AN EVALUATION OF REITER'S MEDIUM AND THREE DIFFERENT POOL SIZES FOR OVIPOOL SURVEILLANCE OF *CULEX TARSALIS*, *CULEX RESTUANS* AND *CULISETA INORNATA* IN MANITOBA.

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Lisa Marie Baspaly

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ABSTRACT

This study was conducted to determine whether hay infusion and smaller ovipools could replace current oviposition surveillance methods. Female *Culex tarsalis* given a choice of oviposition sites in the lab showed a preference for standing tap water over hay infusion (X^2 = 82.9; p<.05). Hay infusion (as described by Reiter in 1983) was not a suitable replacement for the conventional sod infusion in Manitoba. Field experiments were conducted to determine the minimum size of ovipool that could be used for surveillance of *Culex tarsalis*, *Cx. restuans* and *Culiseta inornata*. One point five per cent of egg rafts laid were collected from the smallest pools (10 cm X 10 cm X 15 cm), 47.8% were collected from the medium pools (30 cm X 15 cm X 15 cm) and 50.7 % were collected from the largest pools (40 cm X 30 cm X 15 cm). When frequency of oviposition events were calculated, there was no significant difference in the efficacy of the small, medium, large and traditional meter-square pools used.

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Section 1: General Introduction

Vector-borne pathogens are a major threat to human and animal health (WHO, 1997). Mosquitoes are important vectors of many pathogens, such as those that cause malaria, filariasis, dengue and encephalitis (Kettle, 1984).

There is greater taxonomic diversity in tropical arthropod-borne pathogens. Protozoan and nematode parasites, as well as arboviruses, are more common in tropical areas than in temperate areas (Roberts & Janovy, 1996). Temperate zones such as Canada and the northern United States tend to have less variety in arthropod-borne pathogens, which consist mainly of arboviruses such as Eastern and Western equine Encephalitis (WEE), Saint Louis Encephalitis virus (SLE), Venezuelan Encephalitis (VE), LaCrosse Encephalitis virus and Powassan Encephalitis virus (Artsob & Spence, 1979).

In Canada, during the last 25 years, there have been several outbreaks of encephalitis such as WEE, SLE and West Nile Virus (WNV). For all of those outbreaks, *Culex* spp. and *Culiseta* spp. were the primary targets of mosquito surveillance operations involved in transmission (Helson *et al.*, 1979).

Mosquito surveillance operations are an important part of predicting and controlling encephalitide outbreaks. They are necessary for monitoring the spread of arboviruses and other mosquito-borne pathogens. In Canada, these programs consist of collection and analysis of data from various types of traps including light traps, vertebrate-baited traps (sentinel bird flocks) and oviposition traps (Brust, 1976).

Oviposition traps are an important part of disease surveillance programs by virtue of the biological information they provide (Brust, 1982). Reiter (1983, 1986), Reisen & Meyer (1990) and Weber & Horner (1992) have examined ways of increasing oviposition trap efficiency by searching for a medium that will render an artificial site more attractive than surrounding natural oviposition sites. Oviposition attractants are used in surveillance operations to encourage females to lay eggs in artificial aquatic habitats.

Newly laid eggs are white, but turn black or brown within one or two hours. A rigid, proteinaceous chorion protects the egg. It minimises water loss and is permeable to gases. Females of raft-laying species will lay 30 to 300 eggs per raft, depending on the species, overall nutrition, stage in gonotrophic cycle, and size (Clements, 1999).

Oviposition pools are especially important where *Culex restuans* Theobald, a species of concern for surveillance operations due to its potential capacity to transmit pathogens, is not reliably collected in light traps (Brust, 1990). In Canada, sod infusions contained in 1 m X 1 m X 15 cm pools have been used to monitor *Culex* and *Culiseta* spp. (Brust, 1982; Brust 1990; Buth *et al.*, 1990). There are a number of problems associated with this combination of pool size and infusion. Areas located away from humans, but adjacent to populated areas, are generally chosen as surveillance sites to decrease the risk of tampering. The meter-square frame is large, so it is difficult to carry one or more into an unpopulated area, usually located some distance away from roads or people. Also, a pool of that size requires approximately 75L of water to fill it to a level where it covers the sod (approximately 5 cm from the top of the ovipool). Most relatively remote areas do not have tap water available nearby, and even many outside urban locations are not so equipped. Seventy-five liters is a large amount of water to transport into a site, and is difficult for one person to carry. Road access or the use of ATVs are possible solutions to this problem, but road access will also allow access to the general public, increasing the risk of tampering. Surveillance operations in Canada are typically limited in terms of money and resources, so the use of ATVs may not always be a feasible option. There are also the logistical problems associated with transporting an ATV and possibly fuel out to the limit of the road access in a remote area. Although surveillance is primarily concerned with mosquitoes in proximity to humans, mosquito populations outside urban areas must also be monitored to identify bridging vectors, amplifiers and the presence of any virus.

There are also problems associated with the sod. A large piece of sod has to be cut and carried into remote areas when setting up pools. Smaller pieces of sod tend to float in large pools. Alternatively, the sod can be dug up on site, but this is not legal or desirable in many areas such as City and Provincial Parks. Also, purchasing sod from a local garden centre can be a problem, as the commercial sod may be over-fertilized to the point where it creates a thick algal bloom in the pools after only a few days.

This research was conducted to examine ways in which mosquito ovipool surveillance in Manitoba could be streamlined. The focus for this study was on ovipools, as this was considered to be the most problematic area at the time this research began. The objectives of this research were:

1) to determine whether gravid female *Culex tarsalis* Linnaeus are more likely to oviposit in a hay infusion medium (as described in Reiter, 1983) vs. distilled / standing tap water, and

2) to determine the minimum size ovipool that can be used for surveillance of *Cx. restuans*, *Cx. tarsalis,* and *Culiseta inornata* Williston in Manitoba.

Section 2: Review of Pertinent Literature

The family Culicidae is divided into three subfamilies: the Culicinae, the Anophelinae and the Toxorhynchitinae (Lane & Crosskey, 1993). Worldwide there are about 3200 species of mosquitoes distributed in 38 genera (Harbach & Kitching, 1998).

Location and selection of an oviposition site is an essential part of the mosquito life cycle. Before an oviposition site is selected, a blood meal is needed for eggs to develop in anautogenous species. Autogenous species may lay their first batch of eggs without a blood meal, relying on nutritional reserves left over from earlier life stages. The blood meal is where most pathogens are acquired. Thus, pathogen transmission requires the completion of one oviposition cycle before pathogen transfer can occur with a subsequent blood meal. Oviposition is an important component of the surveillance of mosquito-borne pathogens (Bentley and Day, 1989). It should be mentioned that for host-pathogen interactions where transovarial transmission occurs, transmission occurs from an infected female to her progeny. These progeny are then sometimes able to infect with their bite, and / or continue the cycle of transovarial transmission (Turell, 1988).

In this chapter, I will broadly examine mosquito oviposition strategies, with a focus on the species that lay their eggs as rafts on the surface of water. These species are of particular importance to disease surveillance in Manitoba. It is assumed here that readers will have a general familiarity with culicid biology and only short reviews are provided for the more pertinent aspects of their life cycle.

BASIC LIFE HISTORY

The major events in the life of a female mosquito are hatching, larval development, pupation, emergence as an adult, mating, feeding and oviposition. The rate of embryonic development is dependent on temperature but larvae usually take a few days to several weeks to hatch (Lane & Crosskey, 1993). Eggs laid on the surface of water will hatch as soon as embryonic development is complete. Larvae have modified mandibles / maxillae to obtain food from the water column or the substrate. The larvae of most species eat microorganisims (such as protists), detritus (such as decomposing leaves), algae, and dead as well as living invertebrates. Some species also consume other mosquito larvae (Clements, 1999). During pupation, larval structures and organs eventually degenerate and are replaced with the pupal forms from undifferentiated cells in the imaginal discs. When metamorphosis is complete within the pupal cuticle, air is inhaled to increase the internal pressure. This pressure causes the cuticle to split along the ecdysial sutures. The adult then slowly emerges from this split (Lane & Crosskey, 1993; Clements, 1999).

Mosquitoes have diverse life history patterns. There are several traits commonly used to categorize these life histories: the stage in which diapause occurs, number of generations per year (voltinisim) and type of larval habitat

(Lane & Crosskey, 1993). Larval habitat, though not a life history trait in the classical sense, is widely used to categorize life histories. Larval habitats are especially important to identify during surveillance operations, as they will guide the use and placement of ovipools, and direct control efforts such as larviciding. It is also important to be aware of the preferred larval habitats for vector species, as this will be valuable information for any mosquito control / abatement programs. Larval habitat is the only trait used to describe life history that will be discussed here.

LARVAL HABITATS

Larval habitats are determined by where the female mosquito oviposits (Laird, 1988). Clements (1999) divided mosquito oviposition sites into the following six categories: water surfaces of permanent, semi-permanent, or transient ground waters; soil surfaces subject to transient flooding; leaf surfaces of aquatic plants; water or plant surfaces of phytotelmata; other natural cavities; artificial containers. Only those habitats that are relevant to mosquitoes that lay egg rafts, *i.e.* water surfaces, phytotelmata, and natural or artificial containers, will be discussed here.

The term "ground waters" includes ground pools, rock pools (saline and fresh), puddles, hoof prints, stream or river edges (Clements, 1999), rice fields, saline and fresh water marshes (predominantly vegetation with abundant water), saline and fresh water swamps (predominantly water with abundant vegetation), ponds, ditches, polluted waters, wells and subterranean waters (caves, flooded cellars, etc.) (Lane & Crosskey, 1993). The main differences among ground habitats are size, permanence, exposure to sunlight or shade, chemical composition and aquatic flora. These differences will affect such things as water chemistry and will be a function of environmental factors such as elevation or latitude.

Permanent ground habitats (marshes, swamps, exposed ponds, forest ponds, ditches) usually have permanent vegetation of some sort and are colonized by a variety of mosquito genera, including *Culex, Aedes* Meigen, *Anopheles* Meigen and *Coquillettidia* Edwards (Lane & Crosskey, 1993).

Temporary water is used as an oviposition site for some mosquito species. Small ponds, ruts, hoof prints and puddles are examples of this type of habitat and they are used mainly, but not exclusively, by aedine mosquitoes. These mosquitoes have adapted to these temporary habitats by developing eggs that can resist desiccation (Clements, 1999; Lane & Crosskey, 1993).

Some mosquitoes are found in slow flowing water. In Australasia, larvae of *Culex starckeae* Stone & Knight are found in flowing water, and other species, such as *Anopheles punctipennis* Say, inhabit the edges of shallow streams (Lane & Crosskey, 1993).

Rice fields are another common oviposition site for *Anopheles*, *Culex,* and *Psorophora* species mosquitoes. Rice fields may prolong the developmental season for some species. *Anopheles gambiae* Giles will oviposit in rice fields during the dry season when transient ground pools are unavailable (Jones & Schreiber, 1994).

Polluted waters are used as oviposition sites by *Culex quinquefasciatus* Say, *Culex stigmatosoma* Dyar and some others. Polluted waters range from ponds containing decomposing wood from logging operations, cesspits, cesspools and latrines, to ditches in both urban and rural areas. These species are making use of a habitat where there is less competition, as most other species cannot tolerate even a moderate level of organic pollution (Lane & Crosskey, 1993).

Mosquitoes also colonize subterranean waters (Kay *et al.*, 2000). *Culex pipiens molestus* Linneaus breeds in dark underground waters such as cellars and mine shafts in India and Hungary, and possibly elsewhere. *Culex quinquefasciatus* has also been found in mines, while *Anopheles smithii* Theobald is sometimes found in African caves, along with other *Anopheles* species (Lane & Crosskey, 1993; Beaty & Marquardt, 1996). A few species, such as *Anopheles stephensi* Liston are sometimes found in wells (Lane & Crosskey,1993).

Another category of habitat utilized by raft layers is the phytotelmata (Clements, 1999). These include tree holes, leaf axils of bromeliads and of other plants, floral bracts, bamboo, pitcher plants, leaf pools, fallen bracts and fruit husks.

A natural container habitat is a very small, isolated pool containing some sort of aqueous medium. Tree holes are the most widely used container habitat and certain species of *Toxorhynchites* Theobald, *Ochlerotatus, Sabethes* Robineau-Desvoidy, *Orthopodomyia* Theobald, *Aedes*, some *Anopheles* and a few *Culex* use them. Certain mosquitoes show a preference for a specific type of tree hole. Some, such as *Sabethes chloropterus* Von Humboldt, prefer tree holes with narrow apertures while others utilize a specific tree species (Lane & Crosskey, 1993). Specific preferences, if any, vary among species.

Ground containers such as cocoa pods and coconut husks are utilized by *Anopheles* and *Eretmapodites* Theobald (in Africa) and *Armigeres* Theobald (in Asia) (Lane & Crosskey, 1993). Large fallen leaves, palm fronds, and banana leaves are all utilized by some *Aedes, Culex* and *Eretmapodites* species (Lane & Crosskey, 1993; Clements, 1999).

An additional category used by Clements (1999) to classify mosquito oviposition sites / larval habitats is other natural cavities which includes rock holes, crab holes, and discarded gastropod shells. Empty snail shells are used by *Aedes calceatus* Edwards, *Eretmapodites quinquevittatus* Theobald and *Eretmapodites silvestris* Ingram & De Meillon (Clements, 1999). Rock holes are utilized by *Aedes vittatus* Bigot and *Deinocerites* Theobald breed in tunnels dug by land crabs. Certain species of *Aedes* and *Culiseta* will also breed in this specialized habitat.

The last category relevant to raft layers is artificial containers (Clements, 1999). Man-made containers such as water tanks / cisterns, latrines, septic tanks, water storage pots, discarded tires, cans and bottles can function as oviposition sites for mosquitoes (Lane & Crosskey, 1993; Clements, 1999). *Culex quinquefasciatus* and *Cx. stigmastomata* are just some examples of these kinds of breeders (Bentley & Day, 1989; Clements, 1999).

FEEDING

Most species of mosquitoes are anautogenous, meaning they must take a blood meal to lay eggs. However, some species can use protein they accumulated during the larval stages to provide enough energy to lay the first batch of eggs. These species are called autogenous (Lane & Crosskey, 1993; Clements, 1999). Autogeny is an important factor in vector biology as it can decrease the rate of pathogen transmission. Autogenous females are older when they take their first blood meal. Female mortality increases with age (Reeves *et al.*, 1994), so fewer of these mosquitoes will survive to take multiple blood meals, thus potential for pathogen transmission is lower.

Autogeny is a genetically determined trait among mosquitoes but expression of autogeny is often controlled by environmental factors (Fox, 1994). Poor larval nutrition caused by environmental factors may reduce protein reserves for some individuals. The resulting adult mosquito will be unable to lay the first batch without a blood meal (Gillies, 1972; Brust, 1991).

Mosquitoes feed on plant nectar or other sources of carbohydrates (Nayer & Sauerman, 1975). Female mosquitoes require a blood meal for each batch of eggs they lay, except in the case of autogenous females, which may lay their first batch of eggs without blood. It is this blood feeding that is significant to pathogen transmission, as this is the point where pathogens are transferred to or from hosts.

Once a mosquito has found a suitable feeding site on the host and penetrated the skin with piercing stylets, it will insert its stylet bundle (which forms the salivary ducts and food canal) into the skin. Saliva is injected into the feeding site, along with an anti-coagulant like substance, in some cases, during probing. This injection of saliva is the primary means of pathogen introduction into a vertebrate host. Chemoreceptors on the stylet bundle detect the presence of blood and direct probing into the capillary, or into the haematoma caused by probing. The pumping structures in the head are activated, and blood is sucked into the midgut. A female mosquito can consume up to four times her weight in blood in just a few minutes (Lane & Crosskey, 1993). Feeding is terminated by feedback from stretch receptors in the midgut that respond to distension. Females then digest the blood and develop eggs. If not enough blood was obtained to initiate egg development, the female must search for another host. If the volume of blood obtained was sufficient, host-seeking and biting behaviour are inhibited fully (Klowden, 1986).

In terms of blood-feeding patterns, mosquitoes range from being generalists or specialists (Bentley & Day, 1989). A generalist will feed on a variety of hosts, while a specialist is limited to feeding on specific hosts. This is an important factor in assessing vector potential. Host use patterns may vary seasonally, between geographic regions and as the relative abundance of different hosts changes in an area (Reisen *et al.*, 1997).

OVIPOSITION

Once a blood meal large enough to stimulate ovarian development has been obtained and the eggs have developed, the female must locate a suitable oviposition site.

Nutritional status is known to affect mosquito oviposition (Foster, 1995). Prolonged exposure to sugar inhibits oviposition in the laboratory. Carbohydrate-starved *An. gambiae* females laid an average of 49 eggs per raft, while sugar-fed females laid only 22 eggs per raft (Gary & Foster, 2001). Sugar availability in the field may be important in influencing oviposition behaviour of gravid females. Depleted nutritional reserves may force females to lay eggs in poor or overcrowded habitats (Wallace & Merritt, 1999; Gary & Foster, 2001).

Insemination is known to influence mosquito oviposition behaviour. During copulation, the male introduces a substance produced by his accessory gland, called matrone (Klowden, 1999). Matrone will render the female refractory to any further insemination. This substance is known to be an oviposition stimulant in several mosquito species (Klowden 1999; Lee & Klowden, 1999).

Mosquito egg-laying has been divided into two distinct behaviours: pre-oviposition and the actual deposition of the eggs on the appropriate substrate. Pre-oviposition behaviour is any behaviour involved in the attraction, location, recognition of, and acceptance of an oviposition site. Pre-oviposition behaviour is induced by a hemolymph-borne substance in *Ae. aegypti* (Klowden and Blackmer, 1987). The behaviour can involve extensive searching flights, which often occur during twilight. The initiation of a pre-ovipositional flight is linked with environmental factors, such as rainfall, relative humidity, temperature and wind speed (Bentley & Day, 1989). Where the species exhibit two crepuscular biting periods, two pre-oviposition flights may occur.

Factors affecting site selection

Gonotrophic development may take two to seven days (Clements, 1999), after which a female searches for an oviposition site. Mosquito oviposition behaviour is regarded as similar to host-seeking behaviour. Both require the integration of complex physical and chemical cues. Long-range cues, probably involving vision, allow mosquitoes to identify different habitats and specific host and oviposition site characteristics (Weber & Horner, 1992). As the distance decreases, other cues become more important. Olfactory cues help mosquitoes to identify volatile factors at the oviposition site. These volatile compounds will be discussed in detail later in this chapter. Once an oviposition site has been identified, short-range cues such as temperature and chemical signals received by contact chemoreceptors probably become more important (Weber & Horner, 1992; Weber & Tipping, 1990).

Selection of an oviposition site is a critical factor in mosquito survival and population dynamics. Some species of mosquito will oviposit in almost any aqueous habitat, but others are quite specialized when it comes to site selection. For example, *Culex nigripalpus* Theobald egg rafts have been found in a great variety of habitats, while *Culiseta melanura* Coquillett will only oviposit in cedar or bay-head swamp potholes (Bentley & Day, 1989).

Female site choice does not appear to be driven by larval survival in some *Culex* species. For example, Roberts (1996) found that *Cx. quinquefasciatus* larvae, which are not normally found in brackish water, survived well in concentrations of up to 25% sea water. Gravid *Cx. quinquefasciatus* almost always chose to oviposit on fresh water in the lab trials. Survival in *Culex sitiens* Weidemann larvae was highest in saline water (66% sea water), but oviposition was greatest in concentrations of only 28% sea water.

Oviposition site selectivity is considered to be species dependent but there may be some overlap in habitat preference (Bentley & Day, 1989). Mosquitoes invest considerable energy in selecting an appropriate oviposition site and it is evident from the results of several studies that this selectivity is what determines larval distribution (Bates, 1940). There is no evidence that a species that is selective in oviposition sites is also selective when choosing hosts.

For many species, chemical substances have an effect on oviposition site selection. The origins of these substances are wide-ranging and their chemical structures are varied (Bentley & Day, 1989). An oviposition attractant is any chemical that causes a gravid female mosquito to make oriented movements toward the source. An oviposition stimulant is any chemical that elicits oviposition. Conversely, an oviposition repellent is any chemical that causes the insect to make oriented movements away from the source, while an oviposition deterrent will inhibit oviposition by an insect in an area where oviposition would have occurred in the substance's absence (Bentley & Day, 1989). Only oviposition attractants and stimulants will be discussed in this chapter.

Physical and chemical factors interact to affect oviposition site selection. Physical factors include water qualities (such as depth or movement), visual cues, landscape, soil quality and physical obstructions (Bentley & Day, 1989; Clements, 1999). Chemical factors include substances of egg, larval and pupal origin, vegetable matter, organic compounds, inorganic salts, and presence of predators/parasites (Isoe & Millar, 1995; Isoe *et al.*, 1995).

A mosquito's response to certain visual cues such as colouration (wavelength and frequency) and shade intensity will influence the selection of an oviposition site (Dhileepan, 1997). Colouration is an important cue and can elicit pre-oviposition behaviour from a responsive female (Belton, 1967). Colour preference varies greatly among species. To some day-laying species, such as *Wyeomyia mitchellii* Theobald, colouration of the oviposition medium is important for accepting or rejecting a site (Frank, 1986). Mosquitoes are using wavelength discrimination to select coloured backgrounds (Clements, 1999; Beehler & Mulla, 1993). Still water often has a mirror-like surface that can reflect brightly (*i.e.* has a specular reflection). Females that reject light-coloured oviposition media on dark backgrounds will readily oviposit on a reflective site. The mechanism by which females recognize a specular reflection is not understood.

For certain species, visual cues are more important than chemical cues. *Anopheles darlingi* Root will die without ovipositing when kept in complete darkness, while other species, such as *Cx. quinquefasciatus,* will oviposit regardless of light conditions (Zulueta, 1950).

Shade is an important visual cue for oviposition site selection for many species but is less or not important for others. Shade is defined here as protection from direct sunlight or moonlight. *Culex restuans* will oviposit mainly in shady pools, while *Cx. tarsalis* will oviposit mainly in pools exposed to sunlight (Brust, 1990). In a tire dump in Indiana, Beier *et al.* (1983) found that 21.6% of the larvae in exposed tires were *Aedes atropalpus* Coquillett, while the same species was only present in 1.2% of shaded tires. Conversely, *Aedes triseriatus* Say comprised 14.0% of those in exposed tires and 61.9% of those found in shade. Removing the vegetation that overhangs ponds and streams, thus denying ovipositing females shade, has been an effective control strategy for *Anopheles leucosphyrus* Doenitz in Borneo (McArthur, 1947). The presence or the absence of shade may not be directly affecting the gravid female, but may instead be acting on the water temperature or chemical composition of the medium as a result of leaf fall. On the other hand, some species that oviposit at night will only oviposit in areas shaded from direct starlight (and presumably sunlight during the day) (Clements, 1999).

Elevation of an oviposition site can affect its suitability to a particular mosquito species (Dhileepan, 1997; Jones & Schreiber, 1994). Some species that oviposit in tree holes show a preference for a particular height (*e.g. Toxorhynchites rutilus* Coquillett) (Jones & Schreiber, 1994), while others do not *e.g. Ae. triseriatus* (Wood *et al.* 1979). Different elevations can mean a different brightness (from reflected sun) and / or chemical composition of the oviposition medium or a different humidity. Wetter areas will have more ground-level sites to exploit and the elevation preference for a single species can vary with habitat, *i.e.* grassland versus woods (Downs & Pittendrigh, 1946).

Gravid female mosquitoes are responsive to water and water vapour (Kennedy, 1942). The optical characteristics of water can act as cues to its presence. It has also been hypothesized that mosquitoes may use humidity gradients to find water, but in further studies in the field, researchers have shown that this seems unlikely and clear evidence of this has not yet been demonstrated (Clements, 1999).