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Host-Seeking Activity of *Culicoides sonorensis* Across Seasons in Southern California and Improved Identification of *Culicoides* Species in the Southern California Desert

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ABSTRACT OF THE DISSERTATION

Host-Seeking Activity of *Culicoides sonorensis* Across Seasons in Southern California and Improved Identification of *Culicoides* Species in the Southern California Desert

by

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Culicoides (Diptera: Ceratopogonidae) biting midge is a genus of small hematophagous flies that can transmit multiple disease-causing pathogens to animals and humans. In southern California, *Culicoides sonorensis* is the only known vector of the bluetongue virus, which is of great concern worldwide due to its rapid spread and high morbidity/mortality in ruminant animals. Investigating the diel host-seeking activity of *C. sonorensis* as a function of environmental conditions and exploring the overwintering mechanism of BTV will increase our knowledge of BTV transmission. It is observed that the host-seeking activity pattern of *C. sonorensis* varies among days and most activity starts near sunset though sometimes it starts before sunset during winter periods. The host-seeking activity pattern is influenced by weather,

moon, and seasons. The relatively mild winter in southern California allows *C. sonorensis* to be active throughout the year, but virogenesis requires a certain temperature. Therefore, it remains unclear which mechanisms BTV is utilized for overwintering and needs further investigation. While *C. sonorensis* is recognized as the main vector of BTV in the southern California dairies, other *Culicoides* species may be important vectors of BTV to wild ruminants (e.g., bighorn sheep) in the desert regions of California. However, correct identification of these species becomes an obstacle for investigating *Culicoides* species-related topics in the desert area. Therefore, I develop molecular techniques combined with traditional morphological methods to identify *Culicoides* species in the southern California desert, which contributes to the global *Culicoides* taxonomy and biology. Moreover, studying the *Culicoides* diversity and their host preferences in the inland desert area of southern California may shed light on the relationship between *Culicoides* species and hemorrhagic diseases among wild ruminants and will facilitate studies of the epizootiology of hemorrhagic diseases in the area. Evaluating different trap methodologies increases the knowledge about the appropriate trapping method for targeting different *Culicoides* species and *Culicoides* of different physiological statuses in the desert region. With the sequence information, identification of immature midge species becomes easier and the abundance variation of midge species at two locations will assist future research on immature ecology.

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INTRODUCTION

General Taxonomy and Morphology of the Genus *Culicoides*

Culicoides Latreille, in the family Ceratopogonidae (Diptera), is a genus of tiny blood-feeding flies that causes annoyance to vertebrate hosts due to their nuisance and ability to transmit disease-causing pathogens to animals and humans (Mellor et al. 2000). The infraorder Culicomorpha which *Culicoides* belongs to is a monophyletic group and is closely related to Psychodomorpha (sand flies) (Wiegmann et al. 2011). Some families in Culicomorpha are of medical and veterinary importance, including Ceratopogonidae (biting midges), Culicidae (mosquitoes), and Simuliidae (black flies) (Yeates et al. 2007). According to Wiegmann et al. (2011), using as many as 14 nuclear genes and mitochondrial genes revealed that Ceratopogonidae is the sister group of Chironomidae (non-biting midges) and is more closely related to black flies than to mosquitoes, which is consistent with other studies based on morphological characters of adults and larvae (McAlpine and Wood 2002, Oosterbrook and Courtney 1995).

Ceratopogonidae are further divided into four subfamilies, Forcipomyiinae, Dasyheleinae, Ceratopogoninae, and Leptoconopinae. Three subfamilies, Forcipomyiinae, Dasyheleinae, and Ceratopogoninae, share many adult morphological features, including antennae with 13 flagellomeres and five-segmented maxillary palps. Leptoconopinae differ from the other subfamilies by having four-segmented maxillary palps and eyes that are widely separated instead of approximated at the dorsal midline of the head. Male and female Leptoconopinae vary in the number of antennal flagellomeres, with females having 11 or 12 flagellomeres while males having 13 flagellomeres. *Culicoides* in the subfamily Ceratopogoninae can be separated

from other genera by their small and simple claws, small or vestigial empodia, well-formed wing cells r1 and r2+3, and abundant wing macrotrichia (McAlpine et al. 1981).

In general, adult female *Culicoides* have well-developed mouthparts with a pair of toothed mandibles that can cut through host skin to acquire blood. The third segment of the maxillary palp is usually swollen and bears a sensory pit or several sensilla. In males, the antennae are usually plumose, and the pedicels are enlarged to bear the Johnston's organ for vibration perception (Jobling 1928; Blanton and Wirth 1979; McKeever et al. 1988). Some antennal flagellomeres bear small sensory pits (sensilla coeloconica, SCo), which can be used to assist in species identification (Phillips 2022). Many *Culicoides* species have dense microtrichia on their wings to form a pattern of dark and pale spots, characteristic of most *Culicoides*. The wings of male *Culicoides* are narrower and longer than the females' and bear a similar but often less conspicuous wing pattern (Blanton and Wirth 1979). The genitalia of male *Culicoides* are one of the most useful features for morphological identification. The shape of gonopods, parameres, and the aedeagus are different among *Culicoides* species even when females of different species are morphologically indistinguishable as, for example, in *C. sonorensis* and *C. occidentalis* (Velten and Mullens 1997).

Culicoides Biting Midges and the Viruses They Transmit

Because of their blood-feeding habit, *Culicoides* transmit several animal pathogens including bluetongue virus (BTV), African horse sickness virus (AHSV), epizootic hemorrhagic disease virus (EHDV), equine encephalosis virus (EEV), Akabane virus (AKAV), bovine ephemeral fever virus (BEFV), the Palyam viruses, Schmallenberg virus (SBV), and Oropouche virus (OROV) (Mellor et

al. 2000, De Regge et al. 2012). While most of these viruses are pathogens of ruminants (even-toed mammals with a ruminating stomach), OROV is a pathogen of sloths and humans.

BTV in the genus *Orbivirus* is one of the most important animal disease agents that *Culicoides* transmits. Bluetongue disease (BT) is a non-contagious viral disease that affects both wild and domestic ruminants. It can cause particularly severe symptoms in sheep and white-tailed deer, sometimes leading to death (Gibbs and Greiner 1994). In cattle, the symptoms of BT are less severe and generally less recognizable, which makes cattle a reservoir for BTV (Du Toit 1962, Nevill 1971). Bluetongue disease was classified by the World Organization for Animal Health (OIE) as a list A disease due to its potential for rapid and serious spread and significant impact on the international trade of animal and animal products ("OIE-Listed disease" 2022). It was estimated that $3 billion and $125 million were lost due to BTV directly (disease) and indirectly (trade, vaccines, etc.) worldwide and in the U.S. respectively every year (Tabachnick 1996).

Culicoides also serves as vectors for other viruses in the genus *Orbivirus*, including epizootic hemorrhagic disease virus (EHDV) and African horse sickness virus (AHSV). Like BTV, EHDV can cause morbidity and mortality in domestic and wild ruminants, and white-tailed deer are the most severely affected ruminant species in North America (Savini et al. 2011). The symptoms of EHD and BT are indistinguishable in wild ruminants, thus they are often collectively referred to as hemorrhagic disease (HD). EHDV affects the growing deer farming industry in the U.S. and is estimated to cause $7.9 billion in economic impact annually (McGregor et al. 2019). African horse sickness (AHS) is endemic in sub-Saharan Africa and is an infectious, non-contagious disease that results in high mortality rates for horses in non-endemic areas. Like BT, it is

classified as one of the List A diseases by the OIE (Mellor and Hamblin 2004, "OIE-Listed disease" 2022). The largest recorded outbreak of AHS occurred in southern Africa in 1854-1855 causing the deaths of over 70,000 horses (Barnard 1998). A recent study estimated that the economic impact of AHS is $95 million each year (Redmond et al. 2022).

BTV Distribution Worldwide and in the U.S.

Bluetongue virus is thought to have originated in Africa, as it was first described in South Africa in the late 18th century when Merino sheep were introduced to the Cape region (Gerdes 2004, Maclachlan 2011). The first outbreak of BT in Europe occurred in 1924 in Cyprus, and BT cases have continued to be reported throughout southern Europe since 1943 when a more virulent outbreak in Cyprus caused the death of 60-70% of sheep in some larger flocks (Gambles 1949). By 2016, 29 serotypes of BTV have been described worldwide (Schulz et al. 2016, Mayo et al. 2017), and they are found on all continents except for Antarctica (Tabachnick 2004, MacLachlan and Osburn 2006). The most recent and severe outbreak of BT occurred in northern Europe in 2006, caused by the introduction of BTV serotype 8, resulting in a case fatality rate of 30-50% in sheep and up to 10% in cattle (Darpel et al. 2007, Meiswinkel et al. 2008).

The first record of BT in the U.S. was in Texas in 1948 (Hardy and Price 1952). BT was then reported in California in 1952, affecting approximately 15,000 sheep in the Central Valley of California (McKercher et al. 1953). Since then, BTV has been detected throughout the U.S. (Ostlund et al. 2004) where serotypes 2, 10, 11, 13, and 17 are now considered endemic (Walton 2003, Johnson et al. 2011). In the U.S., BTV serotype diversity is highest in Florida where serotypes 3, 5, 6, 14,19, and 22 have been recently introduced from 1999 through 2006

(Johnson et al. 2011) perhaps as a result of the northward range expansion of *Culicoides* species from the Caribbean (MacLachlan et al. 2013). In California, BTV-10, 11, 13, and 17 were isolated from ruminants including sheep, cattle, and goats, and *Culicoides sonorensis* (formerly included within the *Culicoides variipennis* species complex) (Osburn et al. 1981, Stott et al. 1985, Gerry et al. 2001, Mayo et al. 2012). In 2010, a novel serotype, likely a reassortment of BTV-2 and BTV-6, was detected in the Sacramento Valley of California, which later was confirmed to be closely related to southeastern strains of BTV (MacLachlan et al. 2013, Gaudreault et al. 2014).

BTV Transmission Cycle and its Vectors

Bluetongue disease is not contagious, and thus can only be transmitted through the bite of infected vectors. *Culicoides* vectors become infected through feeding on viremic animals including cattle, sheep, goats, deer, or other ruminants. Viruses must penetrate through multiple barriers within the vector body (i.e., mesenteron infection barrier, mesenteron escape barrier, and dissemination barrier), replicate, and disperse to the whole body of midges (Fu et al. 1999). Eventually, following a temperature-dependent extrinsic incubation period, viruses reach the salivary glands and can be transmitted when the infected midge bites another host animal (Erasmus 1990).

The primary vector of BTV in the U.S. is *C. sonorensis* (Tabachnick 1996), which was previously classified as a subspecies within the *C. variipennis* complex (Holbrook et al. 2000). A recent study evaluating single-nucleotide polymorphisms has shown that *C. variipennis* complex includes five separate species – *C. variipennis*, *C. sonorensis*, *C. occidentalis, C. albertensis,* and an undescribed species (Shults et al. 2022). In the southeastern U.S. where *C. sonorensis* is rare or

even absent (Mellor et al. 2000), other *Culicoides* species are believed to be responsible for BTV transmission. One of these species, *C. insignis,* is abundant throughout the Caribbean and has recently spread across most of peninsular Florida (Greiner et al. 1989, Tanya et al. 1992, Vigil et al. 2018). In recent years, studies have revealed that some other *Culicoides* species may also carry BTV in the southeastern U.S. such as *C. stellifer*, *C. venustus*, *C. crepuscularis*, and *C. debilipalpis* (McGregor et al. 2019, Becker et al. 2020). However, laboratory studies on vector competence (i.e., the ability of a vector to be infected, allow for replication, and transmit the pathogen) need to be conducted to confirm the role of these potential vectors.

In other parts of the world, *Culicoides* species other than *C. sonorensis* are responsible for BTV transmission. In Africa, *C. imicola* is the major vector and *C. bolitinos* is able to transmit BTV as well (Du Toit 1944, 1962, Venter et al. 1998). BTV has also been isolated from *C. milnei* and *C. tororoensis*, suggesting their potential as vectors (Du Toit 1944, 1962; Walker and Davies 1971; Mellor et al. 2000). In Europe, multiple *Culicoides* species are considered potential vectors of BTV in addition to *C. imicola*. *C. obsoletus*, *C. chiopterus*, *C. pulicaris*, *C. dewulfi*, *C. punctatus*, *C. newsteadi* (Mehlhorn et al. 2007, Dijkstra et al. 2008, Goffredo et al. 2015). In Australia, *C. fulvus*, *C. wadai*, *C. actoni*, and *C. brevitarsis* are thought as potential vectors of BTV (Standfast et al. 1985, Mellor et al. 2000). In Asia, studies are patchy but confirmed vectors from other geographical regions exist such as *C. actoni*, *C. fulvus*, *C. wadai*, and *C. brevitarsis* which might also be important vectors, but more potential vectors need to be examined (Mellor et al. 2000).

BTV Overwintering

BTV transmission is highly seasonal in temperate regions (Nevill 1971, Mayo et al. 2016). The outbreak of BT in its enzootic areas of southern Africa always occurs in mid- to late summer and ceases with the onset of winter (Du Toit 1962). BTV was isolated from vertebrates and *C. sonorensis* in the western U.S. from June through December while January through May was free of the virus (Osburn et al. 1981). The period of a year that no BTV infection is reported in animal hosts is called the interseasonal period, and people use overwintering to describe the phenomenon that BTV disappears during the interseasonal period and reappears the following spring or summer as temperature increases again (Nevill 1971, Mayo et al. 2016). Typically, the overwintering period could last from as short as 3 months to as long as eight to nine months (Wilson et al. 2008). How BTV persists through the interseasonal period in the U.S. remains unclear, though researchers have offered many hypotheses. Some of the most accepted hypotheses were from Nevil (1971): 1) Adult *Culicoides* infected with BTV in fall survive through winter and become active again in spring to transmit disease; 2) a low transmission cycle is maintained between *Culicoides* and domestic animals throughout winter; 3) BTV persists in cattle throughout the winter; 4) other reservoir animals maintain BTV during winter; 5) BTV can be transmitted transovarially, that is, from mother *Culicoides* to its offspring.

Mayo et al. (2014) collected a small number of *C. sonorensis* using CO_2-baited traps in northern California during February and March of 2013 and 2014 that tested positive for BTV through reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) suggesting that BTV may overwinter in long-lived adult *C. sonorensis* or by maintaining a low transmission cycle between *C. sonorensis* and their ruminant hosts. Only parous (i.e., female midges that had produced at

least one batch of eggs) *C. sonorensis* were detected with BTV in the Mayo et al. (2014) study, suggesting that BTV cannot be vertically transmitted in *C. sonorensis*. The lack of vertical transmission of BTV from female *C. sonorensis* to her offspring was further supported by Osborne et al. (2015) who failed to recover BTV from the progeny of laboratory-reared *C. sonorensis* infected with BTV or from field-collected *C. sonorensis* larvae at BTV transmission hotspots in northern California.

For other possible hypotheses about BTV overwintering, cattle maintaining infectious BTV throughout the winter seems unlikely because cattle infected with BTV only remain viremic (i.e., infective to vectors) for less than 60 days, though BTV RNA and host antibodies can be detected within host blood for several months after infection (Singer et al. 2001, Bonneau et al. 2002, Mayo et al. 2014). However, another study showed elk could remain viremic for as long as three months (Murray and Trainer 1970) suggesting that other ruminant hosts might assist in BTV overwintering. A separate study demonstrated that ticks could be infected with BTV in the laboratory with the virus persisting in the ticks for a month (Bouwknegt et al. 2010).

Culicoides Surveillance Methods

Vector surveillance is used to identify vector species in a region, acquire individual insects for pathogen detection, and investigate the field activity pattern of vector species. There are many techniques to collect *Culicoides* species, including animal baited traps, light baited suction traps, animal odor baited suction traps, and vehicle-mounted traps. Each of which relies on different attractants and can result in the capture of different *Culicoides* species or different ratios of

these species. In addition, males are common in light baited traps but uncommon or absent in animal odor or animal baited traps with a few exceptions (Gerry and Mullens 1998).

Animal baited traps use host animals to attract host-seeking *Culicoides* with animals often placed into a cage or pen which is covered by a fine mesh net (Mullen et al. 1985, Mullens and Dada 1992a, Carpenter et al. 2008). After exposing the animal for a period, nets are dropped, and aspirators are used to collect any *Culicoides* species inside the net. Animal baited traps can reveal which *Culicoides* species are attracted to the animal, and which will actually feed on the animal. Animal-baited traps are therefore useful to determine host preferences of *Culicoides* species (Mullens and Dada 1992a). Animal baited traps are especially helpful to investigate biting rates of *Culicoides* species and potential vectors for pathogens (Mullen et al. 1985, Carpenter et al. 2008, Gerry et al. 2009); however, this trapping method requires a calm animal to be held in a small place for the collection period which can be difficult and require additional approvals for animal use (Cohnstaedt et al. 2012).

In comparison, light-baited suction traps are much easier to set up and capture midges. There are many suction trap models, such as New Jersey traps, Onderstepoort traps, and CDC traps (Wieser-Schimpf et al. 1990, Gerry and Mullens 2000, Venter et al. 2012). New Jersey traps and Onderstepoort traps are typically used with a light source as an attractant, incandescent light for the New Jersey trap and UV light for the Onderstepoort trap. Both traps produce a powerful downdraft to capture insects and require considerable power to function for a sufficient collection period; requiring them to be either wired into electrical outlets or powered by a vehicle battery. This can be a challenge for insect surveillance in remote areas where wired

electrical current is unavailable (McNelly 1989). In contrast, the CDC-type traps are smaller and can be -powered by smaller batteries (Sudia and Chamberland 1962). The CDC trap can attract insects using light but is often paired with a source of host odor such as carbon dioxide (CO_2). Insects attracted to the trap are captured in a downdraft produced by a motor and fan. A wired mesh at the trap entrance can exclude larger-sized insects such as beetles and moths.

Suction traps can be baited with different light sources such as LED or black light (Wieser-Schimpf et al. 1991, Sloyer et al. 2018) or different animal odors such as octenol (Mands et al. 2004) or CO_2 (Aybar et al. 2011). Animal odors and light sources can be used in varied combinations for trapping to give different efficiencies for various *Culicoides* species or midges of different physiological statuses (Wieser-Schimpf et al. 1990, McDermott et al. 2016, Sloyer et al. 2018). It must be considered that light or odor-baited traps may capture *Culicoides* species that would not be attracted to or feed on a particular host of interest and investigators should take care in interpreting these collections relative to animal disease risk (Carpenter et al. 2008, Gerry et al. 2009), and McDermott et al. (2015) found that BTV infected *C. sonorensis* would not come to UV light traps, emphasizing that appropriate trapping methods must be selected based on the purpose of the study.

Identification of *Culicoides* Species

After *Culicoides* specimens are captured, the next pivotal step is to correctly identify them to species. There are 1347 valid *Culicoides* species in the world (Borkent and Dominiak 2020). *Culicoides* identification relies on local morphological keys, for example, *The Sandflies (Culicoides) of Florida* (Blanton and Wirth 1979) are used by studies in the southeastern United

States, and the recently published *Culicoides Latreille and Leptoconops Skuse biting midges of the southwestern United States with emphasis on the Canyonlands of southeastern Utah (Diptera: Ceratopogonidae)* (Phillips 2022) became a very useful guide for *Culicoides* species in the western U.S. A list of currently available keys for *Culicoides* fauna by biogeographical regions around the world is available (Harrup et al. 2015).

Culicoides biting midges in the southern California desert area are especially difficult to identify because midges are small in size and closely related species have similar characteristics. Many *Culicoides* species have faint dark and pale wing pigmentation patterns or lack these pigmentation patterns altogether, adding to the difficulty of their identification. However, even with keys, identification of *Culicoides* in the southern California desert remains very challenging (Mullens and Dada 1992b). Sometimes, even researchers with rich experience cannot consider their identification final (personal communication with Robert Phillips).

DNA barcoding has been used for the identification of organisms from bacteria to eukaryotes and using DNA barcodes to assist with *Culicoides* species identification is increasing (Harrup et al. 2015). Harrup et al. (2015) listed all molecular markers used for *Culicoides* phylogenetic analysis before 2015 and from which it is shown that the most often used DNA markers are the cytochrome c oxidase subunit I (COI) gene and the internal transcribed spacer 1 (ITS1) gene. However, molecular techniques for the identification and phylogenetic analysis of *Culicoides* species are still being evaluated and may pose some problems. For example, there is not a consensus on the appropriate intraspecific genetic distances (Harrup et al. 2015). Moreover, some studies showed *Culicoides* within one species to have divergent genetics (Gomulski et al.

2006, Pagès et al. 2009, Ander et al. 2013), while some *Culicoides* species with relative distinct morphological characters cannot be separated using genetic analysis (Ander et al. 2013, Bellis et al. 2013).

By 2007, there were 8094 published records of *Culicoides* sequences representing 261 species (Ratnasingham and Hebert 2007); however, there is a huge deficit of available DNA sequences compared to the number of extant *Culicoides* species number. Most of the *Culicoides* sequences online are from *Culicoides* species distributed within Europe or Africa. There are not many sequences for *Culicoides* species from the United States, and none from the southern California inland desert area. Lacking referential sequences within DNA libraries prevents researchers from identifying desert *Culicoides* species even though they obtain their DNA sequences. Thus, there is great value in sequencing *Culicoides* species present in the SW deserts of the United States to enhance GenBank® and The Barcode of Life Data System (BOLD), and to help future genetic studies of *Culicoides* species in this region.

Will et al. (2005) argued that DNA barcoding should not replace the traditional morphological methods in taxonomy and that "the real cutting-edge future for systematics and biodiversity research is *integrative taxonomy*, which uses a large number of characters including DNA and many other types of data, to delimit, discover, and identify meaningful, natural species and taxa at all level." With the development of a non-destructive DNA extraction technique (Truett et al. 2000, Bellis et al. 2013), both morphological and molecular identification can be conducted on a single specimen! Non-destructive DNA extraction enables researchers to extract DNA without

damaging the organism, leaving the intact specimen as a morphological reference to accompany the sequenced DNA barcode (Harrup et al. 2015).

Culicoides Field Activity Pattern

Culicoides species and viruses they transmit have important economic and animal health impacts. To measure these impacts and to assess transmission risk, it is important to understand the activity patterns of these species under field conditions. Host-seeking activity patterns are particularly important as these are the individual midges that can acquire or transmit a pathogen during feeding on the host. Understanding *Culicoides* field activities will help to control the disease.

As the most important vector of BTV and EHDV in the U.S., *Culicoides sonorensis* has received much research attention. This species is described as a crepuscular species (Jones 1961, Rowley 1965, Foulk 1969). In 1965 and 1966, the activity of *C. sonorensis* was examined in southern California during summer and fall using truck-mounted traps, light traps, and CO_2 traps (Nelson and Bellamy 1971). *C. sonorensis* is highly active near sunset and sunrise, but peak activity could be also associated with moonlight. Studies also show that *C. sonorensis* could sometimes continue to be active throughout the night (Nelson and Bellamy 1971, Barnard and Jones 1980, Mullens 1995). Not unexpectedly, light traps are unproductive before sunset and after sunrise even when truck-mounted traps and CO_2 traps still collected large numbers of *C. sonorensis* (Nelson and Bellamy 1971). Flight activity is reported to occur at 10 – 32 °C and when humidity is above 25% (Nelson and Bellamy 1971), which is consistent with later studies by Barnard and Jones (Barnard and Jones 1980) who observed that *C. sonorensis* flew between 7 – 37 °C in

temperature and 18 – 94% in humidity. Daytime catches of *C. sonorensis* occasionally occurred. In another study that investigated the diel flight activity of *C. sonorensis*, daytime activities (i.e., before sunset or after sunrise) were observed in late spring, early summer, and late fall in Colorado (Barnard and Jones 1980). Winter collections in California also captured *C. sonorensis* during the daytime on a few occasions (Mayo et al. 2014).

The activity pattern of other *Culicoides* species was also studied. For example, in southern California, *C. haematopotus* and *C. posoensis* were found to have dusk and dawn activity peaks, which is similar to *C. sonorensis*, while the activity peak of *C. crepuscularis* and *C. freeborni* only had activity peak occurring near dusk but not dawn (Nelson and Bellamy 1971). The activity pattern of *C. crepuscularis* in Colorado was observed to be different from California *C. crepuscularis*, for example, midges were frequently captured during dawn and in daytimes (Barnard and Jones 1980). In contrast to *C. sonorensis*, *C. hieroglyphicus* and *C. palmerae* had flight activities that were mostly associated with daytime (Barnard and Jones 1980). Similar to *C. sonorensis,* the diel activity pattern of *C. furens* was bimodal and the activity peak was near sunset or sunrise in Florida (Lillie et al. 1987). The evening peak of *C. furens* was greater than the morning peak in Florida and North Carolina (Koch and Axtell 1979, Lillie et al. 1987), but another study in Florida observed greater activity in the morning than in the evening (Bidlingmayer 1961). The variable activity pattern of the same *Culicoides* species emphasized that activity patterns could change under variable circumstances. The activity pattern of all the species mentioned above also showed seasonal differences. Therefore, it emphasized that understanding the field activity pattern in a certain geographical location and in different seasons is important for the *Culicoides* species and the virus they transmit.

Compared to the general flight activity, the host-seeking activity of *Culicoides* species was much harder to investigate mostly because it is difficult to determine the purpose of flight – flying midges could be locating a host for blood, or newly emerging midges fly to find nectar sources. Male midges fly to find a chance to mate, and gravid females fly to find an oviposition site. To focus on the host-seeking activity, animal baited traps and host odors baited traps such as CO_2 are likely the most useful (Mullens 1995). Other commonly used traps such as vehicle-mounted and light traps do not target host-seeking midges, therefore they only show general flight activity. Nelson and Bellamy (1971) indicated that host-seeking activity and flight activity of *C. sonorensis* did not always coincide. For example, the truck trap catches showed that *C. sonorensis* increased flight activity during the moonlight period while the host-seeking activity shown by dry ice baited trap did not have a clear relationship with moonlight, and the activity peaks observed by light traps, truck-mounted traps, and CO_2 traps were all different. Many of the flying midges were not host-seeking presumably because they were too young to host-seek or were looking for oviposition sites.

The diel host-seeking pattern has also been observed for *C. furens*, *C. hollensis*, and *C. melleus* in South Carolina using CO_2-baited rotation traps (Breidenbaugh et al. 2009). Similar to *C. sonorensis,* all three *Culicoides* species had distinct dusk activity peaks, except for one collection in April 2004 when *C. furens* were collected throughout the night and after sunrise. However, the study only conducted 24-hour collections in April, August, September, and October, so it is hard to conclude that three *Culicoides* species are only active during sunset and to explain the "abnormal" collection in April 2004. In western France, animal baited traps were used to

observe the diel host-seeking activity of *Culicoides* species from May through October (Viennet et al. 2012). Except for one species – *C. brunnicans* which had a bimodal pattern near sunset and sunrise, all other studied species – *Culicoides obsoletus*, *C. scoticus*, *C. dewulfi*, and *C. chiopterus*, had one activity peak around sunset, with a small or no peak around sunrise.

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CHAPTER 1

Seasonal Changes in Diel Host-Seeking Activity of the Biting Midge *Culicoides sonorensis* at a Southern California Dairy

Abstract

Understanding the diel activity pattern of *Culicoides sonorensis* is important for midge and virus surveillance because the information on when midges start to be active and when they are most active can serve for pest control and disease prevention. On a southern California dairy, the diel host-seeking activity of *C. sonorensis* was studied using a time segregated rotation trap baited with CO_2. Over the three years of observation, the host-seeking activity of *C. sonorensis* mostly occurred from near sunset to near sunrise, but the activity pattern varied greatly among days. Midges mostly started their host-seeking activity near sunset, but sometimes occurred before sunset during winter periods. The activity peak occurred during nighttime in warmer months while near sunset or sunrise in cooler months. The host-seeking activity pattern including the start of host-seeking, peak host-seeking time, and host-seeking period, was related to weather factors including temperature, wind speed, and relative humidity. Moon and sunlight could also influence the activity pattern from some aspects. This study showed that the host-seeking activity of *C. sonorensis* may start early before sunset so using of light traps would miss the daylight portion of active midges. Moreover, this study quantitatively investigated the environmental variables and their influence on the diel host-seeking activity pattern of *C. sonorensis*, which will provide information on future studies of other *Culicoides* species in other geographical locations.

Introduction

Vector surveillance is fundamental to vector-borne disease epidemiology, with surveillance efforts providing critical information on the seasonal and diel activity pattern of vectors. Strategies for pathogen control are often based on knowledge of these vector activity patterns.

As the primary vector of bluetongue virus (BTV) in North America, the geographical and seasonal distribution of *Culicoides sonorensis* Wirth and Jones (Diptera: Ceratopogonidae) has been extensively studied (Barnard and Jones 1980, Gerry and Mullens 2000, Lysyk and Dergousoff 2014, Zuliani et al. 2015). *Culicoides sonorensis* is the predominant biting midge species on dairies in California and has been historically responsible for the transmission of BTV to cattle (Stott et al. 1985).

The activity of *C. sonorensis* is highly seasonal, with midge abundance and biting activity being greatest during late summer and fall in southern California (Gerry and Mullens 2000). Prevalence of BTV infection in cattle generally peaks during early fall following the peak in *C. sonorensis* biting activity (Gerry et al. 2001). Flight activity is reported to peak near sunset (crepuscular activity), though this species may continue to seek hosts throughout the night and occasionally an activity peak is noted near sunrise (Nelson and Bellamy 1971, Barnard and Jones 1980). In Colorado, use of truck-mounted traps to capture flying insects showed *C. sonorensis* flight activity peaks occurred before sunset in April-May and September- November, while flight activity peaked after sunset in June- August when daytime temperature was relatively high (Barnard and Jones 1980). In northern California, *C. sonorensis* was also captured in carbon

dioxide baited traps prior to sunset on winter days (Mayo et al. 2014). These findings suggest that flight and host-seeking activity of *C. sonorensis* might shift from post-sunset during warm seasons to pre-sunset during colder seasons, allowing midges to be active during hours when environmental conditions are suitable for flight.

Environmental and meteorological factors such as temperature, solar radiation, moonlight, and wind speed can affect the flight activity of *C. sonorensis* (Nelson and Bellamy 1971, Barnard and Jones 1980, Gerry and Mullens 2000, Walgama and Lysyk 2018). The suitable temperature observed for *C. sonorensis* flight activity is 7 – 37 °C (Barnard and Jones 1980). Wind speed is usually observed to have a negative correlation with flight activity in midges (Kettle 1969, Walgama and Lysyk 2018), and activity was suppressed at wind speed over 2 -4 m/s for different species (Kettle 1969, Mellor et al. 2000, Sanders et al. 2012). Moonlight is observed to increase the flight activity of midges (Nelson and Bellamy 1971, Linhares and Anderson 1990). Nevertheless, knowledge of how the diel host-seeking activity pattern is influenced by these environmental variables is still lacking, which is important for understanding the dynamic of BTV transmission on a local scale. Suction traps baited with UV light are a common surveillance tool for monitoring *Culicoides* flight activity as part of epidemiological and transmission risk studies. However, light traps are likely ineffective during daylight hours and thus could fail to detect flight activity before sunset. If a shift to pre-sunset host-seeking activity is common during cooler winter months, *Culicoides* host-seeking activity per day may be much greater than is suggested when using light traps for monitoring midge flight activity.

In this study, we studied the diel host-seeking activity of *C. sonorensis* by using a time-segregated trap baited with CO_2, believing that collections by CO_2-baited traps represent better host-seeking activities than collections by light traps or truck-mounted traps. More specifically, we investigated whether the diel host-seeking activity of *C. sonorensis* shifts to before sunset as temperature drops and explored what additional environmental variables influence the host-seeking activity pattern.

Methods

Study Site

The study was conducted at a drylot dairy in the Chino dairy region of southern California (San Bernadino County). Drylot dairies in this region are constructed with dairy wastewater ponds to capture and retain wastewater from milking operations. These manure-polluted wastewater ponds are a common immature development site for *C. sonorensis* (Mullens 1989; Gerry and Mullens 2000). The study dairy maintains a herd of over 1000 cows in several cattle pens with two wastewater ponds (Figure 1.1). The water level in the ponds fluctuates throughout the year with lower water levels typically in the summer. The northernmost and largest pond (pond 1) was heavily vegetated. During year 2 of the study, this pond was partially drained and dredged to reduce mosquito development per requirement of the local health authority. At the end of year 3, this wastewater pond went through reconstruction to deepen the pond and a berm was built around the pond. The southernmost pond (pond 2) was smaller and shallower with gently sloping sides. A high density of immature *Culicoides* was commonly observed each year during visual inspection of this pond. During year 3, the flow of wastewater to this pond was stopped

and the pond eventually dried up during the middle of the third study year. The open land immediately adjacent to pond 2 was used to grow seasonal vegetables. The study dairy was bordered by two neighboring dairies and by a small farm to the south containing a variety of animals including horses, sheep, goats, pigs, and fowl.

Insect Collection

Host-seeking midges were captured over 24 hours starting at 8:45 am (9:45 am during DST) every other week from April 9, 2018, to April 6, 2021, using a battery-operated, time-segregated rotating trap with 18 collection jars that rotated in sequence beneath a Centers for Disease Control (CDC) type miniature suction trap without light (J. W. Hock, Gainesville, FL) mounted at the trap entrance (Mullens 1995). Each collection jar contained approximately 50 ml of soapy water to retain captured insects and each jar was positioned beneath the suction trap for an 80-min time interval. Collection intervals were coded 1 through 18 with the24-hour collection period encompassing the daytime period before sunset and the full nighttime period through sunrise the following day. Carbon dioxide (CO_2) from a compressed gas tank was released near the trap opening at a flow rate of 1,000 ml/min to mimic the breath of a nearly grown Holstein heifer (Roberts 1972, Gerry et al. 2001). The rotating trap was set on a table to put the trap opening ~1.3 m above ground. Host-seeking *C. sonorensis* responding to the CO_2 were captured into collection jars.

The rotating trap was positioned near pond 1 from April 2018 through March 2019, shifted to near pond 2 from April 2019 through October 2020 due to physical alteration of pond 1 during after year 1, and then returned to near pond 1 in late October of 2020 till the end of collection

due to the desiccation of pond during year 3 of the study. Captured insects in each collection jar were sorted and *C. sonorensis* were counted by sex and physiological status of females (nulliparous, parous, blood-engorged, and gravid) (Dyce 1969, Akey and Potter 1979).

Environmental and Weather Information

Weather information including temperature, relative humidity, wind speed, and solar radiation was acquired from a nearby weather station (Station KCACHINO13: 34.01 °N, 117.67 °W) with data accessed using WeatherUnderground ("wunderground.com" 2022). For three collection days when weather data was not available from this station, it was obtained from one of two nearby weather stations (Station KCACHINO71: 34.02 °N, 117.69 °W or Station KCACHINO89: 34.01 °N, 117.67 °W). Time of sunset and sunrise during the collection period, as well as the time of moonrise, moonset, and the moon phase, was obtained from Timeanddate.com ("timeanddate.com" 2022). For collection dates when moonrise occurred prior to sunset, the sunset time is also recorded as the time of moonrise as any light reflection from the moon prior to sunset would not be expected to impact midge activity. Similarly, if the moonset occurred after sunrise, then the time of sunrise is recorded as the moonset time.

Data Analysis

Analyses and visualization of the collection data were conducted in R version 4.1.2 (R Core Team 2013). Host-seeking activity was analyzed as start of host-seeking, peak host-seeking time, and host-seeking period. Host-seeking period is the total number of collection periods in which host-seeking midges were captured excluding non-continuous collection periods with fewer than five midges captured. Start of host-seeking is the first collection period that midge activity occurred.

Peak host-seeking time is the collection period when the greatest number of host-seeking midges were collected and only periods that had more than five midges were considered. To assist analyses, peak activity time was normalized as (peak activity time – sunset time) / (sunrise time – sunset time).

Regression analyses were used to study the relationship between environmental variables and the host-seeking activity pattern. Independent variables that were considered in the analyses were the highest and lowest temperature on the date before collection (odTh, odTl), highest and lowest relative humidity on the date before collection (odRh, odRl), highest and lowest temperature on collection day 1 (hT1, lT1), highest and lowest relative humidity on collection day 1 (hR1, lR1), the lowest temperature on collection day 2 (lT2), the highest relative humidity on collection day 2 (hR2), temperature, relative humidity, and wind speed at sunset (sT, sR, sW), temperature, relative humidity, and wind speed at sunrise (rT, rR, rW), highest solar intensity during collection (hS), highest and mean wind speed during collection (hW, mW), sunset and sunrise time (ST, RT), moonlight start and end time (Ms, Me), night length and moonlight length (nl, ml), and moon phases represented by days to the last new moon day (Mp) (Table 1.1). All time-related variables were coded to range from 0-18 to align with 18 collecting periods of 24 hours. Temperature and relative humidity on the date before collection were considered in the analysis to obtain a more stable seasonal changing pattern in case unusual temperature or humidity occurred during collection (Figure 1.2). Since collections were conducted on days without rain, precipitation was not considered in the data analysis.

Table 1.1: Variables for consideration.

Variables	odTh	odTl	odRh	odRl	hT1	lT1	hR1	lR1	lT2	hR2	sT	sR	sW
Host-seeking period	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Start of host-seeking	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Peak host-seeking time	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Variables	**rT**	**rR**	**rW**	**hS**	**hW**	**mW**	**Mp**	**nl**	**ml**	**ST**	**RT**	**Ms**	**Me**
Host-seeking period	yes	yes	yes	yes	yes	yes	yes	yes	yes	no	no	no	no
Start of host-seeking	yes	yes	yes	yes	yes	yes	yes	no	no	yes	yes	yes	yes
Peak host-seeking time	yes	yes	yes	yes	yes	yes	yes	no	no	yes	yes	yes	yes

Linear regression was used to study how peak host-seeking time and host-seeking period are associated with environmental variables. Since there are many environmental variables in this study, we first selected the potentially important environmental variables using different variable selection methods, including forward, backward, and stepwise selection. Since different variable selection methods may select different environmental variables, for each variable selection method, we applied the following model-building procedure to the variables selected from that particular variable selection method: (1) Build a complete second order model using the first-order terms, quadratic terms and interactions of the selected variables; (2) Remove non-significant terms in the complete second order model step by step based on the p-value until all terms in the model have p-values that are less than 0.05; (3) Perform residual analysis to check if the model assumptions are satisfied.

Following this analysis, the model derived from the stepwise selection which had the fewest variables was used to constructing new models by manually adding variables excluded by model

selection method, but which were considered to likely be important predictors of host-seeking activity according to professional judgment of study authors. Adding a variable went through the same model-building procedures as described above. Lastly, models that satisfied the linear regression assumptions and had relatively high adjusted R-squared value (> 0.5) were retained and then compared to find the best fit model based on the least Akaike Information Criterion (AIC) and by leave-one-out cross-validation.

When studying how the start of host-seeking is associated with the environmental variables, we were not able to find a good linear regression model to fit the data. To circumvent this difficulty, we coded the start of host-seeking into three categories: before sunset (activity started before the period in which sunset occurred), at sunset (activity started in the period in which sunset occurred), and after sunset (activity started after the period in which sunset occurred). Then ordinal logistic regression was used to study the relationship between the start of host-seeking and the environmental variables. Models were built similar to the procedures we used in the above linear regression, except that each fitted model was tested for ordinal logistic regression assumptions and a confusion matrix (i.e., the number of correct and incorrect predictions summarized by each category in a table) was created to show the performance of the model in predicting the start of host-seeking into the correct category. Among all the models that satisfied the model assumptions, the best model was chosen based on the confusion matrix where all three categories could be best predicted.

Results

General Observations on Seasonal Activity

A total of 98222 *C. sonorensis* were captured from April 2018 through April 2021 over 77 collections, comprising 43963 (45% of all *C. sonorensis* collected) parous females, 43124 (44%) nulliparous females, 1028 (1%) blood-fed females, 6 (< 0.01%) gravid females, and 10101 (10%) males. Parous and nulliparous females captured in the trap were considered host-seeking. The number of host-seeking midges captured changed drastically throughout the year. The lowest number of host-seeking midges were collected from December through April. Midge abundance began to increase in April with peak abundance during August or September, before decreasing through December. One exception to this general pattern of abundance occurred in 2020 when abundance had a second (higher) peak in November-December which probably due to the movement of the trap from pond 2 to pond 1 and the higher mean temperature in November and early December of 2020 (Figure 1.3).

Host-seeking midge activity was generally consistent with changing daily temperature (Figure 1.5). July through September were usually the hottest months and corresponded to peak midge activity. Host-seeking midge activity lagged behind change in solar intensity (Figure 1.4) and change in wind speed which also showed a seasonal pattern (Figure 1.5). There was no apparent relationship between relative humidity and host-seeking midge activity as the highest relative humidity of the 24-hour collection period was similar across dates while the lowest relative humidity varied greatly over the collection dates and lacked an obvious seasonal pattern (Figure 1.6). Parity rate (number of parous females/number of parous and nulliparous females) of *C.*

sonorensis fluctuated around 0.5 throughout the three-year sampling period with no observable seasonal pattern (Figure 1.7).

General Observations on Diel Host-Seeking Activity

Host-seeking *C. sonorensis* were captured from near sunset through sunrise from late May through early November with a few exceptions (Figures 1.8- 1.10). During these months, peak host-seeking activity generally occurred during nighttime collection periods. From November through early May, abundance of host-seeking midges was low, and activity was not continuous throughout the night, with peak host seeking activity generally occurring near sunset or sunrise. Although, host-seeking activity was noted to start before sunset during winter months, the first host-seeking females arrived only shortly before sunset rather than in the early afternoon.

When looking at the diel host-activity by date, we found different patterns: 1) continuous activity throughout the night with a distinct nighttime peak (e.g., 2018-04-09); 2) continuous activity throughout the night with multiple small peaks (e.g., 2018-10-10); 3) non-continuous nighttime activity although midge abundance was relatively high (e.g., 2018-10-24); 4) activity occurred only during several periods (e.g., 2018-12-03). The first two patterns were usually observed during warmer seasons, and the last two patterns were usually observed in cooler seasons.

The time that most host-seeking activity occurred varied by month (Figure 1.11). Depending upon collection date, sunset occurred in collection period 5-7, while sunrise occurred during periods 15 and 16. During cooler months (Oct-Feb), a greater proportion of host-seeking midges

were captured during collection periods 6-7 which were near sunset, while during warmer months (Apr-Sep) a greater proportion of host-seeking midges were captured during periods 11-15 which were much later after sunset. When looking at actual counts of host-seeking midges, the higher midge abundance usually occurred during July through September except for several collections in November and December of 2020 which had very a high number of catches (Figure 1.12).

Environmental variables changed in a similar pattern each day (Figures 1.8-1.10). The temperature rose after sunrise and reached a peak in the afternoon. After sunset, the temperature dropped and reached the lowest at late night and just before sunrise. The relative humidity changed in an opposite direction, it dropped after sunrise and reached the lowest in the afternoon. The relative humidity rose after sunset and reached the peak at late night or just before sunrise. The wind speed has more variations among days, but it was generally higher during the daytime than the nighttime. On many days, the wind speed during the night was zero.

Environmental Predictors Associated with Diel Activity

Host-Seeking Period

The variables and their range in the analysis were shown in Table 1.2. The host-seeking period of *C. sonorensis* on the dairy was best described by a linear regression model (Adjusted-R^2 = 0.78; F = 21.14 on 12 and 58 DF, p-value < 0.0001) that included highest temperature on the date before collection (odTh), lowest temperature on collection day 2 (lT2), mean wind speed during

collection (mW), highest solar intensity during collection (hS), and relative humidity at sunrise (rR) (1; Table 1.3). Quadratic forms of odTh, lT2, and mW, as well as interactions between odTh and mW, odTh and hS, lT2 and hS, mW and rR were also included in the model to assist in modeling the activity time range. The cross-validation result (RMSE = 1.90, R-squared = 0.72, MAE = 1.53) showed that this model is also good for prediction.

The odTh, lT2, and mW all had a quadratic relationship with the host-seeking activity period, indicating the relationship of these variables to host-seeking period changed across the range of the measured variables. The relationship of temperature to the host-seeking period varied, with host-seeking period shortening as odTh increased at low temperatures and host -seeking period lengthening as odTh increased at higher temperatures (2). The inflection point for this change occurred at approx. 19 to 20 °C as modified by mW and hS (Table 1.2). For lT2, host-seeking period lengthened as lT2 increased at low temperatures and it shortened as lT2 increased at higher temperatures (3). The inflection point for this change occurred at approx. 10 to 16 °C as modified by hS. Similar to temperature, wind speed has varied relationship with host-seeking period, with host-seeking period lengthening as mW increased at lower wind speed and host-seeking period shortening as mW increased at higher wind speed (4). The inflection point for this change occurred at approx. 0.2 to 1.7 m/s.

The hS generally had a negative correlation with the host-seeking period, although the effect depended on lT2 and odTh (5). The correlation between rR and host-seeking period depended on mW: when mW was larger than approx. 1.8 m/s, rR had a positive correlation with the

activity time range, and when mW was smaller than approx. 1.8 m/s, rR had a negative correlation (6).

$$\hat{Y} = 19.60 + 0.01\ odTh^2 - 0.09\ lT2^2 - 5.15\ mW^2 - 0.14\ odTh + 1.47\ lT2 - 5.38\ mW - 0.0025\ hS - 0.22\ rR + 0.31\ odTh * mW \pm 0.00094\ odTh * hS + 0.0014\ lT2 * hS + 0.12 mW * rR \quad (1)$$

$$\hat{Y} = 0.01\ odTh^2 + (-0.14 + 0.31\ mW - 0.00094\ hS) * odTh + C1\ ^{1} \quad (2)$$

$$\hat{Y} = -0.09\ lT2^2 + (1.47 + 0.0014\ hS) * lT2 + C2 \quad (3)$$

$$\hat{Y} = -5.15\ mW^2 + (-5.38 + 0.31\ odTh + 0.12\ rR) * mW + C3 \quad (4)$$

$$\hat{Y} = (-0.0025 - 0.00094\ odTh + 0.0014\ lT2) * hS + C4 \quad (5)$$

$$\hat{Y} = (-0.22 + 0.12\ mW) * rR + C5 \quad (6)$$

Table 1.2: Summary of possible values for each independent variable.

	ST	RT	Ms	Me	odTh (°C)	odTl (°C)	odRh (%)
Min.	5.95	14.93	5.95	6.45	11.44	4.44	56
1st Qu.	6.34	15.12	6.74	10.91	24.42	10.08	80
Median	7.11	15.56	7.65	14.93	28.94	14.44	86
3rd Qu.	7.56	16.04	11.38	15.48	32.78	16.64	88
Max.	7.76	16.62	16.11	16.62	38.78	21.78	97
	odRl (%)	**hT1 (°C)**	**lT1 (°C)**	**hR1 (%)**	**lR1 (%)**	**lT2 (°C)**	**hR2 (%)**
Min.	9	16.17	1.56	57	10	1.89	38
1st Qu.	26	24.31	9.36	83	29	10.31	83
Median	37	28.67	13.67	86	37	14.33	86
3rd Qu.	46	33	16.11	88	45	16.53	88
Max.	74	37.44	23	97	66	22.33	94
	sT (°C)	**sR (%)**	**sW (m/s)**	**rT (°C)**	**rR (%)**	**rW (m/s)**	**hS (w/m^2)**
Min.	13.78	12	0	0	34	0	282.7
1st Qu.	19.31	44	1.8	10.58	79	0	705.2

[1] In equations, Cs represent all other terms in the model

Median	22.67	55	2.4	14.56	85	0.1	888.3
3rd Qu.	25.08	65	2.9	16.89	86	0.4	968.4
Max.	30.83	83	4.9	22.39	94	0.9	1053.9
	mW (m/s)	**hW (m/s)**	**Mp (days)**	**nl**	**ml**		
Min.	0.1	0.5	0	9.58	0		
1st Qu.	0.9	2.6	6	10.24	2.96		
Median	1.2	3.3	13	11.47	5.18		
3rd Qu.	1.6	3.8	20	13.15	8.43		
Max.	2.4	5.2	28	14.13	14.08		

Table 1.3: Regression coefficients for the best linear regression model for host-seeking activity time range.

	Estimate	Std. Error	t value	P-value	
odTh	-0.14	0.32	-0.44	0.662	
lT2	1.47	0.33	4.44	< 0.001	***
mW	-5.38	4.27	-1.26	0.213	
hS	-0.0025	0.01	-0.42	0.675	
rR	-0.22	0.05	-4.69	< 0.001	***
odTh^2	0.01	0.01	2.23	0.03	*
lT2^2	-0.09	0.02	-5.8	< 0.001	***
mW^2	-5.15	1.28	-4.02	< 0.001	***
odTh : mW	0.31	0.13	2.43	0.018	*
odTh : hS	-0.00094	0.00029	-3.19	0.002	**
lT2 : hS	0.0014	0.00048	2.95	0.005	**
mW : rR	0.12	0.03	3.67	0.001	***

***: P-value < 0.001

**: P-value < 0.01

*: P-value < 0.05

Peak Host-Seeking Time

Peak host-seeking time was best described by a linear regression model (Adjusted R^2 = 0.70; F = 14.03 on 11 and 51 DF, p-value < 0.0001) which contained mean wind speed during collection (mW), highest solar intensity during collection (hS, relative humidity at sunset (sR), moon phases (Mp), and sunrise time (RT) (7; Table 1.4). Quadratic terms of mW, as well as interactions

between mW and sR, mW and RT, hS and Mp, sR and Mp, Mp and RT, also had influences on peak activity time. The cross-validation showed that this model was also good for prediction (RMSE = 0.15, R-squared = 0.59, MAE = 0.12).

Wind speed has a quadratic relationship with peak host-seeking time, meaning that peak host-seeking time moved to a later time as mW increased at low wind speed while peak time moved to an earlier time as mW increased at higher wind speed (8). The inflection point for this change occurred at approx. 0.3 to 1.6 m/s as modified by sR and RT (Table 1.2). Solar intensity and peak host-seeking time always had a positive correlation thought it is affected by Mp: host-seeking peak would move to a later time as hS increased (9).

The relationship between relative humidity and peak host-seeking time depended on other variables – mW and Mp: when mW and/or Mp were larger, sR and peak time had a positive correlation, and vice versa (10). Similarly, the relationship between moon phase and peak host-seeking time varied and was related to hS, sR, and RT (11). Only when all hS, sR, and RT were large enough, would the correlation between Mp and peak time be positive, which means that the host-seeking peak would move to a later time as the Mp increased only when during summer and when relative humidity at sunset was high enough. Overwise, host-seeking peak would move to an earlier time as the moon phase increased. Moreover, the relationship between sunrise time and activity peak time depended on mW and Mp: when mW and/or Mp were large, RT and peak host-seeking time had a positive relationship, and vice versa (12).

$$\hat{Y} = 20.22 + 0.75\,mW^2 - 12.81mW + 0.001hS - 0.01sR - 0.47Mp - 1.14RT + 0.01mW * sR + 0.69mW * RT + 0.0001hS * Mp + 0.0006sR * Mp + 0.02\,Mp * RT \quad (7)$$

$$\hat{Y} = 0.75\,mW^2 + (-12.81 + 0.01\,sR + 0.69\,RT) * mW + C6 \quad (8)$$

$$\hat{Y} = (0.001 + \ 0.0001\,Mp) * hS + C7 \quad (9)$$

$$\hat{Y} = (-0.01 + 0.01\,mW + 0.0006\,Mp) * sR + C8 \quad (10)$$

$$\hat{Y} = (-0.47 + 0.0001\,hS + 0.0006\,sR + 0.02\,RT) * Mp + C9 \quad (11)$$

$$\hat{Y} = (-1.14 + 0.69\,mW + 0.02Mp) * RT + C10 \quad (12)$$

Table 1.4: Regression coefficients for the best linear regression model for host-seeking peak activity time.

	Estimate	Std. Error	t value	P value	
mW	-12.81	2.63	-4.87	< 0.001	***
hS	0.001	0.0003	-3.72	0.001	***
sR	-0.01	0.004	-2.63	0.011	*
Mp	-0.47	0.16	-2.93	0.005	**
RT	-1.14	0.22	-5.26	< 0.001	***
mW^2	0.75	0.11	6.75	< 0.001	***
mW : sR	0.01	0.003	2.59	0.012	*
mW : RT	0.69	0.15	4.69	< 0.001	***
hS : Mp	0.0001	0.00003	3.41	0.001	***
sR : Mp	0.0006	0.0002	4.07	< 0.001	***
Mp : RT	0.02	0.01	2.66	0.010	*

***: P-value < 0.001

**: P-value < 0.01

*: P-value < 0.05

Start of Host-Seeking

The best ordinal logistic model (likelihood ratio = 35.31, p-value < 0.0001) to describe the start of host-seeking included moonlight start time (Ms), relative humidity at sunset (sR), highest

temperature on collection day 1 (hT1), and mean wind speed during collection (mW). The quadratic terms of hT1, as well as interactions between Ms and hT1, sR and hT1, sR and mW, were significant in the model (13 & 14; Table 1.5). According to the confusion table, this model had a 0.70 overall classification rate (i.e., accurately identify activity start time into the correct category), and the classification rate for each category was 47%, 86%, and 50% for before, during, and after sunset, respectively. The reason that classification rates for categories before and after sunset were much lower than the one for during sunset may be due to the fact that most of the start of host-seeking in our data is during sunset.

Since the start of host-seeking was categorized to before, during, and after sunset, the interpretation for the estimated regression coefficients for each variable was the predicted change in log odds of the activity start time being in a later time as opposed to an earlier time per unit change on the independent variable. Temperature has a quadratic relationship with the start of host-seeking: there was a predicted decrease in the log odds of the start of host-seeking falling into a later time (as opposed to an earlier time) as hT1 increased at low temperatures while there was a predicted increase in the log odds of the start of host-seeking falling into a later time as hT1 increased at higher temperatures (15). Another way to interpret is that there is a decreased probability of the start of host-seeking falling into a later time (as opposed to an earlier time) as hT1 increased at lower temperatures while there was an increased probability of the start of host-seeking falling in a later time as hT1 increased at higher temperatures. The inflection point occurred at approx. 4.8 to 46.8 °C as modified by Ms and sR.

The relationship between moonlight start time and log odds of the start of host-seeking depended on hT1 (16). When hT1 was lower than approx. 32.2 °C, there was a positive correlation meaning that there is an increased probability of the host-seeking starting at a later time when Ms increased. On the contrary, when hT1 was higher than approx. 32.2 °C, there was a negative correlation. The relationship between relative humidity and log odds of the activity start time was related to hT1 and mW: when hT1 was large and mW was small, sR and log odds of the activity start time had a negative correlation, and vice versa (17). Similarly, the relationship between wind speed and log odds of activity start time was related to sR: when sR was larger than approx. 62%, mW and log odds of the activity start time had a positive relationship, and vice versa (18).

$$logit\left(\hat{P}(Y \leq 1)\right) = 10.47 + 0.02hT1^2 - 0.23hT1 + 1.61Ms + 0.19sR - 3.1mW - 0.05Ms * hT1 - 0.01sR * hT1 + 0.05sR * mW \quad (13)$$

$$logit\left(\hat{P}(Y \leq 2)\right) = 14.38 + 0.02hT1^2 - 0.23hT1 + 1.61Ms + 0.19sR - 3.1mW - 0.05Ms * hT1 - 0.01sR * hT1 + 0.05sR * mW \quad (14)$$

$$logit\left(\hat{P}(Y \leq j)\right) = 0.02\ hT1^2 + (-0.23 - 0.05\ Ms - 0.01\ sR) * hT1 + C11\ ^2 \quad (15)$$

$$logit\left(\hat{P}(Y \leq j)\right) = (1.61 - 0.05\ hT1) * Ms + C12 \quad (16)$$

$$logit\left(\hat{P}(Y \leq j)\right) = (0.19 - 0.01\ hT1 + 0.05\ mW) * sR + C13 \quad (17)$$

$$logit\left(\hat{P}(Y \leq j)\right) = (-3.1 + 0.05\ sR) * mW + C14 \quad (18)$$

[2] Since there are 3 categories, 1 = host-seeking start before sunset period, 2 = host-seeking start during sunset period, 3 = host-seeking start after sunset period. j = 1, 2.

Table 1.5: Regression coefficients for the best ordinal logistic regression model for host-seeking activity start time.

	Estimate	Std. Error	t value	p value		OR
Ms	1.61	0.45	3.57	< 0.001	***	5.01
sR	0.19	0.08	2.48	0.013	*	1.21
hT1	-0.23	0.27	-0.86	0.388		0.79
mW	-3.1	0.01	-447.57	< 0.001	***	0.05
hT1^2	0.02	0.01	2.24	0.025	*	1.02
Ms : hT1	-0.05	0.02	-3.28	0.001	**	0.95
sR : hT1	-0.01	0.00	-1.99	0.046	*	0.99
sR : mW	0.05	0.01	4.15	< 0.001	***	1.05

OR: odds ratio, obtained by exponentiate estimates (log odds).

***: P-value < 0.001

**: P-value < 0.01

*: P-value < 0.05

Discussion

Analysis of Diel Host-Seeking Pattern

We divided the diel host-seeking activity into three aspects – the start of host-seeking, the peak host-seeking time, and the host-seeking period, to investigate the relationship between them and environmental variables. Since we are mostly interested in the diel activity pattern, the actual number of midges collected at each period is not as important as the proportion of midges being active at each period. For collections considered in the analysis, the host-seeking activity started before sunset in 23 of 69 collection days, and most before sunset activities occurred in late fall, winter, and early spring, which is consistent with Barnard and Jones (1980). In a few cases, midges were collected occasionally during the diurnal time, but the number captured was too low to show on the daily activity bar graph (Figure 1.8-1.10). Most interestingly, in November and December of 2020 when the rotation trap was moved back to

pond 1, host-seeking midges were captured in relatively high numbers during daytime collection periods. The daylight lengths of these days are short, and midges collected during the day is still small compared to the total catch (< 5 %), suggesting that when midge abundance is high and the weather is suitable for flying during the winter period, a few midges may take chances to seek host during short diurnal periods.

Linear regression, as the most straightforward regression, is the first choice for analyzing host-seeking period and peak host-seeking time because it is easier to interpret. Ordinal logistic regression is the most suitable method found when linear regression does not work for start of host-seeking. However, the low classification rate for two of the categories (host-seeking start before and after sunset) implies that some other factors that are affecting the activity start time have not been considered or that the activity start time is relatively random and could not be predicted accurately. The trapping location on the dairy has human and natural creature activities, which could all affect the collection of biting midges and affect our data analysis. Additionally, since only 23 and 8 over 69 observations are host-seeking start before and after sunset, the unbalanced observation would also affect the prediction power of the start of host-seeking.

Temperature

Temperature is related to the start of host-seeking and host-seeking period, but not to the peak host-seeking time for *C. sonorensis*. Many studies show that temperature is an important factor influencing the flight activity of *Culicoides* species (Nelson and Bellamy 1971, Barnard and Jones 1980, Sanders et al. 2012, Walgama and Lysyk 2018). Also, a quadratic relationship between

temperature and midges' activity is found for some *Culicoides* species (Sanders et al. 2012, Walgama and Lysyk 2018), which is also found in this study, meaning that temperature does not only have a simple positive or negative association with host-seeking activity of midges. Temperature could directly affect the flying ability of *Culicoides* species and flight activity only occurred between 7 to 37 °C (Barnard and Jones 1980). Also, low temperature would suppress the flying ability of midges, and high temperature would be lethal to midges.

In this study, temperatures that are related to host-seeking period contain odTh and lT2, and both have quadratic relationships with host-seeking period, meaning that both daily maximum and minimum temperature would have effects on midges' host-seeking activity. The longest host-seeking period would happen when the highest temperature on the date before collection is high and lowest temperature during the collection is suitable (approx. 10 to 16 °C).

The hT1 has quadratic relationship with the start of host-seeking, meaning that it decreases the probability of host-seeking starting later as hT1 increase at lower temperatures while it increases the probability of host-seeking starting later as hT1 increase at higher temperatures. The highest temperature during collection would likely delay the start of host-seeking when temperature is too high. Since the daily maximum and minimum temperature usually change in a seasonal pattern, it is reasonable that high temperature of the day would delay the start of activity and therefore the length of host-seeking would shorten because most of activity happened from sunset to sunrise. Nelson and Bellamy (1971) observed that evening peaks of flight activity of *C. sonorensis* are independent of temperature, which is consistent with our findings in which temperature is not found significant in our analysis for peak host-seeking time.

Overall, the highest temperature during collection is associated with start of host-seeking and lowest temperature during collection is associated with host-seeking period.

Wind Speed

Wind speed is an important factor relating to the start of host-seeking, peak host-seeking time, and host-seeking period. Average wind speed during collection (mW) is significant for three analyses, and quadratic relationships between mW and host-seeking period as well as peak host-seeking time are found. Wind speed is a known factor that affects the flight activity of *Culicoides* species, and many studies indicate that wind speed has a negative effect on the flight activity of many midge species including *C. sonorensis* (Kettle 1969, Blackwell 1997, Walgama and Lysyk 2018). However, this study shows that a low wind speed can have a positive effect on the host-seeking activity and would suppress the activity when it exceeds a certain value. This nonlinear relationship between wind speed and flight activity of midges was suggested by Jess et al. (2018) and was observed for other insects (Messing et al. 1997). Daily wind speed change usually follows a certain pattern: it increases in the afternoon, then decreases during sunset, and remains very low or zero during the night, which likely explain why the host-seeking peak moves to a later time if the mean wind speed increases too high.

Relative Humidity

The relative humidity is found to relate to the host-seeking activity of *C. sonorensis*. The start of host-seeking and peak host-seeking time is influenced by humidity at sunset, and the host-seeking period is influenced by humidity at sunrise. The relationship between relative humidity and midges activity is only described by a few studies as it has less impact on the activity of

midges (Sanders et al. 2012, Grimaud et al. 2019). It is observed that *C. sonorensis* is only collected between 18 to 94% relative humidity (Barnard and Jones 1980). Humidity is important for the survival of adult midges because low humidity would expose *Culicoides* species to the danger of desiccation (Murray 1991, Mellor et al. 2000). Some mosquito studies showed that mosquitoes would avoid extremely low and high relative humidity though their activity is not influenced by a wide range of humidity (Thomson 1938, Rowley and Graham 1968). Relative humidity also interacts with temperature to affect activities of midges and mosquitoes (Rowley and Graham 1968, Mellor et al. 2000), which is found true in analyzing the start of host-seeking of *C. sonorensis*. Additionally, relative humidity strongly interacts with wind speed that influences the start of host-seeking, peak host-seeking time, and host-seeking period, indicating that under different relative humidity, the effect of wind speed on the host-seeking activity pattern of *C. sonorensis* is different.

Moon

Moonlight and moon phases are found to relate to the start of host-seeking and peak host-seeking time, respectively. The flight activity of *C. sonorensis* has been reported to increase when the moon rises at night (Nelson and Bellamy 1971, Linhares and Anderson 1990) and during a full moon night (Lillie et al. 1987). The moon phase is represented by days to the last new moon date (Mp), so the full moon usually occurs on day 14 or 15. Thought with interactions, the relationship between moon phases (i.e., days to the last new moon) is negatively related to the peak host-seeking time. The moon phase is related to the timing of moonrise and moonset, so for each lunar cycle, the moonlight overlaps with night from only evening after sunset (new moon) to most of the night (full moon), and to only early morning

before sunrise (close to the next new moon). If moon phases influence the peak host-seeking time through the part of night the moonlight shines, then a positive rather than a negative relationship between the moon phase and host-seeking peak is expected. However, when looking at diel host-seeking activity pattern by date, many activity peaks do not occur when the moon is present (Figures 1.8 – 1.10), which may explain why the model had a different relationship than previously reported and suggesting that factors other than the moon have stronger impacts on host-seeking activity peaks. Some studies use light traps to collect mosquitoes and find that mosquito catches are greater during the new moon period than the full moon period, which is possibly due to the competition between artificial light and moonlight (Provost 1959, Miller et al. 1970). If the presence of moonlight increases the use of visual cues for attraction leading to fewer midges in a CO_2 trap which does not look like cattle, it might explain a negative relationship between the moon phase and activity peak time. Nelson and Bellamy (1971) observed that activity peaks from truck-mounted traps and light traps were sometimes different from CO_2 traps, which indicates that the host-seeking activity pattern is different from the general flight activity and may explain the unusual relationship between moon phases and peak host-seeking time.

The start of host-seeking is related to the interaction between moonlight start time and temperature (Ms * hT1). It is expected that as moonlight start time moves to a later time, there is an increased probability of activity staring later. However, when a daily high temperature is higher than 32 °C, there is a decreased probability of activity starting at a later time as Ms increase. It is unclear why Ms and the probability of activity starting late has a negative

relationship when hT1 is very high. Nevertheless, the model shows that the moonlight has influence on start of host-seeking, but the effect is modified by temperature.

Seasonality

Sunrise time is an indicator of seasonal change. In comparison, the solar intensity of the day also has a clear seasonal pattern, but unusual cases can occur due to cloud cover or other environmental factors. In this study, we find that sunrise time (RT) and its interactions with wind speed and moon phase are associated with the peak host-seeking time. Solar intensity and its interaction with moon phase are associated with the peak host-seeking time and the interaction with temperature are associated with the host-seeking period. These two environmental variables in the model imply that the host-seeking activity is in association with seasonality, which is also found by other studies in which the activity pattern of some *Culicoides* species has a seasonal shift (Lillie et al. 1987, Viennet et al. 2012). The interaction terms indicate that the seasonal pattern can influence the diel host-seeking activity pattern, but the effect is modified by other environmental factors.

There are many interactions between environmental variables in the regression model, which indicates a complicated relationship between the diel host-seeking activity pattern and these variables. Several studies have looked at the diel flight activity of *Culicoides* species and the environmental factors such as temperature, relative humidity, and wind speed, but did not use quantitative methods to analyze it (Nelson and Bellamy 1971, Barnard and Jones 1980, Lillie et al. 1987). Also, some studies focused on the *Culicoides* abundance and environmental factors (Sanders et al. 2012, Grimaud et al. 2019). The modeling for diel host-seeking activity is difficult

because time of a day works as one of the parameters and the variation among dates is huge. The mix-effect model was firstly considered but did not work for such great variation under current observations. Future studies that obtain multiple years of diel host-seeking data may help perform the mix-effect model analysis and may provide more information on the association between environmental factors and midges host-seeking activity.

Other Observations

Over the 98222 *C. sonorensis* captured, about 10% of midges were males, which is expected because males of *C. sonorensis* are known to respond to hosts for purpose of mating and can be collected in CO_2-baited traps (Gerry and Mullens 1998). The seasonal abundance of host-seeking midges is in accordance with Gerry and Mullens (2000) in which they collected midges in the same dairy region though the parity rate differs a lot. The previous study observed that parity rates were high from late fall through spring and were low from summer through early fall (Gerry and Mullens 2000), while parity rates do not have a clear seasonal pattern and change drastically from month to month in this study. The unclear parity change could be due to the movement of the trap and operations on the wastewater pond. Since only one trap can conduct this time-segregated collection, multiple collection sites during one night are impossible to get an average abundance. On the contrary, Gerry and Mullens (2000) have multiple trapping locations for each trap night, so their number of midges can be averaged to obtain a relatively regular change of parity rates.

Overall, this study attempts to quantitatively investigate the diel host-seeking activity pattern, which has not been studied before. These analyses will help us understand the diel host-seeking

activity pattern of *C. sonorensis* in southern California dairy and will provide information on the study of diel host-seeking activities of other *Culicoides* species in other geographical locations. Additionally, the three-year collection shows that host-seeking activity of *C. sonorensis* would occur early in winter and it is necessary to start the CO_2 trap at least 1.3 hours or even the whole day to capture most active midges during this period.

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Figure 1.1. Overview of the dairy region and adjacent farms. Drylot pens containing cattle (orange mesh), pens with other animals (orange checker), wastewater ponds (blue) and farmland (green) are indicated. Pond 1 (red star) and pond 2 (purple star) are identified.

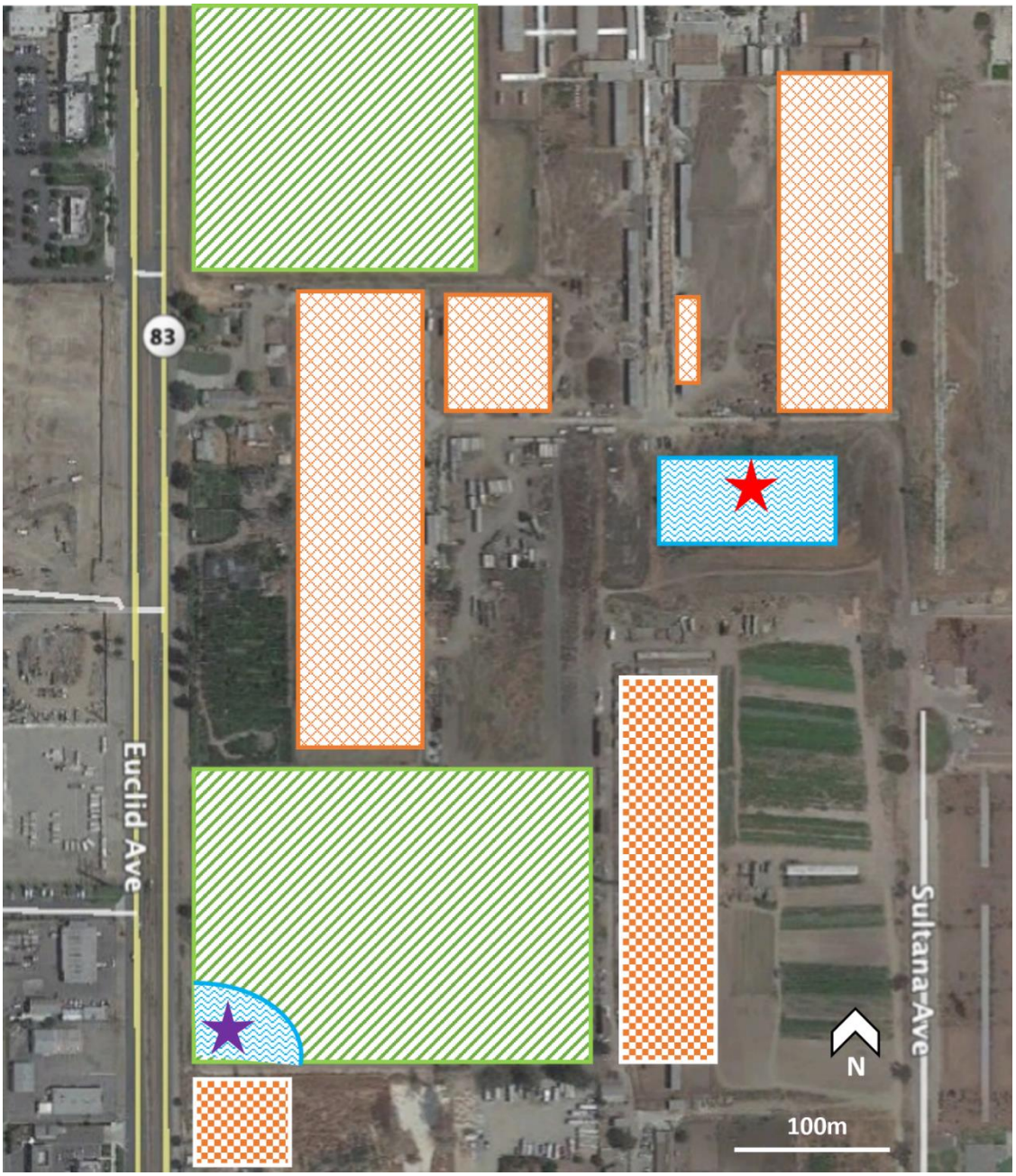


Figure 1.2: The overview of collection timeline and time coding. Weather information is obtained for the date before collection and collection day one and two. The 24-hour collection was conducted from 9:45 on day one through 9:45 on day two. The 24-hour collection was divided into 18 periods.

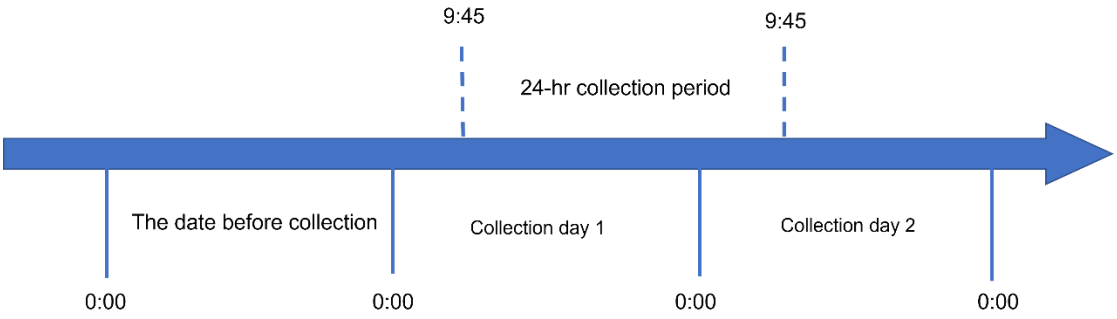


Figure 1.3: Host-seeking *C. sonorensis* collection by days and temperature. Black points are midge counts corresponding to y-axis on the left. Red and blue points are highest and lowest temperatures (°C) of the 24 hours collection period corresponding to y-axis on the right. Temp = temperature

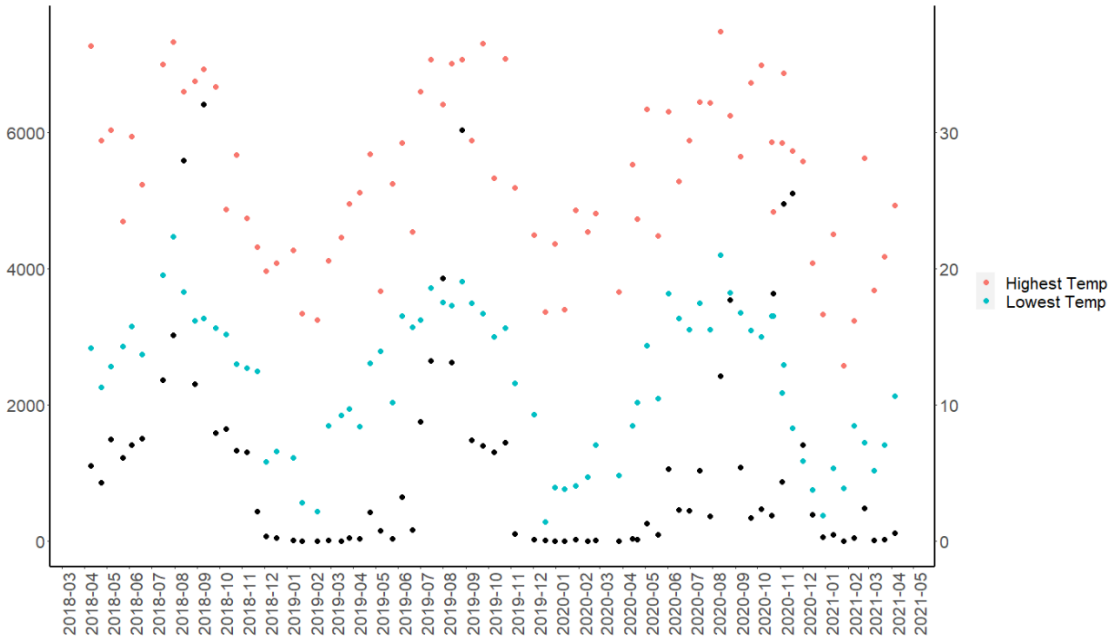


Figure 1.4: Host-seeking *C. sonorensis* collection by days and solar intensity. Black points are midge counts corresponding to y-axis on the left. Red points are highest solar intensity (w/m^2) of the 24 hours collection period corresponding to y-axis on the right. SI = solar intensity

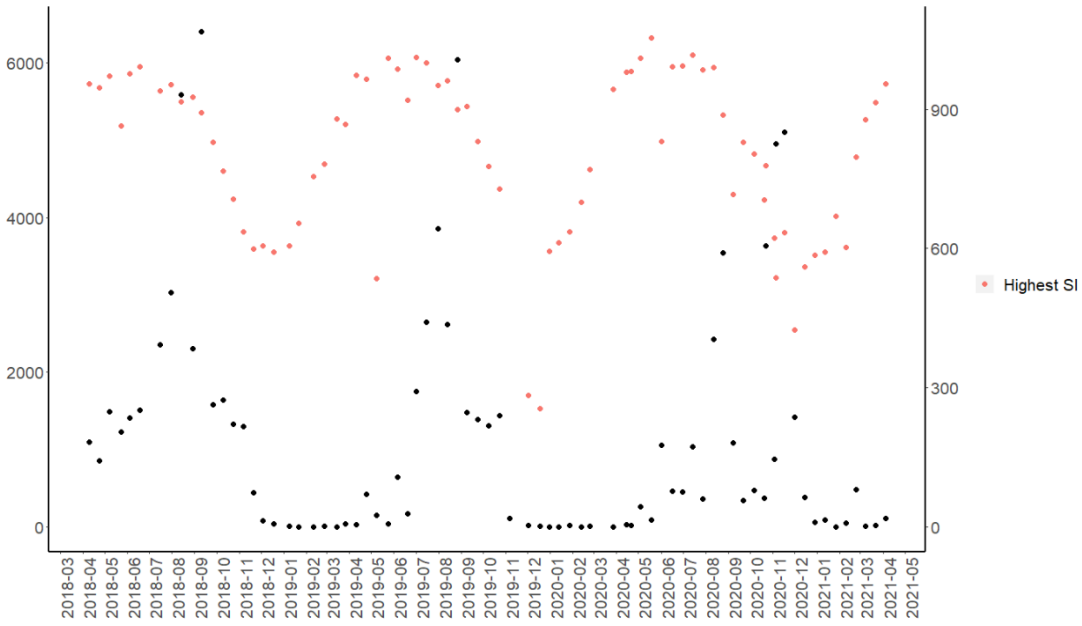


Figure 1.5: Host-seeking *C. sonorensis* collection by days and wind speed. Black points are midge counts corresponding to y-axis on the left. Red and blue points are highest and mean windspeed (m/s) of the 24 hours collection period corresponding to y-axis on the right. WS = wind speed

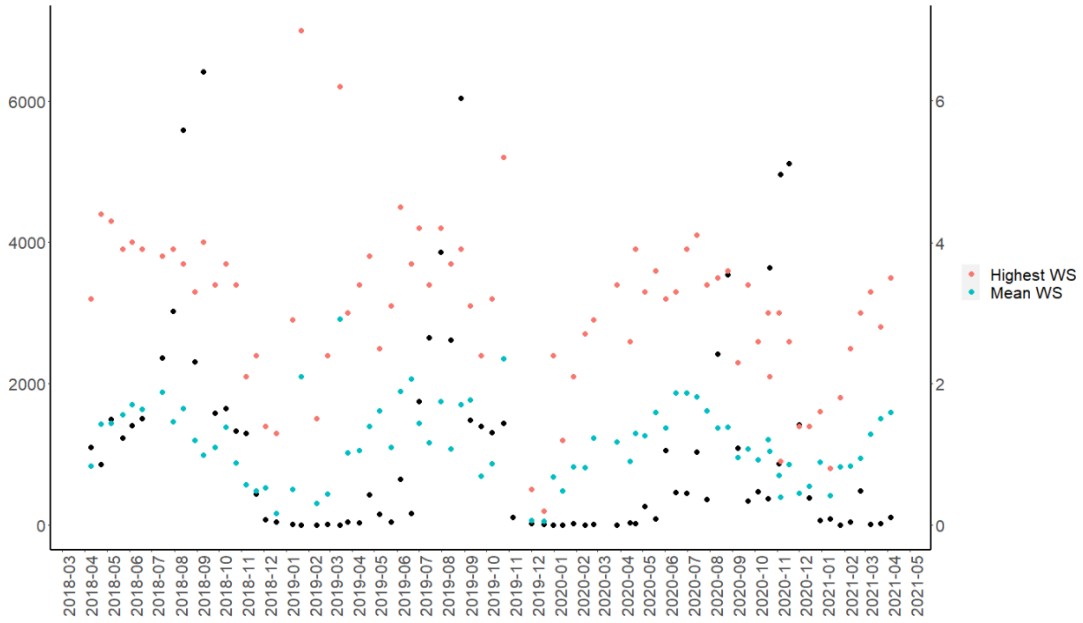


Figure 1.6: Host-seeking *C. sonorensis* collection by days and relative humidity. Black points are midge counts corresponding to y-axis on the left. Red and blue points are highest and lowest relative humidity (%) of the 24 hours collection period corresponding to y-axis on the right. RH = relative humidity

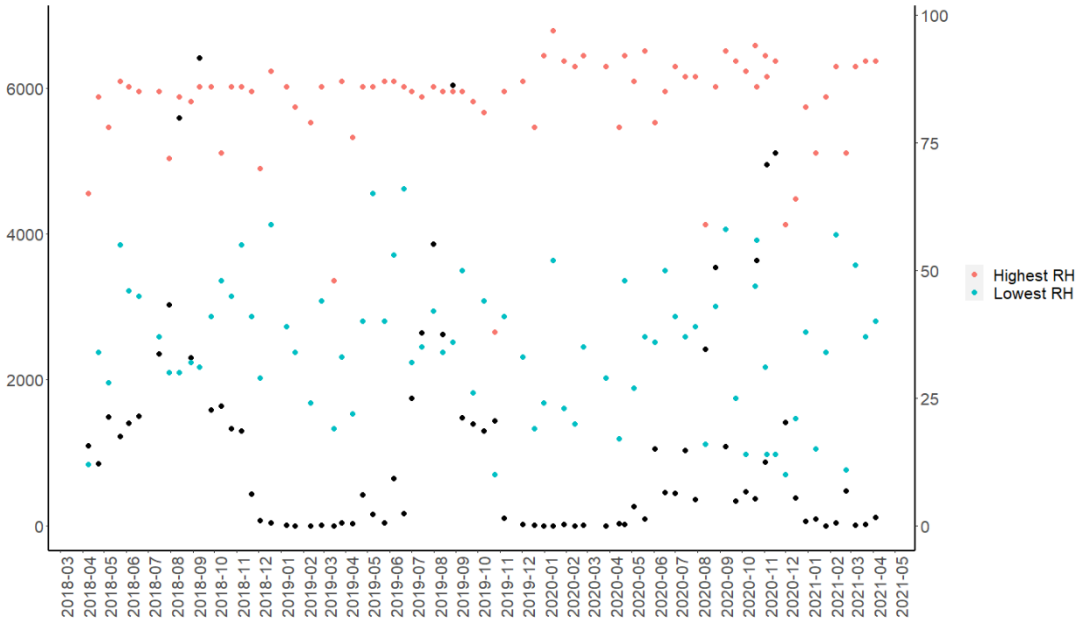


Figure 1.7: Parous, nulliparous, and male midges collection by month. Color bars represent number of midges being collected per trap day corresponding to the y-axis on the left. Black solid lines represent the parity rate corresponding to the y-axis on the right.

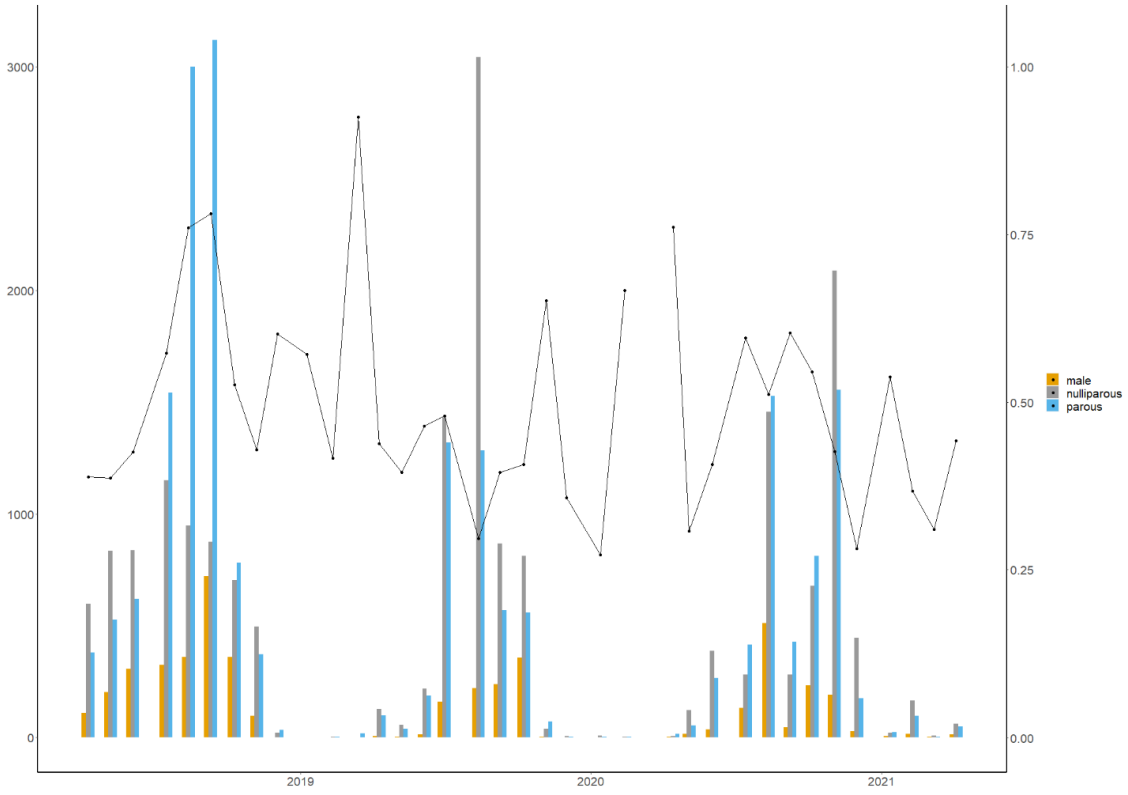


Figure 1.8: Host-seeking *C. sonorensis* activity pattern by date (April 2018 – March 2019). X-axis represents 18 collecting periods from 9: 45 am (0) on day 1 through 9: 45 am (18) on day 2. Bars are the ratio of host-seeking midges captured in each collecting period to the total host-seeking midges captured throughout the 24 hours, representing the proportion of host-seeking midges in each period compared to the total catch. Purple vertical lines represent the sunset and sunrise time, and orange lines represent the moonlight start and end time. For days when only one purple line exists, another purple line is overlapped by orange line, meaning that either moon rises before sunset or moon sets after sunrise. Three color lines in the graph shows the diel pattern of relative humidity (RH), temperature (Temp) and wind speed (Wind) change.

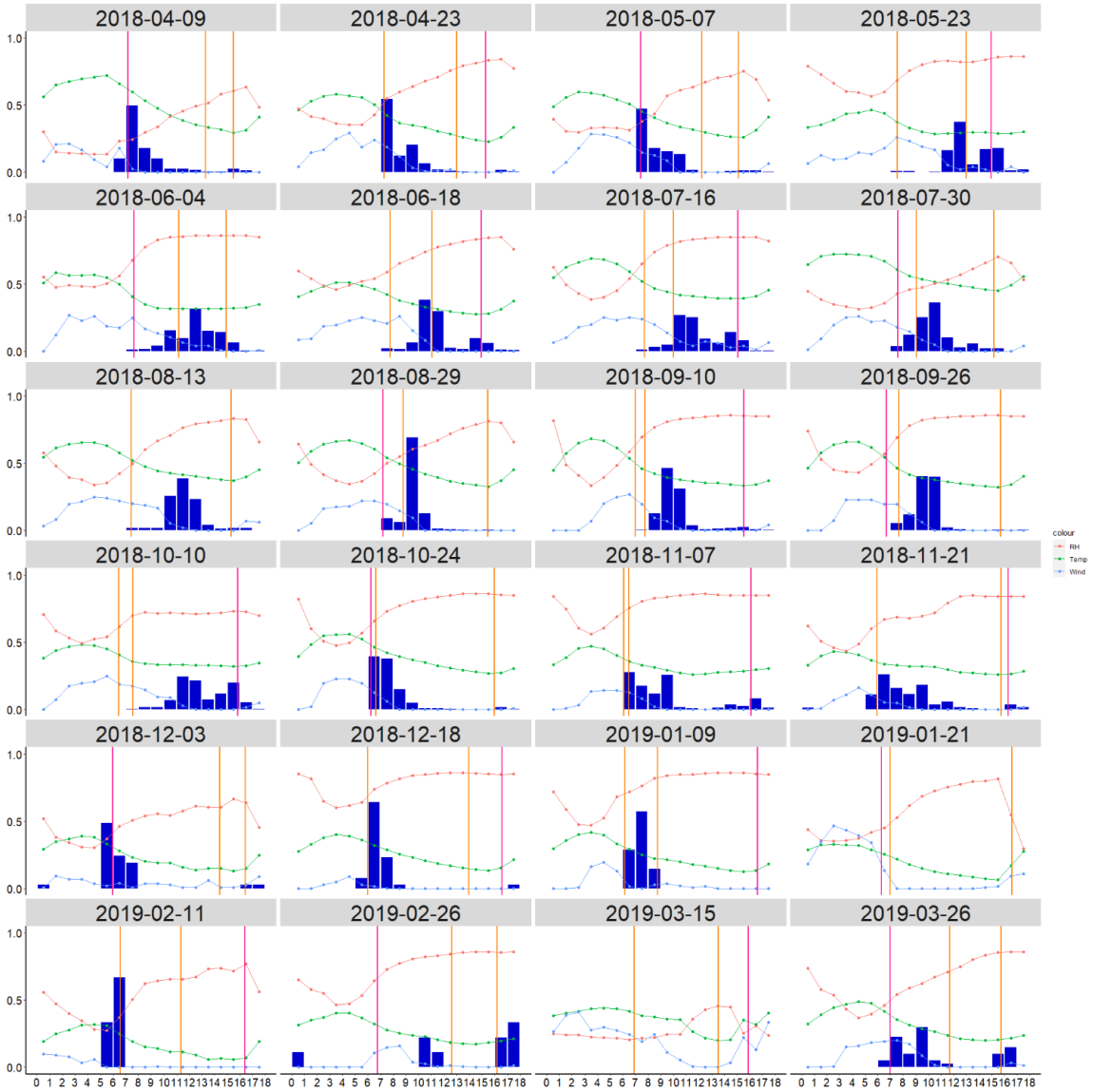


Figure 1.9: Host-seeking *C. sonorensis* activity pattern by date (April 2019 – March 2020). X-axis represents 18 collecting periods from 9: 45 am (0) on day 1 through 9: 45 am (18) on day 2. Bars are the ratio of host-seeking midges captured in each collecting period to the total host-seeking midges captured throughout the 24 hours, representing the proportion of host-seeking midges in each period compared to the total catch. Purple vertical lines represent the sunset and sunrise time, and orange lines represent the moonlight start and end time. For days when only one purple line exists, another purple line is overlapped by orange line, meaning that either moon rises before sunset or moon sets after sunrise. Three color lines in the graph shows the diel pattern of relative humidity (RH), temperature (Temp) and wind speed (Wind) change. The weather information was missing for part of night on November 6, 2019, but did not affect the statistics analysis, therefore it was left blank for this period.

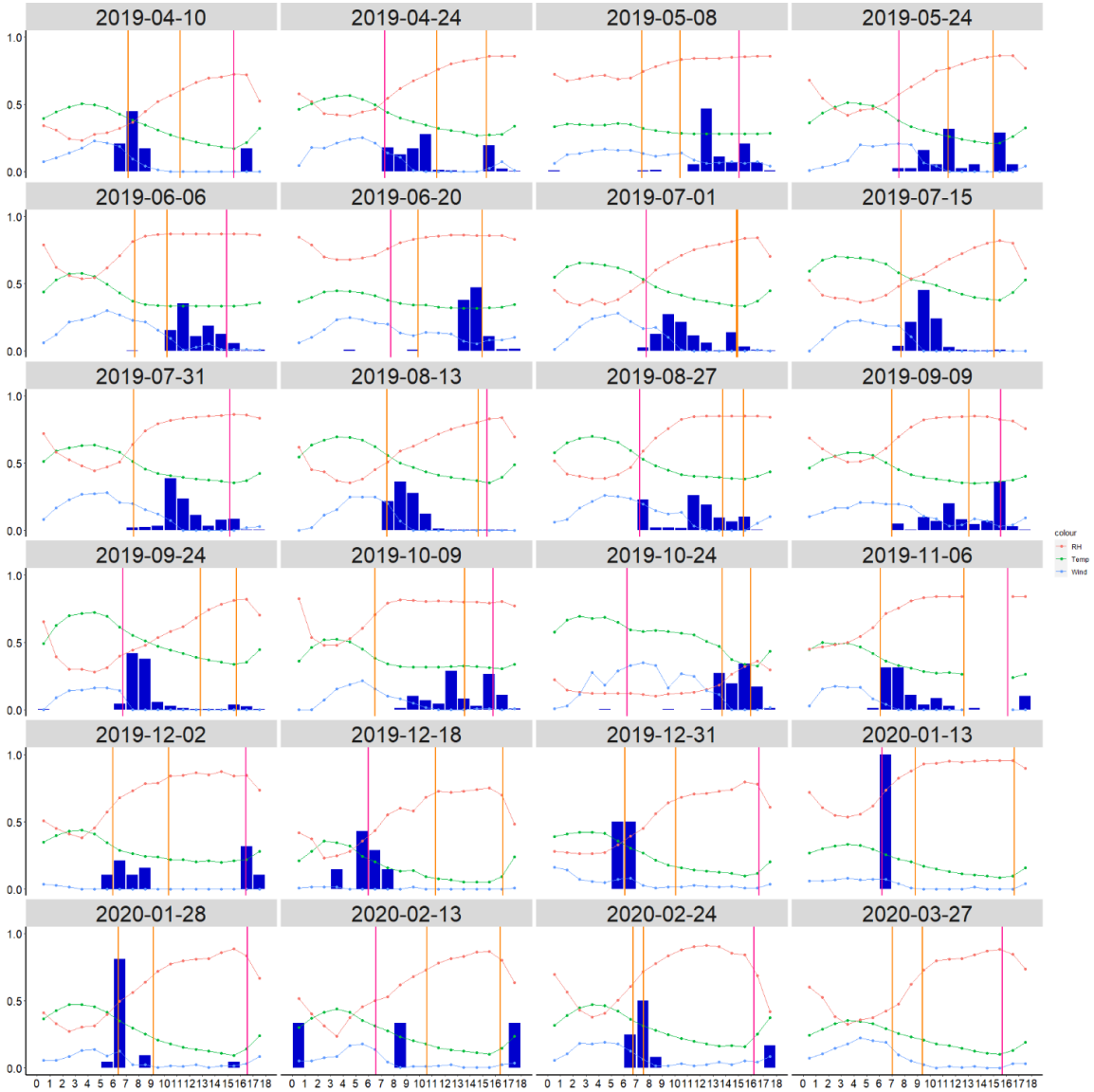


Figure 1.10: Host-seeking *C. sonorensis* activity pattern by date (April 2020 – March 2021). X-axis represents 18 collecting periods from 9: 45 am (0) on day 1 through 9: 45 am (18) on day 2. Bars are the ratio of host-seeking midges captured in each collecting period to the total host-seeking midges captured throughout the 24 hours, representing the proportion of host-seeking midges in each period compared to the total catch. Purple vertical lines represent the sunset and sunrise time, and orange lines represent the moonlight start and end time. For days when only one purple line exists, another purple line is overlapped by orange line, meaning that either moon rises before sunset or moon sets after sunrise. Three color lines in the graph shows the diel pattern of relative humidity (RH), temperature (Temp) and wind speed (Wind) change.

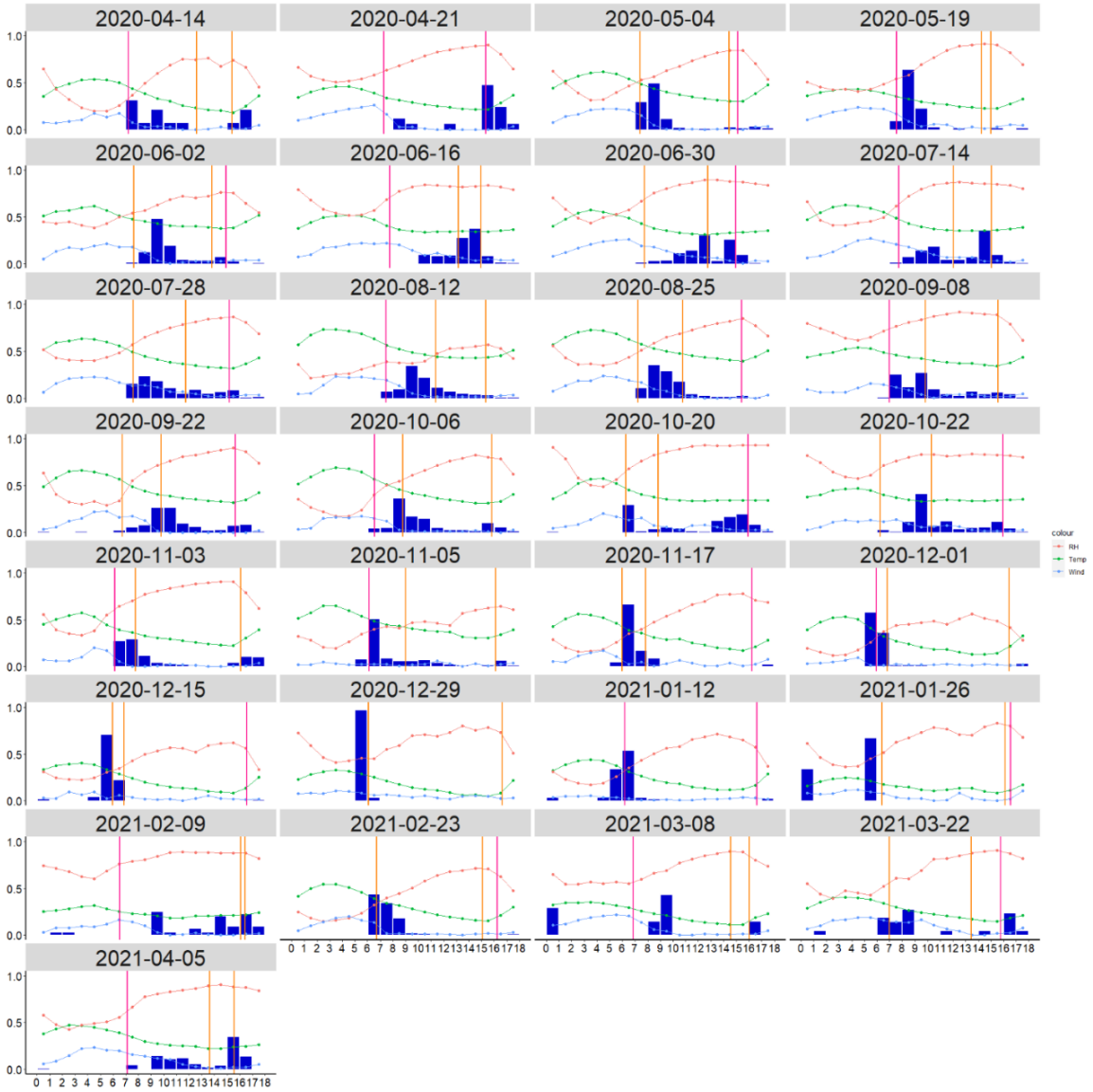


Figure 1.11: An overview of host-seeking *C. sonorensis* diel activity pattern (proportion). X-axis represents 18 collecting periods from 9: 45 am on day 1 through 9: 45 am on day 2. Points are the ratio of host-seeking midges captured in each collecting period to the total host-seeking midges captured throughout the 24 hours, representing the proportion of host-seeking midges in each period compared to the total catch. Different colors represent different months.

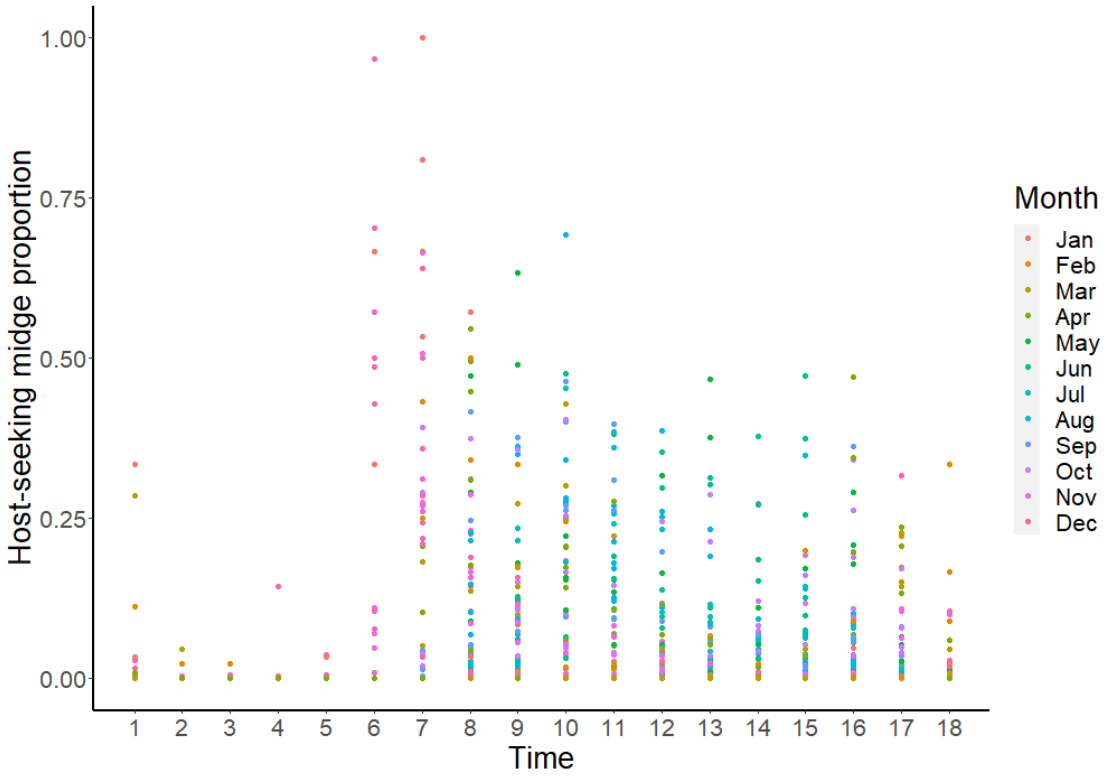
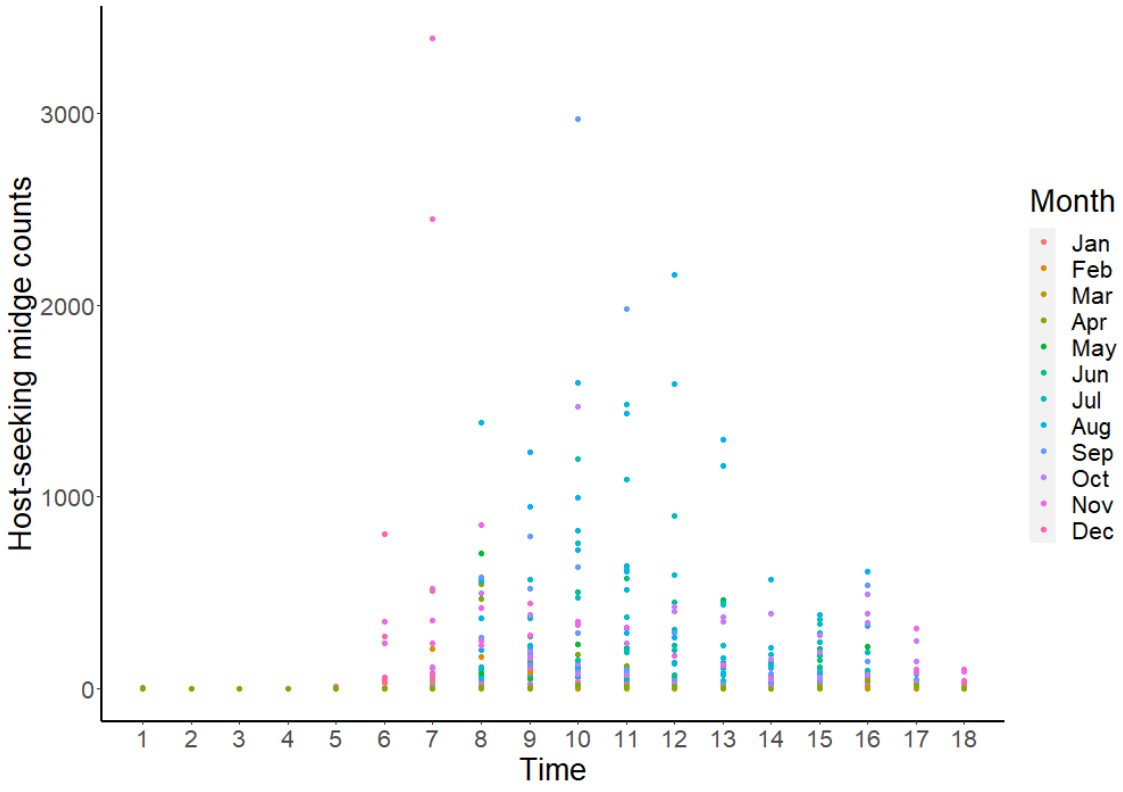


Figure 1.12: An overview of host-seeking *C. sonorensis* diel activity pattern (counts). X-axis represents 18 collecting periods from 9: 45 am on day 1 through 9: 45 am on day 2. Points are the number of midges caught during each collecting period. Different colors represent different months.



CHAPTER 2

Does Bluetongue Virus Overwinter Within the Adult *C. sonorensis* Population in Southern California?

Abstract

The mechanism of BTV overwintering is not well understood. Annual reoccurrence of bluetongue infection in cattle raised in southern California suggests that BTV persists in the region but escapes detection during cooler months, reappearing when the weather gets warmer. The persistence of the virus in the adult biting midge vector (*Culicoides sonorensis*) has been suggested, though it is unknown whether infected biting midges could continue to feed on cattle and transmit the virus during the winter or hibernate in resting locations through the cooler winter months. Understanding the BTV overwintering mechanisms will help understand the seasonal dynamic of BTV transmission and assist in prevention strategies for bluetongue disease. This study collected host-seeking *C. sonorensis* in the southern California Chino dairy region during winter and early spring periods for three years to assess wintertime biting activity and to detect BTV infection in these midges. While *C. sonorensis* were actively seeking host throughout the year, BTV was only detected in midges captured during November and December and not detected in midges captured from January through April. Two possible mechanisms for BTV overwintering at the study location are suggested: 1) BTV was transmitted between midges and cattle throughout the winter but at a level that was too low to be detected in captured midges; 2) BTV infected adult *C. sonorensis* survived through winter without feeding.

The overwintering mechanisms of BTV remain unclear and further research is needed to investigate this question.

Introduction

Among all the disease agents that *Culicoides* Latreille biting midges (Diptera: Ceratopogonidae) can transmit, bluetongue virus (BTV) in the genus *Orbivirus* of the family Reoviridae is one of the most notorious because it can spread rapidly and severely affects the international trade of animal and animal products (Tabachnick 1996). BTV can cause asymptomatic to severe bluetongue disease on wild and domestic ruminants and even cause death during outbreaks (Gibbs and Greiner 1994, Conraths et al. 2009). During the biological transmission of BTV, female midges feed on viremic animal hosts and become infected. BTV replicate and disseminate within the body of midges and eventually infect the salivary gland during the extrinsic incubation period (Fu et al. 1999). Infectious female *Culicoides* then bite another susceptible host and transmit the virus to the host (Mellor et al. 2009, Belbis et al. 2017). Therefore, only parous females that have completed a gonotrophic cycle are able to transmit the virus.

It has been observed that BTV transmission is highly seasonal in temperate zones (Nevill 1971, Gerry et al. 2001, Mayo et al. 2014) with infection of cattle occurring from summer through late fall. The period in which BTV cannot be detected from midges or their hosts is called the interseasonal period, and "overwintering" is the term to describe the unknown mechanism by which BTV escapes from monitoring during this period (Nevill 1971, Mayo et al. 2016). *Culicoides sonorensis* Wirth and Jones, a mammalophillic species, is the only confirmed vector of BTV in California (Foster et al. 1963, Luedke et al. 1967) and is almost exclusively collected on California dairies (Gerry and Mullens 2000, Mayo et al. 2014). BTV infection of cattle usually starts with

low numbers in summer and reaches a peak in late summer through early fall, with new BTV infections ending by late fall when the abundance of host-seeking midges is greatly reduced (Nevill 1971, Gerry and Mullens 2000, Mayo et al. 2014, Mayo et al. 2014). BTV has been occasionally isolated or detected from ruminant hosts or *Culicoides* vectors in winter or spring (Osburn et al. 1981, Stott et al. 1985, Mayo et al. 2014).

Several hypotheses on overwinter mechanisms of BTV have been brought up (Nevill 1971, White et al. 2005, Osmani et al. 2006, Wilson et al. 2008): 1) Adult midges infected with BTV in late fall survive through winter and start to transmit the virus again when weather is suitable; 2) a low transmission cycle is maintained between midges and host animals throughout the interseasonal period; 3) BTV persists in cattle throughout the winter; 4) BTV overwintering in other reservoir animals; 5) Transovarial transmission of BTV (i.e., BTV is transmitted from mother to its offspring); 6) BTV was reintroduced to the region from neighboring areas every year.

The most strongly suggested overwintering mechanism by Mayo et al. (2014) was that BTV can overwinter through long-lived adult *C. sonorensis* by infecting them before the interseasonal period because they captured BTV-infected midges in the middle of the winter period. Compared to Northern California, host-seeking *C. sonorensis* could be captured throughout the winter months instead of occasionally in the southern California dairies, suggesting that these midges may contribute to a low transmission cycle among cattle (Gerry and Mullens 2000). Cattle infected with BTV usually remain viremic for less than 60 days though BTV RNA and antibodies can be detected for a long time after infection (MacLachlan et al. 1994, Maclachlan et

al. 2009, Mayo et al. 2014), so it seems unlikely that cattle can preserve BTV throughout the interseasonal period. Other animals may serve as reservoirs for BTV, for example, it is found that some elk could remain viremic after inoculation for as long as three months (Murray and Trainer 1970), and ticks could be infected with BTV and keep the virus for a month by laboratory feeding (Bouwknegt et al. 2010). Transovarial transmission of BTV was not found in *C. sonorensis* (Osborne et al. 2015) and field collection in Northern California also confirmed that only parous *C. sonorensis* could be detected with BTV (Mayo et al. 2014), suggesting that overwintering through transovarial transmission is unlikely. BTV has been circulated in the southern California dairy region for many years and serotypes 10, 11, 13, and 17 were known endemic in the region (Osburn et al. 1981, Stott et al. 1985, Gerry et al. 2001, Mayo et al. 2012). Reintroduction from the continuous transmission cycles to temperate zones would be more random and unlikely to occur in the same region from year to year (Osmani et al. 2006).

This study aims to test the hypothesis that BTV overwinters through maintaining a low transmission cycle between *C. sonorensis* and cattle in southern California dairies by collecting midge samples during winter months and detect BTV using RT-qPCR.

Methods

Midge Collection

Culicoides sonorensis specimens were collected from the Chino dairy region located in southwestern San Bernadino County, California (Figure 2.1). Collections were conducted on medium to large commercial dairy farms (A, B, and C; Figure 2.1) with locations selected to

cover the entire dairy region from west to east, respectively. Location B was two large, independently operated dairy farms positioned on opposite sides of a narrow road.

Midges were collected every other week from November through April for three years (2018 to 2021). Trapping was conducted using Centers for Disease Control (CDC) type suction traps baited with CO_2 set up on each dairy either near wastewater ponds or near drylot pens containing cows. Traps were hung on poles so that trap openings were positioned at a height of ca. 1 – 1.2m. To avoid midge desiccation during trap operation and increase survivorship, paper towels were placed inside the collection cup to provide shelter to midges.

During the first year (2018-2019) trapping was conducted only at dairy farm A which was the site of a separate study. Traps were set in the morning and run for 24 hours to catch active midges throughout the day. CO_2 was provided by a compressed gas cylinder at a release rate of 1,000 ml/min to simulate the CO_2 output of a calf (Roberts 1972, Gerry et al. 2001). Trapping was expanded during the second year (2019-2020) to include all three dairy farm locations (A-C; Figure 2.1). At locations A and C, *Culicoides* were captured using four CO_2-baited suction traps baited with dry ice while at location B, eight CO2-baited suction traps baited with dry ice were utilized. Trapping was started one to three hours before sunset and continued till one to two hours after sunrise the next morning to capture active midges near sunset and sunrise. In April 2020, trapping was conducted only at sites A and C to minimize close contact with others at the start of the COVID pandemic. During the third year (2020-2021) trapping was conducted only at dairies A and C. which had much higher midge abundance than site B during the previous year. At both dairies, eight CDC-type traps baited with dry ice were spread across the dairy to capture

as many midges as possible. Trapping was otherwise similar to the second year. Upon trap removal, traps were transported at room temperature to the laboratory, and midges were provided with a wet cotton ball on top of the trap to keep moisture before processing.

Midge Sorting and Storage

On the same day of trap removal, captured insects in the catch bags were anesthetized using triethylamine (Work et al. 1990) and *C. sonorensis* were sorted under the dissecting scope by sex and physiological status (unfed, fed, gravid) and parity status (parous, nulliparous) (Dyce 1969). *Culicoides* that were dead, dry, and had wrinkled contracted bodies were briefly placed into 95% ethanol to determine their parity status before being dried on a Kimwipe. Parous, blood-fed, and gravid females were pooled separately by $\leq$ 20 individuals and stored at -80 °C.

Daily Survivorship

Daily survivorship was estimated by $P^{1/u}$ where P is the parity rate (# of parous females/ # of parous and nulliparous females), and u is the gonotrophic cycle length (Davidson 1954, Gerry and Mullens 2000). The gonotrophic cycle represents the length of time between oviposition events. Because hosts for blood and wastewater ponds for oviposition sources were available and abundant at the study dairies, oogenesis is anticipated to be most of the gonotrophic cycle (Gerry and Mullens 2000). The length of gonotrophic cycle was estimated by $-1.98 + 0.07217X + 2516.65X^{-2}$ where X is the temperature (Mullens and Holbrook 1991). In this study, daily

survivorship was estimated by site and day using the average temperature of the day ("Weather History" 2019).

BTV Positive Control

A separate collection was made on dairy A for *C. sonorensis* in fall 2021. Nulliparous females were sorted from the collection on a chill table for virus intrathoracic inoculation. Bluetongue virus serotypes 10, 13, and 17 (at least 7 logs/ml) provided by U.S. Department of Agriculture (USDA) were injected to the thorax of nulliparous *C. sonorensis* by pulled capillary needles, and injected midges were held separately by serotypes in container for seven days. Midges were provided with 10% sucrose water. On the seventh day post injection, live *C. sonorensis* were frozen and stored at -80 °C for later RNA extraction and RT-qPCR.

BTV Detection

RNA was extracted from pooled midges stored at -80 °C using Zymo Direct-zol RNA miniprep kits (California, U.S.). Five extractions were then pooled for quantitative reverse transcription polymerase chain reaction (RT-qPCR) using the SuperScript™ III Platinum™ One-Step qRT-PCR kit (Invitrogen). Each 25µl of reaction mix contained 0.5µl SuperScript™ III RT/Platinum™ *Taq* Mix (kit), 12.5µl 2X Reaction Mix (kit), 0.5µl 10µM forward primer, 0.5µl 10µM reverse primer, 0.25µl probe, 5µl RNA template, and Nuclease-free water to bring the volume to 25µl (Table 2.1). The amplification process included 55°C for 15 min, 95°C for 2 min, and 50 cycles of 95°C for 15 sec and 60°C for 30 sec. Fluorescence was measured at the end of the 60°C annealing/extension step, and the quantification cycle (Cq) value was recorded (Mayo et al. 2012). For RNA pools that had Cq values, individual RNA extractions of the pool were used to

run a second-round RT-qPCR to test which RNA extraction(s) resulted in Cq value. The natural Infection rates (IR) assumed that only one midge was infected per positive pool and was estimated by (Gerry et al. 2001):

$$IR = \frac{number\ positive\ pools}{number\ parous\ midges\ tested} * \ parity\ rate\ .$$

Table 2.1: Primer and probe sequences used for RT-qPCR for BTV detection (Shaw et al. 2007).

BTVuni 291-311F	5' – GCTTTTGAGGTGTACGTGAAC – 3'
BTVuni 381-357R	5' – TCTCCCTTGAAACTCTATAATTACG – 3'
Probe 323	5' – TCCTCCGGATCAAGTTCACTCCAC – 3'

Table 2.2: Report Cq values for positive controls.

Sample ID	Cq value	BTV serotype
1	37.05	BTV-10
2	27.62	BTV-10
3	21.32	BTV-10
4	35.57	BTV-10
5	-	BVT-13
6	36.57	BVT-13
7	36.97	BVT-13
8	30.38	BVT-13
9	23.35	BTV-17
10	30.7	BTV-17
11	22.66	BTV-17
12	35.78	BTV-17

- Minus means BTV was not detected in the sample.

Table 2.3: BTV detection information.

Date	Site	Cq value	No. of midges in pool
11/27/2018	A	31.05	20
11/13/2019	A	33.23	20
11/13/2019	A	13.9	12
11/13/2019	C	29.1	20
11/13/2019	C	34.16	19
11/13/2019	B	37.01	10
12/13/2019	A	38.18	13
11/13/2020	C	24.28	20
11/13/2020	A	27.43	20
11/13/2020	A	28.36	20
11/13/2020	A	18.79	20
11/13/2020	A	35.86	20
11/13/2020	A	33.38	20
11/13/2020	C	38.29	16
11/25/2020	C	25.16	20
11/25/2020	A	37.16	20
11/25/2020	A	33.54	20

Table 2.4: Natural infection rate estimates.

Date	IR
11/27/2018	0.13%
11/13/2019	0.62%
12/13/2019	0.68%
11/13/2020	0.34%
11/25/2020	0.06%

Results

During three years of collection, female *C. sonorensis* were captured throughout the winter and the midge collection varied by dairies (Figure 2.2). In general, site A always had the highest captures over three years and the seasonal trend was clear. The beginning of the winter collection usually had the highest captures and the number of midges collected dropped and

was kept at a low number throughout the collection. On the contrary, site B had the lowest number of captures compared to the other two sites. The number of midges collected at site C did not have a clear seasonal trend: an especially high number of midges were captured in the middle of winter in the second year, and the number of midges captured in the third winter remained relatively constant.

Both parous and nulliparous females were caught throughout the winter collection, but parity rates (# of parous females/ # of parous and nulliparous females) varied among years (Figure 2.2). During the first year of the winter collection, parity rates fluctuated around 0.5 except for one collection in February when only nulliparous midges were collected. During the second and third year, parity rates were generally below 0.5 except for site C in the second year, where parity rates exceeded 0.5 on several occasions.

Daily mean temperature during collection ranged from 7 to 22 °C, and gonotrophic cycle length calculated from temperature ranged from 4 to 47 days (Supp. Table S2.1). Daily survivorship was calculated based on gonotrophic cycle length and parity rate for each site and day. At site A, the daily survivorship varied from 0.82 to 0.97 except for one day on which the daily survivorship was 0. At site B, the daily survivorship ranged from 0.75 to 0.97. At site C, the daily survivorship varied from 0.74 to 0.99 except for two days on which the daily survivorship was 1.

RNA was extracted from 12 BTV inoculated *C. sonorensis* that were held for 7 days, and Cq values ranged from 21.31 to 37.05 (Table 2.2). A total of 414 RNA extractions containing 6760 individuals were tested for the presence of BTV using RT-qPCR. Seventeen of the 414 RNA

extractions resulted in Cq values ranging from 13.9 to 38.29 (Table 2.3). BTV was detected in midges collected in November (all three years) and in December (second year only) and from all three dairy locations. No midges captured from December through April were infected with BTV. The estimated BTV infection rate of *C. sonorensis* on days when BTV was detected ranged from 0.06% to 0.68% (Table 2.4). Since BTV was only detected in November and December, the infection rate was calculated as 0.13% for this period over three years. The overall infection rate for three winters was 0.08% and was calculated by mean parity rate * natural infection rate for parous midges.

Discussion

Host-seeking *C. sonorensis* were captured throughout the interseasonal period which is consistent with previous studies in the same dairy region 20 years ago (Gerry and Mullens 2000). Both parous and nulliparous midges were collected throughout the winter period indicating that immatures continue to emerge during winter periods (Gerry and Mullens 2000). In cooler climates, *C. sonorensis* is reported to survive through winter as mid-late-stage larvae (Barnard and Jones 1980, Vaughan and Turner 1987). In southern California, winter is relatively mild, and the daily mean temperature mostly ranged from 10 to 20 °C.

Parity rates were generally around or lower than 0.5 for three years of winter collections except for site C in the second winter. Parity rates of *C. sonorensis* in Gerry and Mullens (2000) were lower in summer and early fall (approx. 0.2) and higher (around 0.4) from late fall through early summer, suggesting greater survival of adult midges during the interseasonal period. The

especially high parity rate on site C in the second winter is probably caused by the frequent desiccation of wastewater pond. Since immature *C. sonorensis* habits in the muddy interface on the side of wastewater pond (Mullens and Lip 1987), desiccation of the pond would largely impede the development of larvae and therefore reduce their emergence. Daily survivorships are estimated to be similar among the three trapping sites, therefore the lack of emergence of adult midges results in a high parity rate. Additionally, trapping location can determine the parity rate since newly emerged nulliparous midges would disperse upwind before host-seeking while parous midges can search for hosts immediately after oviposition (Zimmerman and Turner 1984, Gerry and Mullens 2000). The trap locations on site C during the second winter collection were near wastewater ponds, so it may explain why the parity rate on site C remain high throughout the winter. During the third winter collection, traps were spread out on the dairy so that the abundance of parous and nulliparous midges was averaged to represent the whole environment.

BTV was detected during each year of the study, indicating that BTV continues to be transmitted in the dairy region annually, which has been observed for years (Gerry et al. 2001). Though *C. sonorensis* were captured throughout the winter in southern California dairy, no BTV was detected after December till the end of collection period in April, which is consistent with Gerry et al. (2001) who also detected BTV from field-captured midges in the same dairy region about 20 years ago. The lack of BTV detection from January through April implies that BTV manages to escape from surveillance and restart transmission after April. Another study in northern California did not capture BTV-infected midges after December based on monthly collections (Mayo et al. 2012), but a more intense collection captured infected *C. sonorensis* in February,

and sentinel cattle also showed weak positive Cq values (i.e., high Cq values) during the interseasonal period (Mayo et al. 2014). According to other studies (Maclachlan et al. 2009, Veronesi et al. 2013, Mayo et al. 2014), Cq values $\leq$ 30 indicate the presence of infectious BTV, while Cq values > 30 may imply the detection of non-infectious viral RNA. Therefore, the Cq value of positive pools in this study ranged from 13 to 38, suggesting the presence of both midges with infectious BTV and midges with non-infectious BTV.

The estimated BTV infection rates varied greatly from 0.06% to 0.68%, which is also shown by Gerry et al. (2001) that field infection rates varied throughout the three collection years. The overall infection rate was 0.08%, which is the same as Gerry et al. (2001). When looking at November and December in which positive midges were collected, the parity rate was 0.13%, which is higher than the overall infection rate.

Regardless of how many parous females were captured during the overwintering period, BTV was never detected from January through April for three years. If BTV positive *C. sonorensis* were captured throughout the interseasonal period, it would suggest that infected midges are still active during this time and are able to transmit the virus to cattle through feeding. Therefore, our findings cannot reject any of these two hypotheses: 1) long-lived parous females are carrying BTV but are not captured during the period; 2) the transmission cycle between midges and their hosts remains very low so that it cannot be detected during this period. Mullens et al. (1995) indicated that temperatures as low as 15 °C does not support virus replication and increased temperature allows virogenesis within *C. sonorensis*. Therefore, adult

C. sonorensis might feed on viremic hosts during the interseasonal period but the temperature is too low to allow for virus replication until the environmental temperature rises.

There might be several mechanisms that BTV can use to survive through winter, and this study only aims at *C. sonorensis* as the vector of BTV. As global climate changes, the overall winter temperature may continue to rise, which may change the primary BTV overwintering mechanism. For now, the mechanism that BTV overwintering in southern California remains unclear, future studies could focus on collecting resting parous *C. sonorensis* to detect virus and adding sentinel cattle to monitor the BTV transmission during the interseasonal period.

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Figure 2.1: Collection sites in the Chino dairy region of southern California.

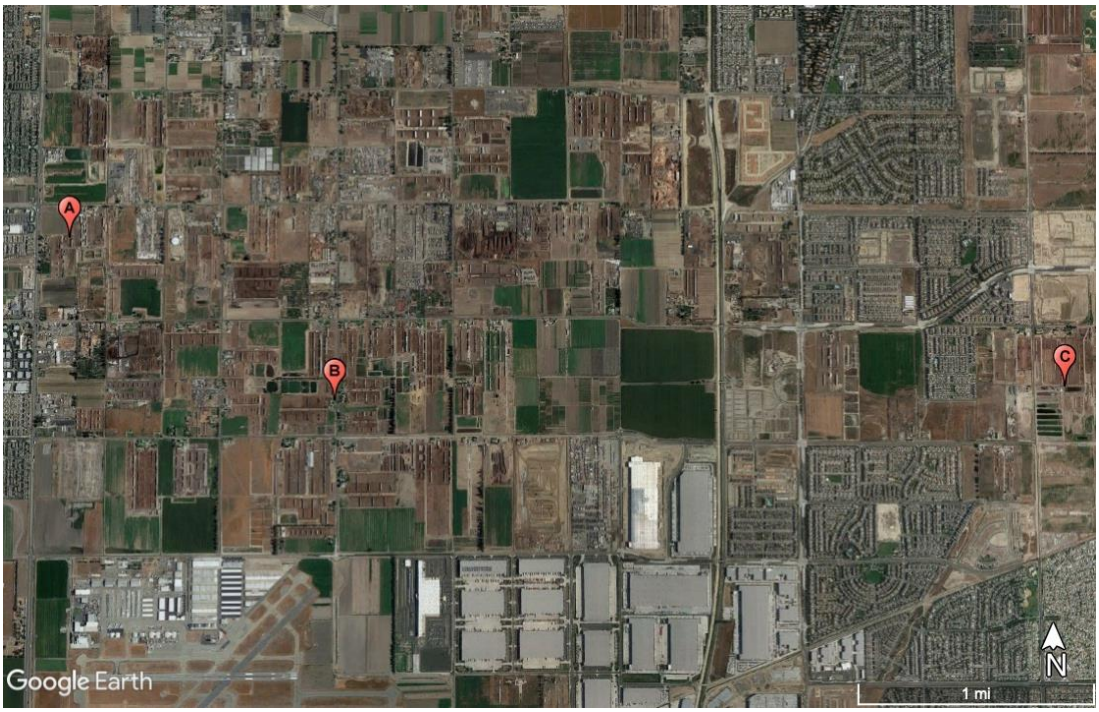
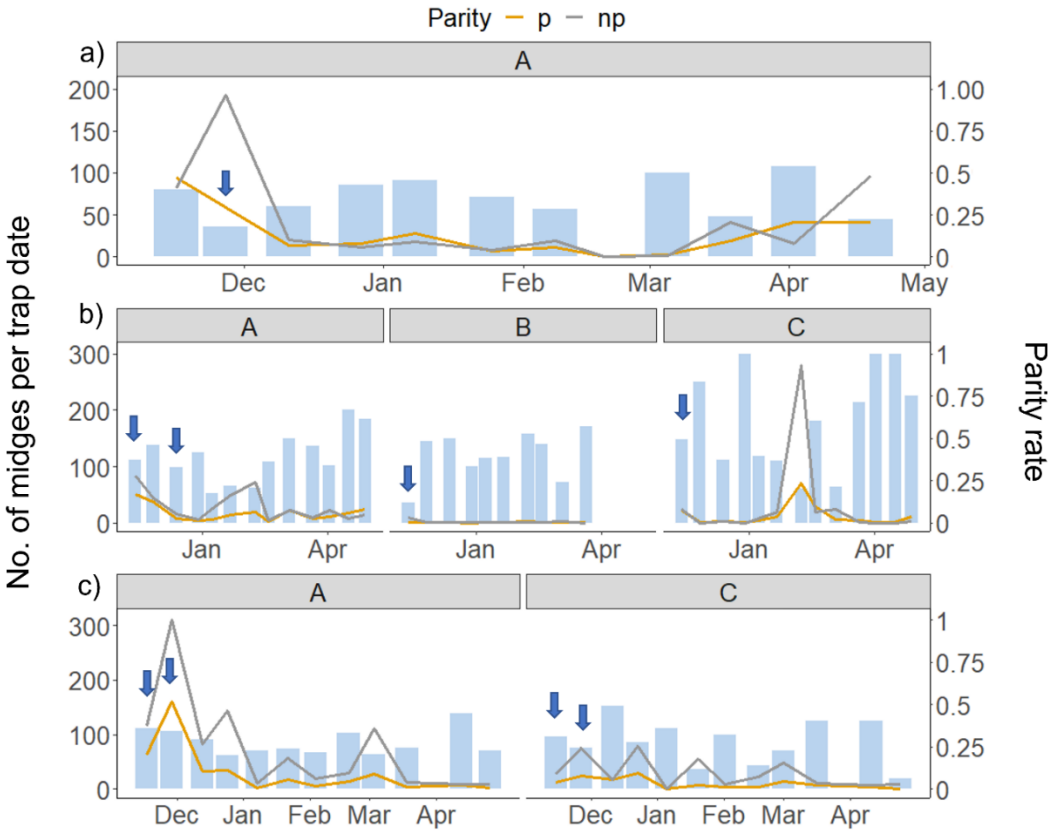


Figure 2.2: Number of parous and nulliparous *C. sonorensis* collected per trap date. Orange and grey lines represent the number of parous and nulliparous females corresponding to the y-axis on the left. Blue columns represent parity rate (No. of parous females / No. of parous and nulliparous females) corresponding to the y-axis on the right. A, B, and C represent locations at which midges were collected. a) year 2018-2019; b) year 2019-2020; c) year 2020-2021. Down arrows mark the days on which BTV was detected from the collection samples.



Supp. Table S2.1: Gonotrophic length and daily survivorship estimated for *C. sonorensis.*

Date	Site	Temperature	Parous	Nulliparous	Parity rate	Gonotrophic length	Survivorship
11/16/2018	A	15.6	94.7	82.3	0.5	9.5	0.9
11/27/2018	A	15.1	59.3	193	0.2	10.2	0.9
12/11/2018	A	13.4	13.3	20	0.4	12.9	0.9
12/27/2018	A	10.9	15.7	11.7	0.6	20	1
1/8/2019	A	14.1	28	18.3	0.6	11.8	1
1/25/2019	A	16	7	7.7	0.5	9	0.9
2/8/2019	A	8.9	11.7	19	0.4	30.1	1
2/19/2019	A	7.2	0	0.7	0	47.5	0
3/5/2019	A	13.3	2	1	0.7	13.1	1
3/19/2019	A	15.5	19	41	0.3	9.6	0.9
4/2/2019	A	16.4	41	16	0.7	8.6	1
4/19/2019	A	19.4	41.3	97	0.3	6.1	0.8
11/13/2019	A	17.7	50.3	83.8	0.4	7.3	0.9
	B	17.7	1.3	9.4	0.1	7.3	0.7
	C	17.7	23.5	24.3	0.5	7.3	0.9
11/26/2019	A	12.4	36.8	43.5	0.5	15.2	0.9
	B	12.4	2	2.1	0.5	15.2	1
	C	12.4	1.3	0.3	0.8	15.2	1
12/13/2019	A	12.4	8.3	16.8	0.3	15.2	0.9
	B	12.4	1.4	1.4	0.5	15.2	1
	C	12.4	2.3	3.8	0.4	15.2	0.9
12/29/2019	A	8.8	5	7	0.4	30.9	1
	B	8.8	0.3	0.5	0.3	30.9	1
	C	8.8	1.3	0	1	30.9	1
1/8/2020	A	10.6	5.8	26.8	0.2	21.4	0.9
	B	10.6	0.6	1	0.4	21.4	1
	C	10.6	4.8	7.3	0.4	21.4	1
1/21/2020	A	12.7	14	49.5	0.2	14.6	0.9
	B	12.7	0.9	1.4	0.4	14.6	0.9
	C	12.7	11.5	19.8	0.4	14.6	0.9
2/8/2020	A	12.6	19	72.8	0.2	14.9	0.9
	B	12.6	2.4	2.1	0.5	14.9	1
	C	12.6	71.3	279.8	0.2	14.9	0.9
2/18/2020	A	14.1	3	5.3	0.4	11.7	0.9
	B	14.1	0.9	1	0.5	11.7	0.9
	C	14.1	29.3	19.3	0.6	11.7	1
3/4/2020	A	14.9	23	23	0.5	10.4	0.9
	B	14.9	0.9	2.8	0.2	10.4	0.9

Date	Site	Temperature	Parous	Nulliparous	Parity rate	Gonotrophic length	Survivorship
	C	14.9	6.8	24.8	0.2	10.4	0.9
3/21/2020	A	13.4	8	9.5	0.5	12.9	0.9
	B	13.4	0.5	0.4	0.6	12.9	1
	C	13.4	5	2	0.7	12.9	1
4/2/2020	A	15.6	11.8	22.8	0.3	9.5	0.9
	C	15.6	1.5	0	1	9.5	1
4/16/2020	A	16.7	17	8.5	0.7	8.2	1
	C	16.7	0.8	0	1	8.2	1
4/28/2020	A	22.4	23.8	15	0.6	4.6	0.9
	C	22.4	11.3	3.8	0.8	4.6	0.9
11/13/2020	A	12.2	64.3	116	0.4	15.7	0.9
	C	12.2	12.8	28.5	0.3	15.7	0.9
11/25/2020	A	13.9	160.6	309.5	0.3	12	0.9
	C	13.9	24.3	74.9	0.2	12	0.9
12/10/2020	A	11.2	33.8	81.8	0.3	19	0.9
	C	11.2	17.6	18.1	0.5	19	1
12/22/2020	A	13.2	35.3	143.8	0.2	13.4	0.9
	C	13.2	29.9	78.4	0.3	13.4	0.9
1/5/2021	A	10.9	3	10.1	0.2	20	0.9
	C	10.9	0.6	1.1	0.4	20	0.9
1/20/2021	A	18	17.8	57.5	0.2	7.1	0.8
	C	18	7.1	54.3	0.1	7.1	0.7
2/2/2021	A	16.1	5.1	18.9	0.2	8.9	0.8
	C	16.1	3.9	8.3	0.3	8.9	0.9
2/18/2021	A	15.1	14.1	28.7	0.3	10.1	0.9
	C	15.1	3.8	23.5	0.1	10.1	0.8
3/2/2021	A	14.4	28.6	111.9	0.2	11.1	0.9
	C	14.4	14.5	49	0.2	11.1	0.9
3/18/2021	A	13.9	3.8	11.8	0.2	12	0.9
	C	13.9	7	10.5	0.4	12	0.9
4/13/2021	A	14.4	6.9	8.5	0.4	11.1	0.9
	C	14.4	4.8	7.1	0.4	11.1	0.9
4/27/2021	A	14.3	2.8	9.3	0.2	11.4	0.9
	C	14.3	0.6	9.8	0.1	11.4	0.8

Parous = number of parous midges collected per trap date; nulliparous = number of nulliparous midges collected per trap date.

CHAPTER 3

Morphological and Molecular Identification of *Culicoides* (Diptera: Ceratopogonidae) Species of the Southern California Desert

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Abstract

Culicoides Latreille biting midges are vectors of important animal pathogens including bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV). While some *Culicoides* species present in the southern California desert are implicated in the transmission of these viruses to ruminant animals, these species have not been extensively studied due in part to the challenge of identifying *Culicoides* to species and to the lack of published gene sequences for these species to support their molecular identification. In this study, *Culicoides* were captured using suction traps baited with either carbon dioxide or UV light from transitional habitat between the southern California peninsular mountain ranges and the Colorado desert of southeastern California. Captured midges were initially identified using traditional morphological methods, with species identification subsequently confirmed by sequence analysis of COI and 28S rDNA genes. Phylogenetic analyses support that some *Culicoides*

subgenera are not monophyletic. Two recognized species (*C. sitiens* Wirth and Hubert and *C. bakeri* Vargas) shared the same COI and 28S sequences. An additional cryptic species may be present within *C. sitiens*. Two additional recognized species (*C. cacticola* Wirth and Hubert and *C. torridus* Wirth and Hubert) may be conspecific or cryptic to each other. A total of 19 *Culicoides* species (or species aggregate) were collected in this study, with genetic sequences published for the first time for 16 of them. Published genetic sequences will support future research on these species, including studies on the ecology and habits of their immature stages which are often tedious to identify using morphology.

Abstract (Spanish)

Los mosquitos mordedores del género *Culicoides* Latreille son vectores de patógenos animales importantes que incluyen los virus de enfermedad hemorrágica. Mientras algunas especies *de Culicoides* presentes en el desierto del sur de California están implicadas en la transmisión de estos virus a animales rumiantes, estas especies no han sido ampliamente estudiadas debido en parte al desafío de identificar *Culicoides* a las especies y a la falta de secuencias genéticas publicadas para estas especies que podrían apoyar su identificación molecular. En este estudio, *los Culicoides* fueron capturados del hábitat de transición entre las cadenas montañosas peninsulares del sur de California y el desierto de Colorado en el sureste de California usando trampas de succión cebadas con dióxido de carbono o luz ultravioleta. Los mosquitos capturados se identificaron inicialmente utilizando métodos morfológicos tradicionales, con la identificación de especies confirmada posteriormente mediante el análisis de secuencia de los genes COI y 28S ADN Ribosómico. Los análisis filogenéticos apoyan que algunos subgéneros *de*

Culicoides no son monofiléticos. Dos especies reconocidas (*C. sitiens* Wirth y Hubert y *C. bakeri* Vargas), aunque morfológicamente distinguibles, compartían las mismas secuencias COI y 28S. Una especie críptica adicional puede estar presente dentro de *C. sitiens*. Dos especies adicionales reconocidas (*C. cacticola* Wirth y Hubert y *C. torridus* Wirth y Hubert) son morfológicamente inseparables y pueden ser conespecíficas o crípticas entre sí. Un total de 19 especies de *Culicoides* (o agregado de especies) fueron recolectadas en este estudio, con secuencias genéticas publicadas por primera vez a 16 de estas especies. Las secuencias genéticas publicadas apoyarán futuras investigaciones sobre estas especies, y pueden incluir estudios sobre la ecología y los hábitos de sus etapas inmaduras que a menudo son tediosos de identificar utilizando la morfología.

Keywords: *Culicoides*, California, Identification, COI, 28S

Introduction

Biting midges in the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) are small blood-feeding flies that are important in the transmission of impactful hemorrhagic viruses of animals including bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), and African horse sickness virus (AHSV) (Mellor et al. 2000). BTV and EHDV are endemic to the United States and cause hemorrhagic disease in cattle, sheep, deer, and other domestic and wild ruminants. In the United States, confirmed vectors of BTV are *Culicoides sonorensis* Wirth and Jones and *C. insignis* Lutz (Jones et al. 1977, Tanya et al. 1992). *Culicoides sonorensis* is considered the primary vector of BTV throughout much of the western United States where it is most abundant (Tabachnick 1996, Schmidtmann et al. 2011), while *C. insignis* is implicated in the transmission of BTV in Florida (Vigil et al. 2018). However, BTV and EHDV occur outside the range of these two *Culicoides* species suggesting that other *Culicoides* species are likely to also serve as vectors of BTV and/or EHDV in North America.

In the peninsular mountain ranges of California, native bighorn sheep (*Ovis canadensis* Shaw) are reported to be infected with BTV and EHDV (Clark et al. 1985, Jessup 1985), but the *Culicoides* species responsible for transmission of these viruses to bighorn sheep within their native habitat has not been confirmed. There are more than 80 *Culicoides* species reported from the southwestern U.S. with diversity reflecting the varied habitat from mountains to deserts (Phillips 2022). At least 19 *Culicoides* species were previously collected from a major drainage system (Deep Canyon) associated with the southern California bighorn sheep habitat (Mullens and Dada 1992a), including five species that were previously undescribed, three of which were

described subsequently (Wirth and Mullens 1992, Breidenbaugh and Mullens 1999a). The well-known BTV vector *C. sonorensis* is present at Deep Canyon and is known to readily feed on bighorn sheep at this location (Mullens and Dada 1992b). However, this species was captured most frequently and in greatest numbers near urban development at the periphery of the bighorn sheep habitat while other *Culicoides* species dominated in the mountainous terrain where bighorn sheep are most active (Mullens and Dada 1992a). At least one of these other species (*C. brookmani* Wirth) will also readily feed on bighorn sheep (Mullens and Dada 1992b). Thus while *C. sonorensis* may be a vector of BTV and EHDV to bighorn sheep in the peninsular mountain ranges of southern California, other *Culicoides* species deserve additional attention as potential vectors of these viruses. Nevertheless, *Culicoides* species in the southern California desert region other than *C. sonorensis* have been understudied due in part to, until very recently (Phillips 2022), the lack of a comprehensive morphological key to the *Culicoides* species in this region and to the difficulty in separating some species based solely on morphological features.

Adult *Culicoides* are often identified by species-specific wing pigmentation patterns. Where wing pigmentation pattern cannot separate species, individual midges may need to be slide mounted and/or dissected to observe microscopic characters of the antennae, maxillary palp, and genitalia (Harrup et al. 2015). These microscopic techniques are time-consuming and impractical with large numbers of individual midges, even when the species are dissimilar. To address this limitation, molecular methods can be used for rapid identification of *Culicoides* species (Nolan et al. 2007, Morag et al. 2012), to separate morphologically similar or cryptic species (Pagès and Monteys 2005, Pagès et al. 2009, Augot et al. 2010, Gopurenko et al. 2015, Shults et al. 2020, Shults et al. 2022), to link morphologically different males and females of the same species

(Blanton and Wirth 1979, Phillips 2022), and to detect new or undescribed species (Nielsen and Kristensen 2015). Molecular methods can also be helpful to identify immature *Culicoides* (Yanase et al. 2013, Bakhoum et al. 2018). Although immature morphology has been described for some *Culicoides* species (e.g., Kettle and Elson 1976; Hribar 1991; Breidenbaugh and Mullens 1999a, b), keys to identify immature midge species present in a geographical location are generally lacking, including in southern California. The ability to identify immature *Culicoides* to species is of critical importance for the study of *Culicoides* species in the southern California desert as the immature habitat for these species is not well characterized, and the identity of species therefore cannot be inferred from the site of immature collection.

Molecular identification of *Culicoides* species relies on variation in DNA sequence within gene regions including cytochrome c oxidase I (COI), cytochrome c oxidase II (COII), nuclear ribosomal DNA internal transcribed spacer 1 (ITS1) and 2 (ITS2), or 28S ribosomal DNA (28S rDNA) (Cêtre-Sossah et al. 2004, Gomulski et al. 2006, Perrin et al. 2006, Henni et al. 2014, Harrup et al. 2015). Intraspecific differences within a given gene region are fewer than the interspecific differences, so specimens belonging to a single species form a cluster on a barcode tree (Hebert et al. 2003, Velzen et al. 2012). Multiple phylogenetic clusters within a morphologically similar group of insects can indicate genetic variation among separated populations of the same species (Gomulski et al. 2006) or the existence of cryptic species (Gomulski et al. 2006, Slama et al. 2014).

Further study of southern California desert *Culicoides* as potential vectors of BTV or EHDV requires improvements to species identification methods, including the development of regional

identification keys and availability of species-specific gene sequence data for molecular identification. Modern methods allow for concurrent species identification using both morphological and molecular methods by extracting DNA using non-destructive methods allowing for the insect to remain intact (Bellis et al. 2013, Martin et al. 2019). In the current study, adult *Culicoides* were collected in southern California from the Santa Rosa Mountains near the earlier study of Mullens and Dada (1992) with individual midges concurrently identified using morphology and gene sequence analysis. Barcode trees were built from variation in gene sequences to evaluate the accuracy of morphological species identifications. Gene sequences for each identified species were submitted to GenBank to support future studies on *Culicoides* of the southwestern United States.

Methods

Study Site

The study was conducted at the Phillip L. Boyd Deep Canyon Desert Research Center (Deep Canyon), one of the largest reserves of the University of California Natural Reserve System. The Deep Canyon research area contains a drainage system (Deep Canyon creek) that runs generally south to north, extending from the Santa Rosa Mountains (2,657 m peak elevation) to the floor of the Colorado Desert (< 290 m elevation). The canyon is narrow throughout the mountains but opens to form a broad alluvial flood plain of sandy washes at its northern edge until reaching a golf course and accompanying residential community in Palm Desert, CA. In its entirety, Deep Canyon is about 25 km^2 though the area sampled for this study extended from just inside the canyon mouth to the end of the alluvial flood plain (approx. 4 km^2) (Figure 1).

Culicoides Collection

Culicoides were collected during six trap nights from May 2018 through July 2020. On the first trap night, trapping was conducted at five locations along Deep Canyon creek from within the narrow canyon mouth to the broad alluvial plain (sites A-E, Figure 1). *Culicoides* were collected using Centers for Disease Control (CDC) suction traps baited with either CO_2 (approx. 1.5 kg of dry ice) or UV light. At each location, one CO_2 and one UV trap were set up and separated by at least 30 m to minimize trap interference (Kirkeby et al. 2013, McDermott et al. 2016). On subsequent trap nights, trapping was conducted at two locations, the canyon mouth (site B) and the lower alluvial plain (site F or G), to maximize the diversity of species captured. At each trapping location, traps were deployed in three positions separated by at least 50 m with a pair of traps, one CO_2 and one UV, placed at each position and separated by approx. 30 m. Thus, a total of six traps (three CO_2 and three UV) were placed at each trapping location during each trap night. Trapping was conducted from before sunset until after sunrise the next day when catch bags were removed from traps, placed on dry ice, and transported back to the laboratory where they were stored at -20 °C for later analysis.

Culicoides Species Identification and Sequence Analysis

Culicoides were identified to species using morphology and molecular analyses as described in the sample-processing flowchart (Figure 2). *Culicoides* species were sorted from other insects and then separated into groups containing midges of each sex with similar morphology, especially similar wing patterning. The number of midges selected from each group for morphological and molecular identification was determined by the number of individual midges

in the group, the distribution of sexes, and the variation in collection dates for individual midges in each group (Supp. Table S3.1). Within each group, midges collected from different seasonal periods were selected for identification to look for cryptic species that might vary in their seasonal activity. Both females and males were examined when both sexes were captured.

Due to the usefulness of wing patterning in the morphological identification of *Culicoides*, the wings from each midge were removed prior to DNA extraction and retained in a separate labeled tube to prevent damage to wings that might occur during the DNA extraction process. DNA was subsequently extracted from the remainder of the midge body using a modified HotSHOT non-destructive DNA extraction method (Truett et al. 2000). Briefly, a single midge was added to 40 µl alkaline lysis reagent (100 mmol NaOH: 0.267 mmol Na_2EDTA = 1:3) and incubated at 95 °C for 30 minutes. After cooling to room temperature, an equal volume of neutralizing reagent (40 mmol Tris-HCl) was added to extract the midge DNA which was transferred to a new tube and stored at -20 °C for later DNA amplification by polymerase chain reaction (PCR). After DNA extraction, the midge body and associated wings were mounted on the same glass slide using Hoyer's or Euparal slide mounting medium.

Table 1: Primers used for COI and 28S rDNA amplification.

COI (Dallas et al. 2003)	C1-J-1718	5'-GGAGGATTTGGAAATTGATTAGT-3'
	C1-N-2191	5'-CAGGTAAAATTAAAATATAAACTTCTGG-3'
28S rDNA (Depaquit et al. 1998)	28S_C'1	5'-ACCCGCTGAATTTAAGCAT-3'
	28S_D2	5'-TCCGTGTTTCAAGACGGG-3'

Slide mounted specimens were morphologically identified using keys to the *Culicoides* of the southwestern United States (Phillips 2022). Morphological identifications were made

independently (independent ID) by XZ and RP and recorded as “morphologically identified” if both XZ and RP agreed on their identification. If they failed to agree, or at least one lacked confidence in their initial species identification, they exchanged identification notes and again attempted to identify the specimen to species (informed ID). If they agreed on the species during informed identification, the specimen was then recorded as morphologically identified. If consensus on species identification for any specimen was not reached or if either XZ or RP lacked confidence in the identification, the specimen was assigned a “tentative ID” as to the most likely species based on the morphology and professional experience. A tentative ID was often due to an important morphological feature being obscured on the prepared slide.

A subset of the morphologically identified *Culicoides* was molecularly identified using the COI gene and/or the 28S rDNA gene (Table 1). The 28S rDNA gene was used for species where COI DNA failed to amplify (*C. byersi* Atchley, *C. californiensis* Wirth and Blanton), for morphologically identified species that could not be separated by COI sequence (*C. cacticola* Wirth and Hubert / *C. torridus* Wirth and Hubert, *C. bakeri* Vargas / *C. sitiens* Wirth and Hubert), and for species that had great morphological variability (*C. ryckmani* Wirth and Hubert). The number of specimens from each morphologically identified species selected for molecular identification was determined by the number of individual midges of each sex captured and the morphological variation within the identified specimens. The PCR conditions for COI amplification were 2 min at 95 °C, then 35 cycles of 95 °C for 30 s, 50.9 °C for 30 s, and 72 °C for 45 s, followed by 5 min at 72 °C. The PCR conditions for 28S rDNA amplification were 3 min at 94 °C, then 35 cycles of 94 °C for 30 s, 58 °C for 90 s, and 68 °C for 60 s, followed by 10 min at 68 °C (Henni et al. 2014). Amplified PCR products were purified using the Illustra ExoProStar (GE Healthcare Life Sciences)

and sent to Genewiz (San Diego, California) for Sanger sequencing. Forward and reverse sequences for each specimen were trimmed and merged using BioEdit (Hall 1999), and the merged sequence was searched against the nucleotide collection (nt) database within GenBank using blastn (Altschul et al. 1990). Sequences were aligned using the MUSCLE program with default settings (Edgar 2004) built-in MEGAX software (Kumar et al. 2018), and ends were trimmed to obtain sequences of sufficient quality for processing. Genetic distance between and within each species was calculated using the Kimura 2-parameter model with default settings by MEGAX software. Maximum likelihood trees were constructed for both COI and 28S rDNA based on the sequence alignment with 1000 bootstrap replication. *Anopheles gambiae* (Diptera: Culicidae) (accession No. DQ792578) and *Forcipomyia* sp. (Diptera: Ceratopogonidae) (accession No. KF286392) served as the outgroup for the COI tree and the 28S rDNA tree, respectively. The best substitution model for the COI tree identified by the MEGAX software based on Bayesian Information Criterion (BIC) was a general time reversible (GTR+G+I) model (Tavaré 1986), and the best model for the 28S rDNA tree was a Tamura 3-parameter (T92 + G) model (Tamura 1992).

We consider our species concepts based on morphological identification corroborated when these specimens formed one clade on the phylogenetic tree. Where morphospecies form non-monophyletic groups (paraphyletic or polyphyletic) in either the COI or 28S rDNA trees, we interpret this as an indication of an undescribed species, a cryptic species, or as misidentification. Some specimens assigned only a tentative ID based on morphology were also identified using molecular methods to determine the accuracy of the tentative identification. To further examine variation within a species, haplotypes for COI and 28S were calculated by DNA

sequence polymorphism analysis using DnaSP6 (Rozas et al. 2017). Slide-mounted *Culicoides* were deposited into the Entomology Research Museum collection at the University of California, Riverside (UCRC_ENT00549860 - 00550182) and amplified COI and 28S rDNA gene sequences were uploaded into GenBank (accession No. OM886391 - OM897428, OM910743 - OM910801; for details, please see Supp. Table S3.1).

Results

A total of 316 *Culicoides* specimens were slide mounted and morphologically examined (Table 2). Of these, 288 (91.1%) were identified to the same species by both XZ and RP, and 28 (8.9%) were only tentatively identified to species because either XZ or RP or both lacked confidence in the identification. Individual midges that received a tentative identification are indicated in Table 2 with a superscript following the species name.

There were 18 distinct species of *Culicoides* identified by morphology: *C. bakeri, C. boydi* Wirth and Mullens, *C. brookman*i, *C. byersi*, *C. californiensis*, *C. copiosus* Root and Hoffman, *C. crepuscularis* Malloch, *C. defoliarti* Atchley and Wirth, *C. freeborni* Wirth and Blanton, *C. jacksoni* Atchley, *C. kettlei* Breidenbaugh and Mullens, *C. lahontan* Wirth and Blanton, *C. luglani* Jones and Wirth, *C. mohave* Wirth, *C. reevesi* Wirth, *C. ryckmani*, *C. sitiens*, and *C. sonorensis*. Some specimens could only be identified by morphology as either of two closely related species, *C. cacticola* or *C. torridus*, due to the similarity of published morphological characters for these species. Thus, these specimens were recorded as *C. cacticola* / *C. torridus* aggregate.

A total of 160 COI and 41 28S sequences were obtained, representing all morphologically identified species captured in this study. All COI and 28S rDNA sequences were combinations of forward and reverse sequences except for the 28S sequence of one *C. sitiens* (No. 296) for which only the forward sequence was obtained. A same-species match in GenBank using a BLAST search was obtained only for COI sequences from species morphologically identified as *C. crepuscularis* (73-81% query coverage, 99-100% identity), *C. reevesi* (95% query coverage, 89% identity), and *C. sonorensis* (95-100% query coverage, 95-100% identity). Other species did not have a same-species match for either COI or 28S genes. For sequencing analysis, COI sequences were trimmed to 409 bp and 28S rDNA sequences were trimmed to 632 bp (gaps included). DNA sequence polymorphisms were identified for several species, with as many as 13 COI haplotypes and 3 28S haplotypes for a single species. Sequence polymorphisms were helpful to confirm species assignment for specimens that were only tentatively identified by morphology. Haplotypes obtained for specimens tentatively identified as *C. brookmani* matched those obtained for morphologically identified *C. brookmani*. Haplotypes obtained for specimens tentatively identified as *C. ryckmani* matched those obtained for morphologically identified *C. ryckmani*, even though some of the tentatively identified specimens had morphological features that appeared similar to *C. copiosus* (Figure 3). The COI and 28S haplotypes obtained for specimens tentatively identified as *C. cacticola* / *C. torridus* matched those obtained for morphologically identified *C. cacticola* / *C. torridus*, even though some of the tentatively identified specimens had morphological features that appeared similar to either *C. ryckmani* or *C. copiosus* (Figure 4). The COI and 28S haplotype for *C. bakeri* and tentatively identified *C. sitiens* matched one of the two haplotypes of *C. sitiens* for each gene sequence.

Genetic distances for COI within a species ranged from 0 to 0.053 (Table 2) while genetic distances between morphologically distinguishable species was > 0.1 except for *C. bakeri* and *C. sitiens* (0.018) (Supp. Table S3.2). Specimens tentatively identified to species had a genetic distance < 0.05 to morphologically identified individuals of the same species (Supp. Table S3.2, bold font) further confirming identification of these tentatively identified specimens. Genetic distances for the more conserved 28S rDNA region were relatively lower than genetic distances for COI both within and between species (Supp. Table S3.3). Within species genetic distances were < 0.006 while genetic distances between species were > 0.009 except for *C. bakeri* and *C. sitiens* (0.003).

Morphologically identified species that were well supported as monophyletic groups in the maximum-likelihood tree based on COI sequences (Figure 5) were *C. boydi* (100% bootstrap support [BS]), *C. defoliarti* (100% BS), *C. freeborni* (100% BS), *C. kettlei* (100% BS), *C. lahontan* (100% BS), *C. luglani* (100% BS), *C. mohave* (100% BS), *C. reevesi* (100% BS), and *C. sonorensis* (100% BS). Due to the collection of only one or two specimens during the study, *C. copiosus, C. crepuscularis*, and *C. jacksoni* were each represented by only one sequence, though each is in an isolated position suggesting separate lineages. *Culicoides brookmani*, and *C. brookmani*[1] formed a single clade with 100% BS. *Culicoides ryckmani*, *C. ryckmani*[1], and *C. ryckmani*[4] also formed a single clade with 100% BS. *Culicoides cacticola* / *C. torridus*, *C. cacticola*/ *C. torridus*[1], and *C. cacticola*/ *C. torridus*[2] formed one clade with 84% support that was further divided into two groups each with 100% support. *Culicoides sitiens, C. sitiens*[5], and *C. bakeri* formed a single clade with 97% support that was further divided into a group with 100% support and a separate

branch with the single *C. sitiens* (No. 296) for which only the forward sequence was obtained for the 28S gene.

The maximum-likelihood tree based on 28S rDNA (Figure 6) showed a well-supported clade for *C. californiensis* (99% BS). *Culicoides ryckmani* formed a clade with 77% BS, and the single sequence for *C. byersi* formed a separate branch on the tree. The same individual *C. cacticola* / *C. torridus* that formed each of the two clades noted in the COI tree also formed two clades on the 28S tree (90% and 68% BS). All *C. sitiens* and *C. bakeri* formed a clade with 100% BS, except for the single *C. sitiens* (No. 296) which had also formed a separate branch in the COI tree.

Discussion

This study resulted in six new species records for the Deep Canyon geographic area, including *C. bakeri*, *C. byersi*, *C. californiensis*, *C. crepuscularis*, *C. luglani*, and *C. reevesi*. Three species, *C. cockerellii*, *C. insolatus,* and *C. vetustus*, previously collected at this site (Mullens and Dada 1992a, Breidenbaugh and Mullens 1999a) were not collected in the current study. The species that were most abundant in the current study (*C. boydi, C. brookmani, C. cacticola / C. torridus, C. lahontan, C. mohave,* and *C. ryckmani*) were similarly most abundant in the previous study (Mullens and Dada 1992a). Except for *C. bakeri*, these *Culicoides* species were known to have distributions in California (Phillips 2022). The single morphologically identified *C. bakeri* collected in Deep Canyon is a new U.S. record for this species (Phillips 2022).

Morphological Identification

Morphological identification was straightforward for species with distinct wing pigmentation patterns. Also, some species have unique morphological features that assisted in their identification, such as the 9th and 10th flagellomeres of *C. reevesi* being much smaller than other flagellomeres (Grogan Jr et al. 2004) (Figure 5). For other species, identification required close examination of slide-mounted specimens under a compound microscope to view smaller characteristics such as spermathecae and sensilla coeloconica (SCo) patterns. Difficulties with morphological identification mostly occurred within the *Culicoides* subgenera *Selfia* Khalaf and *Drymodesmyia* Vargas.

Species within the subgenus *Selfia* lack the dark and pale wing patterning typical for most *Culicoides* species. Though *Selfia* males are readily identified by their genitalia, female identification relies mostly on minute morphological characteristics such as the presence of apical spines on the hind tarsomeres, SCo pattern, and the number of scutellar setae (Atchley 1970, Phillips 2022). Of the 51 *C.* (*Selfia*) examined, all but one was identified or tentatively identified as *C. brookmani*, with a single male *C.* (*Selfia*) identified as *C. jacksoni* (NO. 269).

Species within subgenus *Drymodesmyia* have similar morphological features and these features can be variable within a species making it difficult to separate some species in this subgenus. For example, *C. ryckmani* and *C. copiosus* share a very similar wing pigmentation pattern, with species identification dependent on the shape of a pale spot in the distal portion of the r_3 cell (indicated by arrows in Figure 5) which can be variable among individuals of the same species (e.g., see Figure 3 for *C. ryckmani*) (Phillips 2022). Identification of these species therefore

required microscopic examination of other characteristics such as spermatheca shape and size. Similarly, separation of *C. sitiens* and *C. bakeri* also required microscopic examination of their spermathecae. However, if spermathecae shape is altered during slide preparation for microscopic examination, it can be difficult to confidently identify the specimen to species.

Sequence Analysis

The COI primer used failed to amplify the COI gene for *C. byersi* and *C. californiensis* although this primer worked well for all other species collected at Deep Canyon in this study and for *Culicoides* in several European studies (Dallas et al. 2003, Nolan et al. 2007, Ander et al. 2013). The lack of gene amplification for two of the species in the current study indicates that the primers used by Dallas et al. (2003) are not universal for *Culicoides*. Other common COI primers such as LCO1490 and HC02198 (Folmer et al. 1994) should be tested for molecular identification of *Culicoides* in the peninsular mountains and desert region of southern California. Alternatively, new COI primers could be designed for species present in the southwestern United States. The 28S primers used in this study successfully amplified the 28S gene of both *C. byersi* and *C. californiensis* as well as other species within the subgenus *Drymodesmyia* allowing for phylogenetic comparison of these species. Across species, the number of COI and 28S haplotypes obtained generally increased with greater numbers of individual midges examined. Thus, the number of haplotypes identified in the present study may not represent the true rate of DNA polymorphisms for species present in the Deep Canyon research area.

Culicoides cacticola and *C. torridus* were each described as distinct species by Wirth and Hubert (1960) based on whether the two distal pale spots in the r_3 cell of the wing are conjoined (*C.*

cacticola) or separated (*C. torridus*) in females (Figure 4) or on the aedeagal ratio (the ratio of the height of the basal arch of the aedeagus to the overall length of the aedeagus) and the paramere diameter in males. However, individual midges captured in the current study showed great variation in these features, with the two pale spots in the r_3 cell even appearing in some specimens as a single pale spot (e.g., NO. 97 and NO. 171 in Figure 4) similar to the r_3 pale spot for *C. ryckmani* or *C. copiosus*.

Both the COI and 28S sequence analyses show that morphologically identified *C. cacticola* / *C. torridus* grouped on the tree into two clades (e.g., both supported with 100% BS and sister to each other on the COI tree). Genetic distance for COI within the *C. cacticola* / *C. torridus* aggregate (0.053) was smaller than the between-species genetic distance recorded for morphologically distinct species (> 0.1) but greater than within-species genetic distance for other midge species (0-0.036). The lack of clear morphological distinctions between *C. cacticola* and *C. torridus* suggests these species may be conspecific or very closely related cryptic species. The two well-supported and divergent lineages comprising this species aggregate with each clade containing the same individual specimens on both the COI tree and the 28S rDNA tree suggests the two clades represent cryptic species. This is further supported by the genetic distances between these two clades (0.161 for COI and 0.008 for 28S rDNA) which is similar to the between-species genetic distances in this study. While it is tempting to infer that the two clades are *C. cacticola* and *C. torridus* and simply assign each species to one of the separate clades, there is no morphological support for assigning a particular species to a either clade. Furthermore, we cannot rule out that *C. cacticola* and *C. torridus* as originally described by Wirth and Hubert are conspecific, with *C. torridus* being a junior synonym of *C. cacticola,* based on

morphological similarity and variability of the distinguishing features described for each species. If *C. cacticola* and *C. torridus* are indeed conspecific, the two clades on the phylogenetic trees in this study would be *C. cacticola* and a separate unnamed cryptic species. DNA sequences can reveal genetic differences among species that are morphologically indistinguishable, but confirmation of cryptic species requires further analysis of the morphology and ecology of putative species (Bickford et al. 2007). Additional information on activity patterns, geographical distribution, host-feeding preference, and mating habits of these groups is needed to confirm the existence of a cryptic species.

Though morphologically similar, *C. bakeri* and *C. sitiens* do have a few distinct morphological features. Females of *C. sitiens* have pyriform to elongate saclike spermathecae with broad openings rather than sclerotized necks while *C. bakeri* have subspherical to slightly pyriform spermathecae with narrow sclerotized necks (Figure 5). Additionally, males of *C. bakeri* have spines on the distal portion of the paramere while *C. sitiens* lack these spines (Huerta 2007, Phillips 2022). Only one female (No. 83) was identified morphologically as *C. bakeri* by both XZ and RP in the present study. The single *C. bakeri* was recovered within a clade on both the COI and 28S trees that otherwise comprised only *C. sitiens* suggesting these species may be conspecific but have intraspecific polymorphism in the features of the spermathecae or paramere. That these species may be conspecific is further supported by sharing of haplotypes between them for both the COI and 28S gene sequences. However, given the clearly defined morphological features of these two species, it is possible that *C. bakeri* and *C. sitiens* are very closely related species and that COI and 28S sequences were insufficient to separate them and that other genes should be examined. A single *C. sitiens* (No. 296) that formed a single branch in

both the COI and 28S trees impeded confirmation of species identification. The genetic distances between this single *C. sitiens* and other *C. sitiens* were 0.142 and 0.015 for COI and 28S rDNA, respectively, suggesting the single *C. sitiens* might be a cryptic species. Additional specimens, especially males, would be useful to further investigate this clade.

All *C. ryckmani* were grouped by COI sequence into one clade. Morphologically, *C. ryckmani* and *C. copiosus* were highly similar and variable wing patterning was challenging for identification (Figure 3). However, COI sequences showed that the only confirmed *C. copiosus* formed a single branch different from all *C. ryckmani,* providing confidence in the grouping of *C. ryckmani*.

Morphological identification of subgenus *Selfia* females was time-consuming because females needed to be slide-mounted for examination of small and often indistinct morphological features. Furthermore, some females of other *Selfia* species not collected in this study have no reliable morphological distinctions (Atchley 1970). Identification using DNA sequences proved much easier for *C. brookmani* and *C. jacksoni* collected in this study, and such techniques are likely to greatly aid the identification of other *Selfia* species. COI sequences showed that subgenus *Selfia* is not monophyletic with morphologically identified *C. brookmani* in one clade while the single *C. jacksoni* formed a separate distant branch near *C. boydi* (subgenus Avaritia). This genetic separation within the *Selfia* subgenus was anticipated based on morphological differences reported by Atchley (1970):

“Of the seven species of *Selfia*, the placement of *brookmani* into a phylogenetic scheme is not without some difficulty. This species possesses a number of structures not found in any of the

other species of this subgenus, including the very peculiar pupal respiratory horn, aedeagal complex and the apparent use of spermatophores to transfer sperm."

Atchley did not know of *C. moabensis* Phillips, which is morphologically similar to *C. brookmani* (Phillips 2015) suggesting that these two species may form a group distinct from the other five recognized western North American *Selfia* species—*C. hieroglyphicus* Malloch, *C. denningi* Foote and Pratt, *C. jacksoni*, *C. jamesi* Fox, and *C. tenuistylus* Wirth—and possibly also from the eastern North American *C.* (*Selfia*) *multipunctatus* Malloch.

The maximum-likelihood trees also show that subgenus *Drymodesmyia* is not monophyletic. COI sequence results indicate that *C. ryckmani* and *C. copiosus* are more closely related to species in other subgenera than to other species within the *Drymodesmyia*. Also, 28S sequences suggest that *C. ryckmani* and *C. byersi* are more closely related to *C.* (*Amossovia*) *californiensis* than to other *Drymodesmyia* examined.

Borkent and Dominiak (2020) stated that the phylogeny of *Culicoides* species is not well understood, and that it is highly likely that some subgenera are not monophyletic. This is supported by the phylogenetic analyses in the current study, however this study only used COI and 28S rDNA sequences for sequence analysis and may not allow for confidently reconstructing relationships between species. More work is needed to resolve phylogenetic relationships among subgenera and species of *Culicoides*. Analyzing longer sequences, using multiple genes, and combining additional morphological and molecular data will likely be critical in resolving relationships within the genus *Culicoides*. COI has been used for molecular identification of

Culicoides species in many studies (Nolan et al. 2007, Ander et al. 2013, Hakima et al. 2020), and 28S rDNA has also been used in some studies but always in company with COI gene sequences (Augot et al. 2013, Henni et al. 2014, Slama et al. 2014, Hadj-Henni et al. 2021). In this study, amplification of the COI gene was useful for molecular identification of most species while amplification of the 28S gene was useful as a supplement to identify and separate the remaining species.

The present study added COI and/or 28S gene sequences to the GenBank database for 16 North American *Culicoides* species. Many previous DNA barcode studies did not keep voucher specimens preventing future study of these specimens (Borkent and Dominiak 2020). In the current study, slide-mounted midges including specimens that were identified using gene sequences were deposited as voucher specimens in the University of California at Riverside's Entomological Research Museum providing future researchers access to both the voucher insects and their DNA sequence information.

Accurate species identification is the first step needed for future studies of *Culicoides* in the deserts of southern California. Combining morphological and molecular methods makes identification of desert *Culicoides* species easier and will facilitate future *Culicoides* studies by researchers who are not taxonomists. Publicly available gene sequences will also allow identification of other life stages of *Culicoides* species thereby supporting studies of immature ecology. Phylogenetic relationships of these desert *Culicoides* species might suggest which species should receive further attention as potential vectors of animal viruses to nearby protected bighorn sheep or to other wild or domestic ruminant animals.

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Table 3.2: Summary of specimen examination.

Species	Morphological ID		COI obtained*		COI haplotypes	Genetic distance	28S obtained		28S haplotypes	Genetic distance
	F	M	F	M			F	M		
C. bakeri	1	0	1	-	1	-	1	-	1	-
C. boydi	10	0	5	-	2	0.001	-	-	-	-
C. brookmani	35	14	19	1	4	0.004	-	-	-	-
C. brookmani[1]	0	1	-	1	1	-	-	-	-	-
C. byersi	0	1	-	0	-	-	-	1	1	-
C. cacticola / C. torridus	36	25	21	8	7	0.053	10	7	3	0.003
C. cacticola / C. torridus[1]	2	1	2	0	2	0.005	-	-	-	-
C. cacticola / C. torridus[2]	0	2	-	2	2	0.002	-	2	1	0
C. californiensis	3	2	0	0	-	-	3	2	2	0.001
C. copiosus	2	0	1	-	1	-	-	-	-	-
C. crepuscularis	1	0	1	-	1	-	-	-	-	-
C. defoliarti	9	0	5	-	1	0	-	-	-	-
C. defoliarti[3]	0	2	-	2	2	0.002	-	-	-	-
C. freeborni	3	1	3	1	3	0.01	-	-	-	-
C. jacksoni	0	1	-	1	1	-	-	-	-	-
C. kettlei	2	0	2	-	2	0.002	-	-	-	-
C. lahontan	10	1	9	1	2	0.001	-	-	-	-
C. luglani	4	0	4	-	2	0.007	-	-	-	-
C. mohave	32	13	18	7	13	0.01	-	-	-	-
C. reevesi	8	0	6	-	1	0	-	-	-	-
C. ryckmani	23	24	9	5	3	0.027	2	4	3	0.003
C. ryckmani[1]	3	9	2	1	1	0	-	-	-	-
C. ryckmani[4]	6	1	3	-	1	0	-	-	-	-
C. sitiens	9	8	6	2	2	0.036	6	-	2	0.005
C. sitiens[5]	3	0	3	-	1	0	3	-	1	0
C. sonorensis	6	2	6	2	7	0.027	-	-	-	-

[1] Tentative ID.

[2] Tentative ID. Morphologically similar to *C. ryckmani* or *C. copiosus*.

[3] Tentative ID. Male *C. defoliarti* cannot be separated from male *C. haematopotus*. Because only *C. defoliarti* females were collected, we assume these males to be *C. defoliarti*.

[4] Tentative ID. Morphologically similar to *C. copiosus*.

[5] Tentative ID. Morphologically similar to *C. bakeri*.

*Minus sign (-) means no sample was sequenced or no genetic distance was calculated, 0 means sequencing was unsuccessful.

Figure 3.1: Google Earth image showing *Culicoides* collection sites along Deep Canyon creek.

Figure 3.2: Flowchart of morphological and molecular species identification. The process starts with *Culicoides* specimens being grouped based on similar morphology and appearance.

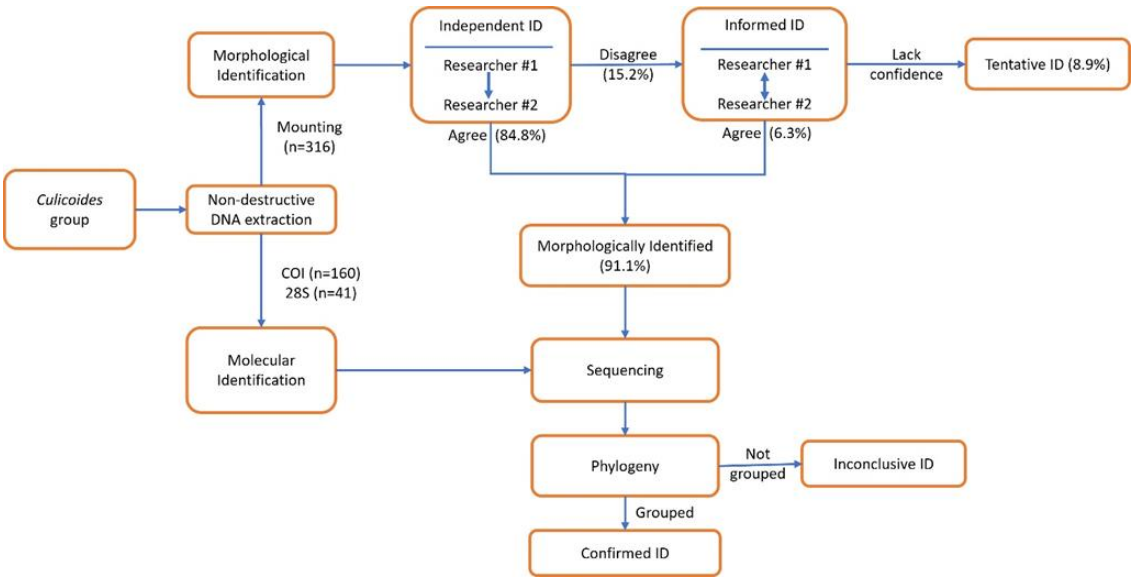


Figure 3.3: Wing images showing color pattern variation of some *C. ryckmani* specimens. The black arrow is pointing to the pale spot in r_3 cell. Specimens No. 99 and 110 are males, and other specimens are females. Specimens No. 43, 53, and 78 are more clearly identified as *C. ryckmani* due to having a clear elongated pale spot in r_3, while specimen No. 82 has a much smaller pale spot in r_3 which might be confused with a more ovoid pale spot. Specimens No. 99, 110, and 222 have the pale spot taking up much of the distal part of r_3 compared to other specimens. Most images were adjusted to increase the color contrast for better observation of the wing patterns.

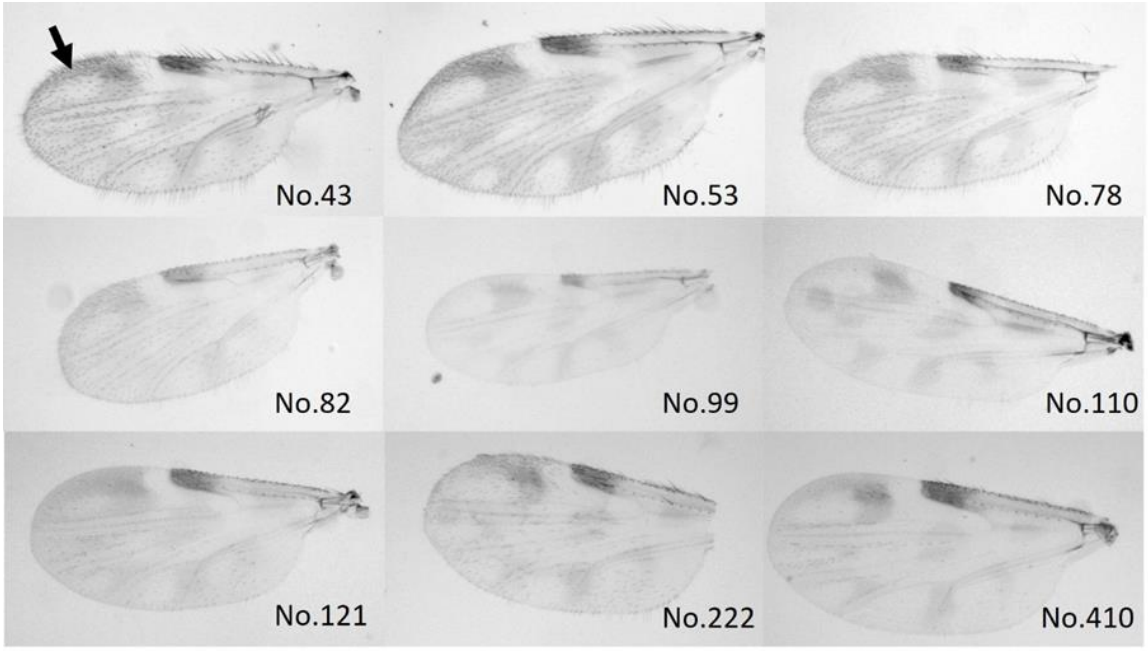


Figure 3.4: Wing images showing color pattern variation of some *C. cacticola* / *C. torridus* specimens. The black arrow is pointing to the pale spots in r_3 cell. Specimens 97, 147, and 171 are males, and other specimens are females. Specimens No. 50 and 85 have two clearly separated pale spots in r_3 as described for *C. torridus*, while specimens No. 147 and 306 has conjoined pale spots in r_3 as described for *C. cacticola*. Specimens No. 89 and 97 have an upper pale spot in r_3 that is less visible. The conjoined pale spots in specimen No. 171 look like a large single pale spot. For specimen 92 (showing both wings), r_3 pale spots in wing image 1 are separated while in image 2 are conjoined.

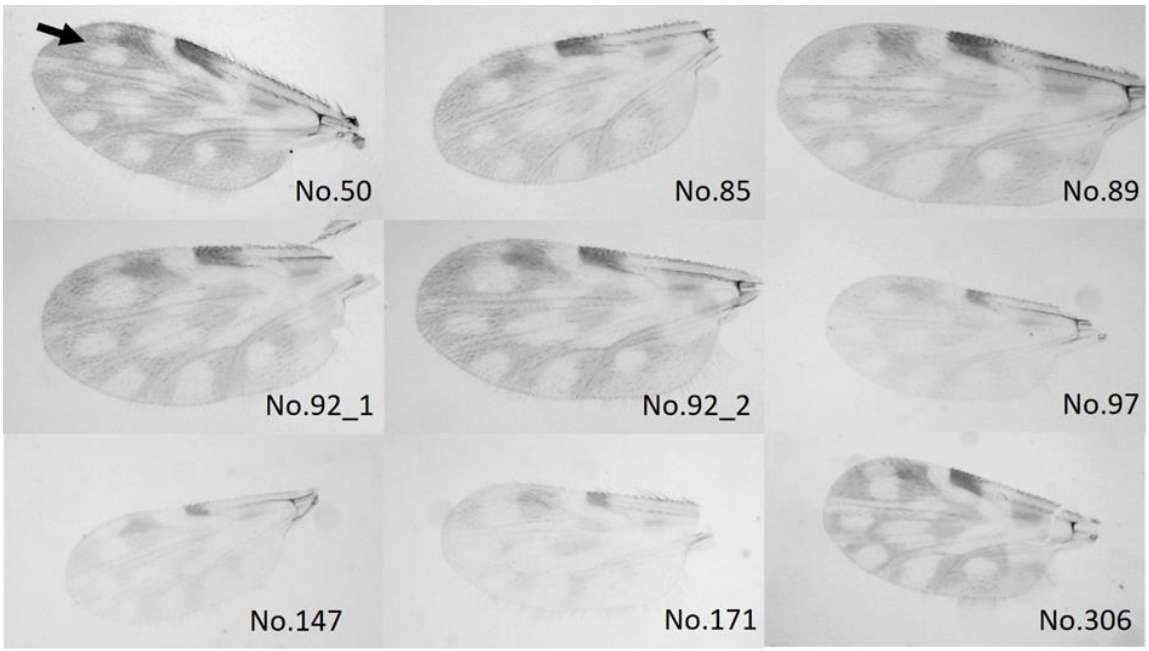
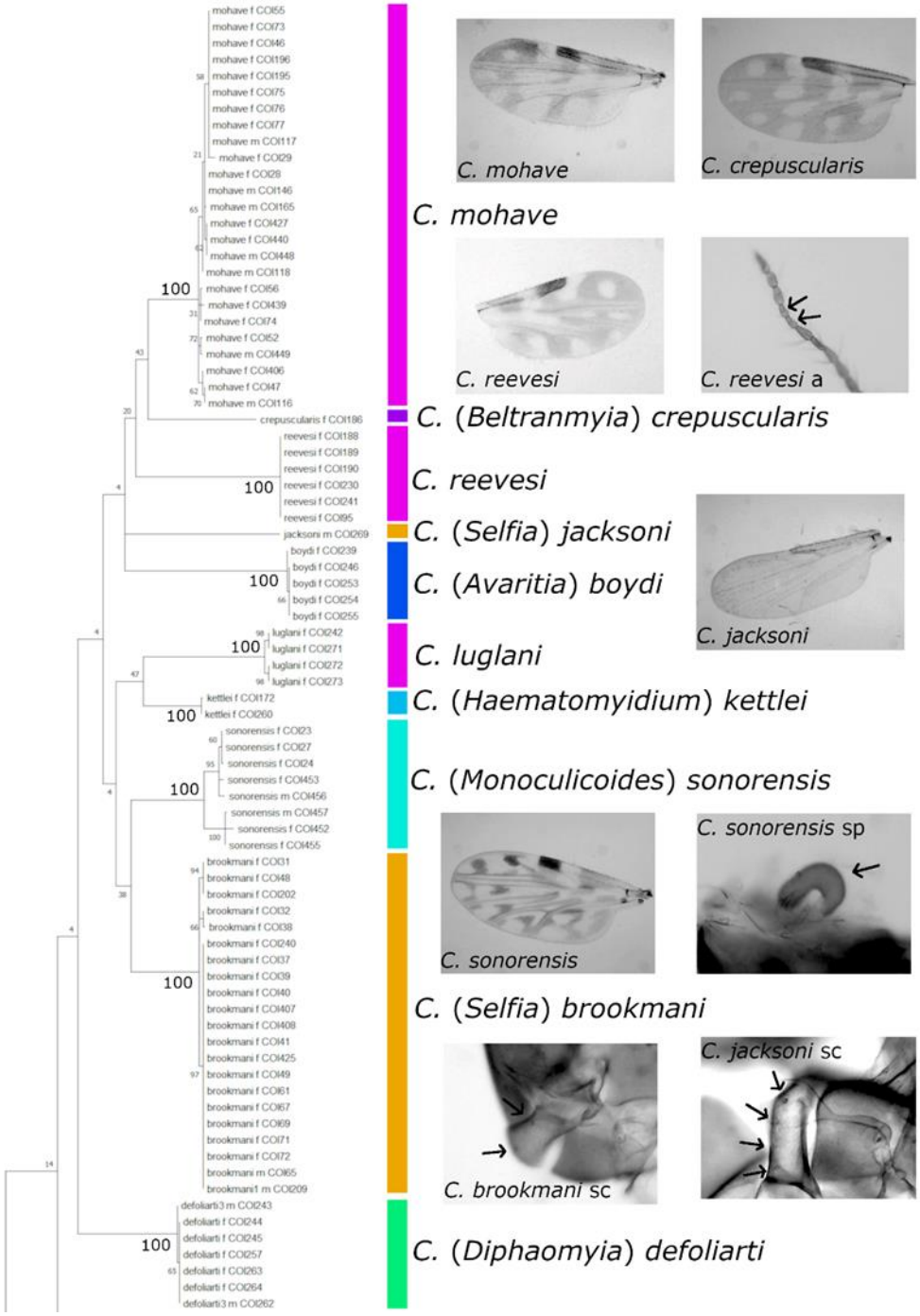


Figure 3.5: Maximum likelihood tree built based on COI sequences using the general time reversible (GTR+G+I) model. *Anopheles gambiae* (accession No. DQ792578) served as the outgroup. Each specimen identification code includes a numeric portion to identify each specimen with COI as the prefix indicating the sequenced gene. Numbers following the species name refer to the species name and associated footnote in Table 3.2. Color bars to the right of the tree indicate the subgenus (in parentheses) of each species or whether a species is unplaced (lacks a subgenus designation; pink bars). Arrows in an image point to the morphological character described in the text as important for identification. Abbreviation: *C. cac*/*tor* = *C. cacticola* / *torridus*, a = antennae, sp = spermathecae, sc = scutellar setae


mohave f COI55
mohave f COI73
mohave f COI46
mohave f COI196
mohave f COI195
mohave f COI75
mohave f COI76
mohave f COI77
mohave m COI117
mohave f COI29
mohave f COI28
mohave m COI146
mohave m COI165
mohave f COI427
mohave f COI440
mohave m COI448
mohave m COI118
mohave f COI56
mohave f COI439
mohave f COI74
mohave f COI52
mohave m COI449
mohave f COI406
mohave f COI47
mohave m COI116
crepuscularis f COI186
reevesi f COI188
reevesi f COI189
reevesi f COI190
reevesi f COI230
reevesi f COI241
reevesi f COI95
jacksoni m COI269
boydi f COI239
boydi f COI246
boydi f COI253
boydi f COI254
boydi f COI255
luglani f COI242
luglani f COI271
luglani f COI272
luglani f COI273
kettlei f COI172
kettlei f COI260
sonorensis f COI23
sonorensis f COI27
sonorensis f COI24
sonorensis f COI453
sonorensis m COI456
sonorensis m COI457
sonorensis f COI452
sonorensis f COI455
brookmani f COI31
brookmani f COI48
brookmani f COI202
brookmani f COI32
brookmani f COI38
brookmani f COI240
brookmani f COI37
brookmani f COI39
brookmani f COI40
brookmani f COI407
brookmani f COI408
brookmani f COI41
brookmani f COI425
brookmani f COI49
brookmani f COI61
brookmani f COI67
brookmani f COI69
brookmani f COI71
brookmani f COI72
brookmani m COI65
brookmani1 m COI209
defoliarti3 m COI243
defoliarti f COI244
defoliarti f COI245
defoliarti f COI257
defoliarti f COI263
defoliarti f COI264
defoliarti3 m COI262
100
C. mohave
C. crepuscularis
C. reevesi
C. reevesi a
C. (Beltranmyia) crepuscularis
C. reevesi
C. (Selfia) jacksoni
C. jacksoni
C. (Avaritia) boydi
C. luglani
C. (Haematomyidium) kettlei
C. (Monoculicoides) sonorensis
C. sonorensis
C. sonorensis sp
C. (Selfia) brookmani
C. brookmani sc
C. jacksoni sc
C. (Diphaomyia) defoliarti

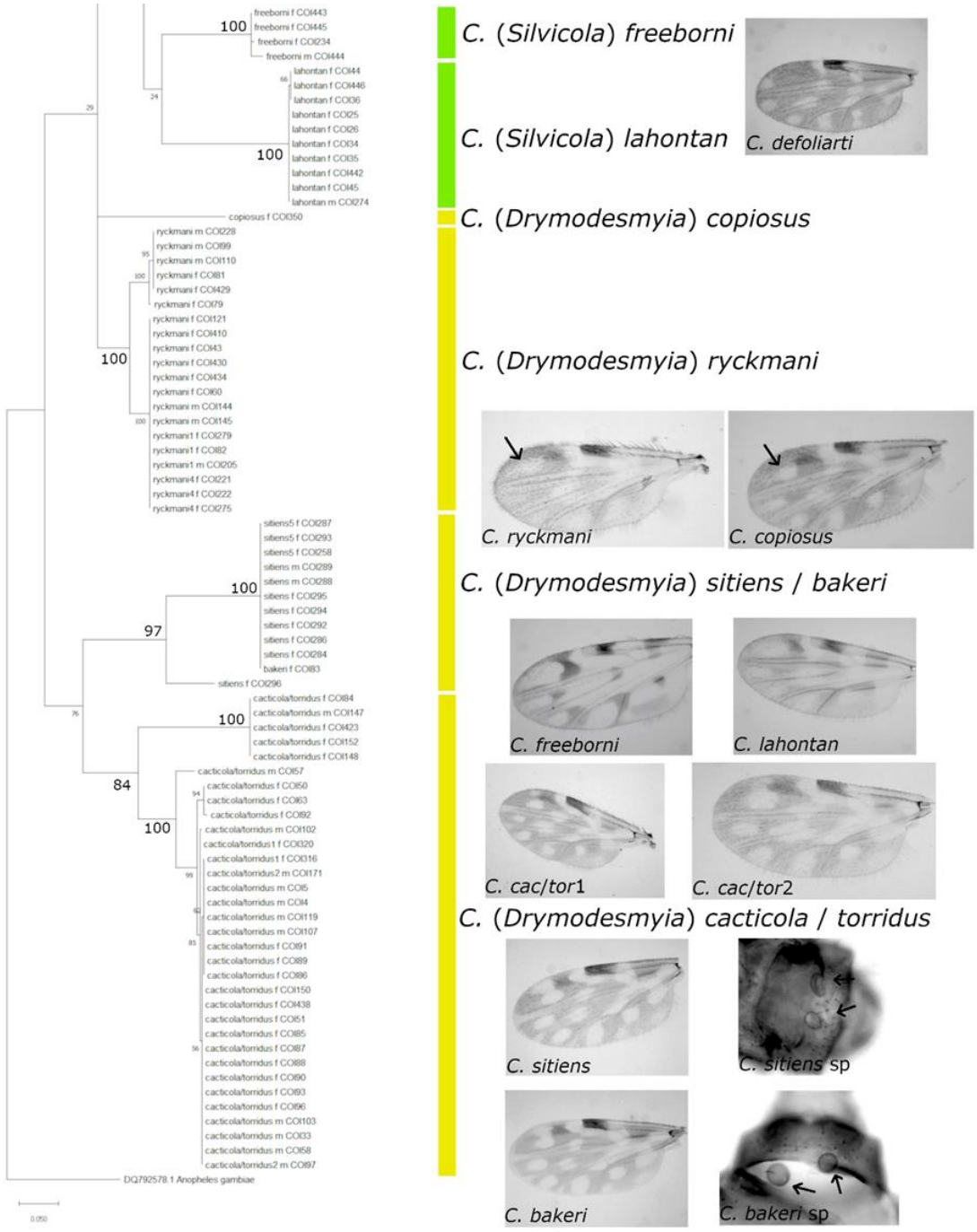
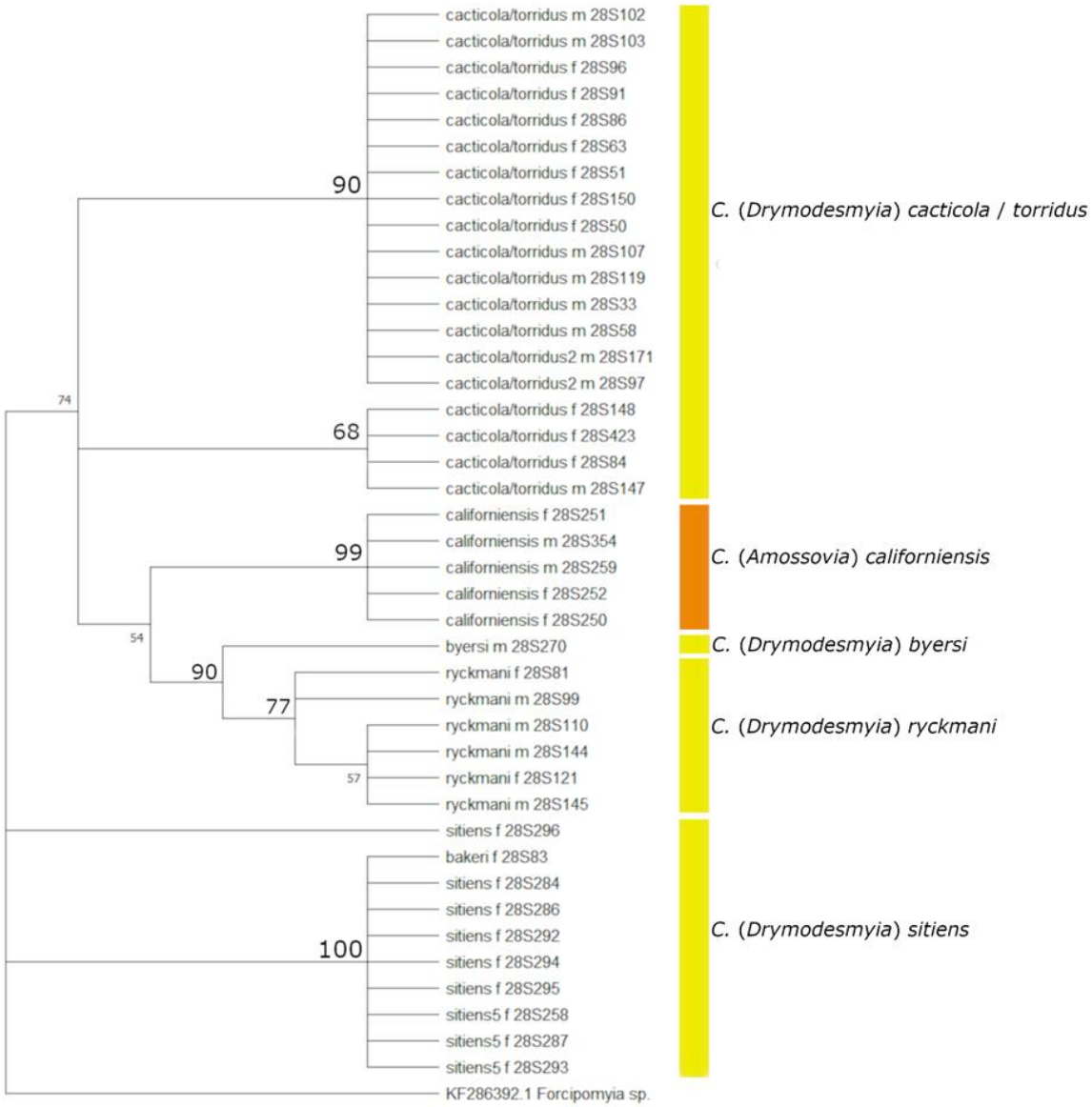

freeborni f COI443
freeborni f COI445
freeborni f COI234
freeborni m COI444
lahontan f COI44
lahontan f COI446
lahontan f COI36
lahontan f COI25
lahontan f COI26
lahontan f COI34
lahontan f COI35
lahontan f COI442
lahontan f COI45
lahontan m COI274
copiosus f COI350
ryckmani m COI228
ryckmani m COI89
ryckmani m COI110
ryckmani f COI81
ryckmani f COI429
ryckmani f COI79
ryckmani f COI121
ryckmani f COI410
ryckmani f COI43
ryckmani f COI430
ryckmani f COI434
ryckmani f COI60
ryckmani m COI144
ryckmani m COI145
ryckmani1 f COI279
ryckmani1 f COI82
ryckmani1 m COI205
ryckmani4 f COI221
ryckmani4 f COI222
ryckmani4 f COI275
sitiens5 f COI287
sitiens5 f COI293
sitiens5 f COI258
sitiens m COI289
sitiens m COI288
sitiens f COI295
sitiens f COI294
sitiens f COI292
sitiens f COI286
sitiens f COI284
bakeri f COI83
sitiens f COI296
cacticola/torridus f COI84
cacticola/torridus m COI147
cacticola/torridus f COI423
cacticola/torridus f COI152
cacticola/torridus f COI148
cacticola/torridus m COI57
cacticola/torridus f COI50
cacticola/torridus f COI63
cacticola/torridus f COI92
cacticola/torridus m COI102
cacticola/torridus1 f COI320
cacticola/torridus1 f COI316
cacticola/torridus2 m COI171
cacticola/torridus m COI5
cacticola/torridus m COI4
cacticola/torridus m COI119
cacticola/torridus m COI107
cacticola/torridus f COI91
cacticola/torridus f COI89
cacticola/torridus f COI86
cacticola/torridus f COI150
cacticola/torridus f COI438
cacticola/torridus f COI51
cacticola/torridus f COI85
cacticola/torridus f COI87
cacticola/torridus f COI88
cacticola/torridus f COI90
cacticola/torridus f COI93
cacticola/torridus f COI96
cacticola/torridus m COI103
cacticola/torridus m COI33
cacticola/torridus m COI58
cacticola/torridus2 m COI97
DQ792578.1 Anopheles gambiae
C. (Silvicola) freeborni
C. (Silvicola) lahontan
C. defoliarti
C. (Drymodesmyia) copiosus
C. (Drymodesmyia) ryckmani
C. ryckmani
C. copiosus
C. (Drymodesmyia) sitiens / bakeri
C. freeborni
C. lahontan
C. cac/tor1
C. cac/tor2
C. (Drymodesmyia) cacticola / torridus
C. sitiens
C. sitiens sp
C. bakeri
C. bakeri sp

Figure 3.6: Maximum likelihood tree built based on 28S sequences using the Tamura-3-parameter (T92+G) model. Each specimen identification code includes a numeric portion to identify each specimen with 28S as the prefix indicating the sequenced gene. Numbers following the species name refer to the species name and associated footnote in Table 3.2. The topology of a condensed tree is shown with only bootstrap support values greater than 50% being retained. Different genera are represented by different colors. *Forcipomyia* sp. (accession No. KF286392) served as the outgroup.



Supp. Table S3.1. Detailed information of *Culicoides* specimens.

UCRC ENT ID	COI	COI GenBank	28S	28S GenBank	trap type	Loca -tion	date	latitude	longitude
00549860	yes	OM897400	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00549861	yes	OM897401	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00549862	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00549863	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00549864	yes	OM892546	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549865	yes	OM892547	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549866	yes	OM892548	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549867	yes	OM892549	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549868	yes	OM892550	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549869	yes	OM892551	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549870	yes	OM892552	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549871	no	na	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549872	yes	OM892553	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549873	yes	OM892554	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549874	yes	OM897402	yes	OM910743	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549875	yes	OM892555	no	na	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549876	yes	OM892556	no	na	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549877	yes	OM892557	no	na	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549878	yes	OM892558	no	na	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549879	yes	OM892559	no	na	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549880	yes	OM892560	no	na	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549881	yes	OM892561	no	na	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549882	yes	OM892562	no	na	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549883	no	na	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549884	yes	OM892563	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549885	yes	OM892564	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549886	yes	OM892565	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549887	yes	OM892566	no	na	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549888	yes	OM892567	no	na	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549889	yes	OM892568	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549890	yes	OM892569	no	na	CO2	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549891	yes	OM897403	yes	OM910744	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549892	yes	OM897404	yes	OM910745	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549893	yes	OM892570	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549894	no	na	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549895	no	na	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549896	yes	OM892571	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W

UCRC ENT ID	COI	COI GenBank	28S	28S GenBank	trap type	Loca -tion	date	latitude	longitude
00549897	yes	OM892572	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549898	yes	OM897405	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549899	yes	OM897406	yes	OM910746	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549900	no	na	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549901	yes	OM892573	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549902	yes	OM892574	no	na	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549903	no	na	no	na	CO2	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00549904	yes	OM897407	yes	OM910747	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549905	yes	OM892575	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549906	yes	OM892576	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549907	no	na	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549908	yes	OM892577	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549909	no	na	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549910	yes	OM892578	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549911	yes	OM892579	no	na	UV	D	22-May-2018	33°39'12.96"N	116°22'25.57"W
00549912	yes	OM892580	no	na	CO2	E	22-May-2018	33°39'35.16"N	116°22'17.19"W
00549913	yes	OM892581	no	na	CO2	E	22-May-2018	33°39'35.16"N	116°22'17.19"W
00549914	yes	OM892582	no	na	CO2	E	22-May-2018	33°39'35.16"N	116°22'17.19"W
00549915	yes	OM892583	no	na	CO2	E	22-May-2018	33°39'35.16"N	116°22'17.19"W
00549916	yes	OM892584	no	na	CO2	E	22-May-2018	33°39'35.16"N	116°22'17.19"W
00549917	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549918	yes	OM892585	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549919	yes	OM892586	yes	OM910784	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549920	yes	OM886391	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549921	yes	OM892587	yes	OM910785	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549922	yes	OM897408	yes	OM910748	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549923	yes	OM897409	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549924	yes	OM897410	yes	OM910749	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549925	yes	OM897411	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549926	yes	OM897412	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549927	yes	OM897413	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549928	yes	OM897414	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549929	yes	OM897415	yes	OM910750	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549930	yes	OM897416	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549931	yes	OM897417	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549932	yes	OM892588	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549933	yes	OM897418	yes	OM910751	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549934	yes	OM886392	yes	OM910777	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549935	yes	OM892589	yes	OM910786	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W

UCRC ENT ID	COI	COI GenBank	28S	28S GenBank	trap type	Loca -tion	date	latitude	longitude
00549936	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549937	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549938	yes	OM897419	yes	OM910752	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549939	yes	OM897420	yes	OM910753	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549940	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549941	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549942	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549943	yes	OM897421	yes	OM910754	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549944	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549945	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549946	yes	OM892590	yes	OM910787	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549947	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549948	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549949	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549950	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549951	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549952	yes	OM892591	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549953	yes	OM892592	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549954	yes	OM892593	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549955	yes	OM897422	yes	OM910755	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549956	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549957	yes	OM892594	yes	OM910788	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549958	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549959	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549960	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549961	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549962	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549963	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549964	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549965	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549966	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549967	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549968	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549969	yes	OM892595	yes	OM910789	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549970	yes	OM892596	yes	OM910790	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549971	yes	OM892597	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549972	yes	OM897423	yes	OM910756	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00549973	yes	OM897424	yes	OM910757	CO2	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W
00549974	yes	OM897425	yes	OM910758	CO2	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W

UCRC ENT ID	COI	COI GenBank	28S	28S GenBank	trap type	Loca -tion	date	latitude	longitude
00549975	no	na	no	na	CO2	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W
00549976	yes	OM897426	no	na	CO2	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W
00549977	no	na	no	na	UV	G	18-Jul-2020	33°40'36.07"N	116°22'13.36"W
00549978	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549979	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549980	yes	OM892598	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549981	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549982	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549983	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549984	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549985	yes	OM886393	yes	OM910778	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549986	yes	OM892599	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549987	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549988	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549989	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549990	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549991	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549992	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549993	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549994	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00549995	no	na	no	na	UV	G	18-Jul-2020	33°40'36.07"N	116°22'13.36"W
00549996	no	na	no	na	UV	G	18-Jul-2020	33°40'36.07"N	116°22'13.36"W
00549997	no	na	no	na	UV	G	18-Jul-2020	33°40'36.07"N	116°22'13.36"W
00549998	no	na	no	na	UV	G	18-Jul-2020	33°40'36.07"N	116°22'13.36"W
00549999	yes	OM892600	no	na	UV	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W
00550000	no	na	no	na	CO2	B	24-Jul-2019	33°38'33.00"N	116°23'2.00"W
00550001	yes	OM892601	no	na	CO2	B	24-Jul-2019	33°38'33.00"N	116°23'2.00"W
00550002	yes	OM892602	no	na	CO2	B	24-Jul-2019	33°38'33.00"N	116°23'2.00"W
00550003	yes	OM892603	no	na	CO2	B	24-Jul-2019	33°38'33.00"N	116°23'2.00"W
00550004	no	na	no	na	CO2	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W
00550005	no	na	no	na	CO2	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W
00550006	no	na	no	na	CO2	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W
00550007	yes	OM892604	no	na	CO2	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W
00550008	yes	OM892605	no	na	CO2	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W
00550009	no	na	no	na	CO2	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W
00550010	no	na	no	na	CO2	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W
00550011	no	na	no	na	CO2	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W
00550012	no	na	no	na	CO2	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W
00550013	no	na	no	na	CO2	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W

UCRC ENT ID	COI	COI GenBank	28S	28S GenBank	trap type	Loca -tion	date	latitude	longitude
00550014	yes	OM892606	no	na	CO2	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W
00550015	no	na	no	na	CO2	G	24-Jul-2019	33°40'36.07"N	116°22'13.36"W
00550017	yes	OM886394	no	na	UV	B	24-Jul-2019	33°38'33.00"N	116°23'2.00"W
00550020	no	na	no	na	UV	B	24-Jul-2019	33°38'33.00"N	116°23'2.00"W
00550021	yes	OM886395	no	na	UV	B	24-Jul-2019	33°38'33.00"N	116°23'2.00"W
00550023	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550024	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550025	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550026	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550027	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550028	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550029	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550030	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550031	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550032	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550033	yes	OM886396	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550034	yes	OM886397	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550035	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550036	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550037	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550038	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550039	yes	OM892607	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550040	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550041	yes	OM892608	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550042	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550043	no	na	no	na	CO2	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W
00550044	no	na	no	na	CO2	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W
00550045	yes	OM892609	no	na	CO2	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W
00550046	no	na	no	na	CO2	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W
00550047	no	na	no	na	CO2	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550048	no	na	no	na	CO2	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550049	yes	OM892610	no	na	CO2	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550050	yes	OM892611	no	na	CO2	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550051	yes	OM892612	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550052	yes	OM892613	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550053	yes	OM886398	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550054	yes	OM892614	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550055	yes	OM892615	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550056	yes	OM892616	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W

UCRC ENT ID	COI	COI GenBank	28S	28S GenBank	trap type	Loca -tion	date	latitude	longitude
00550057	no	na	yes	OM910791	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550058	no	na	yes	OM910792	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550059	no	na	yes	OM910793	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550060	yes	OM892617	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550061	yes	OM892618	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550062	yes	OM892619	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550063	no	na	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550064	yes	OM892620	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550065	yes	OM886399	yes	OM910779	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550066	no	na	yes	OM910794	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550067	yes	OM892621	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550068	no	na	no	na	CO2	B	19-Oct-2018	33°38'33.00"N	116°23'2.00"W
00550069	yes	OM886400	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550070	yes	OM892622	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550071	yes	OM892623	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550072	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550073	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550074	no	na	no	na	CO2	Near F	19-Oct-2018	33°40'25.00"N	116°22'19.00"W
00550075	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550076	yes	OM892624	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550077	no	na	yes	OM910795	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550078	yes	OM892625	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550079	yes	OM892626	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550080	yes	OM892627	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550081	yes	OM892628	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550082	yes	OM886401	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550083	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550084	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550085	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550086	yes	OM886402	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550087	no	na	no	na	UV	C	22-May-2018	33°38'58.52"N	116°22'34.33"W
00550088	no	na	no	na	UV	E	22-May-2018	33°39'35.16"N	116°22'17.19"W
00550089	no	na	no	na	UV	E	22-May-2018	33°39'35.16"N	116°22'17.19"W
00550090	no	na	no	na	UV	E	22-May-2018	33°39'35.16"N	116°22'17.19"W
00550091	yes	OM892629	yes	OM910796	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550092	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550093	yes	OM892630	yes	OM910797	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550094	yes	OM886403	yes	OM910780	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W

UCRC ENT ID	COI	COI GenBank	28S	28S GenBank	trap type	Loca -tion	date	latitude	longitude
00550095	yes	OM892631	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550096	yes	OM892632	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550097	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550098	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550099	yes	OM892633	yes	OM910798	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550100	yes	OM886404	yes	OM910781	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550101	yes	OM892634	yes	OM910799	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550102	yes	OM892635	yes	OM910800	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550103	yes	OM892636	yes	OM910760	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00550104	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550105	no	na	no	na	UV	A	22-May-2018	33°38'28.82"N	116°23'20.75"W
00550106	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550107	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550108	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550109	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550110	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550111	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550112	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550113	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550114	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550115	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550116	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550117	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550118	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550119	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550120	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550121	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550122	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550123	yes	OM892921	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550124	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550125	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550126	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550127	yes	OM892922	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550128	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550129	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00550130	no	na	no	na	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00550131	no	na	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W
00550132	no	na	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W
00550133	no	na	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W

UCRC ENT ID	COI	COI GenBank	28S	28S GenBank	trap type	Loca -tion	date	latitude	longitude
00550134	no	na	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W
00550135	no	na	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W
00550136	no	na	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W
00550137	no	na	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W
00550138	no	na	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W
00550139	no	na	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550141	no	na	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550143	no	na	no	na	UV	B	28-Jun-2019	33°38'33.00"N	116°23'2.00"W
00550144	no	na	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550145	no	na	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550146	no	na	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550147	yes	OM892637	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550148	no	na	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550149	no	na	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550150	no	na	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550151	no	na	yes	OM910801	UV	B	18-Jul-2020	33°38'33.00"N	116°23'2.00"W
00550152	no	na	no	na	UV	G	18-Jul-2020	33°40'36.07"N	116°22'13.36"W
00550153	no	na	no	na	UV	B	24-Jul-2019	33°38'33.00"N	116°23'2.00"W
00550154	no	na	no	na	UV	B	24-Jul-2019	33°38'33.00"N	116°23'2.00"W
00550155	no	na	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550157	no	na	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550158	yes	OM892638	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W
00550159	yes	OM892639	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550160	yes	OM892640	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550161	yes	OM892641	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550162	yes	OM897427	yes	OM910759	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550163	yes	OM892642	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550164	yes	OM892643	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550165	yes	OM892644	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550166	yes	OM892645	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550167	yes	OM892646	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550168	yes	OM897428	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550169	yes	OM892647	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W
00550170	yes	OM892648	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W
00550171	yes	OM892649	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550172	yes	OM892650	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550173	yes	OM892651	no	na	UV	B	21-Aug-2019	33°38'33.00"N	116°23'2.00"W
00550174	yes	OM892652	no	na	CO2	F	19-Oct-2018	33°40'9.00"N	116°22'21.00"W
00550175	yes	OM892653	no	na	CO2	F	19-Oct-2018	33°40'9.00"N	116°22'21.00"W

UCRC ENT ID	COI	COI GenBank	28S	28S GenBank	trap type	Loca -tion	date	latitude	longitude
00550176	yes	OM892654	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W
00550177	yes	OM892655	no	na	UV	G	21-Aug-2019	33°40'36.07"N	116°22'13.36"W
00550178	yes	OM892656	no	na	UV	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W
00550179	yes	OM892657	no	na	UV	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W
00550180	yes	OM892658	no	na	UV	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W
00550181	yes	OM892659	no	na	UV	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W
00550182	yes	OM892660	no	na	UV	G	28-Jun-2019	33°40'36.07"N	116°22'13.36"W

Supp. Table S3.2: Between and within species genetic distance for COI gene.

	C.bakeri	*C. boydi*	*C. brookmani*	*C. brookmani*[1]	*C. cacticola/C. torridus*	*C. cacticola/C. torridus*[1]	*C. cacticola/C. torridus*[2]	*C. copiosus*	*C. crepuscularis*	*C. defoliarti*	*C. defoliarti*[3]	*C. freeborni*	*C. jacksoni*	*C. kettlei*	*C. lahontan*	*C. luglani*	*C. mohave*	*C. reevesi*	*C. ryckmani*	*C. ryckmani*[1]	*C. ryckmani*[4]	*C. sitiens*	*C. sitiens*[5]	*C. sonorensis*
C.bakeri	-																							
C. boydi	0.2069	0.0010																						
C. brookmani	0.2220	0.1707	0.0044																					
C. brookmani[1]	0.2238	0.1688	**0.0026**	-																				
C. cacticola/C. torridus	0.2175	0.2046	0.2045	0.2051	0.0530																			
C. cacticola/C. torridus[1]	0.2184	0.2022	0.2066	0.2071	**0.0331**	0.0049																		
C. cacticola/C. torridus[2]	0.2201	0.2026	0.2082	0.2087	**0.0319**	**0.0025**	0.0025																	
C. copiosus	0.2468	0.2227	0.1941	0.1946	0.2364	0.2309	0.2325	-																
C. crepuscularis	0.2963	0.2007	0.1739	0.1728	0.2376	0.2402	0.2385	0.2302	-															
C. defoliarti	0.2608	0.2096	0.1749	0.1763	0.2302	0.2300	0.2316	0.2141	0.1612	0.0000														
C. defoliarti[3]	0.2591	0.2080	0.1733	0.1747	0.2285	0.2283	0.2300	0.2124	0.1597	**0.0012**	0.0025													
C. freeborni	0.2783	0.2194	0.1657	0.1665	0.2669	0.2695	0.2713	0.1913	0.2228	0.1796	0.1780	0.0099												
C. jacksoni	0.2995	0.2243	0.1763	0.1762	0.2528	0.2437	0.2454	0.2323	0.2014	0.2116	0.2099	0.2525	-											
C. kettlei	0.2977	0.1827	0.1446	0.1446	0.2540	0.2488	0.2505	0.2199	0.1746	0.2030	0.2014	0.1654	0.1944	0.0025										
C. lahontan	0.2481	0.1978	0.2041	0.2052	0.2259	0.2213	0.2229	0.2116	0.1818	0.1936	0.1920	0.1790	0.2482	0.1975	0.0011									
C. luglani	0.2748	0.1921	0.1894	0.1912	0.2582	0.2571	0.2588	0.2290	0.2222	0.1789	0.1774	0.1886	0.2003	0.1513	0.1937	0.0066								
C. mohave	0.2576	0.1667	0.1474	0.1461	0.2153	0.2123	0.2139	0.2151	0.1393	0.1664	0.1649	0.1899	0.1828	0.1630	0.2020	0.1844	0.0100							
C. reevesi	0.2605	0.2202	0.1829	0.1821	0.2374	0.2307	0.2323	0.2313	0.2122	0.2112	0.2129	0.2319	0.2255	0.1944	0.2233	0.2063	0.1675	0.0000						
C. ryckmani	0.2362	0.2287	0.1660	0.1659	0.2136	0.2125	0.2141	0.1712	0.1851	0.1520	0.1535	0.1629	0.2290	0.1951	0.1961	0.1944	0.1802	0.1857	0.0070					
C. ryckmani[1]	0.2434	0.2360	0.1706	0.1695	0.2172	0.2137	0.2153	0.1762	0.1790	0.1545	0.1560	0.1720	0.2221	0.2006	0.1975	0.2024	0.1751	0.1790	**0.0216**	0.0000				
C. ryckmani[4]	0.2434	0.2360	0.1706	0.1695	0.2172	0.2137	0.2153	0.1762	0.1790	0.1545	0.1560	0.1720	0.2221	0.2006	0.1975	0.2024	0.1751	0.1790	**0.0216**	**0.0000**	0.0000			
C. sitiens	**0.0178**	0.2045	0.2187	0.2202	0.2133	0.2131	0.2147	0.2451	0.2893	0.2582	0.2565	0.2763	0.2960	0.2901	0.2455	0.2715	0.2522	0.2580	0.2319	0.2380	0.2380	0.0356		
C. sitiens[5]	**0.0000**	0.2069	0.2220	0.2238	0.2175	0.2184	0.2201	0.2468	0.2963	0.2608	0.2591	0.2783	0.2995	0.2977	0.2481	0.2748	0.2576	0.2605	0.2362	0.2434	0.2434	**0.0178**	0.0000	
C. sonorensis	0.2483	0.1948	0.1352	0.1352	0.2292	0.2334	0.2317	0.2027	0.1618	0.1998	0.1982	0.1712	0.2051	0.1676	0.2018	0.1806	0.1682	0.2168	0.1829	0.1831	0.1831	0.2432	0.2483	

Low genetic distances between species or tentatively identified species are bolded.

Supp. Table S3.3: Between and within species genetic distance for 28S rDNA gene.

	C.bakeri	*C. byersi*	*C. cacticola/C. torridus*	*C. cacticola/C. torridus*[2]	*C. californiensis*	*C. ryckmani*	*C. sitiens*	*C. sitiens*[5]
C.bakeri	-							
C. byersi	0.0334	-						
C. cacticola/C. torridus	0.0296	0.0210	0.0033					
C. cacticola/C. torridus[2]	0.0311	0.0209	**0.0020**	-				
C. californiensis	0.0436	0.0284	0.0322	0.0321	0.0007			
C. ryckmani	0.0369	0.0095	0.0229	0.0217	0.0334	0.0031		
C. sitiens	**0.0026**	0.0328	0.0278	0.0294	0.0413	0.0366	0.0052	
C. sitiens[5]	**0.0000**	0.0334	0.0296	0.0311	0.0436	0.0369	**0.0026**	0.0000

Low genetic distances between species or tentatively identified species are bolded.

CHAPTER 4

Comparison of Trap Efficiency Using Suction Traps Baited with Either UV or CO_2 for the Capture of *Culicoides* Species in the Southern California Desert

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Abstract

Culicoides biting midges are hematophagous flies that can transmit several disease-causing pathogens to animals. Surveillance of *Culicoides* is critical for understanding pathogen transmission risk. The most commonly used traps for midge surveillance are suction traps baited with UV light or CO_2. *Culicoides* species are understudied in the southern California desert region and trapping methods for these desert midges remain largely unexplored. In this study, capture rates of different *Culicoides* species were compared using suction traps baited with either UV or CO_2 placed at two locations at a southern California desert site where a narrow canyon (Deep Canyon) drains the adjacent peninsular mountain range and leads to an expansive flood plain. Over all trap nights and locations, UV-baited traps outperformed CO_2-baited traps for most *Culicoides* species captured at the study site, except for *C. sonorensis* Wirth and Jones and *C. mohave* Wirth. Capture rates varied for each species by trap location, with desert *Culicoides*

species captured in greater numbers at the canyon mouth and *C. sonorensis* and *C. mohave* captured in greater numbers on the flood plain nearer to urban development including a golf course and small zoo. An interaction of trap type with trapping location on the capture rate was noted for some *Culicoides* species, especially for *C. mohave* which was captured in greater numbers using UV traps at the canyon mouth but captured in greater numbers using CO_2 traps in the flood plain. This trap efficiency study will facilitate future research targeting *Culicoides* species in the southern California desert.

Introduction

Biting midges in the genus *Culicoides* (Diptera: Ceratopogonidae) are small blood-feeding flies known to vector several important disease-causing pathogens of animals. Surveillance for *Culicoides* activity is a critical component of risk modeling for pathogen transmission by these flies (Gerry et al. 2001, Wilson and Mellor 2009, Mullens et al. 2015). Trapping methods for capturing adult *Culicoides* include direct aspiration from animals, use of animal-baited traps and suction traps baited with light and/or host odors including carbon dioxide (CO_2) (Bram 1978). Although animal-baited traps provide biting rate data that is most relevant for understanding transmission risk to any individual host species (Gerry and Mullens 1998, Carpenter et al. 2008, Gerry et al. 2009), use of animal hosts as bait for *Culicoides* can be challenging. Therefore, non-animal baited trapping methods such as suction traps baited with UV light and/or host odors such as CO_2 are most commonly used for *Culicoides* surveillance (Cohnstaedt et al. 2012), including for studies of *Culicoides* species diversity (Mullens and Dada 1992a, Veggiani Aybar et al. 2010), population activity monitoring (Smith et al. 1996, Gerry and Mullens 2000, Vigil et al. 2018), and identifying potential disease vectors (McGregor et al. 2019).

Several studies have compared capture of *Culicoides* species using suction traps baited with different light or host odor attractants. UV light has been shown to increase capture of *Culicoides* relative to other light sources (Belton and Pucat 1967, Anderson and Linhares 1989, Venter and Hermanides 2006). For some *Culicoides* species, use of CO_2 as an additional attractant will increase capture rate relative to UV alone (Anderson and Linhares 1989, Sloyer et al. 2018, Walgama and Lysyk 2018). CO_2 also targets the host-seeking population providing more

targeted information on biting activity. Other host odors, such as 1-octen-3-ol, are occasionally combined with CO_2 to increase capture rate (Ritchie et al. 1994, Harrup et al. 2012, Bray et al. 2020). UV light-baited traps can result in higher capture rates of some *Culicoides* species relative to other collection methods, but UV traps can also underestimate some potential vector species (Carpenter et al. 2008, Gerry et al. 2009) with UV perhaps even repellent to virus-infected *Culicoides* thereby impacting pathogen incidence estimates (McDermott et al. 2015). Importantly for pathogen transmission modeling studies, UV traps will also capture non-host-seeking midges resulting in bias to estimated biting rates when using these traps (Cohnstaedt et al. 2012).

The *Culicoides* of the southern California peninsular mountain range and adjacent deserts are surprisingly diverse, perhaps due to the habitat variability associated with the transition zone between these landscapes. Two studies evaluating *Culicoides* diversity in this region reported > 20 *Culicoides* species (Mullens and Dada 1992a, Zhang et al. in press). Feeding preference for some *Culicoides* species in this area was determined using drop traps baited with bighorn sheep (*Ovis canadensis* Shaw), Japanese quail (*Coturnix japonica* Temminck and Schlegel), or domestic rabbit (*Oryctolagus cuniculus* Linnaeus) (Mullens and Dada 1992b). *Culicoides* species that fed on bighorn sheep were *C. brookmani* Wirth, *C. sonorensis* Wirth and Jones, and *C. cacticola* Wirth and Hubert. *Culicoides brookmani* and *C. sonorensis* also fed on rabbits, while *C. cacticola* also fed on quail (Mullens and Dada 1992b).

Understanding the efficiency of different trapping methods is critical to interpret trap collections relative to species biting rates or relative abundance. Also, more efficient traps can be selected

to increase midge capture for virus detection even if traps capture non-host-seeking midges and counts are therefore less related to host-seeking activity. The present study compares capture rates of different *Culicoides* species in the southern California inland desert using CDC-type traps baited with either CO_2 or UV light and overall species richness at two locations associated with a transition zone from native bighorn sheep habitat in the peninsular mountain range to the adjacent lower elevation desert near the city of Palm Desert, California.

Methods

Culicoides Collection

The study was conducted at the Phillip L. Boyd Deep Canyon Desert Research Center (Deep Canyon), one of the largest reserves of the University of California Natural Reserve System. The Deep Canyon research area contains a drainage system (Deep Canyon creek) that extends from the Santa Rosa Mountains (2,657 m peak elevation) south of the research center to the floor of the Colorado Desert (<290 m elevation) north of the research center. The canyon is narrow throughout the mountains, but opens to form a broad alluvial flood plain of sandy washes at its northern edge until it reaches a golf course and accompanying residential community in Palm Desert, CA.

To address variation in species presence along the Deep Canyon drainage system (Zhang et al., in press) *Culicoides* were trapped at two locations: 1) the narrow canyon mouth and 2) the lower alluvial flood plain. *Culicoides* were captured using Centers for Disease Control (CDC) suction traps baited with either CO_2 (approx. 1.5 kg of dry ice) or UV light (spectral peak ~350–368 nm).

At each trapping location (canyon mouth or flood plain), traps were deployed in three positions separated by at least 50 m with a pair of traps, one CO_2 and one UV, placed at each position and separated by approx. 30 m to minimize trap interference (Kirkeby et al. 2013, McDermott et al. 2016). Thus, a total of six traps (three CO_2 and three UV) were placed at each trapping location during each trap night. Suction traps were suspended beneath a black colored insulated paint can containing either dry ice (CO_2 traps) or nothing (UV light traps). The UV light traps were fitted with a small UV bulb placed between the paint can and the opening to the suction trap. Trap openings were at a height of ca. 0.7 – 0.9 m. Trapping was conducted from before sunset to after sunrise the next day once a month in June, July, and August of 2019, and once in July of 2020. Captured *Culicoides* were placed on dry ice for transport to the laboratory where they were stored at -20 °C until processed.

Culicoides Species Identification

Captured *Culicoides* were sorted from other insects under the dissecting scope and morphologically identified to species using a key to the *Culicoides* of the southwestern U.S. (Phillips 2022). A small subset of each identified species was slide mounted for further examination under a compound microscope to look at minute characteristics, and DNA was also extracted from these specimens for gene sequence analyses (Zhang et al., in press).

Bloodmeal Analysis

DNA was extracted from blood-engorged female *Culicoides* using HotSHOT non-destructive DNA extraction (Truett et al. 2000), followed by amplification of 16S ribosomal DNA using vertebrate-wide primers (L2513: 5’-GCCTGTTTACCAAAAACATCAC-3’, H2714: 5’-

CTCCATAGGGTCTTCTCGTCTT-3') (Kitano et al. 2007). The PCR cycling process was 2 min at 95 °C, 35 cycles of 95 °C for 30 s, 57 °C for 15 s, and 72 °C for 30 s, followed by 5 min at 72 °C (Kitano et al. 2007). PCR products with positive results by agarose gel electrophoresis were purified using Illustra Exoprostar (GE Healthcare Life Science) and sent to Genewiz (San Diego, California) for Sanger sequencing. Sequences were then searched against the nucleotide collection (nt) database within GenBank using blastn (Altschul et al. 1990). Vertebrate DNA within blood-engorged *Culicoides* was confirmed to species by having significant sequence alignment with 100% identity to confirmed vertebrate sequences for a vertebrate species known to be present in the Deep Canyon area ("Species lists" 2022).

Statistical Analysis

All statistical analyses were performed in R (R Core Team 2013). For each *Culicoides* species captured, both a negative binomial regression (1) for over-dispersion data and a Poisson regression (2) was performed to model the relationship between the number of midges captured with trap type, trapping location, and collection date.

$$\Pr(Y = y|\mu, \theta) = \frac{\Gamma(\theta+y)}{\Gamma(\theta)y!}\frac{\mu^y\theta^\theta}{(\mu+\theta)^{\theta+y}} \quad (1)$$

$$Pr\ (Y = y|\mu) = \frac{e^{-\mu}\mu^y}{y!}\ ; \quad (2)$$

The main effects (trap type, location, date), two-way interactions (trap type * location, trap type * date, location * date) and three-way interactions (trap type * location * date) were considered in the analysis:

$$\ln(\mu) \sim intercept + trap\ type + location + date + trap\ type * location + trap\ type * date + location * date + trap\ type * location * date$$

Since the Poisson model is nested in the negative binomial model, we used the likelihood-ratio test to determine whether the negative binomial model provided a better fit for the data than the Poisson model, with modeling results reported as the best model with all nonsignificant effects removed. A residual analysis was performed on the final model as a diagnostic check for model assumptions. Statistical analysis was performed only for *Culicoides* species captured in sufficient numbers (*C. brookmani*, *C. cacticola* /*C. torridus* aggregate, *C. mohave*, *C. ryckmani* and *C. sonorensis*).

Results

Culicoides Collection

A total of 9691 *Culicoides* were collected including *C. boydi* Wirth and Mullens, *C. brookmani, C. cacticola* / *C. torridus* Wirth and Hubert, *C. californiensis* Wirth and Blanton, *C. copiosus* Root and Hoffman, *C. crepuscularis* Malloch, *C. defoliarti* Atchley and Wirth, *C. freeborni* Wirth and Blanton, *C. jacksoni* Atchley, *C. kettlei* Breidenbaugh and Mullens, *C. lahontan* Wirth and

Blanton, *C. luglani* Jones and Wirth, *C. mohave* Wirth, *C. reevesi* Wirth, *C. ryckmani* Wirth and Hubert, *C. sitiens* Wirth and Hubert / *C. bakeri* Vargas, and *C. sonorensis* (Table 1).

Culicoides brookmani was the most abundant species at the study site (n=6950; 72% of total midges collected), followed by *C. ryckmani* (n=1097; 11%) and *C. cacticola* / *C. torridus* aggregate (n=876; 9%). A small number of midges identified as male *C. brookmani* under a dissecting scope were actually male *C. jacksoni* when examined under a compound scope (1 of 15 slide mounted males; 6%). None of 35 female *C. brookmani* examined under the compound scope were misidentified. Similarly, a small number of midges identified as female *C. ryckmani* were actually female *C. copiosus* when examined under a compound scope (2 of 32 slide mounted females; 6%), with none of 34 male *C. ryckmani mis*identified. *Culicoides cacticola* and *C. torridus* cannot be separated by morphology or by molecular analyses (Zhang et al., in press.) and therefore are grouped as *C. cacticola* / *C. torridus* aggregate. While *C. sitiens* and *C. bakeri* can be separated by differences in spermathecae shape when slide-mounted specimens are viewed under a compound scope, these species cannot be separated under a dissecting scope or by molecular analyses (Zhang et al., in press.) and are therefore grouped in this study as *C. sitiens* / *C. bakeri* aggregate.

CO_2 traps captured only 10 of the 17 species identified at the Deep Canyon study site, but they captured two species (*C. mohave* and *C. sonorensis*) in large numbers. In contrast, UV traps captured all species identified at the study site, with some species (*C. brookmani*, *C. cacticola / C. torridus*, and *C. ryckmani*) collected in much larger numbers by UV traps than by CO_2 traps. Species captured only by UV light traps were *C. californiensis*, *C. copiosus*, *C. crepuscularis*, *C.*

defoliarti, *C. kettlei*, and *C. luglani.* Males of nine species or species aggregates were captured in UV traps while males of only four species were captured in CO_2 traps. Males captured in the CO_2 traps were mostly *C. sonorensis* (18 of 24 males).

Relative to other trap-location combinations, UV traps within the canyon collected the greatest overall number of midges (n=7413; 76%) and the greatest number of species (16 of 17 species). Although *C. crepuscularis* was not captured by UV trap within the canyon, only a single individual of this species was captured, and it was captured by UV trap in the flood plain. The CO_2 traps within the canyon collected the fewest individual midges (n=715) and the fewest species (6 of 17). Species captured only within the canyon were *C. californiensis*, *C. copiosus*, *C. defoliarti*, *C. kettlei*, *C. luglani*, and *C. reevesi.*

Within a trap location (canyon or flood plain) males and females of each species were captured in similar numbers by UV traps, except that male *C. brookmani* were underrepresented at the flood plain location and male *C. sonorensis* were underrepresented at the canyon location and overrepresented at the flood plain location. Combining both locations for each trap type, species richness ranged from 2.3 to 4.3 per trap night for CO_2 traps and 3.8 to 6.6 per trap night for UV traps (Figure 1A). Combining all traps within a location, species richness (number of different *Culicoides* species) ranged from 3 to 6.3 per trap night in the canyon and from 3.3 to 5.2 per trap night in the flood plain (Figure 1B).

Trap Type and Location Comparison

For the five most abundant species captured during this study, the number of females captured was significantly influenced by trap type, location, and date with significant interaction of these factors for three of these species (*C. brookmani*, *C. mohave*, and *C. sonorensis*) and no significant interactions for two species (*C. cacticola* / *C. torridus* and *C. ryckmani*) (Table 2). Female *C. brookmani, C. cacticola* / *C. torridus,* and *C. ryckmani* were captured in greater numbers in UV traps than in CO_2 traps, and in greater numbers in the canyon than in the flood plain (Figure 2A, B, and D). Female *C. cacticola* / *C. torridus* and *C. ryckmani* were captured almost exclusively by UV traps placed in the canyon. Female *C. sonorensis* were captured in greater numbers in CO_2 traps than in UV traps, and in greater numbers in the flood plain than in the canyon (Figure 2E). Female *C. mohave*, were captured in greater numbers in the flood plain than in the canyon (Figure 2C).

Male *C. brookmani, C. cacticola* / *C. torridus,* and *C. ryckmani* were captured in greater numbers in the canyon than in the flood plain, while male *C. mohave* and *C. sonorensis* were caught in greater numbers in the flood plain than in the canyon. Male *C. californiensis*, *C. defoliarti*, *C. freeborni*, and *C. sitiens* / *C. bakeri* were collected in low numbers only in the canyon. Males of other *Culicoides* species were not collected at either location.

For *C. brookmani* and *C. sonorensis*, the interaction between trap type and location only influenced the degree of the effects, with relative number of female *C. brookmani* captured by UV traps being much greater in the canyon relative to the flood plain while the relative number of female *C. sonorensis* captured by CO_2 traps was much greater in the flood plain relative to the

canyon. For *C. mohave*, the interaction between trap type and location was due to the greater capture of female *C. mohave* by CO_2 traps in the flood plain but by UV traps in the canyon. Females of other *Culicoides* species were not captured in sufficient numbers for statistical analysis.

Blood Meal Analysis

A total of 69 blood-fed females were collected during the study period, comprising 39 *C. ryckmani*, 14 *C. brookmani*, nine *C. cacticola* / *C. torridus,* six *C. mohave,* and a single *C. sonorensis*. All blood-fed females were collected by UV traps in the canyon, except four of the six *C. mohave* which were captured in UV traps in the flood plain.

DNA was successfully amplified and sequenced from 16 of the 69 blood-fed midges with 13 sequences providing a 100% host identity match to vertebrate species present in the Deep Canyon study area. Three *C. brookmani* fed on bighorn sheep or sheep (*Ovis aries* Linnaeus) and one fed on black-tailed jackrabbit (*Lepus californicus* Gray). Bighorn sheep is common in the Deep Canyon study area while sheep could not be excluded because they are present in a nearby zoo located just beyond the flood plain. One *C. cacticola* / *C. torridus* fed on cattle (*Bos taurus* Linnaeus) which were present in a nearby zoo and one fed on loggerhead shrike (*Lanius ludovicianus* Linnaeus). One *C. mohave* fed on either coyote (*Canis latrans* Say) or wolf (*Canis lupus* Linnaeus) and three fed on black-tailed jackrabbit. Coyotes are common in the Deep Canyon study area while wolves are present in the nearby zoo. *Culicoides ryckmani* fed on a variety of bird species including one bloodmeal from cactus wren (*Campylorhynchus branneicapillus* (Lafresnaye)), one bloodmeal from greater roadrunner (*Geococcyx californianus*

(Lesson)) and three bloodmeals from unknown bird species (98-99% identity match to bird species across multiple avian families). One *C. sonorensis* fed on mule deer (*Odocoileus hemionus* (Rafinesque)).

Discussion

This study compared CO_2-baited traps and UV-baited traps for surveillance of *Culicoides* species in the Deep Canyon drainage which leads from the southern California peninsular mountain range to the adjacent Colorado desert. Overall, UV traps collected more species of *Culicoides* and greater numbers of most species than did CO_2 traps, with all species identified at the study site represented in the UV traps. UV light is known to attract a range of insects, and often both sexes, and has been used with different traps such as CDC traps, Onderstepoort traps, and New Jersey traps for surveillance of biting insects including *Culicoides* and mosquitoes (Belton and Pucat 1967, Venter and Hermanides 2006, Shimoda and Honda 2013, Probst et al. 2015). In contrast, CO_2 traps are expected to capture primarily host-seeking female *Culicoides* attracted by CO_2 plumes that signal a nearby host (Nelson 1965, Cohnstaedt et al. 2012). However, male *Culicoides sonorensis* are also attracted to CO_2 as an indicator of a nearby host males may use as a swarm marker or even for on-host mating (Gerry and Mullens 1998).

In the current study, the capture of each *Culicoides* species varied by use of traps baited either with UV or CO_2, with capture rates further affected by collection date and location. The interaction between trap type * days, trap location * days, and trap type * location * days suggests additional unknown factors related to trapping date influenced midge capture at the

study site. Possible explanations could be moonlight competing with UV light in attracting midges (Mellor et al. 2000), presence of nearby hosts, and/or weather factors such as temperature and wind that could affect CO_2 release rate and dispersal (Nelson and Bellamy 1971).

UV light is broadly attractive across many insect groups (Shimoda and Honda 2013) and traps baited with UV light often collect a greater number of *Culicoides* species than traps baited with host odors (Venter and Hermanides 2006, Carpenter et al. 2008, Gerry et al. 2009, McDermott and Lysyk 2020). CO_2 traps mostly attract host-seeking midges, providing a relative estimate of the host biting rate which can be useful for modeling disease transmission (Gerry et al. 2001). However, the CO_2 release rate may also affect the capture of different species that feed on different sizes of animals (Gerry et al. 2009, McPhatter and Gerry 2017) perhaps resulting in the observed skew of CO_2 traps in this study toward capture of *Culicoides* species (*C. sonorensis*, *C. mohave*) known to feed on medium to large mammals.

The topography of the canyon provides great variation in microhabitat leading to varying opportunities for resting and oviposition sites likely leading to the higher species richness for *Culicoides* in the canyon (Mullens and Dada 1992a). In contrast, the flood plain location was near human development including houses, golf courses, and a local zoo that held a variety of desert-dwelling and common zoo animals, perhaps providing anthropogenic development sites for *Culicoides* species more commonly associated with human activity (e.g., *C. sonorensis*).

Male *C. sonorensis* generally disperse a shorter distance from development sites than do females (Zimmerman and Turner 1984, Kluiters et al. 2015). The greater capture of both male and female *C. sonorensis* in the flood plain relative to the canyon suggests that suitable immature development habitat for this species is nearer to the flood plain than to the canyon. In California, *C. sonorensis* is an abundant species on dairies where the immatures develop in organically enriched aquatic sites such as dairy wastewater ponds contaminated with cattle feces (M.J. O'Rourke et al. 1983, Mullens 1989). The flood plain location was not far from a golf course and a local zoo. Both locations contained organically-polluted ponds that might have served as development sites for *C. sonorensis*. More *C. sonorensis* were captured in CO_2 traps than in UV traps at both trapping locations, a similar outcome to earlier studies (Anderson and Linhares 1989, McDermott et al. 2016) suggesting that this species responds strongly to CO_2 concentrations typical of a large mammal (approx. 300 - 1500 ml/min) which are produced from sublimation of dry ice within the trap can (Mullens 1995). *Culicoides sonorensis* is mammalophilic and its host range contains cattle, bighorn sheep, and other mammals (Mullens and Dada 1992b, Gerry et al. 2001, Phillips 2022). One blood-fed *C. sonorensis* captured in the canyon by UV trap fed on a mule deer.

Similar to *C. sonorensis*, a greater number of male and female *C. mohave* was captured in the flood plain than at the canyon, indicating that immature development sites for this species are nearer to the flood plain. Blood-fed *C. mohave* fed on black-tailed jackrabbits and coyotes/wolves. Previously, this species has been captured in animal-baited traps containing bighorn sheep or Japanese quail (Mullens and Dada 1992b) suggesting that *C. mohave is* attracted to a wide range of vertebrate hosts. UV traps and CO_2 traps had different trapping

efficiencies at the two locations, suggesting that the environment may affect the perception of attractants for this species.

Culicoides brookmani was the most abundant species captured in Deep Canyon, with both males and females more abundant in the canyon than in the flood plain, consistent with earlier studies (Mullens and Dada 1992a). Immatures of this species are thought to inhabit the margin of desert streams or creeks (Atchley 1970, Breidenbaugh and Mullens 1999). Blood-fed midges were only collected at the canyon trapping location with hosts identified as black-tailed jackrabbits and bighorn sheep. Mullens and Dada (1992b) also collected blood-engorged *C. brookmani* from bighorn sheep and black-tailed jackrabbits, indicating that this species will readily feed on mammals.

More male and female *C. ryckmani* were collected at the canyon than in the flood plain, an unsurprising outcome given that immature *C. ryckmani* are reported to develop in rotting cactus (Ryckman 1960) which was more abundant near and within the canyon than at the base of the alluvial flood plain. All blood-engorged females were captured at the canyon and all fed on various bird species suggesting an ornithophilic feeding preference. Ryckman (1960) collected blood-engorged females near the nest of a house finch (*Haemorhous mexicanus*) supporting an ornithophilic feeding habit for this species. The capture of few *C. ryckmani* in CO_2 traps in this study may be due to the much greater release of CO_2 from the traps in this study relative to CO_2 released by birds during respiration.

Culicoides cacticola and *C. torridus* cannot be morphologically separated with any confidence and thus are grouped as a species aggregate (Zhang et al. in press). Others have reported these species to be conspecific (Phillips 2022). *C. cacticola* / *C. torridus* are cactus-living species and their larvae develop within rotting cactus (Ryckman 1960). Blood-engorged females were captured in the canyon, with hosts identified as cattle and loggerhead shrike. Mullens and Dada (1992b) collected blood-engorged females from bighorn sheep and Japanese quail, implying that this species aggregate has a wide range of host preference.

More intense sampling will be required to understand the trap attractiveness for rare species. Bloodmeal analysis can only be done when blood-fed midges are captured, so aggressive sampling using UV traps would help. For *C. brookmani*, *C. lahontan*, and *C. boydi* which were thought as potential vectors of BTV or EHDV in Deep Canyon (Mullens and Dada 1992a), developing preferred trapping methods will improve surveillance for epidemiological studies in the future. This study lays a foundation for future research on the natural history of *Culicoides* species in the southern California desert.

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Table 4.1: Summary of *Culicoides* species collection.

Species	Canyon, CO2		Canyon, UV		Flood plain, CO2		Flood plain, UV		Total
	F	M	F	M	F	M	F	M	
C. boydi	2		5						7
C. brookmani[1]	662	2	2712	2647	385	1	521	20	6950
C. cacticola/ C. torridus[2]			442	407	4		6	19	878
C. californiensis			3	2					5
C. copiosus			2						2
C. crepuscularis							1		1
C. defoliarti			3	1					4
C. freeborni			3	1	1		1		6
C. kettlei			2						2
C. lahontan			12		10		2		24
C. luglani			1						1
C. mohave	7	1	22	23	218	1	76	57	405
C. reevesi	3		5						8
C. ryckmani[3]	2		569	518			5	1	1095
C. sitiens/ C. bakeri[4]			9	3		1			13
C. sonorensis	36		19	2	164	18	16	35	290

F: female, M: male.

[1] Midges identified as male *C. brookmani* include a small number of male *C. jacksoni* as these species are indistinguishable under a dissecting microscope, but close examination of a subsample under a compound microscope revealed 6% of males to be *C. jacksoni*.

[2] *Culicoides cacticola* and *C. torridus* cannot be separated morphologically or molecularly (Zhang et al., in prep.) and are therefore grouped.

[3] Midges identified as female *C. ryckmani* include a small number of female *C. copiosus* as these species are indistinguishable under a dissecting microscope, but close examination of a subsample under a compound microscope revealed 6% of females to be *C. copiosus*.

[4] *Culicoides sitiens* and *C. bakeri* are difficult to separate morphologically and cannot be separated molecularly (Zhang et al., in prep.) and are therefore grouped.

Table 4.2: The outcome of negative binomial regression and Poisson regression for female *Culicoides* species.

Culicoides species	Regression method	Variables	Terms	Estimate	Estimate (exp)	Z-value	P-value	
C. brookmani[1]	Negative binomial	Trap type	UV trap	0.874	2.397	2.788	0.0053	**
		Location	Flood plain	-0.917	0.4	-2.849	0.0044	**
		Date	7/24/2019	-1.742	0.175	-4.876	< 0.0001	***
			8/21/2019	0.652	1.919	1.966	0.0493	*
			7/18/2020	0.004	1.004	0.012	0.9902	
		Interactions	UV*Flood plain	-1.263	0.283	-4.259	< 0.0001	***
			UV*7/24/2019	1.123	3.074	2.774	0.0055	**
			UV*8/21/2019	1.079	2.94	2.714	0.0066	**
			UV*7/18/2020	0.635	1.886	1.419	0.1560	
			Flood plain*7/24/2019	2.974	19.57	7.314	< 0.0001	***
			Flood plain*8/21/2019	-0.782	0.458	-1.948	0.0515	.
			Flood plain*7/18/2020	-0.456	0.634	-1.019	0.3083	
C. cacticola/ *C. torridus*[2]	Negative binomial	Trap type	UV trap	4.557	95.284	8.499	< 0.0001	***
		Location	Flood plain	-3.032	0.048	-7.691	< 0.0001	***
		Date	7/24/2019	-3.768	0.023	-6.898	< 0.0001	***
			8/21/2019	-1.249	0.287	-3.445	0.0006	***
			7/18/2020	-1.202	0.301	-2.865	0.0042	**
C. mohave	Poisson	Trap type	UV trap	0.795	2.215	1.657	0.0974	.
		Location	Flood plain	3.439	31.143	8.955	< 0.0001	***
		Date	7/24/2019	-0.523	0.593	-2.873	0.0041	**
			8/21/2019	-0.16	0.852	-0.979	0.3277	
			7/18/2020	-1.099	0.333	-4.944	< 0.0001	***
		Interactions	UV*Flood plain	-2.017	0.133	-4.437	< 0.0001	***
			UV*7/24/2019	-0.061	0.94	-0.162	0.8714	
			UV*8/21/2019	0.715	2.045	2.323	0.0202	*
			UV*7/18/2020	1.06	2.887	2.444	0.0145	*
C. ryckmani[3]	Negative binomial	Trap type	UV trap	5.72	304.872	7.914	< 0.0001	***
		Location	Flood plain	-4.523	0.011	-9.496	< 0.0001	***
		Date	7/24/2019	-3.507	0.03	-8.313	< 0.0001	***
			8/21/2019	-0.066	0.936	-0.284	0.7768	
			7/18/2020	-1.119	0.327	-3.913	0.0001	***
C. sonorensis	Poisson	Trap type	UV trap	-0.625	0.535	-2.204	0.0275	*
		Location	Flood plain	1.2	3.321	5.864	< 0.0001	***
		Date	7/24/2019	-2.42	0.089	-4.639	< 0.0001	***
			8/21/2019	-2.42	0.089	-4.639	< 0.0001	***
			7/18/2020	-2.99	0.05	-4.135	< 0.0001	***
		Interactions	UV*Flood plain	-1.39	0.249	-3.595	0.0003	***
			Flood plain*7/24/2019	1.301	3.671	2.338	0.0194	*

Culicoides species	Regression method	Variables	Terms	Estimate	Estimate (exp)	Z-value	P-value
			Flood plain*8/21/2019	0.713	2.039	1.237	0.2160
			Flood plain*7/18/2020	1.259	3.52	1.641	0.1007

***: P-value < 0.001

**: P-value < 0.01

*: P-value < 0.05

Figure 4.1: Species richness per trap night by A) trap type and B) trapping location.

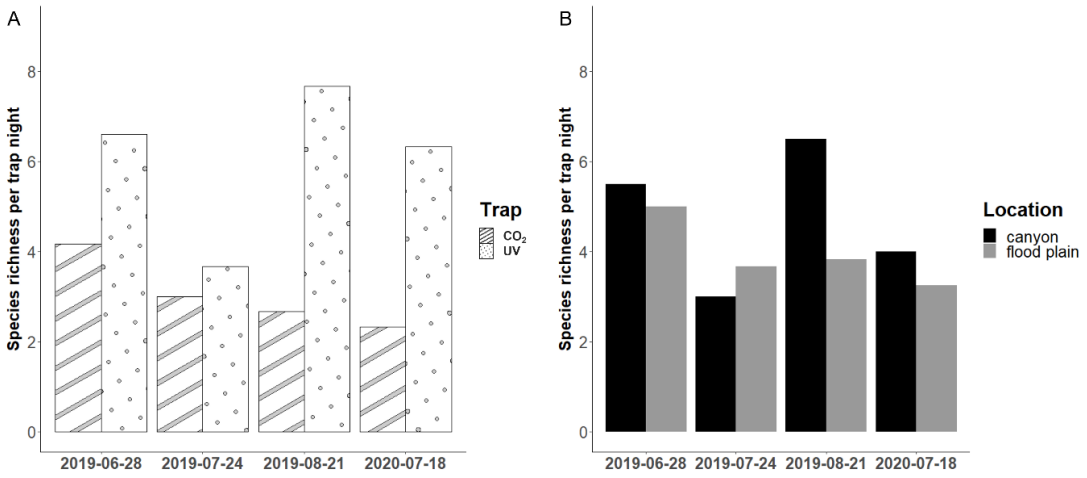
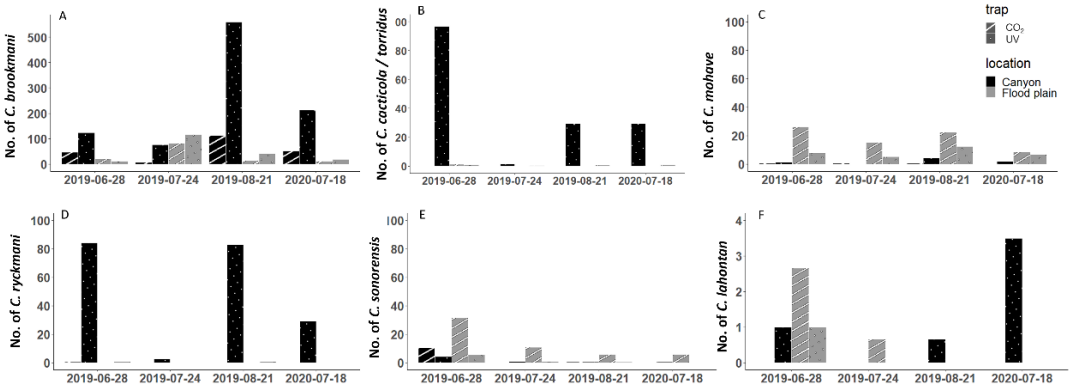


Figure 4.2: Number of female midges collected per trap night by trap type and location. A) *C. brookmani*; B) *C. cacticola* / *C. torridus*; C) *C. mohave*; D) *C. ryckmani*; E) *C. sonorensis*; F) *C. lahontan*.



CONCLUSION

Culicoides (Diptera: Ceratopogonidae) biting midges are notorious for transmitting several viruses to animals or humans, such as bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), African horse sickness virus (AHSV), Schmallenberg virus (SBV), and Oropouche virus (OROV) (Mellor et al. 2000, De Regge et al. 2012). Bluetongue disease, caused by BTV, has received much attention due to its rapid spread worldwide and the great economic impact (Tabachnick 1996).

In southern California, BTV has been transmitted over years between *C. sonorensis* and cattle in dairies (Gerry et al. 2001). Two questions that interested me most were 1) whether the diel pattern of host-seeking activity varied across seasons as a result of changes in temperature and other environmental conditions, and 2) how BTV survives through the interseasonal period in southern California. To study these two questions, I conducted two long-term projects over a three-year period (2018-2021) to observe the diel host-seeking activity of *C. sonorensis* and to detect BTV in *Culicoides* captured during the winter months (the interseasonal period).

Seasonal Change to Diel Host-Seeking Activity

Traps baited with CO_2 on the dairy collected mostly non-fed host-seeking parous and nulliparous *C. sonorensis* and these traps will capture host-seeking midges at any time of the day in contrast to light traps which capture midges only during hours of darkness because they cannot compete with sunlight. While the start of host-seeking did shift forward to an earlier time during the cooler winter months, this shift was not as substantial as I had hypothesized prior to my

research study. I had hypothesized that peak host-seeking activity would occur prior to sunset during the cool winter months and that such a shift in host-seeking activity would be missed by surveillance efforts using light traps or even CO_2-baited traps that were turned on at sunset. However, this study showed that while host-seeking activity did start at an earlier time relative to sunset, during the winter months, peak host-seeking activity mostly occurred during sunset or after sunset.

Diel activity varied across seasons with the start of host-seeking, peak host-seeking time, and host-seeking period having association with environmental conditions including wind speed and relative humidity. Temperature was found to relate to the start of host-seeking and host-seeking period, but not to the peak host-seeking time. Moon was found to relate with the start of host-seeking and peak host-seeking time. Seasonality also influenced the host-seeking period and peak host-seeking time, but the effect was modified by other environmental factors. The interactions found between environmental factors indicated the complexity of predicting the diel host-seeking pattern of *C. sonorensis*.

BTV Overwintering in Adult Midge Population in Southern California

The same serotypes of BTV have been repeatedly detected in southern California cattle across many years (Osburn et al. 19810, Stott et al. 1985, Gerry et al. 2001, Mayo et al. 2012), suggesting BTV has continuously persisted in the region rather than being reintroduced each year. To persist, BTV must survive through the cooler winter season in southern California which last from December through March or so. In locations with extreme winters, such as Colorado, midge activity is absent during the winter months. Winter temperatures are milder in southern

California and midges can be captured throughout the year; however, BTV was detected in these midges only during November and December with no BTV detected in midges captured from January through April. The capture of host-seeking nulliparous midges throughout the December – April interseasonal period suggests that immature midges continue to develop and emerge as new adults throughout the winter months in southern California. Additionally, the capture of host-seeking parous midges suggests that midges do not undergo a diapause during the winter period.

While BTV could potentially remain within vertebrate hosts to overwinter, cattle infected with BTV have infectious viruses for up to 60 days (MacLachlan et al. 1994, Maclachlan et al. 2009, Mayo et al. 2014), after which midges cannot acquire the virus when feeding on these hosts. It is possible that other animal hosts may be infectious for a longer period, but other ruminant hosts are rare in the dairy production regions of southern California where *C. sonorensis* is most abundant. Additionally, BTV is not passed vertically from female midges to their offspring (Osborne et al. 2015). Therefore, combining former findings (Mayo et al. 2014), I suggest the possibility of two BTV overwintering mechanisms in the southern California dairy region: 1) long-lived adult *C. sonorensis* are infected by BTV and survive through the winter; 2) a low transmission cycle continues between *C. sonorensis* and cattle. Further research is needed to explore the primary BTV overwintering mechanisms by increasing midge collection size, targeting resting parous midges, and involving sentinel animals.

Culicoides Species in the Southern California Inland Desert

Despite their ability to transmit several viruses and cause a huge impact on the animal industry, *Culicoides* species overall have not been well studied. For example, very little is known about the *Culicoides* species that live in the southern California inland desert where protected bighorn sheep suffer from infection with BTV and EHDV vectored by one or more of these *Culicoides* species. The known vector species, *C. sonorensis,* is not abundant in this area suggesting that other species are important vectors of these viruses to local wildlife. The investigation of hemorrhagic disease epidemiology, including determining the vectors of these viruses has been impeded by the difficulty of identifying *Culicoides* species in the California deserts. Our study combined morphological and molecular methods to identify *Culicoides* species and provide sequence information for desert *Culicoides* species in the U.S. to support later research on these species. The molecular analysis of desert midges shows that some subgenera of *Culicoides* are not monophyletic and further shows that cryptic species may be present (e.g., within the *C. cacticola* / *C. torridus group*).

Eighteen distinct *Culicoides* species and one species aggregate (*C. cacticola*/ *C. torridus*) were identified from the Deep Canyon, and COI and/or 28S rDNA gene sequences for 16 North American *Culicoides* species were newly added to the GeneBank database. *Culicoides* species of both sexes and immature midges could all be identified easily and linked together using gene sequences. The deposit of midges specimens and their related gene sequences will help future researchers who want to look at desert *Culicoides* species or want to work on related topics such as investigating potential vectors of hemorrhagic disease viruses.

It is not surprising that there are many *Culicoides* species in the southern California desert area because of the variety of topology and plant types that makes a variety of microhabitat for different midge species (Mullens and Dada 1992). The abundance of different vertebrates also provides female midges with a variety of blood meal sources. Our study shows that cryptic or undefined species may exist in the desert region. Further collection in the desert is needed to sample more *Culicoides* specimens to identify cryptic species. Additionally, as there are many *Culicoides* species that habit in the desert, where their immatures live and how to identify immature midge species also attract attention. Future studies to explore the ecology of immature *Culicoides* species will broaden our understanding of desert-dwelling midge species, and molecular identification will assist with the identification of immature midge species which can be much more difficult to identify than the adult stage of these species.

Trap and Location Comparison in Catching Desert *Culicoides* Species

Culicoides species captured in the southern California desert responded differently to traps baited with UV light or CO2. UV light traps collected more midges than CO2 traps for most *Culicoides* species, and exceptions were *C. sonorensis* and *C. mohave*: *C. sonorensis* were collected in greater numbers by CO_2 traps than by UV traps, and *C. mohave* were collected more in CO_2 traps in the flood plain while were collected more in UV traps at the canyon mouth. According to the collection, I would suggest using a combination of both CO_2 traps and UV light traps to collect as many *Culicoides* species as possible unless we are targeting a specific midge species. At the Deep Canyon research station, most midge species were more abundant at the canyon mouth than in the flood plain, while *C. sonorensis* and *C. mohave* were collected more in the flood plain than at the canyon mouth. Biting midges, especially males, captured in greater

numbers at each location are likely developing as immatures in the surrounding habitat. Species that were captured in greater numbers in the flood plain may be associated with human activity since these traps were near a housing area and golf course. Many midge species were only captured in low numbers, which suggests that they were not abundant in the location or they are not attracted by either attractant. For these species, it is necessary to expand the trapping location to include deeper in the canyon or higher in the elevation to see if we can increase their number of catches. Future research to collect less common or abundant *Culicoides* species will also improve our understanding of desert midge species and their general ecology. Understanding how each midge species responds to various trapping systems will assist with surveillance or monitoring programs. Identifying the dominant habitat of *Culicoides* species will help in the control of these midges by providing target locations for control efforts.

Overall, there is much more to explore in the field of *Culicoides* biting midges. Continuing to study the ecology, biology, and related topics of midges will contribute to this field and tell more good stories.

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