Title:

The combination of abundance and infection rates of *Culicoides sonorensis* estimates risk of subsequent bluetongue virus infection of sentinel cattle on California dairy farms

Christie E. Mayo, [1] Christopher M. Barker, [4] Bradley A. Mullens, [3] Alec C. Gerry, [3] Peter P. C. Mertens, [6] Sushila Maan, [6] Narender Maan, [6] Ian A. Gardner, [2] Alan J. Guthrie, [5] N. James MacLachlan[1]

[1] *Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, California, 95616*

[2] *Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, California, 95616*

[3] *Department of Entomology, University of California, Riverside, California, 92521*

[4] *Center for Vectorborne Diseases, University of California, Davis, California, 95616*

[5] *Equine Research Centre, University of Pretoria, Onderstepoort, South Africa, 0028*

[6] *Vector-borne Diseases Programme, Institute for Animal Health, Ash Road, Pirbright, Working, Surrey, GU24 ONF, UK*

*Corresponding author:

Dr. N. James MacLachlan

Department of Pathology, Microbiology and Immunology

University of California

One Shields Avenue

Davis, CA 95626

e-mail: njmaclachlan@ucdavis.edu

Abstract

Bluetongue is an important viral disease of ruminants that is transmitted by hematophagous *Culicoides* midges. We examined the seasonal patterns of abundance and infection of *Culicoides sonorensis* (*C. sonorensis*) at 4 dairy farms in the northern Central Valley of California to develop estimates of risk for BTV transmission to sentinel cattle at each farm. These 4 farms were selected because of their similar meteorological conditions but varying levels of vector abundance and BTV infection of cattle (Mayo et al., 2011). *C. sonorensis* midges were collected weekly at each farm during the seasonal transmission period, using three different trapping methods: traps baited with either carbon dioxide (CO_2) alone or traps with CO_2 and UV light, and by direct aspiration of midges from sentinel cattle. Analysis of BTV-infected midges using group and serotype-specific quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assays confirmed that BTV serotypes 10, 11, 13 and 17 are all present in the region, but that midge infection rates and the number of BTV serotypes circulating differed markedly among the individual farms. Furthermore, more serotypes of BTV were present in midges than in sentinel cattle at individual farms where BTV circulated, and the virus was detected at each farm in midges prior to detection in cattle. BTV infection rates were remarkably lower amongst female *C. sonorensis* midges collected by CO_2 traps with UV light than among midges collected by either animal-baited aspirations or in CO_2 traps without light. A subsample of female midges examined from each collection method showed no overall differences in the proportion of female midges that had previously fed on a host. Findings from this study confirm the importance of using sensitive

surveillance methods for both midge collection and virus detection in epidemiological studies of BTV infection, which is especially critical if the data are to be used for development of mathematical models to predict the occurrence of BTV infection of livestock.

Key words: *Culicoides*, bluetongue virus, vector index

1. Introduction

Bluetongue virus (BTV) is the causative agent of bluetongue (BT), a re-emerging arboviral disease of ruminants that is transmitted by various species of *Culicoides* midges (Spreull, 1905; Verwoerd and Erasmus, 2004; MacLachlan et al., 2009; MacLachlan and Guthrie, 2010). The global distribution of BTV infection coincides with that of competent *Culicoides* vectors and the appropriate environmental ecosystems that support them (Conte et al., 2007; Gibbs and Greiner, 1994; Tabachnick, 2004). Since 1998, multiple serotypes of BTV have invaded and spread throughout extensive portions of Europe, Scandinavia, and the Mediterranean Basin, precipitating an economically devastating epidemic (Gomez-Trejedor, 2004; Gloster et al., 2008; Saegerman et al., 2008). Coincident with this invasion of BTV into Europe, 10 previously exotic serotypes (serotypes 1,3,5,6,9,12,14,19,22,24) have been isolated in the southeastern United States (US) (Johnson, 2007; Ostlund, 2010). Climate change has been implicated as the cause of this dramatic change in the global distribution and nature of BTV infection of livestock, because of its potential impact on the activity, abundance, and vectorial capacity of populations of *Culicoides* insects resident in affected areas (Purse et al., 2005; Purse et al., 2006; Guis et al., 2011).

Culicoides sonorensis (C. sonorensis) is the predominant if not exclusive vector of BTV serotypes 10, 11, 13 and 17 to cattle throughout much of the US, limited by the northern distribution of this species (the so-called “Sonorensis Line”) that extends from approximately Washington State in the west to Maryland in the east (Gibbs and Greiner, 1994; Tabachnick, 2004; Lysyk, 2006; Schmidtmann et al., 2011). In addition to *C. sonorensis*, some other species of *Culicoides* midges are suspected but their contribution,

if any, to the transmission of BTV remains uncertain (Mullen and Durden, 2009). The abundance of vector *Culicoides* midges at specific sites has been traditionally assessed utilizing traps artificially baited with CO_2, light of appropriate wavelength, semiochemicals, or a combination thereof (Du Toit, 1944; Barnard, 1980; Mullens, 1985; Meiswinkel et al., 2007). While collection of insects by these trap methods is relatively cost-effective and convenient, the insect activity and infection prevalence determined using these trap methods may be poorly correlated to the biting rates and infection prevalence of truly host-seeking insects (Gerry et al., 2001; Gerry et al., 2009). These parameters are critical for accurately assessing and modeling the dynamics of virus transmission to livestock (Gerry et al., 2001; Carpenter et al., 2008; Baylis, 2009; Guis et al., 2011). In contrast, enclosure trapping or mechanical aspiration of *Culicoides* midges directly from "bait' livestock has shown biting rates that can be several fold greater than the capture rate obtained using the conventional CO_2 baited suction trap, perhaps because mammalian - feeding *Culicoides* midges also respond to stimuli other than CO_2 (Mullens and Gerry, 1998; Gerry et al., 2009; Viennet et al., 2011).

Given recent changes in the global distribution of BTV infection, we recently initiated an epidemiological study of BTV infection in California, a region where the virus has long been endemic (McKercher, 1953; Metcalf et al., 1981; Osburn et al., 1981; Stott et al., 1985; Uhaa et al., 1990). Results of our studies to date have shown a high incidence of BTV infection of cattle on individual dairy farms that is associated with specific meteorological and anthropogenic factors that promote large populations of *C. sonorensis* midges (Mayo et al., 2010; Mayo et al., 2011). Thus, the objective of the current study was to evaluate the interaction of population dynamics of *C. sonorensis*

midges with the seasonal occurrence of BTV infection of cattle at individual dairy farms in the northern Central Valley of California. Specifically, we determined the seasonal patterns of abundance and infection rates of vector *C. sonorensis* midges at each farm, as estimated using different insect trapping methods. Further, we determined the serotypes of BTV present at each farm, and compared the seasonal occurrence of infection in midges and sentinel cattle. The accurate determination of seasonal midge abundance and host biting rates, coupled with determination of BTV infection rates among both *C. sonorensis* and sentinel cattle using a sensitive quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) assay, allowed us to examine vector indices for prediction of seasonal BTV infection of livestock at each farm.

2. Materials and Methods

2.1. Study design

A longitudinal study of the epidemiology of BTV infection in the northern Central Valley of California began in May, 2010, with the enrollment of 4 dairy farms in the region, as previously described (Mayo et al., 2011). These 4 farms were selected because of their similar seasonal meteorological parameters, but very different prevalence of BTV infection of cattle resident at individual farms. *Culicoides* midges were captured weekly on each farm from May-December 2010, and at monthly thereafter until May 2011, using three trapping methods as previously described (Mayo et al., 2011): CDC downdraft suction traps baited with CO_2 (dry ice) and equipped with or without a 4W blacklight (UV) bulb (All Weather EVS Trap; BioQuip Products, Rancho Dominguez, CA). Insects were also collected by mechanical aspiration directly from five adult dairy cows, using a

modified hand-held household vacuum (DC Insect Vac; BioQuip Products, Rancho Dominguez, CA).

Twelve CDC traps (6 with UV and 6 without UV) were placed at each farm near dairy waste-water lagoons and watering troughs, and within open pasture sites. These traps were deployed 2 hours before sunset and removed 2 hours after sunrise, and the positions of the different types of suction traps were alternated each week to correct for position effects, essentially as previously described (Gerry et al., 2001; Gerry et al., 2009; Mayo et al., 2011). The *Culicoides* midges captured were identified on the basis of their morphological characteristics and wing patterns using a stereomicroscope (Wirth et al., 1985).

Five adult cows on each farm were used as sentinels to monitor occurrence of BTV infection. Sentinel cattle were the same animals from which *Culicoides* were collected by direct aspiration. Sentinel animals were seronegative and virus-free at the beginning of the study. Serum and whole blood were collected weekly from each animal to assay for BTV-specific antibodies and viral nucleic acid, using group-reactive competitive enzyme-linked immunosorbent assay (cELISA; VMRD Inc., Pullman, WA) and RT-qPCR, as previously described (Ortega et al., 2010; Mayo et al., 2010; Worwa et al., 2010; Mayo et al., 2011).

2.2. Detection of BTV infection of Culicoides midges

The harvested *Culicoides* midges were sorted by sex, pooled into groups of 20 midges from the same collection date and collection method, and stored at -80°C. Pools containing female midges were macerated in lysis binding buffer (LBS) (AM8500, Ambion®) with a lysing matrix comprised of (10) 2 mm zirconia beads and (4) 4mm

ceramic beads. Total RNA was extracted from the homogenate using the Microarray Total RNA kit (AM1836, Ambion®) according to the manufacturer's recommendations. A maximum of 10 midge pools from each collection method and date were evaluated from the total midge pools that were harvested at each site. The presence of BTV RNA in each midge pool was detected using the same RT-qPCR assay used for detection of BTV in cattle blood. Optimal midge pool size was determined by dilution analysis of pools of insects infected in the laboratory by oral feeding of BTV serotype 10 and held a maximum of 7 days at 27°C (data not shown). Negative (no template) and positive control samples were included in each RT-qPCR run. Infection rates per 1,000 midges were calculated as bias-corrected maximum likelihood estimates (MLEs) using the Excel Add-In PooledInfRate, version 3.0 (Biggerstaff, 2006).

2.3. Determination of parity among Culicoides midges

To examine potential differences in the parity of midges collected by the 3 trapping methods, a subsample of 50 female midges from pooled collections representing collections of each trap method over five consecutive collection dates (during October, 2010) at one study site (farm A) were removed from storage at -80°C and rehydrated for microscopic examination. Midges were rehydrated following general methods developed for rehydration of mosquitoes (Ungureanu, 1972). Briefly, midges were placed in a rehydration solution of 1 part dish soap to 4 parts deionized water overnight (approximately 10 hours) followed by a rinse with and then storage in physiological saline (150nM NaCl). Following rehydration, parity was determined by the presence of pigment deposited in the abdominal cuticle (Dyce, 1969; Akey and Potter, 1979) and by changes in the pigmentation pattern of the abdominal tergites (Potter and Akey, 1978).

2.4. Determination of BTV serotypes infecting Culicoides midges and sentinel cattle

The serotype of BTV present in each cattle blood sample and insect pool that was positive by BTV group-specific RT-qPCR assay was determined using BTV-serotype-specific RT-qPCR assays (Mertens et al., unpublished). Specifically, blood and insect pools with a Ct value <30 were further evaluated using individual RT-qPCR assays specific for BTV serotypes 10, 11, 13 and 17, which are the only BTV serotypes previously identified in California (Barber et al., 1979; McKercher et al., 1953; Metcalf et al., 1981; Osburn et al., 1981; Stott et al., 1985, Uhaa et al., 1990; Walton, 2004; Mayo et al., 2011).

2.4. Statistical Analysis

Prevalence and 95% confidence intervals were used to characterize midge abundance and BTV infection prevalence of both sentinel cattle and *Culicoides* midges collected at each of the 4 dairy farms. Vector index (mean number of midges captured per trap night x proportion of BTV-infected midges captured on that trap night) was utilized as a standardized measure of BTV transmission potential (Bolling et al., 2009). Infection rates per 1,000 individuals were calculated as bias-corrected maximum likelihood estimates (MLEs) by using the Excel Add-In PooledInfRate, version 3.0 (Biggerstaff 2006). Differences in *Culicoides* midge abundance (mean number of *C. sonorensis* females collected per trap night) at each trapping site location on individual farms were determined by one-way analysis of variance (ANOVA). Means were separated by Tukey post-hoc multiple comparison tests. A chi-square analysis of parous and nulliparous female midges was used to compare by trapping method. For all statistical analyses, $p < 0.05$ was considered significant.

3. Results

3.1. Seasonal patterns of entomological risk measures in relation to occurrence of BTV infection of cattle

Three entomological risk measures were evaluated on each dairy farm to assess their individual relationships to the seasonal pattern of BTV prevalence in sentinel cattle at the same farm, specifically: 1. Vector abundance (mean number of female *C. sonorensis* midges collected per trap night); 2. BTV infection rate per 1,000 female *C. sonorensis* midges; and, 3. Vector index, which reflects the relative abundance of BTV-infected female *C. sonorensis* midges (Figure 1). Abundance of *C. sonorensis* captured by all trapping methods peaked in August on the 3 farms where significant numbers of these midges were collected (farms A, C, D) and declined rapidly thereafter. BTV infection was detected on the first and second weeks of July on farms A and B respectively. BTV infection rates in female midges increased gradually throughout the summer to peak between the last week of September (farm A) and first week of October (farms C & D). The relative abundance of female *Culicoides* midges was much lower at farm B than that at farms A, C, or D, and BTV infection of cattle was not detected at this farm. The vector index peaked between mid and late September. The mean time lag from the onset of increased seasonal abundance of *C. sonorensis* to the index case of a RT-qPCR positive cow (pooled among farms) was 10.5 weeks whereas the mean time lag for the vector index for the same parameters was 4.5 weeks.

3.2. Trap type influences estimates of seasonal infection rates of Culicoides sonorensis midges

When examined across all farms combined, mean BTV infection rates of female *C. sonorensis* midges collected on the 3 farms (farms A, C, D) where significant numbers of midges were collected peaked in October for all trap types (animal- baited aspirations, CO_2 traps without light, and CO_2 traps with UV light) (Figure 2). When combined across the entire season, BTV infection prevalence of female midges was greatest in midges collected by aspiration directly from cattle relative to the other midge capture methods. Notably, infection in midges captured by either direct aspiration or by CO_2 trap without light did not differ statistically, but both collection methods showed a higher infection prevalence ($p<0.05$) than that detected in midges collected by CO_2 traps equipped with UV light (Figure 2).

Given the obvious difference in BTV infection rates amongst midges harvested by each of the 3 trapping methods and the assumption that midges must feed on an animal host to acquire infection with BTV, the proportion of captured female midges that had previously fed on a host (parous) relative to those that had not (nulliparous) was comparedon the farm with the highest midge abundance and infection prevalence (Farm A). Specifically, the parity of female midges collected at farm A on 5 sequential trapping nights was compared during the peak of BTV infection prevalence (October, 2010). Between 240 and 250 females per trap type were compared. Presence of the burgundy-red parous pigment (Mullens and Schmidtmann, 1982) was noted in a blinded fashion by an experienced scorer (ACG) for females from each of the 3 trapping methods. Data from this preliminary analysis indicate that parity of female midges was not significantly different regardless of trapping method. For farm A, percent parity was characterized as:

1. Aspiration from bait cattle-35.6 ± 0.010; 2. CO_2 suction trap- 39.1 ± 0.18; and 3. CO_2 plus UV light trap 33.1 ± 0.18 (p=0.84).

3.3. BTV serotype diversity among farms, livestock, and vector

Only BTV serotypes 10, 11, 13 and 17 were identified in the present study, consistent with historical data from the region (Osburn et al., 1981; Stott et al., 1985). Furthermore, all blood and insect samples with Ct values < 30 as determined with the group RT-qPCR assay were also positive with one of the serotype-specific RT-qPCR assays further confirming the absence of other BTV serotypes. However, there was substantial variation in the serotypes present in vector insects and sentinel cattle at individual farms (Figure 3). Serotype diversity was most apparent at farms A and D, where both vector abundance and prevalence of BTV infection of cattle were markedly higher as compared to farms B and C (Mayo et al., 2011). BTV was detected in midges collected at farm C, but not in sentinel cattle at this site. Furthermore, more BTV serotypes were identified in midges than cattle at both farms A and C, and the virus was detected in midges at each farm before it was detected in sentinel cattle. BTV was detected in neither midges nor cattle at farm B, indeed only small numbers of *Culicoides* midges were collected at this site by any trapping method.

4. Discussion

Findings from this study showed that combining estimates of vector abundance and BTV infection rates of female *C. sonorensis* midges at individual dairy farms in the northern Central Valley of California provides a meaningful risk index (the vector index) for subsequent BTV infection of cattle at each site. Adding vector infection level did not substantially alter the significant relationship of biting rate to the assessment of BTV risk

in an earlier study (Gerry et al., 2001), but the virus detection methods used in the current study were considerably more sensitive than the capture ELISA used previously. Transmission most likely occurred on farms A and D but not farms B or C, due higher vector biting rates on farms A and D. Previous studies have established that biting rates above approximately 60 per night resulted in greater transmission (Gerry et al., 2001). The seasonal abundance of *C. sonorensis* at these farms peaked in August, which was considerably earlier than the peak BTV infection rates of midges in late September/early October. The vector index combines the time - sensitivity provided by monitoring midge abundance and activity with the specificity of virus activity determined by midge infection prevalence, both of which are key components of the vectorial capacity equation (Gerry et al., 2001; Bolling et al., 2009).

The seasonal trends of vector abundance, *C. sonorensis* infection rates, and infection rates of sentinel cattle identified in this study are consistent with results of previous studies (Gerry et al., 2001; Stott et al., 1985). However, measuring these values on several farms where cattle population density and meteorological conditions were similar allowed us to assess the impact of between-farm differences in vector abundance, midge infection prevalence, and vector index, on the prevalence of BTV infection of cattle at each farm. All of these indices contribute to the vectorial capacity equation but virus first must be first introduced with adequate vector-host contact (determined by the biting rate) necessary for ongoing virus transmission. Notably in the current study, BTV infection did not occur in sentinel cattle at farm C despite the presence of BTV-infected female *C. sonorensis* midges. Furthermore, prior serological surveys confirm the absence of BTV infection of adult dairy cattle at this farm (Mayo et al., 2011). This farm also had

a lower vector index than that at both farms A or D where transmission of BTV from midges to cattle occurred frequently, and where seroprevalence is high amongst adult cattle (Mayo et al., 2011).

The midge trapping method and type strongly influenced the estimated BTV infection rate of female *C. sonorensis* midges. Whereas CO_2 traps with UV light captured the greatest number of *C. sonorensis* midges (both males and female) relative to the other trap methods (Mayo et al., 2011), the BTV infection prevalence of female midges captured by these traps was markedly lower than for midges collected by animal-baited aspirations or CO_2 traps without light. Although no difference was identified in overall parity for midges captured by each of the three trap methods during the period of peak *Culicoides* activity, variation in midge infection rate among the trap methods used in this study might also potentially reflect altered behavior between BTV-infected and uninfected midges. Further investigation of these differences is necessary and planned. Because UV light also is the most common attractant used in surveys of *Culicoides spp.*, further evaluation of transmission risk (due to artificially low infection in UV-CO_2 traps relative to hosts) requires further study. Clearly, selection of the trapping method that most accurately determines infection rates of vector *C. sonorensis* midges is central to meaningful prediction of risk of BTV transmission to livestock (Baylis et al., 2009; Guis et al., 2011).

The plurality of BTV serotypes complicates the accurate prediction of risk of BTV transmission to livestock. More virus serotypes were identified in midges than in sentinel cattle at farms A and D where multiple BTV serotypes were circulating, and infection was detected in midges before it was detected in sentinel cattle at each farm.

This might suggest that the midge vector constitutes a reservoir of genetically divergent BTVs that potentially sustain the virus in seasonally endemic areas, essentially as previously proposed (Nevill, 1971). Local selective pressures imposed on the viral population present in each area leads to the emergence over time of region-specific virus genotypes (topotypes), as previously described (Bonneau et al., 2001; Balasuriya et al., 2008). The simultaneous circulation of multiple BTV serotypes at farms with the highest prevalence of BTV infection of midges and cattle might further suggest a genetic "stabilization" process, consistent with the fact that BTV has only become endemic in regions where more than one serotype circulates (MacLachlan, 2010).

5. Conclusion

Data from the current studies confirm the importance of site selection and the use of the most accurate methods for both midge collection and virus detection in epidemiological investigations of BTV infection. Further studies are needed to understand the differences in infection prevalence of female *Culicoides* midges by trap type; however the data does suggest infection prevalence may be misrepresented when female *Culicoides* midges are collected by UV light traps alone, a method most often used in routine vector surveillance. Acquisition of accurate and relevant data is clearly prerequisite to the development of meaningful modeling approaches for risk prediction of BTV transmission among livestock.

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Fig. 1. Seasonal patterns of abundance and BTV infection rates of female C. sonorensis midges collected per trap night, vector index (10×), and BTV infection of sentinel cattle on 4 dairy farms (A–D) in the northern Central Valley of California. Vector index represents the number of infected female C. sonorensis midges (mean number of female C. sonorensis midges captured per trap night × mean proportion of BTV-infected female C. sonorensis midges per trap night). Note that seasonal BTV infection of cattle coincides most closely with increased vector indices, as compared to vector infection rate or abundance alone.

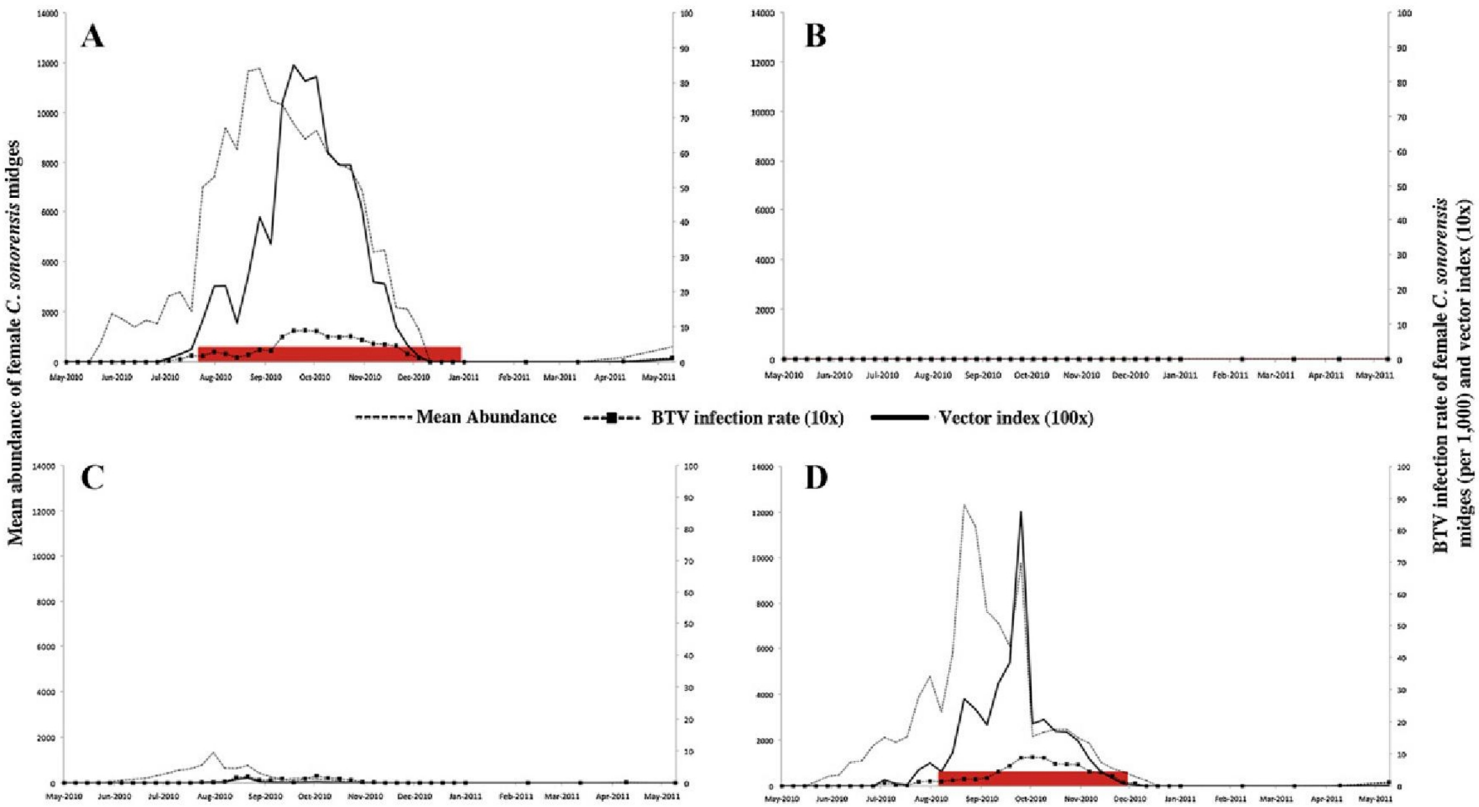

Fig. 2. Seasonal patterns of mean BTV infection rates of female C. sonorensis midges collected by three trapping methods on 3 dairy farms (A, C, and D) where significant numbers of midges were collected: animal baited aspirations; CO2 traps without light; and CO2 traps with UV light. Error bars represent 95% confidence intervals; bias-corrected maximum likelihood estimates (MLEs) were calculated with the Excel Add-In PooledInfRate, version 3.0 (Biggerstaff, 2006). Pink-shaded region is representative of temporal period when sentinel cattle were positive for BTV infection.

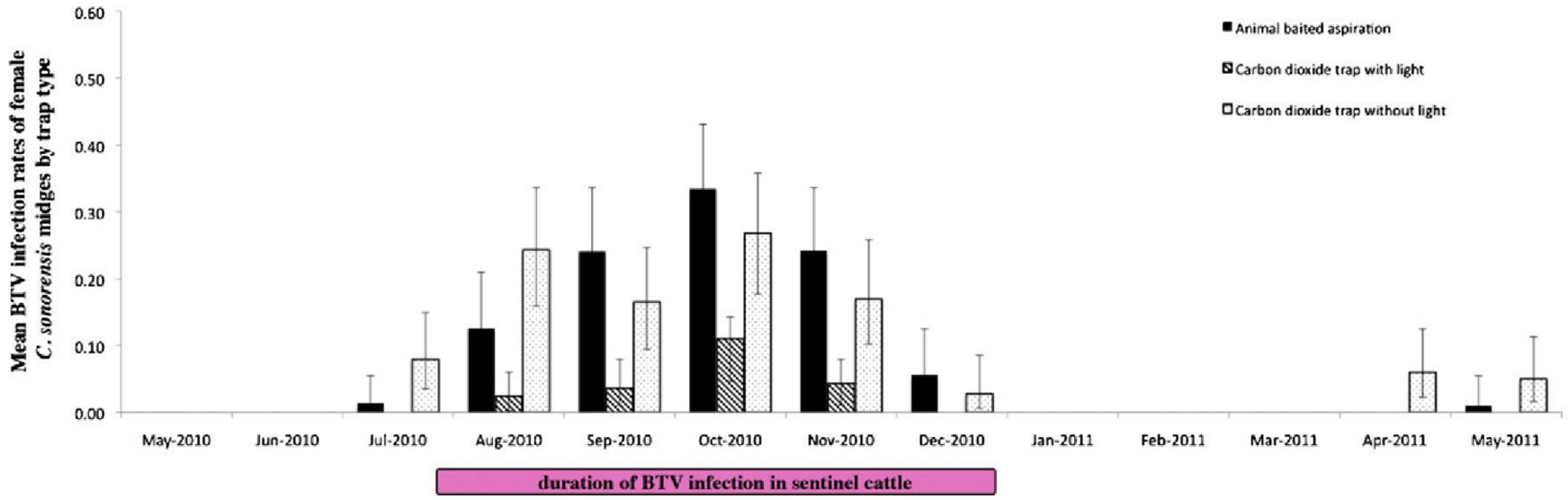

Fig. 3. Prevalence of individual BTV serotypes among adult cattle and female C. sonorensis midges at 4 dairy farms by location in the northern Central Valley of California. Colors indicate individual BTV serotypes, and shaded portions indicate the proportion of the total virus pool in either vector midges or cattle represented by each serotype.