UC Riverside
UC Riverside Electronic Theses and Dissertations

Title
Investigations into the Trans-Seasonal Persistence of Culicoides sonorensis and Bluetongue Virus

Permalink
https://escholarship.org/uc/item/46x392n2

Author
McDermott, Emily Gray

Publication Date
2016

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA
RIVERSIDE

Investigations into the Trans-Seasonal Persistence of *Culicoides sonorensis* and Bluetongue Virus

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Entomology

by

Emily Gray McDermott

December 2016

Dissertation Committee:
Dr. Bradley Mullens, Chairperson
Dr. Alec Gerry
Dr. Ilhem Messaoudi

Copyright by
Emily Gray McDermott
2016

The Dissertation of Emily Gray McDermott is approved:

____

____

____

Committee Chairperson

University of California, Riverside

ACKNOWLEDGEMENTS

I of course would like to thank Dr. Bradley Mullens, for his invaluable help and support during my PhD. I am incredibly lucky to have had such a dedicated and supportive mentor. I am truly a better scientist because of him.

I would also like to thank my dissertation committee. Drs. Alec Gerry and Ilhem Messaoudi provided excellent feedback during discussions of my research and went above and beyond in their roles as mentors.

Dr. Christie Mayo made so much of this work possible, and her incredible work ethic and enthusiasm have been an inspiration to me.

Dr. Michael Rust provided me access to his laboratory and microbalance for the work in Chapter 1.

My co-authors, Dr. N. James MacLachlan and Mr. Damien Laudier, contributed their skills, guidance, and expertise to the virological and histological work, and Dr. Matt Daugherty gave guidance and advice on the statistics in Chapter 4. All molecular work was completed in Dr. MacLachlan's laboratory at the University of California, Davis. Jessica Zuccaire and Erin Reilly helped enormously in counting and sorting thousands of midges.

The Mullens lab helped me keep my colony alive and my experiments going on more than one occasion. Thank you to Dr. Amy Murillo, Natalie Wong, Fallon Fowler, and Diane Soto for making this a great place to do a PhD.

Several dairymen in both California and Colorado generously allowed me onto their property on numerous occasions to collect insects and mud. Without them, none of

this work, but especially Chapters 2, 3, and 4 would have been possible. I hope that someday this work, and my future work, can be translated into practical applications that will help protect their animals and livelihoods.

I didn't get here on my own, and I didn't finish on my own. I'd like to thank my parents who told me they were proud of me when I questioned what I was doing; my cohort who came over to watch football games they had no interest in on many Saturdays and Sundays; Nick, who loved and supported me even when science made me crazy; and Micky, who was my very first friend in California.

The work in this dissertation was supported in part by USDA NIFA Predoctoral Fellowship Award No. 2016-67011-24674.

Chapter 1 was previously published as McDermott, E.G., and B.A. Mullens. 2014. Desiccation tolerance in the eggs of the primary North American bluetongue virus vector, *Culicoides sonorensis* (Diptera: Ceratopogonidae), and implications for vector persistence. Journal of Medical Entomology. 51: 1151-1158.

Chapter 2 has been submitted for publication in the Journal of Medical Entomology as McDermott, E.G., C.E. Mayo, and B.A. Mullens. Low temperature tolerance of *Culicoides sonorensis* (Diptera: Ceratopogonidae) eggs, larvae, and pupae from temperate and subtropical climates.

Chapter 3 was previously published as McDermott, E.G., C.E. Mayo, A.C. Gerry, D. Laudier, N.J. MacLachlan, and B.A. Mullens. 2015. Bluetongue infection creates light averse *Culicoides* vectors and serious errors in transmission risk estimates. Parasites & Vectors. 8: doi: 10.1186/s13071-015-1062-4.

Chapter 4 was previously published as McDermott, E.G., C.E. Mayo, A.C. Gerry, and B.A. Mullens. 2016. Trap placement and attractant choice affect capture and create sex and parity biases in collections of the biting midge, *Culicoides sonorensis*. Medical and Veterinary Entomology. 30: 293-300.

DEDICATION

To my mom- Who always made sure I got enough science

&

To Nick- Who followed me west

ABSTRACT OF THE DISSERTATION

Investigations into the Trans-Seasonal
Persistence of *Culicoides sonorensis*
and Bluetongue Virus

by

Emily Gray McDermott

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, December 2016
Dr. Bradley Mullens, Chairperson

Culicoides biting midges are the primary vectors of some of the most economically damaging pathogens of livestock worldwide, including bluetongue virus (BTV). Bluetongue disease affects mainly domesticated ruminant livestock, especially cattle and sheep. Morbidity and mortality can be very high in susceptible animals. In the United States, production losses and trade restrictions make BTV an economically important pathogen for the cattle industry. Both BTV and the primary North American vector species, *C. sonorensis* Wirth and Jones are endemic to California. Although temperatures in southern California are theoretically high enough to allow year-round transmission, adult vector activity and cattle seroconversions cease or exist at very low levels during the winter. *Culicoides* overwintering has been of considerable interest since BTV expanded into northern Europe in 2006, successfully overwintering in a temperate climate and causing consecutive epizootics that resulted losses of millions of animals and billions of dollars.

Little is known about the immature stages of *Culicoides*. I examined the desiccation tolerance of *C. sonorensis* eggs, and the low temperature tolerance of eggs, larvae, and pupae. Although previous literature had suggested that *Culicoides* eggs would be susceptible to environmental stress, I found that *C. sonorensis* eggs can withstand nearly complete desiccation and exposure to temperatures as low as -20°C without suffering complete mortality. Conversely, larvae succumbed to temperatures less than -4°C. *Culicoides* eggs are likely to be more important in seasonal persistence of vector populations than previously thought.

Our understanding of adult vector activity stems from trap collection data. However, trapping strategies can bias insect collections. I collected *C. sonorensis* on dairies in California using traps baited with either CO_2, UV light, or CO_2+UV, and placed either near cattle, oviposition sites, or in open fields. I found that traps placed in fields were more efficient at collecting adult midges. I also found that traps baited with UV collected insects with significantly lower BTV infection rates than traps baited with only CO_2, suggesting that BTV+ midges are repelled by UV light. The use of UV traps runs the risk of missing active, infected vectors during periods of low activity, like winter, potentially resulting in a misunderstanding of true seasonal disease dynamics.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....iv

LIST OF TABLES.....xii

LIST OF FIGURES.....xiv

INTRODUCTION.....1

REFERENCES.....14

Desiccation tolerance in the eggs of the primary North American bluetongue virus vector, *Culicoides sonorensis*, and implications for vector persistence.....27

ABSTRACT.....28

INTRODUCTION.....28

MATERIALS AND METHODS.....30

RESULTS.....35

DISCUSSION.....39

REFERENCES.....46

TABLES AND FIGURES.....52

Low temperature tolerance of *Culicoides sonorensis* eggs, larvae, and pupae from temperate and subtropical climates.....58

ABSTRACT.....59

INTRODUCTION.....60

MATERIALS AND METHODS.....62

RESULTS.....69

DISCUSSION..73

REFERENCES..82

TABLES AND FIGURES..89

Bluetongue virus infection creates light averse *Culicoides* vectors and serious errors in transmission risk estimates..95

ABSTRACT..96

INTRODUCTION..97

MATERIALS AND METHODS..99

RESULTS..104

DISCUSSION..106

REFERENCES..112

TABLES AND FIGURES..118

Trap placement and attractant choice affect capture and create sex and parity biases in collections of the biting midge, *Culicoides sonorensis*................................124

ABSTRACT..125

INTRODUCTION..125

MATERIALS AND METHODS..127

RESULTS..129

DISCUSSION..132

REFERENCES..139

TABLES AND FIGURES..146

CONCLUSION..152

LIST OF TABLES

Table **Page**

Table 1.1 Mean hatch rate (± SD) and water loss (± SD) of 4-10 h old eggs desiccated for 12-48 h in trials 1 and 2..**52**

Table 1.2 Mean hatch rate (± SD) and water loss (± SD) of 28-34 h old eggs desiccated for 48-60 h. Trials 1 and 2 are pooled..**52**

Table 1.3 Mean hatch rate (± SD) and water loss (± SD) of 25-40 h old eggs desiccated for 12 h in trials 1 and 2..**53**

Table 2.1 Probit model analysis for *C. sonorensis* eggs, pupae, and larvae subjected to subzero temperatures..**89**

Table 2.2 Supercooling points (°C±SE) of different populations of *C. sonorensis* larvae and pupae held at either 27°C or 4°C, n=number of individuals tested. Letters represent significant differences (p< 0.05) between all groups tested...**89**

Table 2.3 Predicted lower lethal temperatures of 50, 90, and 99% of *C. sonorensis* colony eggs, pupae, and larvae after a 1 h exposure, from probit analysis. 95% confidence intervals are reported in parentheses...**89**

Table 2.4 Mean percentage initial and final (through the adult stage) mortality (±SE) of field-collected CA larvae after 1 h exposure at a given temperature, n=number of 25 larva replicates..**90**

Table 3.1 Effects of location, trap and location*trap on infection rate. ANOVA table of final model used for infection rate analysis..**118**

Table 3.2 X^2 Analysis of positive vs. negative pools by trap. Within trap type, the first row of the table shows the observed values and the second row shows the expected values..**118**

Table 3.3 X^2 Analysis of positive vs. negative pools by location. Data from dairies S and D only. Within a location, the first row of the table shows the observed values and the second row shows the expected values...**119**

Table 4.1 Mean (±SE, n trap nights) percentages of parous females, nulliparous females, and males per collection by trap attractant and site at dairies S and D. Significance levels for main effects: (*) p=0.05, (**) p<0.05, (***) p≤0.001. Letters represent significant differences between treatments for each effect per dairy by sex or parity status............**146**

LIST OF FIGURES

Figure **Page**

Figure 1.1 Mean water loss (%) (solid lines) and mean hatch rate (%) (dashed lines) of 28-34 h old eggs desiccated for 12-48 h. Trial 1 is represented by gray lines with closed circles and trial 2 is represented by black lines with open circles. Error bars represent standard deviations..**54**

Figure 1.2 Mean water loss (%) of 28-34 h old eggs subjected to a RH gradient at each 12 h interval. The gray bar represents the net mean water loss experienced by the eggs over 36 h. Bars with the same letter are not statistically different (p>0.05). Error bars represent standard deviations..**55**

Figure 1.3 Comparison of the mean hatch rates (%) of the pooled control eggs and eggs subjected to a RH gradient to trials 1 and 2 of the desiccation time experiment (28-34 h old eggs, desiccated for 12-48 h). Dark gray bars represent trial 1, light gray bars represent trial 2. Bars with the same letter are not statistically different (p>0.05). Use capital letters to compare trial 1 or lowercase letters to compare trial 2. Error bars represent standard deviations..**56**

Figure 1.4 Physical effect of desiccation on the eggs of *C. sonorensis*. (A) Intact egg. (B) Desiccated, collapsed egg..**57**

Figure 2.1 Proportion of final mortality of pupae exposed to 0.0, -5.0, -7.0, -9.0, and -11°C for 1 h. Solid lines represent control pupae, dashed lines represent sub-zero exposed pupae. Black lines represent pupae held at 27°C, gray lines represent pupae held at 4°C..**91**

Figure 2.2 Proportion of a) initial mortality, b) subsequent larval mortality, c) subsequent pupal mortality, and d) final mortality of L4 larvae exposed to -3.0, -3.2, -3.5, -3.8, and -4.5°C for 1 h. Solid lines represent control larvae, dashed lines represent sub-zero exposed larvae. Black lines represent larvae held at 27°C, gray lines represent larvae held at 4°C..**92**

Figure 2.3 Proportion of a) initial mortality, b) subsequent larval mortality, c) subsequent pupal mortality, and d) final mortality of eggs exposed to -10, -12, -15, -17, and -20°C for 1 h. Solid lines represent control eggs, dashed lines represent sub-zero exposed eggs. Black lines represent eggs held at 27°C, gray lines represent eggs held at 4°C...............**93**

Figure 2.4 Proportion of final mortality of L4 larvae from Colorado (dark gray) and California (light gray) exposed to -2.5°C for 1 h and control larvae. Error bars represent standard errors...**94**

Figure 3.1 Proportions of vector pools tested (n=674) for BTV and of the BTV-positive pools (n=126) by trap type...**120**

Figure 3.2 Weekly infection rates. Mean (± standard error) infection rate (per 1,000 insects) by trap type and week...**121**

Figure 3.3 Interaction between trap and location. Mean (± standard error) BTV infection rate by attractant type and trap placement at dairies S and D. Map shows placement of traps on dairy S. Dark green bars represent dairy S, light green bars represent dairy D..**122**

Figure 3.4 A) Ommatidia (eye cross-section) of orally BTV infected *C. sonorensis*, after 10 days. B) Ommatidia of uninfected (un-fed control) *C. sonorensis*. The rhabdom (rh)

and cornea (cor) are heavily infected in BTV-positive insects (punctate green staining).......**123**

Figure 4.1 Layouts of dairies S, D, and V showing locations of cattle enclosures, wastewater ponds/*Culicoides* development sites, fields, and approximate locations of traps on each farm.......**148**

Figure 4.2 Mean (±SE) numbers of parous, nulliparous, and male *C. sonorensis* collected in each trap type for dairies S, D, and V. Top half of graphs shows complete data for all three dairies. Bottom half of graphs shows cut-off (faded) data for dairy V to better show differences between dairies S and D.......**149**

Figure 4.3 Mean (±SE) numbers of parous, nulliparous, and male *C. sonorensis* collected in each trap type for dairies S and D.......**150**

Figure 4.4 Heat map showing mean (±SE, n trap nights) numbers of parous, nulliparous, and male *C. sonorensis* per trap-site combination. Colors represent quartiles: black (top), dark gray (second), light gray (third), white (bottom).......**151**

INTRODUCTION

Culicoides biting midges (Diptera: Ceratopogonidae) are vectors of several important viruses of wild and domestic animals worldwide, including bluetongue virus (BTV), epizootic hemorrhagic disease virus (EDHV), and African horse sickness virus (AHSV). Bluetongue virus infects mainly cattle and sheep, the latter of which are especially susceptible to infection (Parsonson 1990). High morbidity and mortality can occur in susceptible animals. The effects on cattle, which typically do not exhibit clinical disease, are mainly economic, in the form of reduced weight gains and milk production. Pregnant cows may abort, and the calves of infected cows suffer from congenital abnormalities. In the United States, where BTV and EDHV are endemic, the only thoroughly confirmed vector of either virus is *C. sonorensis* Wirth and Jones, though other species have been implicated to various degrees, and may also be competent (Mellor 1990). Throughout the globe, several other species have been implicated especially in BTV transmission, including *C. imicola* Kieffer (Afro-Asia, Europe), and *C. obsoletus* Meigen (Europe).

One of the biggest questions in *Culicoides*/BTV research is that of trans-seasonality. Despite the clear seasonality in transmission and vector populations, overwintering mechanisms for either the virus or its vector are less well understood. It is assumed for many species that the overwintering stage of the vector is the larva (Kettle 1977, Mullen and Hribar 1988, Mellor et al. 2000), but there is very little information on the potential importance of the other immature stages. Theories that have been put forward to explain viral overwintering include transovarial transmission (White et al.

2005), survival of adult midges (Lysyk and Danyl 2007 Napp et al. 2011), and persistent infection in vertebrates (Takamatsu et al. 2003). However, there is a lack of sufficient evidence for these theories, especially transovarial transmission (Osborne et al. 2015), and the question of overwintering also may involve more than one mechanism.

There was renewed interest in this issue in 2006 when there was an unprecedented BTV epizootic in Europe, far above the supposed northern range of the virus (Mehlhorn et al. 2007). Prior to this outbreak, bluetongue was primarily thought of as a tropical and subtropical disease, with periodic temperature incursions. While some endemic *Culicoides* spp. were suspected to be competent for BTV prior to 2006 (Wirth and Dyce 1985; Mellor 1990; Mellor and Boorman 1995), and there had been earlier outbreaks in the Mediterranean region (Purse et al. 2005), no one thought that the virus could be spread as far north as the Netherlands and Germany. Making matters worse, the serotype that was introduced (BTV-8) was particularly virulent, causing high mortality in both sheep and cattle (Elbers et al. 2008a, Elbers et al. 2008b, Conraths et al. 2009). To date, how the virus was introduced is unknown (Meiswinkel et al. 2007). The virus overwintered and caused epizootics in three consecutive years (Carpenter et al. 2009). Consequently, several species of *Culicoides* were identified as new potential vectors of BTV (Meiswinkel et al. 2007, Carpenter et al. 2008, Hoffmann et al. 2009). Because we now know that temperate species play a role in global BTV transmission, it is vital to describe the complete seasonal life cycle of *Culicoides* vectors in order to better understand the BTV transmission cycle.

***Culicoides sonorensis* biology**

In North America, the primary BTV vector is *C. sonorensis*, although other species may be involved in transmission, especially in the Southeast U.S. (Ruder et al. 2015). The biology of *Culicoides* varies somewhat by species, though all utilize moist substrates for oviposition and larval development (Blanton and Wirth 1979). In southern California, *C. sonorensis* is typically associated with dairy cow production, where females will utilize the shallow banks of wastewater holding ponds (sometimes referred to as dairy lagoons) for oviposition. These highly polluted and organically rich habitats are ideal for larval development, and manure loading can significantly increase vector populations (Mullens and Rodriguez 1988). *Culicoides sonorensis* females will lay approximately 100 eggs on the edges of the ponds, above waterline (Mullens and Lii 1987, Mullens and Rodriguez 1989). They prefer gentle slopes, free of vegetation. The species completes four larval instars before pupating. The earliest instars, especially the L1, are likely primarily terrestrial, though later instars (L3 and L4) can be free swimming. Pupae must maintain contact with the air or they will drown. However, if submerged, they are capable of wiggling free of the substrate and floating on the water surface. The generation time is rapid, taking only 26 days from egg to eclosion under summer field conditions (Jones 1960), and laboratory development can be completed in as little as 2.5 weeks (Hunt 1994). Females require a blood meal to develop eggs. In the wild, *C. sonorensis* feeds mainly on medium-large mammals, and is not anthropophillic, though females in colony sometimes bite humans.

Adults are crepuscular. Female flight activity peaks between approximately one hour before to four hours after sunset (Akey and Barnard 1983, Mullens 1995). A smaller activity peak is observed just following sunrise (Mullens 1995). In dissections of the closely related, *C. variipennis* (Coquillett), the presence of sac stage relics (indicating very recent oviposition) in individuals collected from the field during the night and at dawn suggests oviposition after dusk (Mullens and Schmidtman 1982, Mullens and Rutz 1984). Male activity peaks slightly before female activity (Mullens 1995, Gerry and Mullens 1998). Male *C. sonorensis* can be collected in relatively high numbers in carbon dioxide baited traps compared to unbaited, or female-baited traps (Gerry and Mullens 1998). Both the female and male maxillary palps have a large sensory pit with numerous basiconic sensilla (capitate pegs), which house CO_2 receptor neurons (Grant and Kline 2003), further supporting the idea that males are attracted to CO_2. Males are likely locating host animals for mating purposes. Males will swarm several meters downwind of cattle, and will also mate directly on the host with females that are either actively blood feeding, or are fully engorged (Gerry and Mullens 1998). Daytime resting sites for adult *Culicoides* have not been identified, but some species are known to enter buildings and animal housing structures (Meiswinkel et al. 2000).

Bluetongue Virus

Bluetongue virus is a double-stranded RNA orbivirus in the family, Reoviridae with a 10-segmented genome. The virus has seven structural proteins and four non-structural proteins. The capsid is composed of four structural proteins: VP2, VP5, VP3,

and VP7. VP2 is the primary determinant of serotype, and the major protein involved in entering mammalian cells. VP3 and VP7 are the major core proteins of the virus, and VP7 appears to be the protein involved in entering insect cells; the core proteins are 100 times as infectious in *Culicoides* cells as they are in mammalian cells (Mellor 1990). The non-structural proteins, NS1 and NS2 are found in higher numbers in mammalian cells, and are likely important for pathogenesis and viral replication. In comparison, NS3 and NS3a are more abundant in *Culicoides*, and are not associated with pathogenesis. These proteins are most likely involved in the release of virions from insect host cells (Schwartz-Cornil et al. 2008).

After an animal is bitten by an infected vector, BTV is transported to the lymph nodes, where VP2 binds to cell surface glycoproteins, and the virion is taken into the cell via endocytosis. VP5 then fuses with the endosomal membrane and the core is transported into the cytoplasm where viral replication takes place. Structural proteins VP1, VP4, and VP6 serve as the transcription complex of the virus, and replication begins within 2 h of the introduction of the virus. Mature virions are released from the host cell via budding or lysis (Schwartz-Cornil et al. 2008). Secondary infection sites are primarily the epithelial tissues of the lungs and spleen, and mononuclear phagocytes (Barratt-Boyes and MacLachlan 1994).

In the insect host, BTV must overcome the midgut barrier in order for the infection to disseminate. The peritrophic membrane is a physical barrier between the blood bolus and gut wall that can prevent pathogens from leaving the midgut (Hardy et al. 1983). The ability of viruses and other pathogens to breech this barrier may be due

either to the genetics of the host (Hardy et al. 1983, Beernsten et al. 2000), or to the host's environment. It has been hypothesized that under high temperatures, viruses may be able to pass directly into the hemocoel without first infecting the gut cells, known as the "leaky gut" phenomenon (Boorman 1960, Mellor 1998, Mellor et al. 2000). After the virus leaves the midgut and enters the hemocoel it can infect and replicate in the salivary glands (Bowne and Jones 1966), allowing virions to be passed to a potential mammalian host during blood feeding.

Bluetongue virus infects mainly domestic ruminants, including sheep and cattle. In sheep, the clinical symptoms of the disease can be very severe, and include internal hemorrhaging, coronitis (inflammation of the hoof wall), and swelling and cyanosis of the tongue (from which the disease gets its name) (Erasmus 1975). The case fatality rate can be as high as 50% (Elbers et al. 2008b). In cattle, the disease tends to be subclinical, and mortality is very rarely seen. When effects of the disease are seen, they are usually economic in the form of reduced milk yields, weight gain, and quality of calves (Hourrigan and Klingsporn 1975).

Vector Surveillance

Vector and virus surveillance are key to understanding the risk of transmission and the limits of BTV range. Surveillance can be conducted by taking blood samples from sentinel animals on farms, or by collecting and testing insect vectors. These collections also provide valuable information on the size, age structure, and species composition of the vector population. Most *Culicoides* and BTV surveillance is done

using UV light-baited suction traps placed near either animals or development sites, although we know that many variables can affect the composition of the collections. The use of artificial host cues (e.g. CO_2 or 1-octen-3-ol) versus light versus a live host animal as bait can influence which species are attracted to a given trap, as well as the sex and parity status of those insects (Mullens 1985, Bellis and Reid 1996, Gerry and Mullens 2000, Carpenter et al. 2008, Gerry et al. 2009, Viennet et al. 2011, Mayo et al. 2012a, Scheffer et al. 2012). Even the specific wavelength of light used may possibly influence *Culicoides* collections (Bishop et al. 2004).

Due to financial and time restraints on processing surveillance trap collections, and the typically low prevalence of arboviruses in field vector populations, insects are tested for BTV in pools usually using qRT-PCR. Analysis of the results of the PCR assay provides a cycle threshold (Ct) value for each pool. The Ct value is the number of replication cycles required to reach a threshold level of nucleic acid, and is an indication of the amount of starting viral RNA in a sample (Heid et al. 1996). Samples with more virus require fewer amplification cycles to reach the threshold, and so have lower Ct values. For BTV in midges, a Ct value of <30-31 is generally considered a virus-positive sample (Mayo et al. 2012a, Veronesi et al. 2013). Based on the results of PCR, estimates of infection rate can be generated.

There are two main ways of estimating infection rate: minimum infection rate (MIR) estimates and maximum likelihood estimates (MLE). The older of the two methods is MIR, which assumes only one infected individual per pool, and is calculated as the number of positive pools divided by the total number of insects tested. MIR

estimates become problematic when the infection rate of the wild vector population is high or the size of the pools tested is large, both of which increase the odds that more than one infected individual will be present in a given pool (Gu et al. 2003). Because MIR estimates require that all pools have the same number of insects, comparisons of infection rate estimates across studies are often not appropriate (Walter et al. 1980). Vector biologists and epidemiologists are well aware of the shortcomings of MIR estimates for virus surveillance, and so most studies now utilize MLE methods. MLE methods model infection rate based on an assumed binomial distribution, allowing for the relaxation of the restraints of MIR estimates, especially that of pool size (Walter et al. 1980, Gu et al. 2003). In fact, MLE methods are more accurate when variable pool sizes are used (Gu et al. 2004). The result is infection rate estimates that are much closer to the true infection rate (Walter et al. 1980). Several computer programs are now available which make MLE modelling extremely quick and easy to do (Biggerstaff 2006) for even those non-mathematically inclined biologists.

Besides pool size, one of the other issues for estimating infection rate is the parity of collections. For pathogens that are not transovarially transmitted, like BTV (Osborne et al. 2015), only those females who have previously taken a blood meal and completed at least one gonotrophic cycle (i.e. parous) are potentially infected. Although *Culicoides* are relatively easy to sort by parity status visually (Akey and Potter 1979), other insects, like mosquitoes, must be dissected to determine parity (Hugo et al. 2008). Even *Culicoides* are not typically sorted by parity status before pooling for PCR due to the time required for processing, although there is opportunity for computer programs to

automate, or at least partially automate, this process (Osborne et al. 2014). Because many nulliparous females are tested, lots of resources are used to process insects that are not infected, reducing the efficiency of virus surveillance programs. Trapping strategies that optimize the proportion or number of parous females collected are needed. Optimizing surveillance strategies is especially important when the overall adult vector activity is low, like during winter. Because we know that even in temperate zones, low levels of adult *Culicoides* activity can be detected in winter (Gerry and Mullens 2000, Napp et al. 2011, Mayo et al. 2014), trapping strategies that collect higher numbers of parous females are more likely to detect BTV. More efficient winter collecting could provide more information on whether or not BTV may be transmitted trans-seasonally.

Environmental persistence of *Culicoides sonorensis*

The importance of bluetongue as a disease is seen mostly in temperate regions, where livestock are susceptible and outbreaks less predictable (Purse et al. 2005). In tropical and subtropical regions, where BTV is endemic and exhibits predictable seasonal cycles, clinical disease is less apparent. However, there is little understanding, or even serious investigation of those cycles in tropical endemic areas. In California, seroconversions in cattle peak in October, and collections of *C. sonorensis* peak in September, while there is little to no vector activity or transmission from late November or early December through May (Gerry and Mullens 2000, Gerry et al. 2001, Mayo et al. 2012b). A better understanding of *Culicoides* seasonal activity in subtropical regions may aid in understanding temperate transmission patterns. Furthermore, there is concern that

BTV could expand into other temperate regions, including the northeastern U.S. and Canada. *Culicoides sonorensis* is not currently present in these parts of North America, except for seasonal populations in southwestern Canada (Lysyk 2007), but climate change could allow the species to expand its range northward, potentially bringing BTV with it.

Because *Culicoides* spend the majority of their lifespan in one of three immature stages (egg, larva, or pupa), the environmental tolerances and developmental thresholds of these stages are likely to play a pivotal role in determining species range. Compared to the adults, immature *Culicoides* are significantly less mobile and are essentially restricted to the habitat in which they develop. Larvae may have some ability to regulate their temperature by either moving into the mud substrate or out into the deeper water (Vaughan and Turner 1987, Mullens and Rodriguez 1992), but eggs are pupae are restricted to the mud's surface, or just barely below it. In order for an insect to survive periods of challenging environmental conditions, like winter, it must either be able to avoid damage, or to tolerate it.

Desiccation tolerance is often seen in insects that inhabit ephemeral water sources. One of the most commonly referenced examples is that of *Aedes albopictus* Skuse (Diptera: Culicidae) eggs, which are laid in small, temporary water sources, like tree holes. *Aedes albopictus* eggs are extremely tolerant of desiccation and can survive 25-27% water loss (Urbanski et al. 2011). In these and other mosquito species, egg desiccation tolerance is achieved at least in part by the development of the serosal cuticle, a third layer of eggshell below the exo- and endochorion (Rezende et al. 2008, Goltsev et

al. 2009). This occurs approximately 8-14 hours after oviposition, depending on the species (Rezende et al. 2008, Goltsev et al. 2009).

Of course, *C. sonorensis* did not evolve with man-made dairy wastewater ponds. The species likely took advantage of naturally occurring, ephemeral water sources, such as rivulets, puddles or depressions made by host animals, like bison wallows (Pfannenstiel and Ruder 2015). Because of the temporary nature of these habitats, *C. sonorensis* must also have evolved some way of dealing with the risk of desiccation. Intuitively, the life stage where this kind of tolerance would be most critical is the egg, which cannot move with the receding waterline or into the mud to find moisture. Egg diapause and overwintering in *Culicoides* have been discussed rarely in the literature (Parker 1950, Isaev 1975, Glukhova 1979, Breidenbaugh and Mullens 1999), but there is no information related to *C. sonorensis*.

Desiccation tolerance is also closely related to low temperature tolerance. As tissues freeze and cells are essentially dehydrated, cold hardy insects must be able to withstand the loss of much, if not all, of the free water in their bodies. Insects with exceptional desiccation tolerance tend to also be tolerant of exposure to low temperatures; the African rock-pool midge, *Polypedilum vanderplanki* (Diptera: Chironomidae), being the most extreme example (Hinton 1960). No work has been done on *C. sonorensis* immatures in this area, though there has been some interest in the effect of low temperature on adult survival (Nunamaker 1993), and on the regulation of heat shock proteins (HSPs) in adults (Nunamaker et al. 1996). Heat shock proteins are well known to be involved with not only survival of high temperatures, but also survival of

low temperatures in insects (Denlinger et al. 1991, Rinehart et al. 2007). Adult *C. sonorensis* survival in sub-freezing temperatures increases after a brief exposure to low, but above-freezing temperatures (5°C) (Nunamaker 1993). Heat shock proteins are also up-regulated post-exposure to low temperatures (Nunamaker et al. 1996). Development of the serosal cuticle has also been implicated in low temperature tolerance in *Ae. albopictus* eggs (Kreb et al. 2016)

Field studies in Colorado (Barnard and Jones 1980), and in Virginia (Vaughan and Turner 1987) suggested that the larva was the primary *C. sonorensis* overwintering stage in those locations. However, adult females can often be collected in low numbers during the winter, even in temperate climates (Nunamaker et al. 1996, Gerry and Mullens 2000, Napp et al. 2011). The seasonal dynamics of the species in southern California may also be different than in other parts of the country. However, despite the fact that winter in southern California is nowhere near as extreme as it is the northern and eastern extremes of *C. sonorensis* range, we still see a sharp drop in the numbers of adults collected, and in BTV seroconversions in cattle (Gerry and Mullens 2000, Gerry et al. 2001). The two life stages that have been collected during the winter (adults and larvae) are able to physically move to avoid adverse environmental conditions (Mullens and Rodriguez 1992). If eggs and pupae do persist in some areas over winter, they must have physiological mechanisms to survive those same conditions. We have only recently had any success in collecting *C. sonorensis* eggs from the field, and an active winter sampling effort for them may provide more information on *Culicoides* seasonality. If the larvae are in fact the primary overwintering stage of *C. sonorensis*, then we would expect to see

higher survival at low temperatures than other life stages exposed to the same conditions. By determining the environmental tolerances of the understudied immature stages of *C. sonorensis*, and developing the most effective collection methods in the field, we can gain insight on how the virus and vector populations are being maintained across seasons.

The purpose of this dissertation is to explore *Culicoides* and BTV trans-seasonality by gaining an understanding of how the understudied immature stages of the primary North American BTV vector, *C. sonorensis,* tolerate environmental stressors associated with overwintering (Chapters 1 and 2), of how BTV may manipulate vector behavior, resulting in artificially low infection rates, especially during periods of low vector activity, like winter (Chapter 3), and how *Culicoides* trapping methodology can be improved to increase efficiency during such periods (Chapter 4).

REFERENCES

Akey, D. H., and H. W. Potter. 1979. Pigmentation associated with oogenesis in the biting fly *Culicoides variipennis* (Diptera: Ceratopogonidae): determination of parity. J. Med. Entomol. 16: 67-70.

Akey, D. H., and D. R. Barnard. 1983. Parity in airborne populations of the biting gnat *Culicoides variipennis* (Diptera: Ceratopogonidae) in northeastern Colorado. Environ. Entomol. 12: 91-95.

Barnard, D. R., and R. H. Jones. 1980. *Culicoides variipennis*: seasonal abundance, overwintering, and voltinism in Northeastern Colorado. Environ. Entomol. 9: 709-712.

Barratt-Boyes, S. M., and N. J. MacLachlan. 1994. Dynamics of viral spread in bluetongue virus infected calves. Vet. Mirobiol. 40: 361-371.

Beernsten, B. T., A. A. James, and B. M. Christensen. 2000. Genetics of mosquito vector competence. Microbiol. Molec. Biol. Rev. 64: 115-137.

Bellis, G. A., and D. J. Reid. 1996. Sampling bias in determining the parous rate of collection of *Culicoides breivtarsis* Kieffer and *C. wadai* Kitaoka (Diptera: Certatopogonidae). Austral. J. Entomol. 35: 319-322.

Biggerstaff, B. J. 2006. PooledInfRate, Version 3.0: A Microsoft Excel add-in to compute prevalence estimates from pooled samples. Centers for Disease Control and Prevention, Fort Collins, CO.

Bishop, A. L., R. J. Worrall, L. J. Spohr, H. J. McKenzie, and I. M. Barchia. 2004. Improving light-trap efficiency for *Culicoides* spp. with light-emitting diodes. Veterinaria Italiana. 40: 266-269.

Blanton, F. S., and W. W. Wirth. 1979. The sand flies (*Culicoides*) of Florida. Florida Department of Agriculture and Consumer Services, Gainesville, FL.

Boorman, J. 1960. Observations on the amount of virus present in the haemolymph of *Aedes aegypti* infected with Uganda S, yellow fever and Semliki Forest viruses. Trans. R. Soc. Trop. Med. Hyg. 54: 362-365.

Bowne, J. G., and R. H. Jones. 1966. Observations on bluetongue virus in the salivary glands of an insect vector, *Culicoides variipennis*. Virol. 30: 127-133.

Breidenbaugh, M. S., and B. A. Mullens. 1999. Descriptions of immature stages of six *Culicoides* Latreille spp. (Dipetera: Ceratopogonidae) from desert mountain ranges in southern California, with notes on life histories and rearing techniques. Proc. Entomol. Soc. Wash. 101:839-867.

Carpenter, S., C. McArthur, R. Selby, R. Ward, D. V. Nolan, A. J. Mordue Luntz, J. F. Dallas, F. Tripet, and P. S. Mellor. 2008. Experimental infection studies of UK *Culicoides* species midges with bluetongue virus serotypes 8 and 9. Vet. Rec. 163: 589-592.

Carpenter, S., A. Wilson, and P. S. Mellor. 2009. *Culicoides* and the emergence of bluetongue virus in northern Europe. Trend. Microbiol. 17: 172-178.

Conraths, F. J., J. M. Gethmann, C. Staubach, T. C. Mettenleiter, M. Beer, and B. Hoffmann. 2009. Epidemiology of bluetongue virus serotype 8, Germany. Emerg. Infect. Dis. 15: 433-435.

Denlinger, D. L., K. H. Joplin, C. –P. Chen, and R. E. Lee. 1991. Cold shock and heat shock, pp. 131-148. *In* R. E. Lee and D. L. Denlinger (eds.), Insects at low temperature. Chapman and Hall, New York, NY.

Elbers, A. R. W., A. Backx, E. Meroc, G. Gerbier, C. Staubach, G. Hendrickx, A. van der Spek, and K. Mintiens. 2008a. Field observations during the bluetongue serotype 8 epidemic in 2006: I. Detection of first outbreaks and clinical signs in sheep and cattle in Belgium, France and the Netherlands. Prevent. Vet. Med. 87: 21-30.

Elbers, A. R. W., A. Backx, K. Mintiens, G. Gerbier, C. Staubach, G. Hendrickx, and A. van der Spek. 2008b. Field observations during the bluetongue serotype 8 epidemic in 2006: II. Morbidity and mortality rate, case fatality and clinical recovery in sheep and cattle in the Netherlands. Prevent. Vet. Med. 87: 31-40.

Erasmus, B. J. 1975. Bluetongue in sheep and goats. Austral. Vet. J. 51: 165-170.

Gerry, A. C., and B. A. Mullens. 1998. Response of male *Culicoides variipennis sonorensis* (Diptera: Ceratopogonidae) to carbon dioxide and observations of mating behavior on and near cattle. J. Med. Entomol. 35: 239-244.

Gerry, A. C., and B. A. Mullens. 2000. Seasonal abundance and survivorship of *Culicoides sonorensis* (Diptera: Ceratopogonidae) at a southern California dairy, with reference to potential bluetongue virus transmission and persistence. J. Med. Entomol. 37: 675-688.

Gerry, A. C., B. A. Mullens, N. J. MacLachlan, and J. O. Mecham. 2001. Seasonal transmission of bluetongue virus by *Culicoides sonorensis* (Diptera: Ceratopogonidae) at a southern California dairy and evaluation of vectorial capacity as a predictor of bluetongue virus transmission. J. Med. Entomol. 38: 197-209.

Glukhova, V. M. 1979. Larvae of biting midges of the subfamilies Palpomyiinae and Ceratopogoninae of the USSR fauna (Diptera, Ceratopogonidae = Heleidae), pp. 1-85. In Keys to USSR Fauna. Zoological Institute, Academy of Sciences, Leningrad, Russia.

Goltsev, Y., G. L. Rezende, K. Vranizan, G. Lanzaro, D. Valle, and M. Levine. 2009. Developmental and evolutionary basis for drought tolerance of the *Anopheles gambiae* embryo. Dev. Biol. 330: 462-470.

Grant, A. J., and D. L. Kline. 2003. Electrophysiological responses from *Culicoides* (Diptera: Ceratopogonidae) to stimulation with carbon dioxide. J. Med. Entomol. 40: 284-293.

Gu, W., R. Lampman, and R. J. Novak. 2003. Problems in estimating mosquito infection rates using minimum infection rate. J. Med. Entomol. 40: 595-596.

Gu, W., R. Lampman, and R. J. Novak. 2004. Assessment of arbovirus vector infection rates using variable size pooling. J. Med. Entomol. 18: 200-204.

Hardy, J. L., E. J. Houk, L. D. Kramer, and W. C. Reeves. 1983. Intrinsic factors affecting vector competence of mosquitoes for arboviruses. Ann. Rev. Entomol. 28: 229-262.

Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams. 1996. Real time quantitative PCR. Genome Methods. 6: 986-994.

Hinton, H. E., 1960. Cryptobiosis in the larva of *Polypedilum vandkerplanki* Hint. (Chironomidae). J. Ins. Physiol. 5: 286-300.

Hoffmann, B., B. Bauer, C. Bauer, H. –J. Batza, M. Beer, P. –H. Clausen, M. Geier, J. M. Gethmann, E. Kiel, G. Liebisch, A. Liebisch, H. Mehlhorn, G. A. Schaub, D. Werner, and F. J. Conraths. 2009. Monitoring of putative vectors of bluetongue virus serotype 8, Germany. Emerg. Infec. Dis. 15: 1481-1484.

Hourrigan, J. L., and A. L. Klingsporn. 1975. Bluetongue: the disease in cattle. Austral. Vet. J. 51: 170-174.

Hugo, L. E., S. Quick-Miles, B. H. Kay, and P. A. Ryan. 2008. Evaluations of mosquito age grading techniques based on morphological changes. J. Med. Entomol. 45: 353-369.

Hunt, G. J. 1994. A procedural manual for the large-scale rearing of the biting midge, *Culicoides sonorensis* (Diptera: Ceratopogonidae). U.S. Dept. of Agricu., Agric. Res. Serv., ARS-121.

Isaev, V. A. 1975. Photoperiodic induction of the diapause at the egg phase in the blood sucking midge *Culicoides pulicaris punctatus* (Diptera: Ceratopogonidae). Parazitologiya. 9: 501-506.

Jones, R. H. 1960. Mass-production methods for the colonization of *Culicoides variipennis sonorensis*. J. Econ. Entomol. 53: 731-735.

Kettle, D. S. 1977. Biology and bionomics of bloodsucking Ceratopogonids. Ann. Rev. Entomol. 22: 33-51.

Kreb, A. U. Kuch, J. Oehlmann, and R. Muller. 2016. Effects of diapause and cold acclimation on egg ultrastructure: new insights into the cold hardiness mechanisms of the Asian tiger mosquito, *Aedes (Stegomyia) albopictus*. J. Vector. Ecol. 41: 142-150.

Lysyk, T. J. 2007. Seasonal abundance, parity, and survival of adult *Culicoides sonorensis* (Diptera: Ceratopogonidae) in southern Alberta, Canada. J. Med. Entomol. 44: 959-969.

Lysyk, T. J., and T. Danyk. 2007. Effect of temperature on life history parameters of adult *Culicoides sonorensis* (Diptera: Ceratopogonidae) in relation to geographic origin and vectorial capacity for bluetongue virus. J. Med. Entomol. 44: 741-751.

Mayo, C. E., B. A. Mullens, A. C. Gerry, C. M. Barker, P. P. C. Mertens, S. Maan, N. Mann, I. A. Gardner, A. J. Guthrie, and N. J. MacLachlan. 2012a. The combination of abundance and infection rates of *Culicoides sonorensis* estimates risk of subsequent bluetongue virus infection of sentinel cattle on California dairy farms. Vet. Parasitol. 187: 295-301.

Mayo, C. E., I. A. Gardner, B. A. Mullens, C. M. Barker, A. C. Gerry, A. J. Guthrie, and N. J. MacLachlan. 2012b. Anthropogenic and meterological factors influence vector abundance and prevalence of bluetongue virus infection of dairy cattle in California. Vet. Microbiol. 155: 158-164.

Mayo, C. E., C. J. Osborne, B. A. Mullens, A. C. Gerry, I. A. Gardner, W. K. Reisen, C. M. Barker, N. J. MacLachlan. 2014. Seasonal variation and impact of waste-water lagoons as larval habitat of the population dynamics of *Culicoides sonorensis* (Diptera: Ceratopogonidae) at two dairy farms in northern California. PLOS One. 9: e89633.

Mehlhorn, H., V. Walldorf, S. Klimpel, B. Jahn, F. Jaeger, J. Eschwiler, B. Hoffmann, and M. Beer. 2007. First occurrence of *Culicoides obsoletus*-transmitted bluetongue virus epidemic in Central Europe. Parasitol. Res. 101: 219-228.

Meiswinkel, R., M. Baylis, and K. Labuschagne. 2000. Stabling and the protection of horses from *Culicoides bolitinos* (Diptera: Ceratopogonidae), a recently identified vector of African horse sickness. Bull. Entomol. Res. 90: 509-515.

Meiswinkel, R., P. van Rijn, P. Leijs, and M. Goffredo. 2007. Potential new *Culicoides* vector of bluetongue virus in northern Europe. Vet. Rec. 161: 564-565.

Mellor, P. S. 1990. The replication of bluetongue virus in *Culicoides* vectors. Curr. Top. Microbiol. Immunol. 162: 143-161.

Mellor, P. S. 1998. Climate-change and the distribution of vector-borne diseases with special reference to African horse sickness virus. Proc. Int. Symp. Diagn. Control. Livestock Dis. Nucl. Related Tech. Vienna, Austria. 439-454.

Mellor, P. S., and Boorman, J. 1995. The transmission and geographical spread of African horse sickness and bluetongue viruses. Ann. Trop. Med. Parasitol. 89: 1-15.

Mellor, P. S., J. Boorman, and M. Baylis. 2000. *Culicoides* biting midges: their role as arbovirus vectors. Annu. Rev. Entomol. 45: 307-340.

Mullen, G. R., and L. J. Hribar. 1988. Biology and feeding behavior of Ceratopogonid larvae (Diptera: Ceratopogonidae) in North America. Bull. Soc. Vector Ecol. 13: 60-81.

Mullens, B. A. 1985. Sampling bias and the problem of age and survivorship determination in *Culicoides*. Prog. Clin. Biol. Res. 178: 207-211.

Mullens, B. A. 1995. Flight activity and response to carbon dioxide of *Culicoides variipennis sonorensis* (Diptera: Ceratopogonidae) in southern California. J. Med. Entomol. 32: 310-315.

Mullens, B. A., and K. –S. Lii. 1987. Larval population dynamics of *Culicoides variipennis* (Diptera: Ceratopogonidae) in southern California. J. Med. Entomol. 24: 566-574.

Mullens, B. A., and J. L. Rodriguez. 1988. Colonization and response of *Culicoides variipennis* (Diptera: Ceratopogonidae) to pollution levels in experimental dairy wastewater ponds. J. Med. Entomol. 25: 441-451.

Mullens, B. A., and J. L. Rodriguez. 1988. Response of *Culicoides variipennis* (Diptera: Ceratopogonidae) to water level fluctuations in experimental dairy wastewater ponds. J. Med. Entomol. 26: 566-572.

Mullens, B. A., and J. L. Rodriguez. 1992. Survival and vertical distribution of larvae of *Culicoides variipennis* (Diptera: Ceratopogonidae) in drying mud habitats. J. Med. Entomol. 29: 745-749.

Mullens, B. A., and D. A. Rutz. 1984. Age structure and survivorship of *Culicoides variipennis* (Diptera: Ceratopogonidae) in central New York State, USA. J. Med. Entomol. 21: 194-203.

Mullens, B. A., and E. T. Schmidtmann. 1982. The gonotrophic cycle of *Culicoides variipennis* (Diptera: Ceratopogonidae) and its implications in age-grading field populations in New York State, USA. J. Med. Entomol. 19: 340-349.

Napp, S., S. Gubbins, P. Calistri, A. Allepuz, A. Alba, I. Garcia-Bocanegra, A. Giovannini, and J. Casal. 2011. Quantitative assessment of the probability of bluetongue virus overwintering by horizontal transmission: application to Germany. Vet. Res. 42: 1-10.

Nunamaker, R. A. 1993. Rapid cold-hardening in *Culicoides variipennis sonorensis* (Diptera: Ceratopogonidae). J. Med. Entomol. 30: 913-917.

Nunamaker, R. A., V. C. Dean, K. E. Murphy, and J. A. Lockwood. 1996. Stress proteins elicited by cold shock in the biting midge, *Culicoides variipennis sonorensis* Wirth and Jones. Comp. Biochem. Physiol. 113: 73-77.

Osborne, C. J., C. E. Mayo, B. A. Mullens, and N. J. MacLachlan. 2014. Estimating *Culicoides sonorensis* biting midge abundance using digital image analysis. Med. Vet. Entomol. 28: 461-464.

Osborne, C. J., C. E. Mayo, B. A. Mullens, E. G. McDermott, A. C. Gerry, W. K. Reisen, and N. J. MacLachlan. 2015. Lack of evidence for laboratory and natural vertical transmission of bluetongue virus in *Culicoides sonorensis* (Diptera: Ceratopogonidae). J. Med. Entomol. 52: 274-277.

Parker, A. H. 1950. Studies on the eggs of certain biting midges (*Culicoides* Latreille) occurring in Scotland. Proc. R. Ent. Soc. Lond. 25: 43-52.

Parsonson, I. M. 1990. Pathology and pathogenesis of bluetongue infections, pp. 119-141. *In* P. Roy and B. M. Gorman (eds.), Bluetongue viruses. Springer Berlin Heidelberg, Berlin, Germany.

Pfannenstiel, R. S., and M. G. Ruder. 2015. Colonization of bison (*Bison bison*) wallows in a tallgrass prairie by *Culicoides* spp. (Diptera: Ceratopogonidae). J. Vect. Ecol. 40: 187-190.

Purse, B. V., P. S. Mellor, D. J. Rogers, A. R. Samuel, P. P. C. Mertens, and M. Baylis. 2005. Climate change and the recent emergence of bluetongue in Europe. Nat. Rev. Microbiol. 3: 171-181.

Rezende, G. L., A. J. Martins, C. Gentile, L. C. Farnesi, M. Pelajo-Machado, A. A. Peixoto, and D. Valle. 2008. Embryonic desiccation resistance in *Aedes aegyti*, presumptive role of the chitinized serosal cuticle. BMC Develop. Biol. 8: 182.

Rinehart, J. P., A. Li, G. D. Yocum, R. M. Robich, S. A. L. Hayward, and D. L. Denlinger. 2007. Up-regulation of heat shock proteins is essential for cold survival during insect diapause. Proc. Natl. Acad. Sci. 104: 11130-11137.

Ruder, M. G., T. J. Lysyk, D. E. Stallknecht, L. D. Foil, D. J. Johnson, C. C. Chase, D. A. Dargatz, and E. P. J. Gibbs. 2015. Transmission and epidemiology of bluetongue and epizootic hemorrhagic disease in North America: current perspectives, research gaps, and future directions. Vector-Borne and Zoonot. Dis. 15: 384-363.

Scheffer, E. G., G. J. Venter, K. Labuschagne, P. C. Page, B. A. Mullens, N. J. MacLachlan, N. Osterrieder, and A. J. Guthrie. 2012. Comparison of two trapping methods for *Culicoides* biting midges and determination of African horse sickness virus prevalence in midge populations at Onderstepoort, South Africa. Vet. Parasitol. 185: 265-273.

Schwartz-Cornil, I., P. P. C. Mertens, V. Contreras, B. Hemati, F. Pascale, E. Breard, P. S. Mellor, N. J. MacLachlan, and S. Zientara. 2008. Bluetongue virus: virology, pathogenesis and immunity. Vet. Res. 39:46.

Takamatsu, H., P. S. Mellor, P. P. C. Mertens, P. A. Kirkham, J. N. Burroughs, and R. M. E. Parakhouse. 2003. A possible overwintering mechanism for bluetongue virus in the absence of the insect vector. J. Gen. Virol. 84: 227-235.

Urbanski, J. M., J. B. Benoit, R. M. Michaud, D. L. Denlinger, and P. Armbruster. 2010. The molecular physiology of increased egg desiccation resistance during diapause in the invasive mosquito, *Aedes albopictus*. Proc. Roy. Soc. B. 277: 2683-2692.

Vaughan, J. A., and E. C. Turner. 1987. Seasonal microdistribution of immature *Culicoides variipennis* (Diptera: Ceratopogonidae) at Saltville, Virginia. J. Med. Entomol. 24: 340-346.

Veronesi, E., F. Antony, S. Gubbins, N. Golding, A. Blackwell, P. P. C. Mertens, J. Brownlie, K. E. Darpel, P. S. Mellor, and S. Carpenter. 2013. Measurement of the infection and dissemination of bluetongue virus in *Culicoides* biting midges using a semi-quantitative RT-PCR assay and isolation of infectious virus. PLOS one. 8: e70800.

Viennet, E., C. Garros, R. Lancelot, X. Allene, L. Gardes, I. Rakotoarivony, D. Crochet, J. –C. Delecolle, C. Moulia, T. Baldet, and T. Balenghien. 2011. Assessment of vector/host contact: comparison of animal-baited traps and UV-light/suction trap for collecting *Culicoides* biting midges (Diptera: Ceratopogonidae), vectors of Orbiviruses. Parasites & Vectors. 4: 119.

Walter, S. D., S. W. Hildreth, and B. J. Beaty. 1980. Estimation of infection rates in populations of organisms using pools of variable size. Am. J. Epidemiol. 112: 124-128.

White, D. M., W. C. Wilson, C. D. Blair, and B. J. Beaty. 2005. Studies on overwintering of bluetongue viruses in insects. J. Gen. Virol. 86: 453-462.

Wirth, W. W., and A. L. Dyce. 1985. The current taxonomic status of the *Culicoides* vectors of bluetongue viruses. Prog. Clin. Biol. Res. 178: 151-164.

CHAPTER 1

Desiccation tolerance in the eggs of the primary North American bluetongue virus vector, *Culicoides sonorensis*, and implications for vector persistence

Emily G. McDermott[1] and Bradley A. Mullens[1]

[1]Department of Entomology, University of California, Riverside, CA 92521

ABSTRACT

Culicoides sonorensis Wirth and Jones transmits bluetongue virus and develops in a variety of polluted mud habitats. Egg desiccation tolerance was tested by obtaining eggs of known age, drying them, and placing them back on wet substrate. Eggs 4-10 h old failed to hatch after 12 h of drying at 75% RH. Older eggs (28-34 h) survived severe desiccation and over 50% water weight loss. They regained their water within about 2 h of rehydration. Relative to control eggs, average egg hatch was reduced by 36% after 12 h of drying, 79% after 24 h, 91% after 36 h, and 97% after 48 h. Some embryos (1%) survived and hatched after 60 h of drying and water losses of nearly 60%. Eggs in specific 25-40 h age categories did not differ in hatch after a 12 h desiccation stress; critical embryo age to survive drying is between 10 and 24 h. Humidity gradients relieved desiccation stress, and eggs appeared to regain water from saturated RH conditions. Individual, gravid *C. sonorensis* oviposited in 1 liter containers with an artificial mud bank. If they laid eggs, 73% deposited them singly in lines ranging up to 5-6 cm in length (meanderers), while 27% laid eggs in clumps (dumpers). Eggs were positioned an average of 45 $\pm$ 12 mm back from waterline. Younger eggs, if laid in early evening, may not experience severe desiccation. Embryo recovery from such severe desiccation could be adaptive in ephemeral habitats where the species may have evolved.

INTRODUCTION

Culicoides spp. are vectors of several important arboviruses of domestic and wild animals worldwide, including bluetongue virus (BTV) and epizootic hemorrhagic disease

virus (EHDV) of ruminants (Kettle 1977, Mellor et al. 2000). However, very little is known about their immature stages, and especially about the eggs (Kettle 1977). Most *Culicoides* spp., including the primary North American BTV vector, *C. sonorensis* Wirth and Jones, are thought to overwinter in temperate zones as larvae, and their eggs are considered vulnerable to drying (Kettle 1977, Mullen and Hribar 1988, Mellor et al. 2000). However, egg desiccation resistance/tolerance is known in other Dipteran families, including Stratiomyidae (Holmes et al. 2012), Chironomidae (Convey 1992), Culicidae (Minakawa et al. 2001, Roberts 2004, Urbanski et al. 2010, Bader and Williams 2011), and even in other genera of Ceratopogonidae (Rees et al. 1971, Borkent and Craig 2004, Ronderos et al. 2006). Egg diapause and overwintering are well known in some other hematophagous Diptera, most notably the floodwater or tree-hole mosquitoes (Sota and Mogi 1992, Urbanski et al. 2010, Lounibos et al. 2011) and Simuliidae (Shipp 1987, Baba and Takaoka 1992, Stoehr 1994). The same phenomena have occasionally been described in *Culicoides* spp. (Parker 1950, Isaev 1975, Glukhova 1979, Breidenbaugh and Mullens 1999). Because so little research has been done, however, it is difficult to gauge how widespread egg desiccation tolerance or diapause may be in *Culicoides*.

Mechanisms of *C. sonorensis* persistence and overwintering are of particular interest because the details of temperate zone BTV trans-seasonality have yet to be elucidated. In southern California, *C. sonorensis* adults can be collected year round, though their numbers drop significantly during the winter months (Gerry et al. 2001). In

Germany, adult *Culicoides* have been collected during the winter, but the numbers were deemed too small to maintain BTV transmission (Napp et al. 2011).

Regardless of the life stage(s) primarily involved in overwintering, the environmental tolerances of all stages are important to the year round activity and survival of a species, especially those that utilize ephemeral aquatic habitats. The immature stages of *C. sonorensis* are found most commonly in dairy wastewater ponds in southern California, though they can also be found in other, more transient polluted water sources (puddles, trough spillover, etc.) (O'Rourke et al. 1983), more similar to the habitats in which the species likely evolved. In these unpredictable habitats, desiccation tolerance would be advantageous.

We observed in our lab colony that the eggs of *C. sonorensis* were able to survive considerable water loss, and could rehydrate and hatch successfully upon rewetting. To test the extent of this tolerance, we subjected groups of eggs to increasing periods of desiccation before transferring them back to a moist substrate to hatch, and thereby determined their survival. Desiccation tolerance of eggs was then examined in light of the ecology of this species, and its possible adaptive benefits discussed with reference to persistence under suboptimal environmental conditions.

MATERIALS AND METHODS

Eggs were obtained from a colony (VR) of *C. sonorensis*, reared at the University of California, Riverside. This colony was established in 1995 from a southern California dairy and has been maintained using established rearing protocols since that time (Hunt

1994). Oviposition occurred after gravid females were provided with moist filter paper pads (egg sheets) overnight. Egg sheets were collected over specified time periods (known egg age) and kept moist under normal colony conditions (15L: 9D, 25-27°C) until they were used in experiments.

Individual *Culicoides* eggs were beyond the weighing capabilities (nearest 0.001 mg) of the Sartorius M2P microbalance used (Sartorius AG, Goettingen, Germany). At the designated time, therefore, groups of approximately 200-300 eggs were placed into a number of small pre-weighed tinfoil weigh boats, taking care not to transfer skin oils etc. to the foil. Eggs were allowed to air dry for a short period (~10 min) until surface moisture had evaporated before initial weighing. After weighing, eggs were held in plastic relative humidity (RH) chambers over a saturated NaCl solution to achieve a stable 75% RH (Winston and Bates 1960). Four RH chambers (one for each designated time point) were held in an environmental chamber kept at 23 $\pm$ 0.1^{o}C. The egg groups from each RH chamber were removed at the designated time, and reweighed to assess water loss. Eggs were then transferred to a moist substrate (wet cotton on a 1% agarose gel) under colony room conditions. Any clumps of eggs were gently separated using a minuten pin to ensure uniform reabsorption of water, and to allow assessment of individual egg hatch later. Eggs were monitored for hatching at 12-24 h intervals, and the date and time of first observed hatch was recorded. After 3-5 days, the number of both hatched and unhatched eggs was counted per group, and the hatch rate for each replicate was calculated. Six replicates per time point were used in all experiments. Due to logistical considerations (time required for egg handling and experimental setup), some

experiments were done in multiple stages over time. Variations used for each experiment are described below:

Desiccation time: Duration of the egg stage for *C. sonorensis* under laboratory rearing conditions (25-27°C) is approximately 2 d (Hunt 1994), and requires 65 h at 23°C (Vaughan and Turner 1987). Two ages of eggs were tested for desiccation tolerance. By collecting eggs deposited over a 6 h period, eggs 28-34 h old (older eggs) and 4-10 h old (younger eggs) were obtained and held in the RH chambers for 12, 24, 36 and 48 h. After it became clear that some eggs could tolerate 48 h of desiccation, a second group of 28-34 h old eggs was held in the desiccation chamber for 48, 52, 56 and 60 h. This was done twice for each group of time points (12-48 h and 48-60 h). In an attempt to determine more precisely when during development eggs acquired desiccation tolerance, egg sheets were provided to colony *C. sonorensis* and were removed and replaced every hour. Eggs of ages 25, 30, 35 and 40 h old were held in the desiccation chambers for 12 h (enough to cause some impact on egg hatch), replaced on moist substrate, and then checked for egg hatch. This experiment also was done twice.

Responses to a relative humidity gradient: To look at the effect of more gradual humidity changes on egg hatch, groups of 28-34 h old eggs were first held in the normal 75% RH environment. After 12 h, eggs were removed, weighed, and transferred to a second RH chamber over a saturated solution of KCl, to achieve 85% RH (Winston and Bates 1960). After a further 12 h, eggs were removed from the 85% RH environment and weighed, and transferred to a final RH chamber over distilled water, to achieve ~100% RH. After 12 h in this final RH, eggs were weighed and allowed to hatch as

before. The total water loss and the percent change in water loss among the three RHs (and hatch rates) were calculated and compared to the desiccation time experiment eggs (75% RH only). This experiment was done once.

In both the desiccation time and RH gradient experiments, a group of ~500 eggs from the same batch as was used for treatment was set up to hatch in the same manner at the time of the initial weighing (control eggs). Date and time of the first observed hatch was recorded, and hatch rate was calculated in the same way.

Oviposition on natural substrate: Individual gravid colony *C. sonorensis* females were allowed to oviposit on a natural substrate to determine where the eggs would be placed relative to waterline. Surface mud was collected from the edge of a dairy wastewater pond in San Jacinto, CA and frozen (-20°C) to kill any wild larvae. The mud was then thawed and homogenized. Mud (170 g) was added to each of twenty 1 liter, plastic containers, which were 8.5 cm in diameter at the bottom. The mud slope was about 15 degrees, simulating the edge of a wastewater pond. Mud was allowed to set for 1 hour. A 25% "pond water" solution was prepared using the water that had been drained off the mud plus deionized water. Twenty mL of the pond water solution was added back to each cup, providing an area of standing water ~1 cm deep (covering ~25% of the total cup bottom area), leaving 6.5 cm (horizontal distance) of mud above the waterline.

One gravid *C. sonorensis* female was placed in each cup in late afternoon. Twenty individual cups and individual females were used on each of two dates (total n=40). Cups were covered with a fine mesh and kept in ambient laboratory conditions (23.0 ± 2.0°C) overnight. The next day, all cups were frozen briefly to kill females. Dead females were

removed and held individually in 70% ethanol at room temperature for later dissection. Using a dissecting microscope, the mud surface was inspected for oviposition, and observations were made on egg placement. As presented below, females were categorized as either “meanderers” (eggs laid in a linear fashion) or “dumpers” (eggs laid in a dense group). For dumpers, the maximum and minimum horizontal distance of each group of eggs from waterline was measured. The distance from waterline of individual eggs was measured for all meanderers. The number of eggs laid by each female was recorded. Females later were dissected to determine if they retained any eggs.

To determine possible differences in water content of surface mud at different distances from waterline, 5 cups were prepared in the same way as before, but no females were added (blanks). Small strips (1 cm wide and 2 cm long) of surface mud (upper 5 mm) were removed from just above waterline (0 cm), the midpoint (3.25 cm), and the top of the mud incline (6.5 cm). In blanks 1-4, these samples were taken in late afternoon right before females were added to the experimental cups. In blank 5, the samples were taken the next morning just before the experimental cups were frozen. A second group of samples was collected from blanks 1-4 at the same time as blank 5. All samples were weighed, kept in a desiccation chamber for 42 hours, and reweighed to calculate water content.

All data on proportion water loss or hatch rate were transformed to the arc sin of the square root for analysis. The raw data are reported in the figures and tables. In all experiments, analysis of variance (ANOVA) was used to look first at the effect of experimental trial on water loss and hatch rate. If trial effect was found not to be

significant, trials were pooled for further ANOVA. The control eggs (allowed to hatch normally with no experimental manipulation) for each trial of each experiment were used as a pooled baseline control for ANOVA comparisons. As a final check, chi-square analysis was used to compare the mean hatch rates of treatments in each trial (number of eggs hatched versus unhatched) to the control eggs checked for hatch in that trial.

RESULTS

Desiccation time: Younger eggs, 4-10 h old, lost water quickly and uniformly (Table 1.1). There was no significant difference in the mean water loss among any of the treatment (desiccation) times in trial 2 (F=0.49; df=3,20; P=0.691). There were problems with the microbalance in trial 1, and so the water loss data from this trial were not included in the analysis. With the exception of a single hatched egg in trial 2, the youngest *Culicoides* embryos did not survive desiccation for even 12 h, at which time the eggs had lost 36.9 ± 10.5% (trial two) of their original weight. Eggs collapsed essentially completely, and no eggs hatched in any of the treatment groups following rehydration.

For more developed eggs (28-34 h old), however, mean water loss increased steadily with increasing desiccation time (Fig. 1.1). There was no significant effect of experimental trial on mean water loss (F=2.54; df=1,38; P=0.119) so trials were pooled. There was a highly significant desiccation time effect (F=71.39; df=3,42; P<0.001). Eggs desiccated for 12 h were visibly collapsed but lost less weight (22-24%), than eggs desiccated for 48 h lost, which was just over half of their weight (50-51%). Such eggs were severely collapsed.

Collapsed eggs, upon placement back onto moist substrate, regained their normal shape quite quickly, within about 2 h. If they survived, they appeared to hatch on a fairly normal schedule as expected for undried eggs, minus the time dried. Following rehydration, mean hatch rate of the 28-34 h old eggs decreased with longer desiccation times (Fig. 1.1). Control eggs hatched at an overall rate of 67.8 ± 13.9%. For all pairwise comparisons, control egg hatch was significantly different from all of the desiccation time treatments ($X^2 \geq 170.7$; df = 1; $P < 0.001$), so even a 12 h drying time decreased hatch significantly. General linear model analysis showed a significant trial effect on mean hatch rate (F=18.59; df=1,38; P<0.001), so the trials were analyzed separately, but showed similar trends. The effect of desiccation time (up to 48 h) on mean hatch rate was highly significant for both trial 1 (F=62.46; df=4,25; P<0.001,) and trial 2 (F=67.90; df=4,27; P<0.001). On average, hatch rates were reduced by 36.6% (12 h), 79.1% (24 h), 91.0% (36 h), and 96.6% (48 h) compared to the pooled controls.

Despite the large amount of water lost by eggs desiccated for 48 h, some successful hatch (0.83-3.7%) was still observed. When the time of desiccation was extended, we still did not observe complete embryo mortality, even out to 60 h (0.5 ± 0.29% hatch) (Table 1.2). Two-way ANOVA showed no significant effect of trial on either water loss or hatch rate, so the two 48-60 h trials were pooled. Though significantly different overall, the relative mean water loss of these older eggs among the four time points was less variable than was seen in the earlier trials (F=3.63; df=3,44; P=0.020). The mean hatch rates were significantly different over time (F=305.41; df=4,51; P<0.001), with eggs desiccated for 48 h having a higher mean hatch rate than

those desiccated for 60 h. Chi-square analysis showed hatching in all treatments to be highly significantly different from the control ($X^2 \geq 952.9$, df = 1, $P<0.001$).

Eggs that were more precisely categorized within the 25-40 h age range, and then stressed by a 12 h desiccation exposure, showed variability in both water loss and hatch rates. Two-way ANOVA showed significant trial effects on both mean water loss and mean hatch rate, so the two trials were analyzed separately. All treatments resulted in reduced (relative to controls) but still reasonably good embryo survival in both trials (24.2 ± 2.51 to 57.8 ± 10.3% hatch). Mean water loss among the time points differed significantly for both trial 1 (F=9.57; df=3,20; P<0.000) and trial 2 (F=20.83; df=3,20; P<0.001), with the 25 h old eggs having the least amount of water loss in both trials (Table 3). While mean hatch rates differed for trial 1 (F=7.77; df=4,27; P<0.001) and trial 2 (F=23.20; df=4,37; P<0.001), there was no clear relationship to initial egg age. Chi-square analysis showed the mean hatch rate of all treatment eggs to be significantly different from the control of that trial, but the 25 h and 35 h old eggs in trial 1 and the 25 h old eggs in trial 2 were not significantly different from the pooled control.

Responses to a relative humidity gradient: For the RH gradient experiment, first we compared the mean water loss during each of the three desiccation intervals (0-12 h/75% RH, 12-24 h/85% RH, 24-36 h/100% RH) to the net mean water loss over the course of the experiment (Fig. 1.2). The raw data were used for this analysis because the 24-36 h eggs had negative water loss values, indicating that the eggs regained water during this interval, and so could not be transformed. The effect of each interval was significant (F=122.16; df=3,20; P<0.001), though the mean water loss of the 0-12 h eggs

and the net mean water loss were not significantly different. To confirm this, the net mean water loss was compared to the pooled mean water loss of the desiccation time (older eggs, 12-48 h desiccation) experiment, using the transformed data. Again, the net mean water loss was not significantly different from the mean water loss of the 12 h eggs (data not shown). Finally, the mean hatch rate was compared to the mean hatch rates of each trial of the desiccation time experiment (older eggs, 12-48 h desiccation) (Fig. 1.3). Compared to trial 1, the net mean hatch rate was not significantly different from that of the 12 h and 24 h eggs. Compared to trial 2, the net mean hatch rate was not significantly different from only the 12 h eggs.

Oviposition on natural substrate: In the oviposition experiment, the day that the mud samples were taken (afternoon of setup versus the following morning) had no effect on the water content of the mud (F=0.60; df=1,20; P=0.449). The distance from waterline from which the mud samples were taken did significantly affect the water content (F=4.66; df=2,23; P=0.020). Samples from the water edge (25.5 ± 4.05% moisture) and 3.5 cm back from the edge (25.3 ± 5.78% moisture) did not differ, but samples from the furthest point above waterline (6.5 cm) were drier (19.4 ± 4.70% moisture).

The proportion of females that oviposited was 0.75 (30/40). Of those that did oviposit, the proportion described as meanderers (eggs laid in a line) was 0.73, and the proportion described as dumpers (eggs laid in clumps) was 0.27. Most females appeared to have laid their entire clutch at once, with either a continuous line or a cohesive batch. Eggs laid in lines were separated evenly by about 2 mm, and a line of eggs could be as long as 5-6 cm. Occasionally females did lay two different, spatially-discrete egg batches,

or deposit the egg batch in spatially discrete lines. Most females laid the majority of their eggs above midpoint of the mud bank (n=17). Three oviposited below midpoint, 4 oviposited on the surface film of the water and 6 oviposited on the side of the cup. A female's eggs were not included in the analysis of distance from waterline of individual eggs unless the substantial majority of the eggs were laid on the mud. The average distance above water line of eggs laid by meanderers was 45.1 ± 11.7 mm, with the maximum being 65 mm and the minimum being 12 mm. The average distance above water line of eggs laid by dumpers was 44.4 ± 24.2 mm, with the maximum being 62.7 mm and the minimum being 7 mm. The average number of eggs laid per female was 97.4 ± 46.9. Upon dissection, only 2 females that oviposited retained eggs. The first female retained 2 eggs, while the second retained only 1.

DISCUSSION

The eggs of *C. sonorensis* were able not only to tolerate more environmental stress than previously believed, but this study showed that they could tolerate physical collapse (Fig. 1.4), and weight losses of over 50% without complete mortality. This exceeds the desiccation tolerance of even *Aedes albopictus* (Skuse), the eggs of which were found to tolerate water losses of only 25-27% (Urbanski et al. 2010). Most of the lost weight was assumed to be water. Egg survival was dependent upon two main factors: 1) the amount of water lost from the egg (reflecting desiccation time) and 2) the level of development of the egg. Weight loss was rapid over the first 24 h. It appeared that little or no additional water was lost after 48 h, and that the very low hatch rate (viability of

some embryos) was also maintained at least out to 60 h. We therefore have not yet reached the maximum limits of how long some of the *C. sonorensis* eggs may be viable in their desiccated state. Most eggs of this species, even if older and relatively capable of tolerating substantial drying, were killed by desiccation, and 69.0 and 88.0% failed to tolerate 24 h of drying in trials one and two respectively. Still, while most eggs were killed, it could be significant that even a small proportion can tolerate such severe treatment.

Our observation that eggs must reach a certain point in development to achieve this tolerance supports observations of *C. pulicaris* (L.) and *C. punctatus* (Meigen) in England by Parker (1950). He exposed groups of 20 eggs (aged 0-5 d) of *C. pulicaris* and *C. punctatus* (referred to in his paper as *C. pulicaris punctatus*) to severe and rapid drying in a calcium chloride desiccator (32% RH) for 12, 18, 24 and 48 h. Eggs that were treated immediately after being laid experienced complete mortality at all time points, whereas some eggs that were 1 d old or older survived. For *C. pulicaris,* excluding the youngest eggs, hatch rates were the highest for eggs desiccated for 12 h (mean: 80.8%), and decreased as desiccation time increased, with 11.2% hatch after 18 h, and 5.50% hatch after 24 h. For *C. punctatus*, again excluding the youngest eggs, hatch rates were the highest after 12 h (mean: 91.2%) and decreased to 21.0% after 24 h. For both species, he observed survival increase with egg age, with complete mortality after 48 h of drying.

Egg survival of other adverse conditions also increases with age. For example, *C. sonorensis* eggs are only able to tolerate a salinity level of 34.0 $^{o}/_{oo}$ (equivalent to 100% seawater) after they are tanned (Linley 1986). None of the 4-10 h old *C. sonorensis* eggs

in our experiment hatched even after a relatively short 12 h of desiccation, which caused less water loss (trial 2: 36.9 ± 10.5%) than experienced by some older eggs desiccated for 48 h (lost 57.8 ± 2.58% water). However, the 4-10 h old eggs did lose more water over 12 h of desiccation than the 28-34 h old eggs did over the same time period (22-24%). The younger eggs in our experiment appeared to be fully sclerotized, but it is possible that the chorion was either relatively permeable to water loss or that the younger embryo was relatively more susceptible to damage.

We attempted to test whether some of the reduction in hatch rate of the older eggs desiccated for 12 h could be explained by age variation in the groups of 28-34 h old eggs (i.e., perhaps eggs that were closer to 28 h old did not hatch while those that were closer to 34 h old did). We examined the desiccation tolerance of eggs at 25, 30, 35 and 40 h of age. In these trials, both the raw water loss and hatch data gave no indication of one age being more or less tolerant than the others; all could tolerate water loss fairly well. The significant differences between the hatch rates in these trials were likely due to natural variation in hatch rate and might reflect more variable mating efficiency in those groups of insects in the laboratory.

We observed a relatively wide range in the hatch rates of the control eggs (41.3 - 82.0%). Similar variation has also been noted in other experiments involving hatch rates of *Culicoides* eggs. In colony, *C. sonorensis* hatch rates range from approximately 75-95% (Hunt and Tabachnick 1995). The maximum and average hatch rates of eggs from field-collected *C. obsoletus* (Meigen) and *C. impunctatus* Goetghebuer are 82.2 and 44.9%, and 89.6 and 50.5%, respectively (Service 1968). The average hatch rates for *C.*

furens (Poey), *C. hollensis* (Melander and Brues), and *C. melleus* (Coquillett) range from 45.5-90.4% (Koch and Axtell 1978). Natural variation in the response to desiccation was also evident in the differences between trials in our experiment; however, the relative responses were the same.

Regardless, we can conclude that the important developmental point for *C. sonorensis* desiccation tolerance must occur somewhere between 10 and 25 h post oviposition. This observation that desiccation tolerance requires a minimum level of development is supported by work with mosquitoes. In Culicidae, desiccation tolerance is correlated with development of the serosal cuticle, which occurs between 11-13 h post oviposition in *Aedes aegypti* (L.) (Rezende et al. 2008), and 8-14 h in *Anopheles gambiae* Giles (Goltsev et al. 2009). Surface lipids on the chorion have also been implicated in egg desiccation tolerance in mosquitoes (Urbanski et al. 2010).

During the course of the experiment, we noticed that some of the eggs that did not hatch failed to do so because the shells were physically shredded. This damage must have occurred after the eggs had been set up to rehydrate and hatch, because eggs were normally intact immediately after they were placed on the moist substrate. This damage may have been due to excessively rapid reabsorption of water. We tested this hypothesis in the RH gradient experiment, and transferred eggs from 75% to 85% RH before exposing them to 100% RH (saturated conditions). The eggs ultimately (over 36 h) lost water at a level comparable to eggs that had been desiccated at 75% for 24 h. However, their net water loss was similar to that of eggs that had only been desiccated for 12 h. Eggs hatched at a rate comparable to those eggs desiccated at 75% RH for 12 h, despite

being in an environment free of standing water for 36 h. Had these eggs been set up to hatch before the final 24-36 h/100% RH interval, we can assume that they would have hatched at the same rate as the 24 h eggs. The desiccation tolerance mechanism may be more effective with a more natural, gradual reabsorption of water than that experienced by our experimental eggs.

Culicoides eggs are small and cryptic, and so have been difficult to find in the field. Very little information about oviposition can be derived from the few instances where they have been recovered. In one instance, *C. sonorensis* eggs were collected from the edge of a southern California dairy wastewater pond, 6 cm back from waterline (Mullens and Lii 1987). In the laboratory, field collected, gravid *C. vexans* (Staeger) lay their eggs singly, and occasionally in groups, as do members of the *obsoletus* and *pulicaris* groups (Parker 1950, Jobling 1953). *Culicoides paraensis* (Goeldi) females lay their eggs singly, as they walk across the substrate in the laboratory (Roberts et al. 1977). We observed very similar behavior in our experiment.

To our knowledge, this is the most detailed attempt to look at oviposition patterns in *Culicoides*. We observed that *C. sonorensis* females preferred to oviposit away from the waterline, with most of the females laying their eggs at or near the top of the mud arena. This was contrary to what we expected, because the risk of desiccation should be greater further away from the waterline. However, in colony, *C. paraensis* do not lay their eggs on the water surface, except in rare cases (Roberts et al. 1977). We have anecdotally observed that too much moisture may be detrimental to egg survival. In one case where a female had oviposited on the surface film on the water in the arena in our

experiment, the eggs appeared to be split, similar to what was observed in the desiccation experiments after rapid rehydration. It is also possible that the eggs of *Culicoides* are unable to attach to the substrate when there is too much standing water (Becker 1961). We did not investigate this possibility.

We classified the insects in the experiment as belonging to one of two behavioral groups: meanderers and dumpers. Meanderers were females that laid the majority of their eggs singly, along a distinct path, with individual eggs oriented parallel or perpendicular to the waterline. Dumpers were females that laid the majority of their eggs in dense groups, with the orientation of the eggs in each group being more or less the same. Of the 75% of females that oviposited, most were classified as meanderers, supporting earlier observations on *Culicoides* oviposition. Dumping appears to be a much less common behavior and was not observed at all in trial two. Egg dumping has been observed as an emergency reaction to stress (i.e. decapitation) (Linley 1965), and this appears to have been the case with at least one female that was found stuck in the mud next to her clutch. However, we also saw dumpers that laid their eggs in two distinct groups, indicating that the female must have been able to change its position during oviposition.

When thinking about desiccation tolerance as a means of surviving adverse conditions, we should consider the type of habitat in which *C. sonorensis* likely evolved. Obviously, *C. sonorensis* has not always utilized man-made dairy wastewater ponds as oviposition and larval development sites. The species probably originally took advantage of temporary water sources, such as rivulets or puddles, which could dry out and return

quickly. In these types of ephemeral habitats, egg desiccation tolerance would be an advantage.

Culicoides sonorensis activity is mostly crepuscular or nocturnal, with a peak in gravid female flight between sunset and the end of evening nautical twilight (Akey and Barnard 1983). The presence of sac stage relics in females of the closely related *C. variipennis* (Coquillet) caught during the night and at dusk similarly suggests nocturnal oviposition (Mullens and Schmidtmann 1982, Mullens and Rutz 1984). Eggs laid overnight, and especially at dusk, would likely not be subjected to harsh temperatures and desiccation until the embryos were more fully developed. Nocturnal oviposition may be a way of protecting the youngest, and most susceptible, embryos from desiccation. We have yet to determine the limits of how long *C. sonorensis* embryos remain viable in their desiccated state, and future studies should be done to evaluate the potential importance of desiccation resistance as a mechanism to survive unfavorable periods.

REFERENCES

Akey, D. H., and D. R. Barnard. 1983. Parity in airborne populations of the biting gnat *Culicoides variipennis* (Diptera: Ceratopogonidae) in northeastern Colorado. Environ. Entomol. 12: 91-95.

Baba, M., and H. Takaoka. 1992. Dormancy, embryogenesis, and hatching of eggs of *Prosimulium kiotoense* (Diptera: Simuliidae) under natural and experimental conditions. J. Med. Entomol. 29: 430-435.

Bader, C. A., and C. R. Williams. 2011. Eggs of the Australian saltmarsh mosquito, *Aedes camptorhynchus*, survive for long periods and hatch in instalments: implications for biosecurity in New Zealand. Med. Vet. Entomol. 25: 70-76.

Becker, P. 1961. Observations on the life cycle and immature stages of *Culicoides circumscriptus* Kieff. (Diptera: Ceratopogonidae). Proc. Roy. Soc. Edinb. Sect. B. Biol. 67: 363-386.

Borkent, A., and D. A. Craig. 2004. *Austroconops* Wirth and Lee, a lower Cretaceous genus of biting midges yet living in western Australia: a new species, first description of the immatures and discussion of their biology and phylogeny (Diptera: Ceratopogonidae). Am. Mus. Nov. 3449: 1-67.

Breidenbaugh, M. S., and B. A. Mullens. 1999. Descriptions of immature stages of six *Culicoides* Latreille spp. (Dipetera: Ceratopogonidae) from desert mountain ranges in southern California, with notes on life histories and rearing techniques. Proc. Entomol. Soc. Wash. 101:839-867.

Convey, P. 1992. Aspects of the biology of the midge, *Eretoptera murphyi* Schaeffer (Diptera: Chironomidae), introduced to Signy Island, maritime Antarctic. Polar Biol. 12: 653-657.

Gerry, A. C., B. A. Mullens, N. J. Maclachlan, and J. O. Mecham. 2001. Seasonal transmission of bluetongue virus by *Culicoides sonorensis* (Diptera: Ceratopogonidae) at a southern California dairy and evaluation of vectorial capacity as a predictor of bluetongue virus transmission. J. Med. Entomol. 38: 197-209.

Goltsev, Y., G. L. Rezende, K. Vranizan, G. Lanzaro, D. Valle, and M. Levine. 2009. Developmental and evolutionary basis for drought tolerance of the *Anopheles gambiae* embryo. Dev. Biol. 330: 462-470.

Glukhova, V. M. 1979. Larvae of biting midges of the subfamilies Palpomyiinae and Ceratopogoninae of the USSR fauna (Diptera, Ceratopogonidae = Heleidae), pp. 1-85. In Keys to USSR Fauna. Zoological Institute, Academy of Sciences, Leningrad, Russia.

Hair, J. A., and E. C. Turner Jr. 1966. Laboratory colonization and mass-production procedures for *Culicoides guttipennis*. Mosq. News. 26: 429-433.

Holmes, L. A., S. L. Vanlaerhoven, and J. K. Tomberlin. 2012. Relative humidity effects on the life history of *Hermetia illucens* (Diptera: Stratiomyidae). Environ. Entomol. 41: 971-978.

Hunt, G. J. 1994. A procedural manual for the large-scale rearing of the biting midge *Culicoides variipennis* (Diptera: Ceratopogonidae). U. S. Dept. of Agric., Agric. Res. Serv, ARS-121, 68 pp.

Hunt, G. J., and W. J. Tabachnick. 1995. Cold storage effects on egg hatch in laboratory-reared *Culicoides variipennis sonorensis* (Diptera: Ceratopogonidae). J. Amer.. Mosq. Cont. Assoc. 11: 335-338.

Isaev, V. A. 1975. Photoperiodic induction of the diapause at the egg phase in the blood sucking midge *Culicoides pulicaris punctatus* (Diptera: Ceratopogonidae). Parazitologiya. 9: 501-506.

Jobling, B. 1953. On the blood-sucking midge *Culicoides vexans* Stager, including the description of its eggs and the first-stage larva. Parasitol. 43: 148-159.

Kettle, D. S. 1977. Biology and bionomics of bloodsucking Ceratopogonids. Ann. Rev. Entomol. 22: 33-51.

Koch, H. G., and R. C. Axtell. 1978. Autogeny and rearing of *Culicoides furens, C. hollensis* and *C. melleus* (Diptera: Ceratopogonidae) from coastal North Carolina. Mosq. News. 38: 240-244.

Linley, J. R. 1965. The ovarian cycle and egg stage in *Leptoconops (Holoconops) becquaerti* (Kieff.) (Diptera: Ceratopogonidae). Bull. Entomol. Res. 56: 37-56.

Linley, J. R. 1986. The effect of salinity on oviposition and egg hatching in *Culicoides sonorensis* (Diptera: Ceratopogonidae). J. Am. Mosq. Control Assoc. 2: 79-82.

Lounibos, L. P., R. L. Escher, and N. Nishimura. 2011. Retention and adaptiveness of photoperiodic egg diapause in Florida populations of invasive *Aedes albopictus*. J. Amer. Mosq. Cont. Assoc. 27: 433-436.

Mellor, P. S., J. Boorman, and M. Baylis. 2000. *Culicoides* biting midges: their role as arbovirus vectors. Annu. Rev. Entomol. 45: 307-340.

Minakawa, N., J. I. Githure, J. C. Beier, and G. Yan. 2001. Anopheline mosquito survival strategies during the dry period in western Kenya. J. Med. Entomol. 38: 388-392.

Mullen, G. R., and L. J. Hribar. 1988. Biology and feeding behavior of Ceratopogonid larvae (Diptera: Ceratopogonidae) in North America. Bull. Soc. Vector Ecol. 13: 60-81.

Mullens, B. A., and K. Lii. 1987. Larval population dynamics of *Culicoides variipennis* (Diptera: Ceratopogonidae) in Southern California. J. Med. Entomol. 24: 566-574.

Mullens, B. A., and D. A. Rutz. 1984. Age structure and survivorship of *Culicoides variipennis* (Diptera: Ceratopogonidae) in central New York state, USA. J. Med. Entomol. 21: 194-203.

Mullens, B. A., and E. T. Schmidtmann. 1982. The gonotrophic cycle of *Culicoides variipennis* (Diptera: Ceratopogonidae) and its implications in age-grading field populations in New York state, USA. J. Med. Entomol. 19: 340-349.

Napp, S., S. Gubbins, P. Calistri, A. Allepuz, A. Alba, I. Garcia-Bocanegra, A. Giovannini, and J. Casal. 2011. Quantitative assessment of the probability of

bluetongue virus overwintering by horizontal transmission: application to Germany. Vet. Res. 42: 1-10.

O'Rourke, M. J., E. C. Loomis, and D. W. Smith. 1983. Observations on some *Culicoides variipennis* (Diptera: Ceratopogonidae) larval habitats in areas of bluetongue virus outbreaks in California. Mosq. News. 43: 147-152.

Parker, A. H. 1950. Studies on the eggs of certain biting midges (*Culicoides* Latreille) occurring in Scotland. Proc. R. Ent. Soc. Lond. 25: 43-52.

Rees, D. M., P. G. Lawyer, and R. N. Winget. 1971. Colonization of *Leptoconops kerteszi* Kieffer by anautogenous and autogenous reproduction (Diptera: Ceratopogonidae). J. Med. Entomol. 8: 266-271.

Rezende, G. L., A. J. Martins, C. Gentile, L. C. Farnesi, M. Pelajo-Machado, A. A. Peixoto, and D. Valle. 2008. Embryonic desiccation resistance in *Aedes aegypti*: presumptive role of the chitinized serosal cuticle. BMC Dev. Biol. 8: 1-14.

Roberts, D. 2004. Prolonged survival of eggs of the rock-pool mosquito, *Aedes vittatus*, in the extreme heat of the Arabian peninsula. J. Arid. Environ. 57: 63-70.

Roberts, D. R., F. de P., Pinheiro, A. L. Hoch, J. W. LeDuc, N. E. Peterson, M. A. Vasconcelos Santos, and K. A. Western. 1977. Vectors and natural reservoirs of oropouche virus in the Amazon region. U.S. Army Medical Research and Development Command. Washington, D.C.

Ronderos, M. M., G. R. Spinelli, and F. Diaz. 2006. Observations on the immatures of *Dasyhelea necrophila* Spinelli & Rodriguez in laboratory (Diptera: Ceratopogonidae). Trans. Amer. Entomol. Soc. 132: 291-297.

Service, M. W. 1968. Blood digestion and oviposition in *Culicoides impunctatus* Goetghebuer and *C. obsoletus* (Meigen) (Diptera, Ceratopogonidae). Ann. Trop. Med. Parasitol. 62: 325-330.

Shipp, J. L. 1987. Diapause induction of eggs of *Simulium arcticum* Malloch (IIS-10.11) (Diptera, Simuliidae). Can. Entomol. 119: 497-499.

Sota, T., and M. Mogi. 1992. Survival time and resistance to desiccation of diapause and non-diapause eggs of temperate *Aedes* (*Stegomyia*) mosquitoes. Entomol. Experimen. App. 63:155-161.

Stoehr, S. 1994. Respiration and habitat adaptation of winter-dormant eggs of the blackfly *Simulium noelleri* Friederichs, 1920 (Diptera: Simuliidae). Zeitschrift fur Angewandte Zoologie. 80: 181-197.

Urbanski, J. M., J. B. Benoit, M. R. Michaud, D. L. Denlinger, and P. Armbruster. 2010. The molecular physiology of increased egg desiccation resistance during diapause in the invasive mosquito, *Aedes albopictus*. Proc. R. Soc. B. 277: 2683-2692.

Vaughan, J. A. and E. C. Turner. 1987. Development of immature *Culicoides variipennis* (Diptera: Ceratopogonidae) from Saltville, Virginia at constant laboratory temperatures. J. Med. Entomol. 24: 390-395.

Winston, P. W. and D. H. Bates. 1960. Saturated solutions for the control of humidity in biological research. Ecol. 41: 232-237.

TABLES AND FIGURES

Table 1.1 Mean hatch rate (± SD) and water loss (± SD) of 4-10 h old eggs desiccated for 12-48 h in trials 1 and 2.

Trial	Time of Desiccation (h)	Mean Hatch Rate (SD) (%)	Mean Water Loss (SD) (%)
1	12	0.00[a]	N/A
	24	0.00[a]	N/A
	36	0.00[a]	N/A
	48	0.00[a]	N/A
	Pooled Control	67.8 (13.9)[b]	
2	12	0.00[a]	36.9 (10.5)[a]
	24	0.00[a]	31.5 (4.23)[a]
	36	0.045 (0.41)[a]	32.3 (7.92)[a]
	48	0.00[a]	31.5 (11.8)[a]
	Pooled Control	67.8 (13.9)[b]	

Letters indicate significant differences ($p \leq 0.05$) between groups.

Table 1.2 Mean hatch rate (± SD) and water loss (± SD) of 28-34 h old eggs desiccated for 48-60 h. Trials 1 and 2 are pooled.

Time of Desiccation (h)	Mean Hatch Rate (SD) (%)	Mean Water Loss (SD) (%)
48	2.61 (1.99)[a]	57.8 (2.58)[a]
52	1.00 (0.51)[a]	60.0 (2.01)[ab]
56	1.14 (0.84)[ab]	58.6 (2.76)[ab]
60	0.50 (0.29)[b]	60.9 (2.70)[b]
Pooled control	67.8 (13.9)[c]	

Letters indicate significant differences ($p \leq 0.05$) between groups.

Table 1.3 Mean hatch rate (± SD) and water loss (± SD) of 25-40 h old eggs desiccated for 12 h in trials 1 and 2.

Trial	Age of Eggs (h)	Mean Hatch Rate (SD) (%)	Mean Water Loss (SD) (%)
1	25	57.8 (10.3)^{ab}	19.2 (1.61)^{a}
	30	38.7 (9.09)^{b}	27.4 (5.48)^{b}
	35	56.3 (8.96)^{ab}	20.9 (1.94)^{ac}
	40	47.8 (5.20)^{bc}	25.4 (3.42)^{bc}
	Pooled control	67.8 (13.9)^{a}	
2	25	56.0 (8.06)^{ad}	23.0 (3.64)^{a}
	30	24.2 (2.51)^{b}	32.1 (3.18)^{b}
	35	30.8 (4.54)^{bc}	34.0 (0.84)^{b}
	40	43.9 (11.8)^{ac}	33.8 (2.66)^{b}
	Pooled control	67.8 (13.9)^{d}	

Letters indicate significant differences ($p \le 0.05$) between groups.

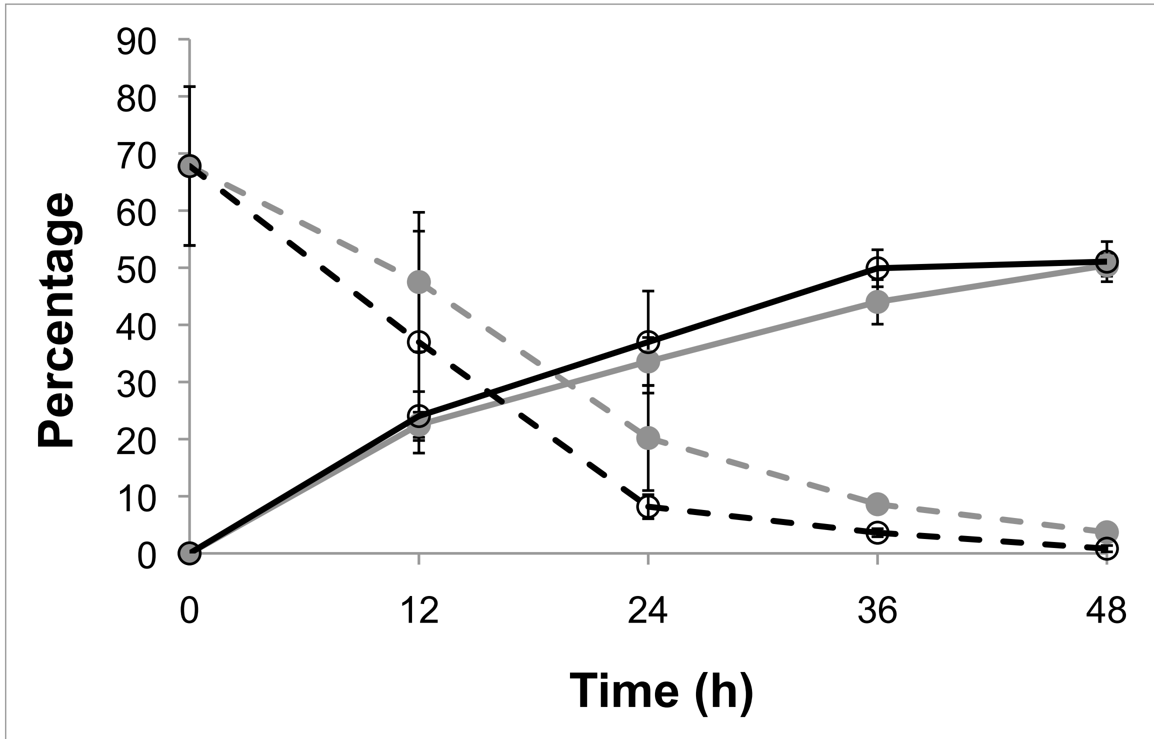

Figure 1.1 Mean water loss (%) (solid lines) and mean hatch rate (%) (dashed lines) of 28-34 h old eggs desiccated for 12-48 h. Trial 1 is represented by gray lines with closed circles and trial 2 is represented by black lines with open circles. Error bars represent standard deviations.

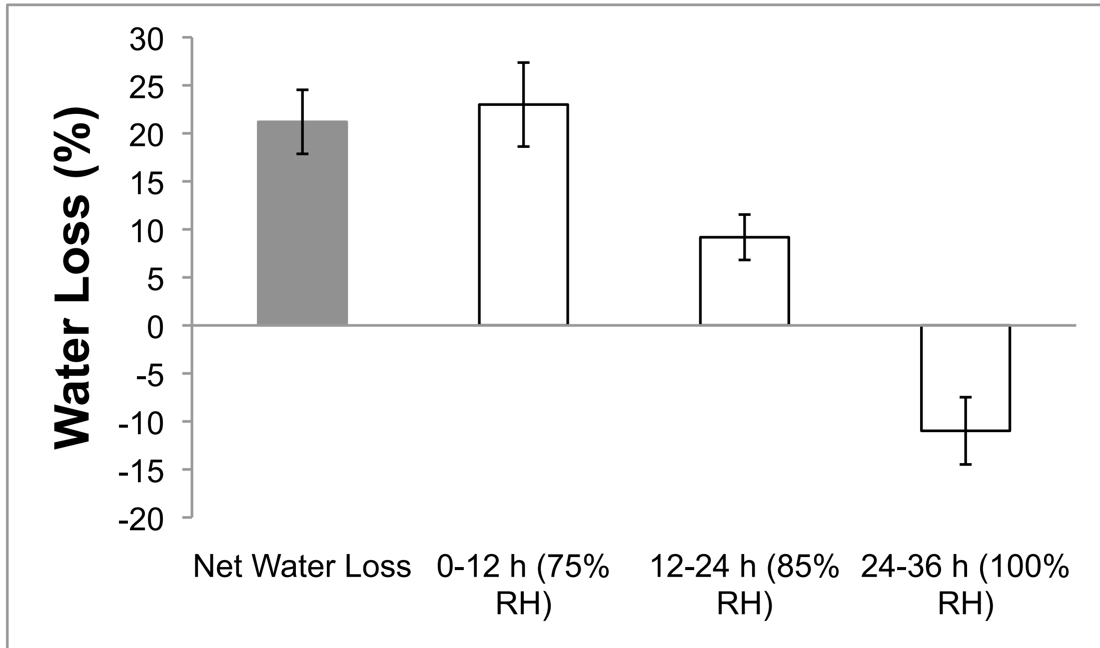

Figure 1.2 Mean water loss (%) of 28-34 h old eggs subjected to a RH gradient at each 12 h interval. The gray bar represents the net mean water loss experienced by the eggs over 36 h. Bars with the same letter are not statistically different ($p>0.05$). Error bars represent standard deviations.

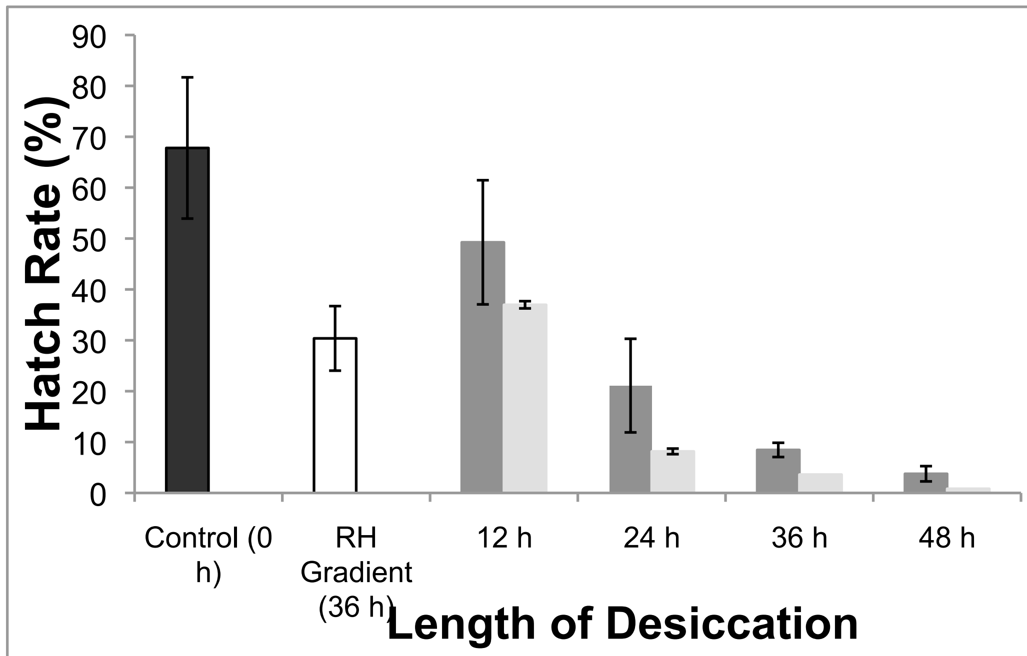

Figure 1.3 Comparison of the mean hatch rates (%) of the pooled control eggs and eggs subjected to a RH gradient to trials 1 and 2 of the desiccation time experiment (28-34 h old eggs, desiccated for 12-48 h). Dark gray bars represent trial 1, light gray bars represent trial 2. Bars with the same letter are not statistically different ($p>0.05$). Use capital letters to compare trial 1 or lowercase letters to compare trial 2. Error bars represent standard deviations.

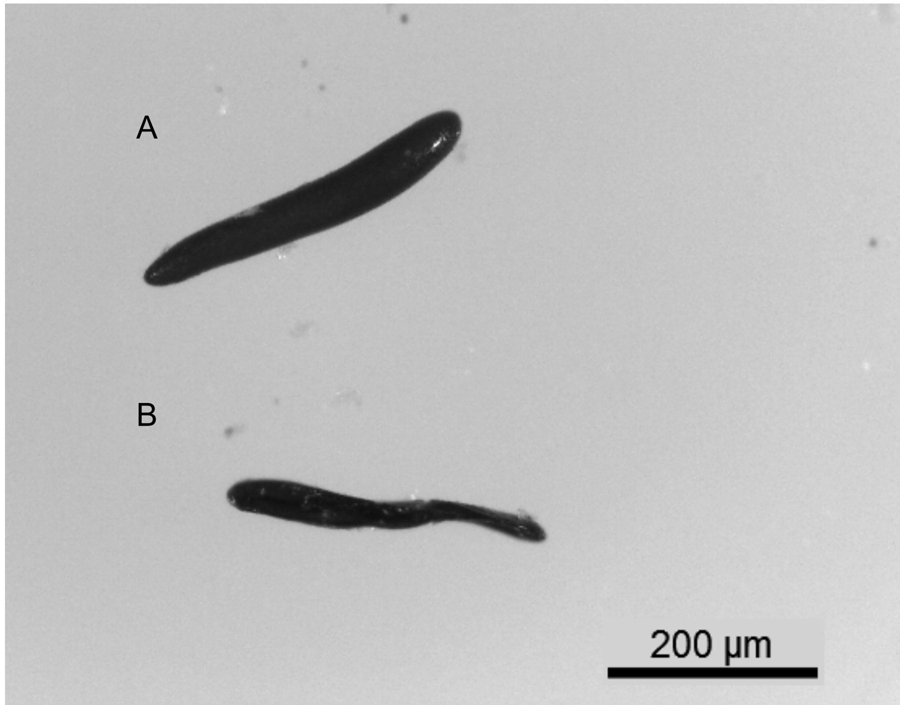

Figure 1.4 Physical effect of desiccation on the eggs of *C. sonorensis*. (A) Intact egg. (B) Desiccated, collapsed egg.

CHAPTER 2

Low temperature tolerance of *Culicoides sonorensis* eggs, larvae, and pupae from temperate and subtropical climates

Emily G. McDermott[1], Christie E. Mayo[2], and Bradley A. Mullens[1]

[1]Department of Entomology, University of California, Riverside, CA 92521
[2]Department of Microbiology, Immunology & Pathology, Colorado State University, Fort Collins, CO 80523

ABSTRACT

Culicoides sonorensis Wirth and Jones biting midges are the primary North American vectors of bluetongue virus (BTV), which infects domestic ruminant livestock and can cause high morbidity and mortality. Both virus and vector exhibit highly seasonal activity patterns, even in subtropical climates like southern California. However, the exact mechanism of BTV/*Culicoides* overwintering has been debated. In this study, we examined the supercooling point (SCP) and lower lethal temperature (LLT) of a laboratory colony of *C. sonorensis* eggs, larvae, and pupae, as well as of field collected larvae and pupae from subtropical (California) and temperate (Colorado) climates. Larvae and pupae succumbed to temperatures higher than their respective SCPs, indicating death from pre-freezing cold injury. Eggs were the most cold-tolerant life stage, and were able to tolerate 1 h exposures to temperatures as low as -20°C without suffering complete mortality. Larvae were the least cold-tolerant life stage and suffered complete mortality at <-4°C, while temperatures of -9 to -10°C were required to kill all pupae. Larvae and embryos suffered chronic cold injury after exposure to subzero temperatures, which increased mortality. Field collected larvae succumbed to slightly higher temperatures (>-3°C) than colony larvae, and did not differ significantly in their survival. *Culicoides sonorensis* immatures did not cold harden when exposed to 4°C for 24 h before freezing. Results of this study indicate that *C. sonorensis* eggs may be more important in vector overwintering than previously thought. Further work is needed on *C. sonorensis* winter survival in the field to corroborate laboratory studies.

INTRODUCTION

In the last decade, a number of tropical vector-borne diseases have expanded their ranges across the globe, causing large-scale outbreaks (e.g. dengue and Zika viruses), and occasionally pushing into temperate zones (Roth et al. 2014, Bogoch et al. 2016). Although global trade and travel have no doubt played a significant role in transporting both pathogens and vectors, there is concern that rising global temperatures are allowing vector insects to persist and transmit pathogens in areas that previously would have been unsuitable for their survival (Purse et al. 2005, Purse et al. 2015).

To assess transmission risk of tropical pathogens in temperate zones, models may incorporate factors such as adult vector temperature tolerance thresholds, extrinsic incubation period, and climate variables (Rogers 2006, Gubbins et al. 2008). These models aim to predict where vectors may establish and transmit pathogens. However, such models are limited in their ability to predict long-term shifts in epidemiology because they typically do not include data on the developmental or tolerance thresholds of insect vector immature stages. Including variables on the environmental tolerances of eggs, larvae, and pupae will greatly improve the ability of these models to predict accurately where vector-borne pathogens will be transmitted on a persistent, seasonal basis.

Pathogens that are transmitted in both tropical and subtropical zones, which border temperate zones, are of particular concern for range expansion, especially when potentially competent vector species may already exist in those areas. Bluetongue virus (BTV) is a good example of such a pathogen. Transmitted by *Culicoides* biting midges,

BTV infects domestic and wild ruminants (e.g. cattle and sheep), causing high morbidity and mortality in susceptible animals (Purse et al. 2015). Introduction of the pathogen into northern Europe in 2006 resulted in a widespread, multi-year epizootic, driven by novel vectors, far above the pathogen's earlier supposed range limits (Sellers and Mellor 1993, Melhorn et al. 2007). The risk of BTV expansion in Europe had been considered serious even prior to the outbreak, but predicative capacity of models was crippled by lack of sufficient biological data (Carpenter et al. 2009). European livestock were completely naïve to the virus, and countries unprepared for an outbreak of BTV were unable to keep its spread under control. Prior to 2006, it was thought that the primary vector driving a persistent, European outbreak would be the well known Afrotropical species, *C. imicola* Kieffer, spreading northwards from the Mediterranean (Gibbs and Greiner 1994). However, it was later shown that endemic north-temperate zone *Culicoides* spp., especially *C. obsoletus* Meigen and *C. pulicaris* L., were responsible for the majority of local European transmission (Ganter 2014).

In North America, BTV is endemically transmitted by *C. sonorensis* Wirth and Jones in the southern and western parts of the United States (U.S.), but both virus and vector are mostly absent from the northeast and Canada (Holbrook et al. 2000, Ruder et al. 2015). In the U.S., *C. sonorensis* inhabits both subtropical regions with extremely mild winters (e.g. southern California), and temperate regions with harsh winters (e.g. Colorado's front range). Few details are known about exactly how species in this group overwinter in either climate. It is assumed that larvae are the main overwintering stage (Vaughan and Turner 1987, Mullens and Lii 1987), although no previous studies have

examined the low temperature tolerances of the immature stages of any *Culicoides* species in detail. Understanding the biology and limits of the environmental tolerance of *Culicoides* immature stages is critical to our understanding of BTV distribution in both time and space (Mullens et al. 2015).

The goals of the present study were to determine 1) lower lethal temperature and 2) supercooling point for eggs, larvae and pupae of *C. sonorensis* from a colony originally established from southern California. We also evaluated the survival of field-collected larvae from subtropical (southern CA) and temperate (northern CO) populations when exposed to low temperatures. We discuss implications for *C. sonorensis* trans-seasonal persistence, and how these data may be used to better predict the potential range of BTV in the light of climate change.

MATERIALS AND METHODS

Lower lethal temperature experiments: Methods necessarily differed slightly for eggs, larvae, and pupae. The general methods for the lower lethal temperature (LLT) experiments are described first, and specifics for each life stage follow. The temperature ranges for each stage were determined from preliminary laboratory studies and were designed to generate a useful range of mortalities that could be subjected to probit analyses to obtain LLT values.

Groups of eggs (approximately 24 h old), L4 larvae, and pupae (approximately 24 h old) from a laboratory colony of *C. sonorensis* (VR colony) were used for the LLT experiments. For each temperature, six replicates per sub-zero temperature treatment

were used. Fifty pupae, 25 larvae, or approximately 30 eggs were used per replicate. In each experiment, two temperature pre-treatments were used. Insects were either held at 27°C (ambient laboratory rearing conditions) or 4°C for 24 h before exposure to sub-zero temperatures. Mud collected from a local dairy wastewater pond (San Jacinto, CA, USA) was used for experiments. Mud was frozen at -20°C for 2-7 d prior to use in order to kill any insects present. Mud was briefly inspected before experiments to ensure no *Culicoides* larvae were present. Experimental insects were held on ~5 ml of this natural substrate in a thin layer (~5 mm deep) in a 118 ml screw top, plastic specimen cup (4MD Medical Solutions, Lakewood, NJ, USA). Any standing water was removed using a pipette. Lids of the cups were screwed on, and then sealed with a strip of Parafilm M® (Bemis Co., Neenah, WI, USA). Cups were attached to a weighted platform using Velcro, and were submerged into a recirculating ethylene glycol chill bath (Model 2006, Forma Scientific, Marietta, OH, USA). The bath was set to a given temperature. After the bath reached that temperature, insects were submerged for 1 h. Cups were then removed from the bath, and insects were returned to ambient laboratory conditions.

Pupae: Pupae were exposed to 0.0, -5.0, -7.0, -9.0, and -11.0°C. Six replicates per temperature of pupae previously held at 27°C and another six held at 4°C for 24 h were used as controls, and were not subjected to sub-zero temperatures. After being removed from the bath, the cups with mud were placed into individual 473 ml cardboard containers with fine mesh lids. Pupae were allowed to continue to develop in the saturated mud substrate. Pupae were held at 27°C for 4 d to complete emergence. After 4

d, cups were frozen at -20°C to kill any live adults. The number of emerged adults was counted and recorded.

Larvae: Larvae were exposed to -3.0, -3.2, -3.5, -3.8, and -4.5°C. Six replicates per temperature of larvae held at 27°C and another six held at 4°C for 24 h were used as controls, as above. After being removed from the bath, mud and larvae were washed into containers using deionized water, and larvae were allowed 1 h to reacclimatize to ambient laboratory conditions. After 1 h, larvae were checked for initial survival. Larvae that were not moving and did not respond to physical stimulation (gentle prodding with a pipette tip) were considered dead. Live larvae from each replicate were transferred as a group into a 55 mm diameter x 20 mm deep petri dish with a 6.3 cm^2 piece of hi-loft polyester batting. Larvae in petri dishes were placed into individual 473 ml cardboard containers with fine mesh lids. Deionized water, supplemented with water from a *C. sonorensis* colony pan to provide nutrition, was added to the level of the top of the batting square each day. Larvae were held at 27°C for 6 d to complete development and emergence. After 6 d, cups were frozen as above, and emerged adults were counted. Due to variation in larval survival between replicates, the larvae experiment was repeated twice.

Eggs: Eggs were exposed to -10, -12, -15, -17, and -20°C. Six replicates of ~30 eggs held at 27°C and six replicates of ~30 eggs held at 4°C for 24 h were used as controls, as above, and all temperature comparisons were made against this baseline (control) egg hatch and survival. Due to logistic and space limitations, all replicates of eggs previously held at 27°C were exposed to sub-zero temperatures on one day, and all replicates of eggs held at 4°C were exposed the next day. Eggs held at 4°C were then

approximately 24 h older than 27°C eggs from the same cohort at the time of freezing, although that extra day was spent at a low temperature (4°C), and thus eggs were probably in developmental stasis. Eggs used for each trial were from separate cohorts, and egg fertility is known to vary somewhat among cohorts (Hunt and Tabachnick 1995, McDermott and Mullens 2014). After being removed from the chill bath, eggs were gently removed from the surface of the mud and transferred to a 7 mm filter paper circle on top of polyester batting in a petri dish (as in larvae experiment). The exact number of eggs used per replicate was counted at this time (mean: 30.7 ± 0.55 (SE)). Petri dishes were then placed into individual containers and held as above. Due to space limitations in the colony room where larvae and pupae cups were held, egg cups were held in a separate room at 25 ± 3°C. Fluorescent lights were suspended 4-5 inches above the cups on a lab bench, to mimic colony room conditions. Cup position on the bench was randomized to account for any possible spatial effects. At 14 d post-exposure, eggs were examined under a dissecting microscope, and the number of eggs that had successfully hatched was counted. Cups were monitored daily for pupation, and each cup was given 6 d from the day of first pupation to complete development and emergence. After 6 d, cups were frozen as above, and emerged adults were counted. Due to variation in final survival, the egg experiment was repeated three times. Egg hatch data from all three trials were used for analysis of initial egg mortality. Due to extremely low larval survival in all cups, including controls, in trial two, all other analyses include only trials one and three.

All analyses were conducted in R (version 3.2.0) For eggs and larvae, effect of trial was examined using a general linear model. If there was no significant interaction

between trial and treatment, data for both trials were pooled for analysis. Differences between controls and treatments for a given temperature were analyzed using Kruskal-Wallis rank sum test or analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test. For eggs, an additional ANOVA was done on the effect of temperature on initial mortality for all eggs (27 and 4°C pre-treatments pooled). Lower lethal temperature for a given proportion of the population of colony eggs, larvae, and pupae was calculated using a probit model and the dose.p function ('MASS' package). Probit model goodness of fit was determined by comparison to a null model using log likelihood and chi-square analysis (Table 2.1). If control mortality was greater than ~10%, Abbott's correction was used for probit models (Abbott 1925).

Supercooling point experiments: Possibly due to the small size of *C. sonorensis* eggs, our instrument was unable to successfully detect the release of the latent heat of fusion (LHF) associated with egg tissue freezing, so SCP was calculated only for pupae and larvae. A single pupa or L4 larva was attached to a copper-constantan (type T) thermocouple using a small amount of petroleum jelly. The thermocouple was then held suspended in air inside of a 32 ml, 1.7 cm diameter glass test tube. A second thermocouple was also held adjacent and inside the tube to measure the ambient temperature of the probe tip. The tube was held inside of a 22.5 cm^3 insulated box that contained a flat piece of dry ice approximately 0.5 kg (covering the bottom of the box to a depth of ~5.5 cm). The tube was suspended 2.5 cm above the dry ice. A third thermocouple measured the ambient temperature inside the box. Once inside the box, the cooling rate inside the test tube was about 1.5°C/min (Salt 1966a, Salt 1966b). The

thermocouples were attached to a thermocouple and voltage data acquisition system (DI-245, Dataq Instruments, Akron, OH, USA), which measured and recorded temperature at a rate of 200 samples/min. The release of the LHF was visualized as a distinct peak in the temperature readout from the thermocouple. The SCP for each sample was recorded as the temperature immediately preceding the release of the LHF. Supercooling points for larvae and pupae held at 27°C (larvae n=42, pupae n=20) and 4°C (larvae n=40, pupae n=20) for 24 h were calculated as means and compared using ANOVA and Tukey's HSD.

Field-collected larvae: *Culicoides sonorensis* larvae were collected from dairy wastewater ponds in San Jacinto, CA (33°51'08.7" N, 117°01'15.2" W, elev. 450 m) and Greeley, CO (40°28'01.5" N, 104°49'12.4" W, elev. 1444 m) in October 2015 and were used for LLT and SCP experiments. These larvae represented subtropical and temperate populations of *C. sonorensis*, respectively. Mud containing larvae was transported back to the laboratory in Riverside, CA on ice. It was necessary to hold bags of mud at 4°C to keep larvae alive in the nutrient-rich substrate for approximately 1-5 d until they could be processed. Larvae were sorted from mud using either deionized water or $MgSO_4$ flotation, and then transferred to development pans. There they were allowed to reach the last larval instar (L4) for testing. *Culicoides sonorensis* is the only *Culicoides* species typically encountered on southern CA dairy farms (Gerry and Mullens 2000). Larvae from CO were identified as *C. sonorensis* by the large pharyngeal armature typical of the subgenus *Monoculicoides* (Blanton and Wirth 1979), and emerged adults were identified by wing pattern to confirm (Wirth et al. 1985). Due to the low number of CO field larvae

available, fewer replicates per treatment and fewer larvae per replicate were used in these experiments.

LLT: California field larvae were exposed to -2.0, -2.5, -3.0, and -3.2°C for 1 h. Four replicates of 25 larvae each, previously held at 27°C, per temperature were used, as well as four control (not exposed to sub-zero temperatures) replicates of 25 larvae, previously held at 27°C.

Because fewer CO larvae were available, we decided to compare CO and CA populations at only one critical sub-zero temperature. Based on the results of the CA larvae LLT experiments, six replicates of 10 CO larvae previously held at 27°C, six replicates of 10 CO larvae held at 4°C, and 10 replicates of 25 CA larvae held at 27°C were exposed to -2.5°C for 1 h. Five replicates of 10 CO larvae held at 27°C, three replicates of 10 CO larvae held at 4°C, and 10 replicates of 25 CA larvae held at 27°C were reared as controls. There was no significant difference in the survival of CO larvae held at 27°C or 4°C, so data from these replicates were pooled for analysis, for a total of 16 CO and 10 CA treatment replicates and 8 CO and 10 CA control replicates. Differences in proportional mortality of field larvae by freeze or control treatments were analyzed using Kruskal-Wallis rank sum test and Welch's t-test.

SCP: Supercooling points of field collected CA larvae (n=12) and pupae (n=10), and CO larvae (n=10) and pupae (n=6) held at 27°C, and CA larvae (n=10) and pupae (n=13), and CO larvae (n=10) held at 4°C were determined as above. Field larvae and pupae SCP was analyzed together with colony larvae and pupae SCP, using ANOVA and Tukey's HSD, as above.

RESULTS

Lower lethal temperature experiments: Lower lethal temperatures were always higher than SCP values, indicating that *C. sonorensis* immatures suffered damage at temperatures that did not actually freeze them. Pupal mortality increased with decreasing temperature, and mortality of pupae exposed to sub-zero temperatures was greater than that of control pupae for all temperatures tested ($F \geq 90.97$, df=3, $P \leq 0.001$) except for 0°C ($F=0.735$, df=3, $P=0.543$) (Fig. 2.1). There was no significant difference in mortality between 4°C and 27°C pre-treatment pupae exposed to any sub-zero temperature. Because control mortality was less than 10%, Abbott's correction was not used for the probit analysis. We observed ~99% mortality at -9.0°C, close to our predicted LLT_{99} of -9.31°C (Table 2.2).

For larvae and eggs, initial mortality after removal from the chill bath, subsequent larval mortality (surviving larvae that failed to pupate), subsequent pupal mortality (surviving pupae that failed to emerge), and final mortality (how many died out of the total starting number of insects) were calculated for each temperature tested. Initial mortality for control larvae was always 0% by default. There was no significant effect of pre-treatment (27 vs. 4°C) on mortality of larvae at any stage of development to the imago ($P \geq 0.134$). There was no significant difference in initial mortality between exposed and control larvae at -3.0°C and -3.2°C ($F \leq 1.578$, df=3, $P \geq 0.181$). Larvae were sensitive to small variations in sub-zero temperatures. Initial mortality after sub-zero exposure increased from not statistically different from controls at -3.2°C to 100% at -4.5°C (Fig. 2.2a). Larvae suffered from chronic cold injury after exposure to low

temperature. Larvae that initially survived exposure to temperatures lower than -3.2°C ultimately had significantly higher mortality than controls (Fig. 2.2b) ($F \geq 19.18$, df=3, $P \leq 0.001$). There was no difference in subsequent pupal mortality between control and sub-zero exposed larvae at any temperature (Fig. 2.2c) ($F \leq 0.809$, df=3, $P \geq 0.496$). Final mortality trends followed larval mortality trends (Fig. 2.2d). Because final control mortality was greater than 10%, Abbott's correction was used for probit analysis. We observed 99% final mortality at -3.8°C, slightly higher than our predicted LLT_{99} of -4.01°C (Table 2.2).

With the exception of one case (below), there was also no effect of pre-chilling (4°C for 24 h) on egg mortality at any stage of development ($P \geq 0.097$). There were significant effects of cold exposure on initial mortality at all sub-zero temperatures tested, but no clear patterns until eggs were exposed to -17° and -20°C, when both groups of eggs (27 and 4°C) had significantly higher mortality than controls ($F \geq 9.839$, df=3, $P \leq 0.001$) (Fig. 2.3a). The theoretically severe -17 and -20°C exposures resulted in < 40% initial egg mortality. Because it was difficult to discern a trend in mortality as temperature decreased, we examined differences in mortality of all eggs (pre-exposure to 27° and 4°C combined) across all sub-zero temperatures tested (-10 to -20°C). There was no significant increase in egg mortality until -20°C (F=13.24, df=1, $P \leq 0.001$). As in the previous larvae experiments, larvae reared from cold-exposed eggs also suffered from chronic cold injury at treatment temperatures lower than -15°C (Fig. 2.3b). There was also no difference in subsequent pupal mortality (reared from the cold-exposed eggs) between sub-zero exposed and control eggs at any temperature, except at -12°C, where

pre-chilled (4°C) eggs had a trend toward significantly lower mortality than sub-zero exposed 27°C eggs (F=2.814, df=3, P=0.0502) (Fig. 2.3c). Final mortality followed larval mortality trends, and eggs exposed to -17° and -20°C had significantly higher mortality through the adult stage than controls did (F≥4.244, df=3, P≤0.0102). In general, eggs held at 4°C before freezing had slightly lower mortality than those held at 27°C, though the difference was not significant (Fig. 2.3d). Because final control mortality was greater than 10%, Abbott's correction was used for probit analysis. We observed ~95% mortality at -20°C, and predicted 99% mortality at -22.9°C. At -20.1°C we predicted only 90% mortality (Table 2.2).

Supercooling point experiments: Exposing larvae and pupae to 4°C for 24h did not generally affect SCP relative to insects held constantly at 27°C (Table 2.2). Colony pupae SCPs were significantly lower than any of the larval populations tested. Mean colony pupae SCPs did not differ significantly from SCPs of CO pupae and CA pupae held constantly at 27°C. Field population pupae SCPs were generally lower than field larvae SCPs, but not significantly. For colony larvae, we initially observed significantly different mean SCPs of -15.8 ± 2.37°C for larvae held at 27°C (n= 22) and -12.1 ± 3.60°C for larvae held at 4°C (n= 20) (F=16.24, df=1, P≤0.001,), respectively. Because it seemed that exposure to 4°C was having a negative effect on the larva's ability to withstand freezing, we repeated the experiment with 20 larvae from each temperature pre-treatment. Larval SCPs during trial 2 were not different (27°C: -13.0 ± 4.21°C; 4°C: -13.7 ± 3.58°C) (F=0.312, df=1, P=0.58). If SCPs for both trials were combined, no significant difference between the two treatments was found. Colorado larvae exposed to

4°C for 24 h also had a slightly, but not significantly, higher SCP than their 27°C counterparts.

Field-collected larvae: To compensate for small sample sizes, some groups of field larvae were pooled for analysis. Colorado larvae held at 27°C or 4°C did not differ in their initial or final mortality (F=2.275, df=3, P=0.119), and so were pooled (n=12 reps, 117 total larvae). Field larvae survival was then analyzed by frozen (pooled) and control treatments.

Field-collected larvae from both CA and CO had lower cold tolerance than colony larvae (by about 1.0-1.5°C), and succumbed to temperatures >-3.0°C. Although a few CA larvae initially survived freezing at -2.5° and -3°C, final mortality was still very high at these temperatures (Table 2.4). Statistical differences in survival of CA larvae at the different temperatures tested were not analyzed due to the low number of replicates available. Final mortality of CO and CA controls (X^2=0.573, df=1, P=0.449) and -2.5°C exposed larvae (X^2=0.207, df=1, P=0.886) did not differ (Fig. 2.4). However, physical freezing of the mud substrate appeared to play a role in larval survival. At the relatively high temperature of -2.5°C, ice crystals did not always form on the mud. When only looking at replicates where the mud froze, mean survival of CO larvae was slightly higher than CA larvae (94.2±0.03% vs. 100±0.0% mortality), though not significantly different (t=1.873, df=8, P=0.098).

DISCUSSION

Low temperature tolerance in insects is dictated by numerous factors including, but not limited to, body size, feeding status, life stage, photoperiod, and previous exposure to cold (Salt 1961, Salt 1966a, Bale 1987). Even within a species, low temperature tolerance can vary greatly between individuals, life stages, and populations. The ability of at least one life stage to withstand or avoid exposure to low temperatures is critical to the overwintering success of that species, especially in temperate or arctic zones. Though the question of how *C. sonorensis* and BTV overwinter in both temperate and subtropical climates has been debated, adult and larval midges have been recovered from the field during periods of virus overwintering (Vaughan and Turner 1987, Nunamaker et al. 1996). Bluetongue is not transovarially transmitted (Osborne et al. 2015), so overwintering immature *Culicoides* do not serve as a virus reservoir, but the ability of these stages to survive or avoid low temperatures is likely critical to determining the geographical range of the species, and the virus itself. In this study, we characterized two measures of low temperature tolerance (SCP and LLT) for the eggs, larvae, and pupae of *C. sonorensis* from temperate and subtropical populations.

The SCP is the temperature at which the insect's body freezes, and is accompanied by the release of the latent heat of fusion due to phase change (liquid to ice) (Lee 1991, Denlinger et al. 1992). The LLT, on the other hand, is the lowest temperature at which an insect can survive, and is often reported as the LLT_{50}, the lowest temperature at which 50% of individuals survive (Hanson and Craig 1995). These two temperatures may not be the same; LLT is sometimes higher than the SCP, indicating that in those

species, death does not occur due to freezing (Bale 1993). In terms of transmission of pathogens like BTV, we may be more interested in temperatures lower than the LLT_{50}, as even a population decrease of 50% may not be sufficient to control disease, and persistence of a small proportion of a population may be epidemiologically relevant (Mullens et al. 2015).

There are two main physiological mechanisms for surviving low temperatures: freeze tolerance or freeze intolerance. Freeze tolerant insects are able to withstand the formation of some level of ice in their tissues, while freeze intolerant species physiologically prevent ice from forming in the first place (Lee 1991). Freeze intolerance is accomplished by the production of hemolymph cryoprotectant compounds that prevent the formation of ice in the body. Glycerol is the most common such compound found in insects (Lee 1991), though other chemicals (including sorbitol, ethylene glycol, and trehalose) may also act as cryoprotectants (Storey and Storey 1991). Cryoprotectants may function to prevent ice recrystallization in cells, prevent ice crystal growth, or to stabilize cell membranes during supercooling (Lee 1991, Dunman 2001). The presence of these compounds in the hemolymph lowers the SCP by creating a differential between the melting and freezing points of the insect, which are equal to each other in the absence of cryoprotectants (Dunman and Horwath 1983). Insects that survive chilling or freezing are often able to increase the concentration of these compounds in their bodies as a response to a drop in temperature. This increase is not instantaneous though, and even freeze intolerant insects can be killed by cold-shock if they are exposed to low temperatures quickly. That being said, even a short acclimation period at a moderately low temperature

(e.g. 0°C) may be enough to permit sufficient accumulation of cryoprotectants in the body (Denlinger et al. 1991).

Another important factor in insect cold tolerance are the heat shock proteins (HSPs), which are highly conserved across insects and play an important role in temperature regulation (Rinehart et al. 2007). Adult *C. sonorensis* rapidly upregulate production of HSPs when exposed to low temperatures, and exposure to 5°C for 1 h is sufficient to increase survival at -10°C compared to control midges (Nunamaker 1993, Nunamaker et al. 1996). The ability of adult *C. sonorensis* to produce glycerol, or other specific cryoprotectants, has not been studied.

Data on the environmental tolerances of immature *Culicoides*, or even details on winter distribution, are severely lacking in the literature. One field study in Virginia (Vaughan and Turner 1987) concluded that *C. sonorensis* primarily overwinters as mid-late stage larvae, deep in saturated mud substrates. This mechanism was also suggested by Barnard and Jones (1980) and Jones (1967), who collected winter larvae of *C. sonorensis* under 50 cm of water in CO. Virginia winter-collected larvae could survive entrapment in winter ice, and had elevated levels of glycerol when compared to larvae collected during other times of the year (Vaughan and Turner 1987). March mud collections of a closely related species, *C. variipennis* (Coquillett), in the relatively severe winter environments of upstate New York (Mullens and Rutz 1983) were comprised of mainly 3rd and 4th instar larvae. Mid-winter 4th instars were observed at that location in a thin layer of melted water on top of ice in one mud habitat (B.A.M., unpublished data) and must have moved there from adjacent mud substrate.

Culicoides occidentalis Wirth and Jones larvae collected in Washington State in November survived extended (6 wks) exposure at -2.2°C in mud, but experienced delayed pupation (Rowley 1967). January field-collected *C. chiopterus* (Meigen) and *C. dewulfi* Goetghebuer immatures in natural cow dung from northwestern Germany did not survive 48 h exposure to -20°C in a laboratory freezer, based on subsequent adult emergence when samples were warmed. Some *C. chiopterus* could survive a 48 h exposure at -18°C, although it was determined from field measurements that pat-dwelling larvae would be unlikely to experience temperatures lower than 0°C in the field (Steinke et al. 2015).

Based on these observations, and previous observations on adult cold hardening (Nunamaker et al. 1996), we expected to see increased survival of immatures held at 4°C for 24 h compared to those exposed to low temperatures directly from colony room conditions (27°C). However, we found that *C. sonorensis* larvae were extremely susceptible to even moderately low temperatures (-3 to -4°C), suggesting that in order to overwinter, larvae must avoid damaging temperatures (less than about -3°C). They may accomplish this by moving into somewhat more moderate mud temperature zones, as discussed earlier. We also did not find a significant difference in survival between 4°C and 27°C pre-treatments for any life stage or temperature tested, indicating that if glycerol or HSPs are up-regulated in immature *C. sonorensis*, the mechanism is more complicated than exposure to 4°C for 24 h.

Eggs were the most cold-tolerant life stage. Whereas we saw nearly complete initial larval and pupal mortality at -4.5 and -9.0°C, respectively, initial egg mortality at -

20°C (as measured by a decrease in egg hatching) was only moderately increased compared to controls. This level of cold tolerance exceeds even *Aedes albopictus* (Skuse) eggs in diapause, which experience nearly complete mortality at -14°C (Hanson and Craig 1995). The ability of *C. sonorensis* embryos to withstand exposure to challenging temperatures is likely related to their desiccation tolerance (McDermott and Mullens 2014) and small size (Salt 1966a). Because both processes (drying and freezing) involve the removal of free water from cells, desiccation and low temperature tolerance in insects are often linked (Zachariassen 1991). Despite not seeing a dramatic reduction in hatch success of cold-exposed eggs, embryos did clearly suffer from chronic cold injury at very low temperatures. Mortality between the larval and pupal stages for cold-exposed, reared eggs was significantly increased compared to controls at -17 and -20°C. Chronic cold injury was also observed in larvae experiments, where larvae that survived the initial exposure were less likely than controls to survive to pupation. It would be beneficial to test how tolerant eggs are of longer-term sub-zero exposures, since our experiments used shorter exposure periods.

Long term exposure to moderately low temperatures (5°C) results in decreased egg hatch when freshly laid (0-8 h) eggs are chilled; 50% mortality at 5°C occurs after 28 d, though some embryos remain viable out to 66 d (Hunt and Tabachnick 1995). Because young (4-10 h) *C. sonorensis* embryos are very susceptible to desiccation (McDermott and Mullens 2014), long-term survival of older eggs at 5°C may be greater than previously described. The embryos used in the present study were 24-48 h old and we did not test younger eggs. The slight increase in survival of pre-chilled eggs in our LLT

experiment may be due to the age of the embryos used, which were 24 h older than the non-chilled eggs.

Emergence success was lower in egg and larvae experiments than in pupae experiments, including for controls, suggesting that some mortality in these experiments should be attributed to our rearing conditions. Rearing eggs in our small experimental containers proved to be difficult. During trials 2 and 3 of the egg experiment, pupation and emergence in all treatments was lower than expected. Because this was observed even in control cups, we believe that it was an effect of rearing conditions rather than delayed development due to cold injury. We accounted for high control mortality (>10%) stemming from rearing conditions in our LLT analyses using Abbott's correction. In the wild, larvae could theoretically have more abundant resources, and less competition than in our small experimental cups, improving their overall fitness. When subjected to low temperatures, wild insects might differ in survival.

Interestingly, larvae from our CA colony population of *C. sonorensis* were able to tolerate lower temperatures (but still within about 1.0°C) than field collected larvae from either CA or CO. We had expected to see greater low temperature tolerance in both field populations compared to the colony, which has not been exposed to naturally low temperatures since 1995. We also expected that CO larvae would have the greatest low temperature tolerance. Insects that inhabit a range of climatic zones may exhibit differences in low temperature tolerances between more and less cold adapted populations (Somme 1982, Kukal and Duman 1989, Hanson and Craig 1995). Colorado larvae were just slightly more tolerant of exposure to -2.5°C than CA larvae, but still

experienced ~78% final mortality at that temperature. In comparison, final mortality of colony larvae at -3.0°C did not differ between sub-zero exposure treatments and controls (~27%).

With any insect colony population, certain phenotypes may become fixed when population bottlenecks occur during the colonization process. It may be that certain individuals in the founding population of the VR *C. sonorensis* colony had naturally greater low temperature tolerance than the population at large, resulting in a colony with greater than average cold-tolerance. Alternatively, the stress of transportation and handling could have made field larvae more susceptible to cold injury, or different food items available to larvae in lab versus field may have influenced cold tolerance (Baust and Lee 1983).

Supercooling points for *C. sonorensis* larvae and pupae were lower than LLTs, indicating that death in the LLT experiments was not caused by complete tissue freezing. Lab colony pupae had the lowest SCPs. Interestingly, field-collected pupae had higher SCPs than our colony pupae, though still lower than any of the larvae tested. We did not test the LLT of field-collected pupae, but we would predict that they succumb to higher temperatures than colony pupae, based on their SCP. Pre-chilling did not lower the SCP of any of the populations tested, indicating that *Culicoides* larvae and pupae do not produce any cryoprotectant compounds that prevent their tissues from freezing when exposed to 4°C for 24 h. In fact, pre-chilled CA pupae had a significantly higher SCP than 27°C CA pupae, and although the difference was not significant, pre-chilled larvae had higher SCPs than 27°C larvae. Based on their LLT, we would surmise that larvae

may be experiencing some level of cold injury, even at temperatures above freezing, that inhibits their ability to withstand freezing. This difference was most marked for colony larvae.

Classification of insect cold tolerance is somewhat difficult, in that many species utilize a variety of survival strategies and may not always exhibit the same level of tolerance depending on life stage or environmental factors (Bale 1987, Kukal and Duman 1989). For our purposes, we characterize *C. sonorensis* immatures as partially freeze tolerant, in that they do not appear to be able to prevent the formation of ice crystals in their tissues (Bale 1987, Sinclair 1999). Fatal cold injury occurs at temperatures higher than the SCP for at least larvae and pupae. Due to their high mortality at temperatures as high as -4°C, *C. sonorensis* larvae may even be further characterized as chill-susceptible (Bale 1993). We were not able to record the SCP of eggs using our experimental design, but we suspect that they might supercool to temperatures potentially as low as even -30°C due to their small size and low LLT (Somme 1982).

Future work should be directed toward looking into the genetic mechanisms of low temperature tolerance in *Culicoides*, and at how these may differ by population. Identification of microsatellite markers that differentiate populations is needed. With the combined knowledge of low temperature tolerance population differences and molecular methods to separate these populations, we can determine whether an introduction of *Culicoides* is “high risk” or “low risk”. For instance, if adult *C. sonorensis* are discovered in Saskatchewan, Canada, we could screen for population specific markers and determine if the introduced insects came from a temperate or subtropical population. Temperate

zone introductions would merit more serious eradication and surveillance responses, as these insects might be more likely to survive and establish in that climate.

Laboratory studies on insect cold tolerance provide an excellent foundation for our understanding of how species overwinter and survive in adverse conditions. The full picture is of course much more complicated. Behavioral adaptations, habitat microclimates, and ambient temperature fluctuations play a role in insect overwintering mechanisms. Though not tested in our experiments, larval survival may be enhanced by their ability to avoid cold injury by physically moving to warmer habitats, such as deeper into mud, or into unfrozen water (Vaughan and Turner 1987). In this study, we defined two important comparative parameters (SCP and LLT) for *C. sonorensis* egg, larva, and pupa low temperature tolerance. Our interpretation of the results of our experiments is limited by the dearth of information on how immature *Culicoides* behave and interact with their environment. The present work should be taken together with future work on the field ecology and genomics of overwintering *Culicoides* to more accurately understand seasonal population dynamics and vector range.

REFERENCES

Abbott, W. S. 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18: 265-267.

Bale, J. S. 1987. Insect cold hardiness: freezing and supercooling- an ecophysiological perspective. J. Insect Physiol. 33: 899-908.

Bale, J. S. 1993. Classes of insect cold hardiness. Funct. Ecol. 7: 751-753.

Barnard, D. R., and R. H. Jones. 1980. *Culicoides variipennis*: seasonal abundance, overwintering, and voltinism in Northeastern Colorado. Environ. Entomol. 9: 709-712.

Baust, J. G., and R. E. Lee. 1983. Population differences in antifreeze/cryoprotectant accumulation patterns in an Antarctic insect. Oikos. 40: 120-124.

Blanton, F. S. and W. W. Wirth. 1979. The sand flies (*Culicoides*) of Florida (Diptera: Ceratopogonidae). Arthropods of Florida and Neighboring Land Areas, Vol. 10, Florida Dept. of Agric. Consumer Services. 204 p.

Bogoch, I. I., O. J. Brady, M. U. G. Kraemer, M. German, M. I. Creatore, M. A Kulkarni, J. S. Brownstein, S. R. Mekaru, S. I. Hay, E. Groot, A. Watts, and K. Khan. 2016. Anticipating the international spread of Zika virus from Brazil. The Lancet. 387: 335-336.

Carpenter, S., A. Wilson, and P. S. Mellor. 2009. *Culicoides* and the emergence of bluetongue virus in northern Europe. Trends in Microbiol. 17: 172-178.

Denlinger, D. L., K. H. Joplin, C. –P. Chen, and R. E. Lee. 1991. Cold shock and heat shock, pp. 131-148. *In* R. E. Lee and D. L. Denlinger (eds.), Insects at low temperature. Chapman and Hall, New York, NY.

Denlinger, D. L., R. E. Lee, G. D. Yocum, and O. Kukal. 1992. Role of chilling in the acquisition of cold tolerance and the capacitation to express stress proteins in diapausing pharate larvae of the gypsy moth, *Lymantria dispar*. Arch. Insect Biochem. Physiol. 21: 271-280.

Dunman, J. G. 2001. Antifreeze and ice nucleator proteins in terrestrial arthropods. Annu. Rev. Physiol. 63: 327-357.

Dunman, J., and K. Horwath. 1983. The role of hemolymph proteins in the cold tolerance of insects. Ann. Rev. Physiol. 45: 261-270.

Ganter, M. 2014. Bluetongue disease- global overview and future risks. Sm. Rumin. Res. 118: 79-85.

Gerry, A. C, and B. A. Mullens. 2000. Seasonal abundance and survivorship of *Culicoides sonorensis* (Diptera: Ceratopogonidae) at a southern California dairy, with reference to potential bluetongue virus transmission and persistence. J. Med. Entomol. 37: 675-688.

Gibbs, E. P. J., and E. C. Greiner. 1994. The epidemiology of bluetongue. Comp. Immun. Microbiol. Infect. Dis. 17: 207-220.

Gubbins, S., S. Carpenter, M. Baylis, J. L. N. Wood, and P. S. Mellor. 2008. Assessing the risk of bluetongue to UK livestock: uncertainty and sensitivity analyses of a temperature-dependent model for the basic reproduction number. J. R. Soc. Interface. 5: 363-371.

Hanson, S. M., and G. B. Craig. 1995. Relationship between cold hardiness and supercooling point in *Aedes albopictus* eggs. J. Am. Mosq. Contr. Assoc. 11: 35-38.

Holbrook, F. R., W. J. Tabachnick, E. T. Schmidtmann, C. N. McKinnon, R. J. Bobian, and W. L. Grogan. 2000. Sympatry in the *Culicoides variipennis* complex (Diptera: Ceratopogonidae): a taxonomic reassessment. J. Med. Entomol. 37: 65-76.

Hunt, G. J., and W. J. Tabachnick. 1995. Cold storage effects on egg hatch in laboratory-reared *Culicoides variipennis sonorensis* (Diptera: Ceratopogonidae). J. Am. Mosq. Contr. Assoc. 11: 335-338.

Jones, R. H. 1967. An overwintering population of *Culicoides* in Colorado. J. Med. Entomol. 4: 461-463.

Kukal, O., and J. G. Duman. 1989. Switch in the overwintering strategy of two insect species and latitudinal differences in cold hardiness. Can. J. Zool. 67: 825-827.

Lee, R. E. 1991. Principles of insect low temperature tolerance, pp. 17-46. *In* R. E. Lee and D. L. Denlinger (eds.), Insects at low temperature. Chapman and Hall, New York, NY.

McDermott, E. G., and B. A. Mullens. 2014. Desiccation tolerance in the eggs of the primary North American bluetongue virus vector, *Culicoides sonorensis* (Diptera: Ceratopogonidae), and implications for vector persistence. J. Med. Entomol. 51: 1151-1158.

Melhorn, H., V.Walldorf, S. Klimpel, B. Jahn, F. Jaeger, J. Eschweiler, B. Hoffmann, and M. Beer. 2007. First occurrence of *Culicoides obsoletus*-transmitted bluetongue virus epidemic in Central Europe. Parasitol. Res. 101: 219-228.

Mullens, B. A., and D. A. Rutz. 1983. Voltinism and seasonal abundance of *Culicoides variipennis* (Diptera: Ceratopogonidae) in central New York State. Ann. Entomol. Soc. Am. 76: 913-917.

Mullens, B. A., and K. Lii. 1987. Population dynamics of *Culicoides variipennis* (Diptera: Ceratopogonidae) in southern California. J. Med. Entomol. 24: 566-574.

Mullens, B. A., E. G. McDermott, and A. C. Gerry. 2015. Progress and knowledge gaps in *Culicoides* ecology and control. Veterinaria Italiana. 51: 313-323.

Nunamaker, R. A. 1993. Rapid cold-hardening in *Culicoides variipennis sonorensis* (Diptera: Ceratopogonidae). J. Med. Entomol. 30: 913-917.

Nunamaker, R. A., V. C. Dean, K. E. Murphy, and J. A. Lockwood. 1996. Stress proteins elicited by cold shock in the biting midge, *Culicoides variipennis sonorensis* Wirth and Jones. Comp. Biochem. Physiol. 113: 73-77.

Osborne, C. J., C. E. Mayo, B. A. Mullens, E. G. McDermott, A. C. Gerry, W. K. Reisen, and N. J. MacLachlan. 2015. Lack of evidence for laboratory and natural vertical transmission of bluetongue virus in *Culicoides sonorensis* (Diptera: Ceratopogonidae). J. Med. Entomol. 52: 274-277.

Purse, B. V., P. S. Mellor, D. J. Rogers, A. R. Samuel, P. P. C. Mertens, and M. Baylis. 2005. Climate change and the recent emergence of bluetongue in Europe. Nature Rev. Microbiol. 3: 171-181.

Purse, B. V., S. Carpenter, G. J. Venter, G. Bellis, and B. A. Mullens. 2015. Bionomics of temperate and tropical *Culicoides* midges: knowledge gaps and consequences for transmission of *Culicoides*-borne viruses. Annu. Rev. Entomol. 60: 373-392.

Rinehart, J. P., A. Li, G. D. Yocum, R. M. Robich, S. A. L. Hayward, and D. L. Denlinger. 2007. Up-regulation of heat shock proteins is essential for cold survival during insect diapause. Proc. Nat. Acad. Sci. 104: 11130-11137.

Rogers, D. J. 2006. Models for vectors and vector-borne diseases. Advan. Parasitol. 62: 1-35.

Roth, A., A. Mercier, C. Lepers, D. Hoy, S. Duituturaga, E. Benyon, L. Guillamumot, and Y. Souares. 2014. Concurrent outbreaks of dengue, chikungunya and Zika virus infections- an unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012-2014. Euro. Surveill. 19: 2-9.

Rowley, W. A. 1967. Observations on larval habitats and the winter bionomics of some common species of *Culicoides* (Diptera: Ceratopogonidae) in the central Columbia Basin. Mosq. News. 27: 499-505.

Ruder, M. G., T. J. Lysyk, D. E. Stallknecht, L. E. Foil, D. J. Johnson, C. C. Chase, D. A. Dargatz, and E. P. J. Gibbs. 2015. Transmission and epidemiology of bluetongue and epizootic hemorrhagic disease in North America: current perspectives, research gaps, and future directions. Vectorbor. Zoonot. Dis. 15: 348-363.

Salt, R. W. 1961. Principles of insect cold-hardiness. Annu. Rev. Entolol. 6: 55-75.

Salt, R. W. 1966a. Factors influencing nucleation in supercooled insects. Can. J. Zool. 44: 117-133.

Salt, R. W. 1966b. Effect of cooling rate on the freezing temperatures of supercooled insects. Can. J. Zool. 44: 655-659.

Sellers, R. F., and P. S. Mellor. 1993. Temperature and the persistence of viruses in *Culicoides* spp. during adverse conditions. Revue scientifique et technique (International Office of Epizootics) 12: 733-755.

Sinclair, B. J. 1999. Insect cold tolerance: how many kinds of frozen? Eur. J. Entomol. 96: 157-164.

Somme, L. 1982. Supercooling and winter survival in terrestrial arthropods. Comp. Biochem. Physiol. 73A: 519-543.

Steinke, S., R. Luhken, and E. Kiel. 2015. Impact of freezing on the emergence of *Culicoides chiopterus* and *Culicoides dewulfi* from bovine dung. Vet. Parasitol. 209: 146-149.

Storey, K. B., and J. M. Storey. 1991. Biochemistry of cryoprotectants, pp. 64-93. *In* R. E. Lee and D. L. Denlinger (eds.), Insects at low temperature. Chapman and Hall, New York, NY.

Vaughan, J. A., and E. C. Turner. 1987. Seasonal microdistribution of immature *Culicoides variipennis* (Diptera: Ceratopogonidae) at Saltville, Virginia. J. Med. Entomol. 24: 340-346.

Wirth, W., A. Dyce, and B. Peterson. 1985. An atlas of wing photographs, with a summary of the numerical characters of *Culicoides* (Diptera: Ceratopogonidae). Contr. Am. Entomol. Inst. 22: 1-46.

Zachariassen, K. E. 1991. The water relations of overwintering insects, pp. 47-63. *In* R. E. Lee and D. L. Denlinger (eds.), Insects at low temperature. Chapman and Hall, New York, NY.

TABLES AND FIGURES

Table 2.1 Probit model analysis for *C. sonorensis* eggs, pupae, and larvae subjected to subzero temperatures.

Life Stage	n[a]	Slope ± SE	X^2 (df)	$logLik_{model}$	$logLik_{null}$
Eggs	3484	-6.08 ± 0.46	>0.001 (1)	-20.2	-165.7
Pupae	3000	-1.96 ± 0.09	>0.001 (1)	-721.0	-1811.8
Larvae	2960	-12.4 ± 1.00	>0.001 (1)	-14.9	-182.5

[a]Number of insects tested per life stage

Table 2.2 Supercooling points (°C±SE) of different populations of *C. sonorensis* larvae and pupae held at either 27°C or 4°C, n=number of individuals tested. Letters represent significant differences (p< 0.05) between all groups tested.

Life Stage (Population)	27°C	4°C
Larvae (Colony)	-14.5±0.56 (n=42)[a]	-12.9±0.81 (n=40)[ab]
Larvae (CA)	-12.1±0.86(n=12)[ab]	-12.4±0.93 (n=10)[ab]
Larvae (CO)	-11.8±1.39 (n=10)[ab]	-10.7±0.78 (n=10)[ab]
Pupae (Colony)	-18.0±0.50 (n=20)[c]	-17.7±0.52 (n=20)[c]
Pupae (CA)	-14.2±1.55 (n=10)[abc]	-9.80±1.00 (n=13)[b]
Pupae (CO)	-15.5±1.17 (n=6)[ac]	Not tested

Table 2.3 Predicted lower lethal temperatures of 50, 90, and 99% of *C. sonorensis* colony eggs, pupae, and larvae after a 1 h exposure, from probit analysis. 95% confidence intervals are reported in parentheses.

Life Stage	LLT_{50}	LLT_{90}	LLT_{99}
Eggs	-16.1°C	-20.1°C	-22.9°C
	(-16.2 to -17.0°C)	(-19.3 to -20.8°C)	(-21.8 to -24.0°C)
Pupae	-4.26°C	-7.05°C	-9.32°C
	(-4.09 to -4.44°C)	(-6.85 to -7.24°C)	(-9.01 to -9.63°C)
Larvae	-3.38°C	-3.73°C	-4.01°C
	(-3.34 to -3.42°C)	(-3.66 to -3.80°C)	(-3.90 to -4.12°C)

Table 2.4 Mean percentage initial and final (through the adult stage) mortality (±SE) of field-collected CA larvae after 1 h exposure at a given temperature, n=number of 25 larva replicates.

Temperature (°C)	Initial Mortality	Final Mortality
Control	0.0±0% (n=4)	26.0±2.58% (n=4)
-2.0	0.0±0% (n=4)	54.8±6.35% (n=4)
-2.5	91.9±17.4% (n=5)	100±0% (n=5)
-3.0	96.7±3.26% (n=4)	100±0% (n=4)
-3.2	100±0% (n=4)	100±0% (n=4)

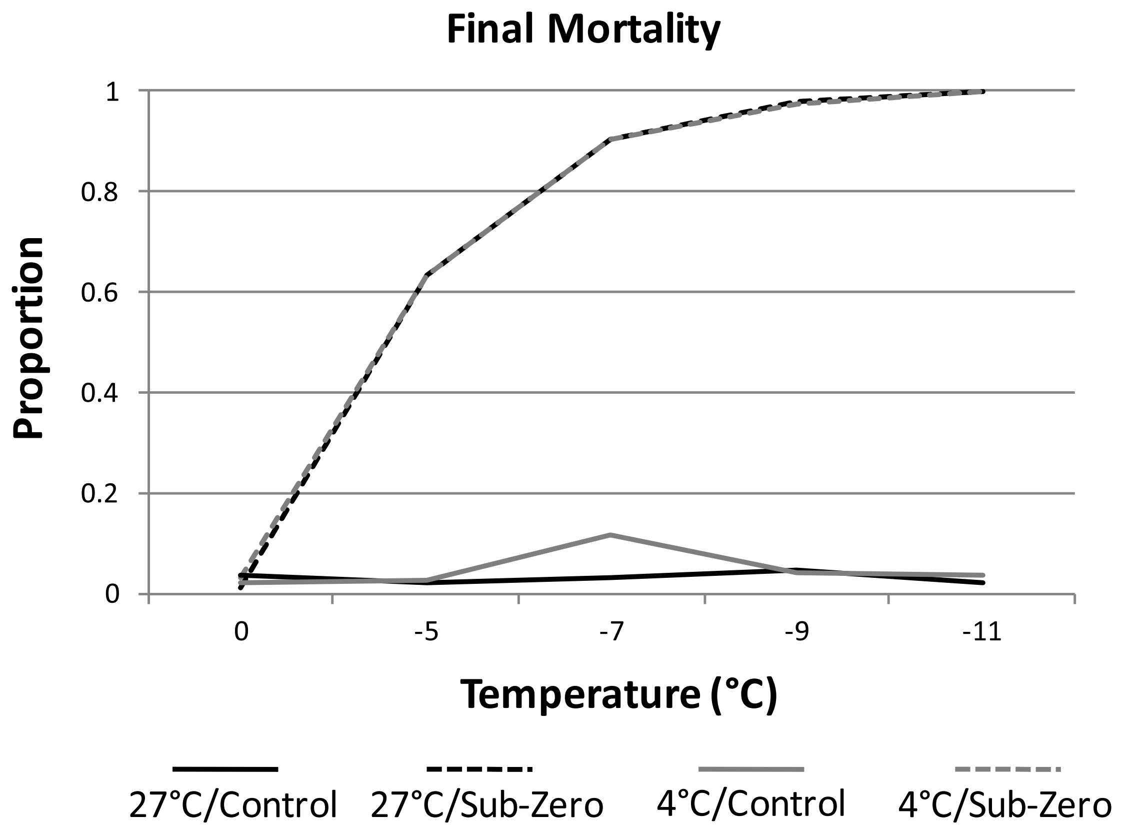

Figure 2.1 Proportion of final mortality of pupae exposed to 0.0, -5.0, -7.0, -9.0, and -11°C for 1 h. Solid lines represent control pupae, dashed lines represent sub-zero exposed pupae. Black lines represent pupae held at 27°C, gray lines represent pupae held at 4°C.

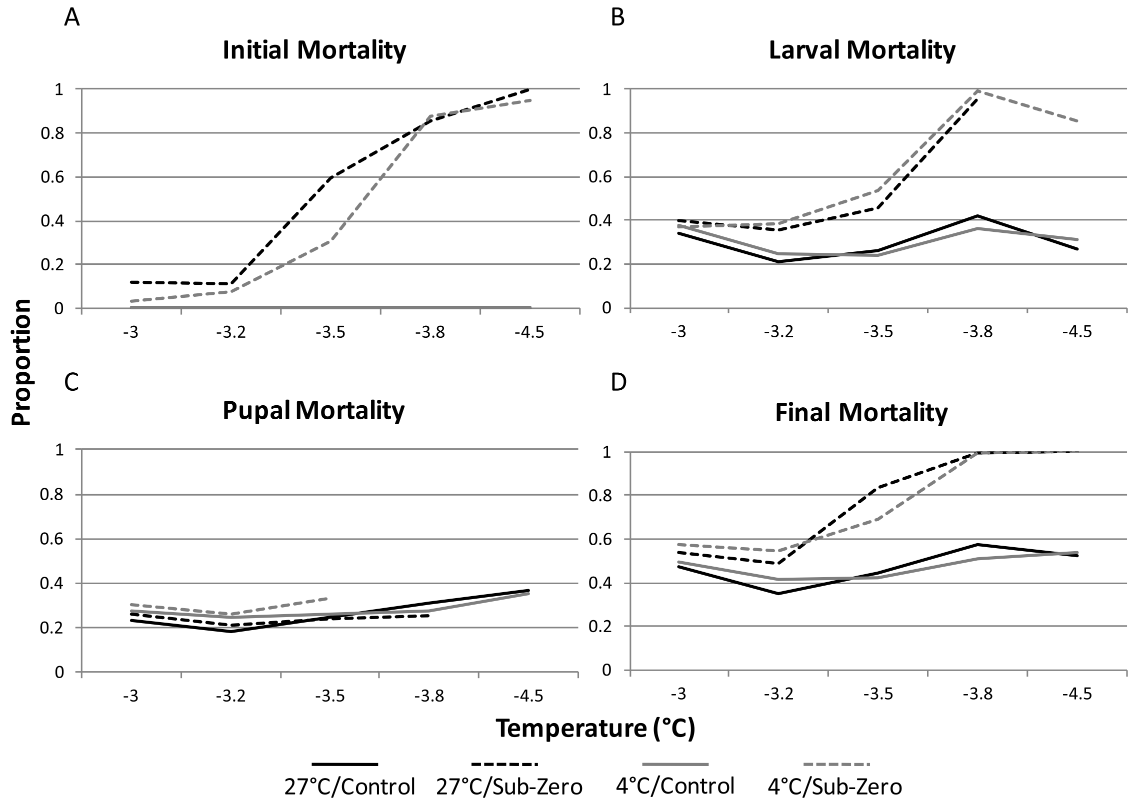

Figure 2.2 Proportion of a) initial mortality, b) subsequent larval mortality, c) subsequent pupal mortality, and d) final mortality of L4 larvae exposed to -3.0, -3.2, -3.5, -3.8, and -4.5°C for 1 h. Solid lines represent control larvae, dashed lines represent sub-zero exposed larvae. Black lines represent larvae held at 27°C, gray lines represent larvae held at 4°C.

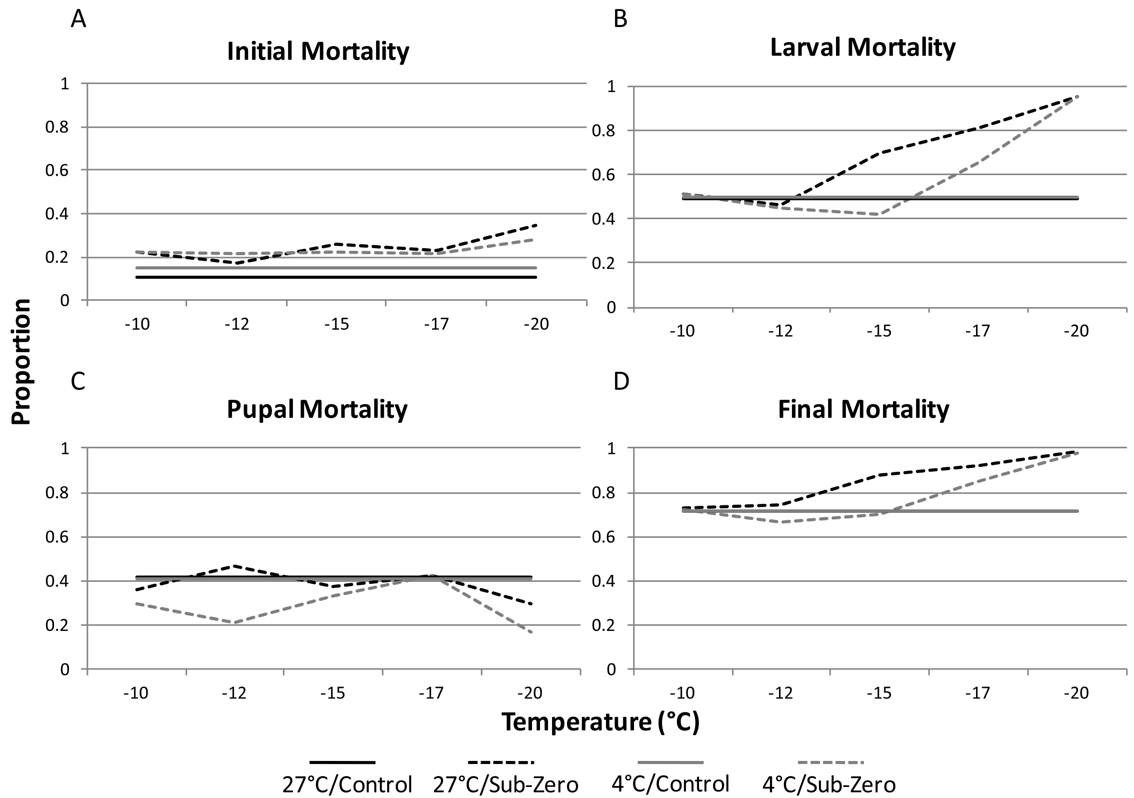

Figure 2.3 Proportion of a) initial mortality, b) subsequent larval mortality, c) subsequent pupal mortality, and d) final mortality of eggs exposed to -10, -12, -15, -17, and -20°C for 1 h. Solid lines represent control eggs, dashed lines represent sub-zero exposed eggs. Black lines represent eggs held at 27°C, gray lines represent eggs held at 4°C.

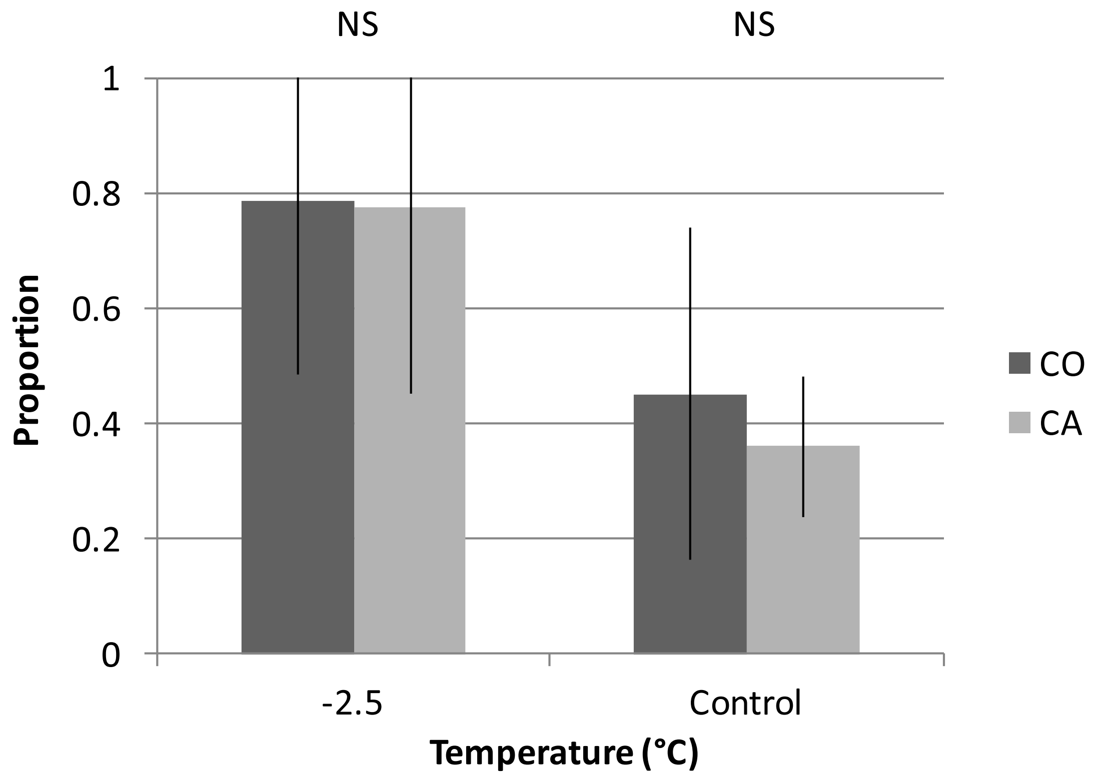

Figure 2.4 Proportion of final mortality of L4 larvae from Colorado (dark gray) and California (light gray) exposed to -2.5°C for 1 h and control larvae. Error bars represent standard errors.

CHAPTER 3

Bluetongue virus infection creates light averse *Culicoides* vectors and serious errors in transmission risk estimates

Emily G. McDermott[a*], Christie E. Mayo[b], Alec C. Gerry[a], Damien Laudier[c], N. James MacLachlan[d], and Bradley A. Mullens[a]

[a]Department of Entomology, University of California, Riverside, CA 92521
[b]Department of Microbiology, Immunology & Pathology, Colorado State University, Fort Collins, CO 80523
[c]Laudier Histology, New York, NY 10024
[d]Department of Pathology, Microbiology & Immunology, School of Veterinary Medicine, University of California, Davis, CA 95616

ABSTRACT

Pathogen manipulation of host behavior can greatly impact vector-borne disease transmission, but almost no attention has been paid to how it affects disease surveillance. Bluetongue virus (BTV), transmitted by *Culicoides* biting midges, is a serious disease of ruminant livestock that can cause high morbidity and mortality and significant economic losses. Worldwide, the majority of surveillance for *Culicoides* to assess BTV transmission risk is done using UV-light traps. Here we show that field infection rates of BTV are significantly lower in midge vectors collected using traps baited with UV light versus a host cue (CO_2). We collected *Culicoides sonorensis* Wirth and Jones midges in suction traps baited with CO_2, UV-light, or CO_2+UV on three dairies in southern California to assess differences in the resulting estimated infection rates from these collections. Pools of midges were tested for BTV by qRT-PCR, and maximum likelihood estimates of infection rate were calculated by trap. Infection rate estimates were also calculated by trapping site within a dairy. Colonized *C. sonorensis* were orally infected with BTV, and infection of the structures of the compound eye was examined using structured illumination microscopy. UV traps failed entirely to detect virus both early and late in the transmission season, and underestimated virus prevalence by as much as 8.5-fold. CO_2+UV traps also had significantly lower infection rates than CO_2-only traps, suggesting that light may repel infected vectors. We found very high virus levels in the eyes of infected midges, possibly causing altered vision or light perception. Collecting location also greatly impacts our perception of virus activity. Because the majority of global vector surveillance for bluetongue uses only light-trapping, transmission risk

estimates based on these collections are likely severely understated. Where national surveillance programs exist, alternatives to light-trapping should be considered. More broadly, disseminated infections of many arboviruses include infections in vectors' eyes and nervous tissues, and this may be causing unanticipated behavioral effects. Field demonstrations of pathogen-induced changes in vector behavior are quite rare, but should be studied in more systems to accurately predict vector-borne disease transmission.

INTRODUCTION

Pathogen manipulation of host behavior is fascinating from evolutionary, ecological, physiological, and epidemiological standpoints (Stafford et al. 2011, Cator et al. 2014). The idea that one organism can live within the body of another and control its actions intrigues scientists and the general public alike. However, when human and animal pathogens alter the behavior of their insect vectors, the result can be increased disease burdens. When behavioral alterations are adaptive to pathogen spread, there should be an increase in some aspect of vectorial capacity (i.e. the efficiency of transmission), such as biting rates. Increased probing, possibly enhancing transmission, was first demonstrated in the laboratory with *Aedes triseriatus* (Say) mosquitoes infected with La Crosse virus (Grimstad et al. 1980). Since then, several other laboratory studies have demonstrated infection-associated behavioral changes that may increase vectorial capacity, including increased frequency of re-feeding (Jackson et al. 2012), increased movement (Lima-Camara et al. 2011, Luz et al 2011), and improved mating efficiency, enhancing transovarial transmission (Reese et al. 2009).

Though less well characterized, changes with no impact on transmission may be important for vector control or vector-borne disease surveillance (Lefevre et al. 2008, Cator et al. 2014). Altered vector behavior as a result of infection affects transmission risk estimates, and surveillance and control measures. While laboratory studies are important, field evidence is ultimately required to understand how pathogen-induced behavioral changes relate to disease control and surveillance. Such field studies are rare or lacking, especially with arboviruses.

Biting midges in the genus *Culicoides* transmit many important animal viruses, including bluetongue virus (BTV), which causes disease in ruminants (e.g. sheep and cattle) with serious economic and animal health impacts (Purse et al. 2015). Globally, most *Culicoides* surveillance for BTV is conducted using UV-light-baited suction traps (Hoffmann et al. 2009, Goffredo et al. 2013), although alternatives include light traps supplemented by CO_2 (Becker et al. 2010, Mayo et al. 2012), traps with CO_2 alone (Gerry et al. 2001, Mayo et al. 2012), or rarely, direct collections from sentinel animals (Mayo et al. 2012). Mayo et al. (2012) showed that BTV field infection rates in *Culicoides sonorensis* (Wirth & Jones), the primary North American BTV vector, were lower in insects collected by suction traps baited with both CO_2 and UV versus traps baited with CO_2 alone or collected directly from cattle.

We collected *C. sonorensis* from three dairy farms in southern California using UV, CO_2, and UV+CO_2 baited suction traps, and tested them for BTV using qRT-PCR to assess differences in estimated infection rates between trap types. We also orally-infected laboratory colony *C. sonorensis* with a BTV-spiked blood meal, and used structured

illumination microscopy (SIM) to look at infection intensity in structures of the compound eye. The present study firmly establishes the light effect on BTV-infected insects, shows major spatial heterogeneity of BTV-infected insect activity, discusses consequences for vector-borne disease surveillance, and provides valuable field evidence for pathogen manipulation of host behavior.

MATERIALS AND METHODS

Field data: Three dairies in southern California were chosen for the study based on their large populations of *C. sonorensis* determined by preliminary collections. Dairy D and dairy V were located in the Chino Basin, east of Los Angeles in San Bernardino Co., California, USA (approximately 34.00 N, -117.65 W), and dairy S was located in San Jacinto in Riverside Co., California, USA (33.85 N, -117.02 W). The Chino and San Jacinto dairies were separated by approximately 69.2 km and the two Chino dairies were separated by 2.7 km.

All dairies were confinement dairies (cattle on dirt lots fed concentrates and hay), but represented different examples of southern California dairies. Dairy S was the largest of the three in terms of number of cattle and size (~1500 head on 1.59 km^2), located in a rural valley area. Dairy S had large fields separating the animals and wastewater ponds by several hundred meters, and there was a considerable distance (about 2 km) separating the dairy from the nearest neighbors. Dairy D was smaller (~900 head on 0.30 km^2). There were two small open fields at dairy D, but feed stalls and open areas separated the animals and wastewater ponds. There were other dairies immediately adjacent to dairy D

on three sides. Wastewater ponds at dairies S and D were sampled to confirm that they did serve as developmental sites for *C. sonorensis* immatures. Dairy V was the smallest of the three dairies, both in area (0.21 km^2) and number of animals (~200 head). During the course of the study there was no wastewater pond in use at dairy V, but runoff water in pastures and along feed stalls proved to be excellent *C. sonorensis* development sites, and large numbers of larvae could be collected from them at any given time.

At each dairy, three CDC-type suction traps (miniature light trap model 512, J. D. Hock and Co., Gainesville, Florida, USA) were used in each of three separated locations (near animals, wastewater ponds, and in fields), except at dairy V, where only two locations were used (animals and fields). The three traps at each location were positioned in a line, 20 m apart, approximately 1 m above the ground and perpendicular to prevailing east-west winds. The traps were baited with CO_2 (0.5 kg dry ice), battery-powered UV light (F4T5BL, 4W black light bulb), or CO_2+UV light. One of each trap type was set up at each location. The initial positions of the traps within the location were randomized at the start of the study and rotated each night afterwards, so that each trap type was in each position twice to reduce autocorrelation. Insects were collected into a solution of deionized water plus 0.5% detergent (Liqui-Nox®), keeping the insects in ideal body condition for identification and especially parity sorting, while still being suitable for virus assays (see below) (Mayo et al. 2012). Traps were set once a week on each dairy for 6 weeks in September and October, which was anticipated to be peak BTV transmission season (Gerry et al. 2001, Mayo et al. 2012). Traps were set approximately 2 h before sunset, and collected no more than 4 h after sunrise.

In the laboratory, insects were immediately transferred into 70% ethanol and stored at -20°C until they were sorted. *Culicoides sonorensis* were identified by wing pattern and size (Wirth et al. 1985), but are essentially also the only *Culicoides* species collected on southern California confinement dairies (Gerry and Mullens 2000). Parity of females was determined visually using a dissecting microscope by the presence or absence of a burgundy-red pigment in the abdominal cuticle of parous midges (Akey and Pitter 1979). Parous midges were usually pooled in groups of 20, or occasionally fewer, and saved in RNAlater® solution (Ambion®) for later viral analysis.

Chi-square analysis was used to look at the numbers of BTV-positive and negative pools from each trap type. Maximum likelihood estimates of infection rates (number infected per 1000 parous females) were calculated using the Excel Add-In, PooledInfRate version 4.0 (Biggerstaff 2009). Differences in infection rate by trap and trapping location were analyzed by permutational multivariate analysis of variance using distance matrices (R version 3.2.0, Vegan package) (Table 3.1). Despite the trapping locations at dairy V not being as separated as they were at the other two dairies, their inclusion in the analysis did not affect the significance levels of either the full model or the pairwise comparisons. For this reason, dairy V samples were included in the analyses of site to increase statistical power.

Week of collection was considered the level of replication and so was not included as a factor in the model. The interactions between dairy and trap or site were not significant, and so dairy was also not included as a factor in the final model. Effect of trap, site, and the interaction between trap and site were significant ($p<0.05$), with the

effect of trap being the most significant ($p=0.001$). Pairwise comparisons were used to look at differences in midge infection rates between traps (averaging all sites) or between sites (averaging all traps). Infection rate error was calculated by dividing the mean infection rate for CO_2 traps on a given week by the mean infection rate for UV traps on that week.

Insect pool processing: Pools of parous females were transferred into microcentrifuge tubes with lysis binding buffer solution (AM8500, Ambion/Life Technologies®) and a 1:1 mixture of 0.5 mm and 0.9-2 mm stainless steel beads, and homogenized using a Bullet Blender STORM (Next Advance Inc.). Viral RNA was extracted using the MagMAX™-96 Viral RNA isolation kit (AM1836, Ambion®). Negative control samples containing only nuclease-free water (n=3) and positive control samples containing pure BTV (BTV-10 ATCC prototype strain) (n=3) were interspersed randomly on each 96-well plate, and were used as controls. The amount of BTV in each sample was quantified by qRT-PCR, using the SuperScript® III Platinum One-Step qRT-PCR kit (11745-100, Invitrogen™). Pools with Ct values <31 were considered BTV-positive (Mayo et al. 2012).

Structured illumination microscopy: Bluetongue virus infection of the tissues and structures of the midge compound eye was examined using laboratory reared *C. sonorensis* (VR colony). Insects were infected with BTV (BTV-17 Tulare, CA strain) orally with an infectious blood meal (n=211). A 1:3 ratio of BTV suspension ($10^{6.7}$ $TCID_{50}$) to defibrinated sheep blood (HemoStat Laboratories, Dixon, CA) was used for the blood meal. After blood ingestion, insects were transferred into cardboard containers,

provided with 10% sucrose solution *ad libitum*, and held at 27°C. At 10 days post-inoculation (dpi), 10 live blood-fed insects were removed and placed into heavy-gel hand sanitizer (62% EtOH) to be processed for structured illumination microscopy (SIM). At this time point, all competent insects should have achieved fully disseminated infections (Carpenter et al. 2011, Veronesi et al. 2013). Additionally, 10 insects injected with saline solution and kept as above, and 10 non-blood-fed, nulliparous insects were placed into hand sanitizer, and used as negative controls. Sectioning and imaging of all samples was done at Laudier Histology (New York, NY).

At Laudier Histology, insect heads were removed for processing, and the remainder of the bodies were saved for possible future use. Samples were fixed for 48 h in a zinc-acetate based fixative (optimized for arthropods), providing optimal morphological preservation for fluorescence immunohistochemistry (IHC).

After fixation, samples were dehydrated, cleared and processed to a custom hydrophobic acrylic resin. Thin sections were cut at 1 micron and placed on charged glass slides. For the IHC procedure, resin was removed from thin sections, and sections were blocked for non-specific antibody binding and auto-fluorescence. All sections on slides (from positive and negative samples) were incubated with a ready-to-use, mouse-origin FITC-conjugated BTV antibody (CJ-F-BTV-MAB, Veterinary Medical Research & Development) for 2 h. For negative controls, both positive and negative sample section slides were incubated with a non-immune mouse serum (in lieu of antibody) and then an anti-mouse-FITC secondary.

Structured illumination microscopy was used for imaging. SIM is a fluorescence microscopy approach that breaks through the 240 nm resolution limit of visible light, generating images based on Moiré fringes combined with image reconstruction in Fourier space (Gustafsson et al. 2008). SIM provides X and Y resolutions of 85 nm for the fluorescein isothiocyanate (FITC) fluorophore.

RESULTS

Because BTV is not transovarially transmitted (Osborne et al. 2015) only previously blood-fed (parous) females were tested for BTV. Of 674 total pools (representing about 13,000 parous female midges), 212 were from CO_2 traps, 145 were from UV traps, and 317 were from CO_2+UV traps. Of the tested pools, 126 (18.7%) were positive for BTV ($Ct < 31$).

Pool sizes (parous insects per pool) did not differ significantly for the three trap types ($P=0.421$). When the data from all three dairies were combined, there was an overall highly significant difference between the traps ($X^2=76.52$; df=2, $P<0.001$) (Table 3.2). Despite comprising similar proportions of the total pools, CO_2 traps and UV traps accounted for vastly different proportions of the positive pools. Infected midges were not well represented in UV-only traps (Fig. 3.1), a trend maintained across all three dairies. All pairwise comparisons were significantly different ($X^2 \geq 4.65$; df=1; $P \leq 0.031$), with CO_2 traps having the highest total number of positive pools (n=80). Relative to UV traps, 10 times as many positive pools came from CO_2 traps. When the collections from each dairy were examined separately, CO_2 traps still had higher numbers of positive pools

($X^2 \geq 8.02$; df=1; $P \leq 0.005$). At two of the dairies (S and V), there was no difference between the UV and CO_2+UV traps ($X^2 \leq 1.74$; df=1; $P \geq 0.188$).

Across dairies, maximum likelihood estimates of infection rates (reported as number of infected insects per 1,000) of UV trap collections (mean=2/1,000) were always markedly lower than those of CO_2 trap collections (mean=15/1,000) ($P \leq 0.021$). UV traps only detected BTV when it was most common, and entirely missed detecting BTV on weeks 1, 2 and 6 (Fig. 3.2). Infection rate estimates of CO_2+UV trap collections (mean=6/1,000) were also significantly lower than those of CO_2 trap collections (P=0.031),

For the two dairies (S and D) that had clear separation of trap locations (near animals, near wastewater ponds where midges developed, or in open fields), chi-square analysis was used to examine the number of BTV-positive and negative pools at each location (Table 3.3). Taken together, there was an overall significant difference among locations ($X^2=17.36$; df=2; $P<0.001$). Positive pools were particularly rare from wastewater pond collections, with significantly fewer positive pools from traps in those locations than from traps near both animals and fields ($X^2 \geq 5.53$; df=1; $P \leq 0.019$), which did not differ from each other ($X^2=2.06$; df=1; P=0.152). At either dairy separately, there was no difference in the numbers of positive pools from animals versus wastewater ponds locations, although there was a stable trend towards more positive pools in traps near animals ($X^2 \geq 2.59$; df=1; $P \geq 0.067$).

The effect of trap placement on infection rate was also examined on dairies S and D (Fig. 3.3). There was a significant interaction between trap and location (P=0.033), with traps

set in fields collecting midges with higher infection rates when baited with CO_2 alone (mean=29/1,000). Traps placed near wastewater ponds consistently yielded almost no BTV, and when they did collect infected insects, the estimated infection rates from those traps were significantly lower than from traps set either near animals or in fields ($P \leq 0.009$). UV traps similarly did poorly, regardless of where they were set (mean≤2/1,000). There was no difference in the infection rates of midges collected near animals or in fields ($P = 0.681$).

Ommatidia in the eyes of infected insects showed positive staining, indicating that BTV infected these tissues. The strongest positive signals were located in the rhabdom and cornea (Fig. 3.4).

DISCUSSION

Vector control and disease surveillance programs worldwide rely on a variety of trapping methods to collect insect vectors in order to assess the risk of pathogen transmission to humans and animals. Pathogen manipulation of host behavior has become a popular research topic, but little attention has been paid to how pathogens may change vector behavior in ways that prevent their detection by our surveillance methods. Although these effects may not be as direct as those on transmission, the consequences of ignoring them could be profound, as we have demonstrated for BTV.

We showed that not only were infection rates significantly lower in collections from UV-only baited traps than from CO_2-only baited traps, they were also significantly lower in the traps baited with both attractants, suggesting that light actually repelled

infected midges. An alternative explanation is that some proportion of uninfected, parous midges are attracted to UV, but not CO_2 (Anderson and Linhares 1989). This could increase the number of total midges collected in the CO_2+UV traps, but decrease the observed infection rates in those traps. However, CO_2+UV traps had only half as many BTV-positive pools as CO_2 traps, supporting the pathogen manipulation hypothesis.

Fully disseminated virus infections of insect vectors may include high infection rates in the head and nervous tissues, including the eyes, and infections of these tissues could entail behavioral modification (Girard et al. 2004, Salazar et al. 2007, Smith et al. 2007). We suspected that BTV also infected *C. sonorensis* eyes during the later stages of infection, and that this may be the root cause of the infection-associated behavior we observed in the field.

The *Culicoides* eye structure closely resembles the dark-adapted arrangement of *Anopheles* mosquito ommatidia (Land 1997), and the strongest positive signals were from the cornea and rhabdom (Fig. 3.4), which collect and focus light to form images (Land 1997). Virus damage in the rhabdom could allow some visible light to essentially be lost, reducing visual acuity. Due to the apparent aversion to light, infection may also be altering how visual signals are processed in the nervous system. Though any adaptive advantage for pathogen spread is unclear, the effect in the field is strong, and it alters our estimates of transmission risk.

Although geographically separate vector populations may show differences in competence (Jones and Foster 1978), it was surprising to observe substantial differences in infection rates among locations on two individual farms. Distances between trap

locations on a farm (200-1,000 m) were well within *C. sonorensis* flight range (1-2 km) (Lillie et al. 1981, Kluiters et al. 2015). We can assume then that the insects belonged to the same interbreeding population, and that infection rate differences among trap locations are unlikely to reflect genetic variation in competence. Further, the same trap location effect appeared on dairies 69 km apart, i.e. in different vector populations.

Detecting higher infection rates in fields and almost no BTV near ponds could reflect an additional virus-mediated behavioral change if infected insects have a longer interval between oviposition and host seeking than uninfected insects. Infected insects may leave oviposition sites (wastewater ponds), ignoring those traps, and disperse further (in this case into fields) before being stimulated by host cues, like CO_2. Though we did not test this hypothesis, this effect could have major epidemiological consequences, and merits further investigation.

Entomological inoculation rates (EIR; infective vector bites per unit time) are field-derived but seldom determined for most vector-borne diseases. Given the many factors that influence vector-host location and biting, collections directly from a host are preferable (Kilama et al. 2014). However, determining actual biting rates is difficult and especially rare in animal vector-borne disease systems (Gerry et al. 2009, Mayo et al. 2012), and true EIR determinations directly from animals in the field have not been done for BTV to our knowledge. In the best-studied vector-pathogen system (*Anopheles* mosquitoes and *Plasmodium*), light traps are imperfect, but provide an adequate representation of EIR in some situations (Kilama et al. 2014). Unlike *Plasmodium*, arboviruses are frequently widely disseminated and capable of infecting many tissues (Fu

et al. 1999, Girard et al. 2004, Salazar et al. 2007, Smith et al. 2007), with possible repercussions for vector behavior. In both human and animal systems, trap-generated EIR data would be far more efficient logistically and would avoid exposing vertebrates to pathogen transmission, but only provided they represent biting and infection adequately.

Surveillance data, including trap-derived EIR estimates, influence our estimates of vectorial capacity and force of infection. An error in vector infection rates would serve as a direct multiplier of overall error for transmission risk estimates. We calculated the error caused by using only UV trap-collected *C. sonorensi*s, assuming that BTV infection estimates in vectors from CO_2 traps accurately represent virus in host-seeking insects (Mayo et al. 2012). When it could be calculated at all, using infection rates from vectors collected in UV traps underestimated those in host-seeking insects (CO_2 traps) by factors of 2.7-8.5. Additionally, UV traps frequently failed to detect BTV when it was present, particularly early in the transmission season, resulting in errors even higher than could be calculated.

Errors generated by reliance on trapping methods that do not accurately represent infection rates in vectors have serious potential consequences for human and animal health, international trade restrictions, and national economies. For example, the massive European BTV outbreak from 2006 through 2008 cost affected individual countries between 32.4 and 175 million Euros (Velthuis et al. 2007, Wilson and Mellor 2010). Estimates from currently BTV-free countries suggest that virus reintroduction would incur similar costs of lost production and control (Fofana et al. 2015). Using only light traps for surveillance, we could fail to detect BTV activity for weeks or longer, missing

critical opportunities for early action to prevent disease spread. Further, UV light traps might not detect BTV during periods when it is not abundant (i.e. potential overwintering in vectors), resulting in misunderstanding of the true activity patterns of BTV in nature.

Biological data from trap collections are also vital for modeling pathogen spread across landscapes (and borders), and for predicting the economic impact of introduction and establishment. Pathogen-associated behavioral changes in vectors should be considered. Using only light trap data may negatively influence predictive models, which inform vaccination campaigns and international trade policy. Surveillance costs should be included in economic impact models, and can be a significant part of total outbreak costs (Hadorn et al. 2009, Souza et al. 2012, Fofana et al. 2015). Therefore, optimizing surveillance strategy is critical.

UV-light baited traps rarely collect BTV infected *C. sonorensis* midges, and in fact, infected midges appear to be UV-light averse. Global reliance on light trapping for BTV vectors may be resulting in transmission risk estimates that are severely understated, and could potentially prevent early detection of BTV outbreaks. Beyond BTV and animal health, light trapping can be used to survey other vector species, including vectors of human pathogens. We have also shown that location of sampling can dramatically impact these estimates, at least for BTV. These data are used by public health officials to time control measures to protect the general public from disease. Little information exists regarding trap influences on estimating infection rates in most other pathogen-vector systems. Accurate prediction of pathogen transmission risk should save human and animal lives, and reduce costs. A better understanding of how pathogens

manipulate vector behavior in the field will help improve our surveillance methods and ultimately reduce transmission.

REFERENCES

Akey, D., and H. Potter. 1979. Pigmentation associated with oogenesis in the biting fly *Culicoides variipennis* (Diptera: Ceratopogonidae): determination of parity. J. Med. Entomol. 16: 67-70.

Anderson, J., and A. Linhares. 1989. Comparison of several different trapping methods for *Culicoides variipennis* (Diptera: Ceratopogonidae). J. Am. Mosq. Contr. Assoc. 5: 325-334.

Becker, M., W. Reeves, K. Dejean, M. Emery, E. Ostlund, and L. Foil. 2010. Detection of bluetongue virus RNA in field-collected *Culicoides* spp. (Diptera: Ceratopogonidae) following the discovery of bluetongue virus serotype 1 in white-tailed deer and cattle in Louisiana. J. Med. Entomol. 47: 269-273.

Biggerstaff, B. 2009. PooledInfRate, Version 4.0: a Microsoft® Office Excel© Add-In to compute prevalence estimates from pooled samples. Centers for Disease Control and Prevention, Fort Collins, CO, U.S.A.

Carpenter, S., A, Wilson, W. Barber, E. Veronesi, P. Mellor, G. Venter, and S.Gubbins. 2011. Temperature dependence of the extrinsic incubation period of Orbiviruses in *Culicoides* biting midges. PLOS One. 6: e27987.

Cator, L., P. Lynch, M. Thomas, and A. Read. 2014. Alterations in mosquito behavior by malaria parasites: potential impact on force of infection. Malar. J. 13: 164.

Fofana, A., L. Toma, D. Moran, G. J. Gunn, S. Gubbins, C. Szmaragd, and A. W. Stott. 2015. An *ex-ante* economic appraisal of bluetongue virus incursions and control strategies. J. Agricult. Sci. doi: 10.1017/S0021859615000015.

Fu, H., C. Leake, P. Mertens, and P. Mellor. 1999. The barriers to bluetongue virus infection, dissemination and transmission in the vector, *Culicoides variipennis* (Diptera: Ceratopogonidae). Arch. Virol. 144: 747-761.

Gerry, A. C, and B. A. Mullens. 2000. Seasonal abundance and survivorship of *Culicoides sonorensis* (Diptera: Ceratopogonidae) at a southern California dairy, with reference to potential bluetongue virus transmission and persistence. J. Med. Entomol. 37: 675-688.

Gerry, A. C., B. A. Mullens, N. J. MacLachlan, J. Mecham. 2001. Seasonal transmission of bluetongue virus by *Culicoides sonorensis* (Diptera: Ceratopogonidae) at a southern California dairy and evaluation of vectorial capacity as a predictor of bluetongue virus transmission. J. Med. Entomol. 38: 197-209.

Gerry, A., V. Sarto I Monteys, J. Moreno Vidal, O. Francinco, and B. Mullens. 2009. Biting rates of *Culicoides* midges (Diptera: Ceratopogonidae) on sheep in northeastern Spain in relation to midge capture using UV light and carbon dioxide-baited traps. J. Med. Entomol. 46: 615-624.

Girard, Y., K. Klinger, and S. Higgs. 2004. West Nile virus dissemination and tissue tropisms in orally infected *Culex pipiens quinquefasciatus*. Vector-Borne. Zoonot. Dis. 4: 109-122.

Grimstad, P., Q. Ross, and G. Craig. 1980. *Aedes triseriatus* (Diptera: Culicidae) and La Crosse virus II. Modification of mosquito feeding behavior by virus infection. J. Med. Entomol. 17: 1-7.

Goffredo, M., F. Monaco, G. Capelli, M. Quaglia, V. Federici, M. Catalani, F. Montarsi, A. Polci, C. Pinoni, P. Calistri, and G. Savini. 2013. Schmallenberg virus in Italy: a retrospective survey in *Culicoides* stored during the bluetongue Italian surveillance program. Prevent. Vet. Med. 111: 230-236.

Gustafsson, M. G. L., L. Shao, P. M. Carlton, C. J. R. Wang, I. N. Golubovskaya, W. Z. Cande, D. A. Agard, and J. W. Sedat. 2008. Three-dimensional resolution doubling in wide-field fluorescence microscopy by structured illumination. Biophys. J. 94: 4957-4970.

Hadorn, D., V. Racloz, H. Schwermer, and K. Stark. 2009. Establishing a cost-effective national surveillance system for bluetongue using scenario tree modeling. Vet. Res. 40: 57.

Hoffmann, B., B. Bauer, C. Buaer, H. –J. Batza, M. Beer, P. –H. Clausen, M. Geier, J. M. Gethmann, E. Kiel, G. Liebisch, A. Liebisch, H. Mehlhorn, G. A. Schaub, D> Werner, and F. J. Conraths. 2009. Monitoring of putative vectors of bluetongue virus serotype 8, Germany. Emerg. Infect. Dis. 159: 1481-1484.

Jackson, B., C. Brewster, and S. Paulson. 2012. La Crosse virus infection alters blood feeding behavior in *Aedes triseriatus* and *Aedes albopictus* (Diptera: Culicidae). J. Med. Entomol. 49: 1424-1429.

Jones, R., and N. Foster. 1978. Heterogeneity of *Culicoides variipennis* field populations to oral infection with bluetongue virus. Am. J. Trop. Med. Hyg. 27: 178-183.

Kilama, M., D. L. Smith, R. Hutchinson, R. Kigozi, A. Yeka, G. Lavoy, M. R. Kamya, S. G. Staedke, M. J. Donnelly, C. Drakeley, B. Greenhouse, G. Dorsey, and S. W. Lindsay. 2014. Estimating the annual entomological inoculation rate for *Plasmodium falciparum* transmitted by *Anopheles gambiae s.l.* using three sampling methods in three sites in Uganda. Malar. J. 13: 111.

Kluiters, G., H. Swales, and M. Baylis. 2015. Local dispersal of palaearctic *Culicoides* biting midges estimated by mark-release-recapture. Parasites & Vectors. 8: 86.

Land, M. 1997. Visual acuity in insects. Annu. Rev. Entomol. 42: 147-177.

Lefevre, T., and F. Thomas. 2008. Behind the scene, something else is pulling the strings: emphasizing parasitic manipulation in vector-borne diseases. Infec. Gen. Evol. 8: 504-519.

Lillie, T., W. Marquardt, and R. Jones. 1981. The flight range of *Culicoides variipennis* (Diptera: Ceratopogonidae). Can. Entomol. 113: 419-426.

Lima-Camara, T. N., R. V. Bruno, P. M. Luz, M. G. Castro, R. Lourenco-de-Oliveira, M. H. F. Sorgine, and A. A. Peixoto. 2011. Dengue infection increases the locomotor activity of *Aedes aegypti* females. PLOS One. 6: e17690.

Luz, P. M., T. N. Lima-Camara, R. V. Bruno, M. Goncalves de Castro, M. H. F. Sorgine, R. Lourenco-de-Oliveira, and A. A. Peixoto. 2011. Potential impact of a presumed increase in the biting activity of dengue-virus-infected *Aedes aegypti* (Diptera: Culicidae) females on virus transmission dynamics. Memorias do Instituto Oswaldo Cruz. 106: 755-758.

Mayo, C. E., B. A. Mullens, A. C. Gerry, C. M. Barker, P. P. C. Mertens, S. Maan, N. Maan, I. A. Gardner, A. J. Guthrie, and N. J. MacLachlan. 2012. The combination of abundance and infection rates of *Culicoides sonorensis* estimates risk of subsequent bluetongue virus infection of sentinel cattle on California dairy farms. Vet. Parasitol. 187: 295-301.

Osborne, C. J., C. E. Mayo, B. A. Mullens, E. G. McDermott, A. C. Gerry, W. K. Reisen, and N. J. MacLachlan. 2015. Lack of evidence for laboratory and natural vertical transmission of bluetongue virus in *Culicoides sonorensis* (Diptera: Ceratopogonidae). J. Med. Entomol. 52: 274-277.

Purse, B., S. Carpenter, G. Venter, G. Bellis, and B. Mullens. 2015. Bionomics of temperate and tropical *Culicoides* midges: knowledge gaps and consequences for transmission of *Culicoides*-borne viruses. Annu. Rev. Entomol. 60: 373-392.

Reese, S., M. Beaty, E. Gabitzsch, C. Blair, and B. Beaty. 2009. *Aedes triseriatus* females transovarially infected with La Crosse virus mate more efficiently than uninfected mosquitoes. J. Med. Entomol. 46: 1152-1158.

Salazar, M., J. Richardson, I. Sanchez-Vargas, K. Olson, and B. Beaty. 2007. Dengue virus type 2: replication and tropisms in orally infected *Aedes aegypti* mosquitoes. BMC Microbiol. 7:9.

Smith, D., N. Arrigo, C. Leal, L. Muehlberger, and S. Weaver. 2007. Infection and dissemination of Venezuelan equine encephalitis virus in the epidemic mosquito vector, *Aedes taeniorhynchus*. Am. J. Trop. Med. Hyg. 77: 176-187.

Souza Monteiro, D., L. Carrasco, L. Moffitt, and A. Cook. 2012. Robust surveillance of animal diseases: an application to the detection of bluetongue disease. Prevent. Vet. Med. 105: 17-24.

Stafford, C., G. Walker, and D. Ullman. 2011. Infection with a plant virus modifies vector feeding behavior. Proc. Natl. Acad. Sci. USA. 108: 9350-9355.

Velthuis, A., H. Saatkamp, M. Mourits, A. de Koeijer, and A. Elbers. 2010. Financial consequences of the Dutch bluetongue serotype 8 epidemics of 2006 and 2007. Prevent. Vet. Med. 93: 294-304.

Veronesi, E., F. Antony, S. Gubbins, N. Golding, A. Blackwell, P. P. C. Mertens, J. Brownlie, K. E. Darpel, P. S. Mellor, and S. Carpenter. 2013. Measurement of the infection and dissemination of bluetongue virus in *Culicoides* biting midges using a semi-quantitative RT-PCR assay and isolation of infectious virus. PLOS One. 8: e70800.

Wilson, A., and P. Mellor. 2010. Bluetongue in Europe: past, present and future. Phil. Trans. R. Soc. B. 364: 2669-2681.

Wirth, W., A. Dyce, and B. Peterson. 1985. An atlas of wing photographs, with a summary of the numerical characters of *Culicoides* (Diptera: Ceratopogonidae). Contr. Am. Entomol. Inst. 22: 1-46.

TABLES AND FIGURES

Table 3.1 Effects of Location, Trap and Location*Trap on Infection Rate. ANOVA table of final model used for infection rate analysis.

Parameter	Df	Sum of Squares	Mean Squares	F. Model	R^2	Pr(>F)
Location	2	0.001	0.0007	4.51	0.067	0.011*
Trap	2	0.003	0.001	8.27	0.123	0.001***
Location*Trap	4	0.002	0.0004	2.68	0.080	0.033*
Residuals	98	0.016	0.0002		0.723	
Total	106	0.021		1.00		

Permutations: 999
Significance codes: 0(***), 0.001(**), 0.01(*), 0.05(.)

Table 3.2 X^2 Analysis of Positive vs. Negative Pools by Trap. Within trap type, the first row of the table shows the observed values and the second row shows the expected values.

Trap	Positive Pools	Negative Pools	Total
CO_2	80	132	212
	39.6	172.4	
UV	8	137	145
	27.1	117.9	
CO_2+UV	38	279	317
	59.3	257.7	
Total	126	548	675

X^2=76.52, df=2, p<0.001

Table 3.3 X^2 Analysis of Positive vs. Negative Pools by Location. Data from dairies S and D only. Within a location, the first row of the table shows the observed values and the second row shows the expected values.

Location	Positive Pools	Negative Pools	Total
Animals	14	68	82
	15.6	66.6	
Wastewater Ponds	7	103	110
	27.1	117.9	
Fields	69	211	280
	53.4	226.6	
Total	90	382	472

X^2=17.36, df=2, $p<0.001$

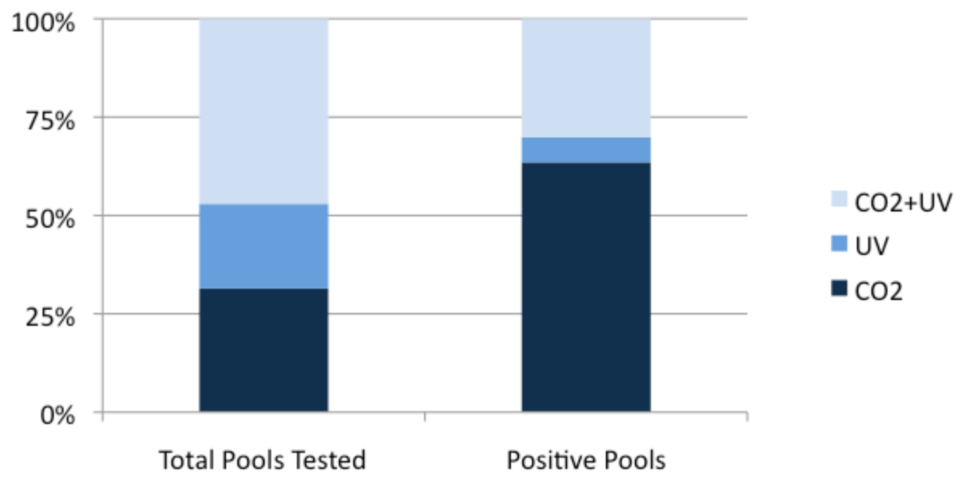

Figure 3.1 Proportions of vector pools tested (n=674) for BTV and of the BTV-positive pools (n=126) by trap type.

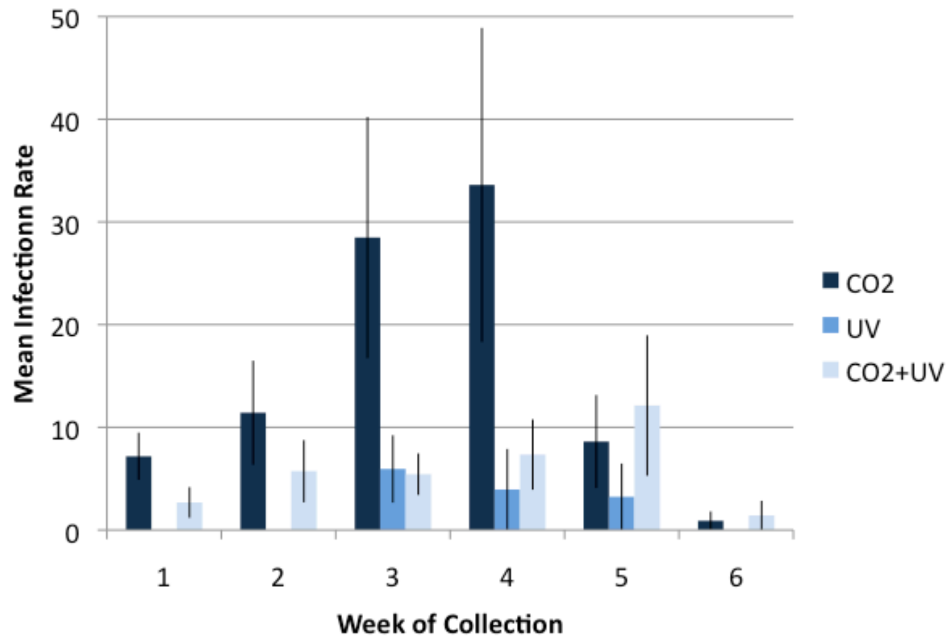

Figure 3.2 Weekly Infection Rates. Mean (± standard error) infection rate (per 1,000 insects) by trap type and week.

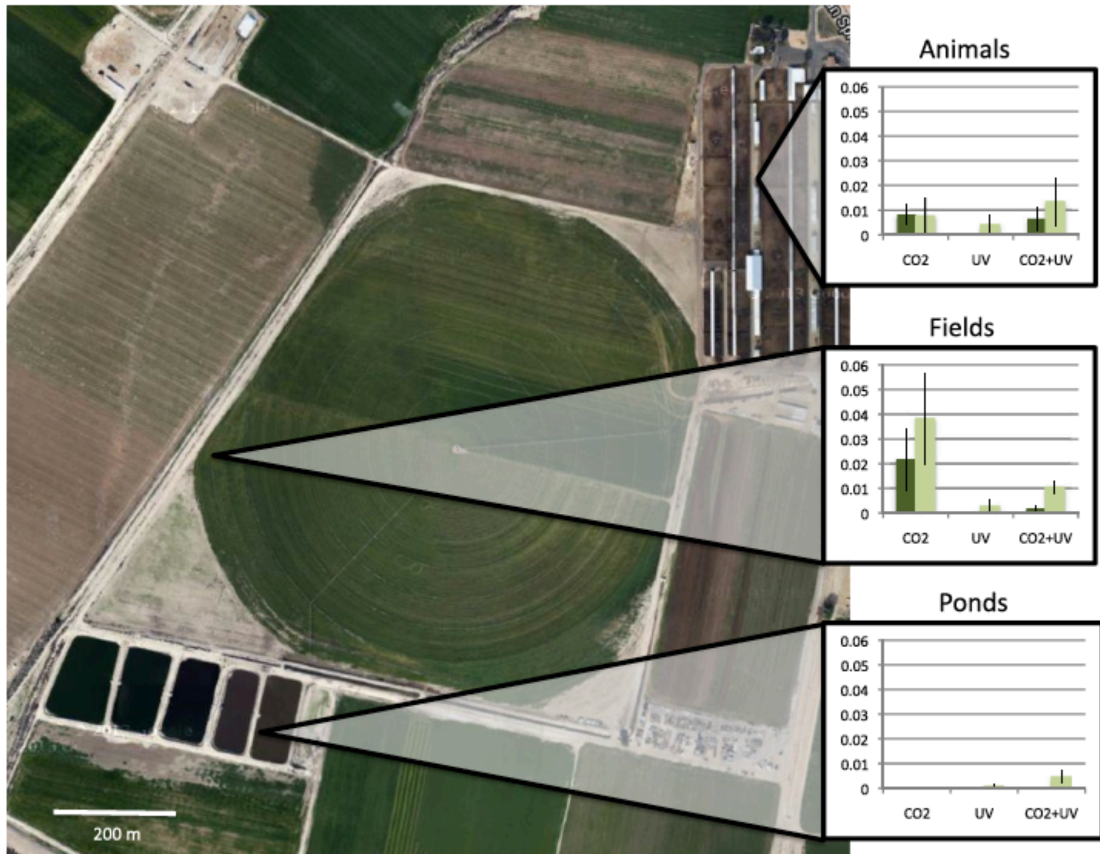

Figure 3.3 Interaction between trap and location. Mean (± standard error) BTV infection rate by attractant type and trap placement at dairies S and D. Map shows placement of traps on dairy S. Dark green bars represent dairy S, light green bars represent dairy D.

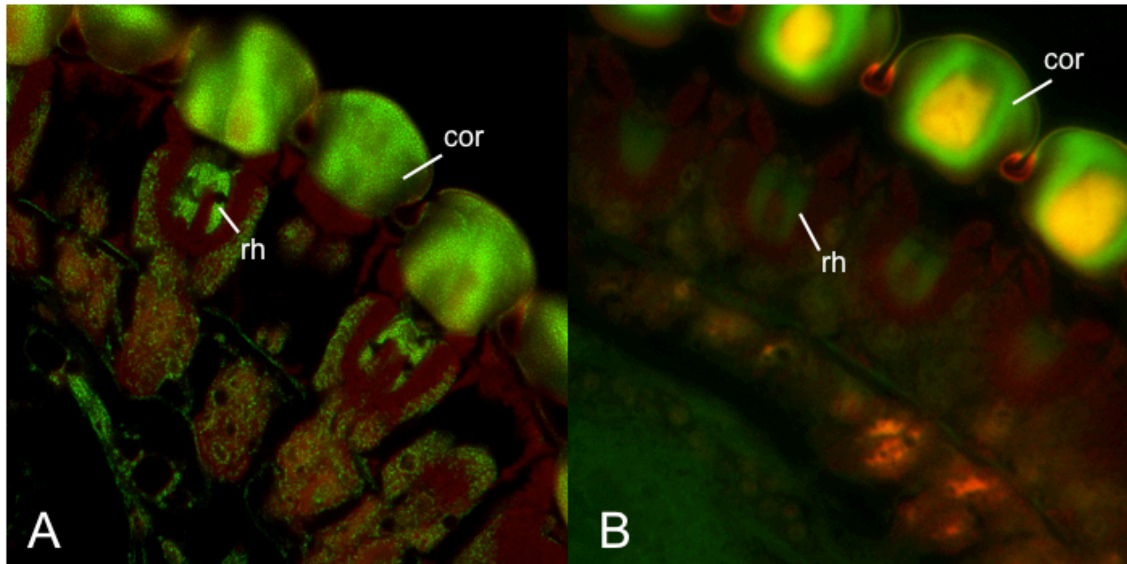

Figure 3.4 A) Ommatidia (eye cross-section) of orally BTV infected *C. sonorensis*, after 10 days. B) Ommatidia of uninfected (un-fed control) *C. sonorensis*. The rhabdom (rh) and cornea (cor) are heavily infected in BTV-positive insects (punctate green staining).

CHAPTER 4

Trap placement and attractant choice affect capture and create sex and parity biases in collections of the biting midge, *Culicoides sonorensis*

Emily G. McDermott[1], Christie E. Mayo[2], Alec C. Gerry[1], and Bradley A. Mullens[1]

[1]Department of Entomology, University of California, Riverside, CA 9252
[2]Department of Microbiology, Immunology & Pathology, Colorado State University, Fort Collins, CO 80523

ABSTRACT

Culicoides sonorensis Wirth & Jones (Diptera: Ceratopogonidae) is the primary North American vector of bluetongue virus (BTV), which can cause high morbidity and mortality in ruminant livestock or wildlife. Worldwide, most *Culicoides* surveillance relies on light (usually UV) traps typically placed near animals or larval development sites. However, trapping method can cause sex, species and parity biases in collections. We collected *C. sonorensis* from three dairies in California using suction traps baited with CO_2, UV light or CO_2+UV placed near animals, wastewater ponds, or in fields. Higher numbers of parous females were collected using CO_2+UV traps, though this difference was only significant on one dairy. UV traps were poor at collecting nulliparous females, but the addition of UV to a trap generally increased the abundance of males in a collection. Traps set in open fields collected significantly higher numbers of males and females than in either of the other two locations. In some cases, there was a significant interaction between the trap type and site. We discuss the limitations of traditional trapping methodologies for *C. sonorensis* and make suggestions for vector surveillance.

INTRODUCTION

Bluetongue virus (BTV) is a widely distributed virus of wild and domestic ruminants and is transmitted by biting midges in the genus *Culicoides* (MacLachlan and Mayo 2013, Purse et al. 2015). Bluetongue disease is of special importance for the production and trade of cattle and sheep, which can suffer high morbidity and mortality (MacLachlan and Mayo 2013). In the United States (U.S.), the primary vector of BTV is

C. sonorensis Wirth and Jones (Tabachnick 1996). This species is associated with livestock habitats, and typically utilizes the shallow, muddy sides of dairy wastewater ponds, or similar habitats, as larval development sites (Mullens 1989). *Culicoides sonorensis* is endemic to the southern and western parts of the U.S., and its distribution overlaps with the endemic range of BTV (Metcalf et al. 1981). *Culicoides sonorensis* is also a confirmed vector of epizootic hemorrhagic disease virus (EHDV), a serious disease of white tailed deer in the midwestern and southeastern U.S. (Foster et al. 1977). However, *C. sonorensis* is absent from much of the current range of EHDV in the U.S. (Ruder et al. 2015), indicating that there are likely other vectors responsible for the much of the EHDV transmission in North America.

Despite evidence that other collection methods (e.g. aspiration off sentinel animals and semiochemical baited traps) can be more epidemiologically meaningful (Gerry et al. 2009, Viennet et al. 2011, Harrup et al. 2012), the majority of *Culicoides* surveillance worldwide is done using suction traps baited with only UV light (Carpenter et al. 2008, Hoffmann et al. 2009, Goffredo et al. 2013). Such traps, commonly Centers for Disease Control (CDC) or Onderstepoort traps (OVI), reliably collect a wide variety of *Culicoides* species, but their shortcomings in assessing vector potential for BTV or similar viruses should be considered.

Traps are typically placed near either livestock or putative *Culicoides* development sites (wastewater ponds, swampy areas, composted manure, etc.), as it has generally been assumed that these locations are the most suitable for collecting midges. Compared to studies on trap design or attractant used, relatively less work has been done

examining how trap placement affects *Culicoides* collections, but there is evidence that placement does matter (Baldet et al. 2008, Baylis et al. 2010, Kirkeby et al. 2013b,c). Trapping biases are also likely to be species-specific (Bellis and Reid 1996), and so it is unwise to develop trapping strategies based on broad generalizations about insect trap response.

Worldwide we have an inadequate understanding of how trap placement and attractant affect the age structure, sex bias, species composition, and abundance of collected *Culicoid*es. Often, we lack an understanding of what trap collections mean epidemiologically, and this impedes our understanding of natural transmission dynamics and development of accurate transmission risk assessment models. The goal of this study was to determine how trap attractant and placement on multiple dairies affected the number of *C. sonorensis* collected, and the sex and parity ratios of those collections. We discuss how the results can be used to improve the efficiency of North American *Culicoides* and BTV/EHDV surveillance, and the implications of relying solely on light traps.

MATERIALS AND METHODS

Materials and methods are described in detail in Chapter 3. Briefly, wastewater ponds and other available *C. sonorensis* development sites on dairies in Chino and San Jacinto, California, U.S. were sampled for larvae to confirm the presence of the species. Three dairies (one in San Jacinto; dairy S, and two in Chino; dairies D and V) were ultimately selected for the study based on large numbers of larvae collected there. Three

spatially discrete trapping sites were chosen on dairies S and D; one near cattle, a second near wastewater ponds, and a third in fields away from either cattle or ponds. On dairy V, only two sites (one in fields and one near both animals and a development site) were chosen because of a lack of separation between the aforementioned locations (Fig. 4.1). At each trapping site, a group of three CDC-type suction traps (miniature light trap model 512, J.D. Hock and Co., Gainesville, Florida, USA) was used, with traps separated by 20 m and arrayed in a line perpendicular to prevailing west-east winds. Traps were baited with UV-light (4W black light bulb, F4T5BL), CO_2 (0.5 kg dry ice), or a combination of CO_2+UV. The position of each trap in each location was randomized at the start of the study and traps were rotated systematically each night thereafter. Each trap attractant treatment occupied each position twice over the course of the study. Insects were collected into a container of water plus 0.05% detergent (Liqui-Nox®), keeping insects in optimal condition for later sorting by parity status. Trapping was conducted once a week on each dairy for 6 weeks from September 10th to October 17th 2013. Traps ran from approximately 2 h before sunset to no more than 4 h after sunrise.

Insects were then transported back to the laboratory where they were immediately transferred into 70% ethanol and stored at -20°C until they were sorted to species, and then by sex and parity status (males, parous females, nulliparous females). *Culicoides sonorensis* were identified by size and wing pattern (Wirth and Dyce 1985). Parity was assessed visually under a dissecting microscope by the presence (parous) or absence (nulliparous) of a red pigment in the abdominal cuticle (Akey and Potter 1979). The

number of males, parous females (pars), and nulliparous females (nullipars) in each collection was recorded.

Count data per trap and night were *ln*(n+1) transformed for analysis using a general linear model (GLM) or standard analysis of variance (ANOVA). Proportions of males, pars and nullipars were transformed using arc sin of the square root for analysis using GLM and ANOVA. The main effects of attractant, site, and the interaction of attractant and site on mean numbers of males, pars and nullipars were examined using GLM on each dairy. Trends on each dairy were examined separately. Collection date was considered a unit of replication and was not included in any of the models. Pairwise comparisons of means by attractant or site were examined using ANOVA followed by a Tukey's test.

RESULTS

With the exception of two female *Culicoides* from a single CO_2+UV trap collection on dairy V, which were identified as *C. freeborni* Wirth & Blanton and *C. crepuscularis* Malloch, all collected *Culicoides* were *C. sonorensis.* The total numbers of *C. sonorensis* caught varied greatly by dairy. Dairy S had the fewest captures averaged over all traps and locations (mean: 168.4±39.8 (SE), n=51 collections; max per trap: 1,293 total *Culicoides*), dairy D had slightly greater captures (mean: 307.6±54.2 (SE), n=50 collections; max per trap: 1,968 total *Culicoides*), and dairy V had the greatest captures (mean: 1,434.3±217.0 (SE), n=24 collections, max per trap: 3,652 total *Culicoides*). Mean numbers of *Culicoides* collected were highest at the start of the study

(mid-September) and the greatest mean captures for each dairy were made by week three (24-27th September).

Overall, attractant had a minimal effect on the abundance of pars in a given trap (Fig. 4.2). At dairy D, significantly more pars were collected in CO_2+UV traps than in CO_2-only traps ($P=0.029$), but there were no differences between attractants at dairies S and V. UV traps collected significantly fewer nullipars than CO_2+UV or CO_2-only traps on all dairies ($P \leq 0.004$), with no differences between CO_2-only and CO_2+UV traps. At dairy D, significantly more males were collected in traps with UV ($P \leq 0.001$), and collections at dairy V followed this trend.

Because the trapping sites at dairy V were not cleanly separated as at the other two dairies (Fig. 4.1), pairwise comparisons of the three trapping locations were performed for dairies S and D (Fig. 4.3). Significantly higher numbers of female (parous and nulliparous) *C. sonorensis* were captured in fields than near ponds ($P \leq 0.026$). With the exception of greater numbers of nullipars in traps near animals at dairy S ($P \leq 0.001$), abundance of females in traps near animals and ponds did not differ significantly. There was also not a significant difference in the number of females captured in traps set near animals or in fields, except for pars at dairy S ($P \leq 0.001$). For males, the only significant difference in midge capture among trapping sites was the greater collection of males in fields at dairy S ($P \leq 0.001$).

The interaction between attractant and trapping site of the number of pars collected was significant at both dairies S and D ($P \leq 0.019$). CO_2 and CO_2+UV traps captured higher numbers of pars, nullipars, and males when placed in fields than when

placed near animals or wastewater ponds at both dairies (Fig. 4.4). Surprisingly, the positive effect of the field sites on capture size disappeared when UV traps were used. In almost all cases, collections from UV traps at the field sites were smaller than from the other trap types. For parous female collection, UV traps were most efficient when placed near ponds, where they generally outperformed both CO_2 and CO_2+UV traps.

We looked at differences in the proportions of males, pars and nullipars collected in each trap and site on dairies S and D. There were significant differences in the proportions between both the traps and sites, and these also differed between dairies, so each dairy was analyzed separately (Table 4.1). At dairy S, midge captures varied more by trapping site than by attractant used, while at dairy D, the opposite was true. Despite this, the trends were generally the same between dairies. On both dairies, UV traps collected the highest proportions of pars and males, and CO_2 traps the lowest. In contrast, CO_2 traps collected the highest proportion of nullipars, and UV traps the lowest. Trends for the effect of trapping site were also similar between dairies. The highest proportions of pars were collected near ponds at dairy S, and at ponds and fields (40.8% and 42.2%, respectively) at dairy D. At both dairies, the lowest proportion of pars and the highest proportion of nullipars were collected near animals, though these differences were only significant at Dairy S. Male collection was more variable between dairies, with the highest proportion of males collected in fields at dairy S (14.8%) but near animals at dairy D (32.9%).

DISCUSSION

Most hematophagous insects will respond to CO_2, which functions as a key host cue (Nicolas and Sillans 1989, Guerenstein and Hildebrand 2008), although other kairomones may be attractive to host-seeking insects, or have synergistic effects when used in combination with CO_2 (e.g. 1-octen-3-ol; Kline et al. 1991, Gibson and Torr 1999, Harrup et al. 2012). The majority of vector surveillance for *Culicoides* still relies on CO_2, UV-light or a combination of the two, and it is common for midges of different physiological states and sexes to be differentially drawn to various attractants (Anderson and Linhares 1989, Gerry and Mullens 1998, Mullens and Gerry, 1998, Venter et al. 2009, Harrup et al. 2012). Because BTV is not transovarially transmitted (Osborne et al. 2015), understanding how trapping strategy affects both the proportion and abundance of parous females collected is important for estimating infection rates and interpreting these results epidemiologically.

Globally, UV traps are most often used for surveillance of *Culicoides* vectors (Carpenter et al. 2008, Hoffmann et al. 2009, Goffredo et al. 2013), and these traps are typically placed either near livestock or putative larval development sites. Light traps are inexpensive to operate, convenient to deploy, and collect a wide variety of *Culicoides* species, including males, which can aid identification. Light traps can be useful in initial faunal surveys, and may be the only available option in areas where the main vector species don't respond strongly to readily deployable host cues, like CO_2, or where CO_2 cannot be obtained. However, they may have serious limitations for epidemiological investigations. UV traps are now well known to result in collections with species or

parity profiles different from host biting collections (Carpenter et al. 2008, Gerry et al. 2009, Viennet et al. 2011, Scheffer et al. 2012). Here, we show that UV traps are less effective at collecting large numbers of *C. sonorensis* than traps that also use CO_2. In general, our results support earlier work comparing CO_2 and light baited suction traps for collecting *C. sonorensis*, (Anderson and Linhares 1989), and recent work on collections of the major BTV vector in Africa, *C. imicola* Kieffer (Venter et al. 2015).

Though not necessarily statistically significant, our CO_2+UV traps generally collected larger numbers of pars relative to CO_2 alone. This may be because some portion of the parous population is not host seeking at a given point in time. Some pars are host seeking while others are ovipositing, or in another behavioral state, and these individuals may be differentially responding to UV. Our observation that UV traps near wastewater ponds collect much greater proportions of pars (including gravids) than CO_2+UV or CO_2-only traps supports this hypothesis. Unlike traps with CO_2, UV traps in fields failed to collect large numbers of *C. sonorensis*, indicating that the parous population in these areas is probably mostly host seeking. Using these two attractants in combination allows for collection of a greater cross section of the parous population. Comparatively, the entire population of nulliparous females should be host seeking, and these insects were collected in lower numbers in UV traps.

UV traps generally collected greater numbers of male *C. sonorensis* than CO_2 traps, and these traps also had the highest proportion of males, which can be difficult to collect in large numbers (Anderson and Linhares 1989). The presence of males in CO_2

traps reflects the fact that although they do not blood feed, male *C. sonorensis* orient towards animals to locate females for mating (Gerry and Mullens 1998).

However, when the aim of a study is to detect BTV, or to examine BTV infection rates or BTV transmission dynamics in the field, the use of traps baited with UV-light is inadvisable for *C. sonorensis*. Suction traps baited with UV-light, with or without CO_2, collect parous *C. sonorensis* with significantly lower BTV infection rates (Chapter 3). Use of UV alone underestimates parous *C. sonorensis* BTV infection rates by 2-8 fold relative to CO_2 alone. The mechanism for this remains to be determined, but the effect in the field is clear. Because CO_2+UV collections also have lower BTV infection rates than CO_2-only collections, UV-light may repel infected *Culicoides*. Further studies are required to confirm this hypothesis. Studies that use UV traps to measure BTV infection in *C. sonorensis* risk inaccurate predictions and conclusions about virus transmission. It is not yet known whether this effect extends to other vector species. Where virus detection is important, artificially low virus infection rates of parous midges in UV traps outweigh the potential benefits of larger collections.

The most surprising result of this study was that in nearly every case (with the exception of males at dairy D), traps set in fields, away from the traditional *Culicoides* trapping locations near animals or wastewater ponds and other development sites, captured many more midges. Compared to the effect of trap attractant, trap placement has received less attention as a factor in the abundance and parity of *Culicoides* collections, though it may be just as important to consider. In our study, trap location influenced *C. sonorensis* numbers more than attractant choice did. Although counterintuitive, placing

traps in fields clearly resulted in increased catches of *C. sonorensis*, with the difference between field captures and pond captures as high as 28x (nullipars in fields vs. ponds at dairy S). Conversely, a recent study in South Africa (Venter et al. 2015) found that more *Culicoides* were collected in OVI traps with UV placed near livestock than in the weaker CDC traps with incandescent light placed 500m from animal enclosures. The present study, however, employed a single consistent trap type to study the effect of attractant and location.

Traps placed near animals are subject to a dilution effect. For traps using light alone, the powerful draw of nearby host animals may enhance *Culicoides* captures (Bellis and Reid 1996, Garcia-Saenz et al. 2011). CO_2 is a potent attractant and greatly increases *C. sonorensis* numbers relative to traps with light (Anderson and Linhares 1989). However, nearby hosts can also compete with suction traps, especially those that employ CO_2 or other host cues. *Culicoides sonorensis* responds positively to increasing amounts of CO_2, but a single mature cow exhales more CO_2 than is emitted by one of our traps (Mullens 1995, Mullens and Gerry 1998). Cattle also emit the full range of host cues (chemical, visual, and thermal), which our traps lacked. Smaller captures of *C. sonorensis* in CO_2-baited traps near cattle have been observed before (Gerry and Mullens 2000). Even when CO_2 output from a trap was matched to the CO_2 output of a bait animal, several times more *C. sonorensis* were collected from the animal than in the trap or its immediate vicinity, indicating the increased attractiveness of a live animal over the animal equivalent concentration of CO_2 (Mullens and Gerry 1998).

There may be fewer nullipars and males in the vicinity of wastewater ponds because they are not seeking oviposition sites. Alternatively, mating swarms forming near traps (as a visual swarm marker) at any site may increase capture of males. We would expect more of the population near development sites to be parous/gravid females seeking an oviposition site, and so not responsive to host cues. Traps set near wastewater ponds did not collect very many females, but parity at these sites was high, as in other studies (Mullens 1985, Anderson and Linhares 1989, Work et al. 1991, Gerry and Mullens 2000).

Although counterintuitive and contrary to common trapping practice, one is likely to collect more *C. sonorensis* in CO_2 traps placed in level, open fields even a few hundred meters from cattle. Notably, however, placing light traps in fields did not necessarily result in more insects collected versus light traps near animals or ponds. Enhanced field captures were seen only in the CO_2 treatments. Habitat features (trees, fences, etc,) can affect structure of odor plumes and thus their range of attraction (Murlis et al. 1992), and this also may have influenced *Culicoides* collections in our study. Light traps might have a more stable range of attraction than CO_2 traps, but that range also may be rather limited (Kirkeby et al. 2013a, Elbers and Meiswinkel 2015).

Other species may differ from *C. sonorensis* in their response to light or CO_2. Work in Louisiana (Wieser-Schimpf et al. 1991) was consistent with ours in showing that parity was higher in *C. sonorensis* collections from UV traps than from CO_2 traps, but parity in *C. stellifer* (Coquillett) did not differ among light or CO_2 treatments. Higher abundance of insects in light traps placed closer to animals (Baylis et al. 2010, Kirkeby et

al. 2013b) also might not be a straightforward effect (Kirkeby et al. 2013b,c), and differences in *Culicoides* response to different trapping strategies are likely species-specific (Bellis and Reid 1996).

Species-specific differences in capture size among trapping locations could reflect their preferences for resting-sites or patterns of local scale abundance and dispersal. Individual farms differ, and so the proximity of livestock to development sites or ungrazed crop fields also differs greatly from one farm to the next. In California, the majority of dairy production features dry lots or free stall barns, which separate the animals from larval development sites (i.e. wastewater ponds) and from fields. This production system makes a study like ours, which used three distinct trapping sites, possible. In other areas, the prominent vector species (mostly subgenus *Avaritia*) utilize intact dung or composted manure for larval development (Purse et al. 2015), and livestock are allowed to graze. In these systems, there may be no clear separation of trapping sites as we have described them. Surveillance programs for *Culicoides* therefore should be customized for the prominent vector species in the area, and for the production and housing systems in place on individual farms. In areas where the main vector species are not well characterized, species identification and an understanding of their biology and behavior are critical for accurate disease surveillance. In the U.S., for instance, trap biases relevant for the capture of putative EHDV vectors should be studied further to improve our understanding of that system.

Most insect trapping systems have intrinsic biases, and so our task is to understand those biases well enough to appreciate how they impact data interpretation. In

this study we showed that trap attractant and trap placement create marked sex and parity biases in collections of *C. sonorensis*, and significantly influence the size of these collections. We showed that the traditional trapping methodology (UV-light traps placed near livestock or wastewater ponds) for *C. sonorensis* resulted in the fewest numbers of insects collected, and that CO_2 traps placed in fields may be a better alternative if the goal is collection of large numbers for virus testing. The ideal goal of any vector surveillance program is to collect a group of insects that accurately represents the relevant vector population in an area, and to collect insects in sufficient numbers for pathogen testing. There are pros and cons to every method, and these must be carefully weighed when making vector surveillance decisions.

REFERENCES

Akey, D. H., and H. W. Potter. 1979. Pigmentation associated with oogenesis in the biting fly *Culicoides variipennis* (Diptera: Ceratopogonidae): determination of parity. J. Med. Entomol. 16: 67-70.

Anderson, J. R., and A. X. Linhares. 1989. Comparison of several different trapping methods for *Culicoides variipennis* (Diptera: Ceratopogonidae). J. Am. Mosq. Contr. Assoc. 5: 325-334.

Baldet, T., J. C. Delecolle, C. Cetre-Sossah, B. Mathieu, R. Meiswinkel, and G. Gerbier. 2008. Indoor activity of *Culicoides* associated with livestock in the bluetongue virus (BTV) affected region of northern France during autumn 2006. Prevent. Vet. Med. 87: 84-97.

Baylis, M., H. Parkin, K. Kreppel, S. Carpenter, P. S. Mellor, and K. M. McIntyre. 2010. Evaluation of housing as a means to protect cattle from *Culicoides* biting midges, the vectors of bluetongue virus. Med. Vet. Entomol. 24: 38-45.

Bellis, G. A., and D. J. Reid. 1996. Sampling bias in determining the parous rate of collections of *Culicoides brevitarsis* Kieffer and *C. wadai* Kitaoka (Diptera: Ceratopogonidae). Austral. J. Entomol. 35: 319-322.

Carpenter, S., C. Szmaragd, J. Barber, K. Labuschagne, S. Gubbins, and P. Mellor. 2008. An assessment of *Culicoides* surveillance techniques in northern Europe: have we underestimated a potential bluetongue virus vector? J. App. Ecol. 45: 1237-1245.

Elbers, A. R. W., and R. Meiswinkel. 2015. Limited attractant range of the black-light suction trap for the capture of *Culicoides* biting midges (Diptera: Ceratopogonidae). J. App. Entomol. 140: 386-394.

Foster, N. M., R. D. Breckon, A. J. Luedke, and R. H. Jones. 1977. Transmission of two strains of epizootic hemorrhagic disease virus in deer by *Culicoides variipennis*. J. Wild. Dis. 13: 9-16.

Garcia-Saenz, A., P. McCarter, and M. Baylis. 2011. The influence of host number on the attraction of biting midges, *Culicoides* spp., to light traps. Med. Vet. Entomol. 25: 113-115.

Gerry, A. C., and B. A. Mullens. 1998. Response of male *Culicoides variipennis sonorensis* (Diptera: Ceratopogonidae) to carbon dioxide and observations of mating behavior on and near cattle. J. Med. Entomol. 35: 239-244.

Gerry, A. C., and B. A. Mullens. 2000. Seasonal abundance and survivorship of *Culicoides sonorensis* (Diptera: Ceratopogonidae) at a southern California dairy, with reference to potential bluetongue virus transmission and persistence. J. Med. Entomol. 37: 675-688.

Gerry, A. C., V. Sarto I Monteys, J. –O. Moreno Vidal, O. Francino, and B. A. Mullens. 2009. Biting rates of *Culicoides* midges (Diptera: Ceratopogonidae) on sheep in northeastern Spain in relation to midge capture using UV light and carbon dioxide-baited traps. J. Med. Entomol. 46: 615-624.

Gibson, G., and S. J. Torr. 1999. The visual and olfactory responses of biting flies to host stimuli. Med. Vet. Entomol. 13: 2-23.

Goffredo, M., F. Monaco, G, Capelli, M. Quaglia, V. Federici, M. Catalani, F. Montarsi, A. Polci, C. Pinoni, P. Calistri, and G. Savini. 2013. Schmallenberg virus in Italy: a retrospective survey in *Culicoides* stored during the bluetongue Italian surveillance program. Prevent. Vet. Med. 111: 230-236.

Guerenstein, P. G., and J. G. Hildebrand. 2008. Roles and effects of environmental carbon dioxide in insect life. Annu. Rev. Entomol. 53: 161-178.

Harrup, L. E., J. G. Logan, J. I. Cook, N. Golding, M. A. Birkett, J. A. Pickett, C. Sanders, J. Barber, D. J. Rogers, P. S. Mellor, B. V. Purse, and S. Carpenter. 2012. Collection of *Culicoides* (Diptera: Ceratopogonidae) using CO_2 and enantiomers of 1-octen-3-ol in the United Kingdom. J. Med. Entomol. 49: 112-121.

Hoffmann, B., B. Bauer, C. Bauer, C., H. –J. Batza, M. Beer, P. –H. Clausen, M. Geier, J. M. Gethmann, E. Kiel, G. Liebisch, A. Liebisch, H. Mehlhorn, G. A. Schaub, D. Werner, and F. J. Conraths. 2009. Monitoring of putative vectors of bluetongue virus serotype 8, Germany. Emerg. Infect. Dise. 15: 1481-1484.

Kirkeby, C., K. Graesboll, A. Stockmarr, L. E. Christiansen, and R. Bodker. 2013a. The range of attraction for light traps catching *Culicoides* biting midges (Diptera: Ceratopogonidae). Parasites & Vectors. 6: 67.

Kirkeby, C., R. Bodker, A. Stockmarr, and P. Lind. 2013b. Spatial abundance and clustering of *Culicoides* (Diptera: Ceratopogonidae) on a local scale. Parasites & Vectors. 6: 43.

Kirkeby, C., A. Stockmarr, R. Bodker, and P. Lind. 2013c. Spatio-temporal optimization of sampling for bluetongue vectors (*Culicoides*) near grazing livestock. Parasites & Vectors. 6: 151.

Kline, D. L., J. R. Wood, and J. A. Cornell. 1991. Interactive effects of 1-octen-3-ol and carbon dioxide on mosquito (Diptera: Culicidae) surveillance and control. J. Med. Entomol. 28: 254-258.

MacLachlan, N. J., and C. E. Mayo. 2013. Potential strategies for control of bluetongue, a globally emerging, *Culicoides*-transmitted viral disease of ruminant livestock and wildlife. Antiviral Res. 99: 79-90.

McDermott, E. G., C. E. Mayo, A. C. Gerry, D. Laudier, N. J. MacLachlan, and B. A. Mullens. 2015. Bluetongue virus infection creates light averse *Culicoides* vectors and serious errors in transmission risk estimates. Parasites & Vectors. 8: 460.

Metcalf, H. E., J. E. Pearson, and A. L. Klingsporn. 1981. Bluetongue in cattle: a serologic survey of slaughter cattle in the United States. Am. J. Vet. Res. 42: 1057-1061.

Mullens, B. A. 1985. Age-related adult activity and sugar feeding by *Culicoides variipennis* (Diptera: Ceratopogonidae) in southern California, USA. J. Med. Entomol. 22: 32-37.

Mullens, B. A. 1989. A quantitative survey of *Culicoides variipennis* (Diptera: Ceratopogonidae) in dairy wastewater ponds in southern California. J. Med. Entomol. 26: 559-565.

Mullens, B. A. 1995. Flight activity and response to carbon dioxide of *Culicoides variipennis sonorensis* (Diptera: Ceratopogonidae) in southern California. J. Med. Entomol. 32: 310-315.

Mullens, B. A., and A. C. Gerry. 1998. Comparison of bait cattle and carbon dioxide-baited suction traps for collecting *Culicoides variipennis sonorensis* (Diptera: Ceratopogonidae) and *Culex quinquefasciatus* (Diptera: Culicidae). J. Med. Entomol. 35: 245-250.

Murlis, J., J. S. Elkinton, and R. T. Carde. 1992. Odor plumes and how insects use them. Annu. Rev. Entomol. 37: 505-532.

Nicolas, G., and D. Sillans. 1989. Immediate and latent effects of carbon dioxide on insects. Annu. Rev. Entomol. 34: 97-116.

Osborne, C. J., C. E. Mayo, B. A. Mullens, E. G. McDermott, A. C. Gerry, W. K. Reisen, and N. J. MacLachlan. 2015. Lack of evidence for laboratory and natural vertical transmission of bluetongue virus in *Culicoides sonorensis* (Diptera: Ceratopogonidae). J. Med. Entomol. 50: 274-277.

Purse, B. V., S. Carpenter, G. J. Venter, G. Bellis, and B. A. Mullens. 2015. Bionomics of temperate and tropical *Culicoides* midges: knowledge gaps and consequences for transmission of Culicoides-borne viruses. Annu. Rev. Entomol. 60: 373-392.

Ruder, M. G., T. J. Lysyk, D. E. Stallknecht, L. D. Foil, D. J. Johnson, C. C. Chase, D. A. Dargatz, and E. P. J. Gibbs. 2015. Transmission and epidemiology of bluetongue and epizootic hemorrhagic disease in North America: current prospectives, research gaps, and future directions. Vector-Borne. Zoonot. Dis. 15: 348-363.

Scheffer, E. G., G. J. Venter, K. Labuschagne, K., P. C. Page, B. A. Mullens, N. J. MacLachlan, N. Osterrieder, and A. J. Guthrie. 2012. Comparison of two trapping methods for *Culicoides* biting midges and determination of African horse sickness virus prevalence in midge populations at Onderstepoort, South Africa. Vet. Parasitol. 185: 265-273.

Tabachnick, W. J. 1996. *Culicoides variipennis* and bluetongue-virus epidemiology in the United States. Annu. Rev. Entomol. 41: 23-43.

Venter, G. J., K. Labuschagne, K. G. Hermanides, S. N. B. Boikanyo, D. M. Majatladi, and L. Morey. 2009. Comparisons of the efficiency of five suction light traps under field conditions in South Africa for the collection of *Culicoides* species. Vet. Parasitol. 166: 299-307.

Venter, G. J., S. N. B. Boikanyo, D. M. Majatladi, and L. Morey. 2015. Influence of carbon dioxide on numbers of *Culicoides* midges collected with suction light traps in South Africa. Med. Vet. Entomol. 30: 117-122.

Viennet, E., C. Garros, R. Lancelot, X. Allene, L. Gardes, I. Rakotoarivony, D. Crochet, J. –C. Delecolle, C. Moulia, T. Baldet, and T. Balenghien. 2011. Assessment of vector/host contact: comparison of animal-baited traps and UV-light/suction trap for collecting *Culicoides* biting midges (Diptera: Ceratopogonidae), vectors of Orbiviruses. Parasites & Vectors. 4: 119.

Wieser-Schimpf, L., L. D. Foil, and F. R. Holbrook. 1991. Effect of carbon dioxide on the collection of adult *Culicoides* spp. (Diptera: Ceratopogonidae) by a new modification of black light New Jersey trap. J. Am. Mosq. Contr. Assoc. 7: 462-466.

Wirth, W. W., and A. L. Dyce. 1985. The current taxonomic status of the *Culicoides* vectors of bluetongue viruses. Progr. Clin. Biol. Res. 178: 151-164.

Work, T. M., B. A. Mullens, and D. A. Jessup. 1991. Estimation of survival and gonotrophic cycle length of *Culicoides variipennis* (Diptera: Ceratopogonidae) in California. J. Am. Mosq. Contr. Assoc. 7: 242-249.

TABLES AND FIGURES

Table 4.1 Mean (±SE, n trap nights) percentages of parous females, nulliparous females, and males per collection by trap attractant and site at dairies S and D. Significance levels for main effects: (*) p=0.05, (**) p<0.05, (***) p≤0.001. Letters represent significant differences between treatments for each effect per dairy by sex or parity status.

	Dairy S			
	Trap Attractant		Site**	
Parous Females	CO_2	40.9% (3.7, 16)[a]	Animals	35.8% (4.1, 16)[b]
	UV	58.8% (7.6, 17)[a]	Ponds	60.2% (8.0, 17)[a]
	CO_2+UV	47.9% (6.6, 16)[a]	Fields	49.0% (5.4, 16)[ab]
	Trap Attractant**		Site***	
Nulliparous Females	CO_2	54.2% (4.5, 16)[a]	Animals	54.6% (4.6, 16)[a]
	UV	27.4% (6.8, 17)[b]	Ponds	27.2% (6.1, 17)[b]
	CO_2+UV	39.7% (5.1, 16)[ab]	Fields	36.1% (5.2, 16)[ab]
	Trap Attractant		Site*	
Males	CO_2	4.8% (1.1, 16)[a]	Animals	9.7% (2.0, 16)[ab]
	UV	13.8% (5.0, 17)[a]	Ponds	6.7% (3.6, 17)[b]
	CO_2+UV	12.4% (3.5, 16)[a]	Fields	14.8% (4.9, 16)[a]
	Dairy D			
	Trap Attractant**		Site	
Parous Females	CO_2	31.8% (2.8, 17)[b]	Animals	32.5% (2.5, 15)[a]
	UV	42.8% (3.8, 16)[a]	Ponds	40.8% (4.2, 17)[a]
	CO_2+UV	42.4% (3.1, 17)[ab]	Fields	42.4% (2.8, 18)[a]
	Trap Attractant***		Site	

Nulliparous Females	CO_2	58.6% (2.6, 17)[a]	Animals	34.6% (5.8, 15)[a]
	UV	11.7% (1.3, 16)[c]	Ponds	33.7% (5.1, 17)[a]
	CO_2+UV	31.8% (2.0, 17)[b]	Fields	35.0% (5.0, 18)[a]
Males	Trap Attractant***		Site*	
	CO_2	9.7% (1.7, 17)[c]	Animals	32.9% (5.0, 15)[a]
	UV	45.5% (3.6, 16)[a]	Ponds	25.5% (4.3, 17)[ab]
	CO_2+UV	25.9% (2.9, 17)[b]	Fields	22.6% (4.2, 18)[b]

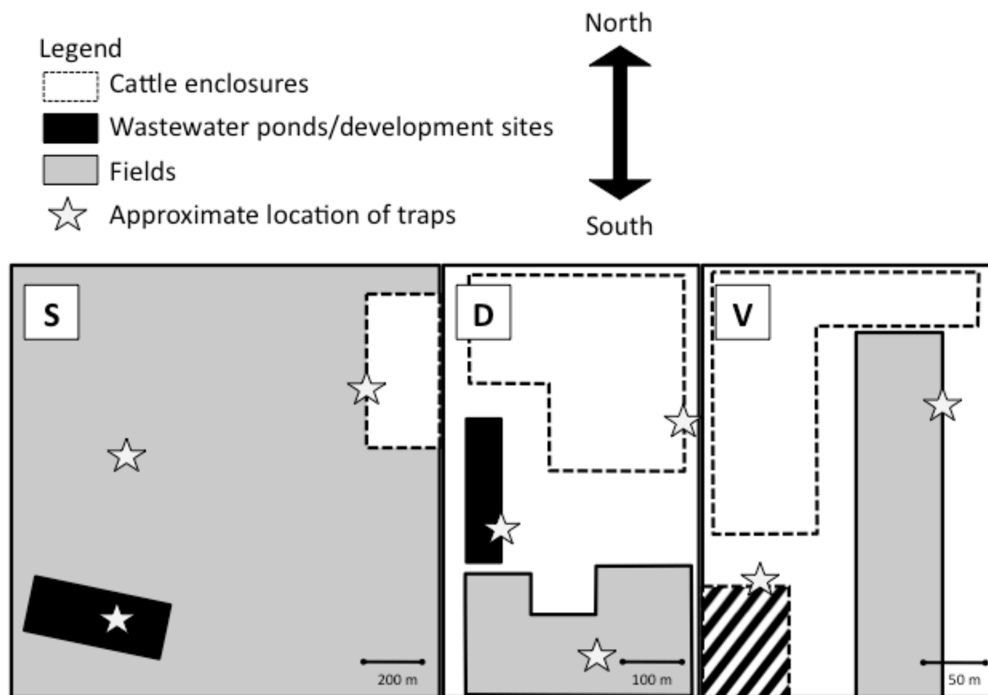

Figure 4.1 Layouts of dairies S, D, and V showing locations of cattle enclosures, wastewater ponds/*Culicoides* development sites, fields, and approximate locations of traps on each farm.

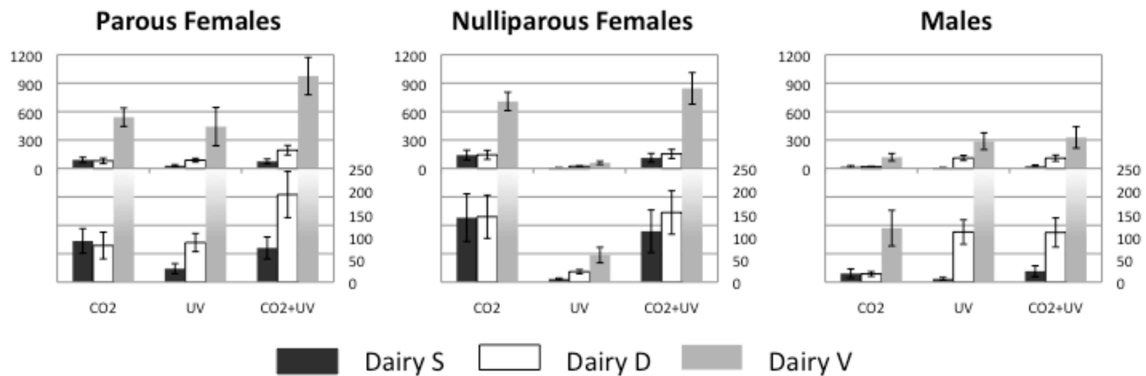

Figure 4.2 Mean (±SE) numbers of parous, nulliparous, and male *C. sonorensis* collected in each trap type for dairies S, D, and V. Top half of graphs shows complete data for all three dairies. Bottom half of graphs shows cut-off (faded) data for dairy V to better show differences between dairies S and D.

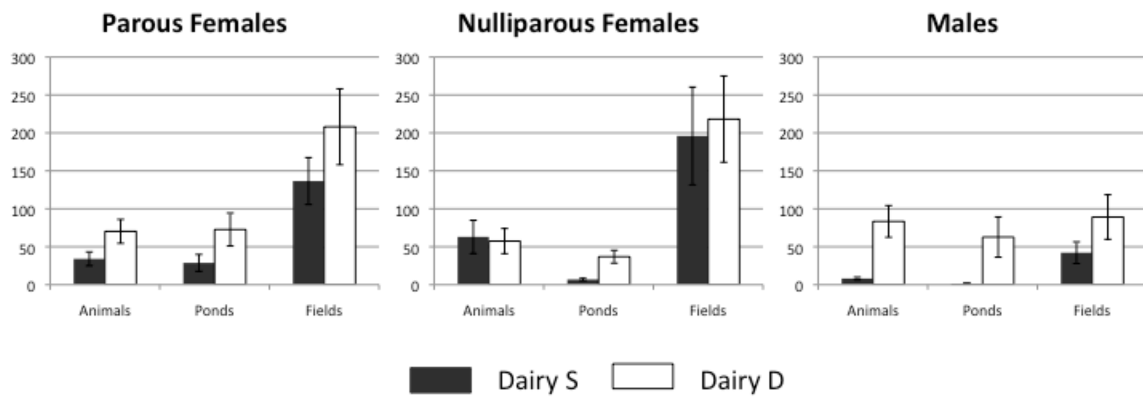

Figure 4.3 Mean (±SE) numbers of parous, nulliparous, and male *C. sonorensis* collected in each trap type for dairies S and D.

		Dairy S			Dairy D		
		Animals	Ponds	Fields	Animals	Ponds	Fields
Parous Females	CO_2	52.7 (19.6, 6)	9.60 (6.2, 5)	196.0 (51.4, 6)	18.8 (7.0, 5)	14.5 (1.8, 6)	198.0 (58.9, 6)
	UV	8.7(3.0, 6)	54.3 (29.2, 6)	25.8 (8.2, 5)	77.8 (24.9, 5)	138.6 (54.7, 5)	51.83 (9.3, 6)
	CO_2+UV	42.4 (14.9, 5)	19.2 (5.2, 6)	176.0 (53.7, 5)	114.2 (27.9, 5)	76.0 (29.6, 6)	375.0 (107.0, 6)
Nulliparous Females	CO_2	98.5 (51.8, 6)	8.8 (2.9, 5)	296.0 (118.0, 6)	32.6 (8.9, 5)	44.7 (21.0, 6)	335.7 (92.3, 6)
	UV	10.2 (3.2, 6)	4.3 (1.6, 6)	7.4 (2.9, 5)	30.4 (8.1, 5)	26.4 (10.1, 5)	14.2 (3.8, 6)
	CO_2+UV	83.8 (34.3, 5)	8.0 (3.4, 6)	265.0 (125.0, 5)	109.4 (41.7, 5)	38.0 (10.2, 6)	305.0 (109.0, 6)
Males	CO_2	8.2 (4.0, 6)	1.0 (0.78, 5)	45.5 (25.4, 6)	5.6 (2.5, 5)	14.8 (9.6, 6)	31.8 (9.3, 6)
	UV	4.5 (2.0, 6)	0.83 (0.65, 6)	19.0 (8.0, 5)	116.2 (22.4, 5)	159.8 (76.7, 5)	63.8 (25.4, 6)
	CO_2+UV	12.4 (3.0, 5)	2.7 (1.2, 6)	61.4 (34.3, 5)	128.4 (42.1, 5)	30.0 (10.9, 6)	172.0 (76.7, 6)

Figure 4.4 Heat map showing mean (±SE, n trap nights) numbers of parous, nulliparous, and male *C. sonorensis* per trap-site combination. Colors represent quartiles: black (top), dark gray (second), light gray (third), white (bottom).

CONCLUSION

Bluetongue is a serious, economically damaging livestock disease. Although it was historically considered a tropical pathogen, the *Culicoides* biting midges that transmit BTV can be found throughout temperate climates, and actual disease outbreaks seem to be detected more often in temperate zones. The European outbreak in 2006 highlighted the risk to livestock in these parts of the world. The outbreak also highlighted the gaps in our understanding of BTV transmission in temperate zones, *Culicoides* biology, and the dynamics of vector and virus overwintering. Today, European nations are considerably more prepared for BTV outbreaks, and while we have a greater appreciation for which species may be involved in transmission, there are still huge knowledge gaps to be filled. Compared to mosquitoes, almost every insect vector group could be considered "neglected." *Culicoides* are no exception, though we are starting to make advances using some of the modern techniques that have been applied to mosquitoes, including so-called "-omics" approaches. While more information on the genomics, proteomics, and transcriptomics of *Culicoides* are vital for controlling the spread of BTV, African horse sickness virus, epizootic hemorrhagic disease virus, and others, there is still a huge need for research on the field ecology of biting midges.

Resting places: Although a seemingly small life history detail, we do not know where adult *Culicoides* rest during the day. Given the amount of time insects spend resting, this is actually quite a critical aspect of their biology. Identification of adult resting sites would provide targets for application of insecticides to control vector populations. Habitats surrounding livestock could be modified to remove potential

harborages, perhaps by removing scrub or tall vegetation. Adult midges may enter building or animal enclosures during winter, or even overwinter underground, as some *Anopheles* mosquitoes do, protecting them from low temperatures. In some areas, shelter against high summer daytime temperatures and low humidity is also important for vector survival.

Immature biology: The biology and ecology of mosquito eggs, larvae, and pupae are recognized as critical to the control of these important disease vectors. The same can be said for the *Culicoides*, but significantly less attention has been paid to immature biting midges. We recognized that studying the environmental tolerances of immature *C. sonorensis* would provide insight into how the species persists trans-seasonally, where the species might be able to establish, and how we might be able to control vector populations. We showed for the first time that the eggs of *C. sonorensis* are much hardier than previously believed, and may play an important role in vector persistence in the field. These eggs are able to tolerate desiccation to the extent of losing over 50% of their weight in water, likely all of their free water, without suffering complete mortality. Such an adaptation would be critical to development in ephemeral water sources. Although *C. sonorensis* eggs appear to be able to survive at least 60 hours in a desiccated state, we do not yet know how long they may be able to persist in an environment free of standing water. Because desiccation and low temperature tolerance are often linked in insects, it makes sense to predict that *C. sonorensis* eggs would also be very tolerant of cold exposure, and indeed, we found this to be true. Eggs were the most cold tolerant immature life stage, and were able to survive exposure to temperatures at least as low as -

20°C with only a minimal decline in hatch rate. However, embryos and late-stage larvae suffered from chronic cold injury after exposure to low temperatures, and so final survival through the adult stage was negatively affected by cold. Despite the increase in mortality between the larval and pupal stages of reared eggs exposed to sub-zero temperatures, we still did not see complete mortality through the adult stage. In fact, we attributed some of the mortality in our experiments to an inefficient rearing system, and speculated that mortality of eggs exposed to similar conditions in the field would actually be higher than what we predicted.

Pupae, and especially larvae were less cold tolerant than eggs. Larvae appeared to be especially susceptible to physical injury from ice crystal formation on the mud surface, perhaps because they are far less sclerotized than pupae or eggs. Larval mortality is higher at relatively high sub-freezing temperatures, and was essentially 100% as soon as the temperature was low enough for ice to consistently form. We did not look at behavioral adaptations that may affect survival at these temperatures, but larvae very well may travel deeper into the substrate or pond to avoid challenging temperatures and cold injury. A change in depth of only a few millimeters could be important. Unlike adult *C. sonorensis*, the immature stages do not appear to cold harden when pre-exposed to lower, but above freezing temperatures.

Control and thresholds: One of the largest challenges for the control of *Culicoides*-borne diseases is the lack of control thresholds and an understanding of how vector population sizes relate to pathogen transmission. Few effective management strategies exist for biting midges other than physical removal of habitats, and even that

may prove to be ineffective. It is likely impossible to successfully eliminate all possible development sites near animals, and we have no data on how many, or what types of development sites, to eliminate in order to reduce vector populations to acceptable levels. In fact, we do not even have an idea of what an acceptable vector population would be, or how far populations would need to be reduced in order to prevent transmission. Studies on the biology and environmental tolerances of immature *Culicoides* will provide us with better insight into how to develop new control measures, and how effective those control measures would be in reducing adult populations. Studies on immatures should involve rearing insects out to the adult stage, since that is ultimately what we're interested in. Manipulative field studies are necessary to relate control of immature *Culicoides* to adult midge populations, and ultimately to pathogen transmission to sentinel animals.

Vector-pathogen interactions: We demonstrated an effect of BTV manipulation on *Culicoides* behavior in the field. Bluetongue infected midges appear to be UV light-averse, and are rarely collected in light baited suction traps compared to traps baited with only CO_2. When the midges collected by UV-baited traps are tested for BTV, MLE of infection rate generated from those data severely underestimate the true infection rate in the field; by as much as eight-fold. Because infected midges are rarely captured in UV traps, there is a good chance of falsely concluding that there is no risk of BTV transmission when vector populations are low, like during winter or initial vector introductions. In areas like Europe, where UV traps are used to determine seasonally vector free periods where livestock can be moved with fewer regulations or inspections, the consequences of missing BTV-positive midges are extremely serious.

Modelling pathogen spread: Using models to predict pathogen and vector spread, especially in terms of climate change, has become increasingly common. These models typically utilize a combination of environmental and climatic variables (e.g. wind speed and direction, normalized difference vegetation index) and biological data related to the survival of the insect vector and extrinsic incubation period. However, these models are limited in their usefulness by the assumptions of the data used. Often, predictions are based on data from laboratory studies on only one vector species. In the case of BTV, this is often *C. sonorensis*, even when models aim to predict pathogen spread in areas of the globe where *C. sonorensis* is not found. These models are also unable to predict where vectors will be able to establish persistently because they typically lack data on the environmental tolerances and limits of the immature stages. Thorough studies on the biology of immature vectors are critical to improving pathogen expansion models if our ultimate goal is to predict where persistent, yearly transmission cycles may occur.

Climate change: Climate change has enormous impact on the emergence, spread, and transmission on infectious disease. Increasing global temperature is associated with species extinction, and loss of diversity is associated with pathogen emergence. Climate change is also associated with habitat loss, which may push wild animals into closer contact with humans and livestock, furthering the opportunity for pathogen spillover and emergence. Models that look at species range have predicted that warmer temperatures at the margins of species range will allow for expansion either north or south. Those species

that inhabit equatorial regions may be driven from their native ranges as temperatures reach the upper limits of development. Although tropical zones are usually considered to be hotspots for disease, we may begin to see a shift of temperate zones becoming emerging infectious disease hotspots. In fact, the northeastern U.S. is already considered as such a hotspot, where emerging vector-borne diseases include human babesiosis (*B. microti*), Lyme disease (*B. burgdorferi*), and human bartonellosis (*B. elizabethae*). As far as vector-borne disease in particular is concerned, expansion of vector range may impact pathogen expansion in two ways. The first is the obvious risk of introduced vectors to introduce and transmit pathogens to naïve host populations. The second is that after introducing a novel pathogen into an area, a second, novel, native vector could begin transmitting the introduced pathogen. This second scenario is one of the proposed hypotheses for how BTV began to circulate in northern Europe. Recently, we have begun to see an expansion of both BTV and *C. sonorensis* in Canada. It is not unreasonable to be concerned about the possibility for native, northern *Culicoides* species, more adapted to temperate zones than *C. sonorensis*, to pick up BTV from southern Canada where invasive and native midge species overlap, and begin to transmit it further and further north.

Culicoides biting midges transmit some of the most damaging animal pathogens worldwide. They are vectors of multiple emerging and re-emerging viruses of livestock and wildlife, and yet there is still so much we don't know about their biology, ecology, and genetics. It is critical that we fill in the existing knowledge gaps on adult behavior, immature biology, species range, vector competence, transmission thresholds, and vector-

pathogen interactions. To do this will require both embracing the –omics tools that have been used to great effect in the mosquito world, and conducting in depth field biology studies, especially of those *Culicoides* species not easily colonized. Innovative, interdisciplinary science and a continued dedication to protecting animal health are the keys to understanding and controlling these neglected, yet important, insect vectors.