On surveillance systems and surveys for bluetongue and zoonotic diseases of ruminants

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On surveillance systems and surveys for bluetongue and zoonotic diseases of ruminants

Over surveillance-systemen en survey's voor blauwtong
en zoönosen bij herkauwers

(met een samenvatting in het Nederlands)

Proefschrift

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CHAPTER 1

Introduction

This thesis documents my experience in studying the epidemiology and control of livestock infectious diseases of veterinary and public health relevance. Epidemiology studies the distribution and determinants of disease (or health-related states or events) in population, and one of the most important source of information for epidemiological investigations is represented by surveillance and monitoring programmes.

This chapter first introduces disease surveillance and monitoring systems and provides a brief overview of some fundamental features of surveillance activities. It places emphasis on the relevance of those elements with respect to certain characteristics of the disease: (i) its presence/occurrence within a territory (e.g. endemic or emerging), (ii) the number and different type of hosts involved in its epidemiological cycle (e.g. human, farm-animal, wildlife) and (iii) the route of transmission of the infectious agent (e.g. direct, vector-borne). The main body of this thesis then, consists of five chapters: (i) as examples of surveillance activities applied to endemic zoonoses (brucellosis, leptospirosis and Q fever) and an emerging vector-borne disease (bluetongue, BT), and (ii) as illustrations of some of the scientific approaches needed to derive useful information from data gathered through surveillance activities and related studies.

1. Surveillance, monitoring and survey

Although extensively used by epidemiologist and (veterinary) public health professionals, there are many existing definitions of the general terms monitoring, surveillance and survey, and different characteristics based on which their activities can be described (Doherr & Audigè, 2001; WHO, 2001; Salman, 2003; ICAHS, 2012).

Surveillance: *'the systematic, continuous or repeated, measurement, collection, collation, analysis, interpretation and timely dissemination of animal health and welfare related data from defined populations, essential for describing health hazard occurrence and to contribute to the planning, implementation, and evaluation of risk mitigation measures'* (ICAHS, 2012).

Monitoring: *'The systematic, continuous or repeated, measurement, collection, collation, analysis and interpretation of animal health and welfare related data in defined populations which is not associated with a pre-defined mitigation plan although extreme changes are likely to lead to action'* (ICAHS, 2012).

Survey: this term describes an investigation or a study in which data related to the health status/disease or group of diseases from a defined animal population are collected systematically in a predefined - and usually short - time frame, for a specific conceptual hypothesis or exploratory purpose (Salman, 2003).

In general, surveys are not considered surveillance activities, because they do not satisfy the continuous time frame typical of the latter. However, it is broadly recognized that they provide very useful and reliable information on the occurrence and distribution of diseases in animal populations, so they might help refining animal or public health priorities (ICAHS, 2012; Salman, 2003). Moreover, if surveys are repeated on a regular basis, they may be used for monitoring purposes and transition into a surveillance system if action is required (Nsubuga et al, 2006).

For the purpose of disease control, improving the health and productivity of livestock, and thereby, the community well-being, information is needed to: (i) monitor and identify changes in infection and/or disease status in a population over time and space (i.e. for endemic diseases), (ii) detect the introduction of a new pathogen (i.e. for emerging diseases), (iii) respond to disease outbreaks, meet reporting requirements of international organizations (e.g. Office International des Épizooties, OIE) and demonstrate disease status to trading partners (i.e. transboundary diseases, with a serious socio-economic and public health impact, significant in international trade).

Generally, for an action to be taken or to evaluate the effects of that action, measurable factors that allow estimating objectively the size of a health problem and the severity of the risk of a health problem to occur, are needed. There are two broad approaches commonly used in surveillance to gather information to derive those measures. The first is called passive or general surveillance. Passive means that no special activity is undertaken by the collecting authorities to generate the information - it usually comes from the farmers/owners. The term general surveillance indicates that surveillance aims to collect information about all sorts of diseases, and is not targeted at a specific one. The second approach is the use of active or targeted surveillance. The term active indicates that the collecting authorities initiate the data collection, usually following a predefined and structured scheme, while targeted means that the surveillance is aiming at detecting one or more specific diseases (i.e. pathogen-specific).

The type of information that can be collected can be related to: (i) the health status (pathogen causing the infection, disease status, clinical signs) and (ii) risk factors for a health-related event (conditions that enhance or reduce the probability of a specific

disease/infection to occur or spread).

Data collection on health status focuses on the detection, identification and quantification of the infectious disease agent in relevant elements of a transmission cycle: susceptible hosts, vectors and environment. Alternatively, the exposure (either past or present) to the pathogen can be detected by checking the changes in the immunological status of the hosts in response to the pathogen (for example seroconversion). This information can be derived by screening the subjects at risk and carrying out relevant laboratory tests; it forms the basis of the targeted surveillance, which is usually applied for example to: endemic diseases, diseases subjected to national control or eradication plans, OIE notifiable diseases. That kind of information can trigger an intervention (when a certain threshold is reached) or measure the impact of a prevention or control measure (e.g. vaccination, contacts restrictions). Lastly, data on clinical signs or group of clinical signs (syndrome or case definition) can be collected and may precede or substitute formal diagnosis. That information may indicate, with sufficient probability, a change in the health status of the population and deserve further investigation or a rapid response. Such kind of information forms the basis of passive surveillance and can be used to detect a variety of diseases or pathogens, including new (emerging) diseases, so it is particularly applicable for early warning.

Data collection on the risk of a health problem focuses on detecting those factors that can potentially enhance the occurrence of a disease/infection, its transmission or spread, and its effect on the host. These data include for example specific behaviour that increases exposure of hosts to a pathogen or a source of infection (e.g. husbandry, animal movements, transhumance, contacts, trading patterns), or information about the presence/absence and abundance of a vector when this is required for a pathogen to be transmitted (as well as data on the conditions influencing vector activity), or natural wildlife migrations. This information can be used to quantify disease threat and inform public health decisions on whether and how to take action (i.e. early warning and preparedness). Such data can also be collected to investigate the unknowns of the epidemiology or ecology of an infectious disease, in order to promote more fundamental scientific understanding.

Surveillance activities require paying careful attention to the methodology used, particularly to having a clear definition of the target population to which the results can be generalized, as well as to the sample size calculation, based on the epidemiological characteristics of the disease under surveillance (validity and representativeness of information). Questionnaires to collect additional information on conditions (e.g. behavioural or environmental), which are considered risk factors for disease

transmission and spread, often accompany surveillance activities. Standardizing these tools, supervising interviewers and maintaining high response rates are then critical to avoid bias. Similarly, accuracy of measures of health status (e.g. sensitivity and specificity of diagnostic tests) or risk is as important as the measurement itself and the decision threshold used. So, project-based experiments specifically designed for evaluating specific aspects of the information collected, or inter-laboratories test (i.e. ring-trial) could improve surveillance activities.

Different elements of those so far described in general assume a pivotal role for each disease. This depends on the respective epidemiological cycle (for example the number and type of hosts involved, their role in pathogen transmission) and the characteristic natural history of the disease (for example susceptibility/exposure, incubation period, clinical disease, recovery/death/carrier status).

1.2 Important elements in surveillance systems for zoonotic diseases

Zoonotic infections (zoonoses) involve ubiquitous pathogens that are sustained in animal populations but can be transmitted to and cause disease in humans. This definition embraces a multitude of pathogens, displaying a broad and diverse range of diseases, and clinical and epidemiological features. Zoonoses encompass some of the oldest known communicable diseases, such as rabies and brucellosis, as well as newly recognized emerging infections, such as hantavirus pulmonary syndrome (HPS) and severe acute respiratory syndrome (SARS). A recent review about agents known to infect humans identified 58% of them as zoonotic in origin; furthermore, 73% of human diseases classified as emerging were zoonotic (Woolhouse & Gowtage-Sequeria, 2005). The global distribution, diversity, clinical severity, economic impacts on communities and potential use as bioweapons all contribute to the importance of zoonotic pathogens for public health.

For the purpose of this thesis, zoonoses include those diseases in which a direct or indirect contact with animals or infected products of animal origin are required for the transmission, propagation or persistence of the pathogen to occur (Figure 1); thus excluding those that require a vector (e.g. arthropod) in their epidemiological cycle.

In case of surveillance activities applied to zoonotic infections acquiring information that characterizes both the infection pattern and risk factors for diseases transmission in animals, offers the opportunity to detect pathogens earlier in the transmission or emergence pathway, before introduction to, and potential spread in, human populations. The observation of animal cases/presence of the infection can be

used to trigger targeted surveillance for high-risk human populations to improve the chances of early detection and prevention.

Indicators of the health status of livestock can be obtained through the isolation and identification of the pathogen from animal samples, for example at farm-level or at abattoirs. It greatly contributes not only to the assessment of the epidemiological patterns and critical foci in a region, but most importantly, the precise knowledge of the aetiologic agent may well influence the choice of the most effective vaccine (when multiple species of a pathogen are involved). However, that information requires time-consuming activities and might necessitates biohazard containment facilities. Therefore, for large-scale surveillance and eradication purposes, serological methods based on antibodies detection make up the most widely used technologies. Several pitfalls should be considered when information is derived from a serological test, either intrinsic to the test or influenced by the prevalence of the infection in the animal population and the immunological response of the animals tested in relation to the evolution of the disease.

Important complement to most surveillance activities aimed at reducing the zoonotic diseases burden is the collection of risk factors for disease transmission and spread. Specifically for directly transmitted zoonoses, animal and herd-level risk factors could be collected through a standardized questionnaire. Possibly, the nature of livestock industry and animal production, demographic factors, animal movements data, and interaction with wildlife, management and husbandry system characteristics are suitable targets of intervention strategies.

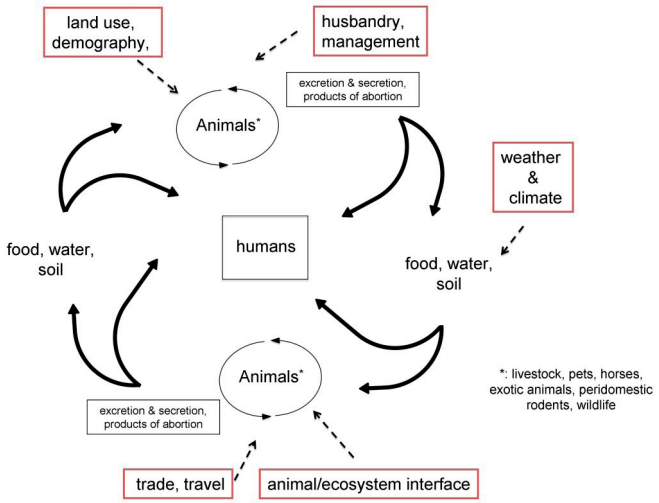

Figure 1. General transmission cycle for zoonotic diseases as defined in the main text. Dashed lines indicate the influence of factors on different elements in the transmission cycle.

1.3 Important elements in surveillance systems for vector-borne diseases

A vector-borne disease (VBD) is one of which the pathogen is transmitted between vertebrate hosts by another organism, called vector. Here, we use the definition in which the bite of an infected arthropod is essential (i.e. true biological vector) for the transmission and propagation of the pathogen and exclude human population from the possible range of susceptible hosts. Because of their complex transmission cycle (Figure 2), vector-borne diseases pose a special challenge to health authorities, as surveillance demands a multidisciplinary approach, which involves experts with different competencies, such as veterinarians, entomologists and ecologists.

Of the general elements that surveillance activities encompass, VBDs require special attention in estimating vector-based parameters, as they are objective measures of the risk of infection and transmission, but are also essential to measure the effect of control strategies directed towards vectors. They are usually derived from data collected actively through a vector-sampling programme. The most important parameters that can be derived are: vector diversity, presence/absence and seasonal abundance. Arthropod vectors are cold-blooded (ectothermic) and thus especially sensitive to climatic factors. Weather influences survival and reproduction rates of vectors, intensity and temporal pattern of vector activity (particularly biting rates) throughout the year and, not least, survival and reproduction of pathogens within vectors. However, climate is only one of many factors influencing vector distribution, such as habitat destruction, land use, pesticide application, and host density. Such information should accompany the insect sampling activity to monitor potential changes in their spatio-temporal distribution. Ideally, the systematic vector collections should be obtained through a consistent sampling frame and standardized collection protocols to allow a more powerful analysis.

A more accurate estimate of the risk of infection for animals can be obtained by combining the vector abundance with its infection rate. So, especially for newly emerging VBDs, the density of infected vectors (through pool testing of vector samples for arthropod-borne pathogens) is a useful complement, but, because of the low infection rate, detecting the pathogen or the exposure to it in susceptible vertebrate hosts (e.g. animal sentinel networks) is preferred as a routine procedure.

Project-based experimental studies on the life parameters, host-preference, seeking activity and the general ecology of the vector species, can provide information to assist health authorities in implementing animal- or vector-based risk mitigating measures, such as: livestock housing and movement restrictions, use of repellents and reducing

larval habitat by removing breeding sites. Besides, some vectors with a broad host feeding preference, might be of relevance to the possible future emergence of new zoonotic diseases.

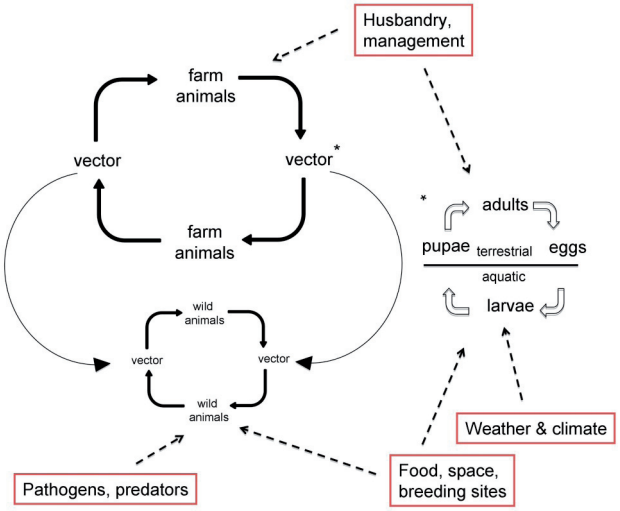

Figure 2. General transmission cycle for VBDs as defined in the main text. Dashed lines indicate the influence of factors on different elements in the transmission cycle.

2. Outline of the thesis

The exposition on surveillance in Section 1, forms the background for the Chapters 2-6 of this thesis as detailed below. In the General discussion (Chapter 7), I return to the broader context to discuss current priorities for livestock infectious diseases.

2.1 Endemic zoonotic diseases in Cameroon

Although it is increasingly recognized that the oldest known zoonotic infections are one of the most important (veterinary) public health priorities in low-income countries, the fundamental lack of knowledge on disease prevalence, incidence and impacts can hardly be overstated. A paucity of region-specific epidemiological data downgrades their importance in the eyes of public health agencies and renders difficult to create the basis for evidence-based disease control policies that are required to protect both human and animal health (Maudlin et al., 2009).

In Chapter 2 and 3, by using existing data from a population-based survey, useful

information are derived on (i) the health status of the cattle population with respect to brucellosis, leptospirosis and Q fever and (ii) the risk factors for exposure to the respective aetiologic infectious agents, amongst animal hosts. The use of an indirect measure of disease occurrence (serological imperfect diagnostic tests), the survey design and a questionnaire, pose challenges for the data analysis and subsequent inference.

2.2 Livestock emerging vector-borne diseases in The Netherlands

Over the last decades, arboviral diseases once confined to the southern parts of the world are emerging beyond the well-known pathogen-vector geographical ranges, particularly at the northern incursional limits (Jones et al., 2008). The BT and its recent emergence in northern Europe represent a clear example. Because bluetongue virus (BTV) transmission requires one of the multiple species of a relatively ubiquitous, but poorly characterized genus of insects (i.e. Culicoides spp.), preparedness strategies for BT incursions would benefit from the knowledge of the basic epidemiological parameters about the potential resident vector population (OIE, 2012).

An active vector-sampling programme is an essential part of the integrated reactive strategy to control BT outbreaks, as provided by EU legislation (Commission Regulation No. 1266/2007). Chapters 4 and 5 are illustrative of the possibility to (i) gather information on the presence/absence, abundance and seasonality of *Culicoides* biting midges, and (ii) study how the presence and activity of BTV vectors are susceptible to weather and environmental conditions, by using the national entomological surveillance dataset during the BT epidemic seasons in The Netherlands.

The use of advanced statistical methods in combination with mechanistic models is a promising tool to investigate the emergence of VBDs. However, modeling vector parameters still remains problematic (Hartemink et al., 2009; Guis et al., 2012). In Chapter 6, a series of field experiments aim at studying which relation exists between the number of biting midges caught by a standardized sampling tool often used during surveillance programmes – suction black-light trap - and the number attracted by the host.

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CHAPTER 2

Serological patterns of brucellosis, leptospirosis and Q fever in *Bos indicus* cattle in Cameroon

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Abstract

Brucellosis, leptospirosis and Q fever are important infections of livestock causing a range of clinical conditions including abortions and reduced fertility. In addition, they are all important zoonotic infections infecting those who work with livestock and those who consume livestock related products such as milk, producing non-specific symptoms including fever, that are often misdiagnosed and that can lead to severe chronic disease. This study used banked sera from the Adamawa Region of Cameroon to investigate the seroprevalences and distributions of seropositive animals and herds. A classical statistical and a multi-level prevalence modelling approach were compared. The unbiased estimates were ~20% of herds were seropositive for *Brucella* spp. compared to ~95% for *Leptospira* spp. and ~68% for Q fever. The within-herd seroprevalences were ~16%, ~35% and ~39% respectively. There was statistical evidence of clustering of seropositive brucellosis and Q fever herds. The modelling approach has the major advantage that estimates of seroprevalence can be adjusted for the sensitivity and specificity of the diagnostic test used and the multi-level structure of the sampling. The study found a low seroprevalence of brucellosis in the Adamawa Region compared to a high proportion of leptospirosis and Q fever seropositive herds. This represents a high risk to the human population as well as potentially having a major impact on animal health and productivity in the region.

Introduction

Zoonoses or diseases transmitted from animals to man, have been recognised as important public health issues for centuries and much of the early history of veterinary science was focused on the control of diseases such as bovine tuberculosis. Ungulates, in particular, are known to carry at least 315 zoonotic pathogens (Cleveland et al., 2001) and many emerging and re-emerging infectious disease problems globally are zoonotic (Taylor et al., 2001). In spite of the clear need to understand these diseases in the animal populations where they may be maintained (Haydon et al., 2002) the veterinary and medical professions need to work closely on infectious disease research in multidisciplinary teams to be successful in tackling many of these diseases. There is a clear and urgent need for this in sub-Saharan Africa (SSA) where the public health and veterinary infra-structures have virtually collapsed through neglect and enforced privatisation.

Brucellosis, caused by bacteria of the genus *Brucella*, is a significant worldwide infectious disease of domesticated animals and wildlife. In animals it is characterized by reproductive failure in females and sterility in males. In man it causes a range of symptoms but typically an undulating fever and is one of the most ancient described zoonosis (Corbel, 1997; Nicoletti, 2002). *B. abortus* is the cattle adapted species and typically is a major abortive agent. It has been the object of successful eradication campaigns in many countries in the developed world. *B. melitensis* may also cause abortion in cattle, although it is mainly associated with sheep, goats and wildlife (Kelly, 2004). Brucellosis is widespread with varying prevalences across Africa, with some areas reportedly having up to 30% seroprevalence. The state of knowledge was recently reviewed by McDermott and Arimi (2002), who highlighted its relative importance in cattle, sheep, goats, pigs and wildlife across the main livestock production systems in SSA.

Leptospirosis is a zoonosis of ubiquitous distribution, caused by infection with pathogenic spirochetes belonging to the genus *Leptospira*. They infect a wide spectrum of hosts, including mammals, reptiles, birds and amphibians. They pose a significant public health problem of increasing concern as well as great impact on the reproductive efficiency of livestock (Hanson, 1982; Ellis et al., 1986; Levett, 2001; Lloyd et al., 2007). Cattle are the maintenance host for *Leptospira borgpetersenii* serovar Hardjo (subtype hardjobovis) and *Leptospira interrogans* serovar Hardjo (subtype hardjoprajitno), which are serologically indistinguishable but genetically distinct (Levett, 2001). A variety of clinical illnesses are seen when a cow becomes infected for the first

time: abortion, mastitis, loss of milk and calves may be stillborn, weak or clinically normal but infected. Infertility associated with persistent infection is the most important economic consequence. Infection is usually transmitted directly by contact with infected urine, run-off water or abortion fluids from infected animals. The situation regarding leptospirosis in Africa is mostly unknown and rarely documented outside South Africa (Hunter & Herr, 2004), although it is associated with high rainfall regions in cattle in South Africa. Symptoms of leptospirosis in man include high fever, severe headache, chills, muscle aches, and vomiting, and may include jaundice, red eyes, abdominal pain, diarrhea, and/or a rash. The symptoms in humans appear after a 4-14 day incubation period following contact with infected urine from animals.

Q fever is a highly contagious zoonotic disease caused by the intracellular pathogen *Coxiella burnetii*. Multiple hosts can serve as a reservoir of infection, but aborting domestic ruminants are typically the main source of the bacterium in humans and animals. The disease has been recognised since the 1930s and has a worldwide distribution with the exception of Antarctica and New Zealand (Woldehiwet, 2004; Arricau-Bouvery & Rodolakis, 2005). All domesticated ruminants are susceptible but, with the exception of reproductive failures such as abortions, stillbirths, infertility and weak offspring, animals are usually asymptomatic and can remain chronically infected (Bildfell et al., 2000; Guatteo et al., 2006; Rodolakis et al., 2007). Infection in man results from inhalation of airborne contaminated particles and from contact with the milk, urine, faeces, vaginal mucus, or semen of infected animals. The most common manifestation in man is a flu-like illness which can progress to an atypical pneumonia, which can result in a life threatening acute respiratory distress syndrome. The chronic form of Q fever is virtually identical to endocarditis which can occur months or decades following the infection. It can be considered the most infectious disease in the world, as a single bacterium is sufficient to cause infection.

This paper presents a serological analysis of exposure to *Brucella* spp., *Leptospira* spp. and *Coxiella burnetti* in cattle in the Adamawa Region of Cameroon in 2000. The presence of antibodies and hence exposure to these pathogens was measured using ELISAs. The study used banked sera from a previous population-based survey of foot-and-mouth disease in the region. We have used both a conventional estimation approach and a Bayesian framework for the analysis. One of the major problems of surveys and surveillance data is that the results are generally based on an indirect measure of disease or exposure such as a serological test. Few studies appear to include any adjustment for the imperfections or uncertainties in the testing systems they use and therefore risk giving both a biased estimate of seroprevalence and a higher degree

of confidence than is actually supported by the data. This may be partly because there is a shortage of reliable test parameter estimates in the literature for well defined populations and also because test parameters are populations specific and the performance of many diagnostic tests in tropical settings is known to be lower (Greiner & Gardner, 2000). Our approach has been to incorporate prior knowledge about the test parameters where available and use these to estimate the true seroprevalence adjusting for both diagnostic test performance and the study design. These diseases are important both because of the direct impact on livestock production but also because of the potential impacts on human health. Understanding the patterns of these diseases in the livestock populations is critical for both the veterinary and public health services if sensible priorities are to be set and controls are to be implemented.

Materials and Methods

Samples

The samples used for this investigation were originally collected as part of a study of foot-and-mouth disease in Cameroon. The study population has been described in detail (Bronsvoort et al., 2003). Briefly, the study area was the Adamawa Region of Cameroon, an area of approximately 64,000 km^2 lying between latitudes 6^0N and 8^0N. It is the main cattle producing region of Cameroon and is divided into five administrative divisions (Vina, Mbere, Mayo Banyo, Djerem and Faro et Deo), with 88 Ministry of Livestock, Fisheriesand Animal Industries (MINEPIA) veterinary centres distributed across it (Figure 1). A database of 13,006 herds constructed from rinderpest vaccination records was used as the sampling frame. A cross sectional study design was used and a stratified, two stage random cluster sample of cattle herds was selected. Sample size was calculated on the basis of an assumed FMD herd seroprevalence of 50% (Bronsvoort et al., 2003).

Herds were visited between April and October 2000. Samples were collected from 146 herds. Five adult (more than 24 months of age) and five juvenile (8 to 24 months of age) samples were collected from the majority of the herds, producing 1377 individual samples in total. Blood was sampled by jugular venepuncture and allowed to clot. At the end of each day the blood samples were centrifuged in the field and approximately 3.5ml of serum was separated from each and divided into two 1.8ml cryovials (Nunc, Thermo Fisher Scientific). The samples were kept at 4^0C in a portable

gas refrigerator until they could be frozen and stored at -20^{0}C, then transported to the UK on dry ice. They have since been stored at the FMD World Reference Laboratory (WRL), Pirbright, at -20^{0}C.

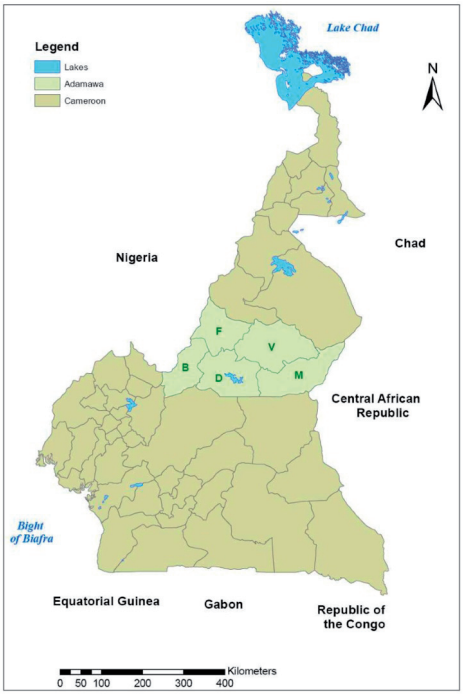

Figure 1. Political map of Cameroon showing the Adamawa Region and the five administrative regions within it (V=Vina; M= Mbere; D= Djerem; B= Mayo Banyo; F= Faro et Deo)

Diagnositic Tests

Brucella **cELISA**. The cELISA *Brucella* diagnostic kit is based on detection of the lipopolysaccharide (LPS) antigen of smooth *Brucella* strains. The immunodominant epitope of the LPS is the O-chain which is a homopolymer of 1,2-linked N-acylated 4-amino-4, 6-dideoxy-a-D-mannopyranosyl residues (Caroff et al., 1984)]. The cELISA was provided and performed by VLA staff according to the O.I.E. Manual of Standards for Diagnostic Tests and Vaccines using the 16M Melitensis strain as antigen and OPD as the chromogen, stopped with Citric acid. The optical density (OD) was read at 450nm and the percentage OD of the conjugate (% OD) were calculated as the average OD of the paired sample wells divided by the average OD of the four

conjugate wells on the plate. The cELISA used a monoclonal antibody specific to the Ochain polysaccharide portion of the *Brucella* LPS (Stack et al., 1999). The standard %OD cut-off of 70% was used initially for interpretation of results but 60% and 50% cut-offs were also explored in the latent class analysis. Using the recommended cutoff and based on the literature, the prior estimates for Se and Sp were 97.8% and 98.6% respectively. All test results were read blind and all results used in this analysis were from the first test unless a plate failed in which case the whole plate was repeated to ensure the controls were within the validation limits.

Leptospira hardjo **ELISA**. The Linnodee Lepto Kit (Linnodee Animal Care, Ballyclare, UK) was used to screen the cattle sera for antibodies to *Leptospira hardjo*. This is a monoclonal antibody capture ELISA kit that detects an antibody response to a LPS outer envelope epitope common to both *Leptospira borgpetersenii* serovar Hardjo bovis and *Leptospira interrogans* serovar Hardjo prajitno (Yan et al., 1999). Sera were diluted 1:50 in the kit dilutent and 100ml was added to a well. Positive and negative controls were run in triplicate on each plate. The plates were incubated at 37^0C for 40 minutes with gentle shaking, then washed with buffer 4 times. 100ml of conjugate was added and the plates covered and incubated at 37^0C for a further 30 minutes with gentle shaking, then washed 4 times with the supplied buffer. Finally 100ml of substrate was added to each well and the plate incubated in the dark at room temperature for 12 minutes. 50ml of stop solution was added and the plates read at 450nm. The test results were expressed as a ratio of the test sample and a mean positive control serum. A sample was recorded as positive if the ratio was greater than the negative cut-off, where the latter was calculated using sera controls using the formula:

$$ratio = \frac{sample\ OD}{mean\ \ positive\ control\ OD} \qquad (1)$$

$$negative\ cut\text{-}off = 2\frac{mean\ negative\ control\ OD}{mean\ \ positive\ control\ OD} \qquad (2)$$

Using the recommended cut-off and based on the literature the prior estimates for Se and Sp were 82.8% and 96.5% respectively. The small sample sizes these are based on is reflected in the higher uncertainty in the priors (Table 1).

Q fever ELISA. A commercial ELISA kit (Chekit-Q-fever, Bommeli, IDEXX Laboratories, Broomfield, CO) was used to screen each serum sample for IgG antibodies to *Coxiella burnetii* based on *C. burnetii* phase I and II purified antigens, where 100ml of 1:400 dilutions of sera were added to the plate with pre-coated *Coxiella burnetii* antigen and incubated for 60 minutes at 37^0C. After incubation the plates were washed 3 times and 100ml of antiruminate IgG conjugate added and incubated for a further 60 minutes. The plates were washed 3 times and 100ml of TMB substrate added to each well and left at room temperature for 15 minutes. The reaction was stopped using the stop solution provided and the plates read at 450nm. Plates where the positive control OD exceeded 2.0 or the negative control OD exceed 0.5 or if the difference between the controls was ≤ 0.3 were rejected and rerun. Samples were run as single spots and 2 positive and 2 negative controls were included on each plate. The % value was calculated using the following formula expressing the OD of the sample as a percentage of the positive controls adjusted for the background OD:

$$\%OD = \frac{OD_{sample} - OD_{neg}}{OD_{pos} - OD_{neg}} 100\% \qquad (3)$$

As recommended by the manufacturer, animals were considered to be positive if they had an optical density percentage (%OD) >40, negative if OD% <30 and ambiguous if between 30 and 40%. Using the recommended cut-off of 40% and based on the literature the prior estimates for Se and Sp were 94.5% and 95.5% respectively. The small samples these are based on is reflected in the higher uncertainty in the priors (Table 1).

Table 1. Priors used for each diagnostic test for modeling true seroprevalence

Parameter	Brucella	Leptospira	Q fever
seA	3428	44	17
seB	77	9	1
spA	7860	217	22
spB	111	8	1

Statistical Analysis

The apparent/test based seroprevalence estimates were calculated using the svy command in Stata 9.0 (Stata Corporation, Texas, USA). The animal-level region-wide seroprevalence variance estimates (P_{animal}), were adjusted using herd as the clustering variable and Division the stratification variable. For the estimates of the proportion of

seropostive herds (P_{herd}), the data set was collapsed to the herd-level and each herd classed as seropositive if one or more animals were test positive for the initial analysis and two or more for the adjusted analysis. Both the P_{herd} and P_{within} variance estimates included adjustment for the study design with veterinary centre as the primary and herd the secondary sampling units, Division as the stratification variable and a weighting to adjust for missing herds from the original sample (Bronsvoort et al., 2003; Lohr, 1999). All confidence intervals are given as 95% intervals for ease of comparison between estimates. None of these estimates include an adjustment for the test sensitivity or specificity.

Modelling

A prevalence model was developed based on the framework used by Branscum et al. (Branscum et al., 2004). Counts of test positive animals in each herd were assumed to be distributed:

$$r \sim binom\,(n_i\,,\, Se\, P_i + (1\text{-}Sp)\,(1\text{-}P_i)) \qquad (4)$$

where r_i is the count of test positive animals in herd i, n_i is the number of animals sampled in herd i, Se and Sp are the test sensitivity and specificity and p_i is the prevalence of seroconversion in herd i. The within herd prevalence, p_i is assumed to be distributed as a mixture:

$$P_i \sim \begin{array}{ll} 0 & Prob\,(1\text{-}\tau) \\ beta\,(a,b) & Prob\,(\tau) \end{array} \qquad (5)$$

In the absence of other data the probability that a herd was sero-positive (τ) was given a vague prior distribution *beta* (1,1). The within herd prevalence used the parameterisation from Branscum et al. (2004) permitting it to be specified with hyper parameters describing the uncertainty of the mean within herd seroprevalence and a term related to its variance.

$$a = \mu + \Psi \qquad (6)$$

$$b = \Psi\,(1 - \mu) \qquad (7)$$

We used a flat (*beta*(1,1)) prior for the mean, μ, within herd prevalence and a vague (*gamma*(0.1,0.1)) prior for the variance related term Ψ.

The prior distributions used for the diagnostic test performances are given in Table 1. The *Brucella* cELISA has been well studied and data from 6 well described studies was use for the priors (Bronsvoort et al., 2009). There was very little published data on the Linnodee test, so estimates were made from the manufacturers data sheet supplied with the kit. A number of publications reported using the CHEKIT Q fever kit e.g. Schelling et al. (2003), however, none of these reported details of the numbers of animals used to validate the test and we have used relatively vague priors with a mean performance of around 92% and 100% for sensitivity and specificity respectfully.

The model parameters were estimated using a Markov chain Monte Carlo methodology with JAGS software (Plummer, 2009) called from R (R core team 2009) using the Rjags package. After an initial burnin period of 200,000 samples a further 300,000 were collected from 3 McMC chains for posterior inference. Apparent convergence of the McMC samples was assessed by visual examination of the sample histories and calculation of the Brooks-Gelman diagnostic (Brooks & Gelman, 1998).

Mapping

Herds had been geo-referenced in the initial (2000) study using hand-held GPS device. The spatial distribution of within herd prevalences, P_{within}, estimated using the Bayesian analysis, were mapped using the R software version 2.9.1 (http://cran.r-project. org/) (Packages 'Sp', 'classInt', 'RColorBrewer' and 'maptools'). Manual jittering was applied to the plotted location of herds with similar recorded locations in order to separate plotting symbols on the published graphics. Mean estimates of prevalence were mapped to a 7 interval colour scale using the same scale for all three pathogens for comparison purposes.

A provisional exploration of global spatial clustering of seropositive herds was carried out using the Cuzick Edwards' *k*-nearest neighbour test (Cuzick & Edwards, 1990). A herd was classed as positive using a cut-off of 1 for *Brucella* and 2 for the *Leptospira hardjo* and *Coxiella burnetii*. For each seropositive herd the test counts how many *k*-nearest neighbours are also seropositive such that if they are ni seropositives and $m_i(k)$ is the number of seropositive herds in the *k* nearest neighbours of herd *i* so that $0 \leq (k) \leq$ k, for $i = 1, \ldots n_r$, a test statistic T_k can be calculated as follows:

$$T_k = \sum_{i=1}^{n_i} m_1(k) \qquad (8)$$

When seropositives are clustered, the nearest neighbour to a seropositive tends to be another seropositive herd and T_k will be large. This is standardised as:

$$\frac{T_k - E(T_k)}{\sqrt{Var(T_k)}} \qquad (9)$$

and the *p*-value reported using the Excel spreadsheet add-in by Carpenter (Spatial Statistics - University of California, 1998). One of the main advantages of this non-parametric test statistic is that it takes account of the heterogeneous distribution of the population at risk as positive and negatives are drawn from the same population.

Herd-Level Sensitivity and Specificity

Using the software tool HERDACC (Jorddan & McEwen, 1998) the herd-level sensitivity (HSe) and herd-level specificity (HSp) were explored for a range of true seroprevalences using the point estimates of the test parameters based on the priors in Table 1.

Ethics Statement

This study used cattle sera biobanked in 2000. The cattle were sampled by a qualified veterinary surgeon with the consent of the animal owner and in accordance with the Cameroonian Ministry of Research (MINREST) guidelines and approval from the University of Liverpool ethics committee in 1999.

Results

Descriptive Test Based Results

A total of 1377 cattle ranging from 8 months to 15 years of age were sampled from 146 herds. The *Brucella* ELISA and Q fever ELISA OD (optical density) values are presented in Figure 2. The distribution of the percentage OD of the conjugate for the *Brucella* cELISA suggests a large negative population with a small test positive population. The distribution of Q fever OD values does not suggest a clear distinction

between the test positive and negative animals at the manufacturers cut-off. The *Leptospira* ELISA does not produce a continuous OD that is comparable between ELISA test plates.

Table 2 shows the estimates of the region-wide animal-level sero-prevalence (P_{animal}), proportion of herds sero-positive (P_{herd}) and within-herd animal-level prevalence (P_{within}).

Prevalence estimates are shown both from test data (i.e. apparent prevalence) and from the Bayesian analysis, which adjusts for diagnostic test sensitivity and specificity. The estimated proportion of seropositive herds is shown using two simple rule based approaches. These rules require either one or more, or two or more test positive animals to classify a herd as seropositive. The apparent P_{animal} of *Brucella* spp. seropositives was 3.1% whereas *Leptospira hardjo* and Q fever had much higher apparent

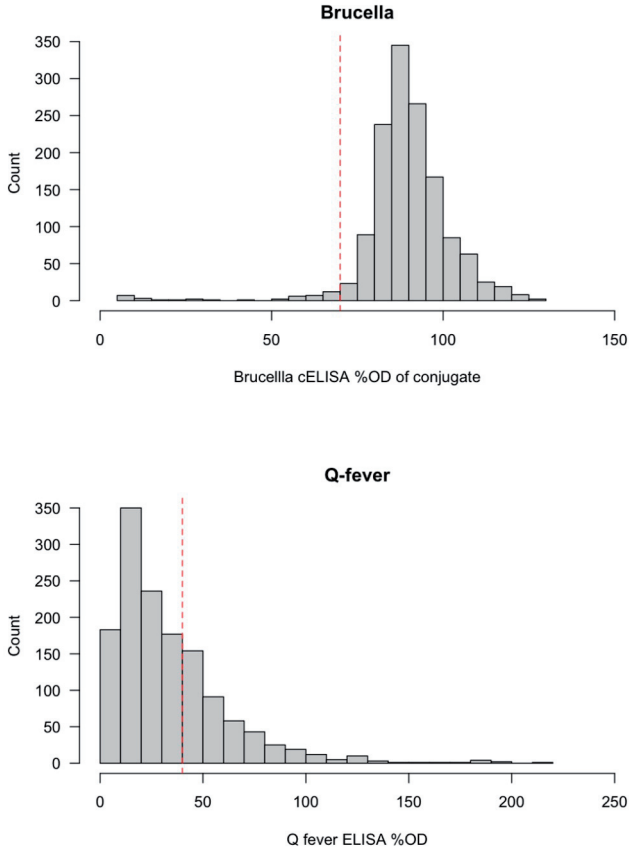

Figure 2. Histogram of optical density values (OD) for the *Brucella* cELISA and Q fever ELISA

P_{animal} seroprevalences of 30.4% and 31.3% respectively.

About 16% of herds (P_{herd}) had at least one test positive animal for *Brucella* spp. compared to 93% for *Leptospira hardjo* and 85% for Q fever. In these test positive herds the apparent P_{within} was ~18% for *Brucella* spp. compared to ~33% for *Leptospira hardjo* and ~36% for Q fever.

P_{herd} for each division was estimated for each of the three infection and are given in Table 3. For each infection, approximately similar proportions of herds are sero-positive across the five administrative divisions (*Brucella* spp. Fisher's exact test p = 0.688; *Leptospira hardjo* Fisher's exact test p = 0.526; Q fever Fisher's exact test p = 0.369).

Seroprevalence Results by Age

The age-stratified apparent seroprevalences for each infection are given in Figure 3. The apparent P_{animal} for *Leptospira hardjo* peaks at around 3 years of age and appears to be steady at ~40% of animals thereafter. The pattern for Q fever is a much more gradual rise possibly peaking at around 45–50% by 8 or 9 years of age. In a closed population with a life long immunity and a non zero force of infection across all ages we would expect seroprevalence to increase asymptotically to 1. A lower asymptotic seroprevalence may be due to loss of immunity or introduction of new animals. However, we would anticipate that the numbers entering are limited and that most of the effect will be due to waning immunity. The pattern for brucellosis is less clear given the very low apparent P_{animal} although there is a suggestion of higher seroprevalences in older animals.

Table 2. Animal-level (P_{animal}), herd-level (P_{herd}) and within-herd (P_{within}) true (model based with 95% highest density intervals) and apparent (with 95% confidence intervals adjusted for study design effects) seroprevalences for cattle in the Adamawa Province of Cameroon to *Brucell*a spp., *Leprospira hardjo* and Q fever.

Disease	Parameter	$Mean_{model}$	LHDI	UHDI	$Mean_{apparent}$	LCI	UCI
Brucellosis	P_{animal}				0.031	0.018	0.044
	P_{herd}	0.203	0.042	0.776	0.159	0.086	0.233
	P_{within}	0.161	0.000	0.345	*0.179	0.141	0.218
Leptospirosis	P_{animal}				0.304	0.276	0.322
	P_{herd}	0.945	0.871	1.000	0.933	0.894	0.972
					+0.760	0.685	0.836
	P_{within}	0.357	0.116	0.577	*0.334	0.304	0.364
Q fever	P_{animal}				0.313	0.273	0.035
	P_{herd}	0.681	0.443	1.000	0.853	0.780	0.926
					+0.629	0.519	0.740
	P_{within}	0.393	0.000	0.725	*0.363	0.324	0.403

*the mean for sub pop with 1 or more test positives in herd. + herd-level seroprevalence estimates using a cut-off of 2

Table 3. Herd-level (P_{herd}) apparent Divisional seroprevalences (with 95% confidence intervals adjusted for study design effects) for cattle in the Adamawa Province of Cameroon to *Brucella* spp., *Leptospira hardjo* and Q fever

Division	Brucella	95% CI	Leptospira	95% CI	Q fever	95% CI
Vina	0.229	(0.111-0.347)	0.958	(0.901-1.00)	0.875	(0.711-0.979)
			+0.813	(0.691-0.934)	+0.604	(0.440-0.769)
Mbere	0.136	(0.00-0.343)	0.881	(0.757-1.00)	0.763	(0.494-1.00)
			+0.814	(0.683-0.944)	+0.610	(0.284-0.936)
Djerem	0.161	(0.017-0.305)	0.935	(0.850-1.00)	0.774	(0.595-0.954)
			+0.742	(0.483-0.984)	+0.613	(0.368-0.858)
Mayo Banyo	0.091	(0.00-0.274)	0.909	(0.815-1.00)	0.939	(0.858-1.00)
			+0.667	(0.483-0.984)	+0.652	(0.428-0.875)
Faro et Deo	0.133	(0.00-0.298)	1.00		0.933	(0.799-1.00)
			+0.733	(0.483-0.984)	+0.733	(0.353-1.00)

in addition herd-level (P_{herd}) apparent Divisional seroprevalences (+) (with 95% CI adjusted for study design effects) *Leptospira hardjo* and Q fever are given after adjusting the herd-level cut-off to b 2 or more test positive animals to class a herd as positive

Herd-Level Sensitivity and Specificity

The original sampling strategy for this survey assumed a 50% within herd prevalence as it was designed to detect foot-and-mouth disease with a 95% herd level sensitivity. Herd-level sensitivity (HSe), which is the probability that a seropositive herd is correctly classified as seropositive, is a function of the sample size, diagnostic test sensitivity, sample interpretation and importantly, within herd animal-level prevalence. The estimated HSe across a range of true within herd seroprevalences are given in Figure 4. Herd-level specificity (HSp) is the probability that a truly seronegative herd is correctly classified as negative by the test system. However, the HSp is simply a function of the sample size and diagnostic test specificity.

Our results suggest that for *Brucella* spp. the expected prevalence is much lower than the design assumption of 50%. For the *Brucella* spp., using the literature based estimates of the cELISA test performance, sampling 10 animals per herd and with an expected within herd prevalence of 15% the HSe was estimated to be ~84% and for a seroprevalence of 20% to be ~91%. Although high, these results mean that unadjusted estimates of P_{herd} in an area will underestimated.

The HSp decreases as the number of animals sampled increases and is ~86% for the *Brucella* cELISA. Therefore, in a completely disease free setting using this testing system we would expect to see on average 21 seropositive herds out of 146. Furthermore, we would expect to only find one false positive animal in a sample of 10 from a herd of 70. Therefore herds with 2, 3 and 4 test positives can more confidently be considered truly seropositive.

The HSe for *Leptospira hardjo* based on the available estimates of diagnostic test

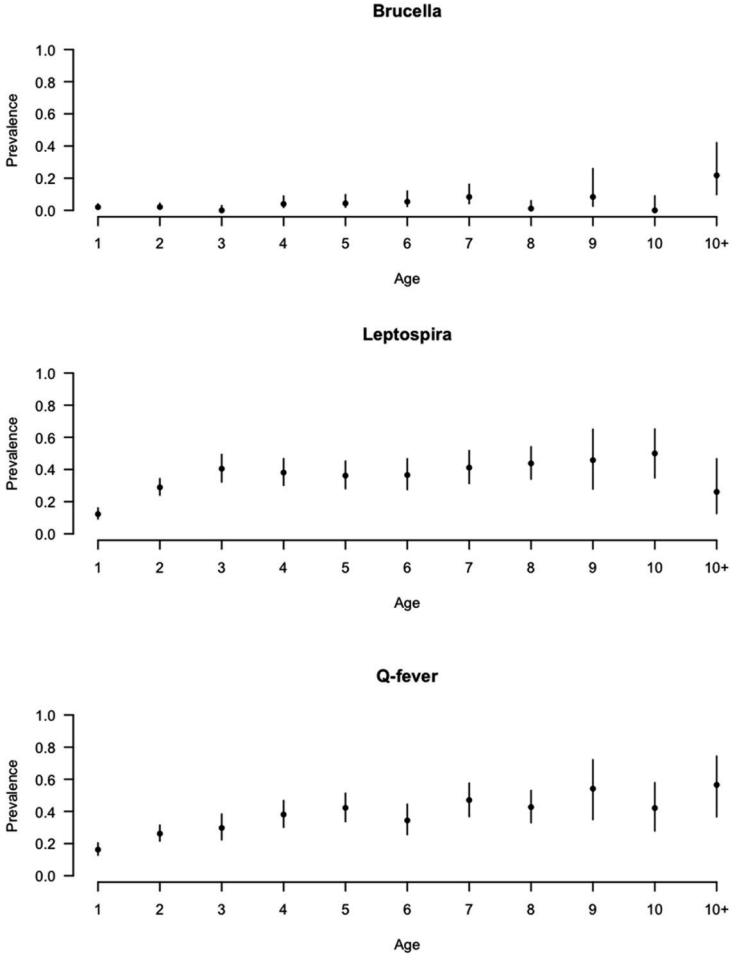

Figure 3. Age stratified animal-level seroprevalence based on raw test results (not adjusted for clustering within herds or diagnostic test imperfections)

performance were ~99.2% for an expected 30% true seroprevalence and ~99.8% at 40%. Therefore at the apparent seroprevalences observed the HSe is high. However the herd level specificity (HSp) is very low at 73.3%. Therefore in a truly negative population using this test we would expect to see 39 test positive herds out of 146. However, the HSp can be greatly improved with minimal impact on the HSe by increasing the cut-point from 1 to 2 test positive animals required to be positive to classify the herd as seropositive. This gives an adjusted estimated HSe of ~97.7% at 30% and HSp of ~98.1%. This approach was used to re-estimate the overall and Divisional P_{herd} (shown in bold in Tables 2 and 3). This resulted in a new estimated proportion of herds seropositive with *Leptospira hardjo* of ~76%, a reduction of 17%.

The HSe for Q fever based on the available estimates of diagnostic test

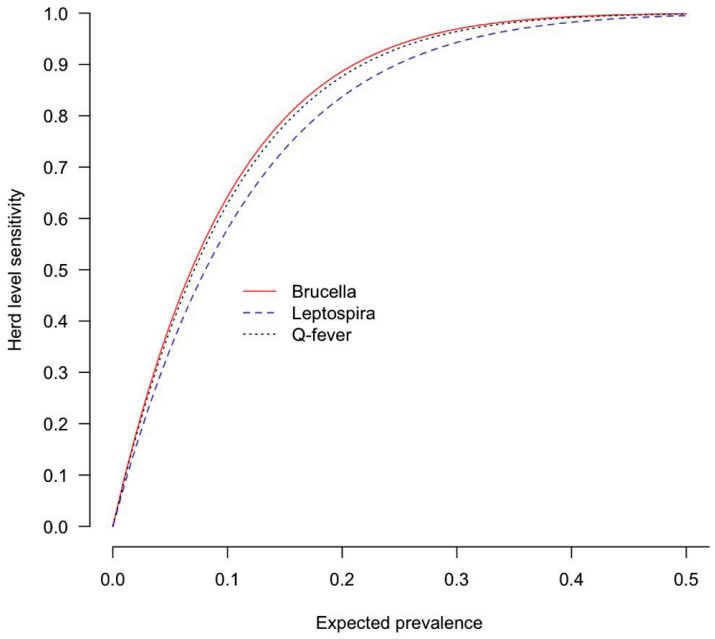

Figure 4. The herd-level sensitivities (HSe) for each of the three infections over a range of true seroprevalences assuming a perfect test specificity

performance were ~99.6% for an expected seroprevalence of 30% and ~100% for 40%. The HSp was low estimated to be ~63%. Therefore, in a truly negative population using this test 55 herds would be classified as seropositive out of 146 sampled herds. However, as with the *Leptospira hardjo* test, the HSp can be greatly improved with minimal impact on the HSe by increasing the cut-point from 1 to 2 test positive animals. This gives an adjusted estimated HSe of ~94.8% and HSp of ~99.3%. The overall and Divisional apparent P_{herd} were re-estimated and are given in Tables 2 and 3. This resulted in a new estimated P_{herd} of ~63%, a reduction of 22%.

Model Based Seroprevalence Estimates Adjusted for Test Performance

Using the hierarchical Bayesian analysis the test imperfections, the uncertainty about their Se and Sp and the study design can all be incorporated to estimate P_{herd} and P_{within}. The overall estimates are given in Table 2 for comparison with the apparent seroprevelence estimates. The model's P_{within} estimates were slightly higher for *Brucella* spp. at 20.3%, similar for *Leptospira hardjo* at 94.5% and lower for Q fever at 68.1%

compared to the apparent estimates. These differences reflect the problems of HSe for *Brucella* spp. using the raw test results and the poor HSp of the Q fever ELISA as already discussed.

The hierarchical model allows for a mixture of sero-negative and sero-positive herds and as well as uncertainty in the test parameters. There will be some herds classed as seropositive falsely by having a false test positive animal and there will be herds that are classified as negative due to the sample failing to pick up a seropositive animal. Furthermore the model based approach enables estimation of P_{within} which can not be done in a conventional analysis after shifting the cut-off.

The model results are summarised for each herd and shown in the caterpillar plots in Figure 5. The posterior mean P_{within} for each herd from the Bayesian analysis is plotted, along with the 95% highest density interval, the apparent seroprevalence from the test results and the probability that the herd was seropositive from the Bayesian analysis.

The graph for *Brucella* spp. still strongly supports the results from the classical analysis and most herds have a low or zero P_{within} and a low probability test negative herds are seropositive. The model estimates for P_{within} for non zero herds is lower than the estimates from the classical approach consistent with a low positive predictive value for a test positive given the low seroprevalence. The probability that a herd is infected increases once the P_{within} rises above ~15%.

The graph for *Leptospira hardjo* is more complicated to interpret. The model estimates for each individual P_{within} suggest a range of P_{within} from ~12% to ~50% compared to the classical estimates that range form 0% to ~70%. There is a switch-over at 35% seroprevalence from the uncorrected test results underestimating P_{within} to overestimating it, reflecting the point where Se and Sp switch their influence. As with brucellosis, once the P_{within} gets above ~20% the probability that the herd is seropositive increases to above 90% and is 100% when P_{within} is above 30%. Using the 2 or more test positive animals cut-off appears to largely classify the same herds with near 100% probability from the model. However the herds with 1 test positive (those with P_{within} of 10%) have a very high probability of being seropositive from the model. There is one herd that due to the small sample of only one animal had a 100% test seroprevalence but the model predicted a more modest 40% true seroprevalence.

The figure for Q fever firstly shows the higher uncertainty in the estimates due to the lack of precision in the Se and Sp estimates. There also appears to be a much wider range of P_{within} from ~5% to ~70%. The use of the higher cut-point reclassifies many of the lower prevalence herds as seronegative; in the model they have a low probability

of being seropositive until P_{within} gets above 30% when the probability the herd is seropositive gets above 95%. This reflects the lack of certainty in the test parameters compounded by the small sample from each herd.

Distribution of Seropositive Herds

The spatial distribution of the P_{within} estimates from the model are plotted in Figure 6. The results of the Cuzick Edwards test statistic are given in Table 4. The spatial distribution of *Brucella* spp. seropositive herds is thinly dispersed across the Region with some suggestion of clustering in the west which is supported by the highly significant test statistic (p<0.001) at all levels up to the third nearest neighbour. In contrast the spatial distribution for *Leptospira hardjo* P_{within} estimates suggest high seroprevalence herds across the entire Region and little statistic evidence of clustering. The pattern for Q fever is the most interesting with a much more variation in P_{within} distribution across the Divisions and possible clustering around the major Divisional towns which was supported by the Cuzick Edwards test statistic (p<0.001) at all levels up to the third nearest neighbour.

Discussion

This serological analysis of exposure to *Brucella* spp., *Leptospira* spp. and *Coxiella burnetti* in cattle is the first report from a well described population based sample of herds in the Adamawa Region of Cameroon for several decades. We have estimated the seroprevalences of these three diseases using both a classical approach which allows for some adjustment for the multi-level design and a model based approach that allows incorporation of the multi-level design of the original sampling, the sensitivity and specificity of the diagnostic tests used and the uncertainties in these tests. The caterpillar plots in Figure 5 summarize most of the information in the results and show that particularly for leptospirosis and Q fever there is a large uncertainty in the individual within herd estimates due to the small sample size from each herd of only 10 animals. The model approach also has the advantage that herds where only a few animals were sampled are adjusted for the general seroprevalence avoiding overestimation. However, for these two diseases it also does confirm the high level of probability that these herds have been exposed. It also highlights the need for high quality diagnostic tests with well described characteristics in order to make reliable interpretation of serological surveys. The lack of sensitivity and/or specificity need to

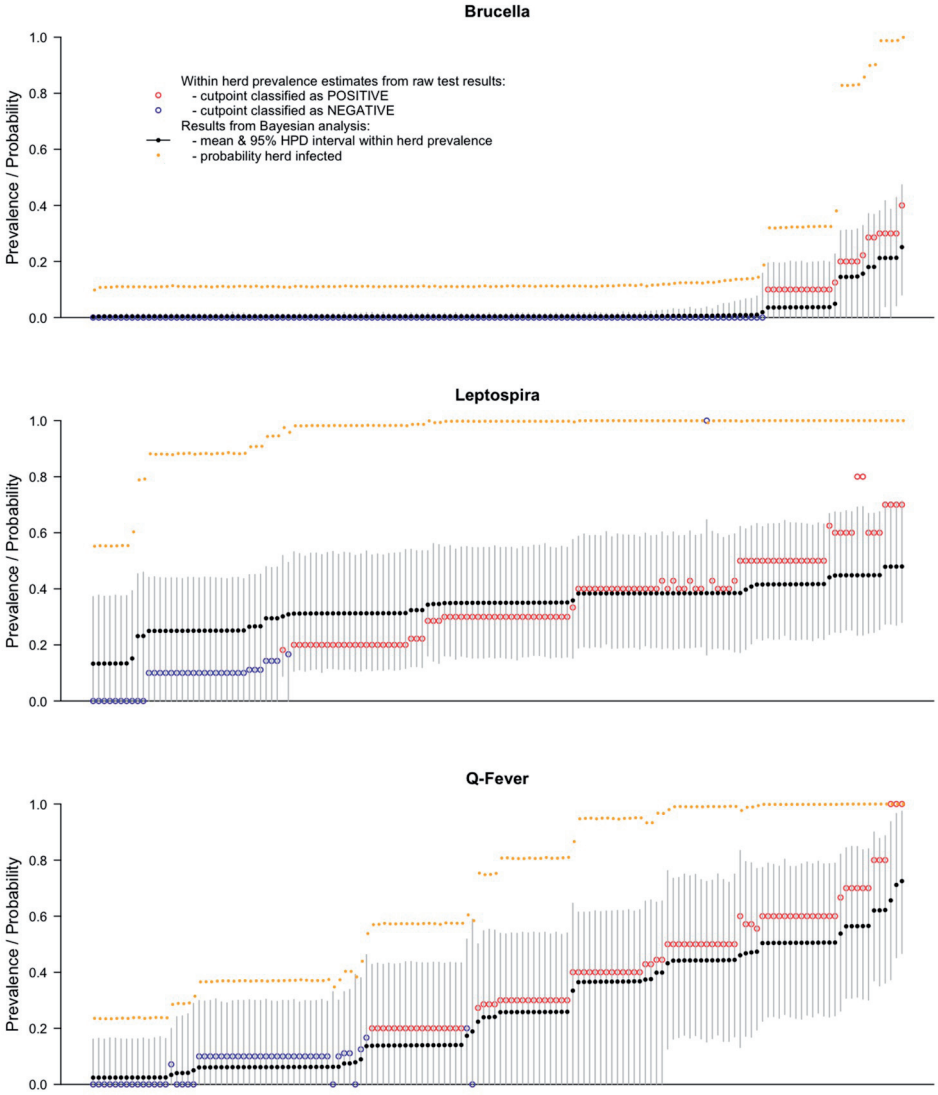

Figure 5. Caterpillar plots showing the classification of each of the 146 herds based on on the raw test results and the Bayesian seroprevalnce model estimates of true within herd seroprevalnce with 95% highest density intervals.

Table 4. Cuzick Edwards k^{th} nearest neignbour analysis resulrts for brucellosis, leptospirosis and Q fever in 146 randomly sampled herds from the Adamawa Region of Cameroon

k	T_k	$E(T_k)$	$V(T_k)$	p-value
Brucella spp.				
1	11	3.8	4.9	<0.001
2	17	7.6	10.0	<0.001
3	24	11.4	15.2	<0.001
Leptospira hardjo				
1	81	84.2	16.4	0.768
2	167	168.4	33.0	0.597
3	245	252.6	63.0	0.832
Q fever				
1	81	59.0	20.6	<0.001
2	147	448.0	41.8	<0.001
3	204	177.0	74.2	<0.001

be adjusted for in order to get unbiased estimates of seroprevalence and as we have shown here that failure to do so can give significantly different estimates.

These analyses estimate the seroprevalence of brucellosis to be much lower than expected even after adjustment for the design and diagnostic test performance. The reasons are not clear. Seropositive herds appear to be focused mainly around the Regional capitol, Ngaoundere, and the western border area next to the North Western Region and Nigeria. The study was under powered to detect seropositive herds at these low within herd seroprevalences and this is therefore likely to be an underestimate of the problem. However, the animal-level seroprevalence is robust.

It is estimated that around 61% of the known 1415 human pathogens are zoonotic (Taylor et al., 2002). The concept of 'one medicine' which is defined as the science of all human and animal health diseases has been around for several decades but its uptake is still generally is lacking in many developing countries where it could have most impact (Schelling et al., 2007). Interestingly Cameroon has a very extensive veterinary infrastructure with 88 centres in the Adamawa alone. Understanding the epidemiology of diseases such as brucellosis, leptospirosis and Q fever are important veterinary issues relating to production losses and abortions. However, the zoonotic nature of these diseases means that it is also important for the medical profession to understand the extent and prevalence of these diseases in the livestock reservoir. All three diseases produce very variable non-specific symptoms in people and are generally believed to be hugely under reported largely due to confusion with malaria in developing countries where 50–80% of malaria cases may suffer fevers resulting from other causes (Amexo et al., 2004).

Brucella seroprevalence in the cattle population of the Adamawa Region appears to be very low with only around 3% of animals in 20% of herds and a mean within herd

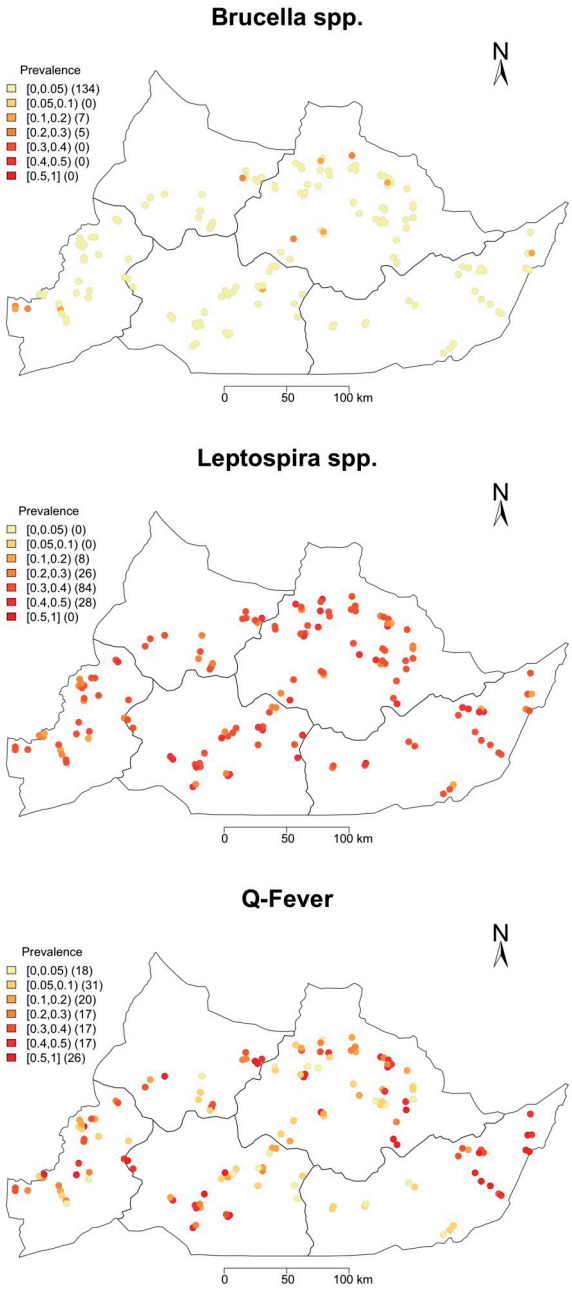

Figure 6. Spatial distribution of sampled herds in the Adamawa Region of Cameroon showing estimated within herd seroprevalnce for brucellosis, leptospirosis and Q fever.

seroprevalence of 16%. Reports from the literature suggest a very variable brucellosis seroprevalence at individual and herd-level across study regions. Estimates include animal-level seroprevalences of 20.2% in Sudan (McDermott & Arimi, 2002), between 0.3% and 8.2% in Eritrea (Omer et al., 2000), 12.3% in Tanzania (Weinhaupl et al., 2000), 6.6% in Chad (Schelling et al., 2003), 3.3% in the Central African Republic (Nakoune et al., 2004), 14.1% to 28.1% in Zambia (Muma et al., 2006). At the herd/unit level estimates range from 2.4% and 46.1% under different husbandry systems in Eritrea (Omer et al., 2000) and in Zambia from 46.2% to 74% across study areas (Muma et al., 2006). Despite the lack of official reports on brucellosis in Cameroon since 1996 (OIE, handistatus II, http://www.oie.int/hs2/), the disease is believed to still be endemic across the country (Shey-Nijla et al., 2005) and the same authors working in Western Province estimated seroprevalaence to be ~10% in cattle sampled at an abattoir. A number of studies have been carried out, mainly in the Northern Province, where seroprevalence values ranging form 7.5% to 31% have been reported (Domenech et al., 1980; Domenech et al., 1982; Bornarel et al., 1987), although these estimates may be largely affected by the sampling method and diagnostic techniques. The low seroprevalence and apparent decline since the 1980s may be due to improved husbandry and awareness but we currently have no knowledge of any systematic control efforts or education campaigns having been carried out.

There does not appear to be any reliable up-to-date information on human brucellosis for the region (Pappas et al., 2006). However, the sub-Saharan African countries included by Pappas (et al.) (2006) appear to have lower annual incidence than North African countries. This may however reflect a poor reporting system in many sub-Saharan regions. There is considerable data on risk factors for human brucellosis and drinking unpasteurized milk Sofian et al., 2008) and handling abortive materials (Samartino & Enright, 1993) from livestock as well as professions such as herdsman and abattoir worker (Swai & Schoonman, 2009) are all higher risk. Currently there are no programs aimed at controlling or eradicating brucellosis from the region. New penside/home test tools are now available for the testing of animals (Bronsvoort et al., 2009; Abdoel et al., 2008) and humans (Abdoel & Smits, 2007) that could greatly speed up identification and of infected animals and people and make control a real possibility.

There are only a few published reports on leptospiosis in African livestock and human populations. Serological studies in cattle in various African countries report overall leptospiral serovars prevalences of 10.4% (Feresu, 1987) to 27% (Feresu, 1992) in Zimbabwe, of 21% (Myburgh et al., 1989) in Malawai and 45% (Niang et al., 1994) in Mali. There is also one report of a seroprevalence of 22% in pigs in South Africa

(Potts et al., 1995). No livestock cases have been reported in Cameroon in the last 10 years (OIE, handistatus II, http://www.oie.int/hs2/). Serological surveillance of human patients in Africa show a similar high seroprevalence with reports from Senegal of a seroprevalence of 35% (Sankale et al., 1976) in hospital patients compared to 37% to 64% in different patient groups in Somalia (Cacciapuoti et al., 1982) and 15.7% in gold miners in Gabon (bertherat et al., 1999).

Q fever has been recently reviewed (Arricau-Bouvery & Rodolakis, 2005) but cites only one paper for Africa (Arricau-Bouvery & Rodolakis, 2005). Malawian zebu cattle have shown seroprevalence ranging from 1.5% up to 5% (Staley et al., 1989); 7%–8.5% for cattle in Transvaal (Gummow et al., 1987); 39% for cattle in Zimbabwe (Kelly et al., 1993); 4% in Chad (Schelling et al., 2003). No livestock cases have been reported in Cameroon in the last 10 years (OIE, handistatus II, http://www.oie.int/hs2/). The seroprevalence in 5 herds in Zambia were 0.9% (Ghirotti et al., 1991). In human populations estimates for the general population are lacking. In a hospital based study in Mali (Steinmann et al., 2005) 40% of patients admitted with fever where positive but none of the individuals had been diagnosed with Q fever at their initial examination.

The Bayesian modeling approach proved useful as this allowed the incorporation of the diagnostic test Se and Sp, the uncertainties in these parameters and the study design features. One of the clear implications of this estimation process is that the within herd sample sizes were small in terms of estimating within herd seroprevalences, which they were never intended for in the first place. However, this approach has allowed unbiased estimates of seroprevelance from a design that was not intended for studying these diseases, allowing maximum information to be extracted from such a survey and the banked material and providing robust estimates for these infections. This is not possible from a classical statistical analysis. This study points to the need for further investigations of these diseases in the Region to confirm the initial and findings and to estimate the levels of clinical and sub-clinical disease in both the livestock and human populations in order to prioritize control strategies. However, control of these diseases in the livestock may be difficult in extensive pastoralist communities in SSA and will need to include education on handling and disposal of abortive materials. The seroprevalence of brucellosis, leptospirosis and Q fever were estimated for the Adamawa Region of Cameroon and brucellosis was found to have a low seroprevalence at both the animal and herd-level compared to leptospirosis and Q fever. The low brucellosis seroprevalence was unexpected based on previous studies from the literature. The high seroprevaelences of exposure to *Leptospira* spp. and *Coxiella burnetti* represent a major challenge both from a veterinary and a public health

view point. It is likely that there is a high incidence of abortion/reproductive failure in affected herds leading to potentially high levels of exposure of livestock owners and their families which is then not being correctly diagnosed. Further studies are clearly needed to study these important zoonoses and to be able to understand the human and animal interactions and the clinical significance of these seroprevalences in both the animal and for the human populations.

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Author Contributions

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CHAPTER 3

Risk factor analysis for antibodies to *Brucella*, *Leptospira* and *C. burnetii* among cattle in the Adamawa Region of Cameroon: a cross-sectional study

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Abstract

Brucellosis, leptospirosis and Q fever are important livestock diseases, commonly responsible for significant production losses, yet their epidemiology in sub-Saharan Africa is largely unknown. Animal reservoirs pose the main risk of transmission to humans, where serious disease can occur. In the developing world setting, the flu-like symptoms of the acute stages of these diseases can be misdiagnosed as malaria, which can result in the administration of the wrong treatment, prolonged disease and increase in antibiotic resistance. Multivariable mixed-effects logistic regression models in this study revealed potential risk factors associated with the aforementioned pathogens in cattle in the Adamawa Region of Cameroon, with wildlife, namely, buffaloes, playing a major role in both *Brucella* and *Coxiella burnetii* seropositivity. Cattle mixing with other herds at night and cattle grazing in an area on a route taken by herds on transhumance appear to be positively associated with *Leptospira* seropositivity, while female cows and whether buffaloes are seen during grazing or transhumance are positively associated with *C. burnetii* seropositivity. On the other hand, animals that have been on transhumance in the past year and animals belonging to herdsmen of the Fulbe ethnic group appear to be protected against *Leptospira* and *C. burnetii*, respectively. Cattle of more than 2 years old appear to have increased odds of being seropositive to either pathogen. Further research is needed to confirm these findings and improve the knowledge of the epidemiology of these three pathogens in Africa, taking particular consideration of the wildlife involvement in the disease transmission.

Introduction

Zoonoses such as leptospirosis, Q fever and brucellosis can cause significant livestock production losses as well as debilitating disease in humans. Such diseases provide a worldwide challenge, which is augmented by the rising population size, globalisation and increased demand for food production. Brucellosis is a zoonosis caused by Gram-negative bacteria of the genus *Brucella* and commonly affects sheep, goats, cattle and humans. Brucellosis can result in abortion, infertility and reduced milk production in cows and varying degrees of sterility in bulls and is mainly caused by *Brucella abortus* (Corbel 1997; Godfroid et al. 2004). Infected animals excrete Brucella in urine, milk and abortive material, and the organism can survive in the environment for up to 80 days (Doganay and Aygen 2003). In humans, the disease is most commonly caused by *Brucella melitensis* followed by *B. abortus* and invariably involves an undulating fever but has otherwise non-specific symptoms. Although, the mortality rate is low, brucellosis can result in long periods of convalescence and residual disability (Namanda et al. 2009). Brucellosis is considered both a foodborne and an occupational disease, and transmission can occur by contact with infected animal parts, consumption of infected unpasteurised milk products and via the airborne route (Pappas et al. 2006, 2008). Despite the disease-free status of a number of developed countries around the world, bovine brucellosis still remains one of the most important zoonoses due to its worldwide distribution and great economic and public health impacts (OIE 2009). In sub-Saharan Africa (SSA), brucellosis is considered particularly important and its prevalence ranges from sporadic cases to as high as 41% in some areas (Domingo 2000; McDermott and Arimi 2002). Risk factors associated with *Brucella* seropositivity suggested by studies carried out in various settings in Africa include high stocking density, common grazing and watering points, older age, herds with multiple livestock species and going on transhumance (McDermott and Arimi 2002; Megersa et al. 2011; Muma et al. 2007; Swai and Schoonman 2010).

Leptospirosis is a neglected disease caused by different species of the spirochete *Leptospira* which has a large variety of carriers and vectors including both wild and domestic animals (Guitián et al. 2001; OIE 2008; Vijayachari et al. 2008). In cattle, it can cause fever, decreased milk production, mastitis or even death and it is also an important cause of abortion and infertility (Hunter 2004; Scolamacchia et al. 2010). Transmission occurs by direct or indirect contact, and excretion is mainly by urine with a variable duration of several weeks to an animal's lifetime (Ellis 1984). The organism may survive in the environment for prolonged periods of time favoured by warm,

humid conditions (Levett 2001).

Transmission to humans occurs via exposure to infected animal urine, either directly or through contaminated environment especially water. Leptospirosis is considered an occupational as well as a recreational disease, and it can vary from a subclinical or mild disease to a life-threatening condition called Weil's syndrome. The disease may initially present as a sudden onset fever mimicking other non-specific febrile diseases and can be misdiagnosed. Mortality rates vary depending on the organ systems involved and have been reported to be between 3 and 54%. In developing countries, mortality rates also depend on diagnostic delays due to lack of clinical suspicion and insufficient infrastructure (Bharti et al. 2003; Esen et al. 2004). The incidence of leptospirosis in the developed world has decreased considerably, while increasing trends have been described in developing countries (Vijayachari et al. 2008). In Africa, with the exception of South Africa, the status of leptospirosis is largely unknown (Pappas et al. 2008). Recent studies have reported a prevalence of leptospirosis of 19.4% in KwaZulu-Natal in South Africa (Hesterber et al. 2009) and 10.8 and 30.3% in Tanga Region of Tanzania (Swai et al. 2005; Schoonman and Swai 2010). Estimates from older studies include 27% in Zimbabwe (Feresu 1987), 21.4% in Malawi (Myburgh et al. 1989) and 44.8% in Bamako, Mali (Niang et al. 1994). Risk factors for bovine leptospirosis, identified mainly in studies outside SSA, include a larger herd size; increased stocking density; access to contaminated water sources; use of an infected bull; co-grazing with infected cattle, sheep or pigs; and older age. Herd type and replacement policy also seem to be important (Alonso-Andicoberry et al. 2001; Lilenbaum 2003; Segura-Correa et al. 2003).

Q fever is a highly infectious re-emerging zoonosis with a worldwide distribution caused by *Coxiella burnetii*, an obligate intracellular Gram-negative bacterium (Maurin and Raoult 1999). It has an extensive range of reservoirs including mammals, birds and ticks. In cattle, the infection is usually asymptomatic but can cause metritis, late-term abortion and stillbirths. In humans, Q fever can present as a flu-like illness, atypical pneumonia or hepatitis in its acute form and as a lifethreatening endocarditis or a chronic fatigue syndrome in its chronic form. Transmission to humans mainly occurs via inhalation of contaminated aerosols originating from excretions and abortive material of farm animals such as cattle, sheep and goats as well as pets. The organism is commonly present at high concentrations in the uterus and mammary glands of infected animals; therefore, transmission is associated with abortion of domestic ruminants (Arricau-Bouvery and Rodolakis 2005; Angelakis and Raoult 2010). Control of transmission is particularly tricky due to the exceptionally low infectious dose, the

long-term survival of the organism in the environment and the ability of the organism to be transported by strong winds (Oyston and Davies 2011). Estimates of prevalence of Q fever in cattle in SSA range from 4% in Chad (Schelling 2003), 14.3 % in the Central African Republic (Nakouné et al. 2004) and 39% in Zimbabwe (Kelly et al. 1993). Literature on risk factor analysis for Q fever seropositivity is limited but includes factors such as large herd size (Ryan et al. 2011) and drinking water from a watercourse or a well, while shed disinfection seems to be protective (Czaplicki et al. 2012).

Despite the endemicity of these three zoonoses in SSA, their prevalence is inadequately documented and their epidemiology is poorly understood. The prevalence of the three zoonoses in this study was presented by Scolamacchia et al. (2010) and was estimated to be 30.4% (95% confidence interval (CI) 27.6–33.2), 31.2% (95% CI 27.3–35.0) and 3.1% (95% CI 1.8–4.4) for *Leptospira*, *C. burnetii* and *Brucella*, respectively.

The current study aims to identify risk factors for seropositivity to the above pathogens and discuss their relevance to previous literature.

Materials and methods

Study background

The current analysis is based on serum samples obtained during a study of foot-and-mouth disease (FMD) in Cameroon. The study was based in the Adamawa Region, which is divided into five administrative divisions and has 88 veterinary centres according to the Ministry of Livestock, Fisheries and Animal Industries.

The sampling frame was created according to a rinderpest vaccination database which included 13,006 herds. A stratified, two-stage cluster sampling design resulted in 1,377 sera being collected from 147 herds. Three herds per veterinary centre were randomly selected without replacement, and approximately five adult (more than 24 months of age) and five juvenile (8 to 24 months of age) cattle from each herd were sampled. In-depth information about this study was published by Bronsvoort et al. (2003). Questionnaire design and data collection On the day of the visit, a 30–40-min pretested questionnaire and a clinical examination of the animals were used to obtain information on possible risk factors. The questionnaire was carried out in Foulfoulde (the local Fulani dialect) and covered a wide range of topics, including information on housing, grazing, watering, ownership or contact with other animals including wildlife, transhumance routines, purchases from markets, treatments used and more.

Additionally, GPS readings were taken in order to match each herd to weather information obtained from the Monitoring Agricultural Resources Unit (http://mars.jrc. ec.europa.eu/mars/About-us/The-MARS-Unit). Lastly, cattle density in each veterinary centre was estimated based on the total number of herds per veterinary centre, the mean herd size for all sampled herds and the area covered. More details on the cattle density calculation can be found in Handel et al. (2011).

Serology

Serology for the three pathogens was carried out on jugular blood samples obtained on the day of the visit and stored at the FMD World Reference Laboratory, Pirbright at -20^{0}C. The Linnodee Lepto ELISA kit (Linnodee Animal Care, Ballyclare, Northern Ireland) and the cELISA *Brucella* diagnostic kit (Brucelisa 400, VLA, Weybridge, UK) were used to screen the sera for antibodies to *Leptospira hardjo* and smooth *Brucella* strains (*B. abortus* and *B. melitensis*), respectively. Lastly, CHECKIT Q Fever ELISA kit (Bommeli, IDEXX Laboratories, Broomfield, CO) was used to screen for IgG antibodies to *C. burnetii*. The tests were performed according to the manufacturer's instructions, and more details can be found in Scolamacchia et al. (2010).

Statistical analysis

Statistical analysis was carried out in R-2.12.1 (R Core Development team 2010). Descriptive analysis was performed in order to identify any missing values or data errors and evaluate the distributions of continuous variables. For categorical variables, a Fisher's exact test was performed using the *epicalc* package (Chongsuvivatwong 2011), and for continuous variables, a univariable logistic regression model was fitted (Hosmer and Lemeshow 2000). Any variables with a p value of less than 0.2 were then used to fit a multivariable mixed-effects logistic regression model shown in Eq. 1 using package *lme4* (Bates et al. 2011). The outcome for the model was individual animal i seropositivity and herd j was used as a random effect. Variables were entered into the model according to their p value, the smallest one entered first, and dropped if the p value was higher than 0.1. The final model was chosen based on the lowest Akaike information criterion (AIC).

$$y_{ij} \sim a + \beta X_{ij} + \mu_j + \varepsilon_{ij} \qquad (1)$$

where a is the fixed intercept, β is the fixed effects, X is the covariate, μ_j is the random effect, ε_{ij} is the error, $\mu_j \sim N(0, \sigma^2_{herd})$ and $\varepsilon \sim N(0, \sigma^2_{animal})$.

Results

Across the five administrative divisions of the Adamawa Region, 1,377 cattle from 147 herds were included in the analysis. Cattle age ranged from 0.33 to 18 years with a median of 3 years and 412 (30 %) of the cattle were male. 70 % of cattle were Gudali, 21 % White Fulani, 7 % Red Fulani and 1 % Cross bred. Results for variables from the univariable analysis used to fit the multivariable model for each pathogen are shown in tables included in the Electronic Supplementary Material.

Brucella

Table 1 describes the modelling process and shows the final model for *Brucella* seropositivity (last row). The final model was chosen using forward selection based on the lowest AIC and includes cattle age and whether they see buffalo during grazing as shown in Table 2. According to this model, cattle of more than 2 years of age show a positive association with an odds ratio (OR) of 2.76 (95 % CI 2.15–3.55) when compared to cattle 2 years old or younger. Additionally, the odds of being seropositive to *Brucella* are almost ten times higher if farmers see buffalo during grazing (95 % CI 1.12–84.58).

Table 1. Comparison of mixed-effects logistic regression risk factor models for *Brucella* seropositivity (bruc)

Model	AIC
bruc~1+(1|hcode)	346.6
bruc~age+(1|hcode)	341.5
bruc~age+buffgrz+(1|hcode)	339.1

buffgrz did you see any buffalo during grazing?
1|hcode herd code (random effect)

Table 2. Final risk factor model for *Brucella* seropositivity in individual cattle in the Adamawa Region of Cameroon (n=1,373; four animals with missing values for buffgrz were dropped)

Variables	Levels	Odds ratio	*p* value	95% CI
Age (years)	0-2	1		
	>2	2.59	0.019	1.17-5.73
buffgrz	No	1		
	Yes	9.72	0.040	1.12-84.58

Leptospira

According to the modelling process followed as shown in Table 3, the final model in terms of *Leptospira* seropositivity included variables 'age', 'trns1vr', 'grzrtrn' and 'nitmix'. Cattle of more than 2 years of age show a positive association with an OR of 2.76 (95 % CI 2.15–3.55) when compared to cattle 2 years old or younger. Additionally, the odds of being seropositive increase in cattle that mix with other herds at night (OR 1.48, 95 % CI 1.05–2.07) and cattle that graze in a grazing area on a route taken by herds on transhumance (OR 1.40, 95 % CI 1.04–1.89). On the contrary, there appears to be a protective effect if any of the animals in the herd went on transhumance this year (OR 0.56, 95% CI 0.42–0.75) (Table 4).

Table 3. Comparison of mixed-effects logistic regression risk factor models for *Leptospira* seropisitivity (lept)

Model	AIC
lept~1+(1|hcode)	1,685
lept~age+(1|hcode)	1,621
lept~age+trns1yr+(1|hcode)	1,612
lept~age+trns1yr+grzrtrn+(1|hcode)	1,608
lept~age+trns1yr+grzrtrn+nitmix+(1|hcode)	1,605

trns1yr Did any of this herd youbrought here todday go on transhumance this year?
grzrtrn Is the grazing area on a route taken by herds on transhumance?
nitmix At night, do youur cattle mix with other herds?; *1|hcode* herd code (random effect)

Table 4. Final risk factor model for *Leptospira hardjo* seropositivity in individual cattle in the Adamawa Region of Cameroon (n=1,377)

Variables	Levels	Odds ratio	*p* value	95% CI
Age (years)	0-2	1		
	>2	2.76	<0.001	2.15-3.55
trans1yr	No	1		
	Yes	0.56	<0.001	0.42-0.75
grazrtrn	No	1		
	Yes	1.40	0.027	1.04-1.89
nitmix	No	1		
	Yes	1.48	0.024	1.05-2.07

Q fever

The modelling process described in Tables 5 and 6 shows the final model for *C. burnetii* seropositivity. Similar to the two previous models, cattle being more than 2 years of age show a positive association with an OR of 2.92 (95 % CI 2.20–3.88) when compared to cattle 2 years old or younger. Female cows seem to have increased odds with an OR of 1.55 (95%CI 1.11–2.15), and the risk also increases if buffaloes are seen during grazing or transhumance (OR 1.89, 95%CI 1.17–3.05). Lastly, when compared to the herdsman

belonging to the Fulbe ethnic group, belonging to Mbororo ethnic group (OR 1.62, 95 % CI 1.00–2.63) and any other ethnic group (OR 2.50, 95 % CI 1.14–5.52), a positive association with *C. burnetii* seropositivity was shown.

Table 5. Comparison of mixed-effects logistic regression risk factor models for *C. burnetii* seropisitivity (qfever)

Model	AIC
qfever~1+(1|hcode)	1,685
qfever ~age+(1|hcode)	1,621
qfever ~age+buffevr+(1|hcode)	1,612
qfever ~age+buffevr+sex+(1|hcode)	1,608
qfever ~age+buffevr+sex+ethngrp+(1|hcode)	1,605

buffevr Did you ever see any buffalo during grazing or transhumance?
ethngrp ethnic group; *1|hcode* herd code (random effect)

Table 6. Final risk factor model for *C. burnetii* seropositivity in individual cattle in the Adamawa Region of Cameroon (n=1,377)

Variables	Levels	Odds ratio	*p* value	95% CI
Age (years)	0-2	1		
	>2	2.92	<0.001	2.20-3.88
buffevr	No	1		
	Yes	1.89	0.010	1.17-3.05
Sex	Male	1		
	Female	1.55	0.010	1.11-2.15
ethngrp	Fulbe	1		
	Mbororo	1.62	0.050	1.00-2.63
	Other	2.50	0.023	1.14-5.52

Discussion

The main aim of this study was to identify potential risk factors associated with cattle seropositivity to *Brucella*, *Leptospira* and *C. burnetii*. According to the three multivariable models described above, cattle more than 2 years old have increased odds of being seropositive to either of the three pathogens. This is in accordance to previous studies, and its explanation lies in the fact that the older an animal is, the longer is the potential exposure to the pathogen (Megersa et al. 2011). The only other risk factor identified by this analysis in terms of *Brucella* seropositivity was if farmers see buffalo during grazing (OR 9.72 ,95 % CI 1.12–84.58).

In addition to age, cattle mixing with other herds at night (OR 1.48, 95 % CI 1.05–2.07) and cattle grazing in an area on a route taken by herds on transhumance (OR 1.40, 95 % CI 1.04–1.89) appeared to be positively associated with *Leptospira* seropositivity. These two factors reflect the increased risk associated with mixing with infected animals. In contrast to what one might expect, animals that have been on transhumance during the past year appear to be protected (OR 0.56, 95 % CI

0.42–0.75). One of the questions in the questionnaire asked 'why did you not take your herd on transhumance this dry season'. One-third of the farmers responded that it was because too many animals die during transhumance. It is possible that farmers with higher disease burdens and hence more deaths do not take their animals on transhumance, and this may act as a confounder. In terms of seropositivity to *C. burnetii*, the multivariable analysis indicated that the odds increase in female cows (OR 1.55, 95 % CI 1.11–2.15) and if buffaloes are seen during grazing or transhumance (OR 1.89, 95 % CI 1.17–3.05). Furthermore, herdman's ethnic group Mbororo (OR 1.62 95 % CI 1.00–2.63) and any other ethnic group (OR 2.50 95 %CI 1.14–5.52) were positively associated with *C. burnetii* seropositivity when compared to the Fulbe ethnic group. This may be due to differences in husbandry regimes in each ethnic group not captured by the current questionnaire, due to its focus on FMD related issues. For example, high concentrations of the pathogen can be found in the placenta of carrier animals so different strategies on disposal of birth products may explain the different risks in each ethnic group (Watanabe and Takahashi 2008). Buffalo seem to be implicated in both *Brucella* and *Coxiella* seropositivity. Antibodies to *Brucella* spp. were found in buffalo (Waghela and Karstad 1986; Chaparro et al. 1990) in Africa, but no recent studies have identified *C. burnetii* in buffalo in Africa, although they were found in buffalo in other areas such as in India (Sodhi et al. 1980). It is therefore possible that seeing buffalo during grazing or transmission reflects the increased risk of a cattle becoming infected when in contact or sharing the same grazing area with infected animals of different species. This finding emphasizes the role of wildlife in infectious disease transmission and highlights the need for inclusion of wildlife in future research.

The high compliance rate, 90.7 % (147/162 herds selected), achieved minimizes non-response bias (Bronsvoort et al. 2003). Another strength of this study is the stratified, two-stage random sampling design used, which provided optimum spatial coverage. Additionally, the use of a well-designed, pretested questionnaire carefully translated into the local language increases the accuracy of the risk factor data. Lastly, the mixed-effects analysis used empowers this study to deal with pseudoreplication that may arise due to the fact that animals may be correlated in space as they belong to the same herd and therefore not completely independent. Using herd as a random effect has the advantage of explaining the variance due to the cluster design, while maintaining the power of the study (Paterson and Lello 2003).

One limitation of this study is the sampling frame used. According to a previous publication providing detailed information on the initial study, the rinderpest vaccination records used from the 1998/1999 MINEPIA campaign were incomplete

and therefore underestimating the number of herds. Nevertheless, this was the best available option for the location and available resources (Bronsvoort et al. 2003). Additionally, the sample size calculation was calculated based on the initial aim of this study, i.e a risk factor analysis for FMD. Lastly, inherent sources of bias in cross-sectional studies using imperfect diagnostic tests include confounding, recall and misclassification bias. Most of these are minimized by the multivariable mixed-effects analysis used. Leptospirosis and Q fever can have life-threatening effects in humans and brucellosis and can cause long periods of convalescence with residual disability. Additionally, during the acute stages of these three diseases, their flu-like symptoms can be misdiagnosed as malaria in the developing world setting, which can result in the administration of the wrong treatment, longer periods of disease and increase in antibiotic resistance (Kunda et al. 2007; WHO 2010). In their review on malaria misdiagnosis based on research mostly located in developing countries, Amexo et al. (2004) have found a mean malaria overestimation by clinical diagnosis of 61 %, highlighting the magnitude of the problem. The main risk of transmission to humans comes from animal reservoirs, and this increases the importance in measuring and identifying ways of decreasing the burden of these diseases in animals. This paper has identified potential risk factors, with wildlife, namely, buffaloes, playing a major role. Further research is needed to confirm these findings and improve the knowledge of the epidemiology of these three pathogens in Africa, taking particular consideration of the wildlife involvement in disease transmission.

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Electronic Supplementary Material

Tables showing the results for variables from the univaraible analysis used to fit the multivariable model for each pathogen

Table 1. Univariable analysis of potential risk factors for *Brucella* seropositivity at the individual animal level (* wald test)

Variable	Level	T+	T-	OR	p-value
Herdsman's ethnic group[b]	Fulbe	20	570	1	0.029
	Laka	4	21	5.4	
	Mbororo	16	636	0.72	
	Hausa	2	8	7.07	
	Sukar	0	10	0	
	Baya	0	16	0	
	Arab choa	0	20	0	
	Dourou	0	10	0	
	Mboum	0	20	0	
	Kapsiki	0	10	0	
	Boute	1	12	3.16	
Did you see any buffalo during grazing or transhumance? [b]	No	24	885	1	0.143
	Yes	19	445	1.57	
Are you the person who looks after the cattle when grazing? [b]	No	33	852	1	0.107
	Yes	10	468	0.55	
Approximately how many herds does your herd mix with during grazing? [b]	0	1	57	1	0.033
	1-6	16	778	1.17	
	>6	22	465	2.69	
Is the grazing area on a route taken by herds on transhumance? [b]	No	11	497	1	0.148
	Yes	32	833	1.74	
Did you see any buffalo during grazing? [b]	No	32	1221	1	0.001
	Yes	11	109	3.84	
Did you see any antelopes during grazing? [b]	No	35	1176	1	0.153
	Yes	8	154	1.74	
Do you feed any cotton seed? [b]	No	37	917	1	0.017
	Yes	6	413	0.36	
At night do your cattle mix with other herds? [b]	No	19	318	1	0.006
	Yes	24	1012	0.4	
How many herds? [b]	0	19	311	1	0.001
	1-3	7	568	0.2	
	4-6	14	330	0.69	
	7-10	3	57	0.86	
	>10	0	54	0	
Do you buy…? [b]	Only cows	7	33	1	0.001
	Only bulls	4	162	0.12	
	Both	17	545	0.15	
Do you keep any goats? [b]	No	39	1065	1	0.082
	Yes	4	275	0.39	
Does your herd mix with, walk amongst, any herds during watering? [b]	No	11	230	1	0.142
	Yes	30	1095	0.57	
What month do the rains normally start here? [b]	February	3	17	1	0.014
	March	4	161	0.14	
	April	25	906	0.16	
	May	8	132	0.35	
Cattle age[a]	0-2 years	13	636	1	0.029
	> 2 years	30	694	2.11	
Cattle sex[a]	Male	7	404	1	0.061
	Female	36	926	2.24	
Cattle breed[a]	Gudali	25	937	1	0.160
	White Fulani	14	279	1.88	
	Red Fulani	3	100	1.12	
	Cross	1	14	2.67	
Average water vapour pressure (hPa) [b]				0.58	0.147*
Number of herds per veterinary area[b]				0.99	0.001*
Physical coverage area of each veterinary centre[b]				1	0.171*
Cattle density in each veterinary centre[b]				0.94	0.026*

T+ test positive, T- test negative, *a* animal level, *b* herd level

Table 2. Univariable analysis of potential risk factors for *Leptospira* seropositivity at the individual animal level (* wald test)

Variable	Level	T+	T-	OR	p-value
Altitude [b]				1	0.002*
Administrative Division [b]	Vina	156	318	1	<0.001
	Mbere	71	154	0.94	
	Djerem	95	158	1.23	
	Mayo Bayo	56	219	0.52	
	Faro et Deo	41	109	0.77	
Herdsman's ethnic group[b]	Fulbe	184	409	1	0.040
	Laka	12	13	2.05	
	Mbororo	189	464	0.91	
	Hausa	0	10	0	
	Sukar	5	5	2.22	
	Baya	5	11	1.01	
	Arab choa	7	13	1.2	
	Dourou	3	7	0.95	
	Mboum	11	9	2.71	
	Kapsiki	1	9	0.25	
	Boute	2	8	0.56	
Is this your herd? [b]	No	120	314	1	0.131
	Yes	299	644	1.21	
Have you ever taken your animals on transhumance? [b]	No	192	354	1	0.002
	Yes	227	604	0.69	
Did any of this herd you brought today, go on transhumance this year?	No	255	475	1	<0.001
	Yes	164	483	0.63	
Are you the person who looks after the cattle when grazing? [b]	No	282	606	1	0.176
	Yes	135	344	0.84	
Does your herd mix with any other herds sharing the grazing area? [b]	No	11	47	1	0.058
	Yes	408	911	1.91	
Approximately how many herds does your herd mix with during grazing? [b]	0	11	47	1	0.051
	1-6	237	558	1.81	
	>6	164	326	2.15	
Is the grazing area on a route taken by herds on transhumance? [b]	No	138	372	1	0.039
	Yes	281	586	1.29	
Did you see any antelopes during grazing? [b]	No	361	853	1	0.147
	Yes	58	105	1.3	
Do you feed any cotton seed? [b]	No	269	687	1	0.006
	Yes	150	271	1.41	
Do you feed any natron? [b]	No	165	418	1	0.155
	Yes	254	540	1.19	
Do you feed any maize? [b]	No	405	942	1	0.069
	Yes	14	16	2.03	
In the night, do you leave your cows to sleep…? [b]	At your wadi	69	151	1	0.087
	In the bush	224	569	0.86	
	Both	126	238	1.16	
At night do your cattle mix with other herds? [b]	No	88	251	1	0.041
	Yes	331	707	1.34	
Do you buy cattle from local markets? [b]	No	162	445	1	0.008
	Yes	257	513	1.38	
Do you keep any goats? [b]	No	323	774	1	0.126
	Yes	96	184	1.25	
How many herds does your herd mix with, walk amongst during watering? [b]	0	66	175	1	0.033
	1-3	139	317	1.16	
	4-6	107	270	1.05	
	7-10	62	124	1.32	
	>10	36	44	2.16	
Do you take the herd to any soda water spring? [b]	No	414	953	1	0.183
	Yes	5	5	2.3	
What month do the rains normally start here? [b]	February	10	10	1	0.103
	March	57	110	0.52	
	April	280	653	0.43	
	May	36	104	0.35	
Cattle age[a]	0-2 years	131	520	1	<0.001
	> 2 years	288	438	2.61	
Cattle sex[a]	Male	109	303	1	0.041
	Female	310	655	1.32	
Average temperature (°C) [b]				1.36	0.008*
Maximum temperature (°C) [b]				1.2	0.008*
Minimum temperature (°C) [b]				1.32	0.027*
Precipitation sum (mm=liters/m²) [b]				0.97	0.007*
Evapo-transpiration sum (mm=liters/m²) [b]				1.06	0.011*
Evapo-transpiration sum (bare soil) (mm=liters/m²) [b]				1.06	0.011*
Evapo-transpiration sum (Penman-Monteith) (mm=liters/m²) [b]				1.07	0.005*
Physical coverage area per veterinary centre [b]				1	0.009*
Cattle density in each veterinary centre[b]				0.98	0.011*

Table 2. continued

Variable	Level	T+	T-	OR	p-value
Global radiation sum (kJ=m² per dekad) *b*	1.81e+05,-1.89e+05	96	266	1	0.053
	1.89e+05, -1.98e+05	96	240	1.11	
	1.98e+05,-2.02e+05	126	228	1.53	
	2.02e+05,-2.07e+05	101	224	1.25	
Climatic water balance (mm=liters/m²) *b*	-5.1, -0.912	112	239	1	0.088
	-0.912, 4	116	229	1.08	
	4, 12.9	107	240	0.95	
	12.9, 28.3	84	250	0.72	

T+ test positive, T- test negative, *a* animal level, *b* herd level

Table 3. Univariable analysis of potential risk factors for *C. burnetii* seropositivity at the individual animal level (* wald test)

Variable	Level	T+	T-	OR	p-value
Administrative Division *b*	Vina	130	344	1	0.020
	Mbere	86	139	1.64	
	Djerem	80	173	1.22	
	Mayo Banyo	79	196	1.07	
	Faro et Deo	56	94	1.58	
Herdsman's ethnic group *b*	Fulbe	154	439	1	0.001
	Mbororo	226	427	1.51	
	Other	51	80	1.82	
Have you ever taken your animals on transhumance? *b*	No	151	3956	1	0.020
	Yes	280	551	1.33	
Did any of this herd you brought today, go on transhumance this year?	No	202	528	1	0.002
	Yes	229	418	1.43	
Did you see any buffalo during grazing or transhumance? *b*	No	255	657	1	<0.001
	Yes	179	289	1.57	
Did you see any antelopes during grazing or transhumance? *b*	No	278	666	1	0.033
	Yes	153	280	1.31	
Does your herd mix with any other herds sharing the grazing area? *b*	No	24	34	1	0.111
	Yes	407	912	0.63	
Approximately how many herds does your herd mix with during grazing? *b*	0	24	34	1	0.066
	1-3	111	266	0.59	
	4-6	138	280	0.7	
	7-10	103	203	0.72	
	>10	45	139	0.46	
Do you feed any cotton seed? *b*	No	320	636	1	0.010
	Yes	111	310	0.71	
Do you feed salt? *b*	No	1	9	1	0.186
	Yes	430	937	4.13	
Do you feed mineral lick? *b*	No	406	921	1	0.005
	Yes	25	25	2.27	
Do you feed any maize? *b*	No	426	921	1	0.109
	Yes	5	25	0.43	
Do you feed hay? *b*	No	430	937	1	0.186
	Yes	1	9	0.24	
How many herds does your herd mix with at night? *b*	0	97	235	1	<0.001
	1-3	211	364	1.4	
	4-6	103	243	1.03	
	7-10	10	50	0.49	
	>10	6	48	0.3	
Where do your cattle get drinking water? *b*	River	407	870	1	0.160
	Pond	2	8	0.53	
	Dammed stream	6	4	3.2	
	Other	2	8	0.53	
	River/dammed stream	7	23	0.65	
	River/pond/trough	1	9	0.24	
	River/pond	6	24	0.53	
How many herds use their water place on an average day? *b*	0	33	60	1	0.191
	1-3	173	322	0.98	
	4-6	128	298	0.78	
	7-10	57	153	0.68	
	>10	33	83	0.72	
How many herds does your herd mix with, walk amongst during watering? *b*	0	78	163	1	0.175
	1-3	160	296	1.13	
	4-6	111	266	0.87	
	7-10	49	137	0.75	
	>10	22	58	0.79	
Do you take the herd to any soda water spring? *b*	No	431	936	1	0.036
	Yes	0	10	0	
Do you borrow a bull from friends or neighbours? *b*	No	366	857	1	0.003
	Yes	65	89	1.71	

Table 3. continued

Variable	Level	T+	T-	OR	p-value
How many years have you lived in this area? [b]	1-3	55	106	1	0.124
	4-6	36	119	0.58	
	7-10	63	132	0.92	
	11-20	99	186	1.03	
	>20	173	391	0.85	
What month do the rains normally end here ? [b]	July	2	8	1	0.024
	September	11	29	1.51	
	October	146	411	1.42	
	November	172	314	2.19	
	December	3	7	1.67	
Cattle age [a]	0-2 years	137	514	1	<0.001
	> 2 years	294	432	2.55	
Cattle sex [a]	Male	94	318	1	<0.001
	Female	337	628	1.81	
Cattle breed [a]	Gudali	291	674	1	0.171
	White Fulani	103	191	1.25	
	Red Fulani	35	68	1.19	
	Cross	2	13	0.36	
No. of herds per veterinary centre [b]				1	0.154*
Precipitation sum (mm=liters/m^2) [b]	42.4, 45	130	215	1	<0.001
	45, 48.7	109	262	0.69	
	48.7, 55.4	77	250	0.51	
	55.4, 68	115	219	0.87	
Evapo-transpiration sum (Penman-Monteith) (mm=liters/m^2) [b]	37.7, 42.3	114	233	1	<0.001
	42.3, 44.8	85	85	0.67	
	44.8, 46.1	90	90	0.73	
	46.1, 48.5	142	142	1.45	
Climatic water balance (mm=liters/m^2) [b]	-5.1, -0.912	141	210	1	<0.001
	-0.912, 4	97	248	0.58	
	4, 12.9	78	269	0.43	
	12.9, 28.3	115	219	0.78	
Physical coverage area per veterinary centre [b]	142, 452	102	272	1	<0.001
	452, 644	114	204	1.49	
	644, 935	132	212	1.66	
	935, 2.62e+05	83	258	0.86	

T+ test positive, T- test negative, *a* animal level, *b* herd level

CHAPTER

4

The Mondrian matrix: *Culicoides* biting midge abundance and seasonal incidence during the 2006-2008 epidemic of bluetongue in The Netherlands

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Abstract

During the northern Europe epidemic of bluetongue (BT), Onderstepoort type blacklight traps were used to capture *Culicoides* Latreille (Diptera: Ceratopogonidae) biting midges weekly between November 2006 and December 2008 on 21 livestock farms in the Netherlands. Proven and potential vectors for the bluetongue virus (BTV) comprised almost 80% of the midges collected: the Obsoletus complex, constituting *C. obsoletus* (Meigen) and *C. scoticus* Downes & Kettle (44.2%), *C. dewulfi* Goetghebuer (16.4%), *C. chiopterus* (Meigen) (16.3%) and *C. pulicaris* (Linnaeus) (0.1%). Half of the 24 commonest species of *Culicoides* captured completed only one (univoltine) or two (bivoltine) generations annually, whereas multivoltine species (including all BTV vectors) cycled through five to six generations (exceeding the one to four generations calculated in earlier decades). Whether this increment signals a change in the phenology of northern Europe *Culicoides* or simply is an adaptive response that manifests during warmer episodes, thus heightening periodically the incursive potential of midge-borne arboviruses, remains to be clarified. *Culicoides duddingstoni* Kettle & Lawson, *C. grisescens* Edwards, *C. maritimus* Kieffer, *C. pallidicornis* Kieffer and *C. riethi* Kieffer are new records for the biting midge fauna of the Netherlands. It is suggested that *C. punctatus* (Meigen) be added to the European list of vector *Culicoides.*

Introduction

The bluetongue (BT) virus (BTV) causes an infectious, noncontagious disease in ruminant livestock, particularly sheep (Verwoerd & Erasmus, 2004). Although confined largely to the tropical and subtropical regions of the world that lie below 45^0N, BTV is able to break out of these warmer zones and move up to about 50^0N (Thomas et al., 1982; Lundervold et al., 2003). Currently, the virus is understood to comprise at least 26 serotypes (BTV-1–BTV-26) (Maan et al., 2012), with serotype multiplicity highest in the tropics, where the ruminant host range is more diverse and where vector *Culicoides* attack them throughout the year.

In August 2006, BTV-8 leapt out of tropical Africa and landed in northern Europe at about 51^0N, near to where the borders of Belgium, Germany and the Netherlands meet (Van Wuijckhuise et al., 2006; Elbers et al., 2008). From there it fanned out rapidly in all directions, spreading at 10–15 km/week (Gerbier et al., 2008). Over the next two seasons >76 000 outbreaks were reported in sheep and cattle. Eventually, 15 countries were affected, including Norway and Sweden, where the virus penetrated to beyond 58^0N (Sternberg Lewerin et al., 2010). After BTV-8 recrudesced in the summer of 2007, the European Commission (EC) opted to contain its further spread through broad scale vaccination (European Council Decision 2008/655/EC) so that by the autumn of 2008 a considerable proportion of the susceptible livestock population had been immunized. The campaign was an unprecedented success: by the end of 2009 residual outbreaks numbered a trifling 275 spread over 7 Member States (MSs). Nevertheless, the financial impact of the epidemic was tremendous and for the Netherlands alone estimated at over €200 million (Velthuis et al ., 2010).

The initial reaction was to classify the incursion of BTV-8 into northern Europe as an 'extraordinary event' and that it involved the simultaneous introduction – or rapid northward movement – of an exotic vector for BTV. *Culicoides imicola* Kieffer from Africa and the Mediterranean region was the prime suspect, but numerous surveys conducted since 2006 have failed to provide evidence for its existence north of the Alps (Meiswinkel et al., 2008b; Clausen et al., 2009; Ander et al., 2012). What transpired instead is that three Palaearctic species, namely *C. obsoletus*, *C. scoticus* and *C. pulicaris*, all incriminated previously as vectors for BTV in southern and eastern Europe, were found to be involved also north of the Alps (Carpenter et al., 2006, 2008; Hoffmann et al., 2009; Vanbinst et al., 2009). Subsequently, a further two, and predominantly northern species, namely *C. dewulfi* and *C.chiopterus*, were implicated as additional vectors (Meiswinkel et al., 2007; Dijkstra et al., 2008). Inclusive of *C. imicola*, this brings

to six the number of species involved in the transmission of BTV in western Europe, the largest agglomeration of potential and proven *Culicoides* vectors found within any one of the five episystems delineated globally for BTV (Tabachnick, 2004).

Takken et al. (2008) were the first to investigate the spatial and temporal occurrence of *Culicoides* species in the Netherlands. Using CO_2-baited traps they found in 2005 that adult midges stayed active until week 45 and in 2006 became active again in week 18. Their study ended just prior to the unexpected advent of bluetongue virus serotype 8 (BTV-8) in August 2006. Its rapid spread subsequently led to a second *Culicoides* survey being conducted in the Netherlands, but on this occasion the more powerfully attractant Onderstepoort-type blacklight trap was used. This culminated in the development of presence–absence 'snapshot' maps for 21 *Culicoides* species captured on 106 cattle holdings sampled across the country (Meiswinkel et al., 2008b). These maps were considered exploratory however as they relied on data emanating from one light trap collection per farm and accumulated over a very limited period (12–28 September 2006). Nevertheless, the Obsoletus Complex (comprising *C. obsoletus* and *C. scoticus*) was found on 93.4% of the farms surveyed, followed by *C. dewulfi* (70.8%), *C. punctatus* (67.9%), *C. chiopterus* (67.0%), *C.* sp. nr. *newsteadi* (44.3%) and *C. pulicaris s.s.* (17.9%).

The 'snapshot' character of the maps did little to dispel certain doubts that arose after the arrival of BTV-8 in August 2006. These doubts included the sense that the heightened seasonal temperatures were the immediate cause for the apparent 'above-average' vector levels; and that these would subside to more 'benign' levels once the weather returned again to 'normal'. In order to throw further light on these questions and because EU regulations make it obligatory to conduct a surveillance programme during outbreaks of a vector-borne disease, a regimen involving weekly lighttrapping, was instituted on 21 of the holdings that had formed part of the original 'snapshot' investigation. The bulk of the results obtained between the beginning of November 2006 and the end of December 2008 are reported upon here. The findings are related to those made historically within the same broad region and defined loosely as 'northern Europe'.

Materials and methods

Study area

The Netherlands, as proposed in the Commission Decision 2005/393/EC, is divided into 21 compartments (Fig. 1) that formed the epidemiological units for all the BT monitoring and surveillance programmes conducted after the advent of BTV-8 (Van Schaik et al., 2008). For the *Culicoides* survey a single light trap was operated on a farm in each one of these 21 compartments; the sites monitored were selected from amongst the 106 originally sampled during the 2006 'snapshot' survey (Meiswinkel et al., 2008b). Each site carried mixed livestock that comprised cattle (>90%), sheep and goats; total livestock numbers are given in Table 1. All 21 compartments were monitored in 2007 and 990 out of a projected 1092 light trap collections made; in 2008, in order to lower costs, only 11 compartments were monitored and 551 of a projected 572 collections made.

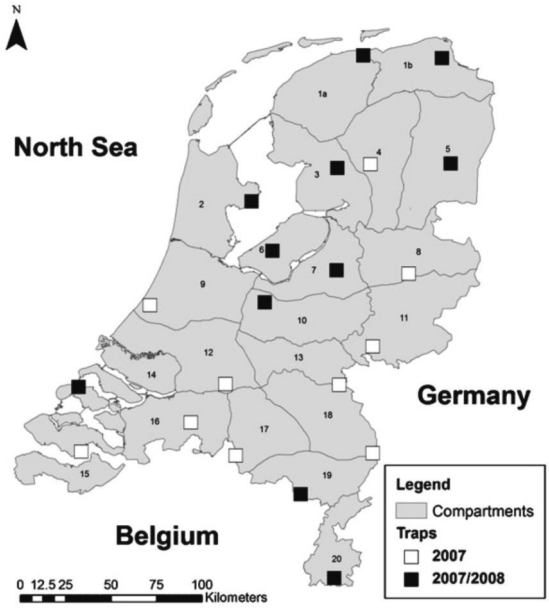

Figure 1. Map of the Netherlands showing 21 compartments (1A, 1B - 20, with compartments 2 and 3 separated by Ijsselmeer) and location of livestock holdings sampled for *Culicodies* biting midges from November 2006 to December 2008 (21 holdings monitored in 2007, 11 in 2008)

Light trapping protocol and Culicoides identification

Culicoides were captured using the Onderstepoort-type downdraught suction trap equipped with an 8w blacklight tube (Venter & Meiswinkel, 1994). The collecting protocol used is outlined in Goffredo & Meiswinkel (2004). The light trap collections were dispatched weekly to the Plant Protection Service (PD) in Wageningen where each catch, prior to analysis, was catalogued and given a unique identification code. Small catches were cleaned and enumerated in their entirety, whereas larger catches were subsampled in an adaptation of the method provided by Van Ark & Meiswinkel (1992).

Under a dissecting microscope *Culicoides* were identified to species based on the wing pattern and other diagnostic features as provided in the works of Campbell & Pelham-Clinton (1960) and Delecolle (1985); in addition, 310 specimens representing 40 species were slide mounted to confirm the identity of the species collected. The respective females of *C. obsoletus* and *C. scoticus* are very difficult to identify accurately based on morphology and because of this and their close phyletic relationship, the data for these two species are combined and presented under the joint taxon 'Obsoletus Complex'. We do not consider *C. grisescens* – a new record for the Netherlands – to be a member of the subgenus *Culicoides*, but instead assign it to the subgenus Silvicola Mirzaeva & Isaev, owing to the posterior margin of tergum nine of the male genitalia being strongly convex and lacking a median notch.

Data analysis

For each *Culicoides* species, its average weekly abundance was calculated by dividing the number of specimens captured by the number of locations sampled in a particular year (21 in 2007 and 11 in 2008). The combined seasonal abundance of five species of 'proven or potential vector *Culicoides*' (*C. obsoletus s.s.* + *C. scoticus* + *C. dewulfi* +*C. Chiopterus* +*C. pulicaris s.s.*) is compared with that of 'all *Culicoides*' (40 species: 24 identified, 16 unidentified) and depicted separately for 2007 (Fig. 2A) and 2008 (Fig. 3A). In order to show them in greater detail, the seasonal abundance of each of the five species of proven and potential vectors is plotted separately for 2007 (Fig. 3A) and 2008 (Fig. 3B). In all four of these figures the data for *C. obsoletus s.s.* and *C. scoticus* are combined and presented as the 'Obsoletus Complex'; the graphs include also the weekly mean of the daily temperature (^{0}C). The annual week Frequency Rate (awFR) is a measure of species incidence and is derived by dividing the number of weeks in

Table 1. List of compartments (1a, 1b-20) sampled, total resident livestock (>90% cattle), and numbers of *Culicoides* captured in 2007 and in 2008 subdivided into: 'proven and potential vector species' ('Obsoletus Complex', '*C.dewulfi*', '*C. chiopterus*', '*C. pulicaris s.s.*') , 'non vector species' ('*C. punctatus*', *C.* sp. nr. *newsteadi*', 'other species') and 'Total *Culicoides*' (40 species). Compartments marked with * were not sampled in 2008

		Proven and potential vector species								**Non-vector species**						**Total** *Culicoides*	
Compartment	**Livestock**	Obsoletus Complex		*C. dewulfi*		*C. chiopterus*		*C. pulicaris s.s.*		*C. punctatus*		*C.* sp. nr. *newsteadi*		Other species			
		2007	**2008**	**2007**	**2008**	**2007**	**2008**	**2007**	**2008**	**2007**	**2008**	**2007**	**2008**	**2007**	**2008**	**2007**	**2008**
1a	237	268	139	13	10	194	37	0	0	1270	3228	42	7	403	26	2190	3447
1b	116	1982	659	183	285	1557	2070	0	2	63	883	11	29	103	192	3899	4120
2	59	2719	4425	1505	4562	11569	6629	0	0	3138	28179	772	1504	688	557	20391	45856
3	248	1326	421	1633	810	1946	2467	1	0	1561	1745	126	6	1684	1949	8277	7398
4*	147	5771	-	3497	-	3524	-	8	-	1215	-	114	-	146	-	14275	-
5	101	2542	1976	666	951	1141	819	1	15	190	3187	0	0	16	5	4556	6953
6	156	15622	11937	6961	4371	1973	1701	86	7	24458	55774	13054	11959	785	404	62939	86153
7	118	188547	44389	8014	5098	13923	53140	71	1	6750	2783	0	1	8983	2461	226288	107873
8*	89	2324	-	5623	-	1374	-	14	-	147	-	0	-	43	-	9525	-
9*	333	734	-	935	-	686	-	4	-	489	-	1454	-	65	-	4367	-
10	68	6200	3380	21005	8579	1173	2246	3	2	147	445	190	45	64	41	28782	14738
11*	435	1698	-	721	-	701	-	3	-	217	-	0	-	37	-	3377	-
12*	170	671	-	121	-	624	-	0	-	346	-	111	-	23	-	1896	-
13*	140	10056	-	1289	-	935	-	59	-	416	-	0	-	774	-	13529	-
14	86	2454	5540	988	509	357	19039	2	4	443	7795	176	287	596	506	5016	33680
15*	130	158	-	38	-	117	-	0	-	36	-	5	-	24	-	378	-
16*	109	821	-	469	-	94	-	2	-	20	-	18	-	22	-	1446	-
17*	170	6928	-	1444	-	560	-	22	-	1204	-	2	-	412	-	10572	-
18*	277	16704	-	3468	-	1960	-	22	-	438	-	0	-	1282	-	23874	-
19	118	14110	5858	14451	9400	10130	2655	88	40	1055	972	56	4	2596	4902	42486	23831
20	113	23847	17055	25988	14787	1241	845	287	224	122	176	58	16	82	39	51625	33142
Total	**3420**	**305482**	**95779**	**99012**	**49362**	**55779**	**91648**	**673**	**295**	**43725**	**105167**	**16189**	**13858**	**18828**	**11082**	**539688**	**367191**

which a species occurred by the total number of sampling weeks, and multiplied by 100. In lieu of numerous species-specific graphs the seasonal abundance and weekly prevalence of each of the 23 identified taxa (representing 24 species) is depicted for both years in a single Mondrian Matrix (Fig. 4); the matrix consists of squares colour-coded according to the $\log_{10}(n+1)$ transformed weekly average abundances of each species.

Results

In the results provided below emphasis is placed on the findings made around the proven and potential vectors for BTV because these, along with 2 non-vector species, were those found to occur most widely and abundantly in the 21 compartments monitored (Fig. 1). Included amongst the 24 species considered below, are 5 new records for the Netherlands: *C. duddingstoni* , *C. grisescens*, *C. maritimus*, *C. pallidicornis* and *C. riethi*. The *Culicoides* checklist of the Netherlands now comprises 26 species; all occur in neighbouring countries and shows the Dutch fauna, although depauperate as a result of landscape homogeneity, to fit what is considered the norm for north-western Europe.

Culicoides abundances

Nearly 1 million *Culicoides* were captured in the 1541 light trap collections made and represented approximately 40 species. Twenty-four species constituted >99% of the *Culicoides* captured and are listed in Table 1 according to year and compartment and subdivided into three categories: 'proven and potential vector species' (i.e. Obsoletus Complex + *C. dewulfi* + *C. chiopterus* + *C. pulicaris s.s.*), 'nonvector species' (i.e. *C. punctatus* + *C.* sp. nr. *newsteadi* +17 remaining species) and 'total *Culicoides*' (40 species). The same 24 species are ranked in Table 2 (according to 2007 abundances) and includes the number of specimens of each sex captured, range in weekly abundances, and the compartment in which the maximum catch of a particular species was made. These include the five species that in north-western Europe are considered proven or potential vectors for BTV and which made up nearly 80% of the biting midges captured.

The vector category was dominated by the Obsoletus Complex and over the 2 years averaged 3904 individuals/week, followed by *C. dewulfi* (1427) and *C. chiopterus* (1418). The fifth potential vector, *C. pulicaris*, averaged only 14 individuals/week, which

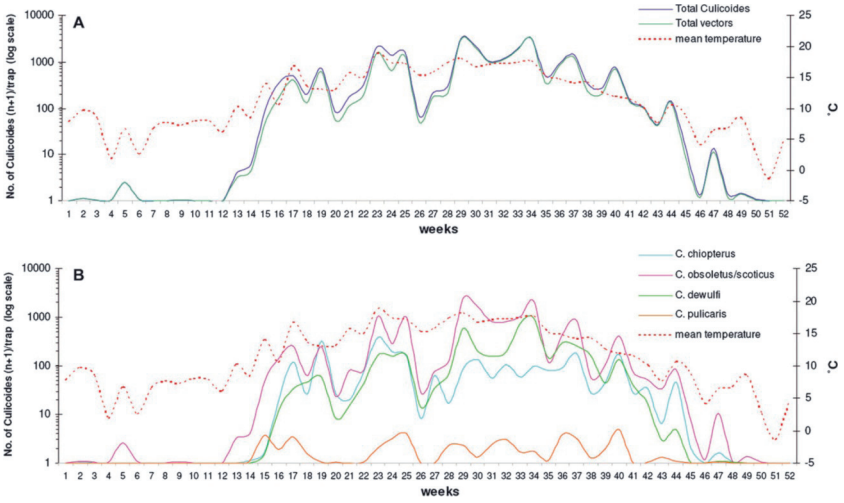

Figure 2a. January to December 2007: averaage weekly biting midges abundances (n+1) on 21 cattle holdings in The Netherlands plotted on a log10 scale for five proven or potential bluetongue vector *Culicoides* species combined (*C. chiopterus*, *C. dewulfi*, *C. obsoletus*, *C. scoticus* and *C. pulicaris*; solid dark green line) compared to that for all *Culicoides* (40 species; solid blue line). The dotted red line depicts the weekly mean of the dadily temperature (^{0}C). **b.** January to December 2007: averaage weekly biting midges abundances (n+1) on 21 cattle holdings in The Netherlands plotted on a log10 scale for each of the proven or potential vectors for bluetongue virus i.e., the Obsoletus Complex (*C. obsoletus/C. scoticus*) (solid pink line), *C. dewulfi* (solid light green line), *C. chiopterus* (solid light blue line) and *C. pulicaris* (solid orange line). The dotted red line depicts the weekly mean of the dadily temperature (^{0}C).

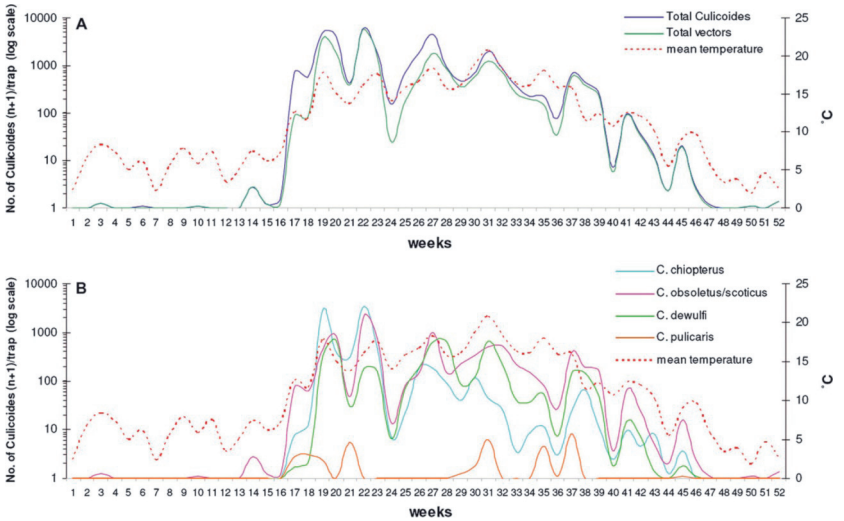

Figure 3a. January to December 2007: averaage weekly biting midges abundances (n+1) on 21 cattle holdings in The Netherlands plotted on a log10 scale for five proven or potential bluetongue vector *Culicoides* species combined (*C. chiopterus*, *C. dewulfi*, *C. obsoletus*, *C. scoticus* and *C. pulicaris*; solid dark green line) compared to that for all *Culicoides* (40 species; solid blue line). The dotted red line depicts the weekly mean of the dadily temperature (^{0}C). **b.** January to December 2007: averaage weekly biting midges abundances (n+1) on 21 cattle holdings in The Netherlands plotted on a log10 scale for each of the proven or potential vectors for bluetongue virus i.e., the Obsoletus Complex (*C. obsoletus/C. scoticus*) (solid pink line), *C. dewulfi* (solid light green line), *C. chiopterus* (solid light blue line) and *C. pulicaris* (solid orange line). The dotted red line depicts the weekly mean of the dadily temperature (^{0}C).

rendered it almost 500-fold less abundant than the Obsoletus Complex + *C. dewulfi* + *C. chiopterus* combined. The most abundant non-vector species were *C. punctatus* (at an average of 1432 individuals/week), followed by *C.* sp. nr. *newsteadi* (289).

The average weekly abundances of the *Culicoides* captured in the 21 compartments during the first year (2007) of the survey are plotted on a $\log_{10}(n+1)$ scale in Fig. 2A, B with the corresponding data for 2008, but obtained for 11 compartments only, shown in Fig. 3A, B. These data are plotted against the weekly mean of the daily temperature (^{0}C), with midge abundances subdivided amongst various species, and groupings of species, as follows: 'Total *Culicoides*', 'Total vectors', '*C. punctatus*', *C.* sp. nr. *newsteadi* and 'Other species'.

Vector distribution and weekly incidence

Four of the five proven and potential vectors for BTV were also the most prevalent of the 24 commonest species captured in the Netherlands: the Obsoletus Complex occurred in 83 of the 104 sampling weeks, followed by C. chiopterus (64weeks) and C. dewulfi (62 weeks). These four BTV vectors had an annual week Frequency Rate (awFR) that ranged from 60% to 80%, double that of the fifth vector C. pulicaris (38%) and which was surpassed by two 'non-vectors', namely C.punctatus (67%) and C. sp. nr. newsteadi (55%) (Fig. 4).

The capture of significant numbers of males of *C. obsoletus* and *C. scoticus* revealed the two species to occur widely across the Netherlands (Table 2). In both years *C. obsoletus* (3050 males) was found at all sites, whereas the less-abundant *C. scoticus* (1741 males) occurred, respectively, at 86% and 73% of the sites monitored. Of the two species that breed obligatorily in cattle dung, *C. dewulfi* dominated in 2007, whereas *C. chiopterus* dominated in 2008 in spite of only half the number of light traps being operated (Table 1, Fig. 4); furthermore, they were consistently dominant at separate sites (*C. dewulfi* at sites 6, 10 and 20, *C. chiopterus* at sites 2, 7 and 14).

The undulating sequence of peaks and troughs in the seasonal data overall (as shown in the four graphs that comprise Figs 2, 3) indicate that the five proven and potential *Culicoides* vectors for BTV completed five or six generations annually; these five species, along with two other multivoltine species i.e. *C. punctatus* and *C.* sp. nr. *newsteadi* , occupy the left third of the matrix (Fig. 4) and highlights the distinction between them and the remaining species that complete only one or two (at most three) generations annually and which form the bulk (>80%) of the *Culicoides* faunal diversity of the Netherlands.

Table 2. Ranked list of 24 identified species of *Culicoides* captured in The Netherlands in 2007 and 2008, showing number (n) and percentage (%) of specimens of each sex, range in weekly abundances, and locality (compartment) in which maximum catch was made

Culicoides species	*Culicoides* (females)				*Culicoides* (males)				Range (min-max)		Locality max catch	
	2007		**2008**		**2007**		**2008**		**2007**	**2008**	**2007**	**2008**
	n	**%**	*n*	**%**	*n*	**%**	*n*	**%**				
Obsoletus Complex	301414	56.68	95136	26.39	4068	51.51	723	10.63	0-36792	0-22162	7	7
C. obsoletus	-	-	-	-	2518	31.89	532	7.82	0-336	0-79	20	19
C. scoticus	-	-	-	-	1550	19.63	191	2.81	0-896	0-84	20	19
C. dewulfi	97225	18.28	48629	13.49	1787	22.63	737	10.83	0-13328	0-6635	10	19
C. chiopterus	55226	10.38	91535	25.39	553	7.00	114	1.68	0-5323	0-33622	19	7
C. punctatus	42914	8.07	100823	27.97	811	10.27	4345	63.87	0-11700	0-21588	6	6
C. sp. nr. *newsteadi*	16181	3.04	13850	3.84	8	0.10	8	0.12	0-2464	0-4200	6	6
C. fascipennis	4758	0.89	706	0.20	1	0.01	4	0.06	0-3024	0-336	7	7
C. impunctatus	2929	0.55	1871	0.52	0	0.00	0	0.00	0-1071	0-756	3	3
C. grisescens	2456	0.46	630	0.17	0	0.00	0	0.00	0-840	0-448	7	7
C. achrayi	2434	0.46	363	0.10	67	0.85	2	0.03	0-728	0-196	13	19
C. nubeculosus	1377	0.26	2629	0.73	235	2.98	556	8.17	0-616	0-1148	19	19
C. pulicaris	671	0.13	279	0.08	2	0.03	16	0.24	0-56	0-77	20	20
C. riethi	583	0.11	200	0.06	49	0.62	7	0.10	0-140	0-84	14	6
C. halophilus	449	0.08	1044	0.29	1	0.01	100	1.47	0-198	0-932	1A	3
C. circumscriptus	276	0.05	555	0.15	32	0.41	24	0.35	0-84	0-316	2	19
C. pallidicornis	236	0.04	7	0.00	14	0.18	0	0.00	0-84	0-7	7	20
C- festivipennis	218	0.04	296	0.08	89	1.13	13	0.19	0-28	0-56	18, 19	7
C. kibunensis	161	0.03	546	0.15	6	0.08	117	1.72	0-28	0-196	20	2
C. duddingstoni	132	0.02	12	0.00	15	0.19	0	0.00	0-98	0-7	6	6
C. maritimus	119	0.02	65	0.02	18	0.23	1	0.01	0-45	0-28	6	2
C. salinarius	102	0.02	134	0.04	1	0.01	0	0.00	0-28	0-112	19	19
C. pictipennis	41	0.01	34	0.01	5	0.06	2	0.03	0-14	0-14	14	14
C. lupicaris	24	0.00	9	0.00	1	0.01	0	0.00	0-7	0-7	7	20
C. stigma	22	0.00	10	0.00	0	0.00	0	0.00	0-14	0-4	7	7
C. indet	1843	0.34	1111	0.31	134	1.69	34	0.50	0-672	0-411	7	10
Total	**531791**	**100**	**360474**	**100**	**7897**	**100**	**6803**	**100**				

To highlight similarities and differences amongst species, the comparative weekly abundances of the 23 commonest Culicoides taxa (representing 24 species) are presented for both years in parallel in the Mondrian Matrix (Fig. 4); the data for 2007 appear in column A and for 2008 in column B. The bolder horizontal mid-line separates the first half of the year from the second, whereas the bolder vertical line separates the proven and potential vectors for BTV (to the left) from apparent non-vectors (to the right); to facilitate their comparison, the relative weekly abundances are colour coded (as explained in the legend to Fig. 4).

The Mondrian matrix shows the midge season in 2008 began approximately 2–3 weeks later than in 2007 and ended also sooner, shortening the 2008 'midge year' by approximately 1 month. During the bridging winter of 2006 and 2007 low levels of activity in the Obsoletus Complex occurred during six of the first 12 weeks of 2007; the 46 specimens captured in the dead of winter were age graded as nulliparous, which means all the midges captured were recent hatchlings.

In 2006 the last parous midge was captured on the 14th of December (data not shown), whereas the first one in 2007 was captured approximately 18 weeks (116 days) later and only 7–14 days after the *Culicoides* mass spring flush had manifested (and which comprised only male and nulliparous female midges). This pattern was repeated in the winter of 2007/2008 and indicates that older, previous season images do not appear to survive the harsher winters that occur above 45–50^{0}N. After the summer had commenced, populations of the five vector species (those to the left of the bold vertical line in Fig. 4) developed rapidly, and thereafter maintained high levels of activity and abundance throughout the duration of the summer, autumn and early winter months (weeks 15–45). The two 'non-vector' species *C. punctatus* and *C.* sp. nr. *newsteadi* (which fall to the immediate right of the bold vertical line in Fig. 4) had abundance and prevalence rates similar to those displayed by all the vector species except *C. pulicaris* (to their immediate left); in surpassing *C. pulicaris*, these two species would seem well placed to act as additional vectors for arboviruses.

Individual midges are not long lived (<30 days); therefore, in order for a species to remain active for 200 days of the year and more, it must be multivoltine (cycling through multiple generations annually). The multivoltine species are grouped in the left third of Fig. 4 and include the five vector and two 'nonvector' species referred to above. In Fig. 4, as one proceeds to the right, the weekly prevalence and abundance rate of each of the remaining 17 species shrinks progressively, these species being either

univoltine or bivoltine (rarely trivoltine); of these only *C. impunctatus* is implicated, albeit tangentially, in the transmission of BTV.

Figure 4. Mondrain matrix of the weekly *Culicoides* (23 taxa representing 24 species) abundances (colour-coded) for 2007 (column A, 25 light traps at 21 cattle holdings) and 2008 (column B, 12 light traps at 11 holdings) in The Netherlands; green = 0 *Culicoides*, light yellow = 1-9; bright yellow = 10-99; orange = 100-999; red = 1,000-9,999 and purple = 10,000-99,999. The border horizontal mid-line separates the 1st half of the year from the 2nd; the bolder vertical line separates nthe known and potential vectos for BTV (to the left) from non-vectors (to the right)

Discussion

Five of the six proven and potential *Culicoides* vectors for BTV in Europe occur in the Netherlands. During the 2006–2008 epidemic they constituted almost 80% of approximately 1 million biting midges captured around livestock, predominantly cattle. Overall, the vector situation in the Netherlands mirrors that reported recently from elsewhere in northern Europe (Balczun et al., 2009; Nielsen et al., 2010; Ander et al., 2012) and includes the observation that the two species that comprise the Obsoletus Complex nearly always dominate light trap collections. The 5 vector species were also amongst the most widespread of the 24 commonest species of *Culicoides* collected and, furthermore, remained active continuously for 27–38 weeks of the year; this included the late summer and autumn when outbreaks of bluetongue peak. Only one of the five vector species, namely *C. pulicaris*, was consistently rare, which raises doubts about its involvement in the transmission of BTV in the Netherlands.

In all probability high livestock densities underpinned the rapid spread of bluetongue in the Netherlands and the newfound involvement of *C. dewulfi* and *C. chiopterus* in the epidemiology of the disease. These two species breed obligatorily in cattle and horse dung (Downes & Kettle, 1952) and so aggregate around these hosts almost wherever they are husbanded. Although the overall abundance levels of *C. dewulfi* and *C. chiopterus* remained similar for the duration of the 2-year survey, they showed differences in their respective geographical ranges and inter-annual weekly incidence rates. It is not clear what caused this difference (given that >10 cattle occurred on each of the farms surveyed), but marked diurnality in *C. chiopterus* may partly account for it (Nielsen, 1971; Townley et al., 1984).

Of the 1 million specimens of *Culicoides* captured, only 46 (0.0005%) were caught during the winter and, furthermore, consisted only of recently hatched, nulliparous females. These included a freshly engorged individual and shows that *Culicoides* will attack livestock during interludes of warm winter weather. No older, parous midges were captured from the middle of December until early April. Their absence during these 100 days and more, and including the first 7–14 days of spring, is significant because it indicates that older, previous season midges do not enter into diapause, probably because they are decimated once temperatures remain below approximately 5^0C. This indicates that the virus to overwinter does not depend solely upon the insect vector, but utilizes a dual pathway: a vertebrate host infected quite late the previous season and which is infective to nulliparous midges active in late winter or early spring. During colder winters disruption of the insect biting cycle is prolonged at increasingly

cooler latitudes and probably explains why *Culicoides*-borne viruses are contained within the tropics and subtropics of the world where vectors remain active throughout the year.

In both years the *Culicoides* season began abruptly. This synchronous 'spring flush', and involving multiple species, is integral to the annual rhythm of many temperate biota; in *Culicoides* it arises directly from the maturation of overwintering larvae, these derived from eggs laid by the parent generation during the second half of the previous season (Hill, 1947; Dzhafarov, 1964). Furthermore, in spring and early summer, the seasonal abundance curve for the 5 vector species combined was found to be separate from that for all 40 species combined. This gap reflects greater species diversity in the first half of the year and is due to the early appearance of most univoltine (single generation) species in spring; their disappearance towards the end of summer is similarly rhythmic, leaving only multivoltine species to 'see out' the year.

After the initial spring flush had occurred, and for the remainder of the season, midge population levels were observed to fluctuate regularly. Such peaks have led many authors (Dzhafarov, 1964; Service, 1969; Birley & Boorman, 1982; Orszagh & Masan, 1992) to conclude that species such as *C. obsoletus* complete three to four generations each season; this number is halved at cooler altitudes and more northerly latitudes (Hill, 1947), but doubled, or even trebled, in the warmer subtropical and tropical areas of the world. In the Netherlands, weekly abundances undulated in near-perfect unison in multiple *Culicoides* species; however, fluctuating temperatures may have had a confounding effect and leaves us uncertain as to whether each of the five to six peaks observed equates to an actual midge generation completed.

In 2008, the 'insect season' was on average 33 days shorter than in 2007. Judging from the literature, such inter-annual oscillations are the norm, some years being comparatively warmer (or cooler) than others. In spite of 2006 being the hottest year since records began in 1706, there is little to suggest that *Culicoides* abundances were anything out of the ordinary. It is necessary to qualify this statement by noting that at cooler latitudes there are significant levels of *Culicoides* activity at dusk and dawn, and also during a part of the day, which render light traps less effective for determining accurately periods of heightened activity and midge abundance levels. On average, inter-annual *Culicoides* abundances appear to have remained relatively stable and comparable for the duration of the study and therefore it cannot be claimed that 2006 was 'extraordinary' in terms of midge population levels as a whole. However, the paucity of longterm *Culicoides* datasets for anywhere in northern Europe, especially ones obtained using a comparable trapping system, render it impossible to establish

whether midge abundances during the 3-year epidemic were either above or below the long-term norm.

At least 8 of the 24 commonest *Culicoides* species collected were multivoltine. They completed three or more generations annually and remained active throughout the summer, autumn and early winter months (between weeks 15–45 and beyond) and as a group included all five of the species that are considered proven or potential vectors for BTV in northern Europe. In being on the wing continuously for 200 days and more, this vector quintet is well placed to initiate and sustain a disease like BT, which builds up slowly, before gathering pace and spreading explosively later in the season (hence the moniker 'autumn disease'). Two of the 'non-vector' species, namely *C. punctatus* and *C.* sp. nr. *newsteadi*, had similar abundance and prevalence rates and therefore deserve scrutiny as additional potential vectors for BTV. While persistent attempts to detect BTV in these two species of *Culicoides* have failed (Goffredo et al ., 2012), it would be prudent to not forget that both Aino virus (AINOV) – which is related to Schmallenberg virus (SVB) – and Ibaraki virus (IBAV), an Orbivirus like BTV, have been isolated from *C. punctatus* in southern Japan (Yanase et al., 2005). Because Japan, like Europe, forms part of the Palaearctic region, it would be appropriate to include *C. punctatus* in the Europe list of vector *Culicoides*.

In the Netherlands at least half of the 24 commonest species of *Culicoides* are univoltine or bivoltine, which means that around the end of summer – after completing a generation or two – they disappear. Significantly, these 'short season' species do not include any of the five recognized vectors for BTV, but do include *C. impunctatus* which, in the laboratory, has been infected artificially with BTV (Jennings & Mellor, 1988). For this reason it is cited as a potential vector for the virus (Carpenter et al., 2006), but as pointed out by Carpenter et al. (2008), this claim is difficult to uphold when particulars of its life-cycle and ecology are taken into consideration.

As mentioned, all the proven and potential vectors for BTV in the Netherlands are multivoltine and in this study appeared to complete five to six generations annually. If correct, it would represent a marked increase over the one to four generations calculated for them in previous decades at similar latitudes. Whether our data mark a (recent) adaptive shift in the seasonal phenology of the multivoltine *Culicoides* species, or whether they simply reflect a response that is inherent and which manifests during warmer seasons when average annual temperatures are more 'tropical', remains to be ascertained. Shifts in phenology reported for other elements of the Palaearctic fauna and flora have been ascribed to a changing global climate that – over the last century – has warmed, particularly in central and north-eastern Europe (Menzel et al ., 2006). It is

to be expected that a continuously warming Earth will lengthen the *Culicoides* biting season, increase the number of insect generations completed (by quickening the larval cycle) and accelerate the vector biting and virus replication rates. These changes, when compounded, are sure to boost the transmission of any *Culicoides*-borne viruses that may be introduced into temperate Europe, now and in the future. The recent advent of the Schmallenberg virus within the region may well illustrate this point.

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CHAPTER 5

Principal climatic and edaphic determinants of *Culicoides* abundance during the 2007-2008 bluetongue epidemic in The Netherlands based on OVI light trap data

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Abstract

Palaearctic *Culicoides* midges represent a vital link in the northward advance of certain arboviral pathogens of livestock such as that caused by the bluetongue virus. The effects of relevant ecological factors on weekly *Culicoides* vector abundances during the bluetongue virus serotype 8 epidemics in The Netherlands in 2007 and 2008 were quantified within a hurdle modelling framework. The relative role of meteorological parameters showed a broadly consistent association across species, with larger catches linked to temperature related variables and lower wind speed. Moreover, vectors abundance was found to be influenced also by edaphic factors, likely related to the species-specific breeding habitat preferences that differed markedly amongst some species. This is the first study on the *Culicoides* vector species of The Netherlands identified during an entomological surveillance programme, in which an attempt is made to pinpoint the factors that influence midge abundance levels and geographic range. In addition to providing key inputs into risk mitigating tools for midge-borne pathogens and disease transmission models, the adoption of methods that explicitly address certain features of abundance datasets (frequent zero count observations and over-dispersion) helped enhance the robustness of the ecological analysis.

Introduction

Culicoides biting midges (Diptera: Ceratopogonidae) are true biological vectors of viruses that cause devastating diseases in domestic and wild ungulates worldwide such as bluetongue (BT), African horse sickness (AHS) and epizootic haemorrhagic disease (EHD) (Mellor et al., 2000). Within European ecological biomes, as defined by Olson and colleagues (2001), various *Culicoides* species are proven or incriminated bluetongue virus (BTV) vectors. In the Mediterranean zone, these comprise the traditional African-Asian vector, *C. imicola* (Kieffer, 1913), and the indigenous Palaearctic species *C. obsoletus* (Meigen, 1818), *C. scoticus* (Downes and Kettle, 1952) and *C. pulicaris* (Linneaus, 1758) (Caracappa et al., 2003; De Liberato et al., 2005; Savini et al., 2005). Moving further north into the temperate zone, from where C. imicola has never been reported, two additional resident species, namely *C. dewulfi* (Goetghebuer, 1936) and *C. chiopterus* (Meigen, 1830), act as novel potential vectors for BTV (Meiswinkel et al., 2007; Dijkstra et al., 2008).

Climatic and other environmental factors are known to modulate the lifecycle of *Culicoides* species and have been linked to the timing and distribution of BT outbreaks at a range of scales (Purse et al., 2008). Furthermore, the general assumption that bioclimatic data are surrogates for *Culicoides* geographical distribution and temporal abundance has been widely adopted to complement survey data. This has led to the development of climate-based models predicting vector distribution in un-surveyed locations and identifying areas at risk to BT outbreaks (Baylis et al., 2001; Wittman et al., 2001; Tatem et al., 2003; Purse et al., 2004a). Despite their general relevance, these models, usually implemented with *Culicoides* data obtained from within a single confined area, have not proved accurate when applied elsewhere (Calistri et al., 2003; Capela et al., 2003). Further, in regions where comparative mapping has revealed the geographic ranges of certain vector *Culicoides* to be markedly disjunctive, this has been shown to be dictated less by climate and more by edaphic factors, especially terrain slope and soil type. Thus in an irregular topography, for example, *C. imicola* is likely to be almost entirely absent where the terrain slope and soil texture induce water runoff and rapid desiccation of the soils' surface layer, due to the fact that moisture is critical for the larvae of this species to survive and complete their developmental cycle (Conte et al., 2007). This caveat has driven later studies and shown that areas suitable for a given species are very specific to the particular landscape being inventoried, giving greater explanatory power to models that incorporate a combination of ground-measured and remotely-sensed climatic variables, edaphic, topographical and host-availability factors

(Calvete et al., 2009; Purse et al., 2012; Silbermayr et al., 2011).

Separate incursions by multiple serotypes of BTV into Europe have characterized the past decade (MacLachlan, 2010). These included a major epidemic - involving a sub-Saharan strain of BTV serotype 8 - that was reported first in The Netherlands in the second half of 2006 and spread rapidly to involve extensive portions of Europe that had not experienced the disease previously (Elbers et al., 2008). Despite BT being transient in a large part of north-western Europe, the probability of recurrence, along with the possibility for incursions by other midge-borne pathogens, remains poorly understood (Backer & Nodelijk, 2011; de Koeijer et al., 2011).

Gauging the influence of ecological correlates on the phenological patterns in Palaearctic *Culicoides* has been the subject of a number of recent studies (De Liberato et al., 2010; Purse et al., 2012; Sanders et al., 2011). For The Netherlands this knowledge is rudimentary at best, relying mainly on a single-point-single-night sampling programme (Meiswinkel et al., 2008) and studies conducted at sub-national level (Takken et al., 2008). Hence, predictive modelling is still fraught with uncertainty (Hartemink et al., 2009). To refine and support risk management tools, such modelling must reflect the diversity of the *Culicoides* population around livestock, as captured by the national entomological surveillance programme. Such models, particularly for newly invaded regions, require knowledge of the wide array of midge-borne pathogens that could be spread by competent vectors, as well as a clearer understanding of the factors that drive *Culicoides* vector distribution and abundance.

The specific aims of the present study were:

1) to describe and quantify the influence of environmental features on the weekly abundance Culicoides vector species across The Netherlands;

2) to assess possible underlying ecological differences between vector species;

3) to implement a statistical model for the study of *Culicoides* spp. surveillance abundance data.

Materials and Methods

The surveillance dataset was combined with freely-available climatic data, host availability information and edaphic characteristics that were found to be effective predictors of the distribution and abundance of different *Culicoides* species (Baylis et al., 1998; Purse et al., 2004b; Conte et al., 2007; Calvete et al., 2009)

Entomological data

Entomological surveillance was established in The Netherlands after the first bluetongue epidemic season, as provided by European Regulation No. 1266/2007. Accordingly, the country was divided into geographical units of about 2000 km^2, which resulted in 21 compartments, in which surveillance and monitoring activities were carried out (van Schaik et al., 2008). One farm per compartment was designated for entomological surveillance (Figure 1), selected from the 106 sampled nationally during the 2006 'snapshot' survey (Meiswinkel et al., 2008). In 2007 all 21 farms were monitored on a year-round basis with a standard weekly Onderstepoort-type (OVI) blacklight trapping protocol (Goffredo & Meiswinkel, 2004), but in 2008 this dropped to 11 in order to lower costs. Light trap records considered in the present study include weekly samples collected from January 2007 to December 2008. Taking into account trap failures and interrupted sampling at some sites, a total of 1541 collections were made (i.e. 990 of a potential 1092 in 2007 and 551 of a potential 572 in 2008). Weekly counts of biting midges were separately recorded for each of the species identified, sorted by sex and with the females classified according to their physiological status, as described in detail in Meiswinkel and colleagues (2013).

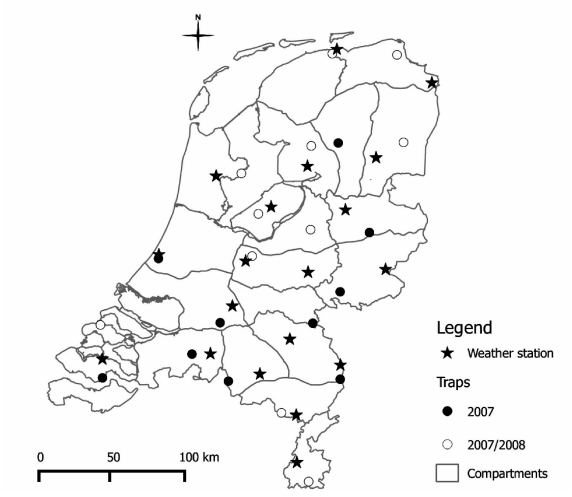

Figure 1. Map showing the location of the 21 trap sitres (one per compartment) an the weather stations

Climatic data

Ground-measured climatic data was provided by the Royal Dutch Meteorological Institute [Koninklijk Nederlands Meteorologisch Instituut (KNMI) (http://www.knmi.nl/klimatologie/)] as recorded from 36 weather stations located across The Netherlands. All meteorological stations were georeferenced, so that the nearest one to each trap site could always be identified (Multiple Euclidean Distance Calculation - http://www.edenextdata.com).

The local topography for each selected weather station (elevation, slope, and other topographic aspects, available from the KNMI website for each location), was comparable to that of the corresponding light trap location. Altitude, while always consistent between the two matched locations, is not a factor of great influence in The Netherlands, which has very little elevation difference and gently slopes from the southeast (322 above m.s.l.) to the northwest (-6 m below m.s.l.); the largest part having more or less flat topography. The terrain between each site sampled and the nearest weather station is not complex, with neither forest nor urban areas; therefore the wind speed measured can be assumed to be a reasonable approximation. For each of the selected stations, weekly means were calculated for the following meteorological variables collected daily: the minimum, maximum and mean for temperature (ºC), relative humidity (%),and air pressure (hPa), the amount of precipitation (mm) and the evapotranspiration (Makkink, %). For wind speed (m/s), we considered the hourly mean on the catch date, as Culicoides flight activity, as captured by OVI light trap, is assumed to be greatly diminished by higher wind speed on a daily basis. The values obtained were attributed to each trap record based on the catch date.

Remotely sensed imagery was used to derive three variables of environmental significance: Normalized Difference Vegetation Index (NDVI), daytime Land Surface Temperature (dLST) and night-time Land Surface Temperature (nLST). NDVI is a common measure of plant growth and photosynthetic activity, which has been shown to correlate with soil moisture, rainfall and vegetation biomass (Campbell, 1996; Huete et al., 2002). LST is a general index of the Earth's temperature in a particular location, whether surface is soil or vegetation (Goetz et al., 2000). The correspondence between daily LST and in situ measurement data is accurate within 1 degree Celsius in the range from -10 to 50 degrees, making LST a widely used quantity (Wan et al., 2002).

The NDVI, dLST and nLST for 2007 and 2008 were obtained from the Avia-geoExplorer composite data set (Avia-geoExplorer DVD Tool, v. Jan2010 - AviaGIS). This software allows generation of time series of the Moderate Resolution Imaging

Spectroradiometer (MODIS) satellite images and the attribution of indices to positions specified by the user. Available with a spatial resolution of 1 km, the NDVI and LST images had a temporal resolution of, respectively, 16 days and 8 days. NDVI, dLST and nLST time series were derived for the 21 trap sites and the weekly mean values attributed to each record in the surveillance dataset.

Soil texture data

Edaphic properties are considered important characteristics influencing the Culicoides population size, because they are a measure of the breeding habitat availability (Braverman et al., 1974; Conte et al., 2007; Foxi & Delrio, 2010; Titeux et al., 2009). For each trap site, the Harmonized World Soil Database (HWSD) Viewer version 1.1, a simple geographical tool to query and visualize a soil information layer to the local scale, was used to obtain the percentage of sand, silt, clay and organic carbon in topsoil (0-30cm) (FAO/IIASA/ISRIC/ISS-CAS/JRC, 2009) (Table 1). The HWSD consists of a 30 arc-second (or ~1 km) raster image linked via the pixel value to the soil information system.

Host availability data

The presence of hosts is a prerequisite for the survival of blood-feeding *Culicoides*. Vector species abundance was found either linked to land use (pastoral), host type and host density (Purse et al., 2011; Sanders et al., 2011), or not affected by the number and type of farmed animals (De Liberato et al., 2010). When included as predictors, host availability data increased the variance explained by the predictive models, but the strength of the association to midge abundance or distribution was considerably lower than that of bioclimatic variables (Calvete et al., 2009; Purse et al., 2011). Very often it is the case that a high amount of variation in *Culicoides* abundance can only be explained by the combined effect of two (or more) factors, due to their complex interactions and relationship with the species specific biological traits (Acevedo et al., 2010).

To explore the role of host availability on *Culicoides* weekly abundances, the number of cattle per compartment (van Schaik et al., 2008), as well as the number of cattle, sheep and goats kept at each trap site were included in the analysis (see Table 1).

Table 1. Summary of host availability and soil texture of the 21 sites sampled (one for each compartment) during the national surveillance programme

Compartment	Compartment	Host			Top soil (0-30 cm) texture			
		Farm						
	No. cattle	No. cattle	No. goats	No. sheep	organic carbon %	silt %	sand %	clay %
Farm 1A	300,000-400,000	237	0	0	< 0.6	> 40	21-50	11-25
Farm 1B	300,000-400,000	79	5	32	< 0.6	> 40	21-50	11-25
Farm 2	75,000-150,000	59	0	0	1.2-2.0	26-40	21-50	11-25
Farm 3	150,000-225,000	130	5	113	> 2	26-40	< 20	26-40
Farm 4	225,000-300,000	79	0	68	1.2-2.0	< 10	> 80	< 10
Farm 5	225,000-300,000	101	0	0	1.2-2.0	< 10	> 80	< 10
Farm 6	0-75,000	156	0	0	< 0.6	> 40	21-50	11-25
Farm 7	300,000-400,000	111	7	0	1.2-2.0	< 10	> 80	< 10
Farm 8	300,000-400,000	89	0	0	1.2-2.0	< 10	> 80	< 10
Farm 9	75.000-150000	280	0	53	< 0.6	11-25	51-80	< 10
Farm 10	300.000-400000	68	0	0	1.2-2.0	< 10	> 80	< 10
Farm 11	300.000-400000	435	0	0	1.2-2.0	< 10	> 80	< 10
Farm 12	150.000-225.000	165	0	5	> 2	26-40	21-50	26-40
Farm 13	75,000-150,000	140	0	0	< 0.6	26-40	21-50	11-25
Farm 14	0-75,000	86	0	0	0.6-1.2	26-40	< 20	> 40
Farm 15	0-75,000	130	0	0	0.6-1.2	26-40	< 20	> 40
Farm 16	150,000-225,000	109	0	0	1.2-2.0	< 10	> 80	< 10
Farm 17	75,000-150,000	170	0	0	1.2-2.0	< 10	> 80	< 10
Farm 18	300,000-400,000	277	0	0	0.6-1.2	26-40	21-50	11-25
Farm 19	75,000-150,000	118	0	0	1.2-2.0	<10	>80	<10
Farm 20	0-75,000	113	0	0	0.6-1.2	26-40	21-50	11-25

Statistical analysis

To obtain the best estimate for potential population size, studies of *Culicoides* population abundance and distribution have relied largely upon different averaging or 'maximum level' measures derived from trap catch data during the peak season of midge activity (Baylis et al., 1997; Conte et al., 2007; Purse et al., 2012; Rigot et al., 2012a). In contrast, to avoid loss of information (i.e. varying activity rate and seasonality), we modelled the actual counts per trap catch.

Culicoides abundance datasets, such as those obtained year-round by the surveillance systems implemented in Europe, show a number of particular features: a) large variation in catch size over a relatively short period of time (e.g. from week to week), sometimes of several orders of magnitude, and possibly reflecting changes in population size, the activity rate of parous midge females (proportion of host seeking females that are active on a given night), or adverse weather conditions (Birley & Boorman, 1982; Baylis et al., 1997); b) frequent zero-count observations (negative catch records) due to the high degree of seasonality in midge activity in the North Temperate

zone (suppressed during the winter); and c) dependency between observations due to the longitudinal study design (repeated measurements on a weekly basis at the same trap sites).

When applied to such datasets, classical Poisson regression to model count data and its extensions (quasi-Poisson and negative binomial), may be of limited use, because for the distribution of counts that exhibit both over-dispersion and excess zeroes, this may not yield a good fit (Zuur et al., 2009). Other studies have shown the utility of applying to *Culicoides* data models that address features like frequent zero-count observations and over-dispersion (Carpenter et al., 2008; Viennet et al., 2011). The abundance data were heavily right skewed, with a variance to mean ratio that ranges between 34 and more than 100, depending on the species, and contained a large proportion of zero counts (between 40% and 95%). Box-and-whisker plots illustrate the weekly abundance and variability of each vector species across the country (Figure 2a-j). Consequently, we used a negative binomial cloglog hurdle model (Cameron & Trivedi, 1998), to explore the relative effects of climatic, edaphic and host availability factors on year-round fluctuations in adult biting midge populations across The Netherlands, while addressing dataset-specific issues. An exploratory analysis by means of contingency tables of *Culicoides* species per week, stratified by year and location, revealed that zero-counts were restricted to certain seasons, typically late autumn, winter and early spring. The only exception was *C. pulicaris sensu stricto* (*ss*) that was equally abundant across all 21 compartments, with little variability between sites and consistently absent in compartments 1A and 2.

The two components of the hurdle model allowed us to investigate whether different factors are responsible for an event (i.e. presence of *Culicoides* in the light trap) and for the strength of this event (i.e. size of weekly counts). The above-mentioned model feature proved to be useful and ecologically sensible, because the presence or absence of midges in the present study appeared, for the majority of BTV proven or suspected vectors, unrelated to the fact that some locations were outside of a species' range or niche, but in contrast more likely due to their strong seasonality. Sampling at certain time of the year, resulted in zero catches everywhere. After the spring flush, all the sampled sites were positive for the species considered, although abundance differed between weeks and between farms. As a consequence of the pattern observed, we fit the model to identify the driving factors that led to the commencement of the adult active season and which were the ones affecting *Culicoides* abundances. This resulted in fitting the zero hurdle model component with time-related and climatic variables only, i.e. only including the covariates that, by changing during the year, might explain the

start of the active season and therefore the probability of observing at least one *Culicoides* in the trap. In contrast, the count component was fitted with covariates that were characteristics of the sites sampled, whether fixed (possibly accounting for the higher prevalence of certain species at specific sites) or variable during the year (possibly accounting for the week to week fluctuations) (Table2). Full details of the model used, its implementation within the hurdle modelling framework and inference, are provided in Supporting information.

Table 2. Summary of the set of covariates considered in the hurdle model components

Name	Definition
	Count model component
Location	Farm (indicator variable), latitude (dd), longitude (dd), altitude (m)
Season	The four annual seasons (spring, summer, autumn and winter) as occurring in the North Temperate zone
Year	Calendar years of the period considered in the present study
Ground-measured variables	Weekly mean values of temperature, relative humidity, pressure, precipitation amount, evapotranspiration and hourly mean of wind speed
Satellite-derived variables	Weekly mean values of NDVI, dLST and nLST
Host availability data	Cattle density, no. of cattle, goats and sheep on the light trap site
Soil texture features	Percentage of sand, silt, clay and organic carbon in topsoil (0-30 cm)
	Zero hurdle model component
Time	Week, season
Ground-measured variables	Weekly mean values of temperature, relative humidity, wind speed, pressure, precipitation amount, evapotranspiration
Satellite-derived variables	Weekly mean values of NDVI, dLST and nLST

dd= decimal degrees, m= meters

A separate hurdle model was fitted for the weekly counts n of the total *Culicoides* spp. and of each of the potential BTV-8 vectors, namely the Obsoletus Complex (comprising *C. obsoletus* and *C. scoticus*), *C. chiopterus*, *C. dewulfi* and *C. pulicaris ss.* To quantify the amount of collinearity between the covariates, we calculated generalized variance inflation factors (gVIFs), sequentially dropped the covariates with the highest VIF, recalculated the VIFs and repeated this process until all VIFs were smaller than 5. Variables related to soil texture (sand, silt and clay), cattle density, the latitude, max, mean and min temperature and 'farm', shown relatively high VIF values, suggesting

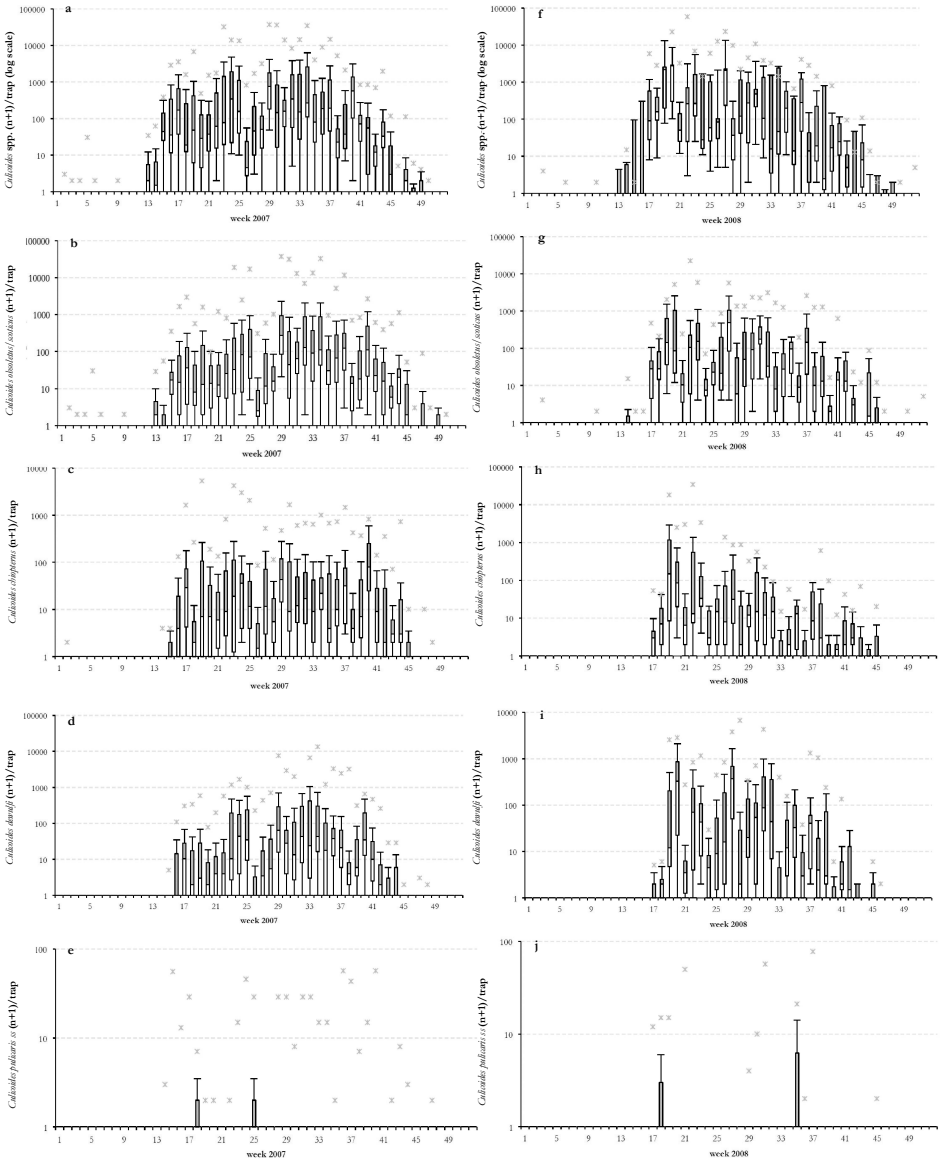

Figure 2. Box-and-whisker plots of biking minge weekly abundances (n+1) plotted on a log scale: *Culicoides* spp. [(a & f) 40 species], *C. obsoletus/scoticus* (b & g), *C. chiopterus* (c & h), *C. dewulfi* (d & i), *C. pulicaris s.s.* (e & j), across The Netherlands in 2007 (a-e) and 2008 (f-j). the ends of the whiskers are set at 1.5*inter quartile range (IQR) above the third quartile and 1.5*IQR below the first quartile. Maximum values exceeding this range are shown as asterisks.

that each of them might be related to one or more of the other covariates. Dropping 'farm', sand, max and mean temperature and latitude, in that order, reduces the VIF values for the other covariates below threshold. The highly heterogeneous *Culicoides* population sizes caught in a light trap, are known to vary in response to local-scale factors (e.g. rate of dung removal from animal holdings, water sources availability etcetera) in addition to climatic ones. Actual on-farm measurements of those factors were not available for the present study. For this reason, and due to the collinearity detected, we started with an a priori set of biologically plausible models, instead of evaluating a single model. We then used measures of information and model uncertainty as a basis for model selection. We fitted two types of model: 1) with indicator 'farm' + climatic variables; 2) with longitude + altitude + host availability + soil texture + climatic variables. For *C. pulicaris* it was possible to fit only models of type 2, because no 'farm' effect can be detected given the little variability between sites. Explanatory variables were removed consecutively in a manually backward-stepwise procedure in order to find the model which, based on a significant reduction of the Akaike's Information Criterion (AIC), best fit the data. We sorted the competing models in terms of their improvements on model fit in comparison to the model with the lowest AIC value, as assessed by the change in AIC (ΔAIC), and we ranked them by AIC weights. The intercept-only model was included as well. The AIC weight corresponds to the relative probability that each model was the best model of those being compared (Burnham & Anderson, 2002). Model checking was carried out by graphical residual analysis instead of numerical methods. The advantage of the first is that it readily illustrates a broad range of complex aspects of the relationship between the model and the data. Graphs of the Pearson residuals versus fitted values and versus each continuous variable were generated to visually assess whether any discernable patterns were present.

To check whether the models were sensitive to outliers, models including and excluding these observations were assessed in terms of the resultant differences in coefficient estimates, SEs and the AIC. The results only differed slightly and did not alter the conclusions (the results shown in Table 4 and 5 refer to the full data set). Statistical analysis was performed using the R software version 2.14.0 (http://cran.r-project.org/), package '*pscl*' (Zeileis et al., 2008). To find evidence of unaccounted-for fine scale spatial variation, plots were examined of the empirical variogram of the standardized residuals for the best performing hurdle model for each *Culicoides* species. This was coupled with the computation of envelopes using data permutation (simulations n=999) under the assumption of no correlation. If the variogram plot

falls within the envelopes, it means the model has considerably reduced spatial autocorrelation. The empirical variogram and the variogram envelopes were estimated using the '*geoR*' package in R (Ribeiro and Diggle, 2001).

To detect any temporal patterns that might violate the assumption of independence between observations, model residuals were tested by using the autocorrelation function (ACF) in the R '*stats*' package (R Development Core Team, 2009).

Results

A total of 906,879 *Culicoides* were trapped in 1541 samples collected during the period of study, representing around 40 species. The twenty-four most common species included the five species that in north-western Europe are considered proven or potential vectors for BTV. The Obsoletus Complex (*C. obsoletus* and *C. scoticus*) predominated in light trap catches and the vector category constituted 44.2% of the midges collected, followed by *C. dewulfi* (16.4%) and *C. chiopterus* (16.3%). The fifth potential vector, *C. pulicaris ss*, was rare (0.1%).

Model checking indicated that in terms of overall fit, the models adequately captured the features of the abundance data (over-dispersion in the non-zero observations, excess of zero observations and the correlation between observations from the same site). Residuals diagnostics showed no pattern or trend. Hence, the model used proved to be suitable for analyzing the Culicoides abundance time-series dataset that resulted from a year round weekly light trapping protocol.

According to our model selection criterion, the models that best describe the data while minimizing information loss appear to be the ones including the 'farm' indicator variable (Table 3).

For all the species, and in both model type 1 and 2 ,'season' and the climatic variable dLST gave the highest probability to observe at least one biting midge in the OVI light trap and are hence likely to govern the commencement of the Culicoides adult active season.

For the count regression component of model type 1 and 2, the most important variables combined different subsets of covariates, even if not all of these were retained in the final model for each species.

Full results of the hurdle models of type 1 and 2 for the total *Culicoides* spp., and for each vector species considered in the present study, are presented in Tables 4 and 5.

Best-fit models of type 1 (Table 4) did identify between-compartment differences in the trap catch abundances of the different species, with the exception of *C. pulicaris*

ss. In general, *Culicoides* appeared to be more abundant in the western (2, 6, 14-16), central (7,10) and southern (18, 19, 20) compartments. This general distribution pattern is broadly consistent for the vector species considered, though the magnitude of the differences between locations did differ. Notably, *C. chiopterus* and *C. dewulfi* also appear to be well established in the north-western (2-4), central (6,7) and south-western (14) compartments and *C. obsoletus/scoticus* in compartments 13, 14 and 17.

Climatic variables associated with the weekly *Culicoides* counts were coherent across species. Of the set of covariates tested, those determining higher numbers of midges per trap catch were related to temperature (dLST), while wind and the evapotranspiration index were associated with lower *Culicoides* counts. Rain, retained only in the count model of *C. chiopterus*, and NDVI, for *C. obsoletus/scoticus*, were associated with increasing numbers of these species.

The factor 'season', when retained and even if not significant at all levels, suggested that after the spring emergence the majority of species were more abundant before the end of summer. The activity of *C. obsoletus/scoticus* appeared to extend across all seasons with a peak of abundance in summer, while for *C. chiopterus* that occurred later in autumn after a decreased activity in summer. In comparison to 2007, vector species were less abundant in 2008.

Best-fit models of type 2 (Table 5) revealed that *C. obsoletus/scoticus* and *C. pulicaris ss* increased from west to east, while *C. dewulfi* decreased in that direction. *C. obsoletus/scoticus* and *C. dewulfi* numbers were positively affected by higher altitude. Seasonal and yearly patterns, as well as effects of climatic variables on the numbers of midges, mirrored the ones described for models of type 1. All *Culicoides* species decreased in abundance with higher cattle density. Host species at a farm, when retained in model, shown differences in their impact on vector abundance: cattle and sheep seemed to limit and goats seemed to increase the number of Culicodies. For higher fraction of organic material in the soil *C. obsoletus/scoticus* and *C. chiopterus* numbers were decreasing, whereas *C. dewulfi* numbers were increasing. Increasing percentage of clay in the soil impacted negatively on *C. dewulfi*.

The variograms of the residuals for each species model considered were all contained by their envelopes (see Figure 1 Supporting information), suggesting that no spatial autocorrelation greater than would be expected by chance remained after adjusting for the covariates. Therefore, geostatistical models that account for spatial dependency were not explored further in this study.

In the autocorrelation plots, with the exception of lag 0, which is always 1 by definition, only a few lags fall slightly outside the 95% CI, which are expected due to random fluctuations (see Figure 2 Supporting Information).

Table 3. Best subset hurdle regression models of type 1 and 2 for each vector species, included AIC, deltaAIC and AIC weight

Culicoides species	Predictors				
	Count component	Zero hurdle component	*AIC*	Δ*AIC*	*AICweight*
Culicoides spp.					
	farm, season, year, dLST, wind	season, dLST	12491.47	0	0.39
	farm, season, year, dLST, wind, mean humidity	season, dLST	12492.37	0.9	0.24
	farm, season, year, dLST, NDVI wind, evapo	season, dLST	12492.81	1.33	0.20
	season, year, wind, dLST, rain, mean humidity, evapo, cattle density, no. cattle, no. sheep, no. goats, silt, clay, organic	season, dLST	12614.07	122.60	<0.001
	season, year, wind, dLST, NDVI, rain, mean humidity, evapo, cattle density, no. cattle, no. sheep,no. goats, longitude, silt, clay, organic	season, dLST	12617.25	125.78	<0.001
	season, year, wind, dLST, rain, mean humidity, cattle density, no. cattle, no. sheep, no. goats, silt, clay, organic	season, dLST	12624.64	133.17	<0.001
	intercept only	intercept only	14134.05	1642.58	0.00
C. obsoletus/scoticus					
	farm, season, year, dLST, wind, NDVI, evapo	season, dLST	10304.71	0	0.60
	farm, season, year, dLST, wind, NDVI, evapo, rain	season, dLST	10305.53	0.82	0.40
	season, year, wind, dLST, NDVI, rain, mean humidity, evapo, cattle density, no. cattle, no. sheep, no. goats, altitude, longitude, silt, clay, organic	season, dLST	10477.83	173.12	<0.001
	season, year, wind, dLST, rain, mean humidity, evapo, cattle density, no. cattle, no. sheep, no. goats, altitude, longitude, silt, clay, organic	season, dLST	10478.30	173.59	<0.001
	season, year, wind, dLST, NDVI, rain, mean humidity, evapo, cattle density, no. cattle, no. sheep, no. goats, longitude, silt, clay, organic	season, dLST	10479.36	174.65	<0.001
	intercept only	intercept only	11896.35	1591.64	0.00
C. chiopterus					
	farm, season, year, dLST, wind, rain	season, dLST	7738.23	0	0.51
	farm, season, year, dLST, wind, rain, evapo	season, dLST	7738.95	0.72	0.35
	farm, season, year, dLST, NDVI, wind, rain, evapo	season, dLST	7740.86	2.63	0.14
	year, wind, dLST, NDVI, rain, mean humidity, evapo, no. cattle, no. goats, silt, clay, organic	season, dLST	7787.26	49.03	<0.001
	year, wind, dLST, NDVI, rain, mean humidity, evapo, no. cattle, no. goats, altitude, silt, clay, organic	season, dLST	7788.18	49.95	<0.001
	year, wind, dLST, NDVI, rain, mean humidity, evapo, no. cattle, no. sheep, no. goats,	season, dLST	7789.62	51.39	<0.001

Table 3. continued

Culicoides species	Predictors				
	Count component	Zero hurdle component	*AIC*	Δ*AIC*	*AICweight*
C. dewulfi					
	farm, year, wind, dLST	season, dLST	7556.97	0	0.95
	farm, year, NDVI, dLST, wind, rain, evapo	season, dLST	7562.68	5.71	0.05
	year, wind, dLST, mean humidity, no. cattle, no. sheep, altitude, longitude, clay, organic	season, dLST	7634.17	77.2	<0.001
	year, wind, dLST, mean humidity, rain, no. cattle, no. sheep, altitude, longitude, clay, organic	season, dLST	7634.93	77.96	<0.001
	year, wind, dLST, mean humidity, rain, no. cattle, no. sheep, no. goats, altitude, longitude, clay, organic	season, dLST	7636.02	79.05	<0.001
	intercept only	intercept only	8635.42	1078.45	0.00
C. pulicaris ss					
	wind, cattle density, no. sheep, no. goats, longitude	season, dLST	1028.309	0	0.44
	year, wind, cattle density, no. sheep, no. goats, longitude	season, dLST	1029.659	1.35	0.22
	year, wind, rain, cattle density, no. sheep, no. goats, longitude	season, dLST	1029.833	1.52	0.21
	year, wind, dLST, rain, evapo, cattle density, no. sheep, no. goats, longitude	season, dLST	1031.111	2.80	0.11
	year, wind, dLST, rain, cattle density, no. sheep, no. goats, longitude	season, dLST	1031.627	3.32	0.08
	year, wind, dLST, rain, evapo, cattle density, no. sheep, no. goats, longitude, clay	season, dLST	1032.427	4.12	0.06
	intercept only	intercept only	1120.61	92.30	0.00

Table 4. Regression coefficients estimates, standard errors (SEs) and p-values for factors retained in the best hurdle model of type 1 for each vector species considered

	Culicoides spp.		*C. obsoletus/ scoticus*		*C. chiopterus*		*C. dewulfi*	
Count model coefficients (truncated negative binomial with log link)								
Name	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)
(intercept)	1.75±0.61	0.001	-1.71±0.78	0.02	-0.30±1.21	0.80	-2.47±0.80	0.002
Farm 1a	*ref*	-	*ref*	-	*ref*	-	*ref*	-
Farm 1b	-0.21±0.37	0.57	1.55±0.39	<0.001	1.62±0.71	0.02	2.08±0.73	0.002
Farm 2	1.53±0.35	<0.001	3.30±0.52	<0.001	3.03±0.75	<0.001	4.54±0.73	0.004
Farm 3	0.41±0.36	0.25	0.69±0.38	0.07	1.70±0.73	0.02	3.21±0.73	<0.001
Farm 4	0.81±0.44	0.06	2.24±0.44	<0.001	2.41±0.82	0.003	4.17±0.79	<0.001
Farm 5	-0.11±0.36	0.75	1.76±0.38	<0.001	0.99±0.67	0.13	2.90±0.72	<0.001
Farm 6	1.74±0.36	<0.001	3.22±0.38	<0.001	1.20±0.72	0.09	4.73±0.73	<0.001
Farm 7	2.70±0.37	<0.001	4.85±0.38	<0.001	3.85±0.76	<0.001	4.85±0.74	<0.001
Farm 8	-0.13±0.46	0.77	1.08±0.46	0.02	0.83±0.84	0.32	4.20±0.82	<0.001
Farm 9	-0.22±0.43	0.60	0.05±0.42	0.90	0.32±0.74	0.66	2.42±0.80	<0.001
Farm 10	0.78±0.39	0.05	1.92±0.38	<0.001	1.06±0.75	0.15	5.42±0.75	<0.001
Farm 11	-1.17±0.46	0.01	0.28±0.47	0.54	0.33±0.83	0.69	2.32±0.95	0.02
Farm 12	-1.50±0.44	<0.01	-0.12±0.43	0.76	0.31±0.78	0.68	0.11±0.83	0.89
Farm 13	0.38±0.49	0.43	2.83±0.50	<0.001	0.49±0.89	0.57	3.20±0.94	<0.001
Farm 14	1.28±0.34	<0.01	2.42±0.36	<0.001	2.96±0.77	<0.001	3.28±0.70	<0.001
Farm 15	-2.34±0.45	<0.001	-0.59±0.47	0.20	-1.50±0.78	0.05	0.003±0.88	0.99
Farm 16	-1.83±0.44	<0.001	0.09±0.44	0.83	-1.47±0.81	0.07	1.61±0.83	0.05
Farm 17	0.19±0.44	0.66	2.23±0.43	<0.001	0.02±0.82	0.97	3.27±0.86	<0.001
Farm 18	0.85±0.45	0.06	3.06±0.45	<0.001	1.19±0.83	0.15	3.53±0.83	<0.001
Farm 19	2.00±0.38	<0.001	3.31±0.39	<0.001	3.23±0.72	<0.001	5.83±0.72	<0.001
Farm 20	1.82±0.37	<0.001	3.88±0.37	<0.001	1.05±0.75	0.16	6.07±0.73	<0.001
spring	*ref*	-	*ref*	-	*ref*	-	-	-
summer	0.39±0.18	0.03	1.17±0.15	<0.001	-0.49±0.28	0.07	-	-
autumn	0.23±0.29	0.42	0.64±0.28	0.02	0.16±0.48	0.72	-	-
winter	-3.00±0.63	<0.001	-1.80±0.62	0.004	-8.74±112.51	0.93	-	-
year 2007	*ref*	-	*ref*	-	*ref*	-	*ref*	-
year 2008	-0.60±0.17	<0.001	-0.75±0.16	<0.001	-0.53±0.26	0.04	-1.05±0.24	<0.001
evapotranspiration	-	-	-0.30±0.11	0.008	-	-	-	-
NDVI	-	-	1.30±0.60	0.03	-	-	-	-
wind	-0.22±0.04	<0.001	-0.15±0.04	<0.001	-0.23±0.07	0.001	-0.17±0.07	0.01
rain	-	-	-	-	0.02±0.01	0.02	-	-
dLST	0.21±0.02	<0.001	0.21±0.02	<0.001	0.16±0.03	<0.001	0.17±0.02	<0.001
log(theta)	-1.37±0.08	<0.001	-1.19±0.08	<0.001	-2.39±0.28	<0.001	-2.06±0.20	<0.001
Zero hurdle model coefficients (binomial with cloglog link)								
Name	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)
(intercept)	-2.80±0.23	<0.001	-2.75±0.22	<0.001	-3.42±0.24	<0.001	-4.28±0.27	<0.001
spring	*ref*	-	*ref*	-	*ref*	-	*ref*	-
summer	0.76±0.11	<0.001	0.64±0.10	<0.001	0.51±0.09	<0.001	0.85±0.10	<0.001
autumn	1.25±0.15	<0.001	1.10±0.15	<0.001	1.19±0.15	<0.001	1.53±0.17	<0.001
winter	-1.49±0.31	<0.001	-1.59±0.32	<0.001	-3.49±1.01	<0.01	-16.3±844.7	0.98
dLST	0.16±0.01	<0.001	0.15±0.01	<0.001	0.16±0.01	<0.001	-0.18±0.01	<0.001

Table 5. Regression coefficients estimates, standard errors (SEs) and p-values for factors retained in the best hurdle model of type 2 for each vector species considered

	Culicoides spp.		*C. obsoletus/scoticus*		*C. chiopterus*		*C. dewulfi*		*C. pulicaris* s.s.	
Count model coefficients (truncated negative binomial with log link)										
Name	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)
(intercept)	4.43±0.73	<0.01	0.03±1.40	0.98	4.39±1.17	<0.001	5.25±1.67	0.01	-3.66±2.41	0.13
longitude	-	-	0.54±0.22	0.01	-	-	-0.50±0.23	0.05	1.52±0.42	<0.001
altitude	-	-	0.01±0.01	0.06	-	-	0.012±0.001	<0.001	-	-
clay	-0.01±0.008	0.10	0.005±0.01	0.62	0.03±0.01	0.06	-0.04±0.01	<0.001	-	-
silt	0.013±0.007	0.07	-0.004±0.01	0.68	-0.02±0.01	0.06	-	-	-	-
organic	-0.01±0.008	0.18	-0.02±0.01	0.03	-0.03±0.01	<0.001	0.06±0.01	<0.001	-	-
cattle density	-0.23±0.06	<0.001	-0.31±0.09	<0.001	-	-	-0.007±0.001	<0.001	-0.72±0.15	<0.001
# of cow	-0.003±0.001	0.01	-0.002±0.001	0.05	-0.001±0.001	0.05	-	-	-	-
# of sheep	-0.006±0.003	0.05	-0.02±0.003	<0.001	-	-	-0.01±0.004	0.05	-0.02±0.01	0.08
# of goat	0.21±0.04	<0.001	0.27±0.04	<0.001	0.25±0.05	<0.001	-	-	0.33±0.12	0.01
spring	*ref*	-	*ref*	-	-	-	-	-	-	-
summer	0.65±0.21	0.01	1.29±0.18	<0.001	-	-	-	-	-	-
autumn	-0.14±0.33	0.66	0.03±0.33	0.93	-	-	-	-	-	-
winter	-3.46±0.66	<0.001	-2.22±0.70	0.01	-	-	-	-	-	-
year 2007	*ref*	-	*ref*	-	*ref*	-	*ref*	-	-	-
year 2008	-0.26±0.19	0.17	-0.67±0.20	<0.001	-0.51±0.26	0.05	-1.36±0.28	<0.001	-	-
humidity	-0.02±0.004	<0.001	-0.01±0.004	0.05	-0.03±0.006	<0.001	-0.01±0.005	0.01	-	-
NDVI	-	-	0.77±0.48	0.11	-2.38±0.72	<0.001	-	-	-	-
evapotranspiration	-0.50±0.14	<0.001	-0.80±0.14	<0.001	-0.31±0.17	0.08	-	-		
wind	-0.26±0.04	<0.001	-0.26±0.05	<0.001	-0.31±0.06	<0.001	-0.26±0.08	<0.001	-0.56±0.18	0.01
rain	<0.001±0.008	0.97	-0.02±0.01	0.07	0.02±0.01	0.08	-	-	-	-
dLST	0.30±0.03	<0.001	0.29±0.03	<0.001	0.23±0.03	<0.001	0.23±0.031	<0.001	-	-
log(theta)	-1.69±0.10	<0.001	-1.71±0.12	<0.001	-3.54±0.80	<0.001	-3.08±0.50	<0.001	-1.05±0.52	0.05
Zero hurdle model coefficients (binomial with cloglog link)										
Name	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)	β ± SE	Pr(>|z|)
(intercept)	-2.80±0.23	<0.001	-2.75±0.22	<0.001	-3.43±0.25	<0.001	-4.28±0.28	<0.001	-4.56±0.67	<0.001
spring	*ref*	-	*ref*	-	*ref*	-	*ref*	-	*ref*	-
summer	0.76±0.12	<0.001	0.65±0.10	<0.001	0.51±0.09	<0.001	0.86±0.10	<0.001	0.02±0.24	0.91
autumn	1.25±0.16	<0.001	1.10±0.15	<0.001	1.19±0.15	<0.001	1.54±0.17	<0.001	-0.18±0.47	0.69
winter	-1.50±0.31	<0.001	-1.61±0.33	<0.001	-3.49±1.01	<0.001	-16.30±844.7	0.98	-15.5±882	0.98
dLST	0.16±0.010	<0.001	0.15±0.01	<0.001	0.16±0.02	<0.001	0.18±0.02	<0.001	0.10±0.03	<0.001

Discussion

Using an objective surveillance system, a valuable amount of data on the distribution and seasonality of *Culicoides* was collected over two years across the Netherlands. Not only will such data be indispensable for predictive modeling, but they might also arm us with a more precise understanding of the ecological niche occupied by each species. The present study attempts to identify the long-term effects of environmental factors on fluctuating *Culicoides* abundance during the epizootic of bluetongue that swept through The Netherlands in 2007 and 2008 (Elbers et al., 2009).

During those years, the proven and potential *Culicoides* vector species constituted nearly 80% of the midges collected. The species remained active continuously for six to nine months, including late summer and autumn when outbreaks of bluetongue peaked. Only one of the five proven vector species, namely *C. pulicaris ss*, was consistently rare. After spring had commenced, *Culicoides* vector populations developed rapidly and thereafter, throughout the summer and autumn, remained continually active and maintained high abundance levels. In particular, *C. obsoletus/scoticus* appeared to peak in summer and *C. chiopterus* in autumn, confirming previous results for farm-associated species in northern Europe (Sanders et al., 2011). As shown by Meiswinkel and colleagues (2013), many univoltine and bivoltine species (completing 1 or 2 generations per year) disappeared towards the end of the summer, which means that only the multivoltine species, including all those acting as vectors for bluetongue, remained active until week 45. This, along with the results from the hurdle regression model, indicates that multivoltine species respond adaptively to any increase in temperature within their respective breeding habitats.

The hurdle model has not been applied previously to analyze *Culicoides* surveillance abundance data collected year-round. By considering count outcomes generated by two systematically different statistical processes, it was sufficiently flexible to describe the relationship between certain environmental factors and weekly *Culicoides* abundances across locations (truncated-at-zero negative binomial regression) and ecologically sensible in the interpretation of the main factors that govern the commencement of *Culicoides* activity at the beginning of each season (binomial regression).

The abundance pattern in vector *Culicoides* species was explained by a combination of climatic – both ground-measured and satellite-derived – and non-climatic factors. The relationships between *Culicoides* vector abundances and meteorological conditions reflect findings made elsewhere in the world and in which the effects of temperature-related variables and wind were confirmed as factors with the most influence on

Culicoides activity and dispersal as measured by suction light traps (Peng et al., 1992; Baylis et al., 1998; Purse et al., 2004b). Moisture availability (NDVI, evapotranspiration and precipitation) is the next most important variable after temperature in the promotions or disruption of *Culicoides* larval development as a result of site-specific edaphic factors and differences in species specific breeding habitats. *C. chiopterus* abundances correlated positively with increased rainfall, whereas *C. pulicaris ss* and *C. obsoletus/scoticus* were affected positively by NDVI and negatively by evapotranspiration, like elsewhere in Europe (Purse et al., 2004b; Kluiters et al., 2013).

The geographical patterns of vector prevalence in The Netherlands are posited not to be entirely disjunctive, but to show overlap because the terrain is largely uniform and humanity has had a tremendous impact on the soil profile and properties (van der Veer, 2006). Soil texture, referring to the relative proportions of organic material, sand, silt and clay particles, is directly linked to its water holding capacity, nutrient retention and drainage. Finely textured clayey soils are known to absorb water very slowly and to retain moisture and nutrients. Coarsely textured sandy soils drain quickly and do not hold nutrients well, while the larger the soil organic matter content the lower the drainage rate will be. The fraction of organic carbon is one of the best indicator of the health status of the soil and a growing percentage is associated with fertile areas with a good structure (e.g. arable land) (HSWD, FAO/IIASA/ISRIC/ISS-CAS/JRC, 2009). Stated broadly, our study indicated that *Culicoides*, in response to moisture availability, occur most abundantly in the low lying coastal areas in the northwest, west and southwest, the central and southern sites; whereas they were less abundant in the elevated north-eastern, eastern and south-eastern parts of the country that are drier and better drained. *C. obsoletus/scoticus* were negatively linked to soil rich in organic carbon, their abundance being lower in arable areas; this support earlier finding that they favour a 'non-arable' habitat, such as forest leaf-litter (Conte et al., 2007). The abundances of *C. chiopterus* and *C. dewulfi*, which both breed in cattle dung, appear to be influenced differently by the organic and clay contents in the top soil. *C. dewulfi* favours less moisture retentive soil, rich in nutrients, whereas *C. chiopterus* prefers soil with a nutrient-retentive texture (clayey), but having a low organic matter fraction. We are unable to compare these results with those obtained during recent studies on Palaearctic *Culicoides*, because these species were not sampled (Harrup et al., 2013), because morphological speciation was done at group level (Purse et al., 2012) and because a different trapping technique was used (Sanders et al., 2011). We cannot exclude the possibility that alternative breeding sites identification necessitates on-site data collection and that habitat suitability may also be revealed by other landscape

components that could be delineated by the use of satellite imagery classified to land use/land cover classes maps (Sithiprasasna et al., 2005).

The present work also revealed *C. pulicaris ss* to be rare overall and be consistently absent from some areas (sites 1A and 2), but whether the absence of this species should be ascribed to a non-favorable niche is unclear. The gaps in the distribution of this species, along with its very low abundances, indicate that *C. pulicaris ss* is unable to penetrate into all areas and to satisfy the three inter-related elements (abundance, seasonal persistence and multivoltinism) that a competent vectors appears to possess. The *C. pulicaris ss* free areas identified in this survey are worthy of being characterized more precisely.

The negative impact of cattle density, number of cattle and number of sheep, as opposed to the positive effect of goats seems paradoxical, given that the presence of hosts a pre-requisite for the survival of blood-sucking midges. Because all the sites sampled during the surveillance programme were medium size livestock holdings and the cattle density was measured at the compartment scale, this does not help explaining whether farmed animals were a limiting factor on fluctuating *Culicoides* species-specific abundance at the local scale or they were a proxy for land use, as suggested previously by others (Sanders et al., 2011). All that may be said is that the present results would indicate an important role of multiple hosts on vector species abundance and prevalence. Hence, the picture that emerges does not alter, but refines the one for farm-associated *Culicoides* species in The Netherlands provided earlier by Takken et al. (2008). They reported species of both the *C. obsoletus* and *C. pulicaris* groups to be strongly associated with livestock farm habitats. Addressing the differences between the two studies is complicated for at least two reasons: firstly, the sites selected for the national surveillance programme considered in the present study had mainly cattle, and contrasts strongly with the greater variety of areas studied by Takken and colleagues (2008); secondly, to treat midges at group instead of at species level might hide some of the key factors that underlie species-specific abundances.

Variance inflation factors were computed to identify relevant collinear variables. However, this resulted in problems identifying which covariates were driving the system, those retained or those eliminated. We used a formal approach from information theory to select, amongst an a priori defined set of biologically plausible models, the ones that best explain the data. The differences in model fitting between the models including the 'farm' indicator variable and those with site specific attributes (related to geography, hosts and soil type), can be hypothesised as being due to farm management factors and characteristics of the site sampled that were not accounted for

in the present study. Example of such factors are : manure storage, the presence of water sources, the presence and type of vegetation surrounding the farm and the pasture (open or wooded) and the distance of the light trap from the livestock (Rigot et al., 2012b; Kluiters et al., 2013). We cannot address this without conducting a controlled experiment. These factors will need to be considered in future attempts to further refine understanding of the webbed environmental processes that underlie complex *Culicoides* communities.

Light trapping protocols usually retrieve abundance data for multiple species from a single night's sampling and, although found not accurate in reflecting the host seeking behaviour for *C. chiopterus* (Carpenter et al, 2008; Viennet et al., 2011), they are considered to represent proportionally the size of the local midge populations found at a sampling site and multiplied by the activity rate and trap efficiency. Up to 22 species have been found to co-occur, but seldom co-dominantly, at the same site in The Netherlands (Scolamacchia F. & Meiswinkel R., personal communication, 2010); similar numbers have been reported from elsewhere in northern Europe (Blackwell et al., 1992). These numbers are considerably lower than those found in the tropics and may simply reflect lower niche diversity at more temperate latitudes. Seasonal periods of peak abundance overlap in many, but not all, species, while there is also evidence for temporal segregation over the 24-hour cycle, with some species becoming active earlier in the day than others (Viennet et al., 2012). Such information, together with detailed seasonal demographic data and records of the respective breeding habitats, should help clarify further the predominance of certain species over others in specific areas.

The present study uses the unique opportunity provided by the national surveillance programme to obtain a broad insight into Dutch *Culicoides* fauna phenology. However, the short time series (only 2 years) and the small number of locations sampled (21 in 2007 and 11 in 2008), renders it difficult to interpret and generalize the findings, so as to better highlight the differences and similarities with what has been observed in *Culicoides* field vectors since midge-borne diseases entered Europe. It is also true that, data referring to a multi-hosts/multi-vectors episystem such as the one that delineated in northern European countries, should include additional hosts to the ones considered in the present study, specifically horses and wildlife species (e.g. roe deer, red deer and wild boar) (Tabachnick, 2010). Finally, including site-level factors such as those related to the microhabitat, artificial breeding sites in the direct vicinity of the trap and farm management might enhance our understanding of species-specific phonological traits that should correlate with the appearance of the relevant disease in the vertebrate host. In fact, where the local vector prevalence and

increased abundance does not seem to be linked to soil, but more intimately to that of hosts, this may favour clustering of BT cases within herds. It might be the case that the latter, in contrast to a totally random distribution of the disease, may depend on the species reproductive biology. A clustering at herd level would be more likely to occur where well-known livestock-associated species are prevalent rather than at to sites where predominant species have to migrate back and forth from the larval habitat to the source of blood (animal).

The processes linked to the emergence and subsequent spread of BT in northern Europe remain difficult to unravel, not least due to a poor understanding of vector dynamics, especially at the local level, and the lack of historical data on midge densities from nearly all the areas affected during an epidemic (Purse et al., 2008). This makes it difficult to establish whether any important changes, if any, have occurred in *Culicoides* with regard to population size and to the number of generations completed annually, and whether these, along with local and annual climatic fluctuations, exert a transient impact on the spread of BT. The implementation of functional risk models for midge-borne diseases is thus of great relevance, given the diverse and valuable livestock population at risk (Hartemink et al., 2009; Backer & Nodelijk, 2011; Guis et al., 2012).

The present study has considerable prospects and its insights could be applied to initiate diverse modelling approaches in order to more accurately predict *Culicoides* vector abundance at different spatial and time scales. Coupled with the recent advent of molecular identification of biting midges, it could strengthen ecological studies on habitat preferences, especially of sibling species, a poorly explored field (Harrup et al., 2013). It is also important to consider how biological and climatic processes that influence adult midge behaviour interact with the dynamics of BTV infection, and the practical applications of this for defining the optimal size of the geographic unit for insect surveillance programmes.

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Supporting Information

Hurdle model

Hurdle regression is also known as two-part model. It considers count outcome generated by two systematically different statistical processes, a binomial distribution determining if a count outcome is zero or non-zero and a truncated-at-zero distribution for count data governing all positive counts conditional on non-zero outcomes (Cameron & Trivedi, 1998; Zuur et al., 2009). The first process is reflected by a binomial regression, while the second one by a truncated Poisson or negative binomial regression. The hurdle model has a useful feature whereby the binomial and counts components can be fitted with a different set of explanatory variables. The hurdle model log likelihood can always be maximized for the two components separately without loss of information.

The idea behind a hurdle model formulation is that, whatever mechanism is causing the occurrence of the event (in our instance the presence of *Culicoides*) it has to cross a hurdle before values become non-zero. So, given an event is occurred - that is 'the hurdle has been crossed' - the conditional distribution of this event is controlled by a truncated at zero distribution. By reflecting a two-stage process, the hurdle model allows to investigate whether different factors are driving the commencement of the *Culicoides* active season to those that are affecting its abundance and what are their relative impacts.

In our study, we defined the probability of crossing the hurdle as p; hence the complementary $(1-p)$ was the probability of not crossing the hurdle (i.e. the probability that a zero is observed). This can be seen as the probability that the hurdle is 'survived'. We defined a Weibull survival function for $(1-p)$. So, the transformation that makes the hazard of surviving the hurdle a linear function of the parameters is the complementary log-log for p: $(\log(-\log(1-p)))$. Fitting the zero hurdle part (i.e. binomial probability model) with a cloglog link and with log (time) as a covariate, corresponds to using a Weibull survival function for the 'hurdle escaping' times.

In the counts component of the model, the non-zero counts were described by a truncated negative binomial. The reason for choosing a negative binomial instead of a Poisson distribution was twofold. Firstly, this distribution can be seen as a Poisson process for the weekly counts per compartment with a gamma random effect, so it is a way to model the dependency in the time series (i.e. repeated measurements at each site year-round). Secondly, the negative binomial distribution is a better suited distribution

for the overdispersed non-zero counts by relaxing the Poisson assumption of equidispersion. The log of the mean of the expected counts was modelled linearly in the variables 'farm', 'year', 'season', soil texture, hosts availability, in several climatic variables and the longitude, latitude and altitude of the sampling sites.

The general formula for the zero hurdle component is given by (1) and for the count component is given by (2a) for model 1 and (2b) for model 2:

$$\text{cloglog}\pi = a + \beta_1 season + \beta_2 wind + \beta_3 rain + \beta_4 min\ temperature + \beta_5 LSTd + ...\beta_n X_n \quad (1)$$

$$\log\mu = a + \beta_1 farm + \beta_2 year + \beta_3 season + \beta_4 wind + \beta_5 rain + \beta_6 NDVI + \beta_7 LSTd + ...\beta_n X_n \quad (2a)$$

OR

$$\log\mu = a + \beta_1 long + \beta_2 alt + \beta_3 year + \beta_4 season + \beta_5 cattle\ density + \beta_6 no.cows + \beta_7 clay + ...\beta_n X_n \quad (2b)$$

where π is the probability of observing an event (presence of Culicoides caught by OVI light trap) and μ is the mean of the non-zero counts (one or more *Culicoides* caught by OVI light trap).

Therefore, the hurdle model was implemented to explore:
-which are the most important environmental variables governing the probability of observing *Culicoides* in the light trap catch;
-which are the most important ecological variables affecting the fluctuations in the number of adult midges caught in the light trap weekly, once the active season has started and according to the vectors species specific emergence pattern.

Violation of independence in space and time

The main issue in any statistical model is the assumptions that underlay any inference procedure. In cases where observations have some spatial or temporal dependence and this is not modelled in the systematic part of the model, the residuals from that model will also exhibit dependence (autocorrelation). Such dependence, if present, would invalidate the theory that produces p-values from test statistics in the model.

The clustering of observations over space has been addressed differently in the systematic part of model 1 and 2. In model 1, an indicator variable representing the

location ('farm') was included as spatial fixed effect in each model to account for the spatial correlated structure. Spatial fixed effects are correctly captured by an indicator variable when individual observations (i.e. OVI weekly catches) are organized into well-delineated groups (farms) and some characteristics of the group are unobserved. The effect of 'farm' is assumed to influence the observations in the group (weekly OVI catches) identically. That is, when observations are grouped by farm, but no data are available to gauge the characteristics of that group, the spatial fixed effects variable may capture how this is reflected in the OVI captures. This simply removes the farm effect, rather than modelling it. This is also known as spatial correlation of the group-wise form (Anselin & Arribas-Bel, 2011 and refs. therein), a special case of spatial dependence, and the only setting where the inclusion of spatial fixed effects corrects for spatial correlation. In model 2, the outcome variable has been modelled as a function of longitude, latitude and altitude of the trapping site. Thus, by including easting and northing coordinates and altitude as covariates, the regression model specifies a tilted plane for the spatial trend in the outcome, with the amount of tilt dependent on the estimated values of the regression coefficients of the topographic variables (Pfeiffer et al., 2008).

Once the first order spatial effects (i.e. large-scale variations in the outcome of interest) due to the location and other explanatory variables has been accounted for, we investigated the second-order effects (i.e. small-scale variations) due to interactions between neighbours. To identify the presence of unaccounted-for second order effects, the residuals from the best hurdle model 1 and 2 for each *Culicoides* species have been examined by computing an empirical variogram. To test the statistical significance of any remaining spatial variation in the data, variogram envelopes were obtained by permutation of the data values across the locations (i.e. envelopes built under the assumption of no spatial autocorrelation). The variogram envelopes are constructed by taking, at each lag distance, the minimum and maximum semivariance values of the variogram of the simulated data. If all points from the empirical variogram fall within its envelopes, then the null hypothesis of spatial independence is not rejected (Figure 1) (Ribeiro & Diggle, 2001).

The dependence over time due to the longitudinal study design have been addressed in the systematic part of model 1 and 2 by the use of a negative binomial distribution (see above for the hurdle model details). To check for the correct specification of our models and to exclude that the residuals from different time points were correlated, we use a formal visualization tool to detect randomness and patterns: the auto-correlation function (ACF). The value of ACF at different time lags gives and

indication whether there is any autocorrelation left in the data. The required R code for the ACF and the resulting graph is given in the 'stats' package version 2.14.0 (http://cran.r-project.org/). ACF was calculated at a maximum lag of $10*\log_{10}(N/m)$, where N is the number of observations and m the number of time series (in our case for N= 1541 and m= 2 max lag= 29). The 95% confidence limits were calculated assuming a white noise series (i.e. serially uncorrelated random variable sequence), so that, if random, autocorrelations should be near zero for any and all time-lag separations and should fall within the bands. With the exception of lag 0, which is always 1 by definition, a few lags slightly outside the 95% CI are expected due to random fluctuations (Figure 2).

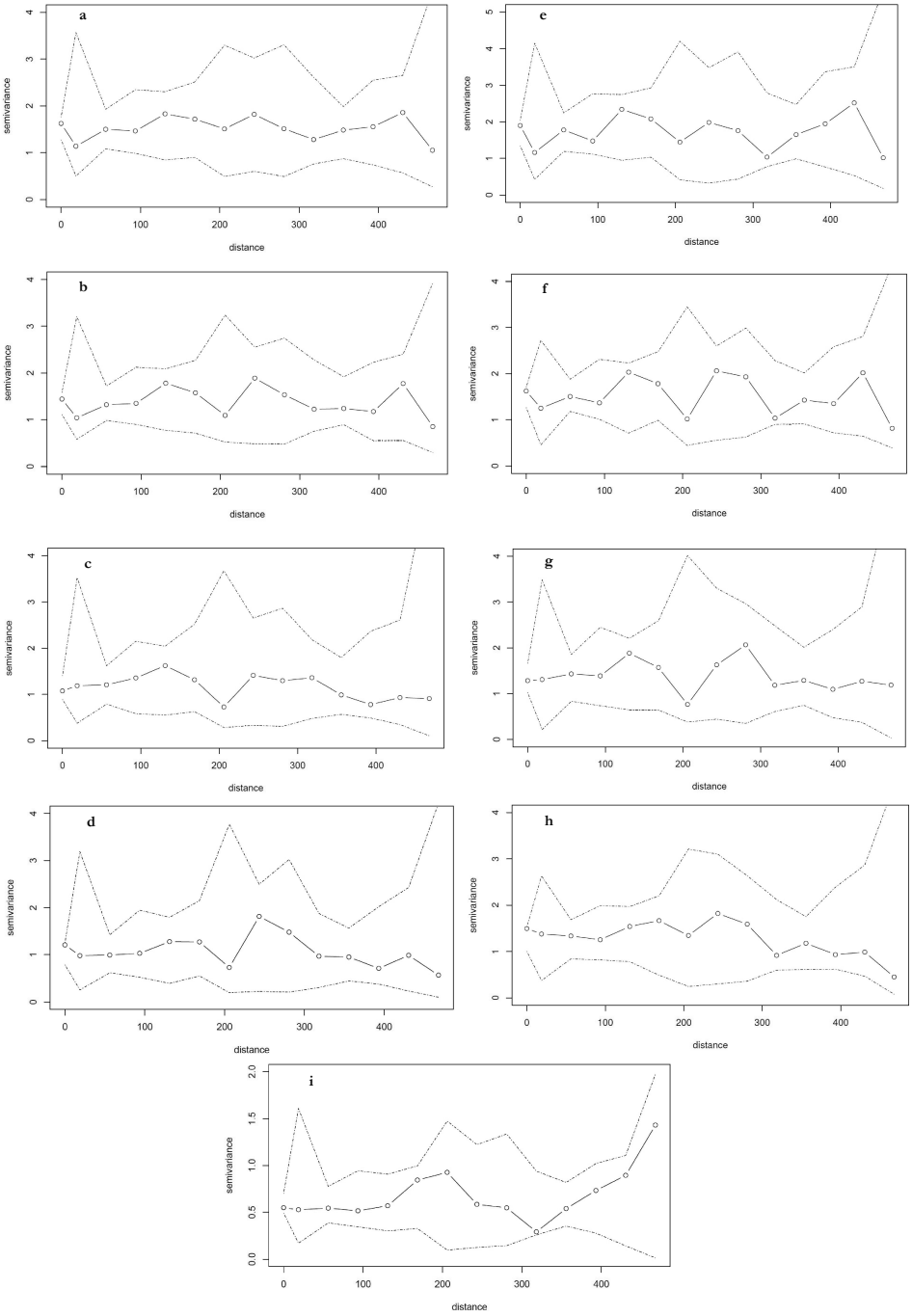

Figure 1. Binned omnidirectional variogram computed using the standardized residuals derived from the best hurdle model 1) and 2), for each species of *Culicoides*. Model 1): a. *Culicoides* spp., b. *Culicoides obsoletus/scoticus*, c. *Culicoides chiopterus*, d. *Culicoides dewulfi*. Model 2): e. *Culicoides* spp., f. *Culicoides obsoletus/scoticus*, g. *Culicoides chiopterus*, h. *Culicoides dewulfi*, i. *Culicoides pulicaris ss*, The pointwise 95% limits (dashed lines) were obtained from 999 simulations where the residuals were randomly allocated to the spatial locations, and the empirical variogram computed for each simulation (solid line). Separation distance is given in Km.

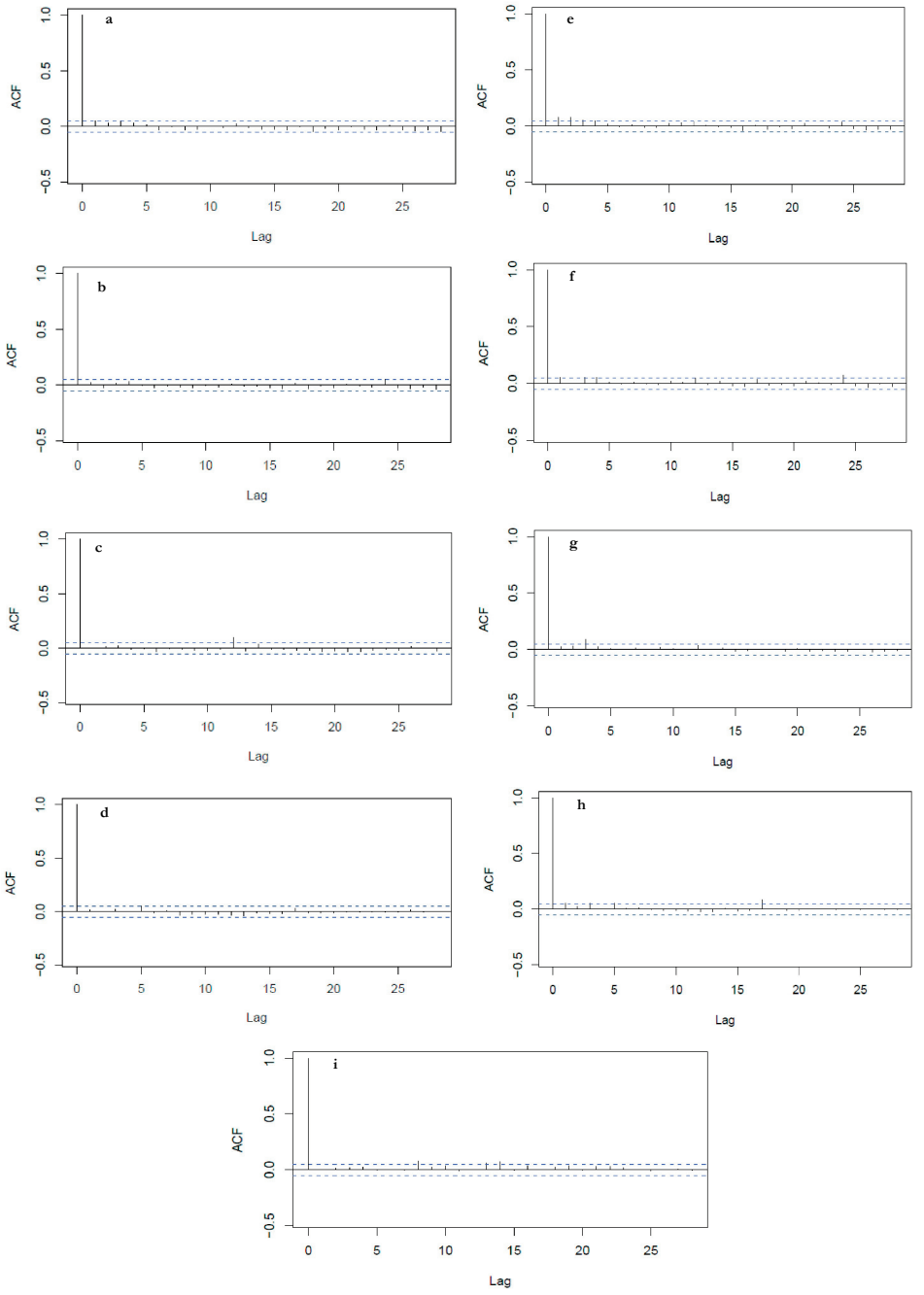

Figure 2. Autocorrelation plot computed using the standardized residuals derived from the best hurdle model 1) and 2), for each species of *Culicoides*. Model 1): a. *Culicoides* spp., b. *Culicoides obsoletus/scoticus*, c. *Culicoides chiopterus*, d. *Culicoides dewulfi*. Model 2): e. *Culicoides* spp., f. *Culicoides obsoletus/scoticus*, g. *Culicoides chiopterus*, h. *Culicoides dewulfi*, i. *Culicoides pulicaris ss*. The blue dashed horizontal lines are 95% confidence limits.

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CHAPTER 6

Studying the relationship between *Culicoides* biting midges caught by OVI light trap and animal-baited trapping methods on cattle in The Netherlands

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in prepapration

Abstract

Epidemiological models of bluetongue (BT) transmission have been seriously hampered by the lack of an objective sampling method that allow for a reliable measure of the vectors activity in the field. The hypothesis that the numbers of *Culicoides* trapped with the standard light trap surveillance method are proportional to the numbers collected on cattle was examined. Collections were made at peak hours (sunset and sunrise) between May and September 2010 using a standardized OVI light trap protocol and two animal-baited sampling methods: mechanical aspiration and sweep net. *Culicoides* specimens were sorted morphologically to species, sex and physiological status for females. A regression method previously applied to study mosquitoes activity was applied to the study the relationship between different trapping methods. In both cases, relationships between animal-baited and OVI light trap catches, appeared to be non-linear, implying that a single straightforward conversion factor could not be calculated. Although the OVI light-suction trap remains a practical and valid method to study the *Culicoides* resident population (presence and abundance), this study suggests that it could not be used to derive parameters of the BT vectors activity and that species-specific and location-specific variations in sampling efficiency deserve further studies

Introduction

The emergence and massive spread of bluetongue (BT) in Western Europe during 2006-2008 had disastrous consequences for sheep and cattle production and confirmed the ability of Palaearctic *Culicoides* spp. (Diptera: Ceratopogonidae) to transmit the BT virus (Velthuis et al., 2010). *Culicoides* spp. are haematophagous insects, which transmit many of the worlds' most significant animal pathogens. Accurate measurement of some ecological aspects of biting midges, especially host-seeking and feeding behaviour, can be difficult to obtain and to date remain insufficiently described. Indirect relative measures can be derived using a trap baited artificially with host semiochemicals, CO_2 or light of an appropriate wavelength. However, the relationship of insect catches by these traps to the true animal biting rate or host-seeking activity, can be largely speculative and seriously hamper the applications of epidemiological models to assess the risk of BT virus transmission and spread in newly invaded areas (Gubbins et al., 2008; Hartemink et al., 2009; Guis et al., 2012). Furthermore, attraction to or capture by semiochemical or light-baited traps may vary substantially among biting insect species, making comparisons of activity between biting insect species problematic. Animal-baited trapping systems (e.g. drop or tent trap, sticky trap, mechanical aspiration, pootering and sweep net), although more difficult to use than the artificially-baited ones, can improve the accuracy of biting rate and host-seeking activity determinations. Additionally, animal-baited trapping is also useful to measure other parameters of vector activity and pathogen transmission, including host feeding preference, pathogen infection prevalence, and biting site preferences.

In survey and surveillance activities for Ceratopogonidae, light traps are often used as they yield a greater number of insects per unit effort, often a prime consideration. Amongst light traps, the Onderstepoort light-suction trap is one of the most effective, as well as the most sensitive, in catching less abundant species. Light traps collections are sensitive to differences in the attractiveness of the light to the different portions of the population, whereas animal-baited collections are sensitive to the time of collection as different portions of the population may be active at different times. Proximity of hosts, however, could affect the composition of collections made by both light traps and nets with collections made using hosts as attractants being more likely to represent what is biting the animals or seeking a blood meal (i.e bloodfed females, nulliparous and parous) than collections made without the use of the hosts.

Several studies involving Palaearctic *Culicoides* biting midges have focused on the comparison of catches derived from direct and indirect trapping methods on different

hosts (Carpenter et al., 2009; Gerry et al., 2009; Viennet et al, 2011) and with different aims such as host preference, circadian rhythms, endo/exophagy and to investigate summer seasonal recurrent dermatitis in horses (Mellor and McCraig, 1974; Townley et al., 1984; Mullens et al., 2005). Light traps have generally been found to underestimate the abundance of biting midges, particularly *C. chiopterus*, and to overestimate others, such as *C. obsoletus* (Carpenter et al., 2008, Viennet et al., 2011). On this basis, it was recommended that alternative/additional methods should be explored to provide a more accurate reflection of the biting population of midges in the field. However, no such study has yet rigorously studied the relationship between the numbers of biting midges caught by light trap and animal-baited methods to evaluate if those sampling methods can be calibrated against each other.

The aim of the present study was to explore the relationship between light trap and animal-baited *Culicoides* spp. collections in order to estimate reliable conversion factors between the two sampling methods.

Materials & Methods

Experimental site and Culicoides trapping protocols

As *Culicoides* spp. activity and animal husbandry are both strongly seasonal in northern Europe, the sampling was carried out between spring and late summer. Experiments were conducted at the didactic farm De Tolakker (52^0 4' 50" N; 5^0 11' 41" E) of the Faculty of Veterinary Science, Utrecht University – Utrecht, The Netherlands. On the premises, dry cows, mainly of the Holstein Friesians breed, are usually kept in the field as soon as the pastures allow for grazing. Autumn is the end of the grazing season and, although cattle are still present in the pasture during the day, the duration of grazing is shortened and they are housed at night. A number of dry cows varying between 4 and 8 were used during the trials.

A total of 10 outdoor experiments were carried out fortnightly from the end of May to the end of September 2010 (May (n=1), June (n=2), July (n=3), August (n=2) and September (n=2)), from 1 hour before sunset to 5 hours after sunrise. *Culicoides* were collected by means of OVI light trap (LT) and two host-baited traps, a mechanical aspirator (MA) and a sweep net (SW), operated at pasture where the cattle were allowed to graze freely.

A 220 V Onderstepoort downdraft suction light trap (manufactured by the Onderstepoort Veterinary Institute in South Africa) operating with an 8W UV-light

tube and on a 12-volt car battery was used for insect collection. The light trap was installed at 1.8-2.0 m above the ground along the fence, which surrounded the cattle grazing area. The collecting protocol used is outlined in Goffredo & Meiswinkel (2004). The light trap was run continuously from 1 hour before sunset to 1 hour after sunrise, with cups collected hourly. A mechanical aspirator based on a rechargeable compact car vacuum cleaner was used. It was equipped with a 50 cm collection tube connected to a plastic container that allowed the insects being blown directly into 200 ml of water with a drop of soap. Collapsible insects net of a very fine mesh fabric (15" ring diameter) with a 24" net handle (BioQuip Products, Inc., USA) was used for sweep netting.

The sweep netting and mechanical aspiration procedures were carried out at pasture, without the help of any artificial light, starting one hour before sunset to a final collection time varying on the day of the trial and limited by visibility (at twilight). Those procedures were resumed 1 hour before sunrise, when the visibility allowed, until the end of the trial and were suspended only during periods of high rainfall. The collecting protocol was hourly based. Each hourly trap period was divided into two consecutive 30-minutes sampling periods consisting of 15-minutes collection period followed by a 15-minutes exposure period. On initiation of the trial two collectors entered the pasture and approached two different cows randomly chosen to carry out the animal-baited collecting activities. The vacuum was consistently passed over the cow for 15 min, from cranial to caudal and moving progressively towards the belly and the legs. At the same time, a sweep net sample was collected by slowly moving around a different cow. After the activities were completed, the collectors moved far away from the pasture and allowed for an exposure period of 15 minutes.

The experimental protocol was examined internally in the Faculty of Veterinary Medicine of Utrecht University, by veterinary surgeons and animal keepers. The protocol procedure did not cause any pain or stress to the animals (i.e. no injection, biological sample taken or surgery procedure). Therefore, according to the Directive 2010/63/EU on the protection of animals used for scientific purposes in Europe, it was not necessary to submit this protocol to an ethical committee. During each step of the protocol qualified and trained staff was present to assure the respect of the standard ethical rules and premises were licensed for animal experiments.

In parallel, an OVI light trap was operated from 1 hour before dusk to 1 hour after sunrise during each collection session to provide an overview of *Culicoides* diversity at De Tolakker site. Besides the field experiment series, weekly sampling by means of OVI light trap was carried out from January to December 2010 on the same premises to monitor the start and end of the biting midges active season for that year. In both

cases, the light trap was placed at 1.8-2.0 m height from the ground underneath a roofed area outside the cattle stable and ran on electricity; it was not visible from the pasture where the cattle were grazing.

Culicoides identification

Insects from the collection cups of the UV-light/suction trap and from the mechanical aspirator were drained and then stored in 70% ethanol until processing. Sweep nets were stored at -20°C for one hour to freeze insects and to allow the collector to handle the catches more easily. Each sweep net sample was then transferred in a separate cup containing water and detergent for 30 minutes to avoid rapid dehydration before storing the sample in 70% ethanol until processing. Each collection was catalogued and given a unique identification code. Small catches were cleaned and enumerated in their entirety, whereas larger catches were subsampled in an adaptation of the method provided by Van Ark & Meiswinkel (1992). Under a dissecting microscope *Culicoides* were identified to species level based on the wing pattern and other diagnostic features as provided in the works of Campbell & Pelham-Clinton (1960) and Delécolle (1985) and sorted by sex. Females were classified as nulliparous, parous, freshly bloodfed and gravid (Dyce, 1969). The respective females of *C. obsoletus* and *C. scoticus* are very difficult to identify accurately based on morphology and because of this and their close phyletic relationship, the data for these two species are combined and presented under the joint taxon 'Obsoletus Complex'.

Statistical analysis

In general, the problem of evaluating agreement between different measurement techniques has been addressed using plots of differences between pairs of measurements against averages of the two methods (Altman & Bland, 1983). This approach has been applied to insects collection data in trap comparison studies previously, but must be modified to allow for zero counts by the simple expedient of adding one to each count. However, Smith emphasized that the log (x+1) transformation of data is highly dependent on insects density, particularly at low values of x, and therefore can be misleading (Smith, 1995).

Random sampling of a homogeneous population of biting midges would give a Poisson distribution of counts (in which the variance is equal to the mean). In addition to this random sampling variation, however, *Culicoides* counts vary considerably as a

result of differences in underlying densities and therefore the observed variance in recorded midges numbers is typically greater than the mean (overdispersion). One modeling approach is therefore to assume the counts to be distributed according to a negative binomial distribution, which has been extensively used to model densities of insects (Taylor, 1984; Scolamacchia et al, in press). Recently, due to the reported weakness of adding one to the counts, several authors (Hii et al, 2000; Mathenge et al., 2005; Overgaard et al., 2012) applied a more rigorous regression analysis to model agreement between sampling methods, based on parameterizing the negative binomial as a gamma mixture of Poisson distributions, assuming both proportionality and non-linear relationship (density dependence).

Here, we used the approach suggested by Hii and colleagues (2000) and modified by Overgaard et al. (2012). Comparison between animal-baited (MA and SW) and artificially-baited trapping methods (LT) was only possible for the time periods during which these activities has been carried out simultaneously, so 3 hours around sunset and 2 around sunrise. Moreover, because the sampling time differed between LT (one continuous sample per hour) and MA/SW (1 sample every 30 minutes), we did correct the data and rescaled the LT catches on 15 minutes, assuming that factors affecting the trap efficiency are averaged on the continuous samples.

It was assumed that the observed counts yielded by LT, MA and SW in month *i* and hour *j* are Poisson distributed, hence:

$$\text{MA or SW counts: } x_{ij} \sim \text{Poisson}(k_{ij})$$
$$\text{LT counts: } y_{ij} \sim \text{Poisson}(\lambda_{ij})$$

Then, the expectation parameter λ_{ij} of y_{ij} is taken to be either linearly or non linearly related to the expected MA or SW counts k_{ij} as follow:

$$\text{Model 1: } \lambda_{ij} = \beta_0 k_{ij} \quad \text{(linearity)}$$
$$\text{Model 2: } \lambda_{ij} = \beta_0 k_{ij}^{\beta 1} \quad \text{(non-linearity)}$$

Further it is assumed that the expected count of x_{ij} depends log-linearly on both hour and month:

$$\log(k_{ij}) = \mu + \theta_i + \gamma_j \ (\theta_i = \text{effect of month}, \gamma_j = \text{effect of hour})$$

The two models were computed for the total *Culicoides* spp. A Bayesian approach using Markov Chain Monte Carlo methods (McMC) was adopted for parameter estimation. The models were implemented in WinBUGS (Lunn et al., 2000) with wide normal priors for all parameters except β_0 for which a wide lognormal prior was assumed to ensure positive expected value of y_{ij}. After an initial burn-in period of 200,000 samples a further 300,000 were collected from 3 McMC chains for posterior inference. Apparent convergence of the McMC samples was assessed by visual examination of the sample histories and calculation of the Brooks-Gelman diagnostic (Brooks and Gelman, 1998). Parameter estimates were obtained as the means of the sampled posterior distribution of each parameter. The model fits were evaluated using 95% credible intervals for the involved parameters and models were compared using the Deviance Information Criterion (DIC), which is readily computed from the McMC runs. The DIC is a model evaluation criterion, which, analogous to the AIC (Akaike Information Criterion) and BIC (Bayesian Information Criterion) criteria, combines a measure of model fit with a penalty for model complexity. When comparing two models, the model with smaller DIC is usually preferred. In a similar manner, posterior means and 95% credible intervals of $\exp \theta_i$ and $\exp \theta_i^{\beta 1}$ were computed for the month and hour effects in model 1 and model 2, respectively. The month of May and the first hour of sampling activities (i.e. 1 hour before sunset), were chosen as references, hence, all other months or hours effects are relative to them.

If β_1 overlap with unit, then Model 1 and Model 2 are equivalent. If β_1 overlap 0, then it cannot be excluded that light trap and MA or SW expected counts are independent. If β_1 is entirely above 1, then the ratio LT:MA or LT:SW increases with increasing insects density. If β_1 is entirely below 1, then the LT is undersampling insects at high density.

Results

Culicoides collections

The *Culicoides* season in 2010 monitored by means of OVI light trap started on week 14 and lasted until week 44. The weekly sampling yielded a total of 26.936 specimens over 52 weeks, comprising 19 species (Table 1), with *C. obsoletus/scoticus* outnumbering the remaining 17. Figure 1 shows the seasonal abundance of the total *Culicoides* spp. for that year.

More than two thousands specimens were collected at pasture (Table 2), 83%

Table 1. Ranked list of 19 identified *Culicoides* species captured during 2010 (52 weeks) by means of OV light trap outside the cattle stable

Culicoides **species**	No. specimens (%)
C. obsoletus/scoticus	12177(45)
C. chiopterus	6075(23)
C. sp nr *newsteadi*	4314(16)
C. dewulfi	2235(8)
C punctatus	970(4)
C pulicaris ss	496(2)
C pallidicornis	261(1)
C festivipennis	184(0.7)
C achrayi	156(0.6)
C smokey punctatus	34(0.13)
C segnis	12(0.04)
C dark obsoletus	6(0.02)
C small lupicaris	6(0.02)
C circumscriptus	4(0.01)
C salinarius	2(<0.01)
C riethi	2(<0.01)
C duddingstoni	1(<0.01)
C subfasciipennis	1(<0.01)
Total	**26936**

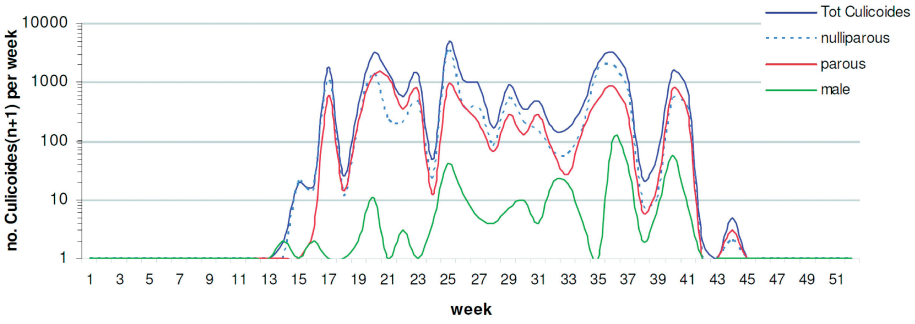

Figure 1 Seasonal abundance pattern for Culicoides spp. as captured by weekly OVI light trap

(n=1890) of which were yielded by the sweep net collections, 11% (n=241) by overnight OVI light trap and 7% (n=153) by mechanical aspiration. Twenty-one species were identified and the proved and potential BTV vectors (*C. obsoletus/scoticus*, *C. chiopterus*, *C. dewulfi*, *C. pulicaris ss*) represented 81% of the total numbers of *Culicoides*. Of those, *C. chiopterus* was the most abundant but not equally captured by the three methods considered, representing 54% (n=1026), 26% (n=40) and 15% (n=35) of the midges caught by SW, MA and LT respectively. Twenty different species were collected by the OVI light trap operated overnight during the experiments outside the cattle stable, for a total of 12652 biting midges (Table 3). Regardless of the trapping method, collections consisted mainly of non-bloodfed parous and nulliparous females, i.e. females actively looking for a blood meal (86% LT stable, 66% LT pasture, 95% SW, 85% MA). Gravid females were mainly detected by the two OVI light traps. Bloodfed *Culicoides* were more common in the OVI light trap close to the stable and in the two animal-baited methods considered than in the OVI light trap at pasture (Table 4). Mean

Table 2. Ranked list of 21 identified *Culicoides* species captured by different trapping methods at pasture

	Method			
Culicoides species	**OVI light trap**	**mechanical aspiration**	**sweep net**	**Total**
	N (%)	**N (%)**	**N (%)**	
C. chiopterus	35(15)	40(26)	1026(54)	1101
C. obsoletus/scoticus	78(32)	47(31)	329(17)	454
C. dewulfi	32(13)	25(16)	206(11)	263
C punctatus	16(7)	15(10)	138(7)	169
C. sp nr *newsteadi*	30(12)	7(4.6)	117(6)	154
C pulicaris ss	1(0.4)	8(5.2)	29(1.5)	38
C pallidicornis	7(3)	8(5.2)	29(1.5)	44
C achrayi	22(9)	1(0.7)	8(0.4)	31
C festivipennis	10(4)	0(-)	2(0.1)	12
C smokey punctatus	0(-)	0(-)	3(0.2)	3
C riethi	3(1.2)	0(-)	0(-)	3
C small lupicaris	0(-)	1(0.7)	2(0.1)	3
C segnis	2(0.8)	0(-)	0(-)	2
C stigma	1(0.4)	1(0.7)	0(-)	2
C salinarius	2(0.8)	0(-)	0(-)	2
C circumscriptus	1(0.4)	0(-)	0(-)	1
C dark obsoletus	0(-)	0(-)	0(-)	0
C large lupicaris	0(-)	0(-)	0(-)	0
C duddingstoni	0(-)	0(-)	0(-)	0
C pictipennis	1(0.4)	0(-)	1(0.1)	2
Total	**241**	**153**	**1890**	**2284**

Table 3. Ranked list of 20 identified *Culicoides* species captured by OVI light trap outside the cattle stable

Culicoides species	No. specimens (%)
C. obsoletus/scoticus	4204(33)
C. chiopterus	3015(24)
C. sp nr *newsteadi*	2513(20)
C. dewulfi	1197(9)
C punctatus	924(7)
C pulicaris ss	364(3)
C pallidicornis	276(2)
C smokey punctatus	38(0.3)
C small lupicaris	32(0.25)
C achrayi	30(0.24)
C festivipennis	25(0.20)
C segnis	18(0.14)
C circumscriptus	6(0.05)
C riethi	3(0.02)
C dark obsoletus	3(0.02)
C stigma	1(0.01)
C large lupicaris	1(0.01)
C haranti	1(0.01)
C dark punctatus	1(0.01)
Total	**12652**

Table 4. Number of *Culicoides* collected by different trapping methods, showing specimens of each sex and physiological status for the females

Collecting method	Female				Male	Total
	nulliparous	parous	gravid	bloodfed		
	N(%)	N(%)	N(%)	N(%)	N(%)	
OVI l.t. stable	5585(89)	5252(78)	1540(94)	78(59)	197(83)	12652
OVI l.t. pasture	89(1.4)	71(1.1)	62(4)	2(1.5)	17(7)	241
MA	69(1.1)	64(1)	2(0.1)	21(16)	0(-)	156
SW	509(8)	1292(19)	36(2)	31(23)	22(9)	1890
Total	6252	6679	1640	132	236	14939

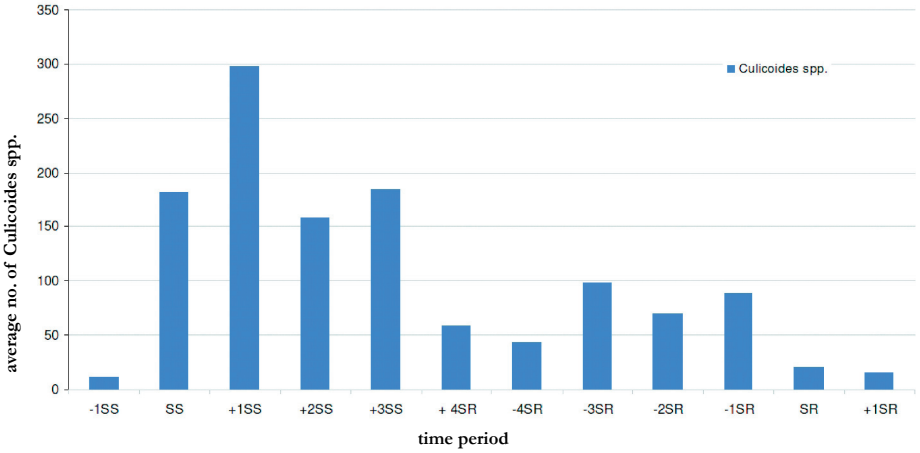

Figure 2. Flight activity pattern of *Culicoides* spp. collected overnight by OVI light trap placed outside cattle stable (SS= sunset, SR= sunrise; collecting time period e.g. -1SS= one hour before sunset)

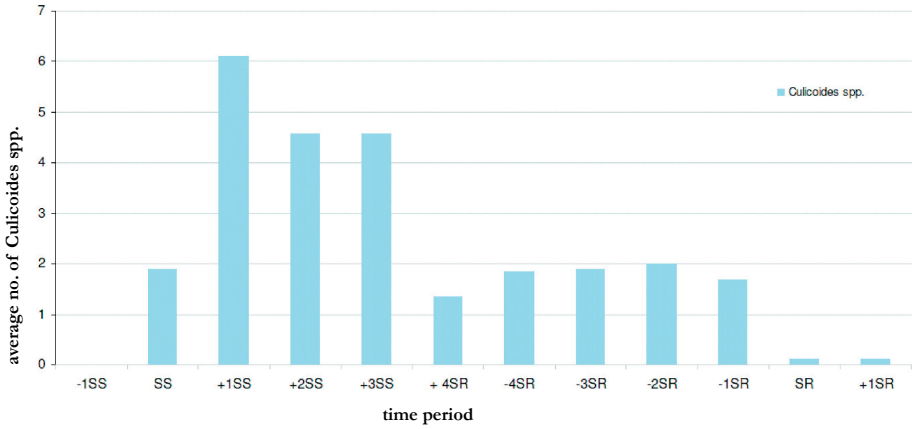

Figure 3. Flight activity pattern of *Culicoides* spp. collected overnight by OVI light trap at pasture (SS= sunset, SR= sunrise; collecting time period e.g. -1SS= one hour before sunset)

number of *Culicoides* per sampling hour collected by OVI light trap close to the stable and the one placed at pasture is provided in Figure 2 and 3, respectively. In both graphs, a bimodal flying activity pattern around sunset and sunrise is clearly shown.

Statistical analysis

When applying the Bayesian approach to study the relationship between artificially- and animal-baited traps, the non-linear model (Model 2) provided a better fit to the data for both comparisons - LT versus MA and LT versus SW- because of a smaller DIC value

(Table 5). In both cases β1 did not include 1, which indicates models equality, hence in both cases, LT and MA or SW counts appeared to be non-proportional. This implies that the ratio LT to MA counts and LT to SW counts is density dependent and a

Table 5. Summary statistics from model estimates

Trapping methods	Model parameters	Model 1	Model 2
LT versus MA	β_0	0.64 (0.43 ; 0.63)	0.89 (0.60 ; 1.21)
	β_1	–	0.01 (-0.65 ; 0.43)
	DIC	**384.15**	**379.00**
LT versus SW	β_0	0.06 (0.04 ; 0.08)	1.89 (0.88 ; 3.87)
	β_1	–	-0.38 (-0.74 ; -0.07)
	DIC	**1719.39**	**1592.42**

For each model and parameter the posterior mean is given with 95% credible intervals (in parenthesis). Model 1 indicates proportionality (linear) and Model 2 density dependence (non-linear) between light trap (LT) collections and direct trapping methods (MA= mechanical aspiration; SW= sweep net). DIC= deviance information criterion

straightforward conversion factor cannot be computed. However, the fact that the credible interval for β1 included zero when comparing LT to MA, indicates that in this case it cannot be excluded that the expected LT counts is independent of the expected MA counts.

Figure 4 showed the monthly and hourly variations in expected LT counts based on the outcome of the two models. Both models comparing LT versus MA showed the lowest expected counts in July, August and September compared to May (reference month). Steadily higher than at the beginning of the active biting midges season are the expected LT counts in the two models comparing LT with SW. As for the peak hour effect, the results from the models comparing LT to SW appeared to be more consistent, showing a decrease in the expected LT counts at sunset, compared to 1 hour before that. The maximum LT counts is expected right after sunrise.

Discussion

Any disease that can be transmitted by *Culicoides* among mammalian host has the potential to cause significant economic hardship. Evaluation of the potential for transmission requires identification of the potential vector complex in an area, the attack rate and biting rate, the vector-to-host ratio.

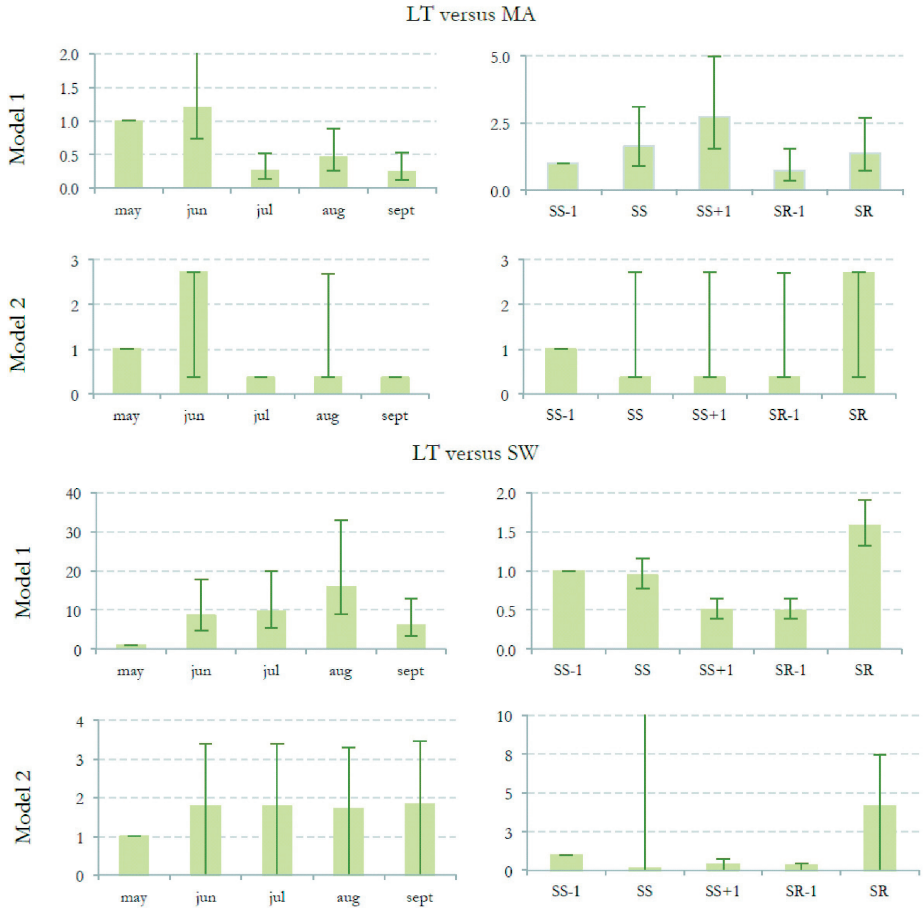

Figure 4. Monthly and peak hours effects on expected light trap (LT) collections using Bayesian analysis. Posterior means and 95% credible intervals from monthly (left panel) and hourly (right panel) effects on *Culicoides* spp. midges collected by mechanical aspiration (MA) and sweep net (SW). SS= sunset, SR= sunrise. May is the reference month; SS-1 (1 hour before sunset is the reference hour).

The present study aimed at evaluating whether it was possible to derive a reliable conversion factor between OVI light trap *Culicoides* collections and one of two different cattle-baited trapping methods – mechanical aspiration and sweep net - which are considered more suitable to study insects biting and host-seeking activity. We applied a method previously used to compare trap efficiency for mosquitoes collections (Overgaard et al., 2012) to biting midges collected around sunset and sunrise, which are known to be peak hours for *Culicoides* host-seeking activity (Blackwell et al., 1997; Viennet et al, 2012). *Culicoides* species collected have been previously identified to occur abundantly near livestock (Nevill et al., 1992; Meiswinkel et al., 2004) and in The

Netherlands (Meiswinkel et al, 2013). The species abundance, however, differed between the trapping devices that were compared (Table 2).

The results from the statistical analyses indicated a possibly non-existent relationship between the expected counts yielded by OVI light trap and mechanical aspiration and a non-linear, density-dependent relative sampling efficiency when light trap was compared to sweep net *Culicoides collections*. In both cases, a simple conversion factor could be calculated.

Jones et al. (1977) noted that results obtained from light traps and aspiration of vector insects from bait animals are not comparable due to differences such as skill of the collector, ineffectiveness of light traps in some portion of crepuscular vector activity periods, and simply the different attractants – light versus animal-generated cues. Braverman (1988) stated that even though light trap results reflect host-seeking, they also reflect other activities of the insects, such as dispersal or looking for oviposition sites or mates. More recently, other studies reported the inaccuracy of light trap catches in reflecting biting rates (Carpenter et al, 2008; Gerry et al., 2009) or found significant differences between UV- and CO_2-baited traps and mechanical aspiration (Harrup et al., 2012). These authors concluded that it is vital to conduct animal-based collections along with the baited trap collections in order to interpret the epidemiological significance of the findings.

Results of the present study are consistent with these findings, confirming that catches obtained from a light trap cannot be used as a proxy for biting rate or host-seeking activity, given the non proportionality between the respective expected counts. However, due to few observations in our dataset, we could not address potential difference in the sampling efficiency of direct and indirect trapping methods for different *Culicoides* vector species. Besides, in evaluating the relative sampling efficiency of artificially-baited trapping methods other factors should be considered. In fact, the different stimuli that trapping methods use to attract insects can strongly influence the fraction of population sampled and therefore the possibility to calibrate one method against the other. For example light traps attract mainly positively photo-tactic flying insects, while animal-baited traps attract females seeking for a blood meal and which respond to the bait offered, other such as sweep net, requires no response from the insects other than for them to be flying at the height of the sweeping. Differences might also reflect location-specific factors, such as availability of breeding sites, or the presence of alternative hosts. Another general limitation in comparing those methods is that some sampling methods obtain data continuously over an extended interval of time, whereas others obtain an essentially instantaneous "snapshot" of a population at

a particular moment. Examples of the latter include sweep-net samples, quadrat samples, and insecticide knockdown samples. Such instantaneous samples are susceptible to the effects of time of the day or weather conditions at the time of sampling, which in turn might influence the number captured. Continuous samples may also be influenced by weather conditions but, because capturing occurs over an extended interval, these effects are averaged over a range of such conditions. The observed monthly and hourly variations in expected light traps counts is probably due to species-specific differences in recruitment rate of nulliparous during the active season and to segregation over time of the day (i.e. circadian rhythm).

These preliminary results emphasize that standardized techniques for measuring the variables of vectorial capacity and vector competence need to be developed and adopted to facilitate interpretation and comparison of data.

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CHAPTER 7

General Discussion

Health of livestock populations is a concern for all communities. This concern arises from the consequences of animal diseases on public health, economy and societal development but also from animal welfare and environmental considerations. The marked differences in economies, environments, husbandry systems and veterinary service capabilities amongst countries, shape the impacts of livestock diseases on societies and priorities that need to be addressed.

Live animals and animal products generate private benefits (income, trade good) and have an important role in global food security, nutritional wellbeing and health. At the same time, livestock production and food supply chains pose serious threats to global health security. They can take the form of pandemic risks, food hazards, high impact livestock epidemics, and a high burden of neglected zoonotic diseases. A central role in minimizing, as far as practicable, the negative effects of livestock health related problems on communities, is represented by (veterinary) public health practice through surveillance and monitoring systems.

Surveillance and monitoring systems for livestock diseases have been broadly depicted for their scope and activities in the Introduction (Chapter 1) and provided the background for Chapter 2 to Chapter 6. In the separate chapters, ample room was given to the discussion of data collected through surveillance activities or related studies, the methods of analysis, and the validity and meaning of the conclusions. In addition to that, however, those examples motivate some cause for reflection for the present chapter, which will focus on animal disease priorities that low- and high-income countries are confronted with, and the common challenges and opportunities.

7.1 Priorities in low-income countries

In low-income settings, animal husbandry is the main livelihood strategy, occurring in a wide range of production systems, from pastoral/grassland systems through mixed crop-livestock and intensive systems in peri-urban/urban areas. People keep livestock as a main asset, because it increases their crop production (manure), provide high quality food (high quality protein, minerals and vitamins), it is crucial in representing a social role and, in the absence of banks, it serves to save and store money and manage risk. Moreover, livestock production is increasing rapidly and uncontrolled in response to growth in population, growth in income, urbanization and changing diets: the so-called livestock revolution (Slingenbergh et al., 2002; Perry & Grace, 2009). The consequent livestock development trajectory differs per animal species and geographical area, with a change in monogastric population representing a proxy for

emerging livestock systems. In much of Asia and Latin America and Caribbean regions, both extensive and intensive animal agriculture co-exist and co-evolve, with an increasing trend to intensification and market-oriented sectors, especially for pigs and poultry. Africa features mainly extensive or traditional village poultry and a growing backyard pig (scavenging animals) production, with a beginning of intensification. Cattle and small ruminants kept by pastoral and agro-pastoral communities and villages still form the major source of subsistence (FAO, 2011a).

In those areas, there have been relatively few changes in the distribution, prevalence and impact of many epidemic and endemic diseases of livestock over the last decades, particularly in sub-Saharan Africa and outside southern Africa (Perry et al, 2011). Over this time, there has also been a general decline in the quality of veterinary services (Leonard, 2004). The international community by means of grants or subsidies has supported heavy investments in animal health services. Most of these activities were designed and applied through the public sector, but the economic plight of many governments has been reflected in the collapse of these institutions, leading to calls for sustainability through decentralization, cost recovery and privatization (Leonard, 2000; McLeod & Wilsmore, 2002; Schelling, 2002; Woodford, 2004). With the exception of a major success story, such as the global eradication of rinderpest (FAO, 2011b), national-level, annual vaccination campaigns routinely, carried out against the most damaging communicable diseases (for example anthrax, blackleg and dipping programmes to prevent ticks and tick-borne diseases), were discontinued and research funding allocated for the development of improved vaccines, was progressively withdrawn (Norval & Deem, 1994; Schelling et al., 2007).

A high under-reporting rate is the major constraint to our ability to understand and prevent animal disease and renders difficult any prioritizing exercise in such countries. Moreover, valuable descriptions and evaluations are published in the grey literature, which is largely online and not easily available. Usually, in sub Saharan Africa, livestock losses do not appear in official reports. At least 50% of these losses are probably due to notifiable diseases, including foot-and-mouth disease, Newcastle disease, African swine fever, classical swine fever, trypanosomosis, East Coast fever and peste de petits ruminants (ILRI, 2012). Recent appraisals, however, agreed that long-standing and persistent zoonoses are responsible for the great majority of human cases of illness and deaths, as well as the greatest reduction in livestock production (WHO & DFID, 2006; ILRI, 2012).

The dual impact of endemic zoonoses on public health as well as on livestock productivity undermines livelihoods both by causing illness in the household and

threatening its livestock and their output, thus perpetuating impoverishment of these societies. Moreover, developing countries usually offer the scene of enhanced ecosystems-agriculture and agriculture-human interfaces. The first featuring a myriad of co-evolving livestock production systems, from extensive to intensive systems, that are at the border with wildlife environments. The second exemplified by a peri-urban production system (i.e. free-range, scavenging animals). The net result of these combined dynamics is a growing diversity of pathogens circulating in domesticated livestock, wildlife and humans; this in turn may lead to zoonotic disease flare-up, spread and persistence.

A difficulty in assessing the changing disease status and priorities in much of the developing world is the lack of data. Hence, one of the critical entry points may well be animal health surveillance. Cutting-edge research to identify predictors of disease emergence and factors underpinning its spread and transmission, innovations in pathogen diagnostics (e.g. DNA fingerprinting and PCR), novel information technologies and data sources, are promising advances that may help surveillance efforts. However, recognizing certain basic constraints in poor resource settings is fundamental if those tools are to be made effective and sustainable. Simply replicating what has been successful in other countries, will not assure that it will work. The reason is that for animal health to positively impact on poverty, it has to be socially feasible and attractive for stakeholders.

Attempts to implement animal health surveillance and information systems in developing countries based on conventional models developed for intensive, sedentary production systems common in wealthy countries (i.e. formal data collection methods, top-down surveillance approach) have proved to be unsustainable projects. As a result, alternative systems of inquiry and learning began to evolve, leading to the development of 'participatory epidemiology' (PE) and community-based animal health workers (CAHW) (Catley & Leyland, 2001). These systems place emphasis on the existing knowledge that people have about the animals that they keep and about the infectious diseases that impact on their health and livelihoods, which results in applying and evaluating new disease control programmes or surveillance systems in partnership with animal owners. Because this approach creates collaborative communities it is both acceptable and effective in the context in which it is applied (Jost et al., 2007). In addition, because one of the key assumptions of PE is that owners report their needs, priorities and problems of the community, this makes PE more flexible to point out 'emerging information' (Jost et al., 2007).

Successful applications of PE-type activities include: (i) identification of the

pockets where rinderpest virus persisted (Jost et al., 2007; Mariner and Roeder, 2010; Vetwork UK, 2011); (ii) surveillance of several important epidemic diseases including foot and mouth disease (FMD), peste de petits ruminants (PPR) and rift valley fever (RVF) (Jost et al., 2007, 2010) as well as to (iii) the surveillance and response to highly pathogenic avian influenza (HPAI) (Jost et al., 2007; Azhar et al., 2010; Catley et al, 2012 and refs therein).

Despite nowadays being recognized as a valuable part of surveillance systems, those methods, and more in general such remote communities, require substantial attention and public investment, which can be seen as an opportunity to national economies rather than a burden or threat.

7.2 Priorities in high-income settings

In high-income resource rich European countries, a high level of intensity, degree of specialization and management control generally characterizes livestock sector, with some production systems now increasing their environmental sustainability and de-intensifying driven by welfare legislation (Compassion in World Farming, 2009). In the last few decades those societies have achieved a general reduction in the burden of livestock communicable diseases as a result of animal science (breeding, nutrition), technology developments (more effective drugs and vaccines, improvements in diagnostic technologies), high disease surveillance and food safety standards and legal acts applying to intra Union trade (Thornton, 2010; DG-Sanco, 2013). As a consequence, infections from major livestock zoonotic pathogens do not represent a major health issue anymore, and they are mainly related to occupational diseases occurring in famers, veterinarians or animal and laboratory technicians. At the same time, transboundary diseases (e.g. FMD, Newcastle disease, classical swine fever) do not normally circulate in those territories. When an outbreak occurs, those nations have the capability to react quickly, limiting the spread of the disease at huge short-term costs and to re-eliminate the pathogen (Thompson et al., 2002; Henzler et al, 2003; Velthuis et al., 2010). However, examples of long-term, widespread presence of zoonoses in certain geographical areas, such as bovine-induced tuberculosis in UK, are still grabbing the headlines and ignite public debates as for the measures applied and the resources devoted to its control in animal populations (Woolhouse & Wood, 2013).

Priorities for animal and public health in these societies are now represented by the so-called emerging and re-emerging diseases, by food safety hazards and anti-microbial resistance. Those threats include: communicable diseases occurring at the

human–animal–ecosystems interface, which lately have been happening with alarming frequency (http://www.who.int for updates), such as highly contagious diseases that have the potential of becoming pandemic (SARS, new influenza viruses); exotic diseases which incursions resulted or may result in highly damaging epidemics (bluetongue, African swine fever, West Nile); as well as the many food scares that arise from animal-source food (BSE, Escherichia coli O157:H7, Salmonella DT104).

Most of the health threats to come are unpredictable and, even though they could be potentially identified, resources are too limited to implement systems dedicated to each of them. Therefore, epidemiologists in recent years have explored new approaches in human and animal surveillance for (near) real-time collection, analysis, interpretation, and dissemination of health-related data (i.e. syndromic surveillance, early warning systems) (Guglielmetti et al, 2006; Stärk et al., 2006; Dupuy et al., 2013). Considerable investments have been made to set up initiatives of international early detection systems for infectious diseases that could integrate surveillance in both animal and human populations. Examples include Global Early Warning Response System (GLEWS) (FAO, OIE & WHO, 2011) and Global Outbreak Alert and Response Network (GOARN) (WHO, 2011), which are technical collaboration of existing international institutions and networks for the rapid identification, confirmation and response to outbreaks of international importance. Others are web-based system aiming at sharing information and resources, such as ProMED, encouraging collaboration and discussion amongst, but not only, health and biomedical professionals (International Society for Infectious Diseases, 2010). Besides, strenuous effort from the EU in trying to harmonize the current legislative framework resulted in an overarching document proposal, which addresses the new challenges under the strategy that 'prevention is better than cure' (EU Commission, 2007).

However, in this scenario, the BT experience in northern Europe, and the following emergence of the Schmallenberg virus (SBV), uncovered one of the weakest points in the current surveillance systems: the arthropod-based surveillance. In general, infectious diseases characterized by an inherent complex epidemiological cycle (e.g. vector-borne disease and zoonoses), often fall through the gaps between different sectors and competencies (i.e. medical doctors, veterinarians, entomologists, ecologists), with the risk of remaining not properly tackled. For example, while data on vector-borne zoonoses cases in humans are provided through the Community networks (Decision No. 2119/98/EC) run by the European Centre for Disease Prevention and Control (ECDC), the correspondent information from animals is not officially collected and analysed at the European level (EFSA, 2012). As well, although the most

suitable surveillance systems might be in place for detecting the disease and/or pathogen in farm-animal and humans, the current state of surveillance to cover other elements in the transmission cycle (i.e. vector, wildlife, companion or exotic animals) is suboptimal if not negligible and accompanied by a very fragmented expertise (RIVM, 2010). Most of the information are collected either (i) during a project, which might be narrow in scope and remain a paperwork exercise; or (ii) in a reactive form, generally following an outbreak and to comply with EU regulation requirements, and then stopped soon after the emergency dies. Thus, this inconsistent planning represents one of the major hurdles to overcome and it may result in discontinuity in action.

The animal and public health demands in resource rich countries will be understanding and managing widening risks. Because in the picture of emerging and re-emerging threats diseases of animal origin are anticipated to become more important, a more comprehensive surveillance approach is needed in a manner that balances the many relevant aspects of these complex present and future disease risks. Surveillance systems already in place can be adapted and serve to react to the new pathogens threats (e.g. the GD-Veekijker in the Netherlands - a free monitor help-desk -; farm animals that are routinely objectives of data collection for other agents; sentinel surveillance networks). Moreover, redirecting efforts on enhancing the under developed surveillance for vectors, wildlife, companion animals (including horses) that share habitat frequentation with people, and exotic animals would provide a key for reinforcing core capacities that are needed for all zoonotic diseases. Lastly, strengthening the collaboration, communication and data sharing between different institutions and research groups with different competencies would benefit the community entirely.

7.3 Challenges and opportunities

Effective surveillance is essential to understand pathogen epidemiology and consequently pivotal to the development of disease control programmes. Whilst there is growing consensus regarding the value of effective surveillance, it is clear that in many parts of the world there is either no ongoing surveillance for many zoonoses (see 7.1), or strong weaknesses in those systems (see 7.2).

The inherent complexity of the threats from old and new pathogens demonstrate the necessity for novel, interdisciplinary surveillance strategies that are both more comprehensive and more flexible than any that have existed previously. An integrated approach between human and animal surveillance would prevent a duplicated effort

and investment in both sectors in a time of budget constraints, whilst making use of the best available resources. At the same time, although few data exist to demonstrate the true burden of neglected endemic diseases, it is clear that good surveillance is required to tackle these disease challenges too, but the schemes must first recognize the need for effectively engender a 'culture of surveillance' and avoid the imposition of locally inappropriate surveillance tools (Soto et al., 2008).

The increasing recognition and awareness of the current priorities, has prompted a proliferation of international workshops and reports that address the existing networks and systems of infectious disease surveillance, the possibility for global surveillance in general and for zoonoses in particular (King et al., 2004; FAO et al., 2008; IOM and NRC, 2009; The World Bank, 2010; Halliday et al, 2012).

The strong evidence that emerges from those collaborative schemes is that there is ample scope for future investments in global surveillance systems to restore global health security and that this could represent a shared challenge for high- and low-income settings. To date, most investment has been directed towards predicting and mapping 'hotspots' for disease emergence, with much less attention given to endemic zoonoses for which cost-effective control tools already exist (Molyneux, 2008; Molyneux et al., 2011). However, there are two fundamental features shared by emerging and endemic zoonoses that provide a strong rationale for grouping these diseases together: 1) successful control requires both veterinary and medical inputs, and 2) emerging pathogens often have their base in neglected or endemic zoonoses areas. So, whereas most of the themes are truly global in nature, the shift towards prevention and safer practices bring the developing world into focus as priority regions. That overlap offers the opportunity to simultaneously address the need for identifying unusual disease events and to respond to them, and would reduce the burden of those diseases that still have a disproportionate impact on developing worlds.

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ADDENDUM

Samenvatting

De gezondheid van de veestapels is van belang in elke maatschappij. Dit vanwege de effecten van dierziekten op de volksgezondheid, de economische gevolgen hiervan en de gevolgen op maatschappelijke ontwikkeling. Niet te vergeten de gevolgen voor het dierenwelzijn en de (potentieel) schadelijke effecten op het milieu. Monitoring- en surveillance systemen van (veterinaire en-) volksgezondheid spelen een centrale rol in het minimaliseren, indien praktisch mogelijk, van de negatieve effecten van dierziekten op de maatschappij.

Meer en meer wordt erkend dat de van oudsher bekende zoönotische ziekten tot de meest belangrijke (veterinaire en-) volksgezondheid prioriteiten in ontwikkelingslanden behoren. Het kan niet vaak genoeg benadrukt worden dat er een fundamenteel gebrek aan ziekte prevalentie en incidentie is, en wat de gevolgen hiervan zijn. Het gebrek aan regio-specifieke epidemiologische data, heeft als gevolg dat het belang hiervan door volksgezondheid instanties wordt onderschat. Dit maakt het moeilijk om een basis te leggen voor op wetenschappelijk bewijs gefundeerde controle beleid, wat nodig is om zowel de dier- als humane gezondheid te beschermen.

In hoofdstuk 2 wordt gebruik gemaakt van reeds bestaande data uit een populatie studie waarin sera-prevalenties tegen, en distributie van Bruceilose, Leptospirose en Q-koorts in seropositieve kuddes in de Adamawa regio van Kameroen werd onderzocht. Geschat wordt dat ongeveer 20% van de kuddes seropositief zijn voor *Brucella* spp. terwijl dit voor *Leptospira* spp. en *Coxiella burnetii* geschat wordt op ongeveer 95 en 68%. Binnen de seropositieve kuddes waren de respectievelijke sera-prevalenties ongeveer 16, 35 en 68%. Daarbij zijn er statische aanwijzingen dat er clustering is van Bruceilose en Q-koorts binnen seropositieve kuddes.

In hoofdstuk 3 wordt gebruik gemaakt van een regressie analyse (generaliseerde lineaire *gemengde* model) om potentiele risicofactoren geassocieerd met de in hoofdstuk 2 genoemde pathogenen te identificeren. Met behulp van het model werden risico factoren in rundvee, in dieren in het wild, als gevolg van contact van besmet rundvee met andere kuddes, verplaatsing van kuddes, en geassocieerd met leeftijd (> 2 jaar) geïdentificeerd.

Gedurende de laatste decennia wordt de opkomst van arbovirale ziekten, ooit beperkt tot de zuidelijke gebieden van de wereld, waargenomen voorbij het geografische bereik (grenzen) van de bekende pathogeen-vectoren, voornamelijk aan de noordelijke vector

verspreidingsbereik. Het blauwtong (BT) virus en het recentelijk opkomen van BT in noordelijk Europa is een duidelijk voorbeeld hiervan. Voor transmissie van het BT virus is één van de vele species van een relatief wijdverbreid voorkomend, maar slecht gekarakteriseerd genus van insecten (i.e. *Culicoides* spp.) nodig. Daarom is ontwikkelen van basiskennis over epidemiologische parameters van de potentiele gevestigde vector populatie, van belang van het opzetten van bestrijding programma's tegen de opkomst van BT.

In hoofdstuk 4, wordt beschreven hoe de data verzameld tijdens de actieve monstername van de ziekte-vector gedurende de epidemische seizoenen van 2006-2008 Blauwtong uitbraak in Nederland werd ingezet om informatie te achterhalen betreffende aanwezigheid/afwezigheid, aanwezig hoeveelheden en seizoen gebondenheid van knutten. Bijna 80% van de gecollecteerde knutten bestond uit potentiele en bewezen vectoren voor BT, te weten; het Obsoletus Complex (*C. obsoletus* and *C. scoticus*) (44.2%), *C. dewulfi* (16.4%), *C. chiopterus* (16.3%) and *C. pulicaris* (0.1%). De helft van de meest voorkomende gevangen *Culicoides* species produceerde maar één of generaties per jaar, terwijl de BT virus vectoren wel 5-6 generaties per jaar opleverden.

In hoofdstuk 5 wordt beschreven hoe met behulp van een negative binomial hurdle model, de relatieve rol van meteorologische parameters op de hoeveelheid aanwezige vectoren gekwantificeerd kunnen worden. Het model toonde een consistente species-brede associatie, waarbij grotere vangsten van de vectoren een gevolg is van temperatuur gerelateerde variabelen en minder wind. Bovendien was er een opvallend verschil tussen de species ten gevolge van de invloed van bodem factoren, waarschijnlijk gerelateerd aan species-specifieke voorkeuren voor voortplanting habitatten.

In hoofdstuk 6 wordt een serie veldexperimenten beschreven waarmee de relatie tussen de hoeveelheelheid gevangen steekmuggen, met behulp van binnen een toezicht systeem gestandaardiseerd steekproef methode (Onderstepoort blacklight val, een val met UV licht), en het aantal tot de host aangetrokken steekmuggen. Ondanks dat de Onderstepoort val een praktische en geldige methode blijft om de aanwezige *Culicoides* populaties te bestuderen (aanwezigheid en hoeveelheden), suggereert deze studie dat het niet gebruikt kan worden als een betrouwbare methode om vector activiteit in het veld te bestuderen. Verder toont het aan dat species-specifieke en locatie-specifieke monstername variatie verder bestudeerd dient te worden.

Riassunto

L'importanza della sanità animale è legata a fenomeni di portata generale come le potenziali conseguenze negative sulla salute dell'uomo, sull'economia e lo sviluppo della società. Il marcato divario economico, nei sistemi di allevamento e nei servizi veterinari, esistente fra paesi in via di sviluppo e paesi industrializzati, determina l'impatto che le malattie animali hanno sulle società e le priorità che devono essere affrontate. Un ruolo determinante nel prevenire la diffusione delle malattie, è rappresentato dalla sanità pubblica attraverso i sistemi di sorveglianza e monitoraggio epidemiologici. Una buona sorveglianza epidemiologica fornisce la base razionale su cui impostare interventi di profilassi efficaci.

Nei paesi in via di sviluppo, le comunità sostengono un pesante fardello dovuto alle malattie infettive endemiche, molte delle quali zoonotiche. In queste aree permangono i ritardi accumulati nel tempo in merito alle conoscenze epidemiologiche e ai dati ufficiali sulla diffusione di queste patologie, ancora molto scarsi su larga scala. Ne consegue che l'importanza reale delle zoonosi non viene sufficientemente enfatizzata, riflettendosi in una generale sottostima e uno scarso interesse da parte dei media, dei politici e dei servizi sanitari nazionali ed internazionali (finanziamenti).

Nel Capitolo 2 al fine di contribuire alla conoscenza della diffusione delle zoonosi in Camerun, i dati raccolti nell'ambito di uno studio epidemiologico trasversale sulla popolazione bovina della provincia di Adamawa, sono stati analizzati per stimare la sieroprevalenza di tre zoonosi: brucellosi, leptospirosi e febbre Q. La prevalenza di allevamenti bovini sieropositivi rilevata è stata di: ~20% per *Brucella* spp., ~95% per *Leptospira* spp. e ~68% per *Coxiella burnetii*. Le sieropositività intra-allevamento riscontrate sono state rispettivamente di: ~16%, ~35% e ~39%. Nella regione, cluster di allevamenti siropositivi sono stati riscontrati per brucellosi e febbre Q.

Nel Capitolo 3, una stima del rischio relativo di sieropositività animale nei confronti degli stessi agenti zoonosici, nelle categorie dei diversi fattori di rischio, è stata calcolata usando un modello finale parsimonioso di regressione logistica multipla ad effetti misti. I fattori di rischio individuati sono stati: contatti con la fauna selvatica per la sieropositività nei confronti di *Brucella* spp. e *Coxiella burnetii*; l'uso di aree pascolative presenti lungo gli itinerari utilizzati per la transumanza e l'interazione con altre mandrie al pascolo per *Leptospira* spp.; il sesso (femmine) e la pratica della transumanza per *Coxiella burnetii*; l'età (> 2 anni) per tutti e tre i patogeni.

Negli ultimi decenni, alcune tra le malattie virali trasmesse da insetti (arbovirosi) storicamente segnalate principalmente nelle regioni tropicali e subtropicali, si sono diffuse ben oltre i limiti geografici conosciuti. Un esempio è rappresentato dalla bluetongue (BT), che recentemente ha espanso il suo areale di diffusione nel nord Europa. Un ruolo centrale nell'epidemiologia della bluetongue è rappresentato da una o più specie di un genere relativamente diffuso, ma poco caratterizzato, di ditteri ematofagi di piccole dimensioni (i.e. *Culicoides* spp.). Pertanto nella pianificazione di strategie di controllo e prevenzione, importanza fondamentale riveste lo studio delle preferenze ambientali e della fenologia di questi insetti.

Nel Capitolo 4, i dati prodotti nell'ambito dalla sorveglianza entomologica effettuata durante le stagioni epidemiche di bluetongue 2006-2008 in Olanda, sono stati analizzati per determinare la presenza/assenza, l'abbondanza e l'attività stagionale delle specie di Culicoides locali. Specie considerate vettori reali e potenziali del virus della bluetongue hanno rappresentato circa l'80% dei culicoidi catturati: Obsoletus Complex (che comprende *C. obsoletus* e *C. scoticus*) (44.2%), *C. dewulfi* (16.4%), *C. chiopterus* (16.3%) e *C. pulicaris ss* (0.1%). Circa la metà delle 24 specie più comuni catturate, hanno mostrato di completare una o due generazioni per anno, contro i più elevati tassi riproduttivi delle specie vettore (5-6 generazioni/anno).

Nel Capitolo 5, l'influenza di fattori ambientali e pedologici sulla abbondanza stagionale e diffusione dei principali vettori del virus della bluetongue, sono stati quantificati usando modelli di regressione per dati sovradispersi e con eccessiva presenza di zeri (negative binomial zero-augmented o hurdle models). Le catture più abbondanti sono risultate positivamente influenzate dalle variabili come temperatura ambientale e basse velocità dei venti. La diversa natura dei suoli e la circolazione idrica associate all'abbondanza di specie vettore, indicherebbero una influenza nel promuovere habitat larvali/siti riproduttivi.

Nel Capitolo 6, i dati raccolti durante una serie di esperimenti eseguiti nella stagione 2010 di attività dei Culicoides, sono stati utlizzati per studiare la relazione esistente fra monitoraggio mediante ovitrappole (numero di insetti per cattura) ed il numero di insetti catturati direttamente sugli animali. I risultati hanno suggerito che, sebbene la OVI light trap rimanga uno strumento fondamentale per lo studio della distribuzione e delle dinamiche stagionali dei vettori della bluetongue, le catture effettuate con ovitrappole non riflettono la reale attività ematofaga degli stessi, e pertanto non possono essere considerate affidabili nel definire una soglia di rischio epidemiologico.

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About the Author

Francesca Scolamacchia was born in Bari, on the 4th of October 1976. She qualified as a veterinary surgeon from the University of Bari in 2004, with a thesis on the epidemiology of tick-borne diseases of cattle in southern Italy.

In 2005, Francesca moved to Rome to collaborate with FAO, before joining one of the ten Italian veterinary public health institutes (Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana), where she worked as a consultant on various aspects of prevention and control of animal infectious diseases. In 2006, Francesca worked as a research fellow at Istituto Superiore di Sanità, participating in a project on the susceptibility of sheep to classical and atypical scrapie.

In 2007, she was awarded a DEFRA founded VTRI (Veterinary Training and Research Initiative) scholarship in Quantitative Epidemiology and she moved to Edinburgh, UK. As part of her scholarship she completed a Master of Science by Research in Infectious Diseases at The University of Edinburgh.

After graduation, in 2009, Francesca moved to The Netherlands to start a PhD in Epidemiology on a joint project between the University of Utrecht and the Central Veterinary Institute of WUR. During those years, she also completed the Postgraduate Specialization Diploma in Infectious Diseases and Preventive Veterinary Medicine at the University of Bari.

Francesca's research and applied interests are in disease surveillance, decision support systems, biology and control of (re-)emerging diseases and influence of the physical environment on pathogen transmission.