Saccharine Ceratopogonids: Sugar feeding ecology and behaviors of *Culicoides* biting midges (Diptera: Ceratopogonidae).

by

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Abstract

Culicoides is a diverse genus of approximately 1,300 species of small biting flies found across the globe (Borkent and Dominiak 2020), containing numerous species that can transmit viral pathogens which cause mortality in livestock and wildlife and can infect people. These diseases have no direct treatment, so prevention through vector control is paramount. Current surveillance and control methods are limited by a lack of ecological knowledge of *Culicoides*, particularly phytophagous (sugar feeding) behavior. The plants fed on by *Culicoides* are largely unknown, as are the driving forces behind choosing these plants. This information could be vital for improving control strategies through increased trap attraction and/or application in other management methods. To determine what plants are being fed on, *Culicoides* were collected from northeastern Kansas and sugar meal analysis performed on plant DNA extracted from midge bodies. Biting midges were found to feed on a diverse group of plant families, including Amaranthaceae, Solanaceae, and Cupressaceae, indicating multiple sugar sources including nectar and fruit are utilized under natural conditions. Using two-choice behavioral bioassays, the role of sight and color preference in sugar feeding choice behavior was analyzed in laboratory colony midges. When presented with differently colored simulated flowers in the lab, *Culicoides* exhibit a preference for red over purple. However, a strong preference also existed for the flower on the right side of the assay chamber, indicating other cues such as inflorescence type or olfactory stimuli may also be important and should be further investigated.

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Dedication

I dedicate this thesis to all the *Culicoides* that contributed to this project. Just because you are small does not mean you are not worthy of being seen.

Chapter 1 - *Culicoides* phytophagy: reviewing ecology and behaviors as an avenue for surveillance and control

1. Introduction to *Culicoides* Biology

Culicoides biting midges (Fig. 1.1) are small flies (1-3mm) in the family Ceratopogonidae. With a near global distribution (Mellor et al. 2000) they are known to transmit several important viruses and even filarial parasites (Carpenter et al. 2013). For most species, females are hematophagous (blood feeding) and will take multiple blood meals from a variety of hosts throughout their adult stage (Fig. 1.2) to produce up to approximately 3,000 eggs (Connelly et al. 2005). Adults mate in swarms (Zimmerman et al. 1982), and eggs are laid on a variety of moist substrates including soil, manure, on the periphery of water sources such as ponds, and even tree holes (Borkent 2014). Larvae are semi-aquatic (Wirth and Hubert 1989) and depending on species and environment, this stage may last anywhere from weeks to months. During this stage, immature *Culicoides* develop through four instars and feed on small semi-aquatic organisms, bacteria, and algae, and (Connelly et al. 2005, Aussel & Linley 1994). Larvae pupate and emerge 2-3 days later (Erram & Burkett-Cadena 2020, Erram & Burkett-Cadena 2021). Overwintering largely occurs in the larval stage, although it has also been reported in adults (McDermott et al. 2017, Rawlings & Mellor, 1994).

2. *Culicoides* as vectors

Bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), and African horse sickness virus, all of which affect hoofstock and wild ruminants at varying severities are the most well-known veterinary pathogens transmitted by biting midges (McGregor and Lewis

2023 McGregor et al. 2019; Riddin et al. 2019). Several species of *Culicoides* have been implicated as important vectors of BTV and EHDV in the U.S., although only *Culicoides sonorensis* Wirth and Jones and *Culicoides insignis* Lutz meet the WHO criteria as competent vectors (McGregor et al. 2022). Criteria include that the pathogen must be detected in field-collected midges, midges must become infected after taking a blood meal from a source containing the pathogen, infected midges must also be able to transmit the pathogen while feeding, and there must be overlap in habitat use (spatially and temporally) between midges and infected hosts in the field (WHO 1967). While other *Culicoides* species have been shown to meet some of these criteria, but more research is needed to determine their role as vectors. Putative *Culicoides* vectors associated with sylvatic (wild) animals include *Culicoides stellifer* (Coquillett), *Culicoides venustus* Hoffman, and *Culicoides haematopotus* Malloch, among others, which occupy a large range of habitats and feed on diverse vertebrate host species (McGregor et al. 2022). None of these sylvatic species have been fully confirmed as virus vectors.

Although the importance of *Culicoides* species as vectors of livestock pathogens has been well-established, there has also been recent interest in their role as vectors of human pathogens. Oropouche virus is a midge-borne human pathogen that has caused outbreaks of Oropouche fever in South and Central America (CDC 2024, Sakkas et al. 2018). The primary putative vector of Oropouche virus is *Culicoides paraensis* (Goeldi), which is found in urban environments and has been shown to be capable of transmitting the virus under laboratory conditions (Pinheiro et al. 1982, Linley et al. 1983). At the time of writing, this outbreak is recent and the vector status of other *Culicoides* species for Oropouche virus has yet to be assessed.

3. *Culicoides* Ecology

Historically, work on *Culicoides* ecology has focused on seasonal and diel activity of species, identifying blood meal hosts and rates of blood feeding, and life history studies investigating larval habitats.

3.1 Seasonality

In North America, surveillance by light traps has shown *Culicoides* to be most abundant during the summer, with population peaking around June for many species (Lysyk 2006). Quaglia et al. (2020) found that in Florida, midge communities do not follow a typical annual four-season phenology, instead cycling through wet and dry seasons with patterns potentially extending over several years. Interestingly, this study also found that species composition and richness follow a nested pattern, with the highest richness and abundance of midges occurring during spring and decreasing throughout the other seasons. Unique species were not seen emerging during other seasons; rather it appeared that different species were "lost" from the community through the year, returning in spring (Quaglia et al. 2020). The seasonality of such a diverse group is understandably varied, potentially aiding biting midge success through temporal partitioning. Throughout the year, *Culicoides* populations generally reach peak abundance in the U.S. from May through October, disappearing during the winter in northern states and persisting in the south (reported in southern California and Florida) (Barnard and Jones 1980b, Zhang et al. 2024, Lillie 1985). A similar trend can be seen in Europe; however, initial emergence of populations differed between species and region, with vector seasons beginning as early as January and as late as September (Cuéllar et al. 2018). Climate change potentially impacts seasonality in midges, with range expansions and lengthening of seasons possibly enhancing midge presence (Hudson et al. 2023).

3.2 Diel activity

Culicoides are largely crepuscular, being most active at dawn and dusk (Barnard and Jones, 1980b). However, trapping methods may bias studies of diel activity. For example, in a study comparing captures of *Culicoides impunctatus* Goetghebuer over time between suction traps and light-suction traps, Blackwell (1997) found two clear peaks of midge activity around dawn and dusk when using the suction-only traps, and a single peak of activity through the night when using light-suction traps. This is possibly due to the competitively attractive nature of a light trap in the dark, although this can be affected by moonlight (Nelson and Bellamy 1971). Nelson and Bellamy found that *C. sonorensis* (reported as *Culicoides variipennis* (Coquillett)) activity was greater during moonlight hours compared to dark night hours. Using truck traps, Sanders et al. (2012) found a clear increase in flight activity around sunrise and more so at sunset for multiple species in southern England. They also noted that environmental conditions such as wind speed and temperature affected *Culicoides* diel activity, with lower activity from all species at wind speeds above $4ms^{-1}$ and most species at temperatures above 21°C. Blood-feeding commonly occurs during dusk and dawn, but this is not the case for all species. While *C. variipennis* and *Culicoides guttipennis* (Coquillett) are crepuscular feeders, *C. paraensis* only feeds during the day (Foulk 1969, Lillie et al. 1988). The rest of the diel period is spent either resting, mating, or sugar-feeding.

3.3 Resting behaviors

Resting behaviors of biting midges are largely unknown. Reported resting sites are varied, including grass, lichen and moss, trees, and stones (Carpenter et al. 2007, Mondal et al. 2022). In Mondal et al. 2022, different-colored sticky resting boxes (initially developed for mosquito surveillance) were introduced in cattle sheds to evaluate their efficacy in capturing

Culicoides and investigate their behaviors. They found that dark, smooth surfaces were preferred, and height preference differed by sex. Interestingly, males were seen in the open more so than females, who were observed sheltering in crevices. Additionally, resting sites differed depending on physiological status, with nulliparous females resting higher than blood-fed or gravid females (presumably due to increased weight of blood meal/eggs). Another study by McGregor et al. (2018) found similar results, with ground traps collecting a greater proportion of gravid individuals than other physiological states compared to traps set in tree canopy. However, all states were collected in both traps and overall abundance of gravid individuals was higher in the canopy traps. In addition to weight playing a role in resting strata, McGregor et al. (2018) proposes that the increased proportion of gravid females in ground traps may be due to females searching for oviposition sites, rather than simply resting.

4. Current surveillance and control

Biting midges are notoriously difficult to colonize. Although 15 species of *Culicoides* have been studied for colonization, only two species' (*Culicoides nebeculosis* and *C. sonorensis*) colonies remain extant (Nayduch et al. 2014). As such, much of the vector biology knowledge for these species has been derived from surveillance studies in the field or from laboratory studies with colonies of a single species, *C. sonorensis*.

4.1 Immature surveillance

Surveillance of immature midges is done by collecting larval substrate (sediment along the edge of water bodies, contents of tree-holes or moist organic matter) and either directly identifying midge larvae/pupae or allowing midges to eclose under laboratory conditions and identifying the adults. Collections of eggs, larvae, and pupae have been made in the field to

better understand female oviposition preferences and larval biology (Wong et al. 2017, González et al. 2013, McDermott and Lysyk 2020). These forms of surveillance are particularly important for understanding overwintering ecology of *Culicoides*, which may help identify ideal times and locations to employ immature midge control (Barnard and Jones, 1980a).

4.2 Adult surveillance

Most adult midge surveillance is conducted using suction traps. While styles and attractants may differ, the basic principle is the same; an attractant (usually light) is positioned near a fan to attract flying insects. The fan pulls insects through a mesh into a container. Some specific examples include CDC miniature light traps, Onderstepoort traps, and Rieb light traps (Venter et al. 2009). Light traps are widely used due to their relative ease of use and high collection capacity, both in terms of midge number and diversity of species (McDermott and Mullens 2018). Although generally effective, light traps may be impacted by temperature, with more midges caught in cold traps. This effect was more pronounced the greater the difference between the trap and ambient temperature (Hochstrasser et al. 2024) and could be due to thermal preference for survival (Wittmann et al. 2002).

Light sources may also influence capture rates (McDermott and Mullens 2018, Bishop et al. 2006, Hope et al. 2015). For example, in a field study conducted by Hope et al. (2015), while most *Culicoides* species were preferentially captured using traps fit with UV bulbs compared to a range of LED wavelengths, *Culicoides brunnicans* preferred green LED to UV bulbs. Among the LED wavelengths, more midges were caught using blue or green LEDs compared to white, yellow, red, or UV LEDs. Differences in light attraction have been shown in the lab as well; Snyder et al. (2016a) found that *C. sonorensis* were more attracted to UV wavelengths than red, green, blue, or no light. The same study also found that attraction to light can be affected by a

light's intensity; with brightness positively correlated with attraction. A midge's infection status with bluetongue virus has been shown to influence light-seeking behavior as well, with infected midges becoming less attracted to UV-light (McDermott et al. 2015). As a result, infection rates in wild caught midges may underrepresent actual rates of infection.

Traps may be baited with other attractants to improve capture rates. Using CO_2 has been shown to increase midge captures overall, with an increase in the proportion of host-seeking females (McDermott and Mullens 2018). Research has also investigated the use of other attractive volatiles, including hexanoic acid and 1-octen-3-ol (Harrup et al. 2012). Active capture surveillance, sweep nets, truck traps, and direct host animal aspiration may also be employed (Sanders et al. 2012, Gerry et al. 2009), although these methods are less common. Accurate surveillance efforts are important to guide where control methods should be deployed and are used to evaluate the efficacy of developed controls.

4.3 Control methods

While several control methods have been proposed and are currently being researched, few have been approved or are widely utilized. A unique challenge of biting midge control compared to other vectors is their size. Nets and barriers are challenging to develop for an insect so small, and little is known about where these physical controls would be most effective.

Insecticidal chemicals are a popular control method for many insect pests, however few pesticides have been specifically labelled for commercial use against *Culicoides* (Lawson and McDermott 2023). Studies have shown the effectiveness of pyrethroids in the lab, especially deltamethrin, against *Culicoides* adults (Venail et al. 2015). Organophosphates were also evaluated, but whereas high mortality rates were achieved, these insecticides were less toxic than the pyrethroids. Although results are promising, more research is needed to determine proper

dosages and applications of these insecticides. Improper use of pesticides not formulated for *Culicoides* may be used by producers as off-label treatments, leading to possible insecticide resistance. Additionally, chemical controls applied to livestock may reduce biting rates by female midges but may not reduce the overall *Culicoides* population due to lack of male and immature control.

Mechanical control methods, such as larval habitat reduction, have proven to be difficult to achieve on a large scale, as *Culicoides* have been shown to utilize a wide range of larval habitats, not all of which are well-understood (Harrup et al. 2016). In areas where multiple midge species are present, multiple larval habitats may require removal. Even then, one study found that habitat reduction had little to no effect on bluetongue virus transmission (Mayo et al. 2014). A common suggestion to stakeholders is to reduce the larval habitat of *Culicoides* to halt population growth and re-establishment. Harrup et al. (2014) however found that this solution is not always effective and reported that *Culicoides* control is complex and challenging. Physical/mechanical controls, namely stabling livestock, have also been investigated. Stabling livestock can be difficult due to practical limitations for certain livestock operations, and *Culicoides*' small size and species-specific preferences for shelter restricts the efficacy of this method (Carpenter et al. 2008).

Alternative control methods such as RNAi have been proposed; however very little is known about these methods in *Culicoides* and how to effectively implement them (Osborne et al. 2023). Chemical repellents have also been explored for use against *Culicoides*. Traditional repellents such as DEET and plant-derived oils have been successful in laboratory assays and field experiments (González et al. 2014). Methyl salicylate and isothiocyanates in particular were shown to have potential in push-pull management systems (Blackwell et al. 1997).

Attractive Toxic Sugar Baits (ATSBs) have been proposed as control/surveillance systems in which midges are attracted to a sugar source that is laced with an insecticide. ATSBs have the potential to control both female and male adult *Culicoides*, as both sexes sugar feed. Pyrethroids, neonicotinoids, and sugar alcohols such as erythritol and xylitol are effective when used in ATSBs, causing up to 100% mortality in some cases (Rochlin et al. 2022, Cohnstaedt and Snyder 2016). Additionally, the diminutive size of biting midges means most non-target organisms can be excluded from the trap simply by use of tightly woven mesh, as with the ATSB design proposed by Cohnstaedt and Snyder (2016). Unfortunately, trap attractiveness was expected to be poor at a distance and the proposed system required weekly maintenance.

With more research, the potential for a *Culicoides*-specific effective ATSB with few non-target effects is achievable. To reach this goal, more research is needed on *Culicoides* sugar feeding behavior and ecology. These ATSBs must be able to compete with the environment to effectively attract midges. One approach to this is to more accurately represent the sugar sources *Culicoides* naturally prefer. However, this is largely unknown, as are the factors driving potential preferences.

5. Phytophagy behavior of *Culicoides*

Very little is known about the sugar-feeding behaviors of biting midges. Sources utilized in the field, preferences, and driving forces are largely unknown, and are likely to vary across habitats and species. In *Culicoides*, several sugar sources have been proposed including floral nectar, honeydew (sugary liquid waste produced by aphids and other insects), extrafloral nectar, and plant sap. While strong evidence exists for midges using floral nectar and honeydew as well

as feeding from dried fruit under laboratory conditions (Kaufmann et al. 2015, Jones 1964), to the author's knowledge no substantive evidence for extrafloral nectary use exists in the literature.

Field studies have focused on how much sugar is present in a population at certain times, rather than where the sugar meal originates (Stewart and Kline 1999). Historically, rates of sugar feeding have been determined by anthrone assays (Stewart and Kline 1999), in which a colorimetric reaction indicates fructose presence (Van Handel 1984). The first record of anthrone analysis in *Culicoides* investigated sugar feeding and follicular development in *Culicoides melleus* and *Culicoides hollensis* (Magnarelli 1981). This study noted rates of sugar fed midges caught were consistently above 50%, regardless of time or site. They also proposed that sugar meals were more important for maintenance and flight rather than for egg development in these species. Magnarelli noted that multiple sugar meals may be taken throughout the course of a day to fuel daily activities. Another study which collected *C. sonorensis* (reported as *C. variipennis*) found sugar feeding rates ranging from 3-67%, lower than Magnarelli, which was attributed to potential differences in timing of feeding between species (Mullens 1985). In Magnarelli's study, midges were collected by aspiration from humans throughout the day whereas Mullens collected midges for a shorter period in the evenings using sweep nets and CDC miniature suction traps. Interestingly, significantly more parous females were found to be sugar positive than nulliparous females and males in this study. In 1999, Stewart and Kline investigated sugar feeding with regards to diel activity, finding that *Culicoides mississippiensis* fed throughout the day and potentially at night. Midges were collected directly from flowering plants from sunrise to sunset, and the rate of sugar fed midges was much more comparable to Magnarelli, at 55.6% (Stewart and Kline, 1999).

6. Phytophagy of related insect systems

Inferences may be made by looking at research conducted in mosquitos, which are related and more studied insects. Mosquitos and biting midges are both Culicomorpha and are both vectors of pathogens. *Culicoides*, like mosquitoes, also have well-developed eyes (McDermott et al. 2015), suggesting that they share some common physiology and behaviors, and implying that mosquito research techniques can be applied to biting midges with some modifications.

Vision, and color in particular, has been shown to play a role in mosquito behavior, with certain colors being attractive or repellent (Brett 1938, Burkett and Butler 2005). For example, Dieng et al. (2018) designed an assay to determine mosquito floral choice behaviors, based on previous research which found that vision is important to mosquito foraging and resting. *Aedes aegypti* mosquitos were presented with four artificial flowers of two different colors in equal choice bioassays and allowed to forage while a human observer monitored their behaviors (Dieng et al. 2018). This study found that *Ae. Aegypti* preferred colored flowers over white flowers for both resting and as a sugar source, and that these mosquitos often approached colored flowers faster than white flowers. This assay has the potential to be very useful for the development of ATSBs for *Culicoides*, with some changes in methodology. For instance, biting midges are much smaller than mosquitos, and so behavioral monitoring by human-eye may be challenging, instead requiring use of a camera and video-tracking software, as is used in Hochstrasser et al. (2024) to track *Culicoides* movement and thermal preference. Patterns and inflorescence of flowers may also be important cues. In 2016, Bernáth et al. investigated the ability of *Anopheles gambiae* to recognize patterned cards associated with either sucrose or sucrose and salt. These mosquitos were able to differentiate between different patterns and even

learn to associate different patterns with different sugar solutions. Differential attraction to flower UV-fluorescence was investigated in 2019 by Peach et al., finding UV patterns to be attractive to *Culex pipiens* mosquitos, driven mainly by UV darkness. Given the importance of visual cues to mosquitoes, it seems likely that visual cues may also be important for midges to find sugar sources for feeding and are worth investigating.

Volatile chemicals such as linalool oxide have been found to be attractive, while citronella is repellant to mosquitoes (Omondi et al. 2019, Kim et al. 2005). Volatile compounds collected from microbes present in nectar sources have also been shown to be attractive to mosquitos and may be a driver in choice of sugar meals (Peach et al. 2021). These chemicals may be good candidates for developing midge attractants/repellants.

7. Concluding remarks

Improved knowledge of *Culicoides* sugar feeding behaviors and ecology may lead to advancements in ATSB use and efficacy. ATSBs are the most promising method for biting midge control and have several advantages including attractiveness to both sexes, specificity of control, high mortality, and potential for implementation into IPM programs. These may also be employed in other integrated pest management techniques, such as push-pull or mating disruption. As well as their importance for vector control, further studies in this area are important for furthering our ecological knowledge of these flies, as pollinators. Mosquitos are not only vectors of pathogens, but also important pollinators (Shannon et al. 2024). *Forcipomyia* biting midges are known to be important pollinators of cacao (O'Doherty and Zoll 2012). A similar case may be seen in *Culicoides*, allowing scientists to focus on sustainable, targeted control, rather than total eradication of this incredibly diverse genus of flies.

Figure 1.1. Life cycle and developmental substrates of *Culicoides* biting midges. (A) *Culicoides sonorensis* female feeding through an artificial membrane; (B) Hatched and unhatched cigar-shaped eggs (length<2mm); (C) Active, semi-aquatic larvae of *C. sonorensis* with vermiform body shape (length <5mm); (D) Pupal stage (length <5mm); (E) Common organically enriched spring habitat in North America; (F): Tree-hole habitat in North America. Fig. A courtesy of the USDA-ARS Image Gallery; Figs. B-F courtesy of Bethany McGregor.

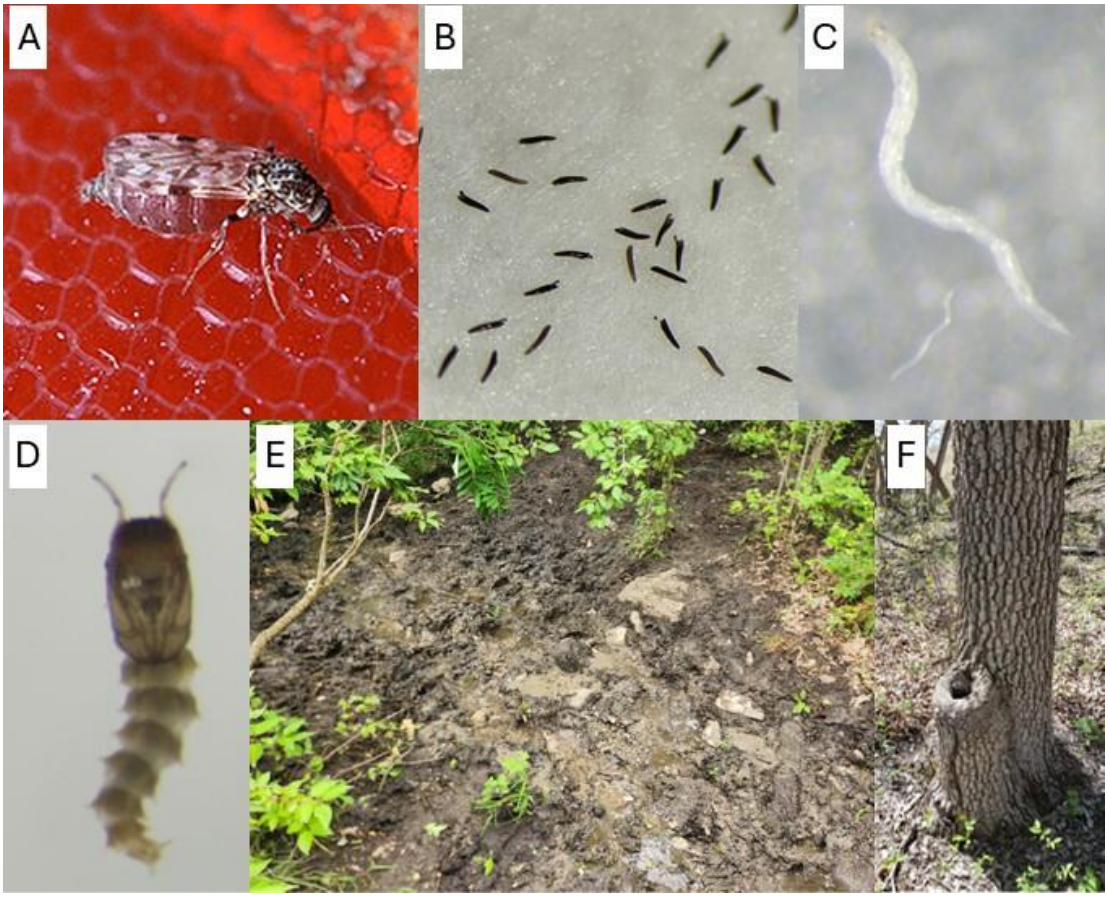
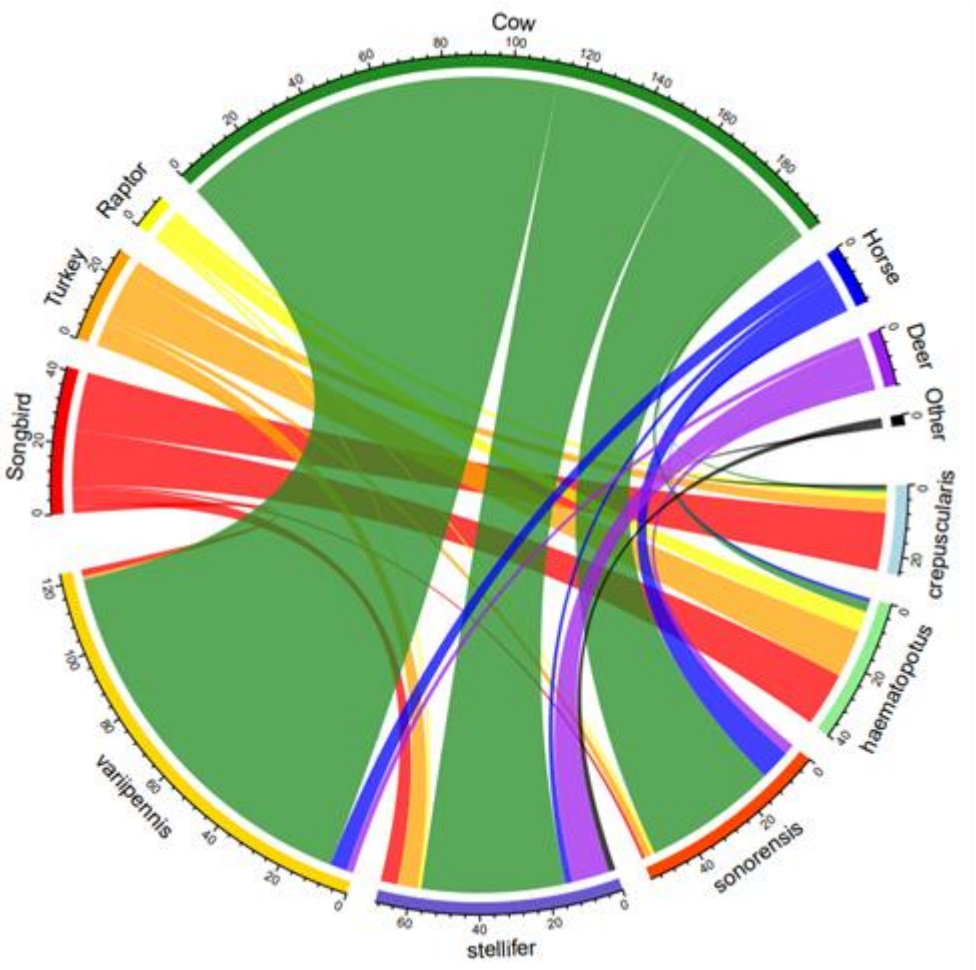


Figure 1.2. Chord diagram of vertebrate hosts fed on by *Culicoides*. Figure courtesy of Bethany McGregor (McGregor and Lewis, 2023).



Chapter 2 - Determining sugar-source associations of *Culicoides* biting midges (Diptera: Ceratopogonidae)

Introduction:

Culicoides biting midges are a diverse genus of small biting flies distributed across the globe, some of which are capable of transmitting pathogens to humans and animals (Purse et al. 2015). Surveillance and control of these flies is important to prevent disease outbreaks; however, efficacy of current control and surveillance methods can be limited due to a lack of ecological knowledge of *Culicoides* (Pfannenstiel et al. 2015). Seasonality and ecology vary across different midge species and this is important to consider for the timing of control applications. Novel control methods such as attractive toxic sugar baits (ATSBs) rely *Culicoides* sugar feeding behavior knowledge to effectively attract and trap midges. However, sugar sources fed on by *Culicoides* under natural conditions are largely unknown, as are the driving forces behind sugar source selection, making it difficult for ATSBs to compete with the natural environment.

ATSBs work by attracting insects to a sugar source laced with a toxic insecticide (Kline et al. 2018). ATSBs have been used successfully to control other Diptera species, especially those whose sugar feeding behaviors are better understood. Several studies have shown this method is effective for controlling mosquitos, with few non-target effects (Fulcher et al. 2014, Khallaayoune et al. 2013, Revay et al. 2014). Both male and female *Culicoides* are known to feed on sugar in nature and laboratory colonies survive much longer when provided a sugar source (Kaufmann 2015). Therefore, ATSBs have the potential to control not just host-seeking females, but males as well. ATSBs can also be carefully designed to exclude non-target organisms from the trap. This can be relatively simple with *Culicoides*, whose small size means

most non-target organisms can be excluded from the laced sugar source by a small-aperture mesh screen. An ATSB design using light as an attractant has been proposed for *Culicoides*, and while effective insecticides have been identified for use (Snyder et al. 2016b), attraction to the trap itself is likely low from a distance, hindering its efficacy (Cohnstaedt and Snyder 2016, McDermot and Mullens 2018). Traps could potentially be made more attractive to sugar-seeking midges if they were made to more closely resemble the sugar sources these midges are seeking in nature, allowing the ATSB to better compete with the surrounding landscape for *Culicoides* interest. However, little is known about how midges make use of sugar sources in a natural setting.

Numerous studies have shown rates of sugar feeding under laboratory and field conditions through use of cold anthrone assays (Van Handel 1967, Van Handel 1972). Anthrone assays give information on the ecology of *Culicoides* sugar feeding by combining crushed midges with a reagent which detects the presence of sucrose and fructose. The first record of anthrone analysis in *Culicoides* found sugar in 66% of *C. melleus* and 64% of *C. hollensis* captured June – August of 1977 (Magnarelli 1981). Sugar was seen in at least 46% of samples regardless of habitat or month, although there was a significant peak in July. Another study which collected *C. sonorensis* (reported as *C. variipennis*) over the course of a year found that sugar meals were more prevalent in the population during winter and spring in California (Mullens 1985). These results may be due to regional/seasonal climate or may be due to differences in daily timing when collecting midges. In 1999, Stewart and Kline investigated sugar feeding with regards to diel activity, finding that *C. mississippiensis* fed throughout the day and potentially at night.

Through these studies we can understand the timing and rates of sugar feeding but less is known about which sugar sources are being used by midges in the field. One study by Kaufmann et al. (2015) found that midges survived longer when provided aphid-produced honeydew than extrafloral nectaries. Anthrone tests were conducted on these midges, confirming ingestion of honeydew. Very few midges consumed any sugar when provided in extrafloral nectaries but did feed on the nectar of other flowering plants. In the anthrone study conducted by Stewart and Kline (1999), *C. mississippiensis* were collected directly from *Ilex vomitoria*, confirming the plant as a nectar source.

No large-scale study has been conducted to assess the community of plants midges may feed on within their natural environment. With so many questions remaining related to *Culicoides* phytophagy (what plant sources are used, where, and why), there is a dire need for more research and analysis in this area. *Culicoides* are notoriously difficult to work with due to their small size and challenges in establishing laboratory colonies (Sick et al. 2019). Under both laboratory and field conditions, molecular techniques offer an option for analyzing sugar-source associations and population trends. Molecular forensics studies in mosquitos and sand flies have been done to determine what plants these flies visit and feed on (Nyasembe et al. 2018, Abbasi et al. 2018). Additionally, the use of molecular techniques is already the standard for *Culicoides* blood-meal analyses (McGregor and Lewis 2023), transmission trials (Rozo-Lopez et al. 2022), disease surveillance, taxonomic identification and population studies (Shults et al. 2022, Zhang et al. 2022). These indicate that the same molecular techniques can be applied to phytophagy studies in biting midges. The purpose of this study, therefore, was to analyze wild caught *Culicoides* and determine the plants utilized as sugar sources in a field setting via molecular sugar meal analysis.

Materials and Methods:

Sample Collection

Established in 1971, the Konza Prairie Biological Station (KPBS, 39.09306°N 96.55861°W) is a 3,487-hectare native tallgrass prairie preserve in northeastern Kansas that provides habitat for many vertebrates and over 500 species of plants. Five sites (C3SA Top, K1A2, N20B, SC Creek, and SC Spring) were chosen based on the presence of suitable *Culicoides* habitat (water levels suitable for larvae, shaded areas for adults to rest), potential vertebrate host presence, and floral presence. These sites were also selected to represent a range of *Culicoides* habitats, with differing water sources and livestock use. The sites used in this study included two ponds, two springs, and one creek.

The pond sites included one grazed by bison (N20B) and one formally ungrazed (access restricted from bison and cattle, but not wild grazing animals such as deer) site (K1A2). K1A2 was a small pond at the top of a hill, with vegetation surrounding the site and abundant organic material observed in the water body. Trees lined the south side of the pond, providing shade and a place for *Culicoides* to rest. Soil along the shaded edge of the pond was consistently moist, and plants were seen flowering nearby throughout the season. N20B was a larger pond at the bottom of a small hill, surrounded on all but one side by trees, tall grass, and shrubs. The east side of the pond was mostly mud, well-trodden by bison with pockets of water formed from hoof prints. A small creek ran from the pond on the opposite side, which was shaded.

Both spring sites were located within the cattle grazed watersheds at KPBS (C3SA Top, SC Spring). C3SA Top was a small spring located within a shady drainage area on the side of a hill, with notably dense and rocky soil. Several flowering plants were noted along the hillside.

SC Spring was a large spring surrounded by grasses, flowering plants, and a few trees with soil that was soft and well-trodden by cattle. SC Spring was much more exposed with less shading than C3SA Top.

SC Creek was a shaded creek situated at the bottom of two hills. There was evidence of frequent cattle use near this site, with manure consistently present and cattle occasionally seen during field work. Water levels fluctuated throughout the season and the creek bed regularly dried out in the summer. Flowering trees and plants were present uphill from the creek.

Using CDC miniature light traps baited with LED black light arrays (Fig.2.2), *Culicoides* midges were collected overnight from these five sites across KPBS (Fig. 2.1) biweekly from August to September 2023 and May to September 2024. Midges were collected into cups, which were then stored at -20˚C until midges were morphologically identified to species (Blanton and Wirth, 1979, Phillips 2022), sex, and physiological state (nulliparous, parous, gravid). In an attempt to examine plant associations across sites, species, and time, 10 midges of each species per site per date were randomly selected and assigned an identifying number, then stored individually in dry 1.5ml microcentrifuge tubes at -20˚C until plant DNA was extracted. In the case of less than 10 individuals of a species collected from a trap, extraction was performed on as many as were present.

Plant DNA Extraction

DNA was extracted from 788 individual midges. Due to optimization the extraction methods required changes resulting in three unique protocols. Initially, DNA was extracted from 80 midges by rinsing with a 1% chlorine bleach solution immediately prior to extraction to remove potential contaminants. Initial extractions were performed using a CTAB-m buffer (25ml Tris(1M), 35ml NaCl(5M), 5ml EDTA(0.5M), 2.5g CTAB, water up to 247.5ml, and 2.5ml β-

mercaptoethanol) and manual disruption of tissues with a sterile plastic pestle. Five µl Proteinase-K (pro-k, 10mg/ml) was added and samples were incubated in a water bath at 65˚C for 3 h. Following incubation, samples were processed using the DNEasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols and eluted in a volume of 200µl. DNA concentrations of samples were assessed using a Qubit dsDNA High Sensitivity kit (Thermo Fisher Scientific Inc, Waltham, MA, U.S.A.) and found to range from 0.10 - 3.29ng/µl.

To improve extraction efficiency, midges 81-300 were processed using method two. Individual midges were rinsed with a 1% chlorine bleach solution, then disrupted with 100µl DNA Shield (Zymo Research Operations, Tustin, CA, U.S.A.) using an OMNI bead mill (Omni International, Kennesaw, GA, U.S.A.) and 2.4mm stainless steel beads (Revvity, Inc, Waltham, MA, U.S.A.) at 3.10m/s for 2 minutes. Then, 5µl pro-k (10mg/ml) was added to each sample and incubated at 65˚C for 1 h in a heating block. Samples were then further processed using Zymo's Quick-RNA MicroPrep Kit (Zymo Research Operations, Tustin, CA, U.S.A.) following the manufacturer's protocols. DNA was eluted in 15µl of DNAase/RNAase-free water. While this method was far more efficient, optimization was hindered by unknown-source contamination determined by PCR (Table 2.2).

Due to this contamination, the remaining 488 midges were processed using method three as follows. A 1% chlorine bleach rinse followed by OMNI bead mill homogenization and pro-k incubation was performed as before. However, downstream processing was performed using a Puregene Tissue Kit (Gentra Systems, Inc., Minneapolis, MN, USA) following the manufacturer's instructions. This is a non-column-based method used to precipitate DNA. After incubation, samples were centrifuged at 16,000 x g for 5 min and the supernatant was transferred to a 1.5ml microcentrifuge tube containing 300µl isopropanol and 1µl glycogen. Samples were

then held at -80˚C for 1h to improve final DNA yield. After thawing at room temperature, samples were inverted 10 times and centrifuged at 16,000 x g for 1 min. Excess isopropanol was then carefully drained by inverting the tube onto absorbent paper, and samples were washed with 70% molecular biology grade ethanol. After centrifuging once more at 16,000 x g for 1 min, samples were carefully drained of excess ethanol as before and allowed to air dry for 30 min. Finally, DNA was eluted by adding 15µl DNase-free water and allowing samples to incubate at room temperature for 20 min. Qubit analysis resulted in DNA concentrations ranging 1.19 - 14.3ng/µl. All DNA samples were stored at -20°C.

PCR, Gel electrophoresis, and PCR product purification

Each DNA sample was amplified by PCR using two different primer sets, *rbcLa* and *trnH-psbA (rbcLa: 5`- ATGTCACCACAAACAGAGACTAAAGC -3`, 3`- GTAAAATCAAGTCCACCRCG -5`; trnH-psbA: 5`- GTTATGCATGAACGTAAYGCTC –3`, 3`- GCRTGGTGGATTCACAATCC –5`*), (Trujillo-Argueta et al. 2021, Loera-Sánchez et al. 2020). Water was used as a negative control and plant DNA extracted from leaf tissue (*Mimosa quadrivalvis* and an unidentified local angiosperm collected from?) as a positive control. In each reaction, 3µl of extracted DNA was used, along with 1µl forward primer (20µM), 1µl reverse primer (20µM), 12.5µl ProMega GoTaq Green master mix (Promega Corporation, Madison, WI, U.S.A.), and 7.5µl water. The PCR for samples with *rbcLa* had the following conditions: 95˚C for 3min followed by 35 cycles of 95˚C for 30s, 58.4˚C for 1min, 72˚C for 1.5min, followed by a final extension at 72˚C for 5 min (Trujillo-Argueta et al. 2021). The PCR for samples with *trnH-psbA* had the following conditions: 94˚C for 4min, 2 cycles of 94˚C for 45s, 53.6˚C for 45s, 72˚C for 1min, followed by 35 cycles of 94˚C for 45s, 53.6˚C for 45s, 72˚ C for 1min, followed by a final extension at 72˚C for 10min (Trujillo-Argueta et al. 2021). Samples were then visually

assessed for the presence of plant DNA by 1% agarose gel electrophoresis at 115V. Gels were stained with SYBR Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific, Waltham, MA) and electrophoresis was considered complete when tracking-dye bands (from GoTaq Green master mix) were 1cm away from the gel's edge. Gels were visualized using a blue light transilluminator. All PCR products positive for plant DNA were purified using QIAGEN's QIAquick PCR Cleanup Kit. In the case of multiple bands present in a lane, QIAGEN's Gel Excision Kit was used to separate and purify DNA from individual bands.

Sequencing and Basic Local Alignment Search Tool

For each sample where plant DNA was amplified, a solution of 5µl purified PCR product and 5µl corresponding forward primer was sent for Sanger sequencing by Eurofins (Eurofins Genomics LLC, Louisville, KY, U.S.A.). Resulting sequences were then trimmed and cleaned for quality using Geneious (version 2023.2.1, GraphPad Software, LLC, Boston, MA, U.S.A.), and compared to the National Institutes of Health (NIH) GenBank database using the National Center for Biotechnology Information (NCBI)'s Basic Local Alignment Search Tool (BLAST). In general, the first 10-150 base pairs were excluded due to quality and dye blobs, and occasionally the last 50-80 base pairs were also trimmed when their quality was poor. Calls were also made in cases where the software failed to call a clear base or miscalled. Sequences were then categorized following the workflow laid out in Figure 2.3, according to sequence quality post-trim. "High Confidence" results were those with a post-trim sequence quality of 50% or greater, "Moderate Confidence" were those with a post-trim sequence quality between 20 and 50%, and "Low Confidence" were those with a post-trim sequence quality between 10 and 20%. Sequences with a sequence quality less than 10% were discarded. A BLAST search was then done for each sample and the top three unique matches with a pairwise identity match of 95% or

greater (Fig. 2.3) were retained and recorded at the family level for all Confidence categories (Low, Moderate, and High). Further classification was made at the genus level for all categories except Low Confidence if the pairwise identity match was 97% or greater and all results provided by BLAST were from the same genus. If this genus is also found on Konza, the sample was recorded at the genus level. For the High Confidence category, if all results provided by BLAST were from the same species (99% or greater pairwise identity match) and the species is found on Konza, the sample was recorded at the species level.

Data Analysis

Total numbers of *Culicoides* collected across sites were analyzed for significant differences in site use using non-parametric Kruskall-Wallis and Wilcoxon Rank Sum tests. The number of sequences resulting in a successful plant family match was compared to the number of each midge species associated with the sequence and assessed for significant associations using non-parametric Kruskall-Wallis and Wilcoxon Rank Sum tests in R (version 4.4.3) and RStudio (version 2024.12.1+563). Packages used include ggplot2 3.5.1 (Wickham et al. 2024), ggpubr 0.6.0 (Kassambara 2023b), bipartite 2.21 (Dormann et al. 2025), rstatix 0.7.2 (Kassambara 2023a), and fmsb 0.7.6 (Nakazawa 2024).

Figure 2.1. Map of *Culicoides* collection sites sampled across KPBS

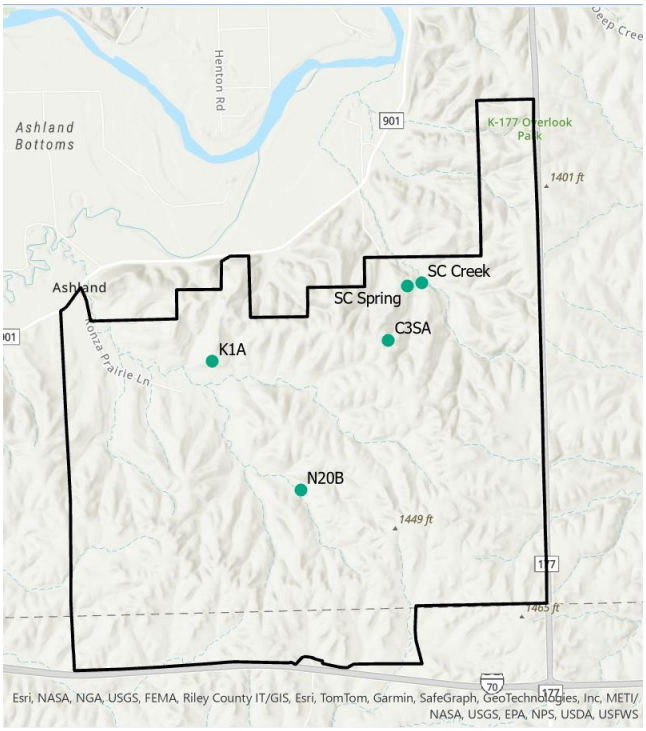
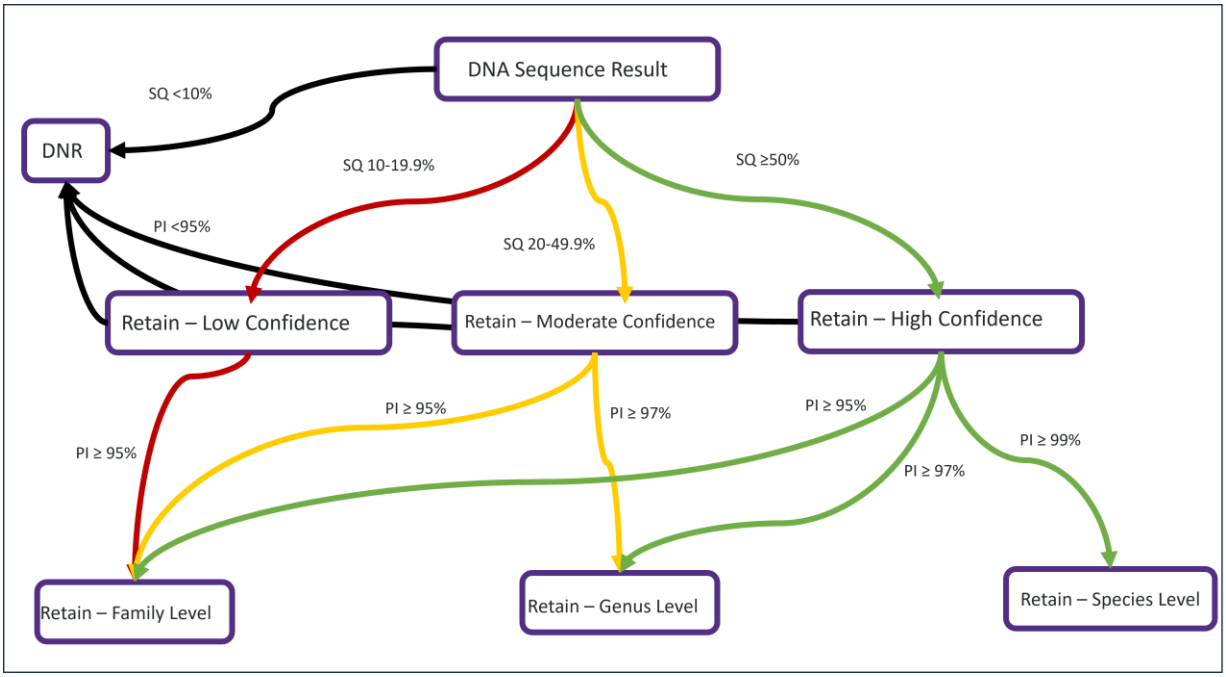


Figure 2.2. CDC Miniature Light Trap at K1A2

Figure 2.3. Geneious Workflow. Sequences were grouped into initial categories based on their quality, and match results obtained from these sequences were either retained with "Low Confidence", "Moderate Confidence", or "High Confidence" at several taxonomic levels or were not retained. DNR = Do Not Retain, SQ = Sequence Quality, PI = Pairwise Identity.



Results:

Konza Midge Population Survey

During the two years of the study, 3,301 midges were collected and identified to species. Midges identified include *Culicoides arboricola, Culicoides bergi, Culicoides crepuscularis, C. guttipennis, C. haematopotus, Culicoides (Selfia), C. sonorensis, C. stellifer, C. variipennis, and C. venustus.* (Table 2.1). Throughout the study, 45 collected midges were unidentifiable due to damage, and labelled "*C. spp*". No statistically significant differences could be found between midge abundance across sites. When including rare species (n<100), abundance was statistically different across species ($p = 0.001$), but no significance was found when these species were removed from analysis ($p = 0.2$) The most abundant species was *C. haematopotus* (n = 1,205),

followed by *C. stellifer* (n = 1,052), and *C. crepuscularis* (n = 640, Table 2.1). *Culicoides sonorensis,* a known vector species, was captured across all sites in lower numbers (29 total individuals). On average, 10% of collected midges were male and 90% were female. Among the female midges, the majority were gravid (46.45%), followed by parous (31.40%), nulliparous (21.94%), and blood-fed (0.21%). Across sites, *C. haematopotus* was the most abundant at all locations except for K1A2 and SC Spring, where *C. stellifer* and *C. crepuscularis* were the most abundant, respectively. K1A2 and SC Spring were also the most diverse sites, both with ten different species identified. Nine species were identified at N20B and SC Creek, and seven at C3SA Top. Throughout the season, peaks of abundance could be seen for multiple species (Fig. 2.6), with *Culicoides crepuscularis* reaching peak abundance in early June and *C. haematopotus* and *C. stellifer* reaching peak abundance July – August. *Culicoides stellifer,* in particular, experienced multiple sharp increases and decreases in abundance.

Table 2.1. Midges collected across KPBS sites

Midge spp	**Sites**					
	C3SA Top	K1A2	N20B	SC Creek	SC Spring	**Spp Total**
C. arboricola	0	4	0	0	4	8
C. bergi	13	6	6	28	13	66
C. crepuscularis	5	139	81	83	331	639
C. guttipennis	0	1	2	8	2	13
C. haematopotus	72	289	280	448	114	1203
C. (Selfia)	32	122	2	14	18	188
C. sonorensis	0	10	2	2	15	29
C. stellifer	29	439	205	142	237	1052
C. variipennis	3	2	4	25	27	61
C. venustus	3	4	8	5	19	39
C. spp	2	8	12	17	6	45
Site Total	159	1024	602	772	786	**3343**

Figure 2.4. Abundance of Biting Midge Species Across Sites

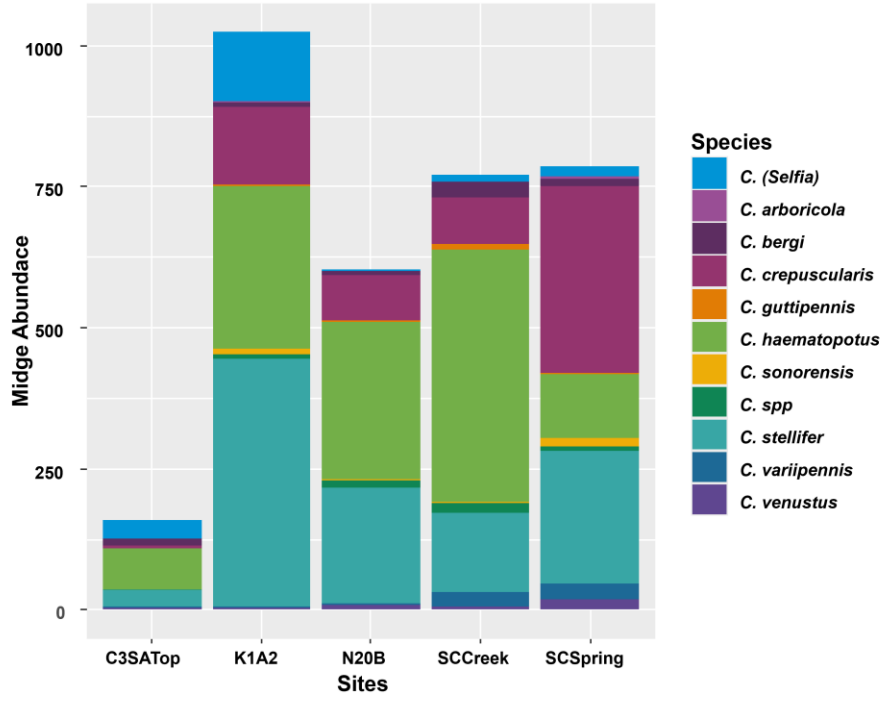
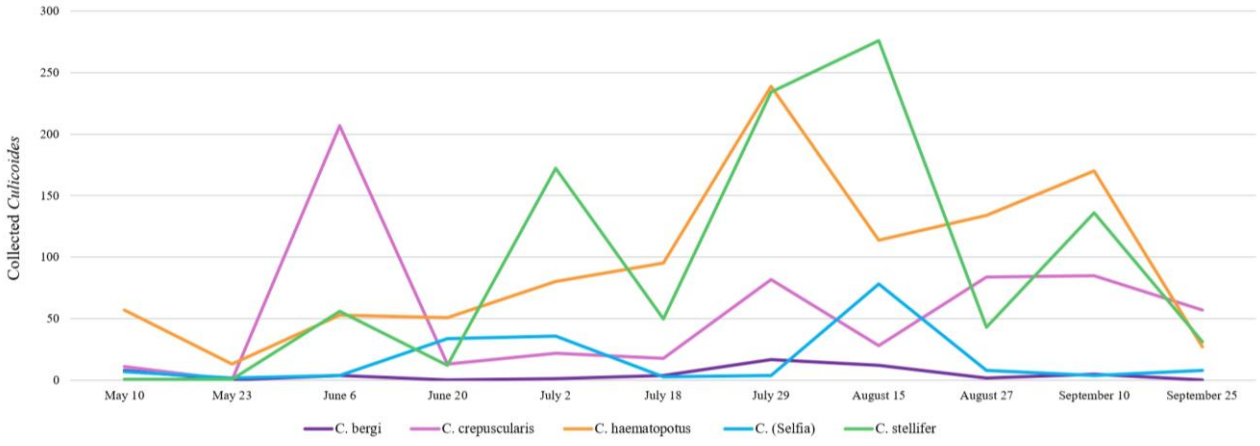


Figure 2.5. Seasonal Abundance of Midges on Konza Prairie



Midge-Plant Associations

Of 788 midges extracted, plant DNA was successfully amplified from 44.67% of samples based on gel electrophoresis (Table 2.2). However, only 11% of midges extracted produced a successful sequence that could be matched to a single plant family (Table 2.3) while a few samples produced sequences which matched multiple plant families. No statistically significant associations were observed between midge species and plant families.

High Confidence sequence (Sequence quality ≥ 50%, ≥95% sequence match, and found on KPBS) results made up 72% of retained sequences, dominated by Verbenaceae and Cupressaceae. Within these two plant families, *C. haematopotus* produced the majority of sequences (n = 42). Additionally, the highest variety of sequences were produced by *C. haematopotus*, with Highly Confident results from nine different plant families. Sequences from *C. crepuscularis* were the second most diverse, with sequences matching seven plant families with High Confidence. Sequence matches recorded at genus level (sequence quality ≥50%, ≥97% sequence match, and found on KPBS) include: *Arachis, Bassia (Kochia), Camellia, Chenopodium, Juniperus, Musa, Pinus, Plantago,* and *Triticum*. No sequences could be matched confidently to species. When comparing results with species present on Konza (Nippert et al. 2024), 28.5% of resulting matches were found to be present at the genus level, and 85.5% at the family level. Double peaks were seen in 48% of sequences in this category.

A higher variety of midge species resulted in plant family matches (sequence quality 20-49.9%, ≥95% sequence match, and found on KPBS) within the Moderate Confidence level compared to High Confidence, although no novel plant families were seen in this category. The majority of sequences again came from *C. haematopotus*. Of sequences in this category, 14.3% of results matched with a plant species, 38% of results matched with genera, and 100% of results

matched with families found on KPBS. Genus matches (Sequence quality 20-49.9%, ≥97% sequence match, and found on KPBS) recorded include: *Chenopodium, Juniperus,* and *Triticum*. Double peaks were seen in 100% of sequences in this category and could account for the lower sequence quality.

Low Confidence results (sequence quality 10-19.9%, ≥95% sequence match, and found on KPBS) included unique plant families as well as more species of midges. Compared to plants identified on Konza, 30% of results produced matches with species, 70% with genera, and 100% with families. Genus matches recorded include: *Amaranthus, Chenopodium, Vitis, and Plantago*. Double peaks were seen in 80% of sequences in this category.

Table 2.2. PCR Product and Ultimate Sequencing Success. This table shows the percentage of positive plant DNA results from extracted midges amplified using *rbcLa* and *trnH-psbA* primer sets, along with the percentage of successful plant matches after sequence processing, in parentheses.

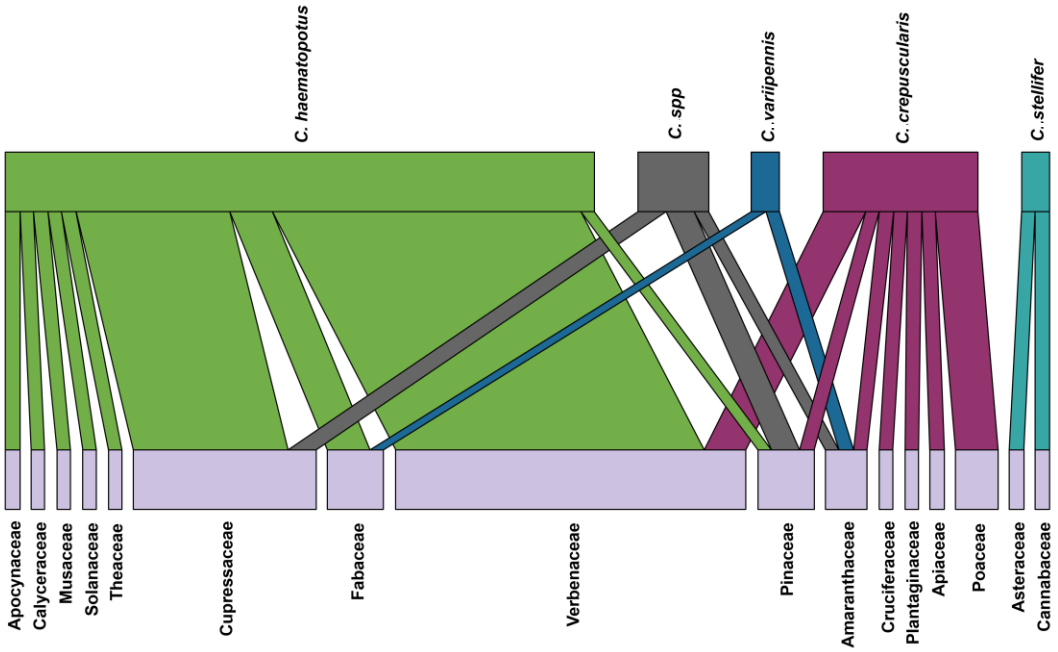
	Positive PCR – *trnH-psbA* % (Successful Match %)	**Positive PCR – *rbcLa* % (Successful Match %)**	**Sample Size**
Midge species	Overall % = 33.5 (3.43)	Overall % = 25.3 (7.49)	Total = 788
C. stellifer	26.67 (2.22)	7.78 (2.22)	90
C. bergi	31.43 (5.71)	14.29 (0)	35
C. sonorensis	31.25 (0)	18.75 (0)	32
C. crepuscularis	31.22 (3.80)	25.32 (2.11)	237
C. haematopotus	44.02 (4.63)	35.52 (16.60)	259
C. variipennis	30.30 (1.52)	10.61 (6.06)	66
C. venustus	11.11 (0)	33.33 (11.11)	9
C. spp	33.33 (4.76)	71.43 (19.05)	21

C. (Selfia)	7.89 (0)	10.53 (0)	38
C. guttipennis	0 (0)	0 (0)	1

Table 2.3. Plant Families Utilized by *Culicoides.* Plant sequences matched to family are summarized, with the associated midge species. Results are included for all Confidence levels, as High/Moderate/Low level results.

Plant Families	**Common *Culicoides* Species (High/Moderate/Low Confidence)**							
	bergi	*crepuscularis*	*haematopotus*	*stellifer*	*variipennis*	*venustus*	*spp.*	**Plant Total**
Amaranthaceae		1/0/0		0/1/1	1/0/1		1/0/0	6
Apiaceae		1/0/0						1
Apocynaceae			1/0/0					1
Asteraceae				1/0/0				1
Calyceraceae			1/0/0					1
Cannabaceae				1/0/0				1
Cruciferaceae		1/0/0						1
Cucurbitaceae						0/0/1		1
Cupressaceae			11/3/0				2/0/0	16
Fabaceae			3/0/0		1/0/0			4
Musaceae			1/0/0					1
Pinaceae		1/0/0	1/0/0				2/0/0	4
Plantaginaceae		1/0/1						2
Poaceae	0/0/1	3 /1/0	0/2/0		0/1/0			8
Polygonaceae			0/0/1					1
Solanaceae			1 /1/0		0/0/1			3
Theaceae			1/0/0					1
Verbenaceae	0/1/0	3/1/0	22/3/2					32
Vitaceae			0/0/1					1
Spp Total	2	14	55	4	5	1	5	86

Figure 2.6. Associations of *Culicoides spp.* with Plant Families



Discussion:

This study identified sources of sugar meals in multiple *Culicoides* species collected from KPBS. Sugar meal analysis found multiple plant families fed on by *Culicoides*, suggesting a generalist strategy for sugar feeding. Associations of midge species with certain plant families and genera provide insight into midge behaviors and site use.

Konza Midge Population Survey

In light trap captures, midge abundance did not differ significantly across sites, although C3SA Top collected the least midges. Although consistently wet, the soil at C3SA Top may have been too dense to provide an ideal larval habitat, leading to lower midge abundance. In terms of diversity, K1A2 and SC Spring both had the highest species richness. At both sites 10 species of *Culicoides* were identified. Seasonality in this study agrees with findings from other papers from

the US, and around the world, with abundance peaking June – September (Barnard and Jones 1980b, Lysyk 2006, Clausen et al. 2009).

It is important to note that some captures were lost at K1A2 and N20B, as the wires connecting the trap to the battery were chewed by wild animals during some overnight collections, with N20B more heavily affected. This may have had an impact on the number and species of midges caught on those dates. Despite this, N20B performed similarly to SC Creek and SC Spring; this is interesting given the fact that N20B is a pond site similar to K1A2. Abundance collected may have been similar to K1A2 if traps had not malfunctioned. These are the only two sites with ponds, which may indicate higher abundance at pond sites rather than springs or creeks. Ponds tend to be the focus of studies when collecting *Culicoides,* which makes sense as dairy wastewater ponds are one the major concerns for larval habitat and disease transmission (Harrup et al. 2014). Ponds are also found to be productive habitats in general, across multiple regions and midge species (Mullens 1989, Clausen et al. 2009, Gerry et al. 2009).

SC Creek and SC Spring species compositions differ despite being close together. *Culicoides haematopotus* is the most abundant species at SC Creek, whereas *C. crepuscularis* is the most abundant species at SC Spring. Several factors may lead to this difference, such as differences in larval habitat. Erram et al. (2019) also found *C. haematopotus* in relatively higher abundance in creek habitat (written as stream) than other water body types. Additionally, the current study revealed that there is little overlap of plant families utilized by the two species, although it is unclear whether this could be driven by preference or spatial arrangement.

Midge-Plant Associations

The majority of sequences matched plants within the Verbenaceae family, with plants in Cupressaceae close behind. Verbenaceae includes trees, shrubs, and herbs, all with clusters of

small, often brightly colored flowers while Cupressaceae is made up entirely of non-flowering coniferous trees and shrubs. While no statistical significance was found between midge species across plant family results, both of these plant families were associated with *Culicoides haematopotus;* this could be due to high abundance of these plant families at the sites sampled, and/or the relatively high abundance of *C. haematopotus* compared to other species captured and extracted. This may also be the reason for the increased diversity of sugar meals; at every Confidence level, *C. haematopotus* fed on more plant families than all other species. Alternatively, it is possible that *C. haematopotus* simply requires more energy to acquire blood meals. Previous research has shown that *C. haematopotus* feed almost exclusively on birds in the U.S. (McGregor et al. 2018, Sloyer et al. 2019), and specifically in Kansas (McGregor and Lewis 2023). Birds are extremely mobile and thus, avian-associated midges may expend more energy securing bloodmeals, necessitating more frequent sugar meals (Magnarelli 1981). *Culicoides haematopotus'* strong avian association could also explain Cupressaceae results being exclusive to this species, as birds tend to rest in trees.

Verbenaceae was associated with multiple midge species. On KPBS there are seven recognized species representing three genera (*Glandularia, Phyla,* and *Verbena*), all of which are native to the prairie (Nippert et al. 2024). The small size of these flowers may present an advantageous niche to *Culicoides* as they are a good source of nectar which midges are small enough to easily access, although there are other insect pollinators with specialized mouthparts that are able to make use of these plants as well (Cruden et al. 1990, Estes and Brown 1973).

Across Confidence categories, several common genera were noted. This consistency provides more evidence that these are plants utilized by *Culicoides. Chenopodium*, commonly known as "goosefoot", for example is seen across all Confidence categories for sequence

matches. These are herbaceous flowering plants which are globally distributed. Three species of goosefoot have been documented on KPBS, two that are commonly found in disturbed habitats and one which is commonly found near or in forested areas (Towne 2002). Goosefoots possess flowers which lack petals found in very tight, small clusters, and nectaries are present below the stamen which produce a small amount of nectar (Abdelbar 2018, Williams 1969) not accessible to other insects (Idris and Grafius 1995). However, *Culicoides* are very small and so may be suited to access these nectaries. While it is possible *Culicoides* could access *Chenopodium* nectar, they may also access honeydew excreted by aphids feeding on more accessible parts of the plant, as is the case with the parasitoid wasp *Diadegma insulare* (Idris and Grafius 1995, Kaufmann et al. 2015).

Triticum is a genus of grasses which includes common wheat (*Triticum aestivum*). This genus was commonly identified in our samples, with the only species of *Triticum* recorded at KPBS being the common wheat. Wheat plants lack nectaries entirely and so do not produce nectar. This result seems then to provide support to the theory of biting midges feeding on honeydew, as it is highly unlikely *Culicoides* are themselves piercing wheat stems to directly feed on phloem.

Juniperus, which contains approximately 70 species of coniferous trees and shrubs (Adams 2014) known as junipers were also shared across Confidence levels. Most notably, junipers are gymnosperms and therefore do not flower or produce fruit. This leaves few sugar sources for biting midges to feed on, other than sap and berry-like seeds. Insects have been documented feeding on these seeds (Chambers et al. 1999) and so it is possible that *Culicoides* are making use of them as well. Biting midges have been documented feeding on dried fruit in

lab (Jones 1964), and with *Juniperus virginiana* being abundant (and the only species of Cupressaceae recognized) across KPBS, these seeds are likely a readily available food source.

Other notable results include four genera: *Arachis, Camellia, Musa* (bananas), and *Pinus* (pines); and one family, Vitaceae (grapes). These four genera met the criteria for sequence matches in the High Confidence category, meaning these are likely accurate results. However, none are recorded as growing on KPBS. These plants may grow rarely in the area, in places hidden from researchers conducting plant surveys, or they may be introduced to the area from outside sources (as is likely the case with *Musa*). While bananas do not grow on KPBS, it is a widely used research prairie, and it is possible that a human discarded banana material. This result is interesting to consider alongside the junipers and grapes, as it indicates decaying fruit and seed material may be a commonly used sugar source. While the only Vitaceae result achieved was of Low Confidence, this family is present on Konza and considering past evidence of fruit feeding by *Culicoides* in lab and by mosquitos (Jones, 1964, Peach and Gries 2019), the theory of fruit feeding by *Culicoides* is well supported.

Pine trees do not flower, and unlike junipers they form a hard-skinned nut as a seed, rather than a soft-skinned "berry". The only available sugar sources the authors are aware of from pine trees are sap and sugars stored in the needles (Devaux et al. 2009, Fischer and Höll 1991). Pine trees are not found on KPBS but they are common in the towns nearby (approx. 5km away). This may suggest a further active flight range for *Culicoides* than previously recorded (Lillie et al. 1981, Kirkeby et al. 2013).

Sequence Quality

In cases where sequences were poor quality or where no sequence could be amplified, sugar meals were likely degraded. This process can happen in as little as several hours or as long as three days, according to Magnarelli (1981).

In the first two extraction methods used, 81 sequence results were removed due to suspected false positive results. All of these sequences were of a suspiciously high match (100% pairwise identity match, 100% query cover, 0 E score) with the same singular species of Fabaceae: *Pisum sativum*, the garden pea. It would make sense for biting midges to be feeding on Fabaceae (and even *Pisum* specifically). Plants in this family often have small, colorful flowers with a unique shape that opens downward, which would complement the midge behavior to rest on the underside of objects. Fabaceae flowers are in this way similar to orchid flowers, which are visited by mosquitos and *Forcipomyia* (Lahondère et al. 2020, Bartareau 1994). However, this plant is not found at KPBS, nor is it grown commercially in the surrounding area. Colony midges were used to validate the presence of *Pisum sativum* contamination resulting from the extraction methods being used. Extraction methods were subsequently altered to decrease false positives. However, it is unclear what caused *Pisum sativum* to incorrectly appear in so many samples.

In some instances, results from the *trnH-psbA* and *rbcLa* primers were in agreement, but frequently they were not. This could indicate that multiple sugar meals were present, and/or different plants were more readily detected by a specific primer set. Many sequences were not retained due to poor quality as many double peaks were present and the sequence could not confidently be called, which may be another indicator of multiple sugar meals. Noise in DNA chromatogram results came in several types, including distortion, mis-spacing of peaks, misleading heterozygous peaks, and true double peaks, among others (Al-Shuhaib and Hashim

2023). These true double peaks are noted by Al-Shuhaib and Hashim to be potential indicators of double infections, where multiple strains of a pathogen are present within the sampled host. Multiple “strains” of plants may also be present in midge sugar meals.

Conclusions:

Culicoides are an incredibly diverse genus, in terms of species, habitat distribution, host use, and as this study has shown, plant diet. These plants provide a valuable energetic resource for biting midges, whether through floral nectar, honeydew, seeds, or decaying fruit material. This study found that *Culicoides* feed on multiple families of plants, suggesting a generalist model for sugar feeding. This generalist behavior means mechanical control methods such as removal of one sugar source may not significantly impact sugar feeding or midge populations (as is seen in the removal of larval habitat). Sugar meal analyses performed in more and diverse habitats (other prairies, urban areas, farmland, etc), comparisons of feeding habits across physiological state, species, and region, and field-based behavioral studies should be conducted to further uncover why and how these sources are being used in the environment. Once these behaviors are better understood, targeted and sustainable control methods can be further implemented in the management of *Culicoides* biting midges.

Chapter 3 - Determining choice behaviors of *Culicoides* biting midges (Diptera: Ceratopogonidae) in sugar-source selection

Introduction:

Culicoides biting midges are small biting flies in the family Ceratopogonidae, containing approximately 1,300 globally distributed species (Borkent and Dominiak 2020). These flies are known to transmit viruses of human and animal concern, as well as some filarial parasites (Ben-Chetrit and Schwartz 2015, Wanji et al. 2019). Hosts are located via olfactory and visual cues, and these senses are highly developed in biting midges (Blackwell et al. 1992, Grant and Kline 2003, McDermott et al. 2015). Bloodmeals are taken from these hosts by *Culicoides* females to provide nutrients for egg development and maintenance. Both male and female *Culicoides* are also known to sugar-feed, and longevity of both sexes is increased when provided sugar under laboratory conditions (Campbell and Kettle 1976, Reeves and Jones 2010, Kaufmann et al. 2015). Sugar meals can also be detected in wild caught midges (Stewart and Kline 1999, Mullens 1985) suggesting that sugar meals are critical to midge survival and fitness. However, the relative importance of visual and olfactory cues in locating these meals is currently unknown.

Sight in other dipterans has been shown to be incredibly important (Allan et al. 1987). Mosquitos are sensitive to light within the UV (323-345nm) and green (523nm) light ranges, and a study by Peach et al. (2019) found that visual cues increased the attractiveness of an olfactory cue. They also found that mosquitos discern between different patterns of UV-absorption and reflection similar to those found on flower petals and prefer low reflection of both UV and visible-spectrum light. The preference for low reflection may help mosquitos locate hosts for blood meals, and the UV patterns may aid in nectar source detection. Similarly, black flies have

been shown to be able to detect UV and green light, preferring low reflectance (Allan et al. 1987).

Comparatively, little is known about midge sight and behavior. It has been shown that vision is important to attraction of midges to traps, as infection with blue tongue virus (which is found in eye tissue of midges) reduces light trap captures and alters midge behavior (McDermott et al. 2015). Midges are also attracted to a variety of colored lights, depending on species, sugar feeding status, and even moon phase (McDermott and Mullens 2018). Bishop et al. (2006) found that while *Culicoides brevitarsis* (among 14 other species studied) preferred green LED light over incandescent light, *Culicoides marksi* preferred blue LED over green, and ultimately UV light was preferred by *C. marksi, Culicoides austropalpalis*, *Culicoides bunrooensis*, and *Culicoides dycei*. In Snyder et al. (2016a), midges provided sugar before exposure to lights in assay arenas were more attracted to light than those without sugar prior to trials. Additionally, *Culicoides* trap captures are significantly reduced during the full moon, likely due to moonlight competing with traplight (Bishop et al. 2000).

Plants and their flowers are light-reflecting objects which may be perceived differently by *Culicoides*. According to data collected in the previous chapter, *Culicoides* have been found to feed on a diverse assemblage of plant families with a variety of appearances. Several of these families (Amaranthaceae, Verbenaceae, Caryophyllaceae, etc.) have striking floral colors, begging the question: Do these colors play a role in *Culicoides* foraging behavior? Important visual cues used by *Culicoides* to find ideal sugar sources may better inform trap design for surveillance and control, especially for attractive toxic sugar baits. The purpose of this study was to quantify midge choice behaviors when foraging for a sugar meal based on color.

Materials and Methods:

Culicoides Rearing

Culicoides sonorensis (Ausman colony, est. in 2001 from a population collected from Brighton, CO) reared according to Hunt (1994) at the U.S. Department of Agriculture Arthropod-Borne Animal Diseases Research Unit (Manhattan, KS, U.S.A.) were used. Midge pupae were collected and split into groups of approximately 700 individuals. These pupae were kept in emergence containers in an incubator at 27°C and ~60% relative humidity with a 12-h light cycle. Once emerged, groups of adults were either maintained in the incubator and provided 10% sucrose solution *ad libitum* or placed into the assay chamber to acclimate with no sucrose solution. Unless immediately used for experimentation, each group of midges was provided with the sucrose solution until 48 h prior to experimentation, at which point the sucrose solution was removed and replaced with de-ionized water *ad libitum*.

Floral Assay and Chamber

Artificial white fabric flowers were acquired from Hobby Lobby Stores, Inc. (Oklahoma City, OK, U.S.A.) and evenly coated with watercolor paints (Crayola LLC, Forks Township, PA, U.S.A.) to simulate different floral colors (white, red, yellow, and purple). Painted flowers were allowed to dry completely prior to experimentation. A 10% sucrose solution was provided via cotton wicks fed through the artificial flowers and a small aluminum bowl (to keep solutions from soaking into flower petals) during experiments (Fig. 3.3). The assay is modified from Dieng et al. (2018) to use a smaller cage and observations taken by camera rather than human observer (Fig. 3.1). The assay chamber was constructed using a cage (52 X 29 X 29cm) covered in small aperture mesh with a clear plastic pane on the top to allow camera visualization. Two flowers were placed within the chamber, one on the left and right end, 6.5cm apart. Six replicates

of each color combination were performed, with flower positions swapped after the first three replicates. Each trial was allowed to run for 8 h inside an environmental chamber maintained at 27°C and ~60% relative humidity with a 12 h light cycle, during which adult midges were allowed to forage from either flower. Post experimentation, each group of midges was frozen within the assay chamber at -20°C for 24 h. Midges were then collected from the assay chamber and counted, and were visually inspected for presence of sugar meals in their abdomens.

Data Collection and Analysis

All observations were collected by a Basler Gig-E IR-filtered camera (supplemented by two IR light sources) and recorded using EthovisionXT v17 (Noldus Information Technology, Leesburg, VA, U.S.A.). Color preference was approximated by measuring percent pixel change in two defined areas, defined as Flower 1 and Flower 2 (Fig. 3.2). Levels of activity were calibrated by human comparison of midge movement in videos at different pixel change levels. Instances of inactivity were defined as pixel change below 0.02%, while instances of moderate activity were defined as pixel change between 0.02 and 0.07%, and high activity instances were defined as pixel change above 0.07%. Activity was calculated every 0.04 s and recorded as "1" for occurrence and "0" for non-occurrence.

Instances of activity for each trial were then summed and compared between Flower 1 and Flower 2 to determine which flower experienced the most midge activity. Due to midge size and camera constraints, exact individual behaviors could not be measured, but group activity was analyzed by comparing activity per midge (to account for potentially different numbers of emerged midges) between color treatments. Activity comparisons were analyzed for significance using AOV (MediumActivityPerMidge~Color+Position) and Tukey's Honest Significant Differences in R (version 4.4.3) and RStudio (version 2024.12.1+563). The potential interaction

effect between floral color and position of flower within assay chamber was analyzed using AOV (MediumActivityPerMidge~Color*Position). Additionally, activity was compared across the number of midges in each trial using AOV (MediumActivityPerMidge~NumberMidges). R Packages used include ggplot2 3.5.1 (Wickham et al. 2024), ggpubr 0.6.0 (Kassambara 2023b), bipartite 2.21 (Dormann et al. 2025), rstatix 0.7.2 (Kassambara 2023a), and fmsb 0.7.6 (Nakazawa 2024).

Figure 3.1. Assay chamber and incubator used for behavioral assays. A camera is positioned above, with two IR light sources positioned behind the camera. Midges are released in the center of the chamber, and their movement around each flower is measured for 8 h. Clear plastic sheeting is used in place of mesh on the top panel of the chamber to allow higher resolution video.

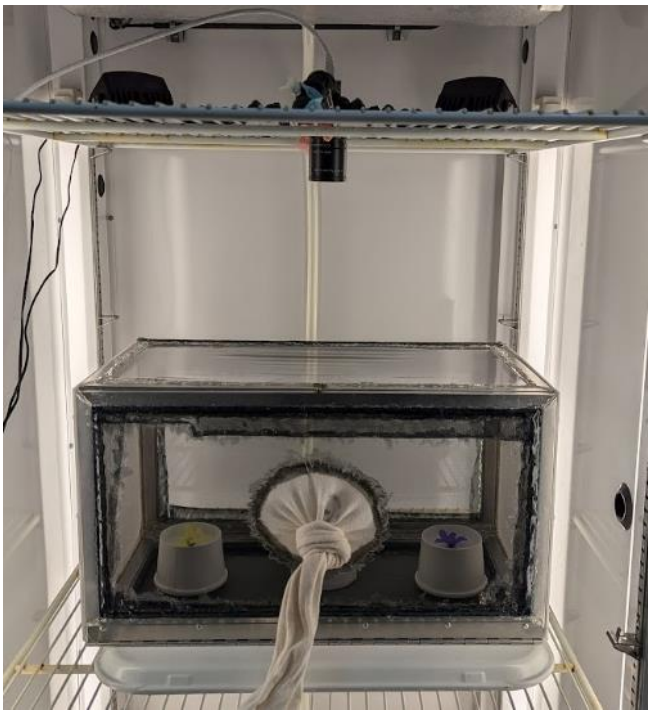


Figure 3.2. Top-down view of the assay chamber. With each color combination, flower position was rotated after the first 3 trials (6 taken total) to account for potential spatial preference in the chamber. Flower 1 is always the left position, and Flower 2 the right (**A**). The same view is shown in Ethovision software, with areas analyzed for activity by *Culicoides* marked with orange circles (**B**).

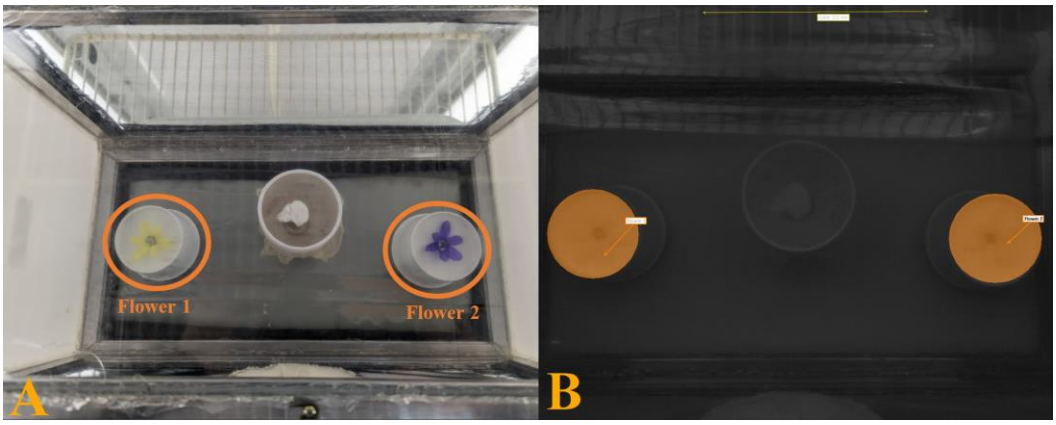


Figure 3.3. Flower design for behavioral assays. Each flower had 2 coats of watercolor paint applied to the petals. A small aluminum bowl was created and hot-glued to the center of each 6-petal flower. A hole was then driven through the aluminum into the hollow artificial pedicel. A thin cotton wick could then be pulled through to supply 10% sucrose solution to the center of the flower, acting as an artificial nectar source (**A**). Once artificial flowers were completed, the cotton wick was fed through a hole in a paper cup which sat over a plastic cup. This plastic cup was filled with approximately 20ml 10% sucrose solution, while the paper cup ensured the midges could only access the solution from the center of the flower (**B**). A range of colors was painted onto flowers, and eventually red, yellow, purple, and white were chosen for assays (**C**).

Results:

Adult midges collected from the assay chamber post-experiment range from 73 to 730 individuals, averaging 250 midges across all trials. To standardize this, activity was divided by midge count. Interestingly, midges were just as active (p = 0.645) in low numbers as in high (Fig. 3.4). In trial 19 there were only 81 midges present; however, they were by far the most active, with 1,077 instances per midge around the left flower and 2,390 instances per midge around the right. This trial was defined as an outlier (Fig.3.5.) and removed from analysis. Boxplots summarizing medium-level activity across treatments are shown below:

Fig. 3.4. Activity Per Midge During Trials. As the number of midges increased, the activity per midge remained relatively the same (p = 0.645).

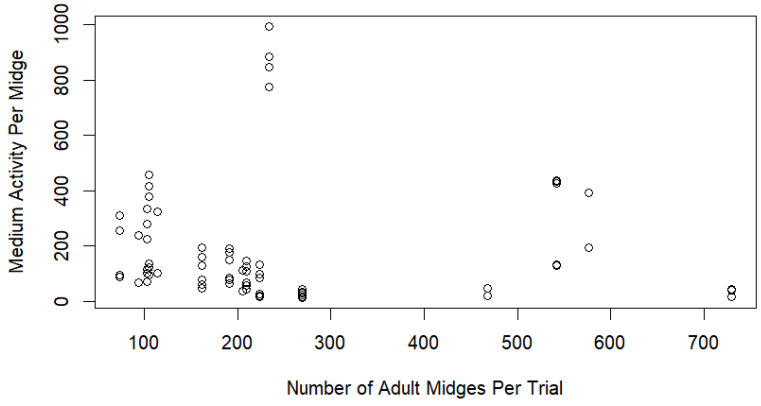


Fig. 3.5. Activity of Midges around Artificial Colored Flowers. The outlier from trial 19 is included for visualization (orange circle).

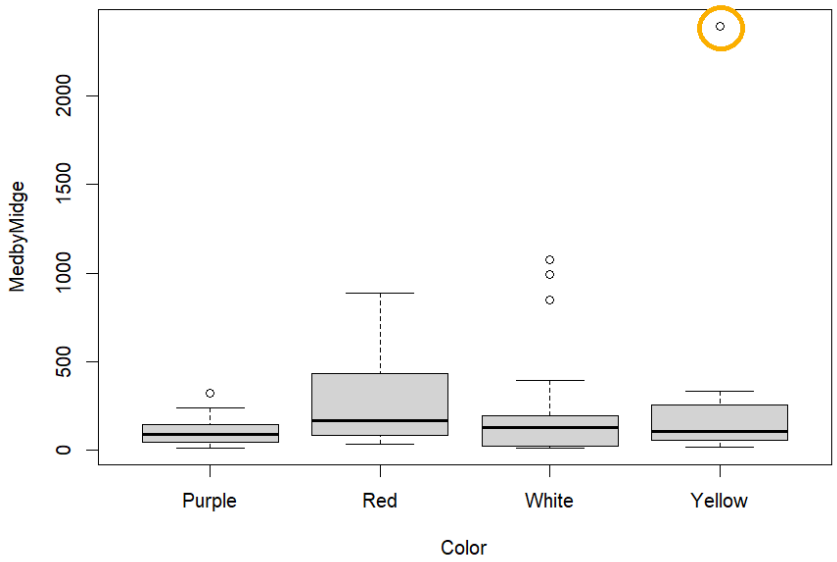
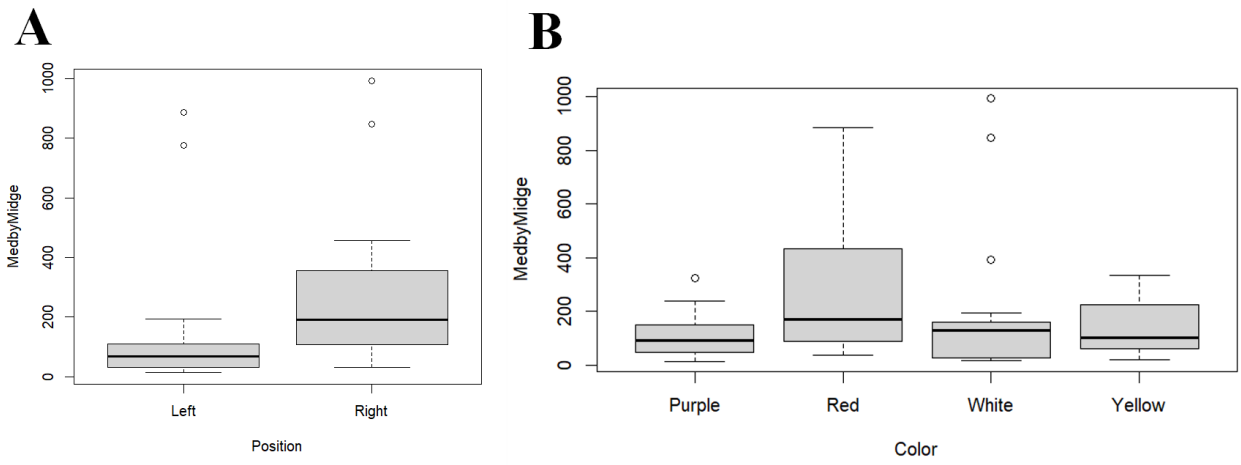


Fig. 3.6. (A) Spatial and (B) Color Preferences of *Culicoides sonorensis* (Ausman). The outlier is removed.



Color and spatial preferences across all trials are shown in Fig. 3.6. There was a clear spatial preference ($p < 0.005$) for whichever flower was on the right side of the assay chamber. A specific significant preference shown for red flowers over purple ($p < 0.03$), but no statistically significant preference was seen between any other color comparisons. While both position and

floral color were significant, there was no significant interaction between these two factors (p = 0.52).

Discussion:

Preliminary observations in the lab showed that the colony midges fed intermittently from early morning till late evening, in agreement with anthrone assays conducted in the field, which found that midges possessed sugar meals throughout the day, with sugar positive rates declining at sunset (Magnarelli 1981, Stewart and Kline 1999). Sugar feeding on the artificial sources used in this study was confirmed by observations of dyed sugar in the abdomen of *Culicoides* after trials were complete (Fig. 3.7). The sugar sources were not initially dyed, however the high humidity of the incubator, along with the force of gravity, led to small amounts of watercolor paint from the underside of the flower petals to seep down into the sucrose solution, which was then drawn up by the cotton wick to where *Culicoides* could access it. Although unintentional, this resulted in a much simpler method for observing the sugar feeding behaviors post-assay.

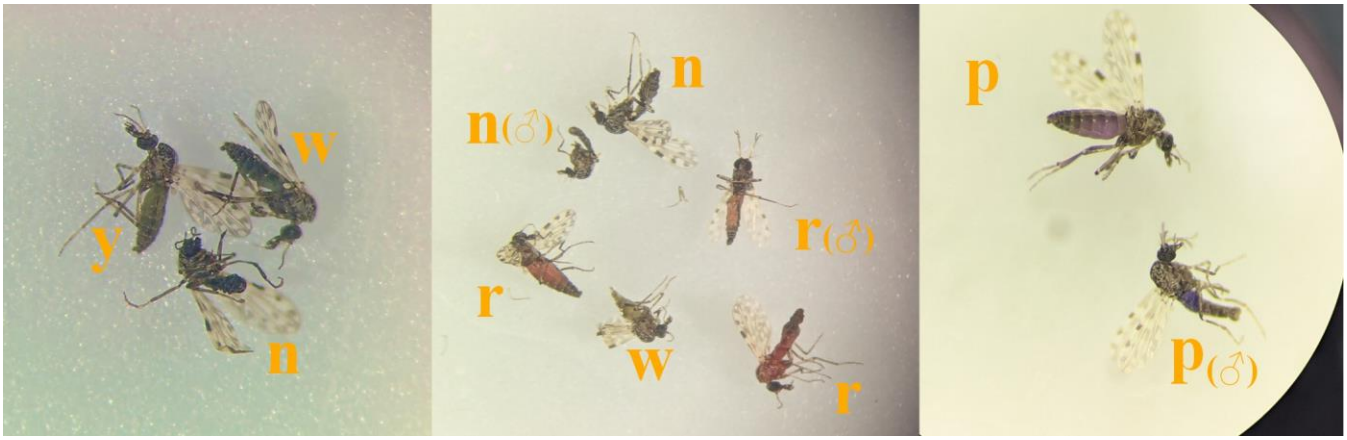
A significant color preference was demonstrated for red over purple. Red flowers generally saw the most midge activity, which is in direct contrast to light trap studies that found red light to be the least attractive (Hope et al. 2015, González et al. 2016), even regardless of sugar feeding status (Snyder et al. 2016b), suggesting *Culicoides* perceive reflected light and emitted light differently, leading to this differential response. These findings align more with Mondal et al. (2022), who found red to be an attractive color for resting box traps. Alternatively, this may also be due to the nature of the midges used in the study. The Ausman colony midges used in this study have been in colony since 2001, and with laboratory reared generation times as short as 24 days, there would have been approximately ~350 generations of *Culicoides* which

have not had plants to respond to (Jones 1964). It is possible that over time and with lack of necessary selective pressures, certain behaviors and genetics of colony insects will diverge from their wild counterparts (Gloria-Soria et al. 2019, Morales-Hojas et al. 2018). For instance, the only color these colony midges regularly interact with as adults is red in the form of blood feeders. While male *Culicoides* were also observed feeding on the red sugar source, female midges may have been responding to the red color searching for a blood meal rather than sugar, inflating the activity rates around the red flower.

The lack of strong preference among other colors agrees with the generalist sugar feeding model proposed in the previous chapter. Although it is likely colony midges may simply not have a preference due to lack of selective pressures, it is also likely when considering the wide variety of plants fed on by wild *Culicoides* that many colors are attractive, or perhaps that color alone does not drive sugar source preference in this group.

In this study, colony *C. sonorensis* also showed a clear preference for the right side of the assay chamber. It is uncertain why this is the case, although airflow within the incubator and/or misreading of activity by camera may have contributed to this. An opening was present in the left side of the incubator, which was covered in an attempt to equalize air movement within the chamber, and the lighting and camera setup was kept as symmetrical as possible to minimize effects due to light. It is also important to note that the incubator had to be moved and rotated during this study, which may have been a confounding variable. As a result of this movement, the assay chamber went from facing south to facing west, which may have affected midge behavior. Additionally, noise from “resting movement” with the plastic sheeting was observed with midges resting on the underside of the smooth plastic sheeting. As a result, not all activity recorded is indicative of feeding behaviors.

Figure 3.4. Midges Fed on Artificial Sugar Sources. Colors are denoted by letters (w = white, y = yellow, r = red, p = purple, n = not fed), and males noted as (♂).



Conclusions:

Attraction to sugar sources in midges is likely influenced by many factors, of which, color may be just one. In this study, a significant association was seen with red flowers over purple. This may be due to a true preference for red sugar sources, such as red flowers, or due to its association as a food source via blood meals in colony. This preference directly contrasts what is seen in light traps, where red is the least attractive wavelength of light (Hope et al. 2015, González et al. 2016). These two visual systems may be perceived differently by *Culicoides*, and thus elicit these different responses. Other factors may include olfactory cues, UV absorption/reflection patterns, floral shape, and even visual contrast. As such, these need to be further investigated for effective ATSB trap design. Additionally, replicates of these assays performed with wild-caught midges may provide more reliable results in terms of actual sugar source preferences in the field. The *Culicoides sonorensis* colony was first established in 2001, meaning this colony has been maintained without access to plants for 24 years. Without related

selective pressures, behavioral responses to attractive sugar source cues may have been lost or changed over time. This study has outlined an assay which may be easily customized to test other visual and non-visual attractive cues, such as floral shape, orientation, height, etc. In the future, researchers should consider combining cues such as floral shape, scent, and color to analyze behavioral responses within context. These cues can then be incorporated into ATSB design to create a targeted control method which is effective and competitive with its surrounding environment.

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