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# Genetic Identification of Culex tarsalis Host Plants from Extracted Gut Contents

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# Genetic Identification of *Culex tarsalis* Host Plants from Extracted Gut Contents

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# Abstract

Since its introduction to the United States in 1999, West Nile Virus (WNV) has become the most prevalent arthropod borne virus (arbovirus) in the Americas. WNV possesses the potential to manifest encephalitic symptoms in both humans and horses, making it an area of constant concern. The most common vector of WNV in the Western United States is the mosquito *Culex tarsalis*, which likely derives WNV from migrating bird populations that act as viral reservoirs. *C. tarsalis* blood feeds specifically for nutrients needed in reproduction, and imbibes floral nectar or other plant sugars for energy. Work with other mosquito species, including members of the genus *Culex*, suggests that there are definite preferences in the kinds of plants for which mosquitoes forage. Using the contents of extracted mosquito guts, it has been demonstrated that the identity of host plants can be determined through genetic methods. This study uses these methods in an attempt to identify popular host plants among *C. tarsalis* females residing in wetlands near Helena, Montana with the hypothesis that *C. tarsalis* selectively forages for floral nectar in a wetland environment, and does not simply feed on the flowers which are most abundant given vegetative data. While no determinations of host plant preference can be made with certainty due to inconclusive PCR results, *Cirsium arvense* is implicated as a possible sugar source due to its high nectar content and relative abundance.

# Introduction

The first diagnosed incidence of West Nile Virus (WNV) in the United States occurred in the summer of 1999 in New York, New York (Petersen et al., 2003). By 2003, WNV had spread to the West Coast of the United States and Canada (Petersen et al., 2003). Since its introduction, WNV has become the most prevalent arbovirus in the United States, amounting to some 95% of diagnosed arboviral cases in 2014 (Lindsey et al., 2015). While WNV does not manifest symptoms in approximately 80% of its hosts (Mostashari et al., 1999), the potential of the virus to result in life threatening conditions such as encephalitis and meningitis (Lindsey et al., 2015) warrants consistent concern.

Livestock, particularly horses, are also susceptible to WNV infection, and Montana is among the states which have suffered the greatest numbers of horse infections (Castillo-Olivares and Wood, 2004), with a peak in 2003 of 191 confirmed cases (Montana Department of Health and Human Services, 2016). While the reported number of horse-based infections has generally decreased in Montana over the last 13 years, occasional spikes in the number of cases, like those seen in 2007 and 2016, are not unusual (Montana Department of Health and Human Services, 2016).

WNV is spread primarily through the bite of competent members of the mosquito genus *Culex* (Petersen et al., 2003), of which *C. tarsalis* is the most prevalent member in the Western United States (Goldberg et al., 2010). Although vertical transmission of WNV from female to young has been observed in nature among members of the genus *Culex* (Nasci et al., 2000), migratory birds serve as the most common introductory reservoirs of WNV for mosquito populations across the United States (Ciota, 2017). While the blood feeding tendencies of disease carrying mosquitoes upon birds and other

competent viral reservoirs is an important and much needed area of study, what is less well understood, and arguably of equal importance from the perspective of mosquito control and management, is the diversity of sugar feeding habits present among mosquitoes.

Female mosquitoes generally rely upon blood feeding for egg production, and often imbibe floral nectar to supplement the reproductive process as well as provide for general energy needs (Foster, 1995). Conversely, male mosquitoes rely entirely upon the energy rendered from plant sugars (Foster, 1995). This knowledge, however, is of limited usefulness for control and management work unless mosquitoes possess definite biases in the kinds of sugars for which they forage. Grimstad and DeFoliart (1974) suggest that such a bias does exist, and that individual mosquito species can be at least partially distinguished in their floral tastes. As a general pattern, Grimstad and DeFoliart (1974) found that lighter colored plants are targeted by the widest variety of mosquitoes, and that individual species tended to specialize in the nectar of approximately five to seven flowering species depending upon the year.

While Grimstad and DeFoliart (1974) employed extensive sampling of known flower groves in order to collect data on mosquito nectar feeding, Junnila et al. (2010) identified mosquito floral preferences through a genetic determination of dissected gut contents of mosquitoes. While the genetic method of determination developed by Junnila et al. (2010) is much faster than that of Grimstad and DeFoliart (1974), as mosquitoes can be easily trapped and tested in bulk, it suffers from a relatively low percentage of mosquitoes (approximately 40%) which test positive for plant DNA.

The purpose of the present study is to use the genetic methods of Junnila et al. (2010) to determine the plant nectar feeding preferences of *C. tarsalis* females caught in a wetland environment near Helena, Montana. A study of this sort has never been conducted in Montana upon any mosquito species, let alone the primary vector of WNV, and might provide valuable insight into the ecology of *C. tarsalis* which could serve to enhance future trapping methods as well as aid in the potential reduction of an environment's suitability to disease carrying mosquitoes. In addition, this study tests the hypothesis that *C. tarsalis* selectively forages for floral nectar in a wetland environment, and does not simply feed on the flowers which are most abundant.

## Materials and Methods

### *Mosquito Trapping*

*Culex tarsalis* were collected via $CO_2$ traps in the wetlands north of Lake Helena adjacent to the intersection of East Lincoln Road and Silo Drive at the coordinates: 46°.704 N, -111°.968 W. Traps were baited using approximately four pounds of dry ice kept in a perforated plastic container hung directly adjacent to the trap opening. Starting from the second week of July and continuing weekly until the third week of August, two traps were deployed each Monday evening at 6PM and collected Tuesday at 9AM. The traps were set on the north and south side of a large cattail stand at a distance of approximately 200 meters from each other. Cattails were chosen as a central frame of reference due to their recognized suitability as *C. tarsalis* habitat (Lothrop and Reisen, 2001). The southern trap location was directly adjacent to standing water while the northern trap site was in a Russian olive tree (*Elaeagnus angustifolia*) approximately 20 meters from the central cattail stand. Collected mosquitoes were euthanized through a

one hour stay at -80°C and stored thereafter at -20°C. *C. tarsalis* were sorted from non-target species and stored at -20°C for later use in DNA extraction.

***Vegetative Data***

Vegetative data were collected through line-intersect transects taken radially extending 30 meters from the edge of the central cattail stand. The starting point of each vegetative transect was determined through a random number generator which correlated to a number of steps taken around the perimeter of the central cattail stand (Figure 1). A half meter squared vegetation frame was used in the above described transects and was deployed every five meters starting from the edge of the cattails. Every plant which was encountered within a given frame, with the exception of grasses, was sampled for later identification and assigned a relative density based upon its percent occupancy within the frame. Fifteen transects were located adjacent to the cattail stand, and 11 more were at a distance of 100 meters from the cattails. The stopping point for transect collections was determined through the generation of a species curve in which each sequential transect was assessed for the inclusion of novel species given previous results. Outer radius transects were completed in order to account for the possibility of *C. tarsalis* migration, which might amount to 90

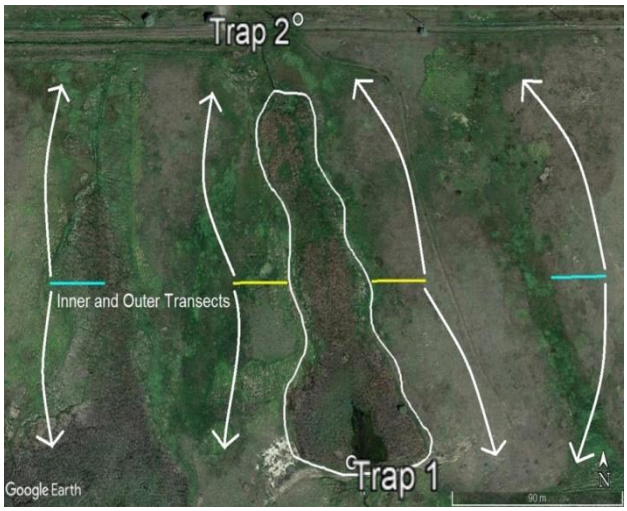


Figure 1. Map of wetland sampling site with a white perimeter around the central cattail stand. To-scale examples are given for both the inner-radius (yellow) and outer-radius (blue) transects. Outer radius transects begin 100 meters from the edge of the cattails.

meters or more in a day (Reisen, 1993). Collected plant samples were pressed in the field shortly after collection.

The relative abundance of encountered plant species was determined through comparing the number of times each species was found across all vegetative transects. Similarly, the relative density of encountered plant species was calculated through a comparison of the number of times any given species was found within a vegetation frame.

***DNA Extraction and PCR Amplification***

Mosquitoes removed from storage at -20°C were given approximately 10 minutes to thaw and subsequently washed in a 0.5% hypochlorite and 0.01uL/mL Triton X-100 solution in order to remove extraneous plant material (Matheson et al., 2008). Washed mosquitoes were suspended in several drops of pH 7.4 phosphate buffer and dissected according to the procedure developed by Coleman et al. (2007). Difficulty was had in directly isolating mosquito midguts, likely as a result of the freezing-thawing process, so intact abdomens were used as a substitute throughout the rest of the extraction. Upon removal, dissected abdomens were homogenized under the conditions provided by Junnila et al. (2010) and left to digest for 8 hours at 55°C while being gently rocked. The genetic material of digested homogenates was extracted using Qiagen DNeasy Plant Mini Kits according to manufacturer instructions.

The target of DNA amplification was a 157 bp fragment of the chloroplast rbcL gene (Junnila et al., 2010). Both primers used in this study, visible in Table 1, were taken from Poinar et al. (1998). The PCR protocol of Junnila et al. (2010) was followed with the exception that each reaction was composed of: 2 uL dNTP, 2.5 uL 10x Taq reaction

buffer, 2.5 uL MgCl, 1 uL Primers Z1 & 19, 0.5 uL Taq, 13.5 uL water, and 2 uL of extracted DNA for a total volume of 25 uL.

Table 1. Primer sequences

| Sequence Name | Sequence 5’ to 3’ |
|---|---|
| rbcl 19 | AGATTCCGCAGCCACTGCAGCCCCTGCTTC |
| rbcl Z1 | ATGTCACCACAAACAGAGACTAAAGCAAGT |

### *Identification and Sequencing*

Amplification of genetic material was verified through gel electrophoresis. Amplified *C. tarsalis* and *Aedes vexans* homogenates, tested in order to verify the initial efficacy of the protocol, were sent to Macrogen, Inc. in South Korea along with positive and negative controls for sequencing. Sequences provided by Macrogen, Inc. were identified using the BLASTn function of the NCBI database.

## Results

### *Mosquito Trapping*

Table 2 details the results of mosquito trapping. Trap 2 consistently yielded larger *C. tarsalis* pools, both in terms of relative proportion to other mosquito species, and also in terms of bulk. A total of 5114 mosquitoes were caught over six weeks, 197 of which were identified as *C. tarsalis* comprising 3.85% of the total mosquito pool.

Table 2. Numbers of *C. tarsalis* trapped each week beginning in the third week of July. Each trap index was calculated from the sum total of collected *C. tarsalis* divided by the number of trap days (6).

| Date (mm/dd) | Trap 1 *C. tarsalis* Yield | Trap 1 % *C. tarsalis* Composition | Trap *2 C. tarsalis* Yield | Trap 2 % *C. tarsalis* Composition |
|---|---|---|---|---|
| 7/16 | 10 | 1.0 | 7 | 1.9 |
| 7/25 | 18 | 2.0 | 52 | 5.3 |
| 8/1 | 5 | 2.2 | 46 | 14.1 |
| 8/7 | 11 | 0.8 | 25 | 9.1 |
| 8/15 | 2 | 0.9 | 19 | 20.2 |
| 8/21 | 0 | 0 | 2 | 2.0 |
| Trap Index | 7.7 | | 25.2 | |

***Vegetative Data***

Flowering plant diversity in the wetland study site was assessed through 26 line-intersect transects. The stopping point for transect collection was determined through the trend observed in Figure 2, which relates a logarithmic decrease in the number of unique plant species observed in each successive transect. A unique plant was one which was not observed in any previous transect.

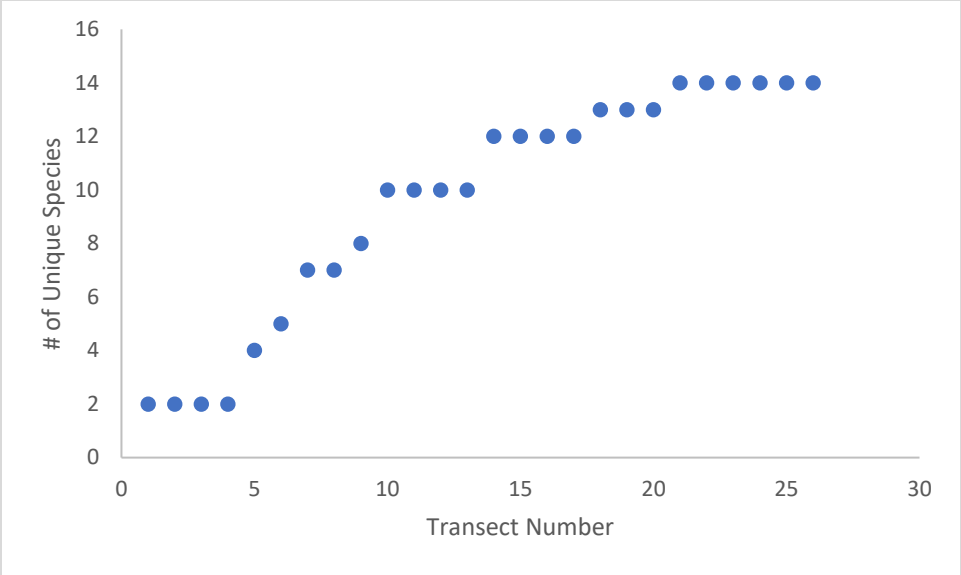


Figure 2. Number of unique, flowering plant species encountered in each vegetative transect.

A total of 15 inner-radius vegetative transects were taken which revealed the presence of at least 10 flowering species. Figure 3 relates the relative abundance of these species. Plant groups which could not be specifically distinguished in the field were given congeneric titles and treated as single species for subsequent analysis.

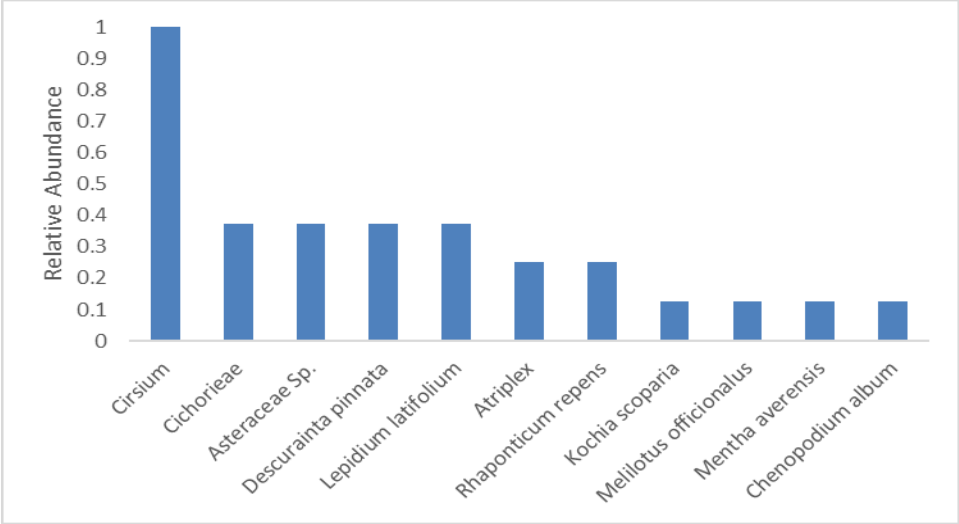


Figure 3. Relative abundance of flowering plants encountered during the inner-radius vegetative transects. *Cirsium* (thistle) was found in eight transects out of 15.

A total of 11 outer-radius vegetative transects were taken in which six species were discovered (Figure 4). Two species, *Vicia americana* (Purple Vetch) and *Lycopus asper* (Rough Bugleweed), were exclusive to the outer-radius transects.

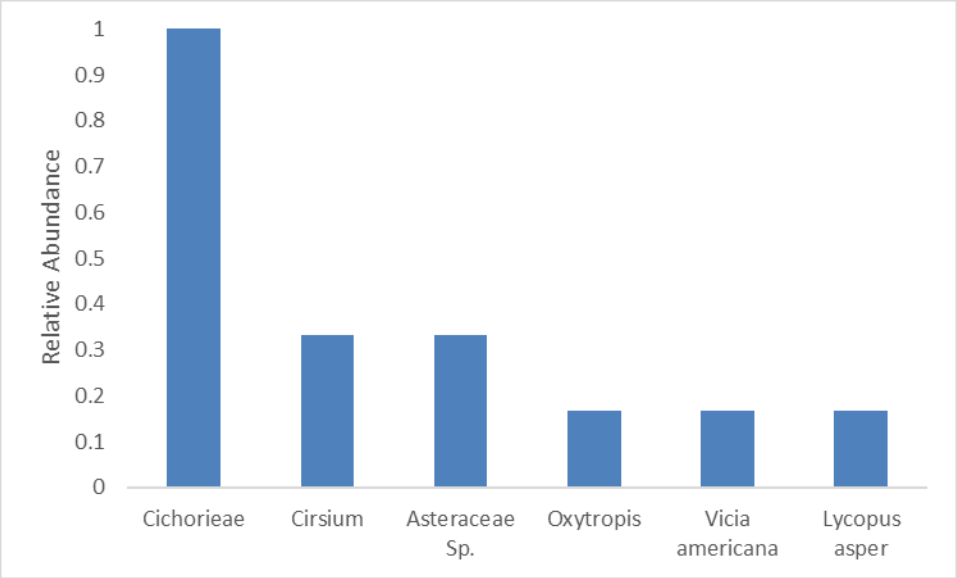


Figure 4. Relative abundance of flowering plants encountered during the outer-radius vegetative transects. *Cichorieae* (dandelion) was found in six transects out of 11.

Figure 5 summarizes the results of all vegetative transects, both inner and outer, in terms of relative abundance. A total of 14 flowering plants were encountered during the course of 26 line-intersect transects. Of these 14 species, members of the genus *Cirsium* were most common with colonies of *C. arvense* comprising the majority of encountered specimens.

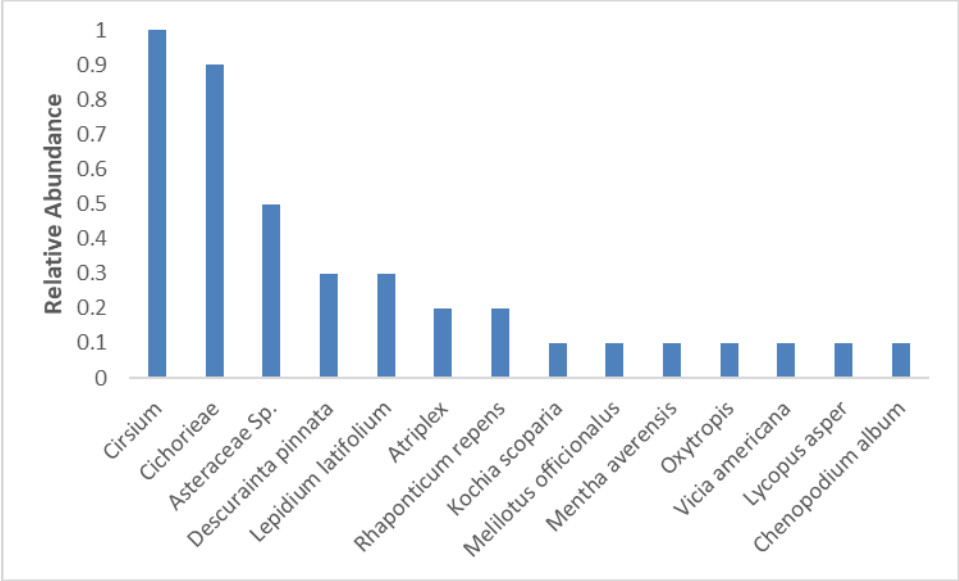


Figure 5. Relative abundance of flowering plants encountered in all vegetative transects. *Cirsium* was found in 10 transects out of 26 total.

Figure 6 details the relative density of flowering plants encountered within all of the vegetative transects. Dwarf Fireweed (*Chamaenerion latifolium)* was observed within 30 meters of the central cattail stand, but was not encountered in any vegetative transect.

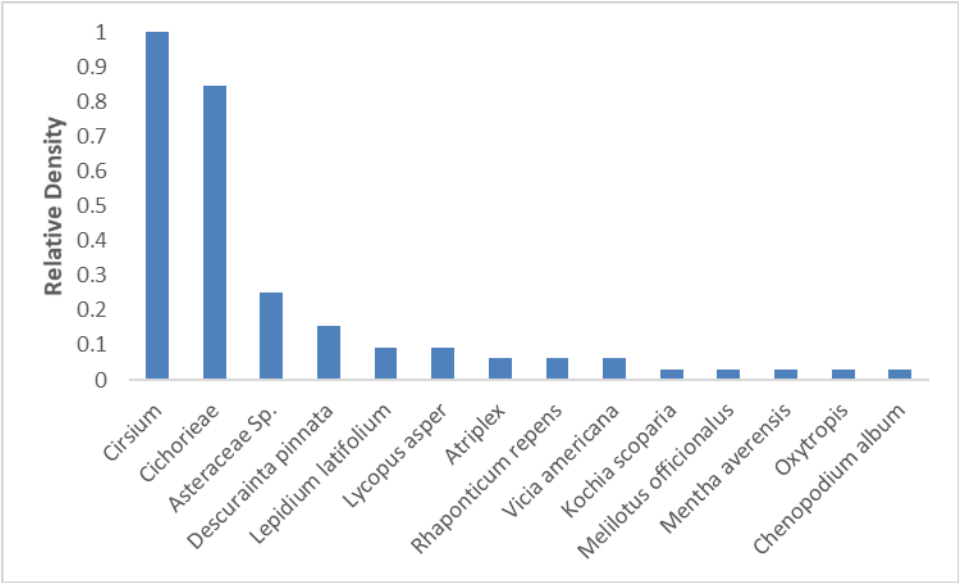


Figure 6. Relative density of flowering plants encountered in all vegetative transects. *Cirsium* was found in 32 vegetative frames out of 182 total.

### *Sequencing*

Twenty *C. tarsalis* as well as 11 *Aedes vexans* individuals were tested using the extraction protocol. None of the 31 tested individuals exhibited strong PCR amplification when compared to positive controls. The amplified results of eight *A. vexans*, three *C. tarsalis*, eight positive controls, two negative controls, and two samples of an unknown forbe encountered during vegetative transects were sent to Macrogen for sequencing. The results of sequenced *A. vexans* and *C. tarsalis* homogenates were not significantly different from negative controls, and were primarily composed of sequence fragments less than 50 bp in length. All but one of the positive controls yielded sequences greater than 120 bp in length, and all of these sequences were associated with the rbcL genes of related plant species from the BLASTn search. The unknown forbe encountered during vegetative transects could only be identified to the family level as a member of the Asteraceae.

## Discussion

The goal of this study was to understand what flowering plants female *C. tarsalis* mosquitoes feed on in a wetland environment North of Lake Helena near Helena, Montana. Given this aim, it is important to understand why *C. tarsalis* populations were concentrated around Trap 2. The high relative proportion of *C. tarsalis* caught in Trap 2 might seem anomalous given its approximately 30-meter distance from the central cattail stand, but two factors might explain this result.

The first, and more likely, is the propensity of preferred avian hosts to take up temporary residence in Russian olives. Mourning Doves and American Robins are both common hosts for *C. tarsalis* across the Western United States from Southern California

(Lura et al. 2012) to Colorado (Kent et al. 2007), and even in Eastern Montana (Bernt, 2013). Both of these species have also been found in surveys across Idaho, Utah, and Colorado to be semi-regular visitors of Russian olives (Knopf and Olsen, 1984). This trend is more representative of Mourning Doves than of American Robins, with Mourning Doves being among the most regularly observed birds perched in Russian olives across the three aforementioned states (Knopf and Olsen 1984).

Somewhat in opposition to the above described trends, the work of McHugh (2017, in review), conducted in parallel with the field portion of this thesis, found both Mourning Doves and American Robins to be infrequent visitors of the wetlands North of Lake Helena.

While both Mourning Doves and American Robins may be minor representatives of the bird population at Lake Helena, other species observed by McHugh (2017, in review) are known to be transient inhabitants of Russian olives (Knop and Olsen, 1984). These include such representatives as the Barn Swallow and the Song Sparrow. *C. tarsalis* were likely found to selectively occupy the area around Trap 2 due to the presence of avian host species, with the possibility of particularly attractive host species, such as Mourning Doves and American Robins, being possible.

Another factor which might explain the relative abundance of *C. tarsalis* at Trap 2 is the possibility of sugar feeding upon the foliage of the Russian olive. This seems unlikely not only because it implies that *C. tarsalis* possesses an attraction to the sugars of the Russian olive, but also because of the abundance of actively flowering species in the area. While mosquitoes have been known to probe leaves in search of phloem sap,

this seems to be a behavior associated with reduced floral nectar availability (Junnila et al. 2010).

Nectar content, among other variables such as flower age, color, and yet unclassified chemical cues, might be important factors in determining attractiveness for plants which are selectively fed on by *C. tarsalis*. Nectar content classifications for the majority of the flowering species identified at Lake Helena have yet to be completed. One notable exception, however, would be the unusually high sugar content (2609 +/- 239ug/inflorescence) within the nectar of Canada Thistle (*Cirsium arvense*), the most common flowering plant in the wetlands (Hicks et al. 2016). Of the 65-flowering species classified by Hicks et al. (2016), *C. arvense* possessed a higher sugar content within its nectar than any representative member of *Cichorieae*, the second most abundant flowering group in the wetlands. While a high relative nectar content and abundance might implicate *C. arvense* as being a prime candidate for selective feeding by *C. tarsalis*, it is important to note that other factors are at play in determining mosquito host-plant preferences. For example, mosquitoes tend towards more lightly colored flowers (white, blue, or yellow; Grimstad and Defoliart, 1974). *C. arvense* is likely to suffer as a potential sugar source for *C. tarsalis* because of its dark purple flower color.

It is impossible, given the results of this study, to make a clear determination of the flower feeding preferences of *C. tarsalis* in the wetland environment North of Lake Helena. While the PCR protocol and selected primers were able to detect extracted plant material from positive controls, they failed to do so in mosquito homogenates, and even produced numerous small strands in negative controls which resulted in the visible smearing of agarose gels during electrophoresis. While initially thought to be the result of

possible contamination, this smearing persisted even after fresh reagents were employed during PCR. It is possible that the primers may have been self-annealing and producing subsequent copies of themselves during the reaction protocol. Given this possibility, a lower concentration of primer may be advisable in future studies as a measure to reduce noise during amplification.

It is also important to note that the resolution of the selected primers was especially poor for members of the Asteraceae family, as was evident in the sequencing of the unknown forbe encountered during vegetative transects. Other work done with rbcL primers supports this result, with resolution failing beyond the family level for the Asteraceae in particular (Gielly and Taberlet, 1994). Given the abundance of Asteraceae representatives within the wetlands, it would be worthwhile to explore alternative primers, such as non-coding chloroplast sequences, that might yield better resolution for this group (Bayer and Starr, 1998).

It is important to recognize the limitations of this study in rendering a complete picture of the host plant preferences of *C. tarsalis*. Far from comprehensive, this study was targeted at deriving the nectar preferences of *C. tarsalis* in a single wetland environment during a relatively short period of the summer. As opposed to adding a further temporal component in tracking the changing nectar feeding preferences of *C. tarsalis* over the whole of summer, it was deemed more pertinent to identify which plants served as primary hosts during peak *C. tarsalis* and West Nile Virus season. This is certainly not to say that additional inquiries into the dynamic nature of mosquito plant feeding preferences would not be valuable, but rather that it was beyond the scope of this study.

The potential benefits of continued research into the exciting field of mosquito sugar dependence are manifold. Müller et al. (2010) has already succeeded in exploiting attractive sugars as potential pesticides by controlling *C. pipiens* populations through chemically treated fruit juices. Ultimately, the ability to quickly and accurately index potential mosquito habitats as being suitable or unsuitable given plant composition and other landcover variables could reduce the prevalence of arboviruses, particularly WNV, through targeted control and avoidance policies.

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