OVIPOSITION BIAS BY *CULEX RESTUANS* THEOBALD IN MANITOBA, CANADA TOWARDS WATER USED BY HORSES (*EQUUS CABALLUS L.*) FOR DRINKING

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ABSTRACT

Culex mosquitoes in North America play important roles in the maintenance/enzootic transmission of arboviruses among animal hosts, as well as in epidemic transmission of these pathogens to humans. Arboviral disease outbreaks in human populations can be serious public health events that require response, often involving vector control. These responses are improved by early-warning information about the enzootic amplification of virus prior to human epidemics. *Culex restuans* is an ornithophilic species known to be commonly infected by avian alphaviruses and flaviviruses in much of North America and plays a significant part in increasing the infected-bird reservoir prior to spill over via bridge vectors such as *Culex tarsalis*, *Culex salinarius* and *Culex pipiens*. Thus, monitoring *Culex restuans* oviposition activity as a proxy for blood feeding and virus transmission may serve as an important form of early-season surveillance. Spring-time observations in Manitoba, Canada in 2014 of *Culex restuans* egg rafts in high numbers from horse troughs relative to other nearby mosquito larval sites (with *Ochlerotatus* spp. and *Culiseta inornata* present) led to experiments that demonstrate a bias in oviposition activity by *Culex restuans* to water in which horses frequently drink. The behavior is somewhat enigmatic given the blood-feeding preference of this mosquito species for bird hosts. Potential mechanisms underlying this bias are discussed.

INTRODUCTION

West Nile virus (WNv) has the potential to cause explosive epidemics of clinical disease in North America. It was first recognized in New York in 1999 and spread within a few years across the continent (Komar 2003; Anderson *et al.* 2015). Rapidly escalating epidemics, within a season, are especially common in western North America (Kramer et al. 2008; Anderson *et al.* 2015). *Culex pipiens* L. (Diptera: Culicidae) (eastern and central North America) and *Cx. tarsalis* Coquillett (central and western North America) are considered major epidemic vectors of WNv during the middle to late summer and early fall, whereas, *Cx. restuans* Theobald is thought to play an important role in early season amplification of the virus in areas of North America where these other species are common. The pre-condition for widespread infection of epidemic vector *Culex* spp. is significant infection of birds early in the season, likely caused by *Cx. restuans*, a widely-distributed, virus-amplifying vector species not caught in large numbers in adult mosquito traps (Johnson *et al*. 2015). The population and virus amplifying dynamics of *Cx. restuans* relative to late season population phenology of *Cx. pipiens* and *Cx. tarsalis* are described by Kunkel *et al.* (2006) and Lampman *et al.* (2006) where the crossover date (the seasonal point at epidemic vector *Cx*. activity starts to exceed that of *Cx*. *restuans*) is demonstrated to be important in epidemic occurrence. These insights support the hypothesis that the biology of *Cx*. *restuans* as an enzootic, amplifying vector is a crucial component of seasonal risk for arboviruses in addition to the dynamics of epidemic vectors.

It is important to gain as much lead time as possible for planning and implementing vector control efforts directed at *Cx. tarsalis*, which is often not within the regular remit of many mosquito control programs in Canada and elsewhere because it is not a significant nuisance. Thus, it is useful to estimate early-season population trends and feeding activity of *Cx. restuans* as an amplifying vector, and thus, later risk of human disease, even if this species is not directly causal of human epidemics.

Small numbers of host-seeking *Cx. restuans* are collected in adult mosquito traps (Manitoba Health: Scott Derham, personal communication, 2018), but there is some question if this index is reliably indicative of the actual population dynamics of the species. *Culex restuans* do lay egg rafts in artificial containers, including tires (McMahon *et al.* 2008) and others, if there is some organic enrichment (Brust 1990). Considerable evidence suggests that organic enrichment alters the olfactory information available to gravid mosquitoes to discriminate between sites within their behavioural sphere of evaluation (Day 2016). Orientation toward aquatic sites for egg-laying is mediated by attractants, although actual egg deposition may or may not happen after contact evaluation has been made by the female mosquito (Day 2016). There is also an emerging body of evidence that mosquitoes, including *Culex*, have relatively specific olfactory sensilla that play a role in assessment of oviposition sites. There are a number of different, published approaches to organic enrichment (Brust 1990; Lampman and Novak 1996). Published accounts of sampling efforts yield significantly variable results, although geographic differences in study area, seasonal timing, and local ecology may contribute to this variation. Brust (1990) used sod-enriched oviposition pools to monitor egg-laying activity of *Culex* species in Manitoba, but that approach is cumbersome and inconsistent. A simple, more consistent oviposition attractant/stimulant for *Cx. restuans* would improve surveillance of this important, virus-amplifying species, especially if a standardized lure could be combined with available ovitraps on the market for other species of *Culex* (Day 2016).

In southern Manitoba in the spring of 2014, large numbers of *Cx. restuans* egg rafts were observed in pools of water where horses were known to drink relative to other available egg-laying spots (grassy pools near a tree line) in close proximity. There were also abundant egg rafts in containers that had been used by horses the previous summer for drinking and which had frozen, then thawed in the spring. This seemed to indicate considerable attractiveness despite no recent contact by the horses and/or addition of fresh organic material. This observation, and preliminary trials with water from horse troughs and control egg-laying sites (fresh well water and untreated water from a nearby wetland) led to more detailed experiments to test the hypothesis that drinking activity of horses at otherwise fresh water made those aquatic habitats more attractive than alternative sites to gravid *Cx. restuans*.

MATERIALS & METHODS

Study Sites

The primary site of experimental work for this project is near Tyndall, Manitoba, Canada (50.084 N, -96.661 W) on a rural property of approximately 16.5 hectares. The property is extensively treed with natural stands of ash, willow, birch, poplar, aspen, tamarack and many species of native shrubs as under story; a vegetation mix typical of transitional zones between short-grass prairie/aspen parkland and boreal forest. There is one human residence on the property with two dogs and two horses. Abundant wild mammals (white-tailed deer, black bear, skunk, raccoon, coyote, fox, porcupine, rabbits, and small rodents) and many species of birds are present in close proximity. The house site and small paddocks for the horses in the natural forest account for approximately 1 hectare. The horses graze/browse (June and July) in the forest but are primarily fed mixed grass hay with no alfalfa the rest of the year. They have access to fresh well water (troughs are rinsed weekly with well water) and standard trace mineral blocks. Five other collection sites assessed for comparison and additional data were rural properties (with some natural ponding suitable for *Culex* mosquitoes) within 30 km of the primary experimental site where horses were present and where it could be verified that no other livestock had access to troughs. Horse numbers varied between properties from two to more than 20.

General Methods

A field experiment was conducted to test whether *Culex* egg rafts are distributed by ovipositing females in a biased pattern with respect to the status of water in potential egg-laying sites. Egg rafts were collected from various oviposition sites with a fine-tipped, hog's-hair, artist's paint brush and placed in individual wells of tissue culture plates until hatched. Larvae from each egg raft were transferred to and reared in individual, plastic, disposable food trays for identification to species at fourth instar using keys in Wood *et al.* (1979).

In 2014, following from initial observations of an apparent concentration of oviposition activity where horses had been drinking, egg rafts were collected at the primary site from May to August from a horse trough (~0.7 m^2) and adjacent aquatic habitat with no horse access for comparison (~8.5 m^2). During the summers of 2016 and 2017, this work was continued at the primary site and extended to one other horse property (with the same trough system and known presence of *Culex* mosquitoes) in more open farmland approximately 25 km away. Records were kept of when the horse troughs were cleaned and replenished with fresh water. Aquatic habitats (~2.5 m^2) used as controls in 2016 and 2017 were replenished with water from the same source as the troughs if they diminished due to lack of rainfall. Collections were made from 21 May to31 August in 2016 and from 19 May to 15 September in 2017.

On three dates in 2014 (July 29, August 7, August 14), nine passenger car-sized tires representing three treatments (three tires per treatment) were used as containers to further evaluate *Cx. restuans* oviposition behaviour with respect to choice of water from different sources. All tires had been thoroughly scrubbed and rinsed multiple times with clean, cold water from the well. Water from a natural, wetland site (control) with documented presence of *Culex* egg rafts was placed in three tires. "Horse" water (from a horse trough filled three days earlier because egg rafts usually appeared starting three to four days after a rinse and refill cycle) filtered through 1 mm mesh cloth was placed in three other tires. Water collected previously from a horse trough that also had *Culex* successfully emerge from it was placed in three other tires. From left to right, the first tire contained control water, the next tire contained water from a horse trough, and the last tire contained horse water that previously had mosquitoes reared in it. That sequence was repeated twice more from left to right. Water surface area in each tire = ~0.03 m^2. The experiment was replicated three times although the start of each replicate differed by a few days. Tires were monitored daily for 1 week and total egg rafts accumulated in each tire was recorded. These data were subjected to ANOVA as sampling could be corrected per unit surface area of each type of habitat. Data from tires with "horse only" water and tires with "horse water that also had *Culex* successfully emerge" were pooled for statistical analysis as each of these types of water yielded similar numbers of egg rafts (no significant difference).

In addition to the observational and experimental efforts described, a survey was conducted of five, separate horse facilities in the surrounding area during summer of 2015. Horse troughs and other watering sources, including valve-controlled, continuous waterers, and adjacent habitat at each of these properties were checked for egg rafts. Owners were shown the egg rafts and mosquito larvae, and recommendations were made to empty and clean horse troughs once per week. This was followed up in 2016 at three of the same sites with a second survey of the same habitats (containers and adjacent areas). The total surface area of water sampled at each location, and classified as "with horse-contact" versus "no horse contact" was not measured accurately although "no horse contact" habitat was always in excess.

RESULTS

During preliminary observations from 2014, >75% of all (27/37) rafts were from habitats where horses had drunk in the last week, despite comparison sites representing the greater surface area (12.1×) of habitat (8.5 m^2 vs 0.7 m^2). All were reared to fourth instar and all were identified as *Cx. restuans*. Most egg rafts observed to accumulate in the tire experiment (94%; 155/165) (mean = 14.05/m^2, ± 2.08 (SE)) were laid in water that horses had contacted (either without or with the previous presence of *Culex* larvae). In contrast, few egg rafts ((6%; 10/165) (mean = 3.91/m^2, ± 2.08 (SE))) accumulated in control tires (most after five days incubation when some organic debris had accumulated). All egg rafts yielded *Cx. restuans*. *Culex restuans* females laid significantly more (T = 3.34; DF = 25; p=0.0026) egg rafts in tires with horse water than tires with control water.

In 2016, 230 egg rafts were collected from troughs and control sites at the primary site and secondary site with horses. Thirty-four egg rafts were not viable (not hatched), whereas, 196 were reared to fourth instar, all of which were identified as *Cx. restuans*. Seventy two percent (166/230) of these rafts were from habitats where horses had drunk in the last week. In 2017, a total of 125 egg rafts were collected from the same sites as in 2016. Thirty-one rafts were not viable (not hatched), 95 were reared to fourth instar, all of which were identified as *Cx. restuans*. Eighty-two percent (103/125) of these rafts were collected from habitats where horses had drunk in the last week. An aggregate Chi^2 analysis of both years data combined shows significant bias in presence of egg rafts toward water where horses have been drinking ($X^2 = 355$, DF = 1; p<0.001).

In 2015, during a one-time survey of five horse properties, more than 90% (75/83) of egg rafts were collected from horse-watering sites versus immediately adjacent (< 10 m away) areas. Chi^2 analysis ($X^2 = 83$, DF = 1; p<0.001) shows significant bias in presence of egg rafts toward water where horses have been drinking. In 2016, the follow up survey found no egg rafts in troughs that had been regularly cleaned based on recommendations from the previous year.

DISCUSSION

I found a significant, behavioural tendency of gravid *Cx. restuans* to lay eggs in wet sites where horses have been drinking for at least two to three days relative to other sites nearby that were presumed to have biological potential for larval development. It is not possible to definitively conclude that the "horse-influenced" sites were more attractive versus less repellent than alternative sites as no direct observations of mosquito orientation and contact-based acceptance/rejection behaviors were made (Day 2016), and thus the underlying mechanism for the bias was not ascertained. Though consistent in general pattern over several experimental and observational approaches, the observations detailed here are somewhat enigmatic, given that most of the published literature characterizes *Cx. restuans* as a primarily ornithophilic or bird-preferring species, at least with respect to blood hosts (Molaei *et al.* 2006). One might reasonably hypothesize that semi-permanent and permanent aquatic habitats with which birds are associated (by proximity or because birds drink there) would be highly attractive because they might signal to female mosquitoes the presence of suitable blood hosts on which to feed following oviposition. Also, many mosquitoes drink from potential oviposition sites immediately prior to egg-laying (Day 2016). It is likely that the selection of oviposition sites is uncoupled, in an olfactory sense, from host seeking, for reasons that have to do with organic enrichment of larval sites suitable for larval survival and emergence to the adult stage. It is well-established that immature mosquitoes in the genus *Culex*, particularly vector species within the sub-genus *Culex* do well in organically enriched water, although the chemical profile of these sites may vary significantly between species within the genus (and certainly among subgenera within *Culex*) (Lampman and Novak 1996; Reiskind and Wilson 2004). Furthermore, studies of other species of mosquitoes indicate that female mosquitoes often seem to choose egg-laying sites favourable for offspring success, though the degree to which that is signaled by the presence of eggs and/or larvae previously deposited seems to vary (Reiskind and Wilson 2004; Day 2016).

Setting aside the question of why *Cx. restuans* may preferentially lay eggs where horses have contacted the water, we may perhaps understand the behaviour mechanistically with respect to the sensory physiology of this and related species as follows. Nonanal is one organic molecule demonstrated to have a stimulatory effect on sensory cells of gravid *Culex* mosquitoes (Syed and Leal 2009). Nonanal is a nine-carbon ester commonly present in the saliva of many mammals, thus, horses may transfer it to drinking water, which may account for the apparent behavioural bias documented for *Culex* mosquitoes in this study. Other volatile organic compounds resulting from chewed forage (and microflora) transferred to fresh drinking water may also change the degree of olfactory stimulation underpinning oviposition site evaluation. Further research is necessary to determine if there is a reproductive advantage to *Cx. restuans* of laying eggs in such places, or if a biochemical change precipitated by horse contact via salivary secretions or microflora introduced during drinking coincidentally produces olfactory stimulation of oviposition.

In Manitoba and by extension, other contiguous parts of the central/western North American prairies, there may be early indicators of the potential for outbreaks of human disease caused by arboviruses such as WNv that are primarily transmitted to humans by *Cx. tarsalis*. In 2003, an epidemic of human disease in Manitoba caused by WNv was correlated with population buildup of *Cx. tarsalis* in July and August (Manitoba Health 2018), but there was likely WNv circulating among birds prior to the presence of *Cx. tarsalis* and human cases, as elsewhere in North America (Komar 2003; Kramer *et al.* 2008). In Manitoba, the seasonal egg-laying activity (Buth *et al.* 1990) (earlier than *Cx. tarsalis*) and known host preferences of *Cx. restuans* for birds (Wood *et al.* 1979; Anderson and Brust 1995) implicate it as an important amplification vector for WNv. Although not collected in large numbers in the same adult traps that collect *Cx. tarsalis*, *Cx. restuans* are often infected with WNv (Manitoba Health 2018) on the southern Canadian prairies. Thus, the ability to detect and accurately monitor the population dynamics of this species before *Cx. tarsalis* become common may provide important lead time to plan for an epidemic and control measures that may become necessary. To this point, it is a potentially viable, but under-used source of early season risk information for potential, later outbreaks of arboviral activity.

Egg laying activity (the deposition of egg rafts into attractive oviposition sites) of *Cx. restuans*, as well as collection of the egressing females after egg laying for virus assay would provide important information about the population build up and the infection status of this amplifying vector before onset of a human epidemic. The extra two to three week lead time available for vector control planning and decision-making versus the usual practice of waiting for a sharp rise in the presence of infected, bridge-vector mosquitoes or human cases may, in most years, improve public health response.

The significant attractiveness of water which has been used by horses for drinking relative to other potential oviposition sites may lead to a sensitive and reliable egg-laying attractant for *Cx. restuans*, and thus a repeatable sampling approach to use *Cx. restuans* egg rafts as one of several tools for forecasting potential outbreaks of human disease caused by avian arboviruses amplified by this mosquito species (Day 2016). This may also yield another attractant that can be incorporated in novel or existing control approaches that involve inducing mosquitoes to lay eggs in artificial containers with insecticides incorporated in their design to prevent the development of new cohorts of immature mosquitoes.

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