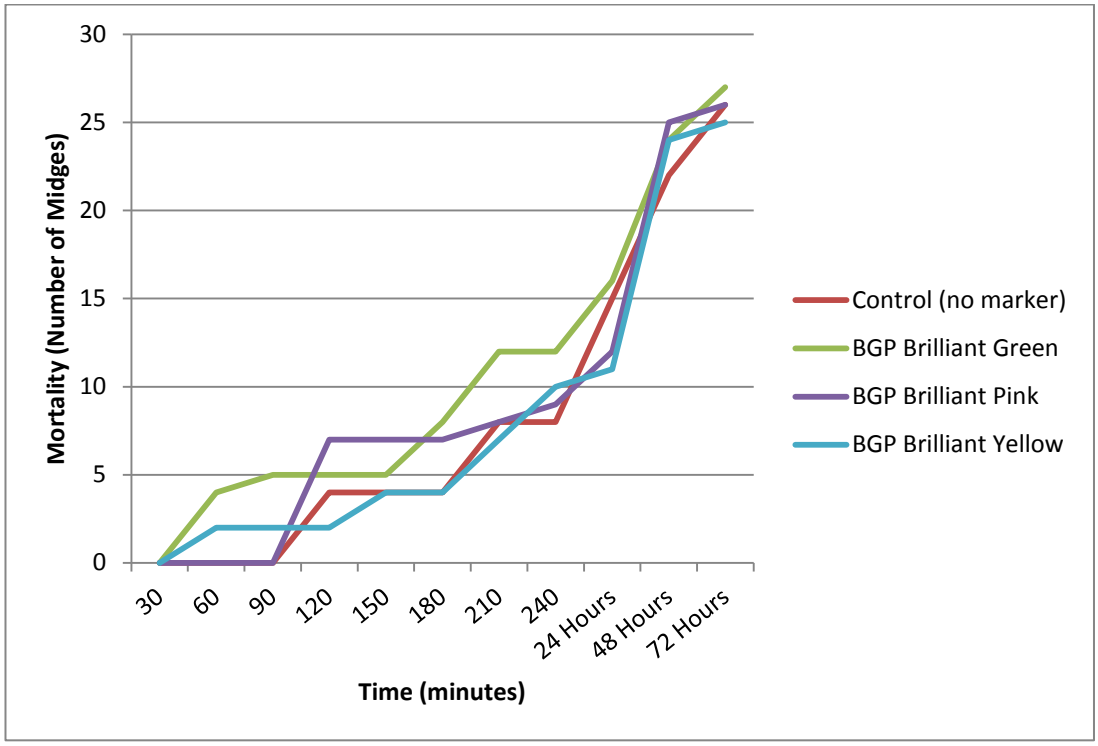
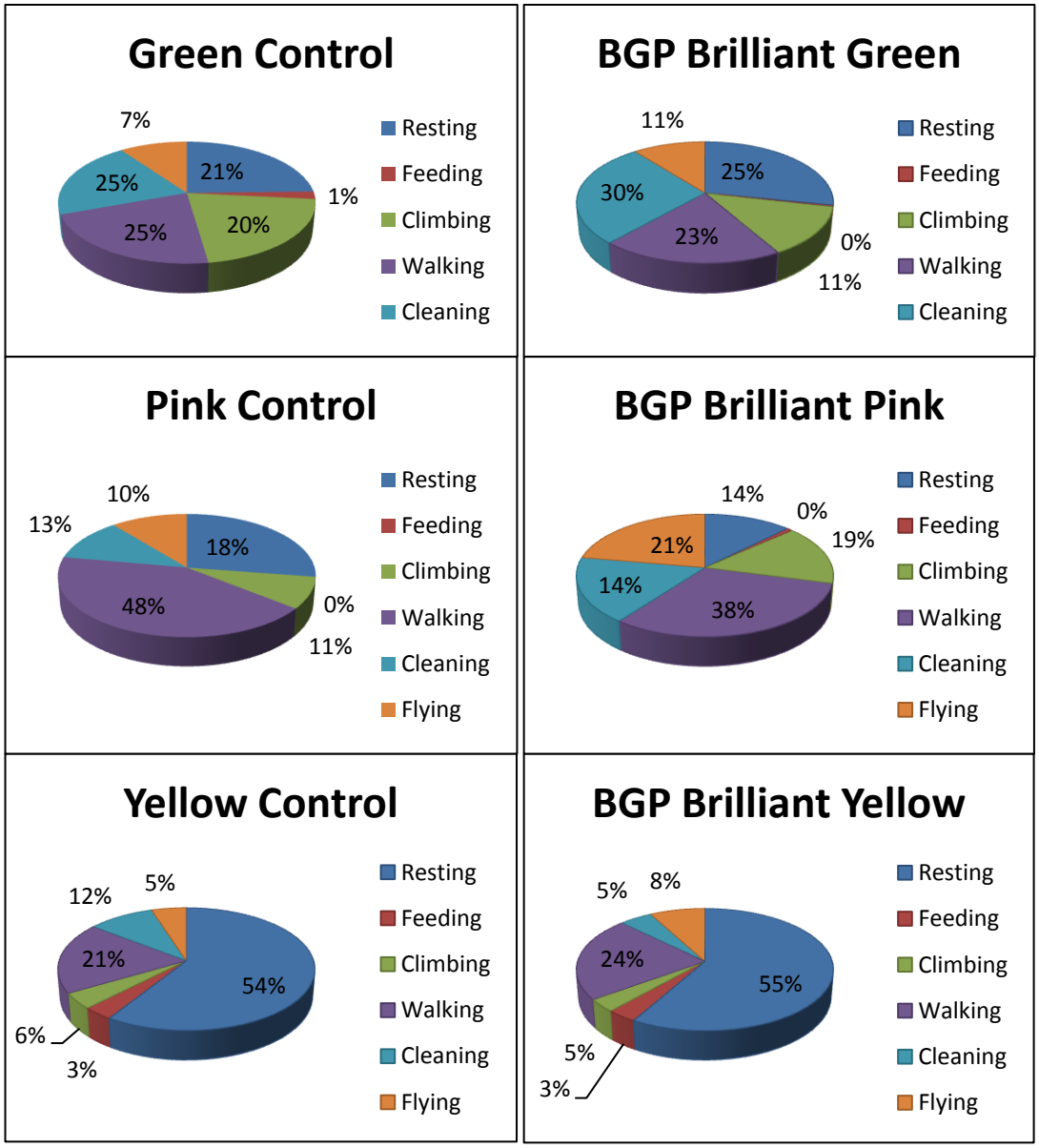


Figure 5.2. Mortality rate of individuals dusted with BGP green, pink and yellow fluorescent dust.

Overall there were no significant differences between the behaviours undertaken between the un-dusted control *Culicoides* and the BGP fluorescent dusted *Culicoides* (chi-square, *P*=0.922). The percentage of *Culicoides* exhibiting the listed behaviours during the experiment can be seen in Figure 5.3. Following Bonferroni correction, the green dust did not significantly alter the behaviour of the *Culicoides* (chi-square, *P*=0.371), but marginally increased flying, resting and cleaning behaviours while decreasing feeding climbing and walking. The pink dust increased feeding, climbing, cleaning and flying, while decreasing resting and walking to a significant degree (chi square, *P*=0.122). The yellow dust did not significantly alter behaviour (chi-square, *P*=0.5), but did increase walking and flying while decreasing cleaning and resting behaviours.

Figure 5.3 Percentage of *Culicoides* exhibiting the observed behaviours of resting, feeding, climbing, walking, cleaning and flying, when dusted with BGP green, pink or yellow fluorescent dusts compared to un-dusted control *Culicoides*.



No dust transfer occurred between dust marked *Culicoides* and unmarked controls, with 15 marked *Culicoides* and 15 unmarked control *Culicoides* identified using a stereomicroscope.

5.4.3 Dust Application

Of the three dust application methods trialled in the laboratory (fine brush technique, syringe injection, pre-dusted containers), the syringe-based method proved the most difficult in terms of accurately measuring the required amount of dust for application, also leading to wastage with dust remaining in the syringe. This method also led to a limited number of individuals being marked within the time limit of the experiment (14 individuals out of 30). Four individuals also exhibited injury to their wings when this method was employed.

Although the required amount of dust was easy to measure when using the fine brush technique, it was difficult to obtain an even coverage of individuals through the mesh gauze of the trapping pot, with some individuals receiving a higher coverage of dust than others (although all 30 midges were marked). The marking method worked best when the pot itself was already pre-dusted with fluorescent dust prior to the *Culicoides* being added (all 30 *Culicoides* marked). Mortality rates were low using all methods.

The number of *Culicoides* trapped during each replicate of the field-marking experiment can be seen in Table 5.1. The total *Culicoides* trapped during the 5 consecutive trapping nights was 880. Of these, 100% of individuals were marked each night, irrespective of the weight of dust used to pre-dust the container. The mortality rate of *Culicoides* did not vary significantly between different dust weights.

Weight of Dust (g)	**Number of *Culicoides* Trapped**	**Number of *Culicoides* Marked**	**Number of Dead *Culicoides***
0.1	134	134	0
0.25	217	217	2
0.5	203	203	0
0.75	167	167	2
1.0	159	159	4

Table 5.1. The number of *Culicoides* trapped, marked and dead during 5 replicates of the field-based, self-marking technique using 0.1, 0.25, 0.5, 0.75 and 1 g weights of fluorescent dust to mark the trapping containers.

5.5 Discussion

This study represents the first attempt to identify a marker for members of the Obsoletus Group, the vectors of bluetongue virus in northern Europe. This technique should be useful in studying the dispersal behaviour of members of the Obsoletus Group under field conditions.

Marking of *Culicoides* midges has historically involved either a fluorescent dust marker (Lillie *et al*., 1981, 1985; Brenner *et al*., 1984) or a rubidium marker (Holbrook *et al*., 1991). Dusts have been used to mark insects for more than 75 years (Darling, 1925) and are one of the most commonly used materials for externally marking a variety of insects (Service, 1993). To our knowledge, there have been no studies published on the use of fluorescent dusts for marking *Culicoides* since the 1980s, and the fluorescent dusts employed in these early studies are now unavailable since the manufacturers, U.S Radium Corporation, are no longer in operation.

The dust properties tested indicate that the fluorescent dusts could be successfully used in mark-release-recapture experiments. As all dusts remained insoluble in all 3 solutions this would allow trapping during field investigations to be undertaken in standard trapping solutions of water with a drop of washing-up liquid in order to break the surface tension. It would also allow storage of insects in 70% ethanol solutions following collection. Similarly, the adherence experiments suggested that not only are the dusts insoluble in these 3 test solutions, but they also remain adhered to the *Culicoides* when they are subjected to either of these solutions. As adherence to *Culicoides*, when stored in ethanol, was only evaluated for 48 hrs in this experiment, more work would need to be undertaken to determine if long-term storage in ethanol would affect the adherence of the dusts.

Dust visibility was not reduced after the marked *Culicoides* were stored in solution. The use of a fluorescent light was not always required to detect marked individuals, as the pink and green fluorescent dusts was easily identifiable on the *Culicoides* without being fluoresced. The use of these dusts therefore provides a quickly identifiable marker, eliminating the need for more complex and time-consuming detection methods as are often needed for studies involving markers of pollen (Jones

et al., 1995), dyes (Coppedge *et al*., 1979; Showers *et al*., 1989), radioactive isotopes, or trace elements (Akey & Burns, 1991).

Transfer of fluorescent dust to the environment in which the marked *Culicoides* were held was not observed over the 24 hour period they were examined for. As it is likely that any superfluous dust would have been removed by the *Culicoides* rapidly, it is unlikely that dust transfer would occur at any time after this 24 hour period. Other researchers have incorporated dusts with gum arabic, so that particles could not easily be removed with preening and wing movements (Sinsko & Craig, 1979; Brust 1980), but that was not necessary in this study.

While visibility and adherence of the marker is of utmost importance, any selected marker must also not have a detrimental effect on the survival or behaviour of marked specimens. (Hagler & Jackson, 2001). Although an increase in mortality of marked *Culicoides* was observed over time, a similar increase was also observed in the unmarked controls. Of the markers tested, there were no significant differences between the behaviours of the un-marked controls and the marked *Culicoides*, highlighting that application of the dusts as a marking agent in the field, would not affect the results obtained by MRR experiments.

In a field study, a range of species would be dusted, not just members of the Obsoletus Group. If the study were to be repeated, it would be useful to test the dust markers on other species of *Culicoides*, such as members of the Pulicaris Group, to determine whether the same results are gained. Further information would also be gained from undertaking a species-specific PCR on the dusted to determine a) if any differences in mortality or behaviours are seen between the Obsoletus Group members, and b) whether the fluorescent dusts interfere with PCR identification.

As adherence to *Culicoides*, when stored in ethanol, was only evaluated for 48 hrs in this experiment, more work would also need to be undertaken to determine if long-term storage in ethanol would affect the adherence of the dusts.

Although many important aspects of a marking agent were tested in this study, the laboratory environment within which the experiments were undertaken is unlikely to be identical to the conditions *Culicoides* would encounter in the field. We did not assess the impact of rain or wind on the adherence of the dusts to *Culicoides*, or their

subsequent transfer to the environment. Similarly, the duration of these experiments was limited due to the difficulties encountered with keeping wild-caught Obsoletus Group members alive in a laboratory environment. Although laboratory colonies of other *Culicoides* species are available (*C. sonorensis* and *C nubeculosus*), the behaviours of these individuals may be markedly different from those of their wild counterparts and as a colony of Obsoletus Group members has not been established, wild-caught individuals were employed here.

Following the series of laboratory experiments on the dusts a small field-trial of a self-marking method was successfully employed. The field-study highlighted the ease of use of the pre-dusted trapping pot method in self-marking *Culicoides* for MRR studies, eliminating the need to manually apply dust to the insects the following morning. As no difference in mortality rates or dust coverage of *Culicoides* occurred when different quantities of dusts were applied to the pots, we recommend that a sufficient quantity of dust is applied so as to fully cover the sides and gauze bottom of the trapping pot, with this quantity varying depending on the size of trapping vessel used.

Relatively small numbers of *Culicoides* were trapped during the field-trial so it would be useful to replicate the trial in an area where larger numbers of insects are likely to be trapped to determine whether dust coverage remains at 100% with large numbers of insects.

In summary, BGP fluorescent dusts are a suitable marking agent for *Culicoides* midges as they do not influence either survival or flight behavior of Obsoletus Group members in the laboratory. Marked midges remained distinguishable for their entire life; dusts did not transfer from marked to unmarked individuals or the environment; the mortality rate of marked midges did not differ from controls under laboratory conditions; and, importantly for trapping and storing *Culicoides*, the dust did not dissolve or wash off in either ethanol or water. Pre-dusting trapping pots with BGP fluorescent dusts prior to trapping provides a fast and reliable method for self-marking *Culicoides* in the field and should prove useful for MRR studies.

CHAPTER SIX

LOCAL DISPERSAL OF PALAEARCTIC *CULICOIDES* BITING MIDGES ESTIMATED BY MARK-RELEASE-RECAPTURE

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Local Dispersal of *Culicoides* Biting Midges Estimated by Mark-Release-Recapture

Georgette Kluiters (GK) conceived and designed the study. GK and Matthew Baylis were awarded a BBSRC-funded Summer Studentship which enabled Harry Swales (HS) to support the fieldwork. GK & HS undertook field samplings and identified marked *Culicoides* to species level. GK undertook statistical analyses on the trapping data. GK wrote the first draft of the manuscript and all authors contributed to approving the final version of the manuscript.

6.1 Abstract

As a key component in BT modelling is farm to farm transmission via midge movement, there is a need to determine dispersal patterns, in particular the distance over which midges fly during a set period; and to identify factors that contribute to the direction and flight distance.

The dispersal of *Culicoides* Obsoletus Group members was studied on 19 farms in the Bala region of north Wales. Field-collected adult *Culicoides* were trapped in an OVI trap and self-marked when deposited into the trap's collecting vessel, which was pre-coated in micronized fluorescent dust. *Culicoides* were released at a central farm and OVI traps set on 18 other farms surrounding the release point, ranging between 0.5 and 4 km from the site. The study was repeated using six colours of fluorescent dust over an 18 day period.

An estimated 61,000 marked *Culicoides* were released during the study and 12 (0.02%) of the marked *Culicoides* were recaptured. Of the females recaptured, six were *C. obsoletus/scoticus*, two *C. dewulfi*, two *C. pulicaris* and one *C. festivipennis*. The male was *C. obsoletus*.

Recaptures occurred 1- 2.5 km from the release site, with the greatest number at 2.5 km. Most recaptures were 2 nights post-release; none were more than 3 nights post-release. Two females were recovered at a distance of 1.5 km on the night of release and one male at 1 km two nights post-release. The mean distance travelled (MDT) for all *Culicoides* through the entire mark-release-recapture was 2.15 km. The MDT for males was 1 km, whereas for females it was 2.21 km. Recaptures were made both downwind and upwind of the prevailing wind direction during the trapping periods, possibly highlighting passive and active dispersal of *Culicoides* between farms.

This is the first study to demonstrate farm to farm movement of the main Palaearctic BT vector species, the Obsoletus Group. Such movement has disease control implications in terms of the vectorial movement of disease between farms. The results suggest that control measures applied at an infected farm will reduce the risk of spread to neighbouring farms, as well as reduce transmission at the source farm itself.

6.2 Introduction

Since the emergence of bluetongue (BT) in northern Europe, the disease had spread to regions where the main Mediterranean vector species, *Culicoides imicola* Kieffer, is absent, and two new vector groups, the Obsoletus Group (*C. chiopterus*, *C. dewulfi*, *C. obsoletus*, *C. scoticus*) and Pulicaris Group (*C. pulicaris* and *C. punctatus*) have been implicated as virus vectors. Relatively little is known about the ecological characteristics of the newly implicated vector species (Conte et al., 2007a), or indeed those believed to be non-vectors, this includes their flight behaviour (Carpenter, 2009), yet this is critical to determining the distance over which an insect may transmit a disease agent (Hocking, 1953) and can be used to determine the size of the area over which control should be applied.

There is, therefore, a need to determine the dispersal patterns of northern European vector *Culicoides* species, in particular the distance over which midges fly during a set period; and to identify factors that contribute to the direction and flight distance.

6.2.1 Why Study Dispersal?

Dispersal is often triggered by factors such as food availability or population density (Danthanarayana, 1986), or other environmental factors which may act as 'surrogates' for habitat change (e.g. temperature, humidity and rain, wind) (Dingle, 2001). Quantifying insect dispersal is an important component of understanding insect population dynamics, and also to answering questions related to insects' behavioural traits, and physiological or genetic constraints.

The major reasons for flight in ceratopogonids and other biting flies are to find food, a mate and a suitable oviposition site (Lillie, 1985). Active dispersal in order to find a food source is of utmost interest due to the mechanism of vectorial transmission of *Culicoides*-borne diseases. Dispersal of *Culicoides* in detectable numbers between farms can have an impact on disease outbreak restriction limits both during times of outbreaks and with regards to scenarios anticipated in virus transmission modelling.

It is widely accepted that wind can assist vectors in dispersing great distances over large bodies of water. However, the sort of long-range (>100 km) spread seen over

the seas has not been detected over land. Hendrickx *et al*. (2008), built a wind density model to quantify the airborne spread of *Culicoides* species during the 2006 European BT outbreak. Their model shows that 50% of cases occur within 5 km of the previous case, while 95% of cases occur within 31 km of it. Turner *et al*.'s (2012) vector transmission model indicates that the majority of parameters affecting the final size of an outbreak of BT relate to vector transmission, and that infection generally cannot be maintained without between-herd vector transmission. This emphasizes the need for further work on native vectors of bluetongue, including the need for more accurate information on how far they can travel in a day (Turner *et al*., 2012).

Underestimation of flight capacity inhibits effective control attempts and overestimation could have severe socioeconomic consequences due to livestock movement restrictions and loss of trade (Gloster *et al*., 2008). To date, one of the best-characterized mechanisms of BT virus spread is via dispersal of infected *Culicoides* (Sellers, 1980). As a key component in BT modelling is farm to farm transmission via midge movement, there is a need to determine dispersal patterns, in particular the distance over which midges fly during a set period; and to identify factors that contribute to the direction and flight distance.

6.2.2 Methods of Studying Insect Dispersal

Insects are generally small, often very numerous, frequently winged and can travel extremely long distances, either actively or passively in the airstream (Osborne *et al*., 2002). Consequently, tracking their movements over space and time presents a great challenge. Reynolds *et al*. (1997) and Southwood & Henderson (2000) provide reviews of techniques to study insect movement, while Turchin (1998) describes measurement techniques and associated analysis.

When characterizing the movements of insect vectors, Reynolds et al. (2006) distinguishes between two types of behaviour, i.e. the 'vegetative' or short-distance movements directed towards resources required for growth and reproduction, and the generally long distance, migratory movements. For *Culicoides*, in particular, Sellers (1992) similarly observed two types of flight:

1. the long-distance dispersal (up to several 100 km) which occurs when the midges are carried by winds with speeds exceeding what can be achieved by unaided flight; and
2. the short-distance flights that occur in any direction (both up- and downwind) at low or zero wind speeds.

Long-distance dispersal studies originally derived solely from the distribution of reported cases of disease (Sellers, 1980; Sellers *et al*., 1978), while short-distance dispersal has been evaluated by collecting unmarked adults near isolated breeding sites or by releasing and recapturing marked specimens.

6.2.2.1 Long-distance Dispersal

The arrival of BTV and other *Culicoides*-borne viruses in naïve areas has long been associated with the long-distance dispersal of *Culicoides* on prevailing winds. Atmospheric dispersion models have been employed to simulate vector movement (and hence likely spread of BTV).

Outbreaks of bluetongue and African horse sickness in several countries have been attributed to this means of dispersal (Sellers *et al*., 1977; Braverman & Chechik, 1996; Alba *et al*.,2004; Ducheyne *et al*., 2007; Gloster *et al*., 2008; Hendrickx *et al*., 2008). Support for many of these attributions has come from modelling studies in which the plausibility of paths of dispersion was assessed through either wind directions (trajectories) or more complex three-dimensional atmospheric dispersion models (Sellers et al., 1977; Braverman & Chechik, 1996; Alba *et al*., 2004; Ducheyne *et al*., 2007; Gloster *et al*., 2008; Hendrickx *et al*., 2008).

The study of long-distance dispersal often involves inferring the movement patterns of *Culicoides* indirectly from the patterns of spread of bluetongue outbreaks between farms and matching this with concurrent wind patterns. Ducheyne *et al*. (2007) determined wind trajectories to and from outbreak sites and compared these with the pattern of the BT outbreaks between 1999-2001 in Greece, Bulgaria and Turkey.

They found that wind trajectories could be matched to the temporal distribution of the outbreak cases.

Sanders *et al*., (2011) investigated the long distance dispersal potential of *Culicoides* and how the risk of wind-borne vector introduction could be predicted. The effects of seasonality and local meteorological conditions upon the daily presence, abundance and activity of *Culicoides* vector species in the UK were examined using a network of 12 m tall suction traps and a vehicle-mounted trap. The presence of *Culicoides* at high altitude (~200 m), conducive to long distance transport on the wind, was established using a balloon-mounted net.

Although the study of long-distance dispersal can be used to implicate virus vectors in the transmission of diseases in the past, such techniques are not generally used to determine new information about the active dispersal of individual species, or indeed distances over which *Culicoides* can disperse unaided by the wind.

6.2.2.2 Short-distance Dispersal

Very few studies have investigated short distance *Culicoides* dispersal, with the most recent work undertaken in 2010 in Denmark (Kirkeby *et al*. 2013), followed by work in the US in the 1980s (Lille *et al*., 1981; Brenner *et al*., 1984; Lille *et al*., 1985). Short-distance or active dispersal of ceratopogonids (<10 km) has been evaluated by collecting unmarked adults near isolated breeding sites or by marking, releasing and recapturing marked specimens (Mark-Release-Recapture; MRR). Most studies are based on the former procedure, which is easier to conduct. MRR techniques are more time consuming, as the researcher must obtain adults by live trapping in the field, collection from laboratory colonies, or by rearing them from the immature stage collected in the field. The collected insects are then marked, typically using radioisotopes (Davies, 1965), fluorescent dusts (Kirkeby *et al*., 2013; Lillie *et al*., 1981), paints (Gillies, 1961), or dyes (Dalmat, 1950), before being released into the field, and recaptured at given time and distance intervals after their release. The recaptured insects are then checked for the presence of the marker to distinguish

them from unmarked insects. MRR studies are particularly useful for mass marking known populations of insects for dispersal studies.

Investigating *Culicoides* dispersal from breeding sites however, does not involve the need of a marker if an isolated breeding site can be identified. In this case a similar set-up is used as in the case of MRR studies, whereby traps are set at varying distances from the breeding site and the distance travelled by insects is determined over a set period of time. The need for an isolated breeding site limits the location that such studies can be undertaken in however, as well as the distance over which such a study can be undertaken. Frequently dispersal from breeding sites, involves the need to mark the breeding sites of individuals so that insects appear marked when they emerge as adults.

6.2.2.2.1 Culicoides *Dispersal from Breeding sites*

A number of studies have attempted to quantify the dispersal of *Culicoides* from isolated breeding sites, making the assumption that it acts as the source of midges caught elsewhere. These studies assume no external interference from other breeding sites and no dispersal of insects from other sources. Such studies have generally found dispersal of 100 m to 3 km for *C. variipennis* (Dyce, 1969; Jones & Akey, 1977; Whitehead, 1935; Zimmerman & Turner, 1984), 1.2 to 3 km for *C. furens* (Bidlingmayer, 1961; Breeland & Smith, 1962; Williams, 1962), and 74 m to 275 m for *C. impunctatus* in Scotland (Hill, 1947; Kettle 1951). Such studies can be useful for quantifying the distance midges may fly, but not for estimating the rate of travel per day.

An alternative is the use of mark-release-recapture (MRR) techniques which, because the date of marking is known, can be used to estimate the rate of dispersal.

6.2.2.2.2 Culicoides *Dispersal in MRR Studies*

Most MRR studies on *Culicoides* have been undertaken in the USA in the 1980s. Such studies are usually undertaken over much greater distances than those studies

investigating dispersal from breeding sites, and as such have led to results suggesting dispersal occurs over much greater distances during certain periods of time.

Although radioactive substances (Davies, 1965; Holbrook *et al*., 1991) and dyes (Campbell & Kettle, 1975) have been employed successfully in both laboratory and field studies investigating *Culicoides* dispersal, the use of micronized fluorescent dusts as a marking agent has been given far more attention in MRR studies (Kirkeby *et al*., 2013; Brenner *et al*., 1984; Lillie *et al*, 1981). Such marking agents are cheap, easy to apply and identify in populations, and have shown little impact on the normal behavior of individuals.

MRR studies have shown that the females of *C. variipennis* can travel at least 4.0 km and the males only 800 m over an 8-day period (Lillie *et al*., 1981). These distances far exceed those identified in dispersal studies from breeding sites (Whitehead, 1935; Dyce, 1969).

In the desert of southern California, (Brenner *et al.*, 1984) *C. mohave* females travelled a mean distance of 1.94 km during a 30 h period following their release. The majority of specimens were recovered in the direction of the prevailing wind, but one individual had dispersed 6.0 km against it. Dispersal studies remain inconclusive regarding wind speed and direction; some studies report recaptures most marked in the direction of prevailing wind (Breeland & Smith, 1962; Brenner *et al*., 1984; Williams, 1962), others state that dispersal was not aided by wind (Lillie *et al*., 1985), and a number note some midges dispersed further against the wind (Brenner *et al*., 1984).

A number of MRR studies have been undertaken in the US, and short-distance dispersal has been evaluated by collecting unmarked adults near isolated breeding sites, including for *C. impunctatus* in Scotland (Kettle, 1951). Only one other study has attempted to determine the dispersal of the Obsoletus Group vector species using MRR techniques on the Palaearctic vector species (Kirkeby *et al*, 2013). This recent study in Denmark investigated a novel technique to mark *Culicoides* in the field, using fluorescein isothiocyanate, and successfully recaptured marked Pulicaris Group members, allowing them to quantify the moment of this species group between farms. Although Kirkeby *et al*. (2013) highlights movement of Pulicaris

Group members up to 1.75 km from their release site, members of the main European BT vector species, the Obsoletus Group, were not successfully recaptured away from the release site and the distances over which they actively disperse during a set period of time is still unknown.

The movement of *Culicoides* in agricultural landscapes is also not well characterized. Past dispersal studies have mainly focused on *Culicoides* dispersal in open landscapes, where the influence of vegetation on trap catches is reduced (Brenner *et al*., 1984). As less than 1% of the number of insects released in a MRR study are usually recovered (Johnson, 1969), this may explain the choice of a more homogenous landscape in terms of MRR studies.

6.2.3 Research Justification

The main means of spread of BT between farms is thought to be via the dispersal of *Culicoides*. A number of MRR studies have been undertaken in the US to determine the dispersal of different species, but as yet none have been undertsken successfully on the main northern European BT vector species, the Obsoletus Group. There is, therefore, still a need to determine this main vector species' dispersal patterns, particularly the distance over which these midges fly during a set period; and to identify factors that contribute to the direction and flight distance.

MRR studies have focused on homogenous landscapes with sparse vegetation to interfere with the ability of traps to attract *Culicoides*; such previous estimates of dispersal are unlikely to be representative of the true extent that the Palaearctic species travel in agricultural landscapes, or those with fluctuating terrains. The dispersal of previously studied *Culicoides* ranges from a couple of hundred meters from breeding sites, to at least 4.0 km during MRR studies, but this may be far greater than *Culicoides* would disperse in a heterogenous landscape.

The Bala region of north Wales in one such region that contains both a high density of farming properties, as well as a landscape containing a wide variety of parcels of land, from fields to forests, residential areas, lakes and mountains. Undertaking a MRR experiment in this region may provide a better estimation of natural dispersal

than previous studies due to the presence of natural barriers that may impact on active dispersal.

As vector transmission models indicate that the majority of parameters affecting the final size of an outbreak of BT relate to vector transmission, and that infection generally cannot be maintained without between-herd vector transmission, there is clearly a need for further work on the Palaearctic BT vectors (such as more accurate information on how far they can travel in a day) in order for accurate simulation models to be built involving these species.

The aim of this study was to investigate the dispersal of *Culicoides* across farms in the Bala region by releasing field-caught midges that are marked with a coloured fluorescent dust, aiming to recapture them at varying distances from the release site. The midges were to be marked using the fluorescent dusts, and the self-marking technique developed in Chapter 5. Marked midges were to be released from a central farm and all other study farms in the area were to have light-traps running constantly to re-capture the midges, determining the distance they flew in a set time-period. Specific aims included exploring the dispersal distance of the Obsoletus Group members over a set period of time in a heterogenous landscape, and determining whether wind plays a role in the direction of dispersal.

The award of a BBSRC-funded Summer Studentship enabled Harry Swales to support the fieldwork undertaken in this chapter.

6.3 Materials and Methods

This study was undertaken during July 2011, in the Welsh province of Bala, situated in Snowdonia National Park. The area primarily consists of extensive sheep and beef cattle farming, with a very hilly landscape comprised of a mixture of forests and field.

6.3.1 Study Farms

As with the fieldwork undertaken in Chapters 3 and 4, the dispersal fieldwork was also undertaken in the Bala area of north Wales, as the spatial distribution of these previously used farms was such that a dispersal study could easily be coordinated in the region.

ArcGIS Desktop 10 (ESRI, Redlands, California, USA) was used to create concentric buffer zones of 0.5 km around the central-most farm within the Bala field-site (farm D3) [see Chapter 2.1 for further details], until the buffers reached a 4 km radius from the central farm (Figure 6.1). Due to a restriction on the number of OVI traps available, 18 of the 24 other study farms in the area, which fell within the 4 km buffer zones were randomly selected to participate within the study. All farms were recruited via personal contact.

6.3.2 Experimental Methodology: Mark-Release-Recapture

Prior to the study, preliminary catches were undertaken on the farms that were to be used to collect marked *Culicoides* (collection farms), in order to estimate the number of *Culicoides* released during each replicate of the experiment.

Culicoides specimens were live-trapped in OVI traps set up on 3 farms (including the release site). Normal collecting beakers were replaced with gauze-bottomed beakers pre-dusted with 1g of BGP micronized fluorescent dust, using the method devised in Chapter 5. Each trap was operated overnight and the self-marked *Culicoides* were released at the release site (see Figure 6.1) at 0900-1000 hrs the following morning. An APRS World (APRS World, LLC, USA) weather station

recording wind direction, speed, temperature and humidity was set up at the release site for the duration of the experiment.

OVI traps, for the recapture of marked individuals, were positioned on all farms except the release site. The traps contained a 23cm 8W black light bulb and *Culicoides* were trapped into a 500 ml beaker containing 200 ml water and a small amount of washing up liquid, to break the surface tension. Light traps were located where they were believed to attract the highest numbers. These were generally near feeding (host) and breeding sites. Other light sources were also avoided so that there was no interference. As marked *Culicoides* were released in the morning, recapture traps were run 24 hours a day to increase the chance of catching marked specimens.

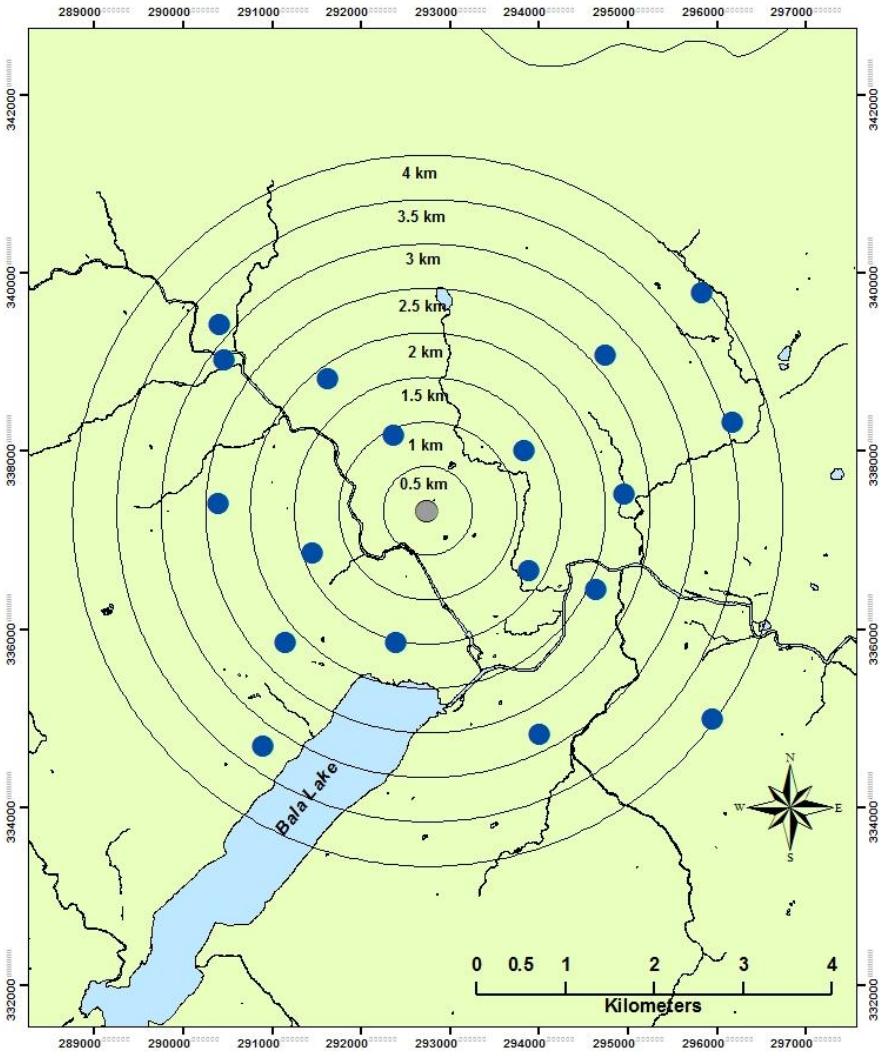


Figure 6.1 The spatial distribution of trapping farms (blue circles) surrounding the release farm D3 (grey circle) using for mark-release-recapture of *Culicoides* in farms around Bala

Every 24 hrs for 3 days following the release, collecting vessels were changed in order for the time period when a marked *Culicoides* was trapped to be determined. This MRR cycle was repeated five more times, so as to maximise the chance of recapturing the released *Culicoides.* Six colours (Pink, Green, Red, Blue, Orange, Yellow) of micronized fluorescent dust were used to allow for replications of the MRR experiment which therefore ran for a total of 18 days between 5th to 23rd July Temperature, humidity, precipitation, wind speed and direction for this period can be seen in Table 6.1.

Table 6.1 Weather variables for the Bala region of north Wales from 5th to 23rd July 2011

Date	**Temperature (°C)**			**Precipitation (mm)**	**Humidity**	**Wind**	
	Max	**Min**	**Average**			**Speed (km/h)**	**Direction**
05/07/2011[a]	20	14	17	0.8	65	17	SE
06/07/2011[a]	20	13	16	0.6	67	13	SW
07/07/2011[a]	20	11	16	2	71	18	SW
08/07/2011[ab]	18	8	13	3	78	16	S
09/07/2011[b]	20	12	16	0	65	13	NW
10/07/2011[b]	17	12	14	15	78	8	NW
11/07/2011[bc]	19	8	14	0	69	8	NW
12/07/2011[c]	20	11	16	0	65	8	E
13/07/2011[c]	18	15	12	0	57	10	N
14/07/2011[cd]	21	7	14	0	61	12	NW
15/07/2011[d]	22	9	16	0.4	64	10	S
16/07/2011[d]	19	13	16	2	72	17	SW
17/07/2011[de]	15	11	13	6	87	14	W
18/07/2011[e]	17	13	15	0.4	78	18	W
19/07/2011[e]	16	13	14	0.2	77	15	NW
20/07/2011[ef]	17	14	11	0	66	11	NW
21/07/2011[f]	18	12	15	0	75	10	N
22/07/2011[f]	18	11	14	1	73	7	N
23/07/2011[f]	17	7	12	0	67	13	NW

Differing colours of micronized fluorescent dusts were used consecutively during the study, whereby [a] indicates pink, [b] is green, [c] is red, [d] is blue, [e] is orange, and [f] is yellow. The first date for each dust replicate indicates the day that marked individuals were released, with the 3 subsequent days representing recapture days.

6.3.3 Sorting and Storage of *Culicoides*

Culicoides from the daily caches were examined for the presence of fluorescent dusts in the field, before being stored in 70% ethanol and later examined under a stereomicroscope at the Leahurst Veterinary School site. Marked individuals were counted and the recapture location and date recorded, before being further identified to species level and age-graded. The number of *Culicoides* trapped during the study at each location, as well as the numbers trapped during preliminary trapping, was determined by sub-sampling the catches, but, in the interests of time, the species composition was not recorded.

6.3.4 Data Analyses

The number and location of recaptured specimens was used to determine the mean distance travelled (MDT) by males and females, using the methods of Lillie *et al*. (1981). The number of recaptured *Culicoides* was corrected to account for unequal trapping areas and unequal trap density in each of the concentric distance bands. The proportion of the total trapping area occupied by each concentric ring was calculated (A_s / A_t), and multiplied by the total number of traps (T_t) used in order to determine the number of traps needed in each trapping area for equal trap density (Table 6.2). The number of midges recaptured at a given distance from the release site was then corrected by multiplying by the ratio of *Expected number of traps/Actual number of traps*.

$$MDT = \frac{\sum(Expected\ no.recovered\ x\ Distance)}{\sum Expected\ no.recovered}$$

Equation 6.1 Formula for the mean distance travelled (MDT)

Table 6.2 Determining the expected numbers of traps in each concentric ring. *Area of circle* is the area of the circle contained within the outer limit of the concentric ring. *Area of concentric ring (A_s)* is the area within the inner and outer limits of the ring. *Actual number of traps* is the number of traps within each concentric ring. *Expected number of traps*, $A_s / A_t \times T_t$, is the number of traps required in each concentric ring to achieve equal density in all rings; where T_t is the total number of traps and A_t is the total trapping area.

Radius of Concentric Ring (km)	**Area of circle (km^2) ^{Aft}**	**Area of Concentric Ring (km^2) ^{As}**	**Actual Number of Traps**	**Expected Number of Traps**
0-0.5	0.79	0.79	0	0.28
0.5-1.0	3.14	2.36	1	0.84
1.0-1.5	7.07	3.93	3	1.41
1.5-2.0	12.57	5.50	2	1.97
2.0-2.5	19.63	7.07	4	2.53
2.5-3.0	28.27	8.64	3	3.09
3.0-3.5	38.48	10.21	2	3.66
3.5-4.0	50.27	11.78	3	4.22
Total	**160.22**	**50.27^{At}**	**18^{Tt}**	**18**

6.4 Results

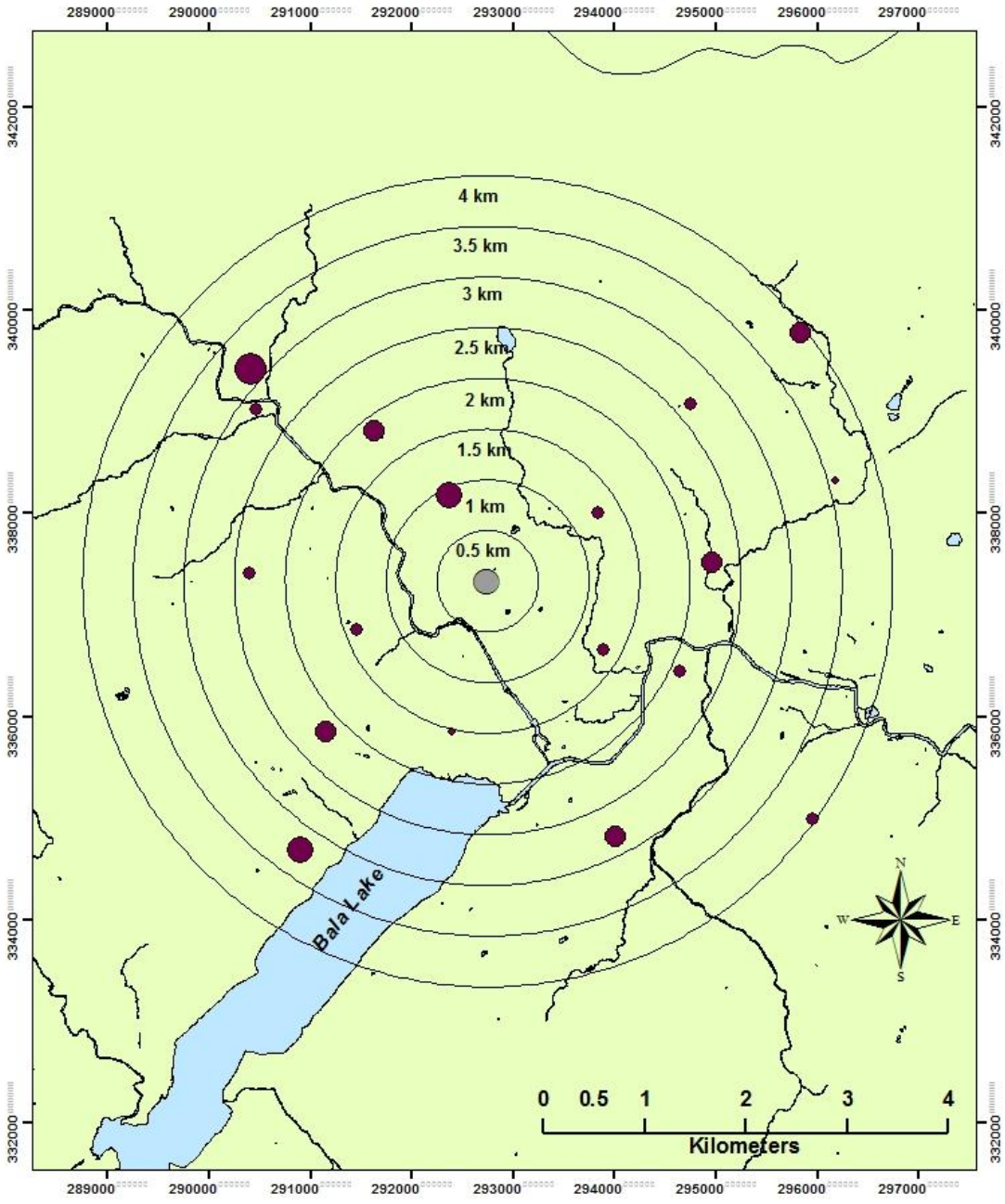
An estimated 10,177 marked *Culicoides* were released per day (61,062 during the total study). A total of 501,094 *Culicoides* were trapped in the recapture traps (Table 6.3), while the maximum catch per night varied on farms between 72 and 33,693 *Culicoides*. The spatial variation in overall catches between the farms can be seen in Figure 6.2.

Table 6.3 *Culicoides* trapped in the recapture traps in Bala. R indicated *Culicoides* marked with red fluorescent dust; B indicates *Culicoides* marked with blue fluorescent dust.

Farm ID	Maximum *Culicoides*	Mean *Culicoides*	Total *Culicoides*	Marked *Culicoides*
A1	33694	8861	175718	0
A6	2550	1162	19966	0
B1	2458	974	13544	0
B2	7494	1438	27330	3 R
B5	3100	538	9650	0
C1	1647	1317	17117	0
C3	7310	2143	40721	1 R
C4	5781	1848	16762	0
C5	4032	1650	23098	4 R + 2 B
C6	142	70	1283	0
D2	2835	1735	18144	0
D4	6016	1145	14881	2 B
D5	1161	367	8824	0
E1	2353	1435	24613	0
E3	72	40	756	0
F1	9979	3002	54043	0
F4	2802	913	19527	0
F6	2142	857	15117	0
Total	**95568**	**29496**	**501094**	**12**

A total of 12 (0.02%) marked *Culicoides* were recaptured, 8 were marked with red fluorescent dust and 4 were marked with blue (Table 6.3). Figure 6.3 shows the spatial distribution of the recaptured *Culicoides* by colour of dust. No recaptures were made of *Culicoides* marked with other colours of dust. Of the females, six were *C. obsoletus/scoticus*, two *C. dewulfi*, two *C. pulicaris* and one *C. festivipennis*. The

male was *C. obsoletus*. Figure 6.4 shows the spatial distribution of the recaptured midges, of each colour, by species.



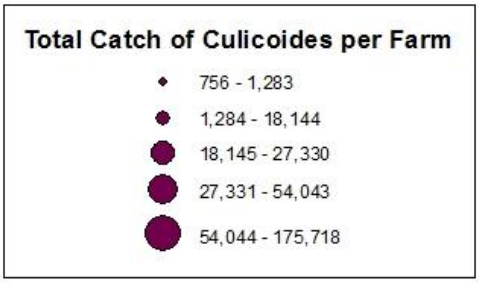


Figure 6.2 Spatial variation in the total trap catches of *Culicoides* on farms with OVI traps set to recapture released *Culicoides*. Grey point indicates release site.

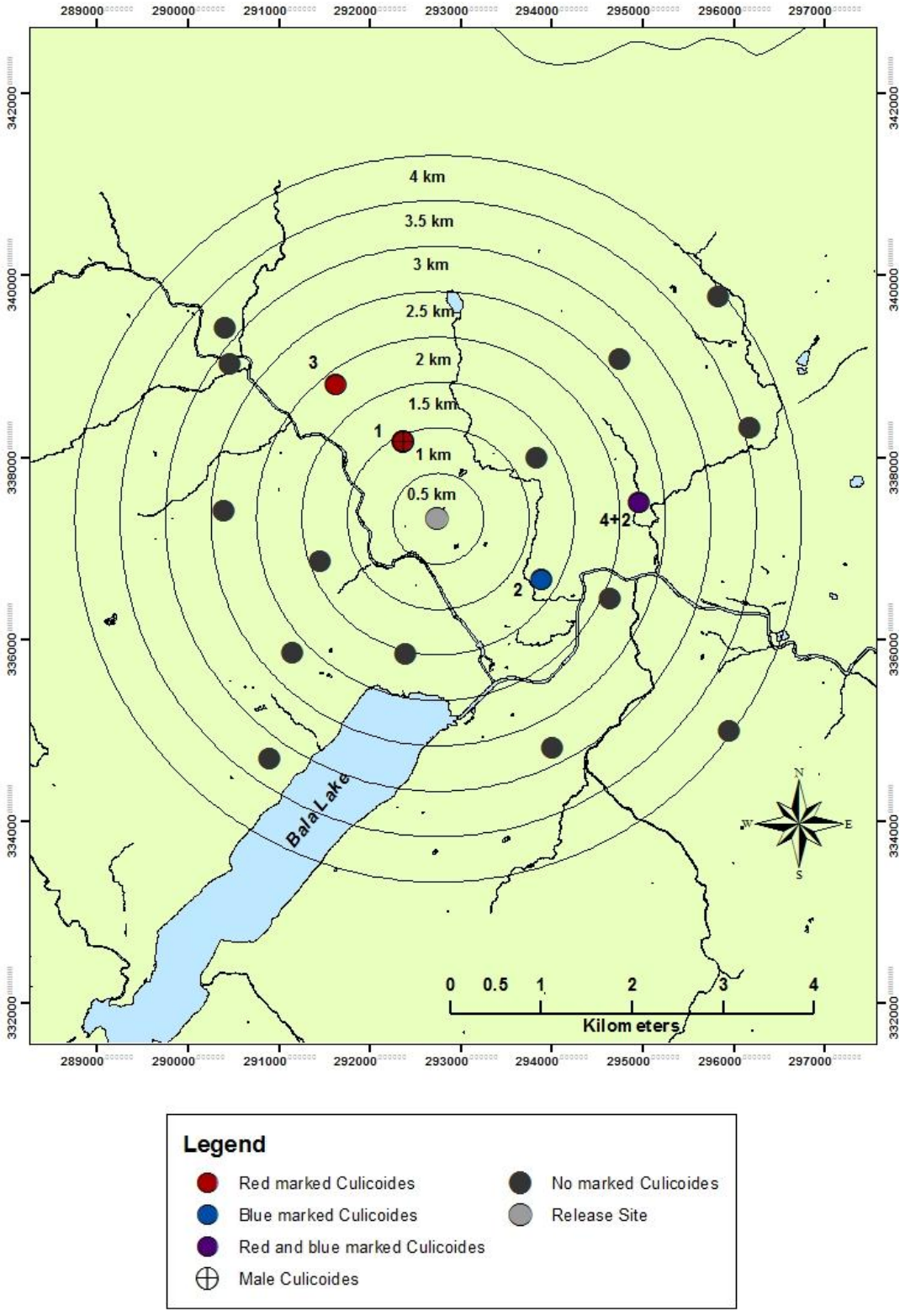


Figure 6.3 Spatial distribution of trapping farms surrounding the release farm, and the location of recaptured marked *Culicoides*. The numbers represent the number of *Culicoides* recaptured at each site.

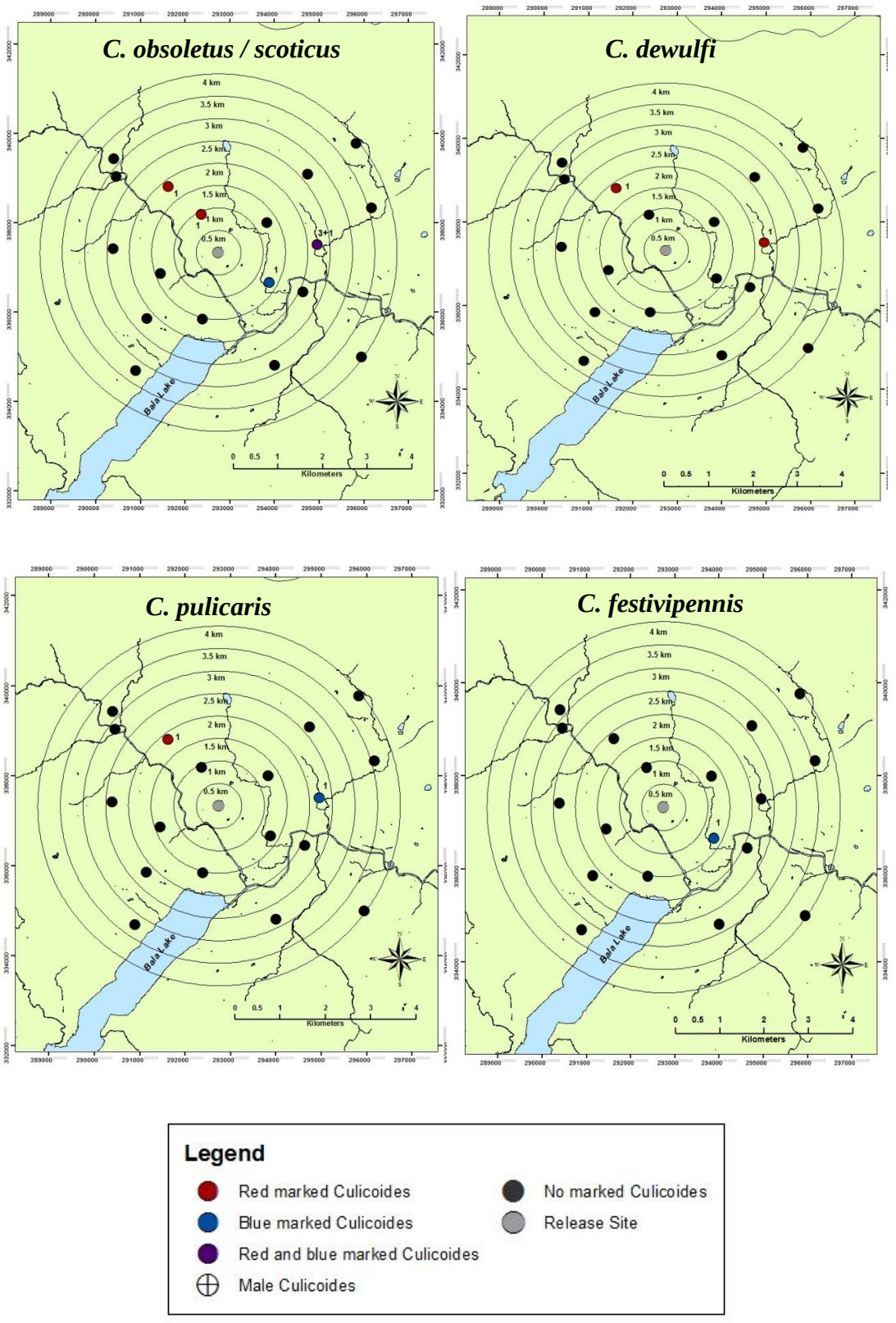


Figure 6.4 Spatial distribution of the species of *Culicoides* recaptured during the MRR. Numbers indicate the number of the species caught at each site.

The marked *Culicoides* were released on a farm with 4,000 sheep and 350 beef cattle present, at an altitude of 223 m. Recaptures were made on two farms with sheep and beef cattle present (550 and 30; and 500 and 20 respectively), one farm with solely sheep present (144), and one site with no animals, at altitudes of 287 m, 214 m, 235 m and 172 m respectively.

The majority of recaptures were 2-2.5 km from the release site and the most numerous recaptures were 2 nights post-release. No marked *Culicoides* were trapped more than 3 nights post-release, following each dusting replicate. Eleven females were recovered up to 2.5 km from the release site, with two of these females recovered at a distance of 1.5 km on the night of release. One marked male was recovered and was trapped two nights post-release at a distance of 1 km. No *Culicoides* were recaptured at distances greater than 2.5 km (Table 6.4).

The *Culicoides* dispersed to greater distances as the post-release time increased (Figure 6.5). Based on the corrected data, the *Culicoides* travelled a mean distance of 1.5 km during the release night (Table 6.4). The MDT increased to 1.79 km for two nights post release (2.15 km for females only) and 2.5 km for three nights post release. There were no *Culicoides* recaptured after three nights post release. A change in the rate of dispersal was noted as the time post release increased. The MDT for females over the first 24 hrs after release was 1.5 km. In the second 24 hrs, the MDT increased by 0.65 km and in the third 24 hours by 0.35 km.

The MDT for all *Culicoides* through the entire mark-release-recapture was 2.15 km. The MDT for males was 1 km, whereas for females it was 2.21 km. Table 6.3 shows the MDT by each species individually during the MRR experiment.

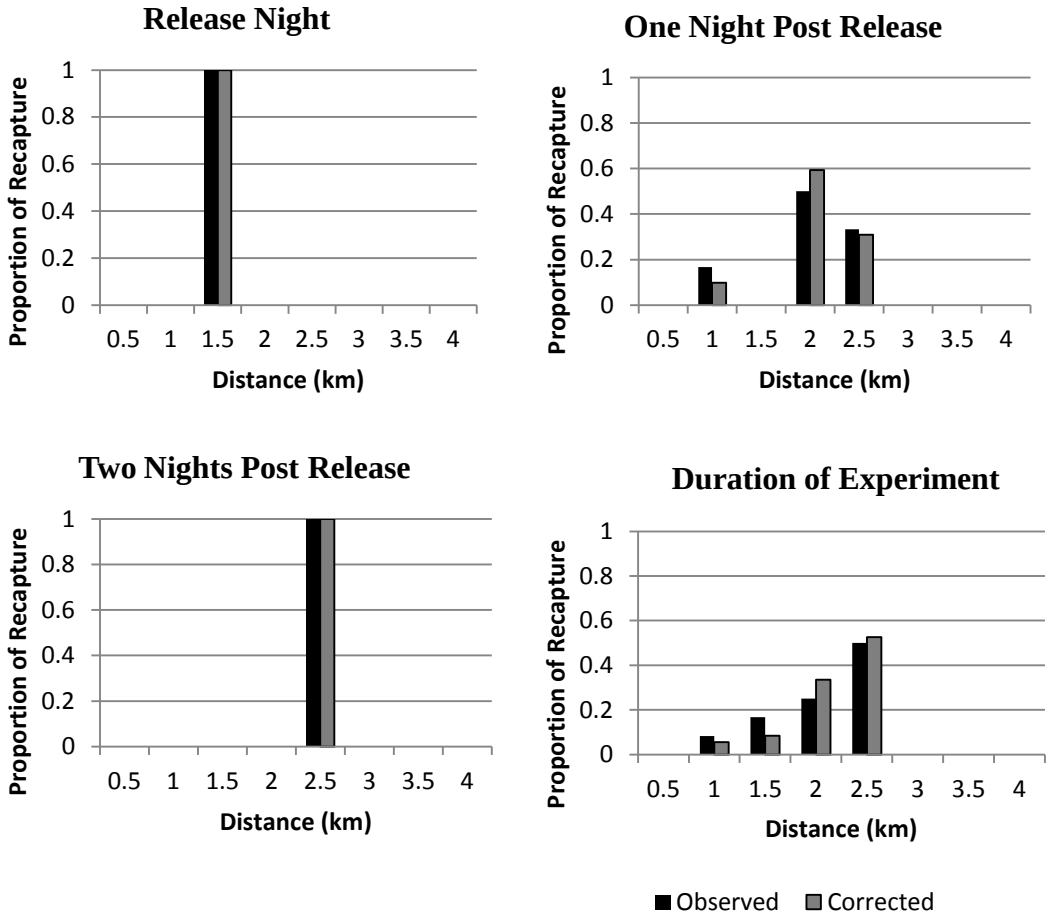


Figure 6.5 The observed and corrected data at three post-release times and pooled for the entire mark-release-recapture period

Table 6.4 Mean distance travelled (km) from release site by individual *Culicoides* species during a mark-release-recapture experiment

Species	**Nights Post-Release**			
	1	**2**	**3**	**Entire Experiment**
C. obsoletus	1.5	2.2	2.5	2.17
C. dewulfi	-	2	2.5	2.2
C. pulicaris	-	2.2	-	2.2
C. festivipennis	1.5	-	-	1.5
C. obsoletus Male	-	1	-	1
All *Culicoides*				
Female	1.5	2.15	2.5	2.21
Male & Female	1.5	1.79	2.5	2.15

6.5 Discussion

This study is the first to successfully demonstrate the dispersal of the Obsoletus Group members between farms. Kirkeby *et al*. (2013) marked and released 607 specimens of the Obsoletus Group on a cattle farm in Denmark yet were unsuccessful in recapturing members of this Group in any of the surrounding traps, highlighting a continued need for the dispersal of the main Palaearctic BT vector species to be investigated.

In other studies, less than 1% of the number of insects released in a MRR study is usually recovered (Johnson, 1969). Kirkeby *et al*. (2013) recaptured 0.75% of *Culicoides* released at sites away from the release point, all of which were Pulicaris Group species. Lillie *et al*. (1981) recaptured 0.5% of *C. variipennis* in Colorado, and 1.5% of *C. mississippiensis* when undertaking a MRR study in Florida (Lillie *et al.*, 1985). An exception to this was seen in the work of Brenner *et al*. (1984) where 13% of *C. mohave* were recovered in southern California, due to the lack of vegetation in the desert environment where the study took place. The landscape in Bala comprises a mixture of forest and field and is an undulating landscape with many natural barriers and sources of water, so low observed recapture rate was expected.

No other dispersal studies on *Culicoides* have been undertaken in such an undulating or vegetation-filled environment. Previous studies focus on dispersal in saltmarsh regions of Florida (Lillie *et al*., 1985), the desert of Southern California (Brenner *et al*.,1984), the South Platte River drainage system in Colorado (Lillie *et al*., 1981), and an open field-landscape in Denmark (Kirkeby *et al*., 2013). However we successfully highlight the dispersal of *Culicoides* between neighbouring farms in this environment.

Our data support the dispersal, or gradual movement, of *Culicoides* away from a release site. Movement of the *Culicoides* was neither unidirectional nor limited to a specific distance. The MDT of the *Culicoides* during the first release night is comparable to that seen for *C. mississippiensis* (Lillie *et al*., 1985), where most individuals were taken at up to 1.5 km at 24 hrs post release. *Culicoides* were not recaptured as far as the 4 km observed for *C. variipennis* over a 36 hr period in

Colorado (Lillie *et al*., 1981). Our estimates of flight range are based on small number of *Culicoides* recaptured and it is therefore not possible to determine if differences exist in flight distances between species. Although *C. festivipennis* was not found further than 1.5 km from the release site, this species is far less abundant than members of the Obsoletus Group, so we would expect to recapture very few. We are able to show that recaptured Obsoletus Group females, as well as *C. pulicaris*, are able to disperse a minimum of 2.5 km in 48hrs.

Data obtained from the MRR study show that *Culicoides* travel between farms in this region and therefore *Culicoides* captured on one farm do not necessarily originate from that farm. Such movement has disease control implications in terms of the vectorial movement of disease between farms. Hocking (1953) stated that the flight range of an insect can be used to determine the distance over which that insect may transmit a disease agent, and recommended treating an area equivalent to the square of the MDT for effective control following a single application. The MDT of the *Culicoides*, and the fact that they appear to freely move from farm to farm, even in such a heterogeneous landscape, highlights the unfeasibility of this method of control.

Previous studies have noted an abrupt decline in recapture following one night post-release (Bailey *et al.*, 1965; Hobbs *et al*., 1935). The change in the rate of dispersal as the time post-release increased is likely to be the result of multi-directional flight patterns, physiological changes, or environmental influences. Females would have been less likely to have been trapped as time elapsed, if the need for a bloodmeal had been fulfilled early on. Instead, they would be searching for an oviposition site rather than a host from which to take a blood meal. This decline in dispersal, or recapture, rates with time has previously been attributed to midges dispersing into areas of low trap density (Lillie *et al*., 1981), as well as mortality, behavioural changes, or a combination of these factors (Kettle, 1951).

The directions that the *Culicoides* took appear to be following the major rivers in Bala and are therefore likely to be related to the landscape. *Culicoides* did not appear to disperse towards farms with larger numbers of livestock present and indeed were trapped on one premises containing no livestock. The red-marked *Culicoides* recaptured on farms to the northwest of the release farm may have been aided by

wind dispersal, with the wind during the day of release heading north-westerly. Similarly the red-marked individuals trapped on a farm easterly from the release site at 2 days post-release may have been influenced by the easterly wind recorded a day following their release. The same cannot be said for the blue dust-marked individuals found in the east and south-east, with the wind heading northwest on the day of release before changing to the southwest, highlighting that these individuals flew upwind. These findings may be explained by Sedda *et al*. (2012) who considered that during the European BT outbreak, upwind midge flight may be a response to wind acting as a carrier of host semio-chemicals, while downwind movement of midges was due to wind transporting the midges themselves.

The recapture of a male *C. obsoletus* at a distance of 1 km from the release site was unexpected. Male recapture is rare in MRR studies with no *C. mohave* recaptured in southern California, despite the homogenous landscape (Brenner *et al*., 1984), and only 2 male *C. mississippiensis* recaptured 0.5 km from their release point by Lillie *et al*. (1985). The maximum distance travelled by male *Culicoides*, prior to this study, was by *C. variipennis* which travelled 0.8 km in Colorado (Lillie *et al*., 1981). The number of males trapped during entomological trapping regimes is generally less than 10% of the females trapped. When considering males do not take a bloodmeal, and therefore remain closer to breeding sites than females, it would be expected that fewer males would be recaptured in a MRR study.

The recaptures of marked individuals occured when maximum temperatures were greatest and humidity lowest during the trapping period, and rain did not hinder the recapture of marked individuals. Further work would need to be undertaken to determine what environmental factors affect dispersal rate and direction.

If the study was repeated, it would be advantageous to use more traps and have equal trapping density in each of the distance bands, reducing the need to transform the recapture data, and maximising the potential of trapping higher numbers of *Culicoides.* Higher recapture numbers would lead to more convincing evidence of the maximum flight distances of each species. Undertaking a species-specific PCR on the Obsoletus Group members recaptures may also provide evidence of differences in flight distances or preferences between the species.

Finally, with such low numbers of marked *Culicoides* identified, it would have been useful to test the sensitivity of identifying marked *Culicoides* from the catches prior to the field study. This could have been achieved by adding a known number of marked insects to differing catch sizes of unmarked *Culicoides* stored in ethanol, and determining how many of these maked individuals were identified by the researchers involved when sorting through those insects.

This study is the first to demonstrate the active dispersal of the *Culicoides* Obsoletus Group from farm to farm. Although the recapture rate was small, we have provided evidence that species of the Obsoletus Group are able to disperse 2.5 km or more, with males able to disperse to a distance of 1 km in 24 hrs. The results suggest that control measures applied at an infected farm will reduce risk of spread to neighbouring farms, as well as reducing transmission at the source farm itself.

CHAPTER SEVEN

MORPHOMETRIC DISCRIMINATION OF *C. OBSOLETUS* AND *C. SCOTICUS*

This work was undertaken as part of a collaborative project sponsored by the EDENext project (Biology and control of vector-borne infections in Europe). Work on this Chapter was undertaken at CIRAD, France, under the supervision of Drs. Claire Garros and Helene Guis.

The study was conceived and designed by Georgette Kluiters (GK), Claire Garros (CG) and Helene Guis (HG). *Culicoides* were collected or supplied by GK and Simon Carpenter (Pirbright Laboratory, Surrey) from the UK, CG (CIRAD, Montpellier) from France, and Nonito Pages (CReSA, Barcellona) in Spain. GK dissected and slide mounted all *Culicoides* before GK and Laetitia Gardes (CIRAD) undertook PCR identifications. GK undertook morphometric measurements on slide-mounted *Culicoides* at the Pirbright Laboratory, Surrey. GK undertook analyses on the data at the University of Liverpool.

7.1 Abstract

Following the introduction of bluetongue into northern Europe in 2006, members of the Obsoletus Group of *Culicoides* biting midges have been implicated as vectors of the disease. Identification of two (*C. obsoletus* and *C. scoticus*) of the four members of this group is considered difficult, if not impossible, when undertaken morphologically. Nolan *et al*. (2007) concluded that the distinction of *C. obsoletus* and *C. scoticus* requires molecular analysis. In many cases female specimens are grouped as an entity in entomological surveys, but species specific identification may be necessary to determine competent vectors of the virus, understand their life history characteristics and assess their relative abundances.

According to Delecolle (1985), *C. obsoletus* and *C. scoticus* females can be distinguished based on the length of the larger of their two functional spermathecae, but this finding was not observed in a similar study undertaken by Pages & Monteys (2005). Augot *et al*. (2010) explored 15 morphometric variables to distinguish the species and found that females of *C. obsoletus* and *C. scoticus* can be accurately distinguished based on the width between their chitinous plates, the length and width of their larger spermatheca and the length of their smaller spermatheca. Nielsen & Kristensen (2011) stated that these methods were time consuming however, and showed how it was possible to separate the females of the four species using a quick microscope method, by combining the shape of the third segment of the maxillary palp and the number and location of hairs on the first abdominal tergite.

The main problem with all of the above studies is that they were undertaken on small populations of *Culicoides* from within one region, therefore these techniques may not be able to be extrapolated to *Culicoides* collected in different countries or indeed during different seasons.

Here we collected Obsoletus Group females from the UK, France and Spain, with two differing geographical locations sampled per country. A total of 759 *C. obsoletus* and *C. scoticus* individuals were identified using the cytochrome oxidase I gene, before 15 morphometric measurements were taken from the head, wings and abdomen of slide-mounted specimens. Multivariate analyses were performed on the morphometric measurements to identify and validate whether a combination of

variables could lead to accurate species identification. Measurements were also compared between the start, middle and end of the vector season on *Culicoides* collected in France to determine whether seasonal variation exists in any of the measurements.

Our results suggest that female *C. obsoletus* and *C. scoticus* individuals can be separated under a stereomicroscope based on abdominal measurements. Seasonal variation in the size of these species, and therefore their morphometric measurements was observed for both head and wing measurements, but not for the abdomen. Geographical variation in the size of individual *Culicoides* was also observed and is likely to be related to temperature at the trapping sites, with smaller *Culicoides* trapped further south. Although we show that the length and width of the spermathecae can be used to differentiate between the species, this can be a time-consuming process and we therefore only recommend undertaking this on a sub-sample of individuals.

7.2 Introduction

Following the introduction of bluetongue into northern Europe in 2006, members of the Obsoletus Group of *Culicoides* biting midges have been implicated as vectors of the disease. The group includes the species *C. obsoletus*, *C. scoticus*, *C. dewulfi* and *C. chiopterus*, and identification of two (*C. obsoletus* and *C. scoticus*) of the four members of this group is considered difficult, if not impossible, when undertaken morphologically. Nolan *et al*. (2007) concluded that the distinction of *C. obsoletus* and *C. scoticus* requires molecular analysis.

Advances have been made in molecular identification of this species group, and three different systems of identification have been established. Cytochrome oxidase subunit I barcoding (COI; Nolan *et al*., 2007), and sequencing of the internal transcribed spacer 1 (ITS-1; Cetre-Sossah *et al*., 2004) and the internal transcribed spacer 2 genes (ITS-2; Gomulski *et al*., 2006) provide the basis for the established methods.

Molecular identification can be expensive and in many cases female Obsoletus Group specimens are grouped as an entity in entomological surveys. However, species specific identification is necessary to identify competent vectors of the virus, understand their life history characteristics and assess their relative abundances.

7.2.1 Problems with Morphological Identification

Taxonomy of the genus *Culicoides* has relied mainly on morphological identification e.g. pigmentation patterning of the wings, length and shape of antennal segments, characteristics of the genitalia in males, distribution of the sensillae on the antennae, and the number and size of the spermathecae in females (Campbell & Pelham-Clinton, 1960; Wirth & Hubert, 1989; Delecolle, 1985; Boorman, 1993; Rawlings, 1996; Boorman & Hagan, 2007).

In the Obsoletus Group, it is difficult to discriminate between the closely related species. Although not involved in disease transmission, the males of all four species can be accurately identified under a microscope on the basis of the shape of their hypopygium (Figure 7.1; Campbell & Pelham-Clinton, 1960). The females of *C.*

dewulfi and *C. chiopterus* can also be differentiated. *C. chiopterus* is a smaller species than the others in the group and the wings of both it, and *C. dewulfi*, are paler in their markings than for *C. obsoletus* and *C. scoticus*, with *C. chiopterus* in particular almost devoid of colour. A pale spot at the distal end of the wing, as well as the pronounced difference in size between the spermatheca seen in *C. dewulfi* specimens further differentiate the two species. *C. obsoletus* and *C. scoticus* however, show no distinguishing markings on their wings to aid in their differentiation. Their spermathecae are also of similar sizes.

Figure 7.1 The shape of the hypopygium in males of a) *C. obsoletus*; b) *C. scoticus*; c) *C. chiopterus*; and d) *C. dewulfi*. [Image taken from Nielsen & Kristensen, 2011]

7.2.2 Previous Studies Investigating Morphometric Identification

Delecolle (1985) proposed that *C. obsoletus* and *C. scoticus* could be differentiated based on the length of their largest spermatheca (BSL; *C. obsoletus* ≤ 59μm; *C. scoticus* ≥ 61μm) and the morphology of their chitinous genital plate (see Figure 7.2

for the location of these features), suggesting that, for *C. scoticus*, the margins of the 2 chitinous plates that face each other, rounding the genital opening, converge posteriorly ending in one conspicuous sharp point, whereas for *C. obsoletus* the margins are parallel and end in an inconspicuous small point (Figure 7.3). These observations were, however, based on 10 *C. obsoletus* individuals (5 from Strasbourg and 5 from Baldenheim) and 5 *C. scoticus* (Strasbourg). Delecolle goes on to state that the antennae, palps and wings are identical for the 2 species.

Figure 7.2 Lower third of the abdomen of a female *Culicoides*. a_1 & a_2) functional spermathecae; a_3) rudimentary spermatheca; b) chitinous ring; c) abdominal sclerites; d) genital opening; e) chitinous plates; f) cerci; VII, VIII, IX and X correspond to the seventh, eighth, ninth and tenth abdominal segments respectively (reproduced from Delecolle, 1985).

Figure 7.3. Lower part of the abdomen, set ventrally, of a) a female *C. scoticus*, and b) *C. obsoletus*, showing the chitinous plate morphology (reproduced from Pages & Monteys, 2005); and chitinous plates surrounding the genital opening of female *C. scoticus* (c) and *C. obsoletus* (d) according to Delecolle (1985).

Such morphological variations were further investigated by Pages & Monteys (2005) who found that the measurement of the size of the spermathecae was not sufficient for precise identification of specimens from Spain, and that there was variation in the chitinous plate morphology within species, such that 15% of samples could not be identified.

Augot *et al*. (2010) undertook a comprehensive analysis of 15 morphometric variables from the head, abdomen and wings of *C. obsoletus* and *C. scoticus* in order to determine whether morphometric identification techniques could be employed to distinguish between the species. Principal component analysis highlighted that females of the species can be accurately distinguished based on four variables: width between the chitinous plates, length and width of the first spermatheca, and length of the second spermatheca.

As morphometric identifications (as identified by Augot *et al*., 2010) generally require insects to be slide-mounted, Nielsen & Kristensen (2011) investigated quick morphological determinants of the species. They state that the Obsoletus Group

members can be differentiated using a stereomicroscope by combining the shape of the third segment of the maxillary palp and the number and location of hairs on the first abdominal tergit. The authors also go on to say that the morphometric measurements of antenna ratio (length of the apical 5 segments divided by the length of the 8 basal segments) and palpal ratio (length of maxillary segment 3 divided by greatest width of that segment) are also able to significantly differentiate between *C. obsoletus* and *C. scoticus*, while wing size combined with the form of the 3rd palpal segment is a valid character to separate the four species.

7.2.3 How can Morphological Identification of the Obsoletus Group Members be Improved?

The previous studies have all been undertaken on small sample sizes of *Culicoides*, or specimens from only one geographical location. In these studies, no mention was made regarding geographical variation in the morphology of *C. obsoletus* or *C. scoticus*. Such techniques can therefore not be extrapolated to identify Obsoletus Group members from other countries, and indeed the use of such techniques may lead to misidentification of specimens.

Similarly, none of the studies take into account the variation in the size of *Culicoides* at different time-points during the vector season. Previous studies on *Culicoides* life history characteristics have highlighted the influence of air temperature on wing length, with individuals exhibiting larger wing sizes in cooler temperatures in February than observed in warmer temperatures during July (Smith & Mullens, 2003).

7.2.4 Research Justification

Different molecular methods have recently become widely used in the identification of the Obsoletus Group members. Currently, three different methods have been established for identifying *Culicoides* species: barcoding of the cytochrome oxidase subunit I (COI -Nolan *et al*., 2007) and sequencing of the nuclear internal transcribed spacer 1 (ITS-1 – Cetre-Sossah *et al*., 2004) and 2 (Gomulski *et al*., 2006) genes.

Although these methods are widely available, the difficulty in differentiating between *C. obsoletus* and *C. scoticus* members morphologically means these species are still often grouped together by researchers without access to molecular identification, or those who do not undertake the methods due to the costs involved.

The main obstacle encountered by the methods of morphological identification, suggested thus far, are that they have each only been tested on *Culicoides* from within one region or country and therefore do not take into account the variations observed between individual species between countries. The *Culicoides* used in such studies have also only been collected from one time point during the year or have been grouped together from seasonal surveillance schemes, so seasonal variation in *Culicoides* size may well have an effect on the use of such techniques in the field.

This study aimed to determine whether morphological or morphometric identification techniques can be applied to *C. obsoletus* and *C. scoticus* individuals trapped in different geographical regions and at different time periods during the vector season.

Specific objectives included exploring the morphological identification methods that were able to differentiate *C. obsoletus* and *C. scoticus* as determined by Augot *et al.* (2010), Delecolle (1965), Nielsen & Christensen (2011), and Pages & Monteys (2005). Each identification method was assessed on *Culicoides* from two differing geographical regions in three different countries (UK, France and Spain). Additionally, the methods were to be employed on *Culicoides* trapped at different time periods throughout a trapping season. Finally, multivariate analyses were to be undertaken to determine whether a combination of variables could be used to discriminate between the two species.

The *Culicoides* specimens used in this study were collected by myself during the fieldwork in Bala in Chapter 6, or during entomological surveys undertaken by the Pirbright Laboratory, Surrey (UK), on a farm in Devon; CIRAD in Montpellier (France), on farms in Calvados and Landes; and by CReSA in Barcellona (Spain), on farms in Avia and Caldes de Malavella. Specimens were provided stored in 70% ethanol.

7.3 Materials and Methods

7.3.1 *Culicoides* Collection

Insects were sampled from two sites in each of the UK, France and Spain between June 2009 and November 2011 (Figure 7.4). In the UK, the first site was a farm in the Bala region of north Wales, with *Culicoides* collected on the 14th July 2011 using an OVI trap. The second site was a farm in Blackmoor Gate, Devon (4th November 2011), where the *Culicoides* were collected using a UV-LED CDC trap (John W. Hock Company, Gainesville, FL, USA). In France, the *Culicoides* from both sites were trapped using an OVI trap. The first site was located on a farm in Calvados, north-western France, and *Culicoides* were taken from 3 trapping periods to sample the start (27th April 2010), middle (12th July 2010) and end (3rd November 2010) of a trapping season. The *Culicoides* from the second site in Landes, south-western France, were trapped on the 26th April 2010. In Spain, OVI traps were used to sample *Culicoides* from Caldes de Malavella (north-eastern Spain) on 16th June 2011, and Avia (northern Spain) on 21st May 2009. *Culicoides* were stored in 70% ethanol prior to morphological and molecular analyses.

7.3.2 Specimen Identification and Mounting

Culicoides were separated from other insects according to their wing characteristics (Mellor *et al*., 2000) using a stereomicroscope, before being identified to the species level. Females belonging to the Obsoletus Complex (*C. obsoletus* and *C. scoticus*) were differentiated from *C. dewulfi* and *C. chiopterus* on the basis of spermathecae size and wing pattern, before a total of 994 specimens were randomly selected from the trapping sites and dates. The individual females were dissected on a slide under Canada Balsam. The head (dorsal side up), wings and posterior abdominal segment (ventral side up) of each of these specimens were subsequently mounted on the slide under three separate cover slips (Figure 7.5). The remaining thorax, legs and anterior abdomen were stored in 75% ethanol for DNA analysis.

Figure 7.4 Location of field-trapping sites for *Culicoides* samples used for morphometric and molecular identification, in France, Spain and the UK. a) Bala; b) Devon; c) Calvados; d) Landes; e) Avia; f) Caldes de Malavella.

Figure 7.5 Location and orientation of slide-mounted segments of *Culicoides*. a) wing; b) head positioned dorsal-side upmost with antennae extended to the right; and c) posterior end of the abdomen situated ventral-side upmost.

7.3.3 Molecular Identification

Prior to DNA extraction, the *Culicoides* were individually removed from their ethanol-filled storage vials and placed on absorbent paper, to remove any remaining ethanol. *Culicoides* were added to a Macherey Nagel round well block with 500 µL of 5% Chelex® 100 resin (Bio- Rad Laboratories, Inc., Hercules, CA, U.S.A.). Lysis was performed using 3mm Qiagen tungsten carbide beads in two cycles of 30 agitations per second for 30 seconds. The beads were removed, and extraction of DNA was achieved by incubating the *Culicoides* at 56°C for 1 hour (700 rpm), then 30 minutes at 96°C (650 rpm) in the 500µL Chelex resin suspension, using an Eppendorf Thermomixer Compact.

Primers and PCR amplifications conditions were as described by Nolan *et al*. (2007), with four forward primers:

- *C. obsoletus:* UOAobsF (5'-TGCAGGAGCTTCTGTAGATTTG-3');
- *C. scoticus:* UOAscoF (5'-ACCGGCATAACTTTTGATCG-3');
- *C. chiopterus:* UOAchiF (5'-TACCGCCCTCTATCACCCTA-3');
- *C. dewulfi:* UOAdewF (5'-ATACTAGGAGCGCCCGACAT-3'); and

one reverse primer:

- C1-N-2191 (5'-CAGGTAAAATTAAAATATAAACTTCTGG-3') (Dallas *et al*., 2003).

The polymerase chain reactions (PCR) of the mitochondrial COI gene were performed in a total volume of 25 µL, containing 2.5 µL buffer, 0.2 µm of 25 µM dNTPs, 0.5 µL of each 10 µM forward primer, 0.5 µm of the 10 µM reverse primer, 18.5 µL H_2O and 0.25 µL 5 u/µL Taq polymerase. The PCR reaction was performed in a PTC-100 Cycler (MJ Research, Inc., Montreal, QC, Canada) under the following conditions: an initial denaturation step at 92 °C for 2 min 15 s, followed by 30 cycles of 92 °C for 15 s, 61°C for 15 s, 72 °C for 30 s, and ending with a final elongation step at 72 °C for 1 min. Results were visualized on a 1% agarose gel after 50 min electrophoresis at 110 V in 0.5× TAE (Tris-acetate-EDTA) buffer, using gel red staining at 1:20,000.

7.3.4 Morphological Measurements

Slide-mounted specimens were observed under a Nikon Alphaphot-2 YS2 compound light microscope (Nikon Instruments, Europe) with a Q Imaging (QI CAM) camera attachment, and measurements were taken using Image-Pro Plus software (Media Cybernetics Inc., Rockville, USA). Morphometric measurements were taken from the head, wings and abdomen of individuals. Fifteen variables were recorded and eight ratios were determined from the variables, based on measurements that had been significant in previous literature.

7.3.4.1 Head Measurements

From the maxillary palps, the length and width of the 3rd palpal segment were measured, and the palpal ratio calculated (length/width). From the antennae, the combined length of flagellomeres 10 and 11 were determined (Figure 7.6), along with the combined length of the five apical flagellar segments and eight basal flagellar segments. The measurements were used to determine the antenna ratio and flagella ratios.

Figure 7.6 Morphometric measurements of the head of *Culicoides*. Where a) is length of eight basal flagellar segments of the antenna; b) is the length of the five apical flagellar segments of the antenna; c) is the length of flagellomere 10; d) is the length of flagellomere 11; e) is the length of the 3rd palpal segment; and f) is the width of the 3rd palpal segment.

7.3.4.2 Wing Measurements

The wing length from arculus to tip, costa length, and width of the wing (from the location of the second radial cell to the base of vein Cu1) were individually measured (Figure 7.7). These measurements were used to determine the wing ratio and costa ratio.

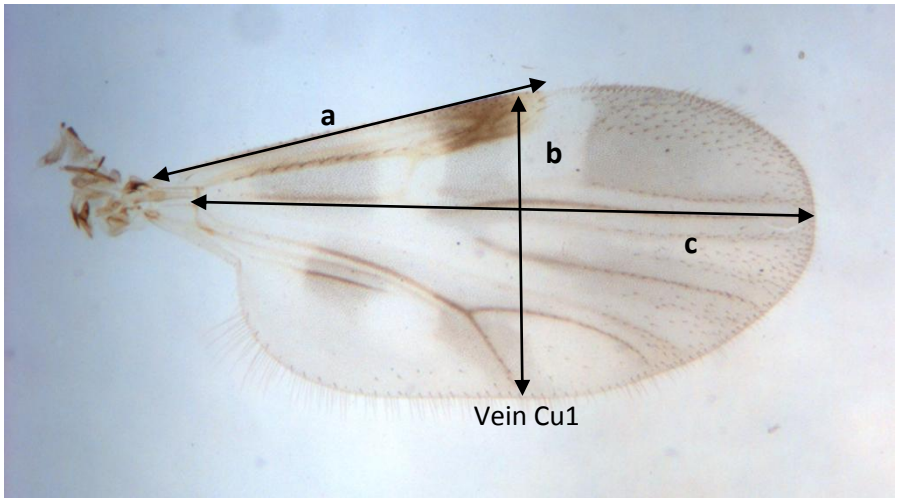


Figure 7.7 Morphometric measurements of the wing of *Culicoides*. Where a) is the costa length; b) is the width of the wing; and c) is the length of the wing (arculus to tip).

7.3.4.3 Abdominal Measurements

On the posterior part of the abdomen, the length and width of both spermathecae were measured along with the length and width between the chitinous plates surrounding the genital opening (Figure 7.8). The spermatheca ratio and the chitinous plate ratio were calculated from these measurements.

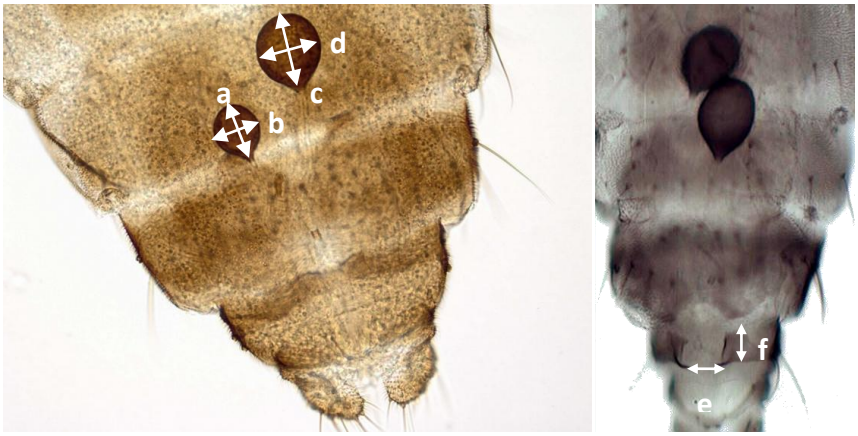


Figure 7.8 Morphometric measurements of the abdomen of *Culicoides*. Where a) is the length of spermatheca 1; b) is the width of spermatheca 1; c) is the length of spermatheca 2; d) is the width of spermatheca 2; e) is the length between the chitinous plates; and f) is the width of the chitinous plates.

7.3.5 Statistical Analyses

All descriptive statistical analyses were preformed in Minitab 16 Statistical Software. Statistical differences between the measurements of *C. obsoletus* and *C. scoticus* taken in every region, between regions, and overall, were determined using the non-parametric Mann Whitney test and an adjustment was made for multiple comparisons, using the Bonferroni correction.

Eight additional variables were also calculated as ratios of the head, wing and spermatheca measurements. The antenna ratio (total length of 5 apical segments (11-15)/ total length of 8 basal segments (3-10)) and flagella ratio (length of flagellomere 11/length of flagellomere 10) were calculated from the head measurements. The wing measurements were used to determine the wing ratio (wing length/width) and costa ratio (costa length/wing width). While the spermatheca ratio (spermatheca length/spermatheca width) was determined for both the larger and smaller spermathecae, and the chitinous plate ratio (length between chitinous plates/width of chitinous plates) was calculated using the abdominal measurements.

Measurements of each variable were evaluated using the coefficient of variation (CV = SD/$\overline{X}$ ×100) and the coefficient of difference (CD = ($\overline{X}_A - \overline{X}_B$) / ($SD_A + SD_B$) for a given variable measured in two groups of individuals, A and B). CV describes the

data heterogeneity whereas CD is linked to the degree of separation between two distributions. Mayr *et al*. (1953) consider 1.28 as the CD critical threshold over which subspecies can be distinguished.

Principal component analysis (PCA) was performed in R 2.10.0 (R Development Core Team, 2009), and used to explore the correlation structure between variables and determine those variables that account for the greatest variance.

7.4 Results

7.4.1 Molecular Analyses

A total of 819 *Culicoides* were identified using the COI gene. Of these, 410 were *C. obsoletus*, 348 were *C. scoticus* and 61 were *C. dewulfi* / *C. chiopterus*.

Molecular identification was possible for 126 (out of 150) *Culicoides* samples from Bala, of these 48 were *C. obsoletus*, 69 were *C. scoticus* and a further 9 were *C. dewulfi*. For the Devon samples, 104 (out of 106) were identified molecularly, with 47 *C. obsoletus*, 48 *C. scoticus* and a further 4 of each of *C. dewulfi* and *C. chiopterus*.

Of the Spanish samples, 24 *C. obsoletus*, and 52 *C. scoticus* were identified in the 77 samples from Avia. With the samples from Caldes de Malavella, 56 *C. obsoletus*, 40 *C. scoticus* and 1 *C. dewulfi* were obtained from the initial 103 samples.

From the French samples, 5 *C. obsoletus*, 9 *C. scoticus* and 2 *C. dewulfi* were initially identified from a total of 91 samples from Landes. A second batch of samples from this location were then analysed to increase numbers. From the second batch, 60 *C. obsoletus* and 10 *C. scoticus* were identified from 98 specimens. From the start of the season in Calvados, 21 *C. obsoletus*, 83 *C. scoticus* and 5 *C. dewulfi* were obtained from 115 samples. In the middle of the season, 40 *C. obsoletus*, 23 *C. scoticus* and 36 *C. dewulfi* were obtained from 124 samples. While at the end of the season, 109 *C. obsoletus* and 14 *C. scoticus* were obtained from 130 samples.

None of the *C. dewulfi* or *C. chiopterus* samples identified molecularly were used to take morphometric measurements.

7.4.2 Morphometric Analyses

7.4.2.1 Descriptive Statistics

Not all morphometric measurements could be undertaken on every sample identified molecularly. Among the 759 *C. obsoletus* and *C. scoticus* individuals, four *C.*

obsoletus and three *C. scoticus* from the UK had damaged flagellae, six *C. obsoletus* from Spain and four *C. scoticus* from France also had damaged flagellae, while one *C. obsoletus* from France had maxillary palp damage (Figure 7.9). The morphometric measurements for these *Culicoides* were not taken from the regions that were damaged, but all other measurements were collected.

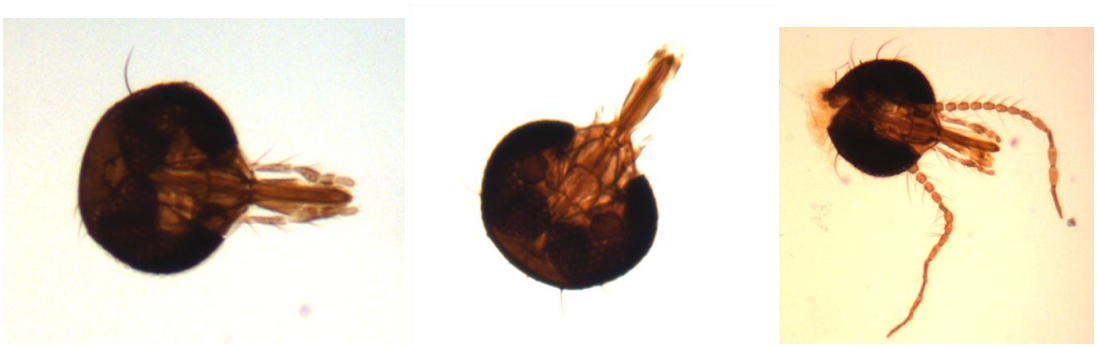


Figure 7.9 Damaged *Culicoides* individuals that had been slide mounted for morphometric measurements to be taken. a) missing antennae; b) missing maxillary palps; and c) damaged antennae.

The mean and standard deviation of all of the *C. obsoletus* and *C. scoticus* measurements can be seen in Table 7.1. Table 7.2 presents CD and CV values for each characteristic. CD values ranged from 0.02 (third palp length) to 4.30 (length of smaller spermatheca). Four variables (length and width of larger spermatheca, and length and width of smaller spermatheca) exhibited CD values over 1.28. CV values for *C. obsoletus* ranged from 2.23 (costa) to 47.02 (wing width), while for *C. scoticus* they ranged from 2.38 (costa ratio in *C. obsoletus*) to 31.23 (chitinous plate ratio). Clear differences can be seen in the means of the four characteristics that showed CD values greater than 1.28.

The Pearson's *r* correlation matrix of the 15 morphometric characteristics, used for the main multivariate analyses, exhibited a high degree of correlation, even when the *P* value was modified following Bonferroni correction ($P \leq 0.0004$; Table 7.3). Most variables exhibited positive correlation, with the strongest positive correlations between the spermathecae measurements ($r > 0.80$).

Table 7.1 Descriptive statistics of morphological characteristics in two members of the *Culicoides* Obsoletus Group (*C. obsoletus* and *C. scoticus*) from 759 *Culicoides* trapped in the UK, France and Spain. Units are expressed in µm, except for wing measurements which are in mm.

Parameter	***C. obsoletus***		***C. scoticus***	
	Mean	**SD**	**Mean**	**SD**
Wing Length	1.27	0.16	1.34	0.15
Wing Width	0.56	0.26	0.58	0.06
Costa Length	0.80	0.10	0.83	0.10
Wing Ratio	2.32	0.15	2.33	0.06
Costa Ratio	1.59	0.04	1.61	0.04
3rd Palp Length	51.94	6.95	51.67	7.08
3rd Palp Width	21.47	3.35	17.87	2.92
Palpal Ratio	2.46	0.39	2.96	0.61
Flagella 10 Length	37.12	3.61	35.89	3.72
Flagella 11 Length	49.32	5.32	50.33	5.10
5 Apical Segment Length	298.30	26.52	299.85	25.36
8 Basal Segment Length	271.94	23.76	259.02	20.94
Flagella Ratio	1.10	0.05	1.16	0.07
Segment Ratio	1.33	0.11	1.41	0.14
Larger Spermatheca Length	47.89	1.74	61.64	2.18
Larger Spermatheca Width	33.26	1.47	41.19	2.48
Smaller Spermatheca Length	47.29	1.31	61.03	1.88
Smaller Spermatheca Width	32.83	1.59	40.35	2.80
Larger Spermatheca Ratio	1.44	0.07	1.50	0.10
Smaller Spermatheca Ratio	1.44	0.07	1.52	0.11
Length between Chitinous Plates	11.91	1.60	20.25	5.84
Width of Chitinous Plates	18.31	2.08	20.68	2.73
Chitinous Plate Ratio	0.66	0.10	0.99	0.31

Table 7.2 The coefficient of variation (CV) and Mayr's coefficient of difference (CD) for 15 measurements and 8 ratios of morphometric parameters in two *Culicoides* species (*C. obsoletus* and *C. scoticus*). The 759 *Culicoides* were trapped in the UK, France and Spain.

Parameter	**Coefficient of Variation (CV)**		**CD**
	C. obsoletus	***C. scoticus***	
Wing Length	12.26	11.32	0.22
Wing Width	47.02	11.05	0.04
Costa Length	12.8	12.36	0.15
Wing Ratio	6.35	2.6	0.05
Costa Ratio	2.23	2.38	0.35
3rd Palp Length	13.39	13.7	0.02
3rd Palp Width	15.61	16.32	0.57
Palpal Ratio	15.68	20.42	0.51
Flagella 10 Length	9.72	10.37	0.17
Flagella 11 Length	10.78	10.12	0.10
5 Apical Segment Length	8.89	8.46	0.03
8 Basal Segment Length	8.74	8.08	0.29
Flagella Ratio	4.62	6.24	0.50
Segment Ratio	8.06	9.96	0.31
Larger Spermatheca Length	3.63	3.54	3.51
Larger Spermatheca Width	4.43	6.03	2.00
Smaller Spermatheca Length	2.77	3.09	4.30
Smaller Spermatheca Width	4.84	6.93	1.72
Larger Spermatheca Ratio	4.84	6.66	0.35
Smaller Spermatheca Ratio	4.87	7.37	0.42
Length between Chitinous Plates	13.41	28.85	1.12
Width of Chitinous Plates	11.37	13.18	0.49
Chitinous Plate Ratio	15.86	31.23	0.81

Table 7.3 Correlation matrix of the 15 morphometric characteristics of *C. obsoletus* and *C. scoticus*

	Costa Length	Wing		Flagella Segments				3rd Palp		Chitinous Plate		Shorter Spermatheca		Larger spermatheca Width
		width	length	8Basal Length	5Apical Length	11 Length	10 Length	Width	Length	Width	Length between	Width	Length	
Larger Spermatheca Length	0.212*	0.062	0.281*	-0.2*	0.086	0.155*	-0.102	-0.479*	0.056	0.462*	0.71*	0.849*	0.945*	0.881*
Larger Spermatheca Width	0.257*	0.078	0.32*	-0.14*	0.112	0.163*	-0.063	-0.435*	0.068	0.472*	0.678*	0.786*	0.886*	
Smaller Spermatheca Length	0.201*	0.06	0.269*	-0.205*	0.091	0.15*	-0.1	-0.486*	0.058	0.461*	0.701*	0.856*		
Smaller Spermatheca Width	0.26*	0.082	0.313*	-0.151*	0.11	0.17*	-0.072	-0.427*	0.061	0.442*	0.614*			
Length between Chitinous Plates	0.237*	0.078	0.279*	-0.153*	0.069	0.113	-0.082	-0.371*	0.029	0.387*				
Chitinous Plate Width	0.284*	0.105	0.308*	0.006	0.113	0.179*	0.023	-0.247*	0.09					
3rd Palp Length	0.207*	0.079	0.223*	0.472*	0.49*	0.45*	0.426*	0.197*						
3rd Palp Width	-0.045	-0.004	-0.064	0.389*	0.27*	0.196*	0.289*							
Flagella 10 Length	0.214*	0.023	0.207*	0.774*	0.701*	0.589*								
Flagella 11 Length	0.296*	0.053	0.315*	0.687*	0.869*									
5 Apical Segment Length	0.302*	0.072	0.315*	0.797*										
8 Basal Segment Length	0.271*	0.08	0.262*											
Wing length	0.983*	0.314*												
Wing width	0.318*													

Significance, determined using the Bonferroni correction, is given where $*P \leq 0.0004$

Table 7.4 shows the Pearsons *r* correlation matrix (with Bonferroni correction) for the eight ratios derived from the morphometric measurements. Almost all of the ratios displayed significant levels of correlation ($P \leq 0.002$) and were positively correlated. The ratios showed weaker correlation than the morphometric measurements in Table 7.3.

Table 7.4 Correlation matrix of the 8 ratios derived from morphometric characteristics of *C. obsoletus* and *C. scoticus*

	Larger Spermatheca Ratio	Smaller Spermatheca Ratio	Chitinous Plate Ratio	Palpal Ratio	Flagella Ratio	Segment Ratio	Wing Ratio
Smaller Spermatheca Ratio	0.083						
Chitinous Plate Ratio	0.193*	0.271*					
Palpal Ratio	0.161*	0.171*	0.274*				
Flagella Ratio	0.199*	0.184*	0.311*	0.179*			
Segment Ratio	0.119*	0.068	0.177*	0.136*	0.458*		
Wing Ratio	0.023	-0.005	0.023	0.008	0.066	0.017	
Costa Ratio	0.157*	0.21*	0.16*	0.089	0.171*	0.119*	0.082

Significance determined using the Bonferroni correction, is given where *$P \leq 0.002$*

7.4.2.2 Differentiation between C. obsoletus and C. scoticus within Regions

Statistical differences in morphometric measurements (Table 7.5) and ratios (Table 7.6) between *C. obsoletus* and *C. scoticus* were determined for each region. The head measurements were the least able to differentiate between the two species in any location, with the length of flagella 11, and the length of the five apical segments only significantly different between the species at the end of the season in France. The width of the third maxillary palp, however, was statistically different between the species in all locations. The abdominal measurements exhibited significant differences between species in every region. The ratios showed a different trend, with the wing ratios least able to differentiate between the two species and the ratios

based on head measurements showing significant differences between the species in all locations.

Table 7.5 A summary of 15 morphological measurements of *C. obsoletus* and *C. scoticus* that exhibited significant differences between the species from sites in the UK, France and Spain.

X indicates a significant difference between the means

Measurement	UK		France				Spain	
	Bala	Devon	Landes	Calvados			Avia	Caldes
				Start	Middle	End		
Wing Length		X	X	X		X	X	X
Wing width		X	X			X	X	
Costa Length		X	X			X		
3rd Palp Length			X			X		
3rd Palp Width	X	X	X	X	X	X	X	X
Length of Flagella 10	X	X	X					
Length of Flagella 11						X		
5 Apical Segment Length						X		
8 Basal Segment Length	X	X	X		X		X	X
Larger Spermatheca Length	X	X	X	X	X	X	X	X
Larger Spermatheca Width	X	X	X	X	X	X	X	X
Shorter Spermatheca Length	X	X	X	X	X	X	X	X
Shorter Spermatheca Width	X	X	X	X	X	X	X	X
Chitinous Plate Length	X	X	X	X	X	X	X	X
Chitinous Plate Width	X	X	X	X	X	X	X	X

Table 7.6 A summary of eight ratios derived from morphological measurements of *C. obsoletus* and *C. scoticus* that exhibited significant differences between the species from sites in the UK, France and Spain

X indicates a significant difference between the means

Ratio	UK		France				Spain	
	Bala	Devon	Landes	Calvados			Avia	Caldes
				Start	Middle	End		
Wing Ratio		X						
Costa Ratio	X	X			X		X	X
Palp Ratio	X	X	X	X	X	X	X	X
Segment Ratio	X	X	X	X	X	X	X	X
Flagella Ratio	X	X	X	X	X	X	X	X
Larger Spermatheca Ratio	X	X	X	X	X		X	X
Shorter Spermatheca Ratio	X	X	X	X	X		X	X
Chitinous Plate Ratio	X	X	X	X	X	X	X	X

7.4.3 Comparisons of *C. obsoletus* and *C. scoticus* between Regions

7.4.3.1 Head

For *C. obsoletus*, the length and width of the third palp, the length of flagellae 10 and 11, as well as the length of the five apical and eight basal segments were significantly longer in *Culicoides* from Landes compared to the other locations. The palpal ratio did not differ within each country and, although not significant, the ratio was smallest for the Spanish sites. The flagella and segment ratios were not significantly different between the sites.

For *C. scoticus* the length of the 3rd palp was significantly smaller for the two UK sites than the collections from Landes. The width of the third palp from Devon was significantly smaller than all other collections. Flagella 10 was significantly smaller in collections from Devon and Caldes de Malavella compared to those from Bala and Landes. Flagella 11, and the five apical and eight basal segments were significantly larger in Landes than any other region. The palp ratio of the Spanish collections was significantly smaller than those from Bala and Landes. For the segment ratio, the collections from Avia were significantly larger than those from the two UK sites. There were no differences in the flagella ratio between the regions.

7.4.3.2 Wings

For *C. obsoletus*, the wing length, wing width and costa length for samples from Caldes de Malavella were significantly smaller than for samples from other sites. These measurements (excluding wing width) for Landes were significantly larger than the other sites. The costa length also differed between the UK sites.

For *C. scoticus* the wing length, width, and costa length was significantly smaller for Caldes de Malavella than the other sites. There were no differences in wing ratio between the sites, but the costa ratio was significantly higher for the two Spanish sites than the others.

7.4.3.3 Abdomen

For *C. obsoletus*, the length of the larger spermatheca was significantly longer in samples from Landes than from the other regions. The spermatheca length was significantly smaller from the two Spanish sites than from the French sites or Bala. In terms of larger spermatheca width, samples from the Spanish sites were significantly smaller than those from Bala or France. The length of the smaller spermatheca was also significantly different for the *Culicoides* from Spain compared to those from the UK or France. The length between the chitinous plates was significantly shorter for the Spanish samples than for the UK or French samples. The width of the chitinous plates was significantly shorter for Caldes de Malavella than for the other sites. There were no significant differences in the larger spermatheca ratio between locations, while the smaller spermatheca ratio was significantly

smaller in samples from Landes than for samples from Devon or Caldes de Malavella.

For *C. scoticus*, the length of the larger spermatheca was significantly smaller in samples from Caldes de Malavella than Landes. The width of the larger spermatheca was significantly smaller in midges from Caldes de Malavella than those from other regions, except Avia. The shorter spermatheca width was significantly shorter in samples from Caldes de Malavella than those from the UK and Landes. The width between the chitinous plates was significantly smaller for the Spanish midges compared to those from Bala and France. The spermatheca ratios and chitinous ratio weren't significantly different between sites.

7.4.4 Comparisons between Seasons in France

7.4.4.1 Head

For *C. obsoletus*, there was no difference in third palp width, or the length of flagella 10 and 11 between the start, middle and end of the season. The length of the five apical segments was greater at the start than in the middle of the season.

For *C. scoticus* flagella 10 and 11, the five apical segments and the eight basal segments of the antenna were all significantly smaller in collections from the middle of the season compared to the start and end of the season.

7.4.4.2 Wings

For *C. obsoletus*, the wing length for the middle of the season was significantly smaller than that at the start and end of the season. The wing width and costa length for the start, middle and end of the season were all significantly different from each other. The wing ratio was significantly larger for the middle of the season compared to the start.

For *C. scoticus* the costa length in the middle of the season was significantly smaller than at the start and end.

7.4.4.3 Abdomen

For both *C. obsoletus* and *C. scoticus*, there was no difference between seasons in the abdominal measurements.

7.4.5 Principal Component Analyses

Size differences were studied through PCA on the measurement data. Kaiser's stopping rule states that only the number of axes with eigenvalues over 1.00 should be considered in the analysis. The initial analysis of the 15 morphometric measurements indicated that three axes had an eigenvalue of 1.00 or higher (Table 7.7) and together, these factors accounted for 71.5% of the variance.

Table 7.7 The eigenvalues, percent variance and cumulative variance of the axes from the principal component analysis of 15 morphometric measurements of *C. obsoletus* and *C. scoticus*.

Axes (principal component)	Initial Eigenvalues		
	Total	% of Variance	Cumulative %
1	5.142	34.283	34.283
2	3.991	26.608	60.891
3	1.592	10.610	71.501
4	0.812	5.412	76.913
5	0.688	4.585	81.498
6	0.664	4.424	85.921
7	0.603	4.020	89.941
8	0.433	2.886	92.827
9	0.399	2.657	95.484
10	0.212	1.411	96.895
11	0.182	1.213	98.108
12	0.114	0.758	98.866
13	0.102	0.683	99.548
14	0.054	0.359	99.907
15	0.014	0.093	100.000

The scree plot (Figure 7.10a) confirms (Bryant & Yarnold, 1995) the relationship between the relative magnitude of the eigenvalues and the number of axis.

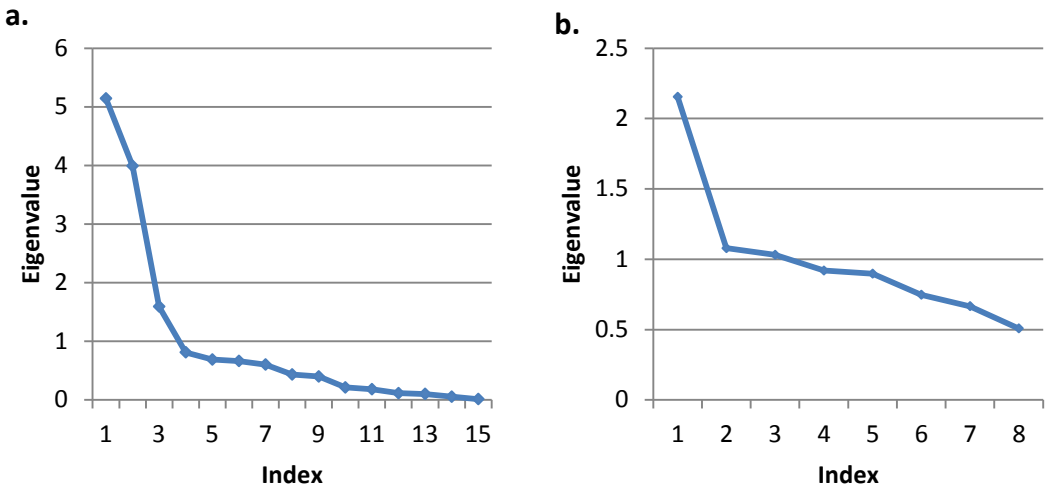


Figure 7.10 A scree plot highlighting the relationship between the eigenvalues and the number of axis in a principal component analysis of a) 15 morphometric measurements of *C. obsoletus* and *C. scoticus*; and b) eight ratios derived from morphometric measurements of those individuals.

The PCA scatterplot unambiguously separated *C. obsoletus* and *C. scoticus* (Figure 7.11). The first axis (PC1) was highly negatively correlated to the lengths and widths of the larger and smaller spermathecae (loadings ≥ 0.8), as well as the length between, and width of, the chitinous plates (loadings ≥ 0.55), and fairly correlated to wing length, costa length and 3rd palp width (loadings ≥ 0.45). The second axis (PC2) was positively correlated with antennal segment lengths (Table 7.8).

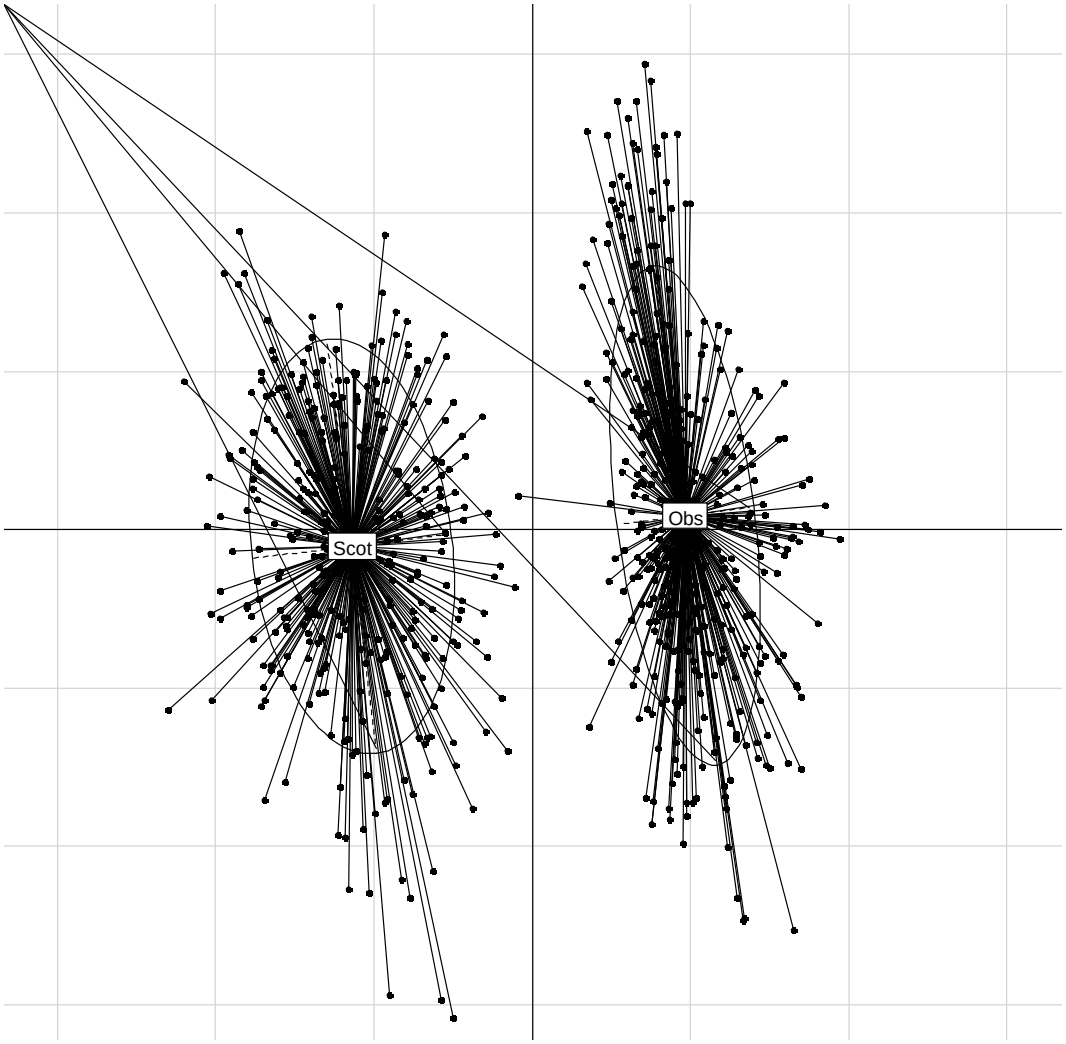


Figure 7.11 Results of principal component analysis on morphological measurements of *C. obsoletus* (Obs) and *C. scoticus* (Scot).

Table 7.8 Characterisation of *C. obsoletus* and *C. scoticus* using the loadings of principal component analyses on 15 morphometric parameters. PC1 = first axis; PC2 = second axis; and PC3 = third axis. Loadings of fair correlation, or above, are highlighted in **bold**.

Parameter	**Principal Component Analyses**		
	PC1	**PC2**	**PC3**
Larger Spermatheca Length	**-0.914**	-0.212	-0.190
Larger Spermatheca Width	**-0.895**	-0.158	-0.146
Smaller Spermatheca Length	**-0.913**	-0.215	-0.202
Smaller Spermatheca Width	**-0.870**	-0.155	-0.133
Length between Chitinous Plates	**-0.767**	-0.163	-0.083
Width of Chitinous Plates	**-0.600**	0.019	0.056
3rd Palp Length	-0.168	**0.600**	-0.150
3rd Palp Width	**0.476**	**0.483**	-0.012
Flagella 10 Length	-0.032	**0.812**	-0.209
Flagella 11 Length	-0.310	**0.787**	-0.259
5 Apical Segment Length	-0.248	**0.866**	-0.252
8 Basal Segment Length	0.030	**0.910**	-0.123
Wing Length	**-0.539**	**0.414**	**0.669**
Wing Width	-0.180	0.148	**0.556**
Costa Length	**-0.481**	**0.424**	**0.705**

In a second PCA, the eight morphometric ratios were analysed in order to look for differences in shape between *C. obsoletus* and *C. scoticus*. Kaiser's stopping rule suggested the inclusion of the first three axes (Table 7.9), while the scree test (Figure 7.10b) suggested inclusion of only the first axis. The structure of the data was also weak, with seven of the eight axes accounting for similar amounts of variance each (6 – 13%). This was confirmed by a scatterplot of the first three axes, which was unable to separate the two species (Figure 7.12). PC1 was negatively correlated to the majority of ratios (Table 7.10).

Table 7.9 The eigenvalues, percent variance and cumulative variance of the axes from the principal component analysis of eight ratios derived from morphometric measurements of *C. obsoletus* and *C. scoticus*.

Factor	Initial Eigenvalues		
	Total	**% of Variance**	**Cumulative %**
1	2.154	26.930	26.930
2	1.078	13.479	40.409
3	1.031	12.889	53.299
4	0.919	11.482	64.781
5	0.897	11.210	75.991
6	0.747	9.337	85.328
7	0.666	8.323	93.651
8	0.508	6.349	100.000

Table 7.10 Characterisation of *C. obsoletus* and *C. scoticus* using the loadings of principal component analyses on eight morphometric ratios. PC1 = first axis; PC2 = second axis; and PC3 = third axis. Loadings of fair correlation, or above, are highlighted in **bold**.

Parameter	Principal Component Analyses		
	PCA1	**PCA2**	**PCA3**
Larger Spermatheca Ratio	**-0.457**	-0.057	-0.048
Smaller Spermatheca Ratio	**-0.488**	**-0.533**	-0.008
Chitinous Plate Ratio	**-0.652**	-0.227	0.122
Palpal Ratio	**-0.502**	-0.262	0.234
Flagella Ratio	**-0.707**	**0.425**	0.054
Segment Ratio	**-0.564**	**0.636**	0.142
Wing Ratio	-0.105	0.161	**-0.861**
Costa Ratio	**0.450**	-0.244	**-0.442**

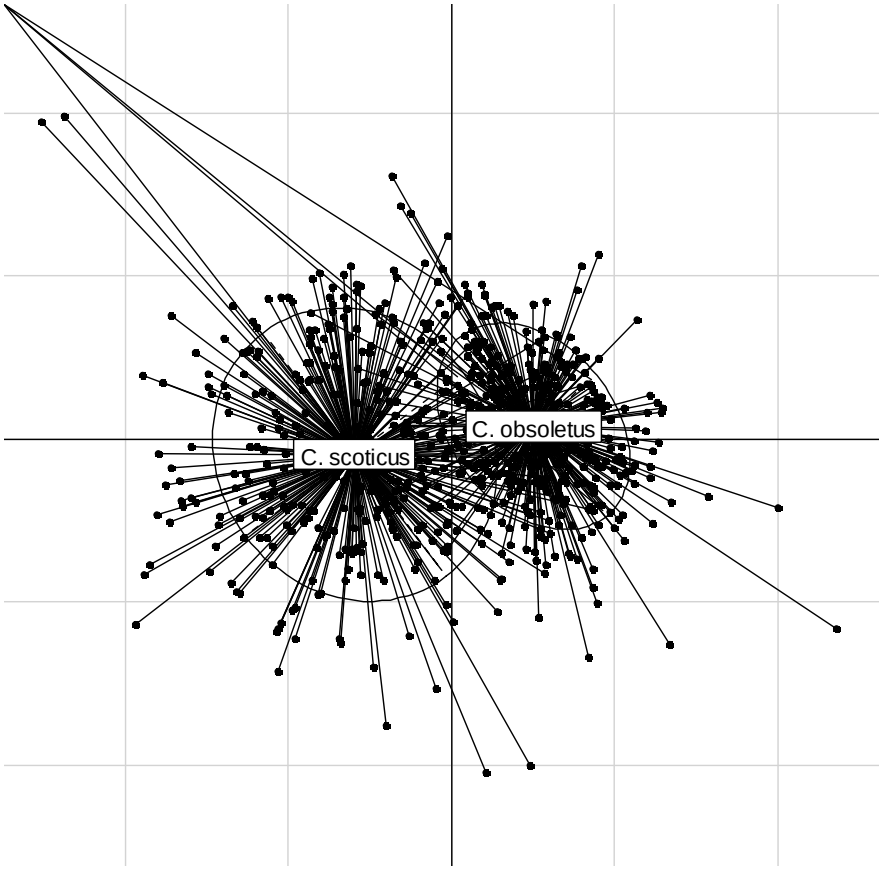


Figure 7.12 Results of principal component analysis on morphometric ratios of *C. obsoletus* and *C. scoticus*.

7.5 Discussion

When large numbers of insects need to be identified to species level, it is important to determine reliable morphological characteristics that can be visualised under a stereomicroscope. In the case of members of the Obsoletus Group of *Culicoides*, although *C. dewulfi* and *C. chiopterus* can be reliably identified to species level, the identification of the females of *C. obsoletus* and *C. scoticus* has remained problematic. As such, the identification of Obsoletus Group members has often included the use of molecular techniques, which have been employed to differentiate between these two cryptic species.

A number of studies have begun investigating whether there really is a need to employ molecular techniques to differentiate *C. obsoletus* and *C. scoticus*, or whether some previously undetermined morphological characteristics can be used (Augot *et al*., 2010; Nielsen & Kristensen, 2011). However, these studies do not take into account geographical differences in *Culicoides* morphology, or indeed the effect of seasonal variation on morphology.

Here, the diagnostic ability of 15 morphometric variables and eight ratios, that have previously been identified as characteristics able to differentiate between *C. obsoletus* and *C. scoticus* individuals, (as determined by Augot *et al*. (2010); Nielsen & Kristensen (2011); and Pages & Monteys (2005)) were investigated on samples with known species identified molecularly using the COI gene sequence.

Greater numbers of *C. obsoletus* were trapped during the start of the season in France compared to *C. scoticus*, but this relationship was reversed at the end of the season, with more *C. scoticus* trapped than *C. obsoletus*. This observation has also been noted by Viennet *et al*. (2012), where the abundance of *C. scoticus* decreased progressively from the first to the last collection period during a season.

The *C. scoticus* means were greater than those for *C. obsoletus* for all wing and abdominal measurements, but smaller for almost half of the antenna and palp measurements, suggesting that although this species tends to have a larger body, the antenna and palps are not comparatively so. The abdominal measurements showed greatest discriminatory power between *C. obsoletus* and *C. scoticus*, with significant differences seen in their means within all locations. The head and wing

measurements showed fewer significant differences in means between the two species within each location sampled.

When comparing geographical differences in the size and shape of the two species between trapping locations, the *Culicoides* from Spain appeared smaller overall than the samples from other locations, with significantly smaller palps, flagellae, wings, spermathecae and chitinous plates. This was especially prevalent for samples from Caldes de Malavella. *Culicoides* from Landes on the other hand exhibited means significantly larger than the other locations for many of the morphometric measurements. Temperature is a driving force for immature development rates of *Culicoides* (Smith & Mullens, 2003) and an inverse relationship has been established between wing length and larval rearing temperatures (Kitaoka, 1982), which would perhaps explain when the *Culicoides* from Spain were smaller than those from other locations.

Seasonal variation was observed in the morphometric measurements from the *Culicoides* trapped in Calvados. All flagella measurements were significantly smaller in the middle of the season than at the start and the end of the season. The same trend was seen for wing and costa length. These seasonal variations are likely to be due to variation in temperature between these time-points, with the peak in temperature exhibited at the middle of the season, and therefore smaller measurements occurring at this time than at the start and end of the season, when the temperatures would be cooler. The abdominal measurements however, did not show any significant differences between seasons, once again highlighting the reliability of using the spermathecae to discriminate between the species.

Four variables (the length and width of the larger and smaller spermathecae) exhibited CD values greater than 1.28, indicating that these characteristics can be used to distinguish between subspecies. The means of these variables did not overlap. None of the ratios exhibited values above the critical threshold. The lack of overlap between spermatheca length was also highlighted by Delecolle (1985), who concluded that all females with a larger spermatheca length $\leq$ 59µm were *C. obsoletus* and those with a larger spermatheca length were *C. scoticus* (overall range of measurements 50-67µm). In this study, larger spermatheca lengths $\leq$ 52µm were *C. obsoletus* and $\geq$57µm were *C. scoticus* (overall range 44-66µm).

Since there is a gap of 5 µm between larger spermatheca length in the *C. obsoletus* and *C. scoticus* individuals measured here, it would be advisable to molecularly identify any individual that falls within this range (>52 µm, but < 57 µm).

According to Delecolle (1985), *C. obsoletus* and *C. scoticus* females can also be distinguished by the formation of the two chitinous plates surrounding the genital openings. As Pages & Monteys (2005) highlighted that *C. scoticus* had widely variable margins that did not always conform to the morphological formations determined by Delecolle (1985), and could therefore be left to individual interpretation, the formation of these was not examined here.

Correlation was observed between the morphometric measurements, as well as the ratios derived from the morphometric characteristics. This was particularly true in the case of spermatheca lengths and widths, whereby strong positive correlations were observed between these measurements as well as between the larger and smaller spermathecae. A multivariate PCA was undertaken to transform the correlated variables into a smaller number of linearly uncorrelated variables (principle components) to explain the variability in the data.

Considering the PCA, the distributions of the length and width of the larger and smaller spermathecae, as well as the length between, and width of, the chitinous plates are the most reliable characteristics to differentiate between *C. scoticus* and *C. obsoletus*. These results confirm the four parameters that exhibited Mayr's coefficient of difference (CD) values above the critical threshold, while the length between chitinous plates was verging on the critical value.

Augot *et al*. (2010) also undertook a PCA on morphometric measurements of *Culicoides* collected in France. Their results agree that the length of the larger and smaller spermatheca, as well as the length between the chitinous plates would be the most reliable characters to differentiate between *C. scoticus and C. obsoletus*, but the width of the smaller spermatheca was not included. The lack of diagnostic ability of the morphometric ratios, as determined by the weak data structure and overlapping scatterplots of a PCA on this data, was also confirmed by the analysis undertaken by Augot *et al*. (2010) on their ratio data.

Although both this study, and that of Augot *et al*. (2010), determined that it is possible to distinguish between these two species based on four characteristics, these characteristics are still time-consuming to measure and may require the use of slide mounting individuals, as it is not always easy to obtain direct access to the spermathecae of individuals with their abdomens attached.

Nielsen & Kristensen (2011) showed how it is possible to separate the females of the four species by combining the shape of the third segment of the maxillary palp. The *Culicoides* used for these identifications however, were all collected from Sweden or Denmark, so do not take into account the possible geographical differences in *Culicoides* morphology, or indeed the effect of seasonal variation on morphology (all samples were collected between July and September). The palpal ratio from our study, while able to differentiate between *C. obsoletus* and *C. scoticus* within each of the trapping locations, was unable to discriminate between these species when the data from all locations was combined. This would suggest that although the shape of the third palp does exhibit differences between the species, geographic variation in the *Culicoides* is such that this characteristic cannot be used reliably to differentiate between individuals from different regions.

If the study was repeated, the *Culicoides* trapped in Spain should be from differing geographical areas, with a sample taken from the south of Spain, rather than both from northern Spain. As the size of the *Culicoides* was smaller further south it would have been interesting to see if this trend was continued. Further work could be undertaken to determine whether the size differences in the Group members are seen throughout the year, or whether there is a time-lag between the counties by undertaking year-round trapping in each country on a monthy basis.

Similarly, follow-up studies should focus on *Culicoides* from the eastern and western limits of the Obsoletus Group members' range to determine if further differences occur in their morphology. Ideally the samples used in this study should have been collected at the same time of year in each location to determine how comparable the samples would be for larger-scale entomological trappings that cover several countries.

Although PCA confirmed four parameters as the most reliable characteristics to distinguish between *C. obsoletus* and *C. scoticus*, the use of discriminant analysis would further allow us to make precictions on the species identifications of newly trapped specimens, based on these characteristics. This would therefore be a useful follow-up study that could be performed on the significant measurements from the PCA.

In conclusion, we have shown that female *C. obsoletus* and *C. scoticus* individuals can be separated under a stereomicroscope based on abdominal measurements. Seasonal variation in the size of these species, and therefore their morphometric measurements was observed for both head and wing measurements, but not for the abdomen. Geographical variation in the size of individual *Culicoides* was also observed and is likely to be related to temperature at these sites, with smaller *Culicoides* trapped further south. Although the length and width of the spermathecae can be used to differentiate between the species, this can be a time-consuming process and we therefore recommend undertaking this on a sub-sample of individuals.

CHAPTER EIGHT

FINAL DISCUSSION AND CONCLUSIONS

A further paper was published [see Appendix E] using the *Culicoides* trapping data from Chapter 3, and is discussed during this Chapter:

Charron, M. V. P., **Kluiters, G**., Langlais, M., Seegers, H., Baylis, M., Ezanno, P. (2013). Seasonal and spatial heterogeneities in host and vector abundances impact the spatiotemporal spread of bluetongue. *Veterinary Research*, *9*;44:44. doi: 10.1186/1297-9716.

8.1 General Discussion

The incidence and prevalence of BTV infection is highly dependent on the distribution and abundance of its *Culicoides* vectors. Quantification of the drivers of vector abundance is therefore required for accurate estimates of transmission risk and for the development of cost-effective targeted vector-control strategies. The extremely small size of *Culicoides* and the cryptic nature of the Palaearctic BT vector species, however, make investigations into their ecology and interactions with susceptible hosts and pathogens exceptionally difficult. This has resulted in a paucity of detail regarding their distribution and abundance at both the local and within-farm scales. This thesis proposed to address this issue by using a multidisciplinary approach combining statistical modelling and GIS techniques with detailed ecological surveys of *Culicoides* populations on UK farms. As a result of this work, drivers of Palaearctic BT vector occurrence and abundance were identified at the local scale, while the active dispersal of the main vector species, the Obsoletus Group, was recognised from farm to farm for the first time.

Maximum catches during the summer-time have been shown in previous studies to be consistently related to the annual abundance of *Culicoides* in Morocco (Baylis *et al*., 1997), highlighting that year-round trapping may not be necessary to determine species abundance. A number of previous studies have attempted to produce climatic models of BT vector abundance, based on only one or two trap catches per sampling point (Calistri *et al*., 2003; Pili *et al*., 2006), but failed to explain a significant level of the observed variation in *Culicoides* populations. Although the inclusion of additional landscape, host and soil characteristics have improved predictions in other countries (Calistri *et al*., 2003; Conte *et al*., 2007a), Harrup (2010) found that a combination of climate, host, and landscape factors failed to explain a large degree of the observed variation for both the Palaearctic vector species (the Obsoletus Group 37%; Pulicaris Group 19%), as well as for *C. imicola* (32%), abundance in Greece and Bulgaria when using a snapshot sampling approach. A commonality between all these studies is the low spatial scale at which samples were taken, with sampling mainly undertaken as part of large-scale nationwide surveillance schemes.

The work contained in Chapter 3, however, highlighted that not only can a very high level of variation be seen when trapping *Culicoides* at the local-scale, but that it is

possible to build a strong model explaining this variation using a mixture of host and environmental variables, with satellite-derived ecological correlates. It is clear from this that the high level of variation in midge catches present between farms, as close as 1 km from each other, undermines attempts to record their nationwide distribution in larger scale models. The variance explained was consistently high for the Obsoletus Group (81%), Pulicaris Group (80%; *C. pulicaris* 73%; *C. punctatus* (74%), as well as for the non-vector models (65-87%). The models for each species differed in their associations with satellite variables and uniquely highlighted an increase in the abundance of all vector species with increasing sheep numbers on farms, yet the non-vector models lacked this relationship. This was particularly interesting given that none of the species concerned are known to utilise sheep-associated larval development sites, but may be related to feeding preferences of the species. *C. impunctatus*, closely related to *C. pulicaris* and *C. punctatus*, may well have lacked this relationship due to its autogenous nature, and it therefore not requiring a bloodmeal to develop the first batch of eggs (Blackwell *et al*., 1992; Boorman & Goddard, 1970), highlighting less of a reliance on hosts for bloodmeals than its vector counterparts. The differential responses to environmental factors between species, was to be expected due to their differing ecological characteristics (McPherson & Jetz, 2007) and emphasises the need to investigate relationships at the species, rather than group level.

With no strong Obsoletus Group abundance models published for the UK and no previous work highlighting a difference in host involvement between vector and non-vector models, the vector species models arising from this work are of utmost value. Our study highlighted the high variation in *Culicoides* abundance that can occur between neighbouring farms, but work still needed to be undertaken to determine the level of variation present at the smaller, within-farm scale, and whether that variation was consistent between years.

Analysing a large number of explanatory variables relative to the number of data points creates a danger of overfitting. Leave-one-out regression analysis is a useful technique for examining overfitting, as overfitted models often show poor predictive ability. The leave-one-out regressions of our models show good predictive ability and therefore, while we accept there was a risk of overfitting, our models show no

evidence of it. Although the leave-one-out regression analyses provide strong evidence that these vector models would have strong predictive power when applied to another area of north Wales, external validation involving sampling in another region will be the ultimate test.

In light of these problems, the research within Chapter 4 was undertaken to assess our model's robustness in a number of ways. Just as the high-variation observed at the local, between farm-scale, in Chapter 3 undermines large-scale models, an equal high variation within a farm would suggest models at local, or greater scales, could be influenced by the choice of trapping site on a farm. Mean *Culicoides* densities on farms were consistently higher from catches in traps located near trees, in comparison to catches from the traps set up in a built environment or in an open area. Although significant differences were observed between catches near trees and those in other environments for three of the non-vector groups, significant differences were only observed for the *C. pulicaris* vector and no differences seen at the Group level. There was also little difference observed in the proportions of the different species caught in each environment.

The results suggest that trapping in areas of high vegetation is not better for one individual species, but equally beneficial for increasing the numbers of all species. Trapping in this environment may well, therefore, be beneficial when trying to capture rare species that generally have a low prevalence. The reasoning behind this remains unclear; on the one hand there may truly be a larger population of midges in this location, but on the other hand the location is likely to be more sheltered, with less wind therefore increasing levels of activity and also trapping efficacy. The level of variation present at the within- farm scale appears to be, therefore, less than at the between-farm scale. A conclusion is that the optimal sampling scheme for *Culicoides* surveys may be a maximal number of sampling farms (determined by logistics and funding) and a standardised location of a single trap on each sampling farm.

Although a number of other studies have already investigated the role that trap type (Scheffer *et al*., 2012; Venter *et al*., 2009b), height (Braverman & Linley, 1993; Venter *et al*., 2009a) and bulb colour (Venter *et al*., 2006) have in trapping *Culicoides*, our results highlight that more work is needed in order to understand the

role of trap location in increasing the numbers of midges caught, which may be particularly useful for studies aiming to trap novel species.

Chapter 4 also successfully confirmed the robustness of the local-scale model produced in Chapter 3, by highlighting both a consistency in the spatial distribution of trapped *Culicoides* and also the ranking of farm catches between years. The lack of significant annual variation observed in midge abundances, with a correlation in trap catches observed on farms between 2008 and 2010, highlights that trapping data collected from one year can be assumed to be fairly consistent between years, even when using a snapshot sampling approach. The remarkably high level of variation in *Culicoides* catches observed in 2008 was not as great in 2010, although the numbers of each species trapped were significantly higher. This may be due to more stable weather conditions during the trapping period in 2010 compared to those in 2008, therefore providing a greater level of consistency in results between farms.

Multi-level modelling would provide a good follow-up method to analyse the results we have produced here. Such an approach could be used to estimate separately the variance between trapping sites within the same farm, and the variance between farms. The high level of local-scale variation captured in the model from Chapter 3, in combination with the lack of significant variation both between years and at the within-farm level, highlights the robustness of this model in predicting the distribution of the BT vectors species, and could prove useful for exploring targeted surveillance and control methods.

Indeed, the 2008 trapping data was further used to develop a model to represent BTV-8 spatiotemporal spread at the local scale. As the spatiotemporal heterogeneity in host and vector abundance and distribution has a strong impact on pathogen spread and persistence (Smith *et al*., 2004; Backer & Nodelijk, 2011), the spatial distribution of the 2008 *Culicoides* trap catches and on-farm host data was used by Charron *et al*. (2013) to assess how spatiotemporal heterogeneities in host and vector abundance and distribution impact on the occurrence and amplitude of local and regional BT epidemics. Here, the abundance in vectors had a significant impact on the date and observed prevalence of BTV at the epidemic peak, with spatial heterogeneities in hosts and vectors delaying the epidemic peak and decreasing the infection prevalence, while local epidemics were shown to occur earlier and be

larger in scale far from the primary case, rather than close to it. This model, highlights the importance of vector dispersal between farms in the spread of BT.

Although the local-scale model produced in Chapter 3 captures a high amount of variation between farms, Sedda *et al*. (2012) emphasises that *Culicoides* distributions do not remain static, and therefore an understanding of their flight behaviour is critical to determining the distance over which an insect may transmit a disease agent (Hocking, 1953) and can further be used to determine the size of the area over which control should be applied. Investigation of insect flight patterns frequently involves the use of dispersal studies. Although numerous methods have been used to mark insects for use in dispersal studies, by far the easiest method to mark numerous insects is by the use of fluorescent dusts. Such techniques, using highly-visible fluorescent dusts have not been undertaken since the 1980s in the USA (Lillie *et al*., 1981; Brenner *et al*., 1984; Lillie *et al*., 1985) and those dusts are no longer available. Chapter 5 therefore aimed to determine whether a readily-available brand of micronized fluorescent dusts (Brilliant General Purpose, BGP, USA) available in variety of colours, proved to be a suitable marking agent for *Culicoides* midges for use in mark-release-recapture techniques.

The three dust colours tested were all insoluble in water, 10% washing up liquid and 70% ethanol, suggesting that trapping and storage of specimens marked with fluorescent dusts would not be compromised using this marker. The dusts were all readily visible down a microscope, with and without the use of a black light, and the pink and green dusts were highly visible without the need for a microscope, an important feature of a marking agent when the number of trapped insects is numerous. The dusts remained adherent to the marked *Culicoides* for the duration of the experiments, did not transfer between marked and unmarked individuals or the environment, and remained adherent when the *Culicoides* were stored in an ethanol or water-based solution. The adherence of the dusts was of utmost importance, not only in ensuring that any *Culicoides* recaptured during a MRR experiment could be identified as marked, but also in ensuring that marked *Culicoides* could not transfer the dusts to unmarked individuals and provide false positive recaptures. The dusts had no effect on the mortality rate of the insects over the 48 hrs of the experiment and there were no significant differences between the recorded behaviours of marked

individuals and un-dusted controls. Although the effects of the dusts were only studied for a short period of time (≤ 72 hrs), the difficulties in keeping wild-caught *Culicoides* alive under laboratory conditions precluded the experiments lasting longer. These experiments could be replicated on laboratory-reared *Culicoides* colonies, but such no such colonies exist for the main Palaearctic vector Groups. Colonies of *C. sonorensis*, the North American BT vector, are available but the behaviours of these insects, which have been maintained for more than 30 years in the laboratory, are likely to be very different to wild-caught midges. Overall, the BGP fluorescent dusts provided a quick and effective method of marking *Culicoides* for both field and laboratory studies, and a 'self-marking' technique was conceived, whereby trapping vessels could be pre-dusted with the fluorescent dust prior to trapping the individuals to be marked.

The use of the field-based 'self-marking' technique led to the first demonstration of farm to farm movement of the main Palaearctic BT vector species, the Obsoletus Group. Chapter 6 employed this technique to study the dispersal of *Culicoides* species between a network of farms over a set period of time and although a small proportion of marked midges were recaptured (0.02%), this is comparable to the small numbers recaptured during similar studies, where generally less than 1% of release insects are recovered (Johnson, 1969; Kirkeby *et al*., 2013; Lillie *et al*., 1981; 1985). The only exception to this has been by Brenner *et al*. (1984) who recaptured 13% of *C. mohave* in southern California, due to the lack of vegetation in the desert environment where the study took place. Although another study has recently been undertaken on the dispersal of the Palaearctic BT vectors, Kirkeby *et al*. (2013) were unsuccessful in recapturing members of the Obsoletus Group from any traps surrounding the release site. In Chapter 6 we were able to show that recaptured Obsoletus Group females, as well as *C. pulicaris*, are able to disperse a minimum of 2.5 km in 48 hrs, with male Obsoletus Group members dispersing 1 km in 24 hrs. This knowledge of flight speed and distance is of utmost value as a critical component in the modelling of BT disease and other *Culicoides*-borne diseases.

Dispersal of *Culicoides* was recorded both upwind and downwind of the prevailing wind direction during the trapping period and was the first empirical support for inferences about *Culicoides* flight behaviour obtained from analysis of the spread of

the wave fronts of bluetongue and Schmallenberg published by other authors (Sedda *et al*., 2012). These results highlight the importance of exploring active dispersal of vectors upwind during disease outbreaks simulations, rather than purely the downwind movement of midges aided by wind transport. This movement of vectors between farms has disease control implications, with our results suggesting that control measures applied at an infected farm will reduce the risk of spread to neighbouring farms, as well as reduce transmission at the source farm itself.

One limitation of the reaction-diffusion model of the 2008 Bala data, employed by Charron *et al*. (2013), was that wind-dispersal of vectors was neglected. They state that "the coupling of both short and long-distance dispersal is primarily necessary to study arbovirus spread in animal populations once the spatial scale is large enough that host movements and passive movements of vectors cannot be neglected anymore" (Murray, 1987; Mellor *et al*., 2000; Finlaison *et al*, 2008). Yet, the dispersal study highlights that short distance dispersal aided by wind may similarly occur over short distances (≤ 3 km). The model could therefore further be used to explore the impact of wind dispersal in the region, as well as how control measures applied at the farm level impact on the spread of BTV to neighbouring farms.

The Obsoletus Group contains four members (*C. obsoletus*, *C. scoticus*, *C. chiopterus* and *C. dewulfi*) which are difficult to differentiate down a microscope; indeed some regard *C. obsoletus* and *C. scoticus* as cryptic species (Nolan *et al*., 2007), while others identify distinguishing features which they believe differentiate between the species (Delecolle, 1985; Nielsen & Kristensen, 2011). Currently, there is no significant evidence that these different species of the Obsoletus Group differ in characteristics relevant to their ability to act as disease vectors (i.e. vectorial capacity). Accordingly, the studies performed throughout this thesis explore the Obsoletus Group members at the Group-level, rather than as individual species.

Previous studies exploring both morphological and morphometric differentiation of *C. obsoletus* and *C. scoticus* have been undertaken on small populations of *Culicoides* from within one region, meaning any discriminating techniques may not be able to be extrapolated to *Culicoides* collected in other countries or indeed during different seasons (Delecolle, 1985; Pages & Monteys, 2005; Augot *et al*., 2010;

Nielsen & Kristensen, 2011). Chapter 7 attempted to rectify these problems by undertaking morphometric analyses on *Culicoides* collected from multiple regions, and time-points during the year, in the UK, France and Spain, and comparing these measurements to molecular identifications of those specimens.

The results suggest that female *C. obsoletus* and *C. scoticus* individuals can be separated under a stereomicroscope based on abdominal measurements (the length and width of the larger and smaller spermathecae (≤ 52µm were *C. obsoletus* and ≥57µm were *C. scoticus*), agreeing with previous comparisons by Delecolle (1985). Seasonal variation in the size of these species, and therefore their morphometric measurements, was observed for both head and wing measurements, but not for the abdomen, once again highlighting the reliability in using an abdominal measurement for differentiating the species. Geographical variation in the size of individual *Culicoides* was also observed however, and is likely to be related to temperature at the trapping sites, with smaller *Culicoides* trapped further south. This result was not surprising given that a higher temperature leads to faster development rates and smaller sized insects, but this has not been evaluated in the context of morphometric identifications previously.

Although the results highlight that the length and width of the spermathecae can be used to differentiate between the species, this can be a time-consuming process, and is therefore only recommend to be undertaken on a sub-sample of individuals from catches. As Chapter 7 was one of the final studies undertaken as part of this thesis, the differentiation of *C. obsoletus* and *C. scoticus* was not undertaken using this method in the other Chapters. Although this method now provides a cost-reducing alternative to molecular identification of the species within this Group, researchers should make their own decision as to whether species-level data on these BT vectors is needed given the lack of information on the differing vectorial capacities of these species and apparent ability of local-scale models to explain such a high amount of variation even when the four species are grouped together.

8.2 Final Conclusions

This thesis has provided significant amounts of novel data concerning the life history and ecology of *Culicoides* species, but has also demonstrated the complexities in working with them in the field and laboratory. Many critical components in bluetongue modelling are regrettably allied with our lack of basic understanding of BT vector ecology, with funding for *Culicoides* research having peaked and troughed in line with the appearance of *Culicoides*-borne diseases in Europe, making long-term data-set collection, or sustainable basic field-based research scarce. Studies such as those contained in this thesis, therefore, are of utmost value in providing information on critical components in the modelling of BT disease and other *Culicoides*-borne diseases.

The accurate characterisation of drivers of population abundance, which would aid in both risk assessment and response to disease incursion appear to be best realised at the local-scale. While the introduction of free and readily available satellite imagery able to provide ecological correlates, in combination with farm-level data on host distribution and the surrounding environment, produce local-scale models able to explain the high amount of variation seen at this scale, the successful prediction of species distribution in a different geographic location will ultimately be the true test of this model.

This thesis, while presenting significant amounts of novel data, has raised many questions and identified yet more gaps in our understanding of both the vectors and non-vectors of BT. Particular attention should be focused on disentangling the uncertainties that arise from the imprecise understanding of the biology of *Culicoides* in the UK. In particular studies on their mortality rate and biting frequency, their feeding preferences, and their distribution within farms, and how these variables relate to climate; in combination with a better understanding of environmental effects on light-trap catches and how local-scale modelling can aid large-scale models; will provide a way forward to reducing uncertainties in critical components of BT modelling in future years.

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APPENDICES

APPENDIX A

Journal of Applied Ecology

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Modelling the spatial distribution of *Culicoides* biting midges at the local scale

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Summary

1. *Culicoides* midges (Diptera: Ceratopogonidae) are ubiquitous on farms in the United Kingdom (UK), but little research has explored their abundance, an important determinant of disease risk. Models to explain and predict variation in their abundance are needed for effective targeting of control methods against bluetongue virus (BTV) and other *Culicoides*-borne diseases. Although models have been attempted at the national scale (e.g. Scotland), no investigations have taken place at a finer spatial scale.

2. Midge abundances were estimated using light traps on 35 farms in Bala, north Wales. *Culicoides* catches were combined with remotely sensed ecological correlates, and on-farm host and environmental data, within a GLM model. Drivers of local-scale variation were determined at the 1-km resolution.

3. Local-scale variation in abundance exhibited an almost 500-fold difference (74–33 720) between farms in maximum Obsoletus Group catches. The Obsoletus Group model explained 81% of this variance and was dominated by normalized difference vegetation index (NDVI). This is consistent with previous studies suggesting strong impacts of forest cover and vegetation activity on distribution, as well as shaded breeding site requirements.

4. The variance explained was consistently high for the Pulicaris Group, *C. pulicaris* and *C. punctatus* (80%, 73% and 74%), the other probable BTV vector species in the United Kingdom. The abundance of all vector species increased with the number of sheep on farms, but this relationship was missing from any of the non-vector models. This is particularly interesting given that none of the species concerned are known to utilize sheep-associated larval development sites. Performance of the non-vector models was also high (65–87% variance explained), but species differed in their associations with satellite variables.

5. *Synthesis and application.* At a large spatial scale, there is significant variation in *Culicoides* Obsoletus Group abundance, which undermines attempts to record their nationwide distribution in larger-scale models. Satellite data can be used to explain a high proportion of this variation and, if shown to be generalizable, they may produce effective predictive models of disease vector abundance. We recommend undertaking a prior survey for farms with high *Culicoides* catches within the sampling area and checking stability in catch size between seasons and years.

Key-words: abundance, bluetongue, *Culicoides obsoletus*, GLM, MODIS, satellite-derived, spatial modelling

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Introduction

Bluetongue (BT), a vector-borne, viral disease of ruminants has undergone an unprecedented emergence following the entry of bluetongue virus (BTV)-8 into northern Europe in 2006. The Palaearctic Obsoletus and Pulicaris Groups of *Culicoides* biting midges are implicated in virus transmission (Carpenter, Wilson & Mellor 2009). Although BT has long been notifiable to the World Organization for Animal Health (OIE), the recent introduction and establishment of BTV-8 into northern Europe has intensified interest in understanding the drivers of *Orbivirus* outbreaks.

Recent advances have been made in understanding larval development sites, taxonomy and molecular recognition of *Culicoides*. Little is currently known, however, about certain ecological characteristics of the vector and non-vector species (Conte *et al.* 2007a), including their distribution and abundance, making disease risk assessment and management difficult. As BT and other *Culicoides*-borne diseases are transmitted almost entirely by the bites of their vector species (Mellor, Boorman & Baylis 2000), their distribution and infection intensity are dependent on the distribution and abundance of *Culicoides*.

Statistical models facilitate informing opinions where ecological knowledge is lacking, by identifying relationships between climatic and environmental factors, and the known distribution of vectors (Baylis & Rawlings 1998; Baylis, Meiswinkel & Venter 1999). Suitability maps can then be produced for vector occurrence across regions where vector distribution is unknown, on the basis of their climate and environment (Rogers & Randolph 2003). Analysing patterns in *Culicoides* abundance with different ecological characteristics in the same model framework (Conte *et al.* 2007a; Calvete *et al.* 2008) can aid understanding of biological mechanisms underlying the sensitivities of midge species to particular environmental factors, despite limited ecological knowledge of these species.

The spatial scale at which these techniques have been used varies greatly, but larger-scale studies have primarily been the focus, while local-scale, high-resolution models are less common. Country-wide risk mapping, as was undertaken in Switzerland prior to the BTV-8 incursion, highlights the use of regression analyses together with GIS techniques in determining vector suitability maps for the major European vector group *C. obsoletus* (Racloz *et al.* 2007), whilst discriminant analysis has been used in terms of continent-wide mapping in Europe and Africa (Tatem *et al.* 2003). Discriminant analysis and regression have also been used in terms of country-wide vector distribution and risk mapping in Sicily (Purse *et al.* 2004b), various regions in the Mediterranean (Baylis *et al.* 2001) and Morocco (Baylis *et al.* 1998), Iberia (Baylis & Rawlings 1998; Wittmann, Mellor & Baylis 2001) and Italy (Conte *et al.* 2003), respectively.

The spatial scale and intensity of a study impacts the temporal frequency of trapping, because of the limited resources, mostly time, available for processing catches. At the extreme ends of the scales, national-level surveys have undertaken single nights of catches at a large number of sites (Meiswinkel *et al.* 2008; Hartemink *et al.* 2009), while other studies report daily catches over several months at a single site (Birley & Boorman 1982; Gerry & Mullens 2000). Many studies adopt protocols in between these extremes: in Morocco, 22 trap sites were sampled twice weekly for 2 years (Baylis *et al.* 1997). The trade-off between spatial and temporal resolution employed by such studies has rarely been overcome, except in recent years where national-level BT concerns led, in some instances, to country-wide surveillance using government resources (Calvete *et al.* 2006; Conte *et al.* 2007a).

The lack of local-scale modelling is likely to be due to climate-driven models performing poorly when validated at the local scale with independent data (Capela *et al.* 2003), as non-climatic factors such as landscape configuration, farm husbandry, host availability and microclimate also influence abundance. Local-scale distribution of *Culicoides* and BTV are best explained by models that incorporate landscape (Guis *et al.* 2007), topographical (Conte *et al.* 2007b) or host factors (Calvete *et al.* 2008) alongside climate.

Until now, few attempts were made to model the relationship between climate, host and environmental factors and the distribution of current and potential bluetongue vectors within the United Kingdom. Although all *Culicoides* species share the same basic habitat requirements, that is, the presence of host for bloodmeals and breeding sites (Mellor, Boorman & Baylis 2000), they differ in their life-history characteristics and therefore the extent to which their distribution and abundance is affected by environmental factors. Purse *et al.* (2012) investigated landscape, host and climate on *Culicoides* in Scotland, yet failed to produce a strong model for the major BT vectors, the Obsoletus Group, highlighting the need for strong models of this species group.

Seasonal climatic variables derived by temporal Fourier processing are good predictors of vectors and vector-borne disease patterns, including tsetse flies and trypanosomiasis (Rogers 2000), malaria (Rogers *et al.* 2002), tick-borne diseases (Randolph *et al.*, 2000) and bluetongue and its vectors (Tatem *et al.* 2003; Purse *et al.* 2007). Previous models indicate that climatic determinants of distribution differ between *Culicoides* species, with Purse *et al.* (2004a,b) finding the distributions of *C. obsoletus* and *C. newsteadi* were primarily related to remotely sensed temperature variables [land surface temperature (LST), air temperature (TAIR)], while normalized difference vegetation index (NDVI) was the most important for *C. pulicaris*, and *C. imicola* was modelled using a combination of LST, NDVI and middle infra-red reflectance (MIR) in Sicily.

This study aimed to measure the light trap catch of *Culicoides* at a local, farm-level, scale and identify determinants of the abundance. The Bala region of north Wales was selected as the study site. Here, the distribution and abundance of known BT vectors and other *Culicoides* species are modelled in relation to satellite-derived ecological correlates, and host and environmental variables at the 1-km scale, with determinants of distribution compared between species. Specific objectives included building a model for Obsoletus Group (*C. chiopterus*, *C. dewulfi*, *C. obsoletus* and *C. scoticus*), determining whether predictors could be generalized across midge species with particular ecological characteristics (groups) and investigating the use of satellite imagery at the local spatial scale.

Materials and methods

TRAPPING DESIGN

This study was undertaken in the Welsh province of Bala, situated in Snowdonia National Park. This area primarily consists of extensive sheep and beef cattle farming, with a very hilly landscape comprising a mixture of forests and field. A 6 × 6 km grid was overlaid on a 1 : 25 000 ordinance survey map of the area just north of Bala lake, north Wales. In each grid square (36 in total), one farm or smallholding was selected to participate in the study, although due to the nature of the terrain, two squares contained no properties. One further control farm, outside the gridded region, also participated (35 farms in total). All farms were recruited via personal contact.

A thrice-replicated randomized trapping grid allocated the selected trapping sites to a 12-night trapping schedule between 7 July and 18 July 2008. Each farm was therefore sampled for a total of three trapping nights.

QUESTIONNAIRE DESIGN

A short questionnaire was designed to capture data on (i) host characteristics – number and distance of dairy, beef, sheep, horses and other animals; use and frequency of insecticide administration on animals, buildings and dung heaps; (ii) Farm surroundings/environment – presence of breeding sites (dung or manure heaps, leaf litter and food heaps) within 250 m radius of traps; water sources (standing and running water, waterlogged ground, artificial sources such as troughs) within 250 m of traps. Altitude of each trapping site was measured using a Garmin eTrex® H GPS receiver (Olathe, Kansas, USA).

TRAPS AND MIDGE COLLECTION

Trapping was undertaken using 15 Onderstepoort-type down draught black light traps connected to either a mains power source or car battery. Such traps measure a mixture of *Culicoides* abundance in an area as well as their activity and attraction to light. Traps were positioned as close to livestock as possible, and the number of livestock within 50 m was recorded each night. Midges were collected in 500-ml beakers containing *c.* 200 ml of water and a small amount of washing-up liquid to break the surface tension of the water. Traps were set between 1600 and 1800 h and collected between 0800 and 1000 h the following morning when collections were transferred to 70% ethanol for storage.

INSECT SORTING

Culicoides sorting and counting was undertaken at the Onderstepoort Veterinary Institute Agricultural Research Council, South Africa. Large collections were subsampled (Van Ark & Meiswinkel 1992), and females were age-graded into nulliparous, parous, gravid or blood-fed based on abdominal pigmentation (Dyce 1969). Males were also counted, but all other insects were stored uncounted. For the Obsoletus Group, the females of four constituent species (*C. chiopterus*, *C. dewulfi*, *C. obsoletus* and *C. scoticus*) were counted together, while males were counted separately. Only females were considered in the analyses as males do not take blood meals and consequently do not act as vectors of disease between vertebrates. For the Pulicaris Group, *C. pulicaris*, *C. punctatus* and *C. impunctatus* catches were modelled together, as well as separately. In Europe, members of the Obsoletus Group, as well as the *C. pulicaris* and *C. punctatus* members of the Pulicaris group, are considered the most important vectors (Mellor & Pitzolis 1979; Carpenter, Wilson & Mellor 2009); we have considered the other *Culicoides* species trapped to be non-vectors.

SATELLITE-DERIVED CLIMATE DATA

Seventy remotely sensed variables were derived from MODerate-resolution Imaging Spectroradiometer (MODIS) imagery from the NASA Terra satellite (Scharlemann *et al.* 2008). Five variables with environmental significance were extracted at 1-km grid resolution between 2001 and 2005: NDVI, MIR, day and night land surface temperature (dLST and nLST) and enhanced vegetation index (EVI). NDVI is a measure of chlorophyll abundance, correlated with soil moisture, rainfall and vegetation biomass, coverage and productivity (Campbell 1996). MIR is correlated with water content, surface temperature and vegetation canopy structure (Boyd & Curran 1998). EVI is similar to NDVI, measuring vegetation activity correlated with levels of soil moisture (Chen *et al.* 2006; Waring *et al.* 2006), but has improved sensitivity in wet zones with high biomass. For each of these factors, 14 temporal Fourier-processed (Rogers 2000) predictors were produced (Table 1).

DATA ANALYSIS

For Pearson Product-Moment correlations between trap catches of different *Culicoides* spp., the critical value for significance was adjusted to a lower threshold using the Bonferroni correction to take account of multiplicity of P values.

Nightly species, or group, catches were $\log_{10}(n + 1)$ transformed, and the maximum of the three catches per farm was used in model building (hereafter, log-max catch). The maximum catch was preferred to the mean because *Culicoides* catches are readily reduced by weather conditions, and, arguably, the maximum provides a better measure of abundance over a short time period (Baylis *et al.* 1997). Abundance models were not parameterized for seven of the 19 species due to low catches. For the 12 other species, none of the distributions differed significantly from normality (Anderson-Darling Test for Normality, $P \geq 0{\cdot}4$ for all species). Log-max catches for these species were related to satellite-derived ecological correlates, host, and environmental

Table 1. Temporal Fourier-processed predictors of five environmentally significant variables derived from MODIS imagery

MODIS variable	Explanation
a0	Overall mean amplitude
a1	Amplitude of the annual cycle
a2	Amplitude of the biannual cycle
a3	Amplitude of the triannual cycle
p1	Phase (peak timing) of the annual cycle
p2	Phase (peak timing) of the biannual cycle
p3	Phase (peak timing) of the triannual cycle
d1	Proportion of variance explained by the annual cycle
d2	Proportion of variance explained by the biannual cycle
d3	Proportion of variance explained by the triannual cycle
da	Proportion of variance explained by the annual, biannual and triannual cycles combined
mn	Minimum of the seasonal cycle
mx	Maximum of the seasonal cycle
vr	Variance

variables using General Linear Models (McCullagh & Nelder 1989) in R version 2.8.1 (R Development Core Team, 2011).

Predictor sets were (i) host factors – on farm sheep, dairy, beef, horse and other animal density; insecticide use – on animals, buildings and dung heaps; (ii) environmental factors – presence of breeding sites composed of leaves, dung heaps, food piles and number of breeding sites within 250 m; presence of running, standing, poor draining, artificial or other water sources and number of water sources within 250 m and (iii) 70 remotely sensed variables – 14 NDVI, 14 EVI, 14 MIR, 14 dLST and 14 nLST variables derived from MODIS satellite data (as described by Scharlemann *et al.* 2008).

The number of explanatory variables available for multivariable modelling was reduced, in order to minimize the risk of overfitting the model. Explanatory variables were examined for univariable Pearson Product-Moment correlations with log-max catch. Only variables with a probability of correlation <0·2 were retained. Collinear variables least correlated with log-max catch were removed.

For model development, a best subsets approach was first used to select a subset of variables (within each of the three predictor sets) that best explained the variation in abundance of each species, where the R^2 and adj-R^2 were the highest possible, while each variable was significant at the $P \leq 0{\cdot}05$ level individually. Finally, the subsets of variables were combined across predictor sets into a global model and the selection procedure, based on R^2 (adj-R^2) values and individual variable significance, repeated to produce a final model for each species. When multiple models fulfilled these criteria, the final model was determined on the basis of having a lower Akaike's Information Criterion value (AIC, Akaike 1973).

The small number of sampled sites precluded partitioning the data into a calibration and evaluation data set. Therefore, to evaluate likely generalization errors of the final models (i.e. overfitting), leave-one-out regression analysis allowed cross-validation to occur, whereby each data point in turn is left out of the analysis and the final model refitted. The stability of variance explained, coefficients and fitted abundance values were evaluated across leave-one-out models.

Variograms were computed from the models' residuals to identify residual spatial autocorrelation, or second-order (local) effects. Second-order effects describe small-scale variation due to the interactions between neighbours (Pfeiffer *et al.* 2008). The Moran's I correlation coefficient (Moran 1950) was employed, with fixed neighbourhood sizes of 1·5, 2 and 3 km, to evaluate spatial patterns and examine residual spatial autocorrelation between farms. Moran's *I* is one of the most established indicators of spatial autocorrelation, and like the correlation coefficient, its values range from 1 (strong positive spatial autocorrelation), through 0 (a random pattern), to -1 (strong negative spatial autocorrelation).

Results

TRAPPING

The 175 trap catches produced a total of 357 233 *Culicoides* of 19 species in the Bala region. The single largest catch was 65 763 midges in one trap over one night, while the mean of the maximum catches was 2706 midges per trap per night. One catch contained zero *Culicoides* due to trap malfunction. A total of 61·9% of the *Culicoides* trapped across sites belonged to the Obsoletus Group and 31·6% to the Pulicaris Group. Of the latter, *C. punctatus* and *C. impunctatus* were the most abundant species, making up 15·4 and 14·4% of the total *Culicoides* sampled, whilst *C. pulicaris* comprized 1·8%. Of the other species, *C. achrayi* contributed 5·5%, while the others made up <1% each (Table 2). Due to the low catches of *C. brunnicans*, *C. circumscriptus*, *C. kibunensis*, *C. minimus*, *C. nubeculosis*, *C. pictipennis* and *C. stigma* models of abundance were not parameterized.

Spatial variation in maximum abundance of the Obsoletus and Pulicaris Groups, along with the three Pulicaris Group species individually, can be seen in Fig. 1. The Obsoletus Group exhibited an almost 500-fold difference in maximum catches (74–33,720) between farms, but *C. punctatus* displayed the highest variation with an almost 4000-fold difference (6–23 656) across sites, while the Pulicaris Group exhibited the lowest 330-fold difference (85–28 423). Similar spatial patterns are seen between the male Obsoletus Group members in Fig. 2.

High correlation emerged between trap catches of females of certain *Culicoides* spp. on farms (Table 3). Obsoletus Group abundance was highly correlated to that of the vectors *C. pulicaris* and *C. punctatus*, but also the non-vector *C. achrayi*, which was associated with five of the eight species examined. The vector species, *C. pulicaris* and *C. punctatus*, were significantly associated. The non-vector species *C. impunctatus*, although a member of

Table 2. *Culicoides* species trapped around Bala

Species trapped	Female (% of total catch)	Male (% of total catch)
Obsoletus group total	211 927 (64·92)	9246 (30·02)
By Species		
C. chiopterus	–	14
C. dewulfi	–	1694
C. obsoletus	–	6576
C. scoticus	–	962
Pulicaris group total	97 262 (29·80)	15 615 (50·71)
By species		
C. impunctatus	37 229	14 225
C. pulicaris	5992	574
C. punctatus	54041	816
Other *Culicoides*	17 250 (5·28)	5933 (19·27)
By Species		
C. achrayi	14 111	5458
C. albicans	1135	346
C. brunnicans	23	0
C. circumscriptus	5	0
C. delta	402	1
C. fascipennis	1249	56
C. festivipennis	168	26
C. kibunensis	45	41
C. minimus	1	0
C. nubeculosus	101	5
C. pictipennis	9	0
C. stigma	1	0
Total	326 439 (100)	30 794 (100)

the same subgenus as *C. pulicaris* and *C. punctatus*, was most strongly associated with the other non-vector species *C. achrayi*. Males of the individual species were highly correlated to the females (except *C. pulicaris* where $P = 0{\cdot}08$), apart from *C. fascipennis* and *C. festivipennis* which were not correlated. Males of the Obsoletus Group constituent species (*C. chiopterus*, *C. dewulfi*, *C. obsoletus and C. scoticus*) were also highly correlated to each other ($P = 0{\cdot}001$) except for *C. chiopterus* with only 11 males in the total maximum catch.

Mean parous rate and range for the eight species modelled were for the Obsoletus Group 0·84 (0·4–0·94), *C. achrayi* 0·83 (0·36–0·94), *C. albicans* 0·64 (0–1), *C. fascipennis* 0·79 (0–1), *C. festivipennis* 0·31 (0–1), *C. impunctatus* 0·83 (0·49–0·99), *C. pulicaris* 0·34 (0–0·72) and *C. punctatus* 0·78 (0·31–0·99). The parous rates of four of these species were significantly correlated to their abundance (*C. achrayi*: $r = 0{\cdot}46$, $P = 0{\cdot}008$; *C. albicans*: $r = 0{\cdot}35$, $P = 0{\cdot}06$; *C. impunctatus* $r = 0{\cdot}39$, $P = 0{\cdot}03$; *C. pulicaris* $r = 0{\cdot}35$, $P = 0{\cdot}05$).

QUESTIONNAIRE DATA

Of the 35 farms, 22 (62·9%) kept sheep, 14 (40%) beef cattle and two (5·7%) horses, while only one kept dairy cattle (Table 4). Other animals included pigs, dogs and chickens. Three farms had sheep present, but did not own them and were unaware of the exact numbers. As the 'number of sheep' was determined as an important variable early on, it

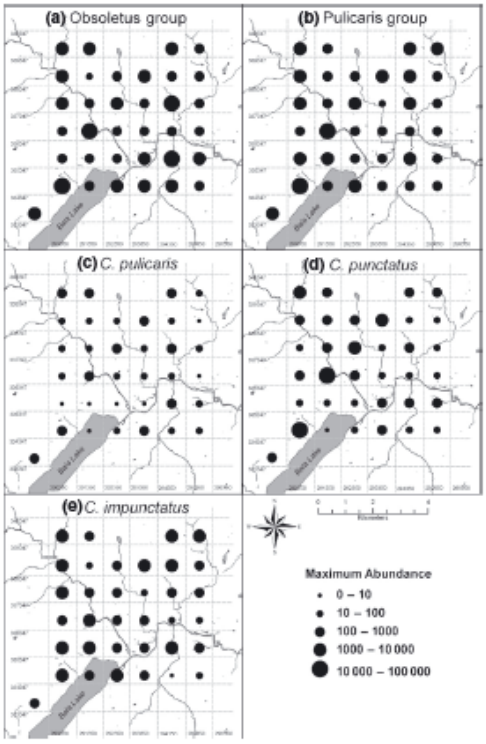

Fig. 1. Spatial variation in maximum log(n + 1) abundances of the (a) Obsoletus Group, (b) Pulicaris Group and the Pulicaris Group constituent species: (c) *C. pulicaris*, (d) *C. punctatus*, (e) *C. impunctatus*, across trapping sites. Abundances have been centred in each grid square for anonymity.

was decided that data from those three farms would be omitted from model building, leaving 32 observations.

Nineteen farms (54·3%) used insecticides regularly on animals, but none on buildings or for dung management, although 21 (60%) farms had dung piles within 250 m of the trapping site (mean 18·29 m, range 1–100 m). In terms of other breeding sites, 24 (68·57%) farms had leaf litter between 1 and 75 m (mean 22 m), and eight (22·86%) had food heaps within 20–150 m (mean 54 m) of the traps (Table 5). The mean altitude on the farms was 237·9 m, but varied between 169·1 and 335·1 m.

MODIS SATELLITE DATA

In the 2001–2005 MODIS data, day-time temperatures peaked in Bala in early to mid-June, and night-time temperatures in late June. Site temperatures ranged from −0·8 to 20·8 °C (mean 10·9 °C) in the day-time and −6 to 9·2 °C (mean 0·9 °C) at night-time. The peak of NDVI occurred between late June and early August, and the seasonal range in this index varied from 1·617 to 1·871. The peak of EVI occurred between early and late June, and

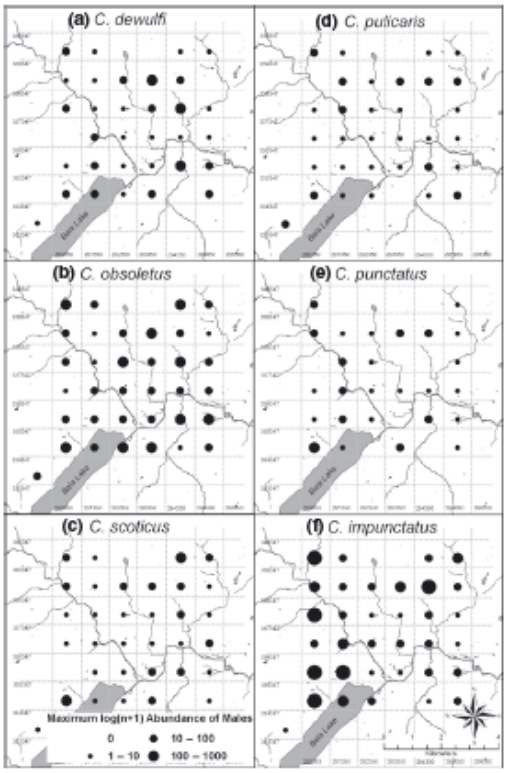

Fig. 2. Spatial variation in maximum $\log(n + 1)$ abundances of the male Obsoletus Group species (a) *C. dewulfi*, (b) *C. obsoletus*, (c) *C. scoticus;* and the Pulicaris Group constituent species: (d) *C. pulicaris*, (e) *C. punctatus*, (f) *C. impunctatus*, across trapping sites. Abundances have been centred in each grid square for anonymity. No map was drawn for the *C. chiopterus* member of the Obsoletus Group as there were only 11 males trapped across four sites in the maximum catches.

the seasonal range at sites varied from 1·140 to 1·739. For MIR, a peak occurred across most sites between early March and May, although the peak on one farm occurred in late February. The seasonal range varied from 0·036 to 0·129.

OVERALL PERFORMANCE OF MODEL VARIABLES

The variables included in the final models are shown in Table 6. Overall, NDVI was the only explanatory variable selected in all models, while MIR was seen in six. The only host variable within the final models was 'number of sheep' on a farm, while for environmental variables the number of water sources, food heaps and dung heaps were included. The vector group models (Obsoletus and Pulicaris Groups) both contained a host variable (sheep), as did the two individual vector species models (*C. pulicaris* and *C. punctatus*), while this remained absent in the non-vector models. See Table S1 in Supporting Information for the coefficients of the model parameters.

MODEL EVALUATION

Moran's I tests for spatial autocorrelation were insignificant at all neighbourhood sizes for all species and groups, except *C. festivipennis* which exhibited negative spatial autocorrelation at the 1·5-km and 2-km neighbourhood sizes (in a 1·5-km neighbourhood $I = -0{\cdot}39$, $P = 0{\cdot}003$). The Obsoletus Group model verged on, but did not reach significance at the 1·5-km neighbourhood size ($I = -0{\cdot}2596$, $E[I] = -0{\cdot}0323$, $P = 0{\cdot}06$). The insignificant Moran's I values suggest spatial autocorrelation has little, or no, influence on patterns of midge trap catches at these scales.

Leave-one-out regression analysis was used for cross-validation due to the small number of sampled sites. Figure 3 shows the fitted abundance values from the leave-one-out regressions for the six most abundant species. Due to the stability of the fitted abundance values and variance explained, low generalization errors would be expected if our predictions were extended to another region of north Wales.

Discussion

This study represents the first attempt to explain and predict trap catch patterns of the Obsoletus Group, Pulicaris Group species and other potential UK vectors at a high

Table 3. Pearson Product-Moment correlation coefficients of the abundance of females of different species of *Culicoides* spp. on farms around Bala

	Obsoletus Group	*C. pulicaris*	*C. punctatus*	*C. impunctatus*	*C. delta*	*C. fascipennis*	*C. festivipennis*	*C. albicans*
C. pulicaris	0·828*							
C. punctatus	0·745*	0·838*						
C. impunctatus	0·401	0·408	0·414					
C. delta	0·372	0·312	0·326	−0·288				
C. fascipennis	0·336	0·393	0·606*	0·293	0·306			
C. festivipennis	0·395	0·278	0·268	0·139	0·503	0·261		
C. albicans	0·337	0·348	0·482	0·531	0·124	0·385	0·369	
C. achrayi	0·637*	0·605*	0·593*	0·703*	0·104	0·407	0·342	0·582*

Significance (determined using the Bonferroni correction) is given where $*P \leq 0{\cdot}001$.

Table 4. Host animals on farms around Bala

Host variable	Number of farms (%)	Mean* number of animals	SD	Range
Sheep	22 (62·86)	541·41	688·92	8–2600
Beef Cattle	14 (40)	90·57	91·29	2–350
Horses	2 (5·71)	3	1·41	2–4
Dairy Cattle	1 (2·86)	70	NA†	NA†
Pigs	1 (2·86)	40	NA†	NA†

SD, standard deviation.
*Mean of those farms with animals.
†Not applicable.

Table 5. Breeding sites and water sources on farms around Bala

Predictor variable	Number of farms (%)	Mean distance (m)*	SD	Distance range (m)
Breeding sites†				
Dung	21 (60)	18·29	25·85	1–100
Leaf Litter	24 (68·57)	21·83	21·80	1–75
Food Heaps	8 (22·86)	54·29	44·01	20–150
Water sources†				
Running	24 (68·57)	104·22	91·12	2–250
Standing	9 (25·71)	197·22	104·79	5–250
Wet Ground	18 (51·43)	70·15	86·06	2–250
Artificial	22 (62·86)	12	16·77	1–50

SD, standard deviation.
*From light trap.
†Within 250 m of the trapping site.

resolution (1 km) in relation to a range of ecologically relevant satellite, environmental and host factors. *Culicoides* inhabit a wide range of moist microhabitats in agricultural and natural ecosystems (Mellor, Boorman & Baylis 2000). As such, mapping potential areas of disease risk and targeting disease control measures requires models that can explain and predict local-scale variation in abundance, rather than simply occurrence, of species.

The Obsoletus and Pulicaris groups were the most abundant *Culicoides* caught in the Bala area, in agreement with previous studies in north Wales (McCall & Trees 1993; Baylis *et al.* 2010). *Culicoides achrayi* and *C. impunctatus* were the most abundant non-vector species. *Culicoides achrayi* is also highly prevalent on Belgian farms (Haubruge 2008). In contrast, relatively few *C. achrayi* were trapped at Chester Zoo (Vilar *et al.* 2011) and instead a high-proportion *C. kibunensis*. The reasoning behind this is unclear, but apparent opposing preferences for a farm or zoo environment are likely due to differences in these species' life-history characteristics.

Large differences between *Culicoides* catches on neighbouring farms highlight the fact that catches on one farm should not be deemed representative of a region. This raises questions about the validity of nationwide entomological surveillance schemes which, inevitably, trap at coarse resolutions. Our results suggest large-scale surveillance should consist of multiple trapping sites in each sampling area, or undertaking a prior survey of multiple farms in each region and proceeding with those that yield the highest catches.

A trade-off exists between temporal and spatial aspects of most surveys. In our study, the intense spatial detail (one trap per km^2) and high-frequency nightly trapping required a degree of effort and generated a volume of midges that precluded undertaking trapping longer than three nights per site and 12 nights in total. This may limit our ability to generalize findings in both space and in time. We cannot be sure that the same farm-to-farm heterogeneity occurs elsewhere in the United Kingdom or at Bala at other times of year or in other years. However, a study near Bala in May–June 2007 reported significant heterogeneity in *Culicoides* catches between just four farms over 12 nights trapping on each farm, suggesting that our findings are robust, at least for Bala. If verified, the small-scale variability observed in this study should highlight an area of concern for those interpreting large-scale studies with scarce sampling points.

High local spatial variation may also explain the difficulty of building strong large-scale models for the Obsoletus Group (Purse *et al.* 2012). We have successfully modelled high spatial variation at the small spatial scale for several species, including the Obsoletus Group. One reason for the success of high-resolution models is that nearby farms may differ in the levels of important explanatory variables while distant sites spread across large areas may differ in the nature of those explanatory variables.

Abundances of several vector species (Obsoletus Group, *C. pulicaris* and *C. punctatus*) were correlated, suggesting common predictors of abundance due to similar life-history characteristics, such as the presence of live hosts. Similarly, male abundances of three of the Obsoletus Group species were correlated, again suggesting similar ecological factors favour abundance.

Calvete *et al.* (2008) found incorporating host variables into bioclimatic models vastly increased the variance explained in BTV-4 occurrence in Spain. In Italy, both biotic (forest and vegetation activity) and abiotic (topography, temperature and aridity index) axes were found to govern the occurrence of the *C. obsoletus* group (Conte *et al.* 2007a). Half of our models contained satellite variables only, and there was no significant difference between the explanatory power of these or the mixed predictor models. This concurs with Calvete *et al.* (2008) who, from the superior performance of climate only models, inferred that bioclimatic variables were the main ecological factors driving BTV occurrence across Spain. Satellite-derived ecological correlates dominated in number in the final models for all species in Bala, highlighting that the importance of these ecological drivers extends to the local scale.

For the Obsoletus Group, four species (*C. chiopterus*, *C. dewulfi*, C. *obsoletus* and *C. scoticus*) with a mix of host and breeding-habitat preferences were modelled together. Even so, the model explained a high amount of

Table 6. Abundance models for each species or group, including percentage of variance explained (R^2 and adjusted R^2) and model AIC (Akaike's Information Criterion). After each variable, the () indicates the operator of the correlation coefficient

Species/Group	NDVI	EVI	MIR	LST		Other	AIC (Null Model AIC)	Mean R^2(%) (adjusted R^2(%))
				dLST	nLST			
Obsoletus Group	p3 (+) d1 (+) da (–) mx (+)	–	a3 (+) d3 (–)	d2 (–)	p3 (+)	Sheep (+) Water (–)	40·035 (73·587)	81·23 (72·29)
Pulicaris Group	p3 (+) d1 (+) da (–)	a3 (+) d1 (+) d2 (+) da (–)	p2 (+)	–	–	Sheep (+)	28·462 (62·533)	80·41 (72·39)
C. impunctatus	p2 (–)	a1 (–) a3 (+) d3 (–) mx (–)	–	a0 (+) p1 (+)	p3 (–) d2 (+) d3 (–)	–	35·565 (68·759)	80·93 (71·85)
C. pulicaris	p3 (+) d3 (–) mx (+)	p1 (–)	–	p3 (–)	p3 (+)	Sheep (+)	48·876 (76·84)	73·10 (65·25)
C. punctatus	p3 (+) d1 (+) mx (+)	p1 (–) d1 (–)	–	–	–	Sheep (+)	44·518 (75·851)	74·17 (67·97)
C. scirpyi	a1 (+) a3 (–) d1 (–) d3 (+) da (+) vr (–)	a3 (+) p2 (–) d1 (+) d2 (+) da (–) mx (–)	–	–	p1 (+) p2 (+) p3 (–)	Breeding Environments (–)	31·712 (58·435)	84·10 (67·15)
C. albicans	a1 (–) p3 (+) d1 (+) d3 (–) vr (+) mn (+)	a0 (–) mx (+)	p1 (+) da (–) mx (+)	–	p3 (+)	Water (+)	41·030 (50·362)	65·78 (41·07)
C. delta	vr (+) mx (+)	–	a1 (–) p2 (–) mn (–) mx (+)	a0 (+) a1 (+) mx (–)	a3 (+) p3 (–) mn (–)	–	28·469 (63·01)	84 (73·90)
C. fascipennis	a1 (+)	a1 (–) d1 (+) da (–) vr (+) mx (+)	–	a1 (–)	a1 (+) d1 (–) d2 (+) vr (–)	–	15·671 (61·5)	87·97 (81·35)
C. festivipennis	a1 (–) p1 (+) d1 (+) da (–) vr (+) mn (+)	a0 (+) da (+)	p1 (–)	a1 (+) a2 (+) a3 (–) vr (–)	p3 (+)	–	23·014 (44·433)	78·57 (60·93)

Remotely sensed variables: NDVI, normalized difference vegetation index; EVI, enhanced vegetation index; MIR, middle infra-red reflectance; dLST, day land surface temperature; nLST, night land surface temperature.
MODIS variables: a = amplitude; p = phase (peak timing); d = proportion of variance explained by the 1 = annual cycle, 2 = biannual cycle and 3 = triannual cycle. da = proportion of variance explained by all three cycles combines; mn = minimum of the seasonal cycle; mx = maximum of the seasonal cycle; vr = variance.

variance (81%) and was consistent with other studies detecting large impacts of landscape factors, such as forest cover and vegetation activity, on the distribution patterns of the Obsoletus Group or Complex (Purse *et al.* 2004a; Conte *et al.* 2007a; Calvete *et al.* 2008). The goodness of fit of the group model could either indicate that one species is highly dominant (and thus the model most influenced by its requirements) or that all species have similar habitat requirements. Further work should be undertaken to explore whether these species' habitats differ.

The distributions of the Obsoletus Group were dominated by remotely sensed NDVI variables. Most NDVI variable coefficients were positive, indicating a preference for microclimates with high levels of moisture, favouring vegetation growth. This is supported by

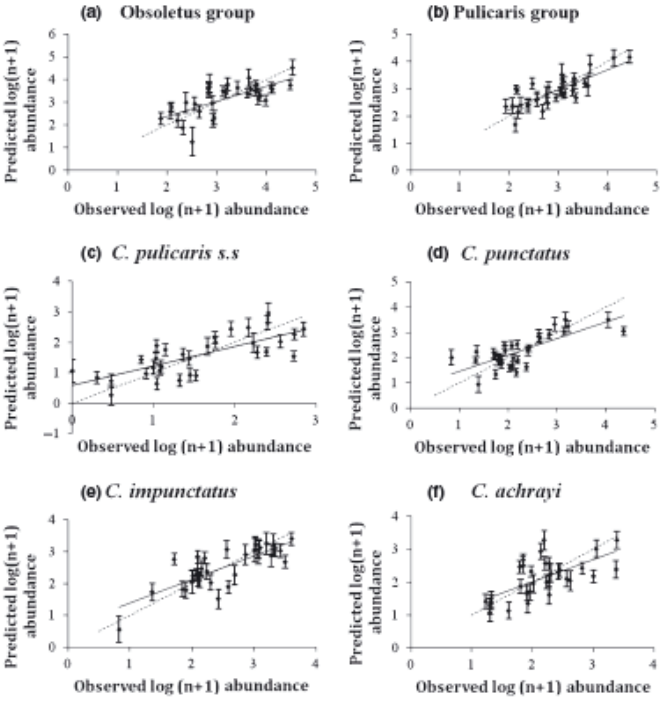

Fig. 3. Predicted vs. observed log(n + 1) abundance for the (a) Obsoletus and (b) Pulicaris Groups and for (c) *C. pulicaris*, (d) *C. punctatus*, (e) *C. impunctatus* and (f) *C. achrayi*. Error bars indicate the magnitude of standard errors, solid black line indicates line of best fit and dashed black line indicates line of equality.

previous observations that *C. obsoletus* breeds preferentially in forest litter (Amosova 1956; Dzhafarov 1964). Conte *et al.* (2007a) also found significant correlation between areas of deciduous and mixed broadleaved/coniferous forests and the Obsoletus Complex. The inclusion of two MIR variables, also correlated with vegetation levels (Boyd & Curran 1998), also supports this theory. The correlation of 'number of water sources' with the Obsoletus Group indicates that moist habitats are favourable, likely due to their semi-aquatic larval stage, and, indeed, *C. obsoletus* has been reared from wet areas of Scotland (Kettle & Lawson 1952).

Obsoletus Group trap catches also increased with the number of sheep on farms. This is in agreement with Garcia-Saenz, McCarter & Baylis (2010) who found a linear increase between *C. obsoletus* trap catches and sheep number.

Molecular identification was deemed unnecessary as there is currently insufficient information to determine vector competence of the Obsoletus Group species individually. If it becomes clear that there are important differences between vector competences, molecular identification of the Obsoletus Group species would provide further evidence of their ecological differences. Whilst sufficient numbers of males were caught and could be used to model the Obsoletus Group species, there is little evidence their relative abundance is proportional to the females of each species.

Like the Obsoletus Group, *C. pulicaris* and *C. punctatus* abundances were positively correlated with NDVI variables and sheep number. Catches of *C. pulicaris*, a wet-soil and bog species, have been associated with high, stable, levels of moisture (high, less variable NDVI) elsewhere in Europe (Purse *et al.* 2004b, 2005). The *C. punctatus* model is similar to that of *C. pulicaris*, only lacking in LST, highlighting the close relationship of the two species. The inclusion of LST variables in the *C. pulicaris* model may highlight the species' need for more stable temperatures, as highlighted by Parker (1950) who found that *C. punctatus* eggs are less adversely affected by above-normal temperatures than *C. pulicaris* eggs.

The *C. impunctatus* model, unlike the other Pulicaris Group species models, was dominated by LST variables. The annual mean of the dLST indicates a preference for warmer temperatures and a later peak in day-time temperatures (positive coefficient for p1). EVI featured heavily in this model, with an increased trap catch in locations with low variation in vegetation activity throughout the year and, in converse to the Obsoletus Group model, less densely covered areas with more access to sunlight. This is likely to represent this species' preference for organically enriched, soil-breeding sites with high water content (Blackwell, Young & Mordue 1994; Blackwell *et al.* 1999). Kettle & Lawson (1952) describe that immature stages of *C. impunctatus* are commonly found in bogland sites in wetter areas of moorland where *Sphagnum* and

Polytrichum moss growth is thin enough to permit penetration by *Juncus articulates*, a species of rush that thrives in hot overhead sunlight. The exclusion of 'number of sheep' in the *C. impunctatus* model may be attributable to its autogenous nature (Boorman & Goddard 1970), making the species less reliant on host blood meals than anautogenous vector species. In turn, the only models incorporating host factors are those that have, currently, been implicated as BT vector species.

Four of the species had parous rates that were significantly correlated to their abundance. A high parous rate is an indication of survivorship, and the correlation for these four species suggest that survivorship, as opposed to proximity to breeding sites, is the reason for the high population sizes, reinforcing the reliability of this data.

Analysing a large number of explanatory variables relative to the number of data points creates a danger of overfitting. Leave-one-out regression analysis is a useful technique for examining overfitting as overfitted models often show poor predictive ability. The leave-one-out regressions of our models show good predictive ability, and therefore, while we accept there was a risk of overfitting, our models show no evidence of it.

Our study highlights the high variation in *Culicoides* abundance that can occur between neighbouring farms, but work still needs to be undertaken to determine the level of variation present at the smaller, within-farm scale and the factors driving that variation. Given the remarkable heterogeneity detected, we recommend that large-scale surveillance includes multiple sites per region or that a prior survey of each region is undertaken to determine those farms with the highest *Culicoides* catches.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Abundance models for each species/group, including coefficients of the model parameters.

APPENDIX B

I) Farm details:

Farm name: ..... Farm grid square: .....

Farm owner: ..... Phone number: .....

Farm address: .....

.....

.....

GPS point				Picture
Point Nb	East (long)	North (lat)	Altitude (m)	Picture Nb

II) Farm characteristics:

What animals are present today on your farm and how many?

Dairy	Beef	Sheep	Horses	Others (what + nb)

Insecticide use and dung management:

Do you use any insecticides

- on the animals? Y N
  - If yes : name of insecticide: .....
  - On which animals: .....
  - Method of administration (pour-on, bath, spray, oral, injected): .....
  - Frequency: .....
  - Date of last use: .....
- on the buildings? Y N
  - If yes : name of insecticide: .....
  - Frequency: .....
  - Date of last use: .....
- on the dung heap? Y N
  - If yes : name of insecticide: .....
  - Frequency: .....
  - Date of last use: .....

Please describe any methods of dung management (turning...) used: .....

.....

.....

.....

III) Farm surroundings:

Potential midge breeding sites:

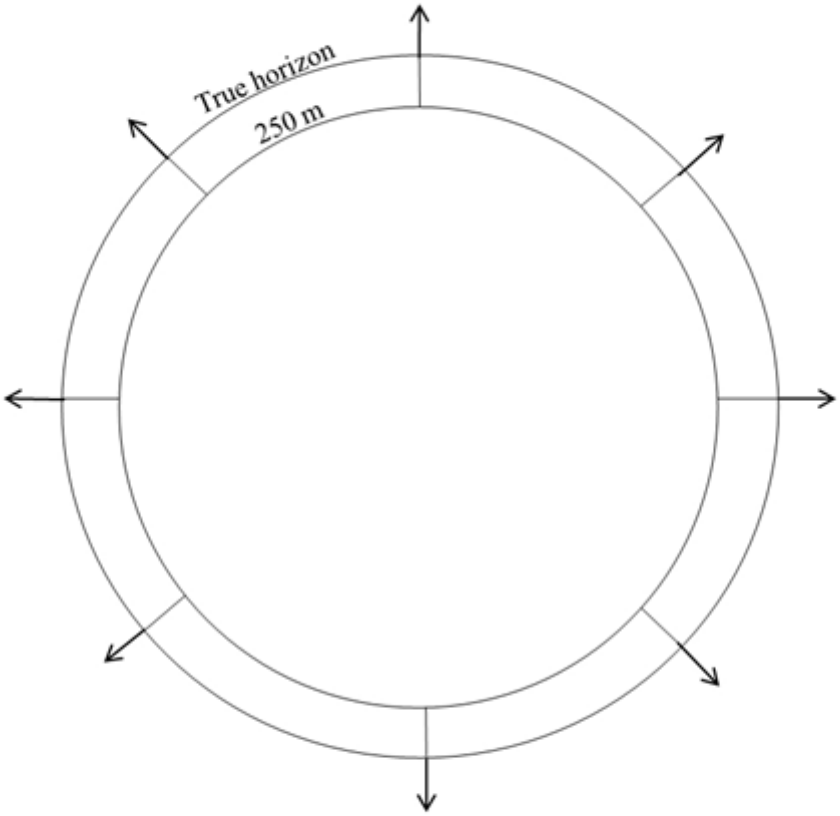
Are any of the potential breeding sites listed below present within 250 m radius of the trap?

Type	Presence	Distance
Dung/manure heaps		
Leaf litter under trees		
Food heap (beet or other)		

Water sources:
Are any of the water sources listed below present within a 250 m radius of the trap?

Type	Presence	Distance (to nearest one)
Natural running water (stream, river)	Y N	
Natural standing water (pond, swamp, lake)	Y N	
Large natrual areas of poorly drained land which are sometimes flooded (marshes)	Y N	
Other patches of ground that are regularly wet in summer	Y N	
Are any artificial water sources (e.g. water troughs, containers, leaking drains...) causing wet ground	Y N	

Landuse and degree of openness:
Imagine the trap as the centre of the circle.
1. Looking no further than 250m, mark within the two concentric rings the circumference of the horizon taken up by buildings, deciduous trees, coniferous trees, water, open field, other (name).
2. On the outside of the ring, mark approximately the distance to the true horizon from the trap for each of the 8 compass points (categories: a: <5m; b: 5-20m; c: 20-100m; d: 100-500m; e: >500m).



APPENDIX C

UNIVERSITY OF LIVERPOOL

Farm Questionnaire: **Midge Trapping**

LUCINDA

This Project: Midges (*Culicoides spp.*) are known to be important vectors of disease, in particular the Bluetongue virus. Our previous studies have shown that midges are abundant in the Bala region, and the numbers caught seemed to vary greatly between farms. The main aim of this study is to determine the variation in the number of midges caught at different sites on a single farm.

Please read each question carefully and answer in BLOCK CAPTIALS.

FARM DETAILS

Reference Number: _____ Latitude (N): _____ Longitude (E): _____ Altitude: _____

Name:

Farm Name:

Address:

Postcode:

Telephone:

What is the size of your farm? ☐☐☐☐☐ acres / hectares *(delete as appropriate)*

Type of farm: *(Tick all that apply)* Dairy ☐ Beef ☐ Sheep ☐ Goats ☐ Arable ☐ Pigs ☐

Other ☐ - *please state:*

Main Species:

How many of the following animals are on your farm?

Sheep	☐☐☐☐	Cattle	☐☐☐☐	Goats	☐☐☐☐	Horses	☐☐☐☐
Lambs	☐☐☐☐	Pigs	☐☐☐☐	Dogs	☐☐☐☐	Other	☐☐☐☐

Using the following satellite picture of your farm as a reference please use the attached maps to mark out your land and where your animals are currently located (examples are included):

Satellite Image Removed for Confidentiality

INSECTICIDE USE

Do you use insecticides on your farm? YES ☐ NO ☐

If yes;

Name of insecticide(s) used: Crovect ☐ Dectomax ☐ Vetrocin ☐ Trodax ☐ Virbamec ☐
Clik ☐ Ectoforce ☐ Cydectin ☐ Enovex ☐ Frontline ☐

Other: ☐ *(please state)* _____

Administration method: Pour-on ☐ Spray ☐ Injection ☐
Dip ☐ Oral ☐ Bath ☐

Do you believe you have an insect/ midge problem at your farm? YES ☐ NO ☐

If yes, briefly why?

What species do you treat with insecticides? *(Tick all that apply)*

More details of types of insecticides and administration methods can be found in the question above.

	YES	Insecticide	Frequency *(per year)*	Administration method	Date of last use
Dairy Cattle					
Beef Cattle					
Sheep					
Lambs					
Goats					
Pigs					
Dogs					

Any other information:

Completion of this questionnaire will be taken as consent to use the information provided. Data submitted to this study will be kept strictly confidential and only be accessed by the researchers involved. During the write up data will be presented completely anonymously, without referring to individual farmers or farms. All farms will be sent a summary of the main findings at the end of the project. Participation is entirely voluntary and you may withdraw at any time, without determent to you or your farm.

UNIVERSITY OF LIVERPOOL

Thank you for your time.

LUCINDA

APPENDIX D

MATERIAL SAFETY DATA SHEET

BGP SERIES FLUORESCENT PIGMENTS

VERSION: 001
ISSUE DATE: October 23, 2008

SECTION 1: PRODUCT AND COMPANY IDENTIFICATION

PRODUCT NAME:	BRILLIANT GENERAL PURPOSE FLUORESCENT COLORANTS
SYNONYMS:	BGP SERIES
PRODUCT CODES:	BGP-PK111, BGP-CE112, BGP-RD113, BGP-OR114, BGP-OG115, BGP-OY116, BGP-YE117, BGP-GR118, BGP-BL119, BGP-VT120, BGP-MG121, BGP-BL129
MANUFACTURER:	BRILLIANT GROUP, LLC
ADDRESS:	PO BOX 590128 SAN FRANCISCO, CA, 94159-0128
EMERGENCY PHONE:	+1-415-771-4757
FAX:	+1-415-789-4150
CHEMICAL NAME:	DYED PROPRIETARY POLYMER
PRODUCT USE:	COLORATION OF PAINTS, COATINGS, INKS AND PLASTICS
PREPARED BY:	DARREN BIANCHI

SECTION 2: COMPOSITION/INFORMATION ON INGREDIENTS

COMPOSITION:	DYED PROPRIETARY POLYMER
INGREDIENT:	CONTAINS NO REPORTABLE HAZARDOUS INGREDIENTS

SECTION 3: HAZARDS IDENTIFICATION

EMERGENCY OVERVIEW: NON HAZARDOUS

ROUTES OF ENTRY: INHALATION, INGESTION

POTENTIAL HEALTH EFFECTS

EYES:	MAY CAUSE IRRITATION
SKIN:	MAY CAUSE IRRITATION
INGESTION:	SWALLOWING SMALL AMOUNTS IS NOT LIKELY TO CAUSE INJURY. NOT A HAZARD IN NORMAL INDUSTRIAL USE.
INHALATION:	DUST MAY BE A NUISANCE. FORMALDEHYDE VAPORS CAN BE IRRITATING.

ACUTE HEALTH HAZARDS: NONE KNOWN

CHRONIC HEALTH HAZARDS: NONE KNOWN

MEDICAL CONDITIONS GENERALLY AGGRAVATED BY EXPOSURE: NONE KNOWN

CARCINOGENICITY

THIS PRODUCT CONTAINS LESS THAN 0.1% FREE FORMALDEHYDE AND MAY BE CAPABLE OF EMITTING FORMALDEHYDE AT LEVELS IN EXCESS OF OSHA'S ACTION LEVEL UNDER SOME CONDITIONS OF USE AND STORAGE. FORMALDEHYDE IS A POTENTIAL CANCER HAZARD.

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MATERIAL SAFETY DATA SHEET

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SECTION 4: FIRST AID MEASURES

EYES: RINSE COPIOUSLY WITH CLEAN, FRESH WATER FOR AT LEAST 10 MINUTES, HOLDING THE EYELIDS APART. SEEK MEDICAL ADVICE.

SKIN: WASH SKIN THOROUGHLY WITH SOAP AND WATER.

INGESTION: GIVE TWO GLASSES OF WATER TO DRINK. DO NOT INDUCE VOMITING. IF SYMPTOMS PERSIST, SEEK MEDICAL ADVICE.

INHALATION: REMOVE TO FRESH AIR, KEEP PATIENT WARM AND AT REST. IF BREATHING IS IRREGULAR OR HAS STOPPED, ADMINISTER ARTIFICIAL RESPIRATION. GIVE NOTHING BY MOUTH. IF UNCONSCIOUS SEEK IMMEDIATE MEDICAL ATTENTION.

SECTION 5: FIRE-FIGHTING MEASURES

FLAMMABLE LIMITS IN AIR, UPPER: N/A
(% BY VOLUME) LOWER: N/A

FLASH POINT: N/A

AUTOIGNITION TEMPERATURE: N/A

NFPA HAZARD CLASSIFICATION
HEALTH: 1 **FLAMMABILITY:** 1 **REACTIVITY:** 0
OTHER:

HMIS HAZARD CLASSIFICATION
HEALTH: 1 **FLAMMABILITY:** 1 **REACTIVITY:** 0
PROTECTION:

EXTINGUISHING MEDIA: FOAM, CARBON DIOXIDE, AND WATER FOG

SPECIAL FIRE FIGHTING PROCEDURES: NONE

UNUSUAL FIRE AND EXPLOSION HAZARDS: DUST EXPLOSION HAZARD.

HAZARDOUS DECOMPOSITION PRODUCTS: OXIDES OF SULPHUR, CARBON, NITROGEN AND OTHER TOXIC FUMES.

SECTION 6: ACCIDENTAL RELEASE MEASURES

ACCIDENTAL RELEASE MEASURES: DO NOT LET PRODUCT ENTER DRAINS OR WATERWAYS. IN THE EVENT OF A SPILL INTO LAKES, RIVERS, OR WATERWAYS, INFORM APPROPRIATE AUTHORITIES IN ACCORDANCE WITH LOCAL REGULATIONS.

SECTION 7: HANDLING AND STORAGE

HANDLING AND STORAGE: AVOID DUST FORMATION. TAKE PRECAUTIONARY MEASURES AGAINST STATIC DISCHARGES. DO NOT HEAT UNLESS FUME CONTROL IN PLACE. FORMALDEHYDE EMISSION WILL OCCUR.

OTHER PRECAUTIONS: STORE IN A DRY, WELL VENTILATED PLACE AWAY FROM SOURCES OF HEAT AND DIRECT SUNLIGHT. KEEP AWAY FROM SOURCES OF IGNITION. KEEP AWAY FROM STRONG OXIDIZING AGENTS, AND ALKALINE AND ACIDIC MATERIALS. CONTAINERS WHICH ARE OPENED SHOULD BE CLOSED OR FOLDED OVER AND KEPT UPRIGHT TO PREVENT LEAKAGE AND CONTAMINATION. KEEP IN ORIGINAL PACKAGING.

SECTION 8: EXPOSURE CONTROLS/PERSONAL PROTECTION

ENGINEERING CONTROLS: PROVIDE LOCAL EXHAUST VENTILATION IF REQUIRED. SEE EXPOSURE LIMITS.

VENTILATION: LOCAL EXHAUST VENTILATION.

RESPIRATORY PROTECTION: PROVIDE LOCAL EXHAUST VENTILATION. IF EXPOSURE LIMITS ARE LIKELY TO BE EXCEEDED THEN ENSURE THAT APPROPRIATE RESPIRATORY PROTECTION IS USED.

EYE PROTECTION: WEAR SAFETY GLASSES OR GOGGLES

MATERIAL SAFETY DATA SHEET

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SKIN PROTECTION: WEAR GLOVES

OTHER PROTECTIVE CLOTHING OR EQUIPMENT: COVERALLS, LAB COAT, ETC. TO KEEP PRODUCT OFF CLOTHES

WORK HYGIENIC PRACTICES: STANDARD GOOD HYGIENE PRACTICES FOR THE SAFE HANDLING OF CHEMICALS SHOULD BE OBSERVED. DO NOT HEAT UNLESS FUME CONTROL IN PLACE.

EXPOSURE GUIDELINES:	**SHORT TERM EXPOSURE LIMIT**	**LONG TERM EXPOSURE LIMIT**
TOTAL INHALABLE DUST	10mg/m3	10mg/m3
RESPIRABLE DUST	5mg/m3	5mg/m3

SECTION 9: PHYSICAL AND CHEMICAL PROPERTIES

APPEARANCE:	BRIGHTLY COLORED FINE POWDER
ODOR:	SLIGHT CHARACTERISTIC ODOR
PHYSICAL STATE:	FINELY DIVIDED POWDER
pH AS SUPPLIED:	N/A
BOILING POINT:	N/A
MELTING POINT:	
F:	320
C:	160
FREEZING POINT:	N/A
VAPOR PRESSURE (mmHg):	N/A
VAPOR DENSITY (AIR = 1):	N/A
SPECIFIC GRAVITY (H2O = 1):	1.3
EVAPORATION RATE:	N/A

SECTION 9: PHYSICAL AND CHEMICAL PROPERTIES (con't)

SOLUBILITY IN WATER:	INSOLUBLE
PERCENT SOLIDS BY WEIGHT:	100%
PERCENT VOLATILE:	0%
VOLATILE ORGANIC COMPOUNDS (VOC):	0%

SECTION 10: STABILITY AND REACTIVITY

	STABLE	**UNSTABLE**
STABILITY:	X	

CONDITIONS TO AVOID (STABILITY): EXCESSIVE HEAT

INCOMPATIBILITY (MATERIAL TO AVOID): STRONG OXIDIZING AGENTS, STRONG ALKALINE OR ACIDIC MATERIALS

HAZARDOUS DECOMPOSITION OR BY-PRODUCTS: FUMES MAY CONTAIN OXIDES OF SULPHUR, CARBON OR NITROGEN, OR OTHER TOXIC FUMES.

HAZARDOUS POLYMERIZATION: WILL NOT OCCUR

SECTION 11: TOXICOLOGICAL INFORMATION

TOXICOLOGICAL INFORMATION:

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ACUTE ORAL TOXICITY LD50:	>16,000mg/kg
ACUTE DERMAL TOXICITY LD50:	>23,000mg/kg
ACUTE DUST INHALATION LC50:	>4mg/L (4 HOURS)
EYE IRRITATION:	NO SIGNIFICANT IRRITATION

SECTION 12: ECOLOGICAL INFORMATION

ECOLOGICAL INFORMATION: THERE IS NO DATA AVAILABLE ON THE PRODUCT ITSELF.

SECTION 13: DISPOSAL CONSIDERATIONS

WASTE DISPOSAL METHOD: WASTE AND EMPTIED CONTAINERS SHOULD BE DISPOSED OF IN ACCORDANCE WITH CURRENT STATE, LOCAL AND FEDERAL REGULATIONS.

RCRA HAZARD CLASS: NON-HAZARDOUS

SECTION 14: TRANSPORT INFORMATION

TRANSPORTATION INFORMATION: CONSIDERED AS NON-HAZARDOUS UNDER TRANSPORT REGULATIONS.

SECTION 15: REGULATORY INFORMATION

U.S. FEDERAL REGULATIONS

TSCA (TOXIC SUBSTANCE CONTROL ACT): ALL COMPONENTS OF THIS PRODUCT ARE INCLUDED ON THE TSCA INVENTORY IN COMPLIANCE WITH THE TOXIC SUBSTANCES CONTROL ACT, 15 U.S.C. 2601 ET. SEQ.

CERCLA (COMPREHENSIVE ENVIRONMENTAL RESPONSE COMPENSATION, AND LIABILITY ACT): THIS IS NOT A REGULATED MATERIAL UNDER 40 CFR 117.302. NOTIFICATION OF SPILLS IS NOT REQUIRED.

SARA TITLE III (SUPERFUND AMENDMENTS AND REAUTHORIZATION ACT): THIS PRODUCT CONTAINS NO KNOWN CHEMICALS CONTAINED ON THE LIST OF TOXIC CHEMICALS SUBJECT TO THE REPORTING REQUIREMENTS OF SECTION 313 OF THE EMERGENCY PLANNING & COMMUNITY RIGHT TO KNOW ACT OF 1986 & OF 40 CFR372.

311/312 HAZARD CATEGORIES: NONE KNOWN

313 REPORTABLE INGREDIENTS: NONE KNOWN

STATE REGULATIONS: CALIFORNIA PROPOSITION 65: WARNING: THIS PRODUCT CONTAINS A CHEMICAL KNOWN TO THE STATE OF CALIFORNIA TO CAUSE CANCER.

INTERNATIONAL REGULATIONS:

CANADA DSL: ALL COMPONENTS OF THIS PRODUCT ARE INCLUDED ON THE DOMESTIC SUBSTANCES LIST.

EEC EINECS: ALL COMPONENTS OF THIS PRODUCT ARE INCLUDED ON THE EUROPEAN INVENTORY OF EXISTING CHEMICAL SUBSTANCES (EINECS) IN COMPLIANCE WITH COUNCIL DIRECTIVE 67/548/EEC AND ITS AMENDMENTS.

SECTION 16: OTHER INFORMATION

OTHER INFORMATION: NONE KNOWN.

PREPARATION INFORMATION: ISSUE DATE: October 23, 2008

DISCLAIMER: IT IS THE RESPONSIBILITY OF THE USER TO ENSURE THAT THEIR EMPLOYEES ARE AWARE OF THE CONTENT OF THIS MSDS, AND ALSO TO ENSURE THAT ANY ADDITIONAL LOCAL ORDINANCES ARE SATISFIED.

THE INFORMATION CONTAINED IN THIS MSDS IS PROVIDED IN ACCORDANCE WITH THE CURRENT LEGAL REQUIREMENT AND SHOULD NOT BE CONSIDERED AS A GUARANTEE OF THE PRODUCT'S PROPERTIES OR PERFORMANCE.

APPENDIX E

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http://www.veterinaryresearch.org/content/44/1/44

VETERINARY RESEARCH

RESEARCH Open Access

Seasonal and spatial heterogeneities in host and vector abundances impact the spatiotemporal spread of bluetongue

Maud VP Charron[1,2,3,4*], Georgette Kluiters[5], Michel Langlais[3,4], Henri Seegers[1,2], Matthew Baylis[5] and Pauline Ezanno[1,2]

Abstract

Bluetongue (BT) can cause severe livestock losses and large direct and indirect costs for farmers. To propose targeted control strategies as alternative to massive vaccination, there is a need to better understand how BT virus spread in space and time according to local characteristics of host and vector populations. Our objective was to assess, using a modelling approach, how spatiotemporal heterogeneities in abundance and distribution of hosts and vectors impact the occurrence and amplitude of local and regional BT epidemics. We built a reaction–diffusion model accounting for the seasonality in vector abundance and the active dispersal of vectors. Because of the scale chosen, and movement restrictions imposed during epidemics, host movements and wind-induced passive vector movements were neglected. Four levels of complexity were addressed using a theoretical approach, from a homogeneous to a heterogeneous environment in abundance and distribution of hosts and vectors. These scenarios were illustrated using data on abundance and distribution of hosts and vectors in a real geographical area. We have shown that local epidemics can occur earlier and be larger in scale far from the primary case rather than close to it. Moreover, spatial heterogeneities in hosts and vectors delay the epidemic peak and decrease the infection prevalence. The results obtained on a real area confirmed those obtained on a theoretical domain. Although developed to represent BTV spatiotemporal spread, our model can be used to study other vector-borne diseases of animals with a local to regional spread by vector diffusion.

Introduction

There is significant concern regarding the resurgence or emergence of vector-borne diseases of animals with serious consequences for animal health and economics [1-3]. Climate change and socio-economic change are both believed to contribute to the emergence and spread of such diseases [4]. A recent example is the unexpected introduction of the serotype 8 of the bluetongue virus (BTV) in northern Europe in 2006. Bluetongue is a non-contagious vector-borne disease affecting domestic and wild ruminants with high direct and indirect economic consequences [5,6]. It spread for three years with an annual epidemic peak followed by the disappearance of clinical cases.

A better understanding of the temporal and spatial spread of BTV has direct consequences for the disease prevention and control. The recent incursion of BTV in Europe has been controlled using a massive vaccination. To propose alternative to such a massive strategy if BTV incursions were to occur, we need to better identify where and when targeted strategies should be implemented. Therefore, the occurrence and amplitude of both local (a few km^2) and regional epidemics should be more precisely predict.

The spatiotemporal heterogeneity in abundance and distribution of hosts and vectors generally has a strong impact on pathogen spread and persistence [7,8]. In a seasonal environment such as in Europe, bluetongue is characterized by strong seasonal variations in incidence related to the seasonality of the vector population [9,10],

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whose lifecycle largely depends on environmental factors, such as humidity and temperature. As a result, clinical cases almost disappear during the unfavourable season for the vector. In addition to the temporal heterogeneity in vector abundance, the heterogeneity in the spatial distribution of vectors and hosts may also impact bluetongue spread [11,12]. Such heterogeneities in vector and host abundance and distribution can differ between geographic areas. In livestock populations, they relate to the landscape structure as well as to farming practices as animal populations are managed by farmers.

Mathematical modelling is a relevant approach for investigating the spread of vector-borne diseases [8,13-16]. As hosts and vectors are mobile entities, a spatial component in vector-borne disease models should be taken into account to better consider the evolution of the biological system [17-19]. This spatial component is not only due to space structuring in terms of density and location of host and vector populations, but also to host and vector movements over space. Different methods of various levels of complexity exist to include this spatial component in epidemiological models. To study the spread of vector-borne diseases, spatially explicit models are generally preferred [13,20]. They permit to take into account both vector active and passive movements and host movements that occur at different scales. Moreover, such models have been used also to describe the velocity of travelling waves of epidemics [8,14,16].

Such a modelling approach has been used to represent the spatiotemporal BTV spread in specific areas [21-24]. If the published models took into account the heterogeneity in distribution and abundance of livestock populations, they tend to assume uniform densities of vectors (i.e. the same number per farm) or uniform host: vector ratio (i.e. more midges on bigger farms) [21-24]. Recently, it has been shown that these are probably unrealistic [25]. Therefore, models that take full account of vector heterogeneity both in space and time have to be developed.

Our objective was to assess, using a modelling approach, how spatiotemporal heterogeneities in abundance and distribution of hosts and vectors impact the occurrence and amplitude of local and regional BT epidemics. We first studied different hypothetical scenarios of spatial heterogeneity in host and vector populations, and then illustrated the theoretical results in a real geographic area.

Material and methods

Model description

Three actors are necessary for bluetongue spread: the virus (here BTV8), the vector (a midge, here *Culicoides*), and the host (a ruminant, here cattle). As the virus is not excreted, we have assumed all transmission is vectorial. The developed model is based on a more complex model of the seasonal temporal spread of bluetongue in cattle [26]. This model has been simplified and made spatial. Here, the vertical transmission (in utero) has not been taken into account, as this hypothesis has not been shown to influence the infection [26]. The temporal dynamics of the vector population has been modified using a more flexible function. We used a standard compartment model (Figure 1) to describe the transmission of a pathogen between a vertebrate host population (*HP*) and a vector population (*VP*). The parameters of this model are defined in Table 1. The host population (*HP*) is divided into three health states: susceptible (*SH*), infectious (*IH*), and immune (*RH*). It is assumed to remain constant: the entry rate (b_H) compensates the exit rate (m_H). The vector population (*VP*) is divided into three health states: susceptible (*SV*), exposed (*EV*), and infectious (*IV*). For vectors, a latency period ($1/\rho_E$; Table 1) was taken into account as it is of the same order as life expectancy. At the disease-free state and with seasonality, the vector population was assumed to have a logistic growth with $K(t) = (b_V - m_V)/k_V(t)$ (Table 1), the carrying capacity of the environment depending on the vector fertility (b_V), mortality (m_V) and density-dependant mortality (k_V) rates. In periods favourable for vectors, the K function is a sinusoidal function with a maximum h. In unfavourable periods, the K function is constant and equal to Nb. The vectorial transmission takes place when an infectious vector (*IV*) bites a susceptible host (*SH*)

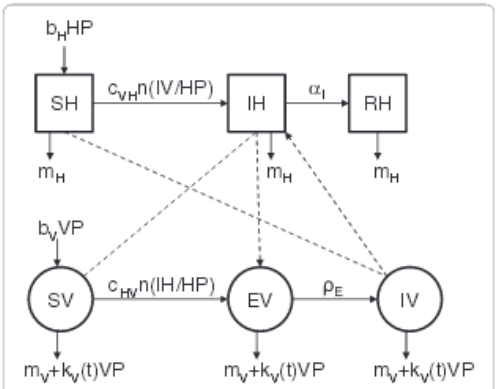

Figure 1 Conceptual model of BTV8 spread. Flow diagram describing the model used for BTV8 spread in midge and cattle populations. Squares represent the health states of hosts (H), circle those of vectors (V), with S for susceptible, E for latent, I for infectious, R for recovered. The descriptions, values and sources of all parameters in the epidemiological model are found in Table 1.

Table 1 Parameters of the model of BTV8 spread in midge and cattle populations

Host parameters	Description	Value	References
b_H	Birth rate (per day)	$6{,}94.10^{-4}$	a
m_H	Exit rate (mortality, selling, culling) (per day)	b_H	
$1/\alpha_I$	Duration of viremia (days)	60	[43]
c_{VH}	Probability of transmission from vector to host	0.92	[44,45]
Vector parameters	**Description**	**Value**	**References**
c_{HV}	Probability of transmission from host to vector	0.15	[46,47]
n	Biting rate (per day)	0.25	[45]
b_V	Fertility rate (per day)	6.1	[45]
m_V	Mortality rate (per day)	1/21	[34,45]
$K(t)$	Carrying capacity	*	
k_V	Density-dependence mortality rate (per day)	$(b_V - m_V)/K(t)$	
h	Maximum of $K(t)$	variable	
d	Duration of favourable period (days)	243	
Nb	Number of vectors during the unfavourable period	100	
$1/\rho_E$	Duration of extrinsic incubation period (days)	10	[4,45]
D	Diffusion coefficient (km^2/day)	$1{,}25.10^{-2}$	

* $K(t) = T_{(t\ ;\ d)}(t)*[\ h*sin(|\pi(365-t)/d|)] + T_{(d+1;365)}(t)*Nb$.
a: agricultural statistics.

which becomes infectious (IH), or when a susceptible vector (SV) bites an infectious host (IH) and then becomes exposed (EV). Incidence functions were frequency dependant for hosts and vectors. The mean host viremia duration (i.e. the time spent in IH) was $1/\alpha_I$. With recovery, animals move from state IH to RH.

Let Ω be the square spatial domain. $X = X(x, y, t)$ represents time dependant population densities in $(x, y) \in \Omega$. During the epidemic, host movements are controlled, therefore the spatial spread of the epidemic is due to vector movements rather than host movements. Moreover, we focus on a local to regional scale, and therefore assume that the spatial spread of BTV8 is exclusively due to local movements of vectors. BTV8 having no detrimental impact on vectors, thereby the diffusion process is similar whatever the health state. Therefore, the diffusion process follows the first Fick's law:

$\vec{q}(x, y, t) = -D(x, y).\nabla SV(x, y, t)$, where $\vec{q}(x, y, t)$ is the diffusion flux of the vector population and $D(x,y)$, the positive diffusion matrix. Appling the conservation law (i.e. the variation of the amount of species in a volume is equal to the balance of entering and outgoing flux), we obtain the second Fick's law: $\frac{\partial SV(x,y,t)}{\partial t} + div\,\vec{q}(x, y, t) = 0$. Combining these two laws, we obtain: $\frac{\partial SV(x,y,t)}{\partial t} = -div\,\vec{q}(x, y, t) = -div(-D(x, y).\nabla SV(x, y, t))$. We consider that the dispersion of vectors is homogeneous in space; therefore $D(x,y) = D$. Then, $\frac{\partial SV(x,y,t)}{\partial t} = D\,div(\nabla SV(x, y, t)) = D\Delta SV(x, y, t)$.

By adding the reaction term, we obtain the following system of equations (Eq. 1) describing the spatiotemporal spread of the BTV8, for $(x, y) \in \Omega$ and $t > 0$:

$$\begin{cases} \frac{\partial SH}{\partial t} = -c_{VH} n \frac{IV}{HP} SH + b_H HP - m_H SH \\ \frac{\partial IH}{\partial t} = c_{VH} n \frac{IV}{HP} SH - (\alpha_I + m_H) IH \\ \frac{\partial RH}{\partial t} = \alpha_I IH - m_H RH \\ \frac{\partial SV}{\partial t} = D\Delta SV - c_{HV} n \frac{IH}{HP} SV - (m_V + k_V VP) SV + b_V VP \\ \frac{\partial EV}{\partial t} = D\Delta EV + c_{HV} n \frac{IH}{HP} SV - (m_V + k_V VP) EV - \rho_E EV \\ \frac{\partial IV}{\partial t} = D\Delta IV + \rho_E EV - (m_V + k_V VP) IV \end{cases} \quad (1)$$

The initial time corresponds to the first day of the favourable season for the vector population. The spatial domain (Ω) is discretized into cells; each cell of surface area of 1 km^2. Initially, all hosts and vectors are susceptible, except the primary case which corresponds to an infected host, placed in the centre of the grid, in a cell where there are hosts. We set non-negative initial conditions (Eq. 2). The flow of individuals across the domain boundary is assumed to be zero, i.e. we do not consider immigration or emigration of individuals and set Neumann boundary conditions (Eq. 2).

$$\begin{cases} SH(x, y, 0) = SH^0(x, y), SV(x, y, 0) = SV^0(x, y) \\ IH(x, y, 0) = 0 \forall (x, y) \neq (21, 21); IH(21, 21, 0) = 1 \\ RH(x, y, 0) = EV(x, y, 0) = IV(x, y, 0) = 0 \\ \frac{\partial SH}{\partial n} = \frac{\partial IH}{\partial n} = \frac{\partial RH}{\partial n} = \frac{\partial SV}{\partial n} = \frac{\partial EV}{\partial n} = \frac{\partial IV}{\partial n} = 0, \forall (x, y) \in \partial\Omega, t > 0 \end{cases} \quad (2)$$

To discretize the problem (Eq. 1 and 2) we used the finite difference method in space, and we converted the continuous time model into a discrete time one by using the semi-implicit Euler method, that we implemented in Scilab 5.1.

Hypotheses of spatial heterogeneities in hosts and vectors

Assumptions are described by increasing level of complexity in Table 2. A first reference hypothesis (H1) considers a homogeneous spatial distribution and abundance in hosts and vectors. Four scenarios were studied for four maxima of the carrying capacity in vectors ($h1$ to $h4$).

Table 2 Hypotheses of spatial heterogeneities in abundance and distribution of hosts and vectors

	H	Host	Vectors	Results figure
Homogeneous in hosts and vectors (4 Scenarios)	H1	500 S/C 1 I in central cell Total number of hosts = 840 501	100 S/C *h1*=10^6, or *h2*=10^7 or *h3*=10^8, or *h4*= 10^9	5
Heterogeneous in hosts Homogeneous in vectors (16 scenarios)	H2	Total number of hosts S≈ 840 501 Four densities of occupied cells (Figure 2): 90% OC: 554 S/OC75% OC: 666 S/OC50% OC: 1000 S/OC25% OC: 2025 S/OC 1 I in central cell	H1	6 and 7
Heterogeneous in vectors Homogeneous in hosts (2 scenarios)	H3	H1	100 S/C Grid divided into four sub-areas of different maximum carrying capacities (*h1*, *h2*, *h3* and *h4*) (Figure 3a)	8
	H4		100 S/C 25% C: *h1*, 25% C: *h2*, 25% C: *h3*, 25% C: *h4* (Figure 3b)	9 and 10
Heterogeneous in hosts and vectors (6 scenarios)	H5	Crossing hypotheses: H2-H4		10 and 11
	H6	Real area (Figure 4b)	Real area (Figure 4a) Multiplication of the number of trapped vectors by 100 or 1000	12

We set *H* hypothesis, *C* cell, *OC* occupied cell. *S* susceptible individual, *I* infected individual.

From this assumption, four levels of heterogeneity were analyzed and compared.

First, a heterogeneous distribution of hosts was considered (H2), vectors being homogeneously distributed. We kept the total number of hosts on the grid constant. We tested five densities of occupied cells from 25% to 100%, where H1 is 100% of cells occupied (Figure 2). For each density, we randomly drew the occupied cells among the 1681 grid cells. For each scenario, three draws of occupied cells were compared. Results were identical whatever the distribution; therefore it did not influence the spatiotemporal spread of the virus on the grid. Thereafter, only one distribution was kept (Figure 2).

Secondly, a heterogeneous distribution of vectors was considered (H3 and H4), hosts being homogeneously distributed. In H3, the domain (Ω) consisted of four sub-areas, each having a different maximum of the carrying capacity in vectors (*h1* to *h4*, Figure 3a). In H4, we considered a random equiprobable distribution of the same four maxima of the carrying capacity in vectors on the grid (Figure 3b).

Thirdly, we considered a heterogeneous distribution of hosts and vectors simultaneously (H5). This hypothesis crosses previous hypotheses H2 and H4.

Fourth, a last hypothesis (H6), based on real data, served to illustrate this theoretical work, in particular hypothesis H5.

Data

The *Culicoides* trap catches used for modelling were collected in the Welsh province of Bala, situated in Snowdonia National Park (for full methods, see [25]). The

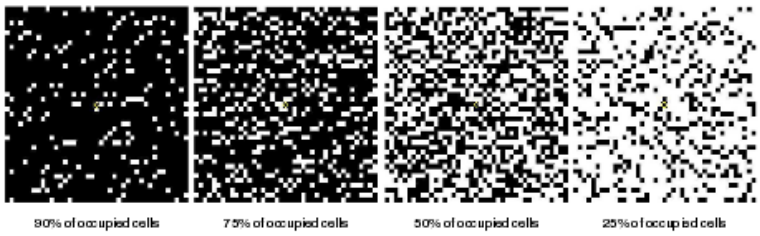

Figure 2 Spatial distribution of cells occupied by hosts for four density levels (hypotheses H2 and H5). Black cells correspond to occupied cells; white cells correspond to empty cells; the yellow and crossed cell corresponds to the cell wherein the primary case occurs.

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http://www.veterinaryresearch.org/content/44/1/44

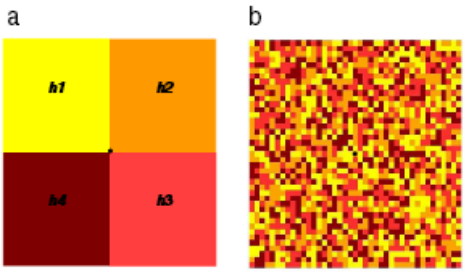

Figure 3 Spatial distribution of the four maximum carrying capacities in vectors. a) Hypothesis H3, **b)** Hypotheses H4 and H5. Yellow cells correspond to cells having a maximum carrying capacity in vectors given by $h1 = 10^6$, orange cells correspond to cells having a maximum carrying capacity in vectors given by $h2 = 10^7$, red cells correspond to cells having a maximum carrying capacity in vectors given by $h3 = 10^8$, brown cells correspond to cells having a maximum carrying capacity in vectors given by $h4 = 10^9$. The black cell corresponds to the cell wherein the primary case occurs.

trapping farms were selected using a 6 × 6 km grid, whereby one farm was selected from each grid square (36 in total, Figure 4). Each farm was sampled for three nights using Onderstepoort-type down draught black light traps positioned as close to livestock as possible. Large collections were sub-sampled [27] and only females were considered in the analyses as males do not take blood meals or, consequently, transmit disease between vertebrates. The maximum trap-catch of *Culicoides* per farm, out of the three trapping nights, was used for modelling purposes (Figure 4a). Due to the nature of the terrain, two squares contained no properties. *Culicoides* counts for these grid squares were estimated using the models of [25]. The vector abundance is difficult to quantify. Therefore, we considered two scenarios, one multiplying the number of vectors per cell by 100, and another by 1000. The numbers of cattle per farm were recorded on questionnaires during data collection (Figure 4b). For farms with unknown numbers of cattle, values were interpolated using the known values of farms in adjacent grid squares.

Outputs

The date and the prevalence at the epidemic peak in each cell were analysed, as well as the total prevalence on the grid over time. Thereafter, these three outputs are respectively named peak date, local prevalence and total prevalence. The peak dates were compared among the cells located on the four lines between the central cell (the half-diagonal), i.e. the cell of virus introduction, and the corners of the grid. This enabled us to numerically calculate an effective speed of the virus spread. We calibrated the diffusion coefficient (D) and the initial conditions ($SH00^0$ and SV^0) to have an effective speed of the virus spread similar to the estimated velocity by Pioz et al. [28], for hypothesis H1 and a maximum of the carrying capacity in vectors equals to 10^7. The theoretical grid is a 41 × 41 km square, each half-diagonal measuring about 29 km. For hypotheses H3 to H6, peak dates and local prevalences were studied for comparable cells, i.e. equidistant and having the same maximum of the carrying capacity in vectors.

Results

Homogeneous in abundance and distribution of hosts and vectors (H1)

For the lowest maximum of carrying capacity in vectors ($h1 = 10^6$), there is no epidemic. For the other values tested, there is an epidemic peak in all grid cells. A larger maximum of carrying capacity in vectors leads to an earlier peak and a faster speed of virus spread (Figure 5). For $h2 = 10^7$, five days are necessary for the virus to cover the half-diagonal from the cell of the virus introduction, with the epidemic peak occurring in the central cell and in the cells at the end of the half-diagonals at 96 and 101 days respectively after virus introduction. As expected in such a homogeneous environment, results are identical for the four half-diagonals. For $h3 = 10^8$ and $h4 = 10^9$, only two

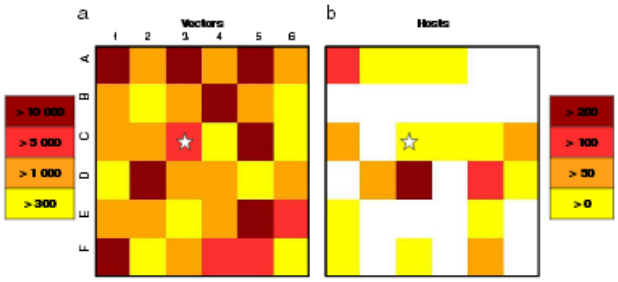

Figure 4 Vector (a) and host (b) spatial distribution in the real area (in the Welsh province of Bala). From yellow to brown, increasing numbers of individuals (white: empty cells); the cell with the star corresponds to the cell wherein the primary case occurs.

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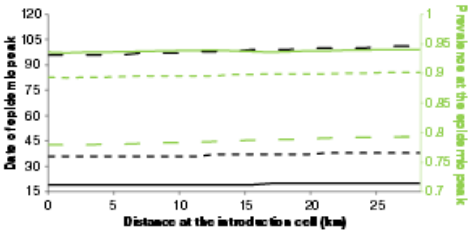

Figure 5 Peak date and prevalence as a function of the distance from the primary case. When hypothesis H1 holds. Black line corresponds to the peak date, green line corresponds to the prevalence. Solid lines correspond to a maximum carrying capacity in vectors given by $h4 = 10^9$, dashed lines correspond to a maximum carrying capacity in vectors given by $h3 = 10^8$, dashed-dotted lines correspond to a maximum carrying capacity in vectors given by $h2 = 10^7$.

and one day, respectively, are necessary for the virus to cover the half-diagonals, the epidemic peak occurring 36 days (19 days, respectively) after virus introduction. A larger maximum of carrying capacity in vectors leads to larger local prevalences (Figure 5). Local prevalences are almost constant over different cells of the grid and are worth 78%, 90% and 94% for *h2*, *h3* and *h4* respectively. The total prevalence on the grid over time confirms these results. A larger maximum of carrying capacity in vectors leads to an earlier and larger epidemic peak (Figure 6).

Heterogeneous in abundance and distribution of hosts and homogeneous in abundance and distribution of vectors (H2)

For all maxima of carrying capacity in vectors (except *h1* whatever the density of occupied cells and *h2* for a density of occupied cells equal to 25%, for which there is no epidemic) a smaller density of occupied cells leads to a later epidemic peak and a smaller local prevalence in all grid cells. For example, for a density of occupied cells

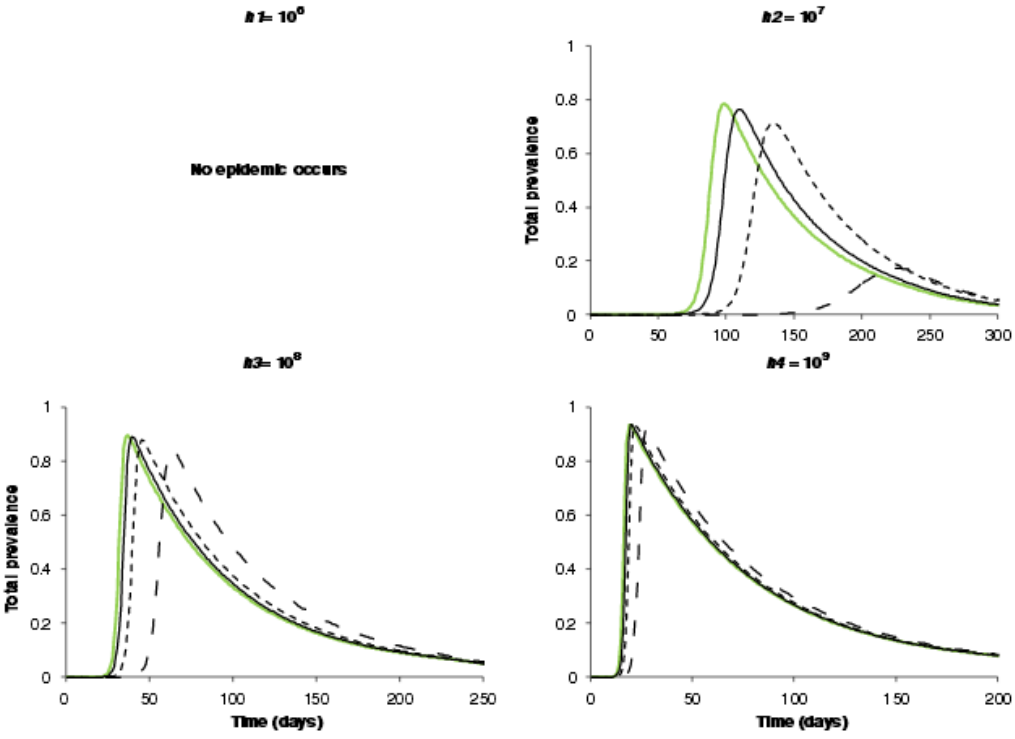

Figure 6 Total prevalence over time. In green when hypothesis H1 holds, in black when hypothesis H2 holds. Solid lines correspond to the case where 90% of cells are occupied, dashed lines correspond to the case where 75% of cells are occupied, in dashed-dotted lines correspond to the case where 50% of cells are occupied and dotted lines correspond to the case where 25% of cells are occupied.

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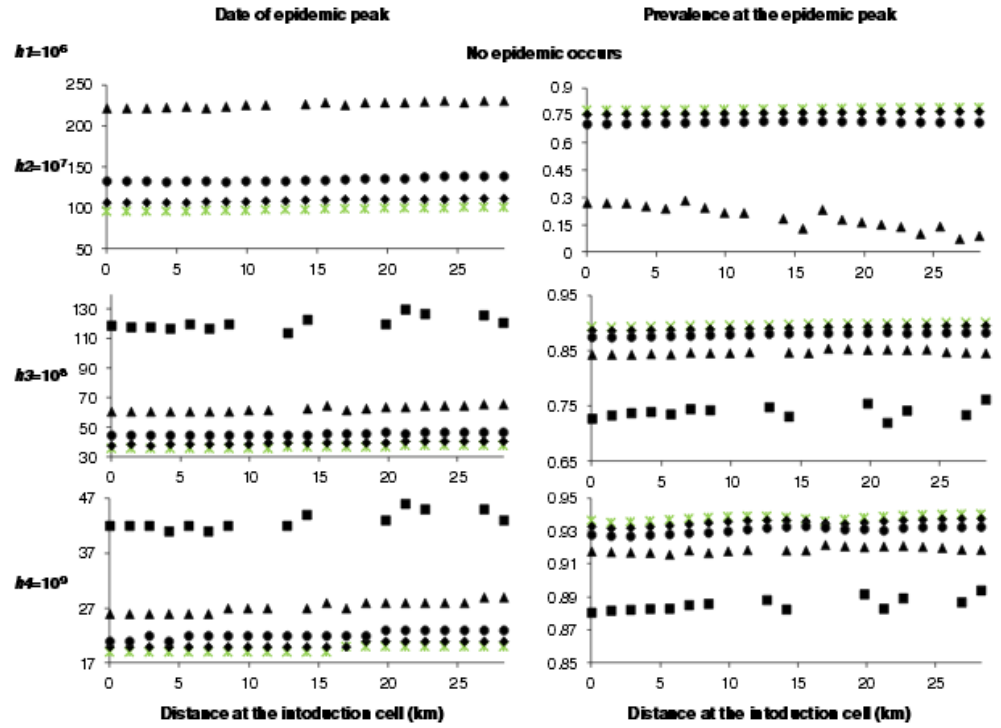

Figure 7 Peak date and prevalence as a function of the distance from the primary case. When hypothesis H2 holds. Green star correspond to the case where 100% of cells are occupied, lozenge correspond to the case where 90% of cells are occupied, circle correspond to the case where 75% of cells are occupied, triangle correspond to the case where 50% of cells are occupied and square correspond to the case where 25% of cells are occupied.

equal to 75% and a maximum carrying capacity of *h2*, the epidemic peak was 37 days later than in H1, occurring at day 133 in the central cell and at day 139 in the cell at the end of the half-diagonal. Indeed, we kept the total number of hosts on the grid constant, so the number of hosts per occupied cell increases when the density of occupied cells decreases. As incidence functions are frequency dependent for hosts and vectors (Eq. 1), this increase in the number of hosts per occupied cell has little effect on the frequency of infection. However, the number of unoccupied cells increases, and therefore the virus transmission slows. The local prevalence is almost constant in all grid cells but is decreased by 9% compared with H1. For a maximum carrying capacity of *h3* and *h4*, the epidemic peak was respectively 9 and 2 days later than in H1 (Figure 7). Similarly, the local prevalence is decreased by about 2% and 1%, respectively, compared with H1 (Figure 7).

The total prevalence on the grid over time confirms these results. A delay of the epidemic is observed as the density of host-occupied cells decreases. There is also a decrease of the total prevalence compared with H1 (Figure 6).

Heterogeneous in abundance and distribution of vectors and homogeneous in abundance and distribution of hosts (H3, H4)

Hypothesis 3: definition of four subareas

Compared with H1, an epidemic peak is observed in all grid cells, even for the lowest maximum of carrying capacity, *h1* (Figure 8). The further the distance from the grid centre, the more the epidemic peak is delayed and the more the local prevalence decreases. For the carrying capacity *h2*, the observed epidemic peaks are earlier than for H1, including in the cell at the end of the half-diagonal. The local prevalence slightly decreases with increasing distance from the grid centre, to reach an equilibrium equal to the observed infection prevalence at the epidemic peak for H1 (Figure 8). For the two largest maxima of carrying capacity in vectors, *h3* and *h4*, the peak

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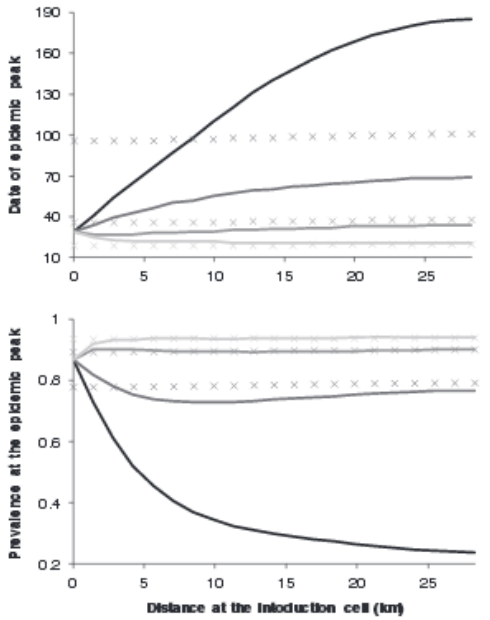

Figure 8 Peak date and prevalence as a function of the distance from the primary case and the vector maximum carrying capacity. When hypothesis H3 holds (to help for the comparison, crosses correspond to the homogeneous case, hypothesis H1). Black lines correspond to a maximum carrying capacity in vectors given by $h1 = 10^6$, very dark grey lines correspond to a maximum carrying capacity in vectors given by $h2 = 10^7$, dark grey lines correspond to a maximum carrying capacity in vectors given by $h3 = 10^8$ and light grey lines to a maximum carrying capacity in vectors given by $h4 = 10^9$.

dates and the local prevalences are similar to H1 results (Figure 8). This hypothesis highlights the influence of diffusion in each subarea on the diffusion in others.

Hypothesis 4: variable maximum of carrying capacities in vectors

Compared with H1, an epidemic peak is observed in all grid cells, even for the lowest maximum of carrying capacity, *h1* (Figure 9). However, the peak dates are delayed. Indeed, epidemic peaks in cells having the same maximum of carrying capacity and equidistant ranged between 96 and 134 days after virus introduction, with no effect of distance on the peak date, (Figure 3, Figure 9). Therefore, cells near the introduction cell can be infected later than cells more distant (Figure 9). While the distribution of the maximum of carrying capacity in vectors is random, clusters of peak dates and of local prevalence arise (Figure 9). The local prevalences vary between 63% and 82% whatever the maximum of the carrying capacity and the distance from the introduction cell (Figure 9). A balance is observed between the peak dates and the local prevalences (Figure 9).

The total prevalence on the grid over time confirms these results. A delay of the epidemic and a decrease in the total prevalence are observed compared with H1 (Figure 10).

Heterogeneous in abundance and distribution of hosts and vectors (H5, H6)

Hypothesis 5: theoretical landscape

As for H2, although later, peak dates are delayed as the density of host-occupied cells decreases (Figure 11). Likewise, the local prevalence decreases when the density of occupied cells in hosts decreases. However, for a density of occupied cells equal to 25%, there is no epidemic; and for a density of occupied cells equal to 50%, the epidemic is small. By comparison with H2 and H4, the same tendencies are observed, with a delay in peak dates and a decrease in local prevalences. The distance has no effect on the peak date, unlike the maximum of carrying capacity in vectors. By comparing the distribution of the maximum of carrying capacity in vectors (Figure 3) with the peak dates and the local prevalences (Figure 11), a balance is observed. Cells with the highest maximum of carrying capacity have an earlier peak date and a larger local prevalence, and vice versa for cells with the lowest maximum of carrying capacity. This is true whatever the distance to the primary case.

The total prevalence on the grid over time confirms these results. A delay in the epidemic is observed as the density of host-occupied cells decreases, as well as a decrease in the total prevalence compared with H1 (Figure 7, Figure 10). A balance is observed between H2-H5 and H4-H5. As for H2 and for a maximum of carrying capacity in vectors of *h2*, the lower the density of host-occupied cells, the longer the peak date is delayed and the lower is the total prevalence (Figure 7, Figure 10). However, the total prevalence for the highest density of occupied cells is similar with hypothesis H4 (Figure 10). Therefore, as soon as the distribution and the abundance of vectors are heterogeneous, they strongly influence the global epidemic dynamics.

Hypothesis 6: application to a real landscape

As in the theoretical case, for realistic distribution and abundance of hosts and vectors on a grid, the peak dates and the local prevalences are affected by the maximum of the carrying capacity in vectors in each cell (Figure 12). For roughly equal numbers of hosts and for a high maximum of carrying capacity in vectors (compared to other cells) (cells: A3 and F1, Figure 4), the peak dates and the local prevalences are close (e.g. cells A3 and F1; Figure 12). On the contrary, for roughly equal number of hosts but different maximum of carrying capacity in vectors (e.g.

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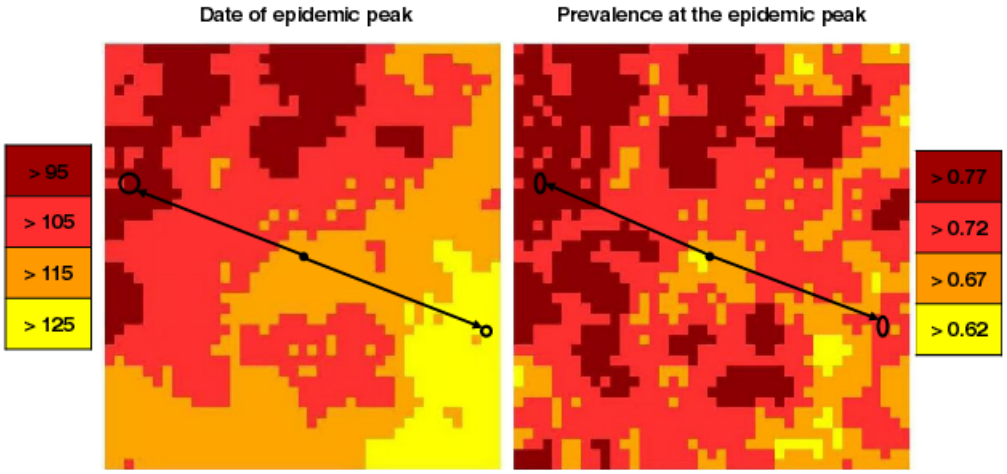

Figure 9 Spatial distribution of peak dates and prevalence. When hypothesis H4 holds. The black cell corresponds to the cell wherein the primary case occurs; rounds correspond to cells having the same maximum carrying capacity. From yellow to brown: earlier peak date and increasing prevalence.

cells C4 and C5; Figure 4), cells with the highest maximum of carrying capacity in vectors have an earlier peak date and a higher local prevalence (e.g. cells C4 and C5; Figure 12). These trends are observed even if the observed number of vectors is multiplied by 100 or 1000, although the peak dates are earlier and the local prevalences higher.

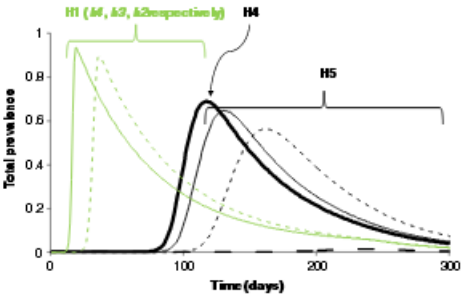

Figure 10 Total prevalence over time. In green results when hypothesis H1 holds (same curves as in Figure 6, given here to help for comparison among hypotheses); solid lines correspond to a maximum carrying capacity in vectors given by $h4 = 10^9$, dashed lines correspond to a maximum carrying capacity in vectors given by $h3 = 10^8$, dotted lines correspond to a maximum carrying capacity in vectors given by $h2 = 10^7$. In thick black results when hypothesis H4 holds, in black results when hypothesis H5 holds; solid lines correspond to the case where 90% of cells are occupied, dashed lines correspond to the case where 75% of cells are occupied, dashed-dotted lines correspond to the case where 50% of cells are occupied and dotted lines correspond to the case where 25% of cells are occupied.

Indeed, in our mathematical model, multiplying the observed number of vectors by 100 or 1000 is equivalent to multiplying the maximum carrying capacity of vectors in each cell; that is, to divide k_V by 100 or 1000 in Eq. 1. This has a scaling effect on the model: it does not modify the time periodicity in the population dynamics, but it does change the abundance of vectors. This causes an earlier increase in the number of available infectious vectors and, therefore, the peak dates occur earlier together with higher local prevalences.

In addition, the number of hosts in each cell has an impact on the peak date and the local prevalence. For equidistant cells having the same maximum of the carrying capacity in vectors (e.g. cells A2 and C1; Figure 4a), the cell having the largest number of hosts (C1) shows a later peak date and a lower local prevalence than the cell having the lowest number of hosts (A2; Figure 12).

Discussion

A mathematical modelling approach allowed us to assess the impact of spatiotemporal heterogeneities in abundance and distribution of hosts and vectors on the spatiotemporal spread of BTV8. Individually and jointly, the heterogeneities in abundance and distribution of hosts and vectors delay the peak date and decrease the total infection prevalence. The different hypotheses of heterogeneity that we have tested allowed us to highlight the importance of the maximum of the carrying capacity in vectors and its influence on the spread of BTV8 within each cell. Indeed, cells next to the primary case can become infected later than more distant cells, if the maximum of carrying capacity is

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lower. Moreover, the density of cells occupied by hosts plays an important role in cases where the maximum carrying capacity in vectors is low (for homogeneous conditions for vectors) and when hosts and vectors are heterogeneous.

The spatial heterogeneity in host and/or vector abundances influences the infection frequency [7,17,29]. In our study, a decrease in the density of cells occupied by hosts results in a delay of the peak date and a decrease in the infection prevalence if vectors are homogeneously distributed and their population is large. In the case where hosts are homogeneously distributed, the same trend is observed for cells where the maximum of carrying capacity in vectors is large. However, for cells where this maximum is the lowest, an epidemic can occur, in contrast to the case with homogeneous vector and host populations. The coupling of heterogeneities in hosts and vectors increases the delay of the epidemic and decreases the prevalence.

Different models of the spatial spread of bluetongue have previously been published for specific geographic areas [21-24]. Szmaragd's model describes the BTV spread within and between farms in Great Britain via a generic kernel, which includes both animal and vector movements. Ducheyne's model was calibrated with data from the BTV1 and BTV8 epidemics in Southern France. It describes the spatiotemporal BTV spread between farms assuming that the number of new cases per week is half attributable to local dispersion (active) of vectors, and half to long-distance dispersion (passive) of vectors by the wind. Graesboll's model describes the BTV spread with a high spatial resolution, which includes both animal and vector movements and the seasonality of vectors. Turner's model is a network model. It takes into account explicitly the spatial dispersal of both hosts and vectors and a seasonal vector to host ratio [24]. It studies the BTV spread between farms in England. Taking into account climatic and environmental data, all of these models consider the spatial heterogeneity of the landscape. Our model advances the field by representing spatial heterogeneity in both hosts and vectors. Here, we highlighted how such heterogeneities concretely impact BTV spread. Moreover, the seasonality of the vector population is managed by a simple function that can be easily related to observed data of vector abundance.

We chose to model the spatiotemporal BTV8 spread by a reaction–diffusion model. Several shapes of the transmission kernel are possible, but it is difficult to choose the most appropriate one to describe the dynamics of a pathogen spread. Indeed, Szmaragd et al. showed that a Gaussian kernel was the most appropriate to describe the BTV8 spread in northern Europe during 2006 [21]. If this kernel shape, comparable to reaction diffusion models, has been shown to underestimate the probability of the long-distance transmission, and thus is not appropriate to describe the pathogen spread on a larger scale [30], it can be

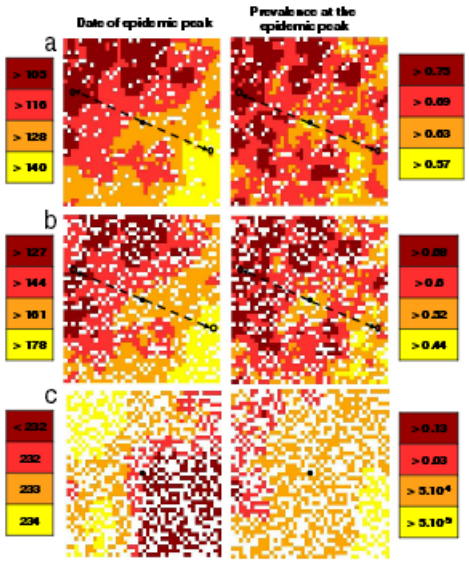

Figure 11 Spatial distribution of peak dates and prevalence. When hypothesis H5 holds. **a)** 90% of occupied cells, **b)** 75% of occupied cells, **c)** 50% of occupied cells. The black cell corresponds to the cell wherein the primary case occurs; rounds correspond to cells having the same maximum carrying capacity. From yellow to brown: earlier peak date and increasing prevalence.

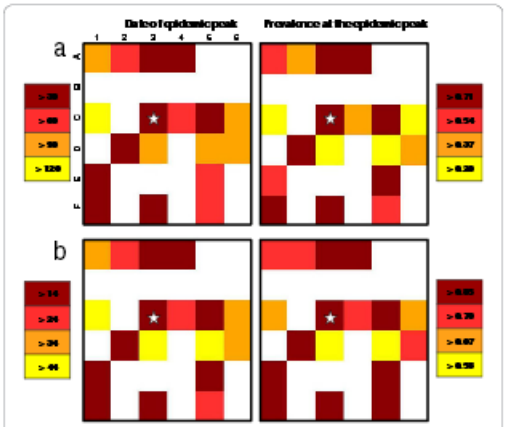

Figure 12 Spatial distribution of peak dates and prevalence in a real area. When hypothesis H6 holds. Multiplication of the number of trapped vectors by: **a)** 100; **b)** 1000. The cell with the star corresponds to the cell wherein the primary case occurs. From yellow to brown: earlier peak date and increasing prevalence.

used on a smaller scale. Graesboll et al. used a Gaussian kernel too, but coupled this approach with the wind dispersion [23]. In our study, the theoretical spatial domain is a 41 × 41 km square. The primary case is always located in the centre of the square, i.e. at 29 km from the domain edges. One limitation is that long-distance dispersal has been neglected; the wind dispersal responsible for the passive movements of vectors generating dispersal up to several hundred kilometres [31,32]. Coupling short and long-distance dispersal is necessary to study arbovirus spread in animal populations once the spatial scale is large enough that host movements and passive movements of vectors cannot be neglected anymore [33-35].

Observational studies have been conducted to assess risk areas and to predict the spatiotemporal spread of BTV in BTV-free areas [11,36,37]. These studies have shown that the landscape heterogeneity, climatic conditions, the distribution and the density of host populations and the abundance of vectors were linked and influenced BTV spatiotemporal spread. Our model highlights similar findings but also allows us to distinguish between the impact of vector versus host heterogeneity. Maps of the basic reproduction number have highlighted the link between vector abundance and BTV spread [12]. However, the vector abundance is difficult to quantify. Hartemink et al. have used trapping data, multiplying the number of trapped *Culicoides* by 100 to obtain a local density of vectors [12]. Our real geographic area illustrates these differences in local abundance. On a small scale, large differences may exist between cells, whether they are occupied by hosts or not. Entomological studies identify and quantify the different vector species present in different geographic locations [9,10,38]. However, the real number of vectors remains difficult to approximate. As shown in our results, the abundance in vectors has a significant impact on the date and on the observed prevalence at the epidemic peak. However, by multiplying the number of vectors by 100 or 1000, we obtained the same qualitative findings on the real geographic area.

Modelling is a relevant approach to investigate the concept of spatiotemporal heterogeneities on the dynamics of virus spread. The distribution of hosts and vectors, and vector abundance strongly influence the dynamics of BTV spread. The application of our model on a real geographic area, although of limited size, allowed us to illustrate the conclusions drawn from a theoretical domain. The reaction–diffusion models are classically used in plant epidemiology [39-41], with the modelled movements generally being for highly volatile entities, and the short *versus* long-distance movements being taken into account via different diffusion coefficients [40,42]. Although developed to represent BTV8 spatiotemporal spread at a local to regional scale, our model can be used to study other vector-borne diseases of animals and its extension to a larger area remains possible.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MC and PE conceived of the study, carried out the model development and analysis, and drafted the manuscript. ML and HS conceived of the study, participated in its design and coordination, and drafted the manuscript. MB and GK provided data used in this research, and both commented on draft and final manuscripts. All authors read and approved the final manuscript.

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