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OF VECTOR ECOLOGY

Theme: Achieving Sound Vector Control Through Ecological Understanding

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The Second International Congress of Vector Ecology will be held in Orlando, Florida, U.S.A., on October 19-24, 1997. It is being sponsored by the Society for Vector Ecology. Facilities for the Congress will be in the Holiday Inn International Drive Resort, which is 15 minutes from the airport and downtown Orlando.

The City of Orlando is world renowned for its Disney World Resort, Universal Studios, and Sea World. A short distance away is Cape Canaveral and the Kennedy Space Center. It offers a subtropical climate. Florida is a state that offers leading edge technology, great agricultural production, and a wide variety of vector problems.

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The Scientific Program will include plenary sessions, symposia, and poster sessions. Only invited papers will be presented at plenary sessions and symposia. Submitted papers will be accepted for poster sessions. Among the topic areas to be presented are:

- Changing Patterns of Vector-Borne Disease
- Parasitic Disease Trends
- Viral and Bacterial Disease Trends
- Vector Control Programs Worldwide
- Vector-Host Interactions
- Biogeography
- Systematics of Vectors
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- Molecular Biology
- Genetics of Vectors
- Vector-Pathogen Interactions
- Surveillance and Reporting Systems
- Immunology and Vaccine Development
- Educational Programs
- New Approaches to Control
- Future Predictions and Needs

For information contact: Gilbert L. Challet, Secretary, 2nd International Congress of Vector Ecology, P. O. Box 87, Santa Ana, CA 92702, Phone (714) 971-2421, ext. 148, Fax (714) 971-3940.
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SOCIETY FOR VECTOR ECOLOGY

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The Society of Vector Ecology lost a leader and a central figure when Russell E. Fontaine passed away on August 6, 1996 after an extended illness. He was most active as the President of the Society in 1982 and thereafter editor for the all-important Newsletter. Using his global network from his previous experiences, he played a major role enabling the Society to reach the level of national and international prominence that it currently enjoys. Most recently he had served very effectively as Coordinator of the University of California Mosquito Research Program from 1976 until his retirement in 1986.

Russ was born on October 9, 1914 in Worcester, Massachusetts. He is survived by his wife Vera, sons Bob (M.D.) and Ted, daughter Lola Craft, four grandchildren, and brother Leo.

Russ did his entire undergraduate work at the University of Toronto and graduated in 1939 with a B.Sc. in agricultural sciences. Through his contact with Richard Peters, he became involved in medical entomology in the Armed Forces during World War II in Australia. He continued to serve as a medical entomologist in the U. S. Army, first in Seoul, Korea, from 1947 through 1948 and subsequently with the
Caribbean Command from 1948 through 1950. In 1950 he was enticed to join Richard Peters again, this time with the Bureau of Vector Control, California Department of Public Health. It was during this tenure that he was dubbed “the Senator” because of his statesman-like, professional approach to conducting business at hand. In 1958 he left to serve as Senior Malaria Advisor in Ethiopia where he directed a malaria pilot project and assisted the host government in planning, developing, and implementing a countrywide malaria eradication program. From 1960 to 1964, he served as Regional Malaria Advisor, Latin America for U.S.A.I.D. Subsequently, he joined CDC as Assistant Chief, Aedes aegypti Eradication Program from 1964 through 1966 and later appointed Chief, Operations Section Malaria Program International (1966-1969). He continued his involvement in global malaria control programs with the World Health Organization from 1969 through 1976. In 1976 he left to accept a statewide responsibility for coordinating the mosquito research program in the University of Californian system where he remained until his retirement in 1986.

Dr. Robert K. Washino, University of California, Davis.
IN MEMORIAM

E. Paul Catts
1930-1996

The scientific community lost one of its brightest and most talented members when E. Paul Catts, 66, professor of entomology at Washington State University (WSU), died 5 April 1996 after suffering a heart attack while playing lacrosse with the university team. Paul was one of the finest, most creative people I have known. He squeezed more "life" out of his 66 years than most of us could only dream of. Just being around him almost always made a person feel happier.

Paul was born 3 April 1930 to Helen Gleason and E. Paul Catts, Sr., in Elizabeth, New Jersey. He was graduated from Cranford High School, spending his summers raising tomatoes for the Heinz Company. It was during this labor that Paul developed the work ethic that he practiced throughout the rest of his professional and personal life.

He married Margaret Seavy in 1952. The marriage, which ended in 1978, produced two sons, Glen and Wade. During the Korean conflict, Paul was a commissioned officer in the United States Army, serving as a Battery Commander of an antiaircraft artillery unit.

Catts earned his B.S. and M.S. degrees in entomology from the University of Delaware (UD) and a Ph.D. in parasitology from Berkeley. In 1964 he was hired by UD as an assistant professor and, within 10 years, was promoted to professor. Paul taught and lived...
the importance of ecology. While at UD, he led field trips to the Delaware marshes, the Okefenokee Swamp, and the Hawaiian Islands while part of his research examined how wildlife adapted to infringements of human activity in woods, swamps, and marshes.

He married Dana Ketner in April 1979 and, in keeping with “Cattsian tradition,” the wedding was in an old church (built in 1773!) that had no heat and the reception was in the local fire house hall! In 1980 they moved to Pullman, WA, where Catts served as chair of the entomology department at WSU. For the next 16 years, Paul taught various courses in entomology while he and Dana enjoyed the great outdoors with their daughter, Summer and son, Ketner.

The dictionary defines ‘Renaissance Man’ as follows: a present-day person with many broad interests who has the opportunity to indulge in them so as to acquire a knowledge of each that is more than superficial. Paul Catts was such a person. He was a scientist. He was internationally recognized in medical and forensic entomology, having published over 40 papers in both areas. Paul worked on over 50 homicide investigations, attempting to use insects to clarify murder investigations.

He was an author. He coauthored two editions (1970 and 1982) of the Manual of Medical Entomology; Entomology and Death: A Procedural Guide; and Insects Did it First, all of which he illustrated. He authored two Annual Review of Entomology articles. Many of his writings have been published in the Delaware Conservationist.

He was a teacher. He taught by and through inspiration, thereby instilling in his students an unending desire to learn the subject. He made a point of photographing students in his class so he could call them all by name. One of his most popular courses, “Insects and People,” was highlighted by a bug luau featuring such culinary delights as fried grubs and bee pupae muffins!

He was an artist. In addition to illustrating his scientific publications, he was an accomplished wildlife and historical artist. A number of his works remain on display at the Delaware Nature Education Society, the National Headquarters for Sigma Nu, and the WSU James Entomology Museum. He also created several duck and trout stamps in Delaware and Washington. Even his class handouts carried one or more of his drawings.

He was a creator/inventor. One of his maxims was “why buy it when you can make it.” During our bot fly research together, Paul decided we should build a fake horse to try and figure out what exactly about a horse attracted the adult flies. To this end, I met him at his house early one Saturday. Having never constructed a horse myself, I was curious, to say the least. After several hours of sawing, hammering, bending, cutting, etc., it was done. And a beauty it was although I would suggest that it was no threat to the Trojan Horse! I regret to inform the readers that five minutes after placing our fake horse in the field, it was unceremoniously smashed to pieces by the true equines. Paul’s comment at the time was “nothing beats a trial but a failure.”

He was a giver. He was truly concerned about the way we treated Mother Earth. He spent much time with youngsters and adults at a church camp called Camp N-Sid-Sen in Washington. His story-telling skills were legendary, especially around a campfire. He was an active scout leader and a member of the Community Congregational United Church of Christ. Other little-known talents included building turtle boats, archery, sand sculpting, skier, birder, reciting poetry, and playing the ukulele.

He was humble. Although outspoken at times, he was well-liked. His popularity did not breed arrogance, however; it was not his nature. One of his descriptive words was “shirty,” a term he used to describe people whom he thought took themselves much too seriously. He practiced simple yet active living, reusing what most would discard, and making needed items from scratch. He would undoubtedly be amused and perhaps slightly embarrassed by all the nice things that have been written about him.

He was a friend; a friend to the earth, a friend to the arts, a friend to his students, and a friend to all those who were lucky enough to know him.

Paul’s efforts did not go unrecognized. In 1977 he received Order of the Arrow, Order of Merit, and the Silver Beaver Award from the Boy Scouts of America for his distinguished service to youth. In 1972 he was recognized as Outstanding Educator in America. In 1975 and 1989 the Entomological Society of America presented him with its Excellence in Teaching Award and Award for Outstanding Service respectively.

Michael Costello, a research technician at WSU, wrote the following about Paul Catts: “It’s difficult to convey the magnitude of the void Paul Catts’ passing leaves behind. He was the smartest, most intellectually honest, generous, and sincerely humble person I have ever known. I always had difficulty calling him my friend because to do so seemed like immodest, self-congratulatory name dropping. If he knew that, it would have embarrassed him. He was the sort of person I want to be when I grow up. And I miss him terribly.”

That sums up the feelings for a lot of us about Paul Catts.

By Stanton E. Cope, Ph.D., Lieutenant Commander, United States Navy.
JOURNAL OF VECTOR ECOLOGY

Guidelines for Contributors

The Journal of Vector Ecology is an international journal published by the Society for Vector Ecology. It is concerned with all aspects of the biology, ecology, and control of arthropod vectors and the interrelationships between the vectors and the disease agents they transmit. The journal publishes original research articles and research notes, as well as comprehensive reviews of vector biology based on presentations at Society meetings. All papers are reviewed by at least two referees who are qualified scientists and who recommend their suitability for publication. Acceptance of manuscripts is based on their scientific merit and is the final decision of the editor, but these decisions may be appealed to the editorial board.

Manuscripts intended for publication should be sent to Dr. Marc J. Klowden, Editor, Division of Entomology, University of Idaho, Moscow, Idaho 83844-2339, U.S.A. Manuscripts must be double spaced on a single side of bond paper with 25 mm margins. An original and two clear copies are required. Draft mode dot matrix type should not be used. Submission of text on a 3-1/2” computer diskette formatted in MS-DOS is encouraged. Microsoft Word, Word Perfect, or Wordstar formats are acceptable, as well as unformatted text files. Please indicate the type of format on the diskette label. Papers must be organized under the following headings, each on a separate page, in order: Title page, abstract, text, acknowledgments (if appropriate), references cited, tables, figure legends, and figures. The title page should contain the names of all authors, their affiliations and the identification and address of the corresponding author. It should also include a keyword index containing no more than five words that best describe the paper. Pages should be numbered consecutively starting with the title page. References should conform to the style in recent volumes. Illustrations that are submitted must be of high quality and remain legible after reduction.

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A Concise Review of Chironomid Midges (Diptera: Chironomidae) as Pests and Their Management

Arshad Ali

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Received 18 May 1995; Accepted 25 October 1995

ABSTRACT. Increasing eutrophication of inland urban and suburban natural and man-made aquatic ecosystems resulting from natural and anthropogenic factors in recent years has been conducive to population increases of aquatic chironomid midges in many parts of the world. Adult midges from these habitats frequently emerge in large numbers, causing nuisance problems, human allergies, and also have severe adverse economic impact. Globally, nearly 100 of the 4,000 known chironomid species are documented as pestiferous. Numerous laboratory and field studies to reduce midges by physical and cultural, biological, and chemical means have been conducted. Organochlorines, organophosphates (OPs), pyrethroids, and insect growth regulators (IGRs) have been evaluated against midge larvae in the laboratory and/or used in the field. The best results for chemical control have come from OP insecticides (chlorpyrifos and temephos), and IGRs (diflubenzuron, methoprene, and pyriproxyfen). The OP insecticides have generally provided larval field control for two to five weeks at rates <0.56 kg AI/ha resulting in insecticidal concentrations of <1 to 5 ppm, but increased tolerance by midge larvae to some materials has been reported. Insect growth regulators (especially pyriproxyfen) have provided >90% suppression of midge emergence for several weeks at <0.25 kg AI/ha. Pyriproxyfen, methoprene, and diflubenzuron warrant registration for chironomid control in the USA. Attempts at physical and cultural control of chironomids have given mixed results. Biological control agents, such as Bacillus thuringiensis serovar. israelensis, the flatworm Dugesia dorotocephala, as well as several fish species are useful but only in smaller habitats. The midge habitats, which cover hundreds or thousands of ha, require appropriate investigations on the biology, ecology, and behavior of larval and adult pestiferous midge species to formulate suitable control strategies.

Keyword Index: Chironomidae, midges, pests, nuisance problems, control, management.

INTRODUCTION

Chironomids (non-biting midges) are one of the most ubiquitous, diverse, and ecologically important groups of aquatic macroinvertebrates (Coffman and Ferrington 1984), the most widespread insects in freshwater (Thienemann 1954), and occur on all continents including Antarctica. The larvae occupy a broad spectrum of aquatic and semi-aquatic habitats including brackish and marine waters, and even inhabit some semi-terrestrial and terrestrial biotopes (Oliver et al. 1990).

In inland natural or man-made aquatic ecosystems, chironomid larvae are usually abundant in terms of numbers of species, biomass, or both. However, in recent years, chironomid densities amounting to thousands of larvae/m² of habitat have been reported from many countries of the world (Ali 1991a). Prevalence of such high midge densities in many habitats are generally due to increasing eutrophication of the habitat from anthropogenic sources, which causes a decline of macrofaunal species diversity. As a result, populations of some pollution-tolerant organisms, including some species of Chironomidae, increase greatly in such habitats. Rapid human population growth and related increases in human activities around the globe in recent decades necessitate construction or creation of a variety of new temporary or permanent aquatic habitats amid
urban and suburban population centers. Some of these habitats, such as reservoirs and channels used for some industrial discharges, ponds and effluent discharge channels at sewage processing facilities, residential-recreational lakes, irrigated agriculture, and others remain heavily infested with chironomid larvae. Consequently, frequent emergence of adult midges in large numbers from these habitats causes a variety of nuisance, economic, and in some situations medical problems for the human populations within the dispersal range of these insects.

**NATURE OF PROBLEMS**

Chironomid males typically produce aerial swarms. Females usually do not swarm, but rest on marginal shore vegetation and structures until they fly into a swarm of males, select a male, and copulate. Size of the swarms may vary from as few as 10 to many millions (Sublette and Sublette 1988). Dense midge swarms often limit human activity outdoors because the adults can be inhaled or fly into the mouth, eyes, or ears; adult swarms may cause asphyxia in cattle (Grothaus 1963). The adults deposit meconion or release egg masses on resting surfaces which require frequent washing or repainting. Midges soil automobiles, cover headlights and windshields, and the swarms may create risky driving conditions. They are hazardous to passengers and crews on trains, buses, cargo vessels, and boats in Venice, Italy (Ali et al. 1992). Attraction of adult midges to light causes great discomfort in residential areas because the adults swarm around lighted outdoors electrical fixtures and other objects which serve as swarm markers. Small-sized adult midges enter homes and cause a variety of nuisance and economic problems indoors (Ali 1995). They may cause a considerable economic loss to the hotel and tourism industry in many situations worldwide. An economic impact study in Florida, USA (Anonymous 1977), revealed that at least 10 counties in Florida were economically affected by chironomids; and specifically swarms emanating from Lake Monroe, Sanford, central Florida, inflicted a business loss of $3 to 4 million annually on the City of Sanford, with just one lakewfront motel spending nearly $50,000 each year on property maintenance and control attempts of the pest. Similar, but unquantified, economic loss caused by adult midges has been reported in the literature from many parts of the USA, Japan, Italy, and Australia. Midges also can be a problem for paint, pharmaceutical, and food processing industries where hordes of adults may contaminate the final products. There can be a considerable disruption and loss of working time because of midge swarms, impairing work, and/or causing complete stoppage of operation. Accumulations of dead adult midges and the unsightly webs spun by the predaceous spiders around resting midges on buildings, stucco, and fascia deface structures and require frequent cleaning and washing of properties for proper maintenance. Adult midges clog air-conditioning wall units and automobile radiators. Some species stick to car paint causing damage. Dead midges can have the odor of rotting fish, which persists for several days after removal of the dead adults. At times, the dead adults accumulate on roads in such quantities that they make the roads slippery and dangerous for traffic. Such conditions often prevail in the multistoried car-parking lots along waterfronts in Venice, Italy, where also at the Marco Polo Airport, there is great concern regarding the possibility of airplanes skidding over massive accumulations of dead midges on waterfront runways. Entry of adult chironomids into delicate equipment mounted on airplanes poses danger and additional economic loss to the aviation industry (Barbato et al. 1990).

Chironomid larvae are not known to be vectors of any disease organism. However, adult midges are associated with human allergic reactions, such as asthma, rhinitis, and conjunctivitis (Cranston 1988). The adults emerging from some polluted habitats may transport bacteria (Steinhaus and Brinley 1957) and organic insecticides (Larsson 1984) to terrestrial environments.

Globally, there are estimated 20,000 species of chironomids, with 4,000 species presently known (Cranston 1995). Of these, nearly 100 species in the subfamilies, Orthocladiinae, Tanypodinae, and Chironominae have been reported to emerge in large numbers and pose pest problems. A list of the nuisance midge species was provided by Ali (1995).

Chironomid larvae also can produce certain undesirable effects, such as contamination of drinking water supply systems (Langton et al. 1988). Midge larvae transported in potable water find their way into bottled beverages; they have also been seen in toilet bowls, causing concern for homeowners (Mulla, pers. commun.). Larvae of some midge species are agricultural pests, damaging rice seeds and plants (Stevens and Warren 1992) and several horticultural and aquatic plants as reviewed in Ali (1991a).

**MANAGEMENT ATTEMPTS**

Numerous chemical, biological, and physical and cultural techniques to control midges in a variety of habitats have been employed in the past 50 years. Of these, chemical control has been the most practiced. Midge control in some natural or man-made habitats by mechanical means, such as application of surface oils to
trap emerging adults (Lewis 1957), removal of midge egg masses from habitats, and dredging and mixing of substrate materials (Shimizu 1978) proved ineffective, while removal of substrate materials containing midge larvae (Ali et al. 1976) and continuous water spraying to prevent midge oviposition (Flynn and Bolas 1985) caused considerable midge reductions. Electrocutor traps of various sizes and designs are perhaps the most commonly used means of adult midge control worldwide, but their effectiveness in reducing midge nuisance has not been documented in any quantitative manner. Also, these traps often malfunction in situations where midge swarms are dense and recurring (Ali 1991a).

Behavioral manipulations of some midges through attraction of the adults to various light intensities (Ali et al. 1984, 1994a) or wavelengths (Kokkinn and Williams 1989, Hirabayashi et al. 1993) could reduce midge nuisance in some situations. The adults could be drawn from heavily populated residential and business centers to relatively less-inhabited areas by using brighter lights at the latter sites where suitable adult control may be implemented. Keeping the lights off in some situations, where possible, may provide some relief from midge nuisance (Bay, pers. commun.). For light spectrum-specific attraction, commercially available lamps which emit light with peaks in these parts of the spectrum may be employed for adult midge diversion, trapping, or decoy purposes. In case of adulticiding, applications of insecticides could be synchronized with emergence periodicity of the adults (Ali 1980), their range of dispersal (Ali and Fowler 1983), and short-term and long-term patterns of abundance (Ali et al. 1983, 1985a). This practice should not only reduce the land area to be treated, but also the amount of insecticide needed.

Reduction of midge larvae in some habitats is possible through habitat management, such as source reduction by alternate operation of sludge lagoons (Anonymous 1963), and rotational flooding and drying of partial areas of spreading systems used for aerifer recharge (Anderson et al. 1964). Proper designing of new reservoirs and man-made lakes would be conducive to low midge production (Magy 1968). In some habitats, midge populations may be manipulated by understanding their interaction with prevailing macrophytes (Johnson and Mulla 1983), or by reducing some larval food components (Ali 1990). Environmental management to reduce midge populations is highly desirable in large natural midge habitats, each spread over thousands of hectares. In such habitats, physical and chemical composition of substrate materials and chemistry of overlying water in relation to spatial and seasonal abundance of larvae of pestiferous midges may provide a clue to the ecological basis of midge production. Their proliferation could be discouraged by manipulating physical, chemical, and/or biological conditions conducive to chironomid breeding and rapid propagation.

Attempts to control midges biologically are relatively limited. The reported pathogens of chironomids include viruses (Harkrider and Hall 1979, Majori et al. 1986), rickettsiae (Federici et al. 1976), and fungi (Weiser and McCauley 1971). Protozoan parasites of chironomids are the microsporidia (Hunter 1968), and the ciliophores (Corliss 1960). Several nematodes, Gastrorhynchus, Hydromeremis, Octomyomermis, Orthohermis, and Paramermis have a variety of chironomid hosts (Johnson 1963, 1965; Parenti 1966; Poinar 1964, 1968). However, none of the above-mentioned pathogens and parasites has thus far been studied objectively enough to develop for midge control purposes.

The bacteria, Bacillus thuringiensis serovar. israelensis (B.t.i.) and Bacillus sphaericus have been evaluated as midge larvicides in the laboratory. The LC50 values for Glyptotendipes paripes, Chironomus crassicaudatus, Chironomus decorus, and Tanytarsus spp. ranged from 4.6 to 47 ppm for three wettable powder (WP) and one flowable formulation of B.t.i. containing 1,000 to 3,500 international toxicity units (ITU)/mg (Ali et al. 1981). The former two midge species were insensitive to several toxic strains (to mosquitoes) of B. sphaericus, with L50 values exceeding 50 ppm in the laboratory (Ali and Nayar 1986). In outdoor ponds in Florida, a WP of B.t.i., containing 1,000 ITU/mg, gave 18 to 88% larval reductions of Chironominae (Chironomini and Tanytarsini) for two weeks at rates ranging from 1 to 10 kg/ha. The same formulation applied at 3 kg/ha to a pond on a golf course in central Florida, yielded up to 67% reductions of larval Chironomini for four weeks posttreatment (Ali 1981a). In a man-made lake in southern California, a technical powder (Vectobac®, 5,000 ITU/mg) of B.t.i. produced >90% larval reductions of C. decorus for two to four weeks at rates of 4.4 to 6.7 kg/ha; a flowable formulation (Vectobac® 12 AS, 1,200 ITU/mg) applied to the same lake also resulted in excellent control of the midge for four to five weeks when applied at 9.4 and 18.7 liters/ha (Mulla et al. 1990). At a lower rate of 5.2 liters/ha, the flowable formulation provided little or no control of the midge. In other studies in California, Rodcharoen et al. (1991) used technical powders (5,000 and 12,430 ITU/mg), corn grit granules (200 ITU/mg), and an aqueous suspension (Vectobac® 6AS, 600ITU/mg) formulations of B.t.i. in 30 m3 and 0.3 m deep ponds and/or in man-made lakes ranging from 8.4 to 21.4 ha and 1.8 to 2.1 m in depth. In these studies, >80 to 100% control of chironomine midges was achieved for two to three
weeks at the rather high rates of treatment employed, but Tanypodinae in general had remained unaffected. Specifically, a technical powder (5,000 ITU/mg) at 6.7 kg/ha gave complete control of Chironomus midges in a lake (Rodcharoen et al. 1991). Thus, B.t.i. is effective mostly against Chironominae midges, but relatively high rates of treatment (at least 10X or higher than the rates established for mosquito larvicidal activity) are required to achieve satisfactory chironomid control in some situations. The use of such elevated rates of B.t.i. for midge control in California, Florida, and elsewhere may be possible in habitats ranging up to 100 ha, but would not be economical or practical in natural large lakes spread over thousands of hectares. Field studies on B. sphaericus in California (Rodcharoen et al. 1991) confirmed the ineffectiveness of this bacterium against chironomids when applied at a rate as high as 22.4 kg/ha. Therefore, the presently available strains of B. sphaericus do not appear to offer any potential for midge control. There is a need to discover more toxic and effective strains of B.t.i. and B. sphaericus against chironomid larvae.

The invertebrate predators of chironomid larvae and pupae include a wide range of predators, both small [Hydra (Grzybkowska 1988)] and large [Odonata (Johnson 1985)]. Armitage (1995) lists 11 invertebrate groups that consume aquatic stages of chironomids in a wide range of aquatic habitats, and also specifies terrestrial invertebrates predaceous and scavengers on adult midges. Although numerous invertebrates have been reported in the literature as predators of chironomid larvae and pupae, the planarian, Dugesia dorotocephala, is the only invertebrate studied in a quantitative manner for the biological control of chironomids under field conditions (Ali and Mulla 1983). Since mass rearing and maintenance of D. dorotocephala seems feasible (Tsai and Legner 1977), this flatworm, and perhaps other Dugesia species, such as tigrina may have potential for chironomid control in some habitats.

Chironomid predation by fish has been reported in the literature by a large number of aquatic biologists. Armitage (1995) has provided selected references citing chironomids as food of at least 19 fish groups (families), including bream, bullhead, char, grayling, gudgeon, minnow, perch, pope, tench, Tilapia, brown and rainbow trout, walleye, whitefish, and mormyrids. Additionally, young bass, carp, catfish, mosquito fish, desert pupfish, gilthead seabream, and sunfish were reported to feed on chironomids (Ali 1991a). Despite the numerous accounts on chironomid predation by fish in the literature, field assessments revealing any successful biological control of Chironomidae through the use of fish are limited. In quantitative field studies, only the carp (Bay and Anderson 1965) and whitefish (Rasmussen 1990) produced short-term reduction of midge larvae. Mosquito fish did not produce any significant midge reduction even when stocked at 276 kg/ha (Bay and Anderson 1966). In one situation, the introduction of catfish remained ineffective in reducing midge nuisance because of the immediate recolonization by midge larvae, replacing the supply of fish food as it was consumed (Hayne and Ball 1956). The use of exotic fish, such as Tilapia spp. for chironomid control (Legner and Medved 1973) involves the risk of causing temporary or permanent environmental and faunal alterations in the aquatic ecosystem (Hurlbert et al. 1972). In general, short-term and partially effective biological control of chironomids through predatory fish alone may be possible only in small (<20ha or so) and closed habitats. In open (connected to river systems) and large lakes, which extend over thousands of hectares, the use of predatory fish would have to be considered as one component in the overall integrated approach to chironomid management.

There are a few reports of chironomid larvae and pupae forming significant proportions of food consumed by amphibian newts (Avery 1968, Strohmeier et al. 1989). Frogs and toads also were reported to consume adult midges (Wilson 1969).

Among birds, the groups that consume chironomids include flycatchers, gulls, martins, plovers, quail, rails, swallows, swifts, terns, waders, wagtails, waterfowl, and others. Armitage (1995) provided Latin equivalents of English common names of some birds which consume chironomids as food. In northern latitudes, chironomids formed a large part of the diet of mallard ducklings (Chura 1961), so much so that in one artificial lake high mortality of mallards in their first week of life was attributed to the low availability of chironomids (Street 1977).

The mammals which consume chironomids include bats and humans. Large numbers of Chironomidae in stomach and fecal samples of bats have been reported, and it was suggested that the bats cue in on the humming sound emanating from large aggregation of swarming midges (Griffith and Gates 1985). Swarming diptera (including chironomids) are used as food by humans living around some large African lakes (Armitage 1995). The flies that are attracted to lights, fall to the ground and are collected, boiled, and made into small cakes (Kungu cake) which are said to taste similar to caviar or salted locust.

The above given account on amphibians, birds, and mammals are merely reports of natural predation on Chironomidae by these animal groups. None of these animals has been assessed or developed in a quantitative
manner to reduce or control midges biologically.

The chemical control of midges primarily by larviciding has been attempted mostly in the USA and Japan (Ali 1991a, Tabaru et al. 1987). A few scattered and limited chironomid chemical control studies in the laboratory and/or field have also been conducted in Europe, United Kingdom (Edwards et al. 1964), France (Sinegre et al. 1990), Germany (Buchmann 1932), Italy (Ali and Majori 1984, Ali et al. 1985b, 1992), Africa (Abul-Nas et al. 1970, Brown et al. 1961), and Australia (Stevens 1992, Trayler et al. 1994).

The use of pyrethrum powder against midge larvae was attempted in Germany more than six decades ago (Buchmann 1932). In the USA, pyrethrins, rotenone, orthodichlorobenzene, and trichlorobenzene were the first chemicals used as midge larvicides (Fellton 1940, 1941). Later, organochlorines, such as DDD, DDT, dieldrin, BHC, and others were used against midge larvae (Brown et al. 1961, Patterson 1964, Anderson et al. 1964). However, the problems of bio-magnification of organochlorines in the aquatic food chain, occasional fish mortality, and development of resistance to organochlorines in midge larvae (Lieux and Mullenann 1956) necessitated the use of organophosphates (OPs), such as Diperex, DDVP, EPN, and malathion which proved highly toxic to *Chironomus plumosus* at 0.11 kg AI/ha in laboratory and field trials (Hilsenhoff 1959, 1962). The OPs, fenitrothion, and temephos also were successfully used nearly 30 years ago as larvicides of *G. paripes* in some small-sized natural lakes in central Florida (Patterson and Wilson 1966).

In the past 25 years, the majority of midge chemical control studies have been conducted in the USA (particularly California and Florida), Japan, Italy, and Australia. These studies include OP insecticides and insect growth regulators (IGRs), such as chitin synthesis inhibitors (CSIs) and juvenile hormone analogs (JHAs) (Ali 1995). Several pyrethroids also were highly effective against midge larvae in the laboratory (Ali 1981b). Larval susceptibility of selected chironomid species to various OPs and pyrethroids in the laboratory in different geographical regions of the world are summarized in TABLE 1.

Data in TABLE 1 reveal that susceptibility of a chironomid species to the OPs or the pyrethroids (including numbered experimental materials) varies considerably, and different chironomid species or genera occurring in the same habitat may respond differently to a test compound in terms of susceptibility. For example, *Chironomus tesserri* populations in rice fields in Australia were highly susceptible to chlorpyrifos (LC<sub>90</sub> = 0.0019 ppm), but were tolerant to fenitrothion (LC<sub>90</sub> = 0.447 ppm) (Stevens 1992). Similarly, *Chironomus yoshimatsui* in Japan were susceptible to chlorpyrifos (LC<sub>90</sub> = 0.0139 ppm) (Tabaru 1985a), but tolerated fenitrothion (LC<sub>90</sub> > 4 to 8.87 ppm) (Sato and Yasuno 1979, Tabaru 1985a). Species of *Chironomus* and *Tanytarsus* in the Santa Ana River water percolation system, California, were highly susceptible to chlorpyrifos and temephos (LC<sub>90</sub> < 0.005 ppm), but *Cricotopus* spp. in the same habitat were tolerant to the two insecticides as well as to the other OPs tested (LC<sub>90</sub> = 0.12 to 2.1 ppm) (Ali and Mulla 1976). Chironomidae fauna of concrete sewage channels and storm drains in California were generally tolerant to chlorpyrifos, fenitrothion, malathion, and temephos (Ali and Mulla 1980). In natural lakes of Florida, *G. paripes* were susceptible to most OPs while *C. crassicaudatus* in the same lakes tolerated chlorpyrifos, fenitrothion, malathion, and temephos, with LC<sub>90</sub> ranging from 0.14 to 0.48 ppm (Ali 1981b). In the lagoon of Venice, Italy, *Chironomus salinarius* were generally susceptible to the OPs tested (TABLE 1). The test pyrethroids were highly toxic to many chironomid species occurring in a variety of habitats (TABLE 1), but use of pyrethroids as midge larvicides in natural or man-made lakes would involve a great risk because they likely would have simultaneous adverse effects on nontarget invertebrates and fish (Mulla et al. 1978a, 1978b).

However, pyrethroids may be useful for midge control in sewage ponds, wastewater channels, and perhaps rice fields (Stevens 1993) where nontarget biota would be of minimal concern. Pyrethrins have been successfully used to control chironomid larvae infesting public water supply systems (Burfield and Williams 1975).

In field studies, many OP insecticides including chlorfenvinfos, chlorpyrifos, chlorpyrifos methyl, diazinon, fenitrothion, fenitrothion, malathion, methyl parathion, phenthion, temephos, and trichlorfon have been used since the early 1970s to reduce midge larvae in a variety of habitats worldwide (Mulla et al. 1971, 1975; Tabaru et al. 1987; Stevens and Warren 1992). Among these insecticides, chlorpyrifos and temephos have been the most extensively used because of their higher levels of effectiveness against a variety of midge species. In California, chlorpyrifos applications at rates of 0.11 to 0.28 kg AI/ha to shallow (1 to 5 m deep) man-made lakes covering <10 to 100 ha resulted in excellent midge control for over one month (Mulla et al. 1973, Ali and Mulla 1977b). The same insecticide at 0.25 kg AI/ha also was successfully used for control of *Chironomus riparius* in a sewage effluent channel in Chicago, Illinois (Polls et al. 1975). In Japan, chlorpyrifos methyl concentrations up to 2 ppm maintained for 20 to 30 minutes provided satisfactory control of *C. yoshimatsui* in wastewater gutters, disinfectant tanks, and discharge
TABLE 1. Activity of organophosphorus and pyrethroid insecticides against 4th instar laboratory-colonized and/or field-collected chironomid larvac from a variety of habitats located in different geographical regions of the world.

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>24 hr LC₅₀ values in ppm</th>
<th>Larval source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chironomus tepperi</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>0.0019</td>
<td>Rice fields</td>
<td>Stevens 1992</td>
</tr>
<tr>
<td>Diazinon</td>
<td>0.0647</td>
<td>(Australia)</td>
<td></td>
</tr>
<tr>
<td>Fenthion</td>
<td>0.447</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malathion</td>
<td>0.0129</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temephos</td>
<td>0.0045</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trichlorfon</td>
<td>0.103</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifenthrin</td>
<td>0.00054</td>
<td>Rice fields</td>
<td>Stevens 1993</td>
</tr>
<tr>
<td>Cyhalothrin</td>
<td>0.00038</td>
<td>(Australia)</td>
<td></td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>0.00025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Permethin</td>
<td>0.00087</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fenvalerate</td>
<td>0.0169</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chironomus yoshimatsui</strong></td>
<td></td>
<td>Laboratory colony</td>
<td></td>
</tr>
<tr>
<td>Bromophos</td>
<td>1.627</td>
<td>(Japan)</td>
<td>Tabaru 1985a</td>
</tr>
<tr>
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<td>0.0139</td>
<td></td>
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</tr>
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<td>Dichlorvos</td>
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</tr>
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<td>Diazinon</td>
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<tr>
<td>Fenthion</td>
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</tr>
<tr>
<td>Fenitrothion</td>
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<td>Temephos</td>
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<table>
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<tr>
<th>Insecticide</th>
<th><strong>Chironomus yoshimatsui</strong></th>
<th><strong>Polypedilum nubifer</strong></th>
<th><strong>Psectrocladius sp.</strong></th>
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<tr>
<td>Dichlorvos</td>
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<td>0.098</td>
<td>0.076</td>
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<tr>
<td>Fenitrothion</td>
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<td>0.0031</td>
<td>0.01</td>
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<td>0.0012</td>
<td>0.009</td>
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<td>0.00032</td>
<td>0.0042</td>
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<tr>
<td>Resmethrin</td>
<td>0.202</td>
<td>0.033</td>
<td>0.01</td>
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### TABLE 1 - continued

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>24 hr LC$_{50}$ values in ppm</th>
<th>Larval source</th>
<th>Reference</th>
</tr>
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<tr>
<td><strong>Chironomus salinarius</strong></td>
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<td></td>
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<td>0.0071</td>
<td>Lagoon of Venice</td>
<td>Ali et al. 1985b</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>Temephos</td>
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<td></td>
</tr>
<tr>
<td>Cypermethrin</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Permethrin</td>
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<td></td>
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<tr>
<td><strong>Glyptotendipes paripes</strong></td>
<td><strong>Chironomus decorus</strong></td>
<td><strong>Chironomus crassicaudatus</strong></td>
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<tr>
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<td>0.30</td>
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<tr>
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<tr>
<td><strong>Dicrotendipes californicus</strong></td>
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<td>—</td>
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<td>—</td>
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<td>Insecticide</td>
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<td>Larval source</td>
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<td>-------------</td>
<td>--------------------------------</td>
<td>---------------</td>
<td>-----------</td>
</tr>
<tr>
<td></td>
<td>Chironomus utahensis</td>
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<td>Parathion</td>
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<td>0.001</td>
</tr>
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</table>

*LC$_{50}$ values
channels at sewage treatment facilities (Tabaru 1985b). More recently, an emulsifiable concentrate (EC) and a pellet formulation of chlorpyrifos at rates of 0.07 ppm (EC) and 0.2 to 0.6 kg Al/ha (pellets) yielded up to 100% control of chironomid larvae for several days in rice fields in Australia (Stevens and Warren 1992, 1995).

Temephos has also been used in California as a midge larvicide at rates ranging from 0.17 to 0.84 kg Al/ha in water percolation basins and man-made lakes. These rates yielded a wide range of midge control depending upon the nature of the habitat and its midge composition. For example, in water percolation basins, temephos at 0.27 to 0.38 kg Al/ha gave a maximum of 78% control of Tanytarsus, Chironomus, and Procladius midges for one week posttreatment (Johnson and Mulla 1980), while rates of 0.56 to 0.84 kg Al/ha in man-made recreational lakes controlled midges for four to five weeks (Mulla et al. 1971, 1975). By contrast, much lower rates of 0.17 to 0.28 kg Al/ha were needed to achieve satisfactory control of midges in water percolation basins (Ali and Mulla 1976) and recreational lakes in California (Ali and Mulla 1977b) and Florida (Xue et al. 1993). However, repeated use of temephos in a lake in California resulted in poor or lack of control of C. decorus and Procladius spp. even at application rates of 0.33 to 0.56 kg Al/ha (Johnson and Mulla 1981).

In Japan, temephos at concentrations of up to 2 ppm maintained for 60 minutes gave satisfactory control of C. yoshimatsui in polluted rivers (Tabaru 1975, Tabaru et al. 1978, Ohno and Shimizu 1982). In cereal crop growing ponds, chironomid populations were significantly reduced by 0.05 to 1 ppm of temephos (Ohkura and Tabaru 1975, Yasuno et al. 1982). In wastewater habitats, 2 ppm of temephos maintained for 20 to 30 minutes produced excellent control of C. yoshimatsui (Inoue and Mihara 1975, Tabaru 1985b). In Italy, temephos at rates of 0.2 to 0.4 kg Al/ha reduced C. salinarius populations by 82 to 92% in the saltwater lagoon of Venice (Ali et al. 1992). In shallow wetlands in Australia, granular temephos at 0.26 kg Al/ha gave 72 to 92% control of Polypedilum nubifer after seven days (Pinder et al. 1993), and in 80 m² experimental rice bays, a pellet formulation of temephos applied at rates ranging from 0.042 to 0.73 kg Al/ha significantly suppressed C. teppei-type larvae for at least 13 days at all treatment rates utilized, with the higher rates yielding control for longer periods (Stevens and Warren 1994).

Temephos, in general, yields control of midges for shorter durations than chlorpyrifos even when applied at higher rates than the latter insecticide and in most situations is innocuous to Tanypodinae at field-use rates. Overall, field use of chlorpyrifos and temephos has indicated that these OP larvicides were effective and suppressed larval populations of midges for at least two weeks and longer, at rates below 0.56 kg Al/ha, resulting in insecticidal concentrations of <1 to 5 ppm. Granular and pellet formulations of these OP compounds have produced better chironomid control in terms of magnitude and duration in various habitats than the EC formulations.

There are no specific data in the literature on chironomid adult control although in Florida in the 1950s and 1960s, malathion and malathion-lethane or naled applied as thermal aerosol fogs from trucks, boats, and airplanes at 0.14 to 0.27 kg Al/ha (malathion) were effective, giving up to four days control of G. paripes (Patterson et al. 1966). In Japan, there are no significant reports on the use of insecticides as midge adulticides; but in Italy, deltamethrin and malathion have been employed to control adult C. salinarius in Venice (Ali et al. 1992). Presently, no specific insecticide is registered in the USA for the sole purpose of adulticiding midges. However, some OP compounds and synthetic pyrethroids, such as Scourge®(resmethrin) labeled for adult mosquito control (Rathburn 1988), can be used for midge adulticiding as included on the label.

In the past two decades, IGRs have provided additional options for midge control. In laboratory evaluations, several JHAs (including methoprene and pyriproxyfen) and CSIs (including diflubenzuron) were effective at ppb levels against a variety of midge species in the USA (California and Florida), Japan, and Australia (TABLE 2). Among the JHAs, pyriproxyfen and methoprene were the most active. In Australia, pyriproxyfen caused 90% inhibition of adult emergence of P. nubifer at 10 ppb (Trayler et al. 1994); similar results were obtained with methoprene against Chironomus sp. 51 and Tanypus grodhausi midges in California (Mulla et al. 1974). In Japan, 0.65 ppb of methoprene resulted in 50% inhibition of adult emergence of C. yoshimatsui (Kamei et al. 1982). The benzoylphenylurea CSIs including diflubenzuron, Bay SIR-8514, and several other experimental (numbered) compounds were highly active with LC₅₀ values in the range of 2.0 to 22 ppb (TABLE 2). A comparative laboratory study on the activity of methoprene and diflubenzuron against C. yoshimatsui revealed diflubenzuron to be more toxic than methoprene (Tabaru 1985c).

Field assessments of IGRs against chironomids have previously been reported from the USA (California and Florida), Japan, and more recently from Australia. In California, Pelsue et al. (1974) reported 60 to 86% midge control for one to three weeks with methoprene (Altosid®, SR-10) and diflubenzuron (25% WP) concentrations of 0.1 ppm in water spreading basins.
TABLE 2  Activity of selected juvenile hormone analogue and chitin synthesis inhibitor insect growth regulators (IGRs) against fourth-instar laboratory-colonized or field-collected chironomid larvae exposed continuously to the IGRs in the laboratory.

<table>
<thead>
<tr>
<th>IGR</th>
<th>LC₉₀ (90% Inhibition of Adult Emergence) Values in ppb</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chironomus sp.51</td>
<td>Chironomus crassicaudatus</td>
</tr>
<tr>
<td>Pyriproxyfen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methoprene (ZR-515)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>RO-20-3600</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>Methoprene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-20458</td>
<td></td>
<td>240</td>
</tr>
<tr>
<td>MV-678</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Bay SIR-8514</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>Diflubenzuron</td>
<td></td>
<td>7.4**</td>
</tr>
<tr>
<td>UC-62644</td>
<td></td>
<td>5.7</td>
</tr>
<tr>
<td>UC-84572</td>
<td></td>
<td>2.0**</td>
</tr>
<tr>
<td>UC-75150</td>
<td></td>
<td>9.4</td>
</tr>
<tr>
<td>UC-76724</td>
<td></td>
<td>4.5</td>
</tr>
</tbody>
</table>

*LC₉₀ value.
**Data of Ali and Nayar 1987 (Florida, USA).
Several formulations of these IGRs were employed for chironomid control in man-made lakes, while diflubenzuron also was utilized in flood control channels and spreading basins (used to replenish subsurface water) in California. In the lakes, methoprene at 0.11 to 0.34 kg Al/ha inhibited adult emergence of Chironominae and Tanytarsinae midges by 47 to 100% for 8 to 19 days (Mulla et al. 1974, 1976), while diflubenzuron at 0.11 to 0.28 kg Al/ha induced 48 to 100% midge control for five to eight weeks (Mulla et al. 1976), and >80% for two to five weeks (Ali and Mulla 1977b, Ali et al. 1978). In flood control channels and spreading basins, diflubenzuron at 0.11 kg Al/ha gave 88 to 100% (four weeks) and 59 to 64% (three weeks) control of midges in a channel and spreading basins, respectively (Ali and Mulla 1977a). Evaluation of 25% wettable powder (WP) and 0.5% granular (G) formulations of the IGR, SIR-8514 at 0.11 and 0.28 kg Al/ha in ponds on a golf course in California resulted in 50 to 100% and 70 to 100% control of Chironomus spp. and Procladius sp. midges for five weeks with the WP and the G formulation, respectively (Johnson and Mulla 1982). The level and duration of control given by the lower rate of each formulation were almost the same as produced by the higher rate. In Florida, evaluations of methoprene, diflubenzuron, and several new experimental IGRs in various formulations against natural populations of chironomids in experimental ponds showed that among SIR-8514, MV-678, diflubenzuron, UC-62644, UC-84572, methoprene, and pyriproxyfen, UC-62644 was the most active, inhibiting 94 to 99% adult emergence of midges for four weeks at a rate as low as 28 g Al/ha. Other IGRs, SIR-8514, diflubenzuron, and UC-84572, also caused significant reductions of adult midge emergence for several weeks posttreatment at rates ranging from 24 to 200 g AI/ha (Ali et al. 1994b, Ali 1995). Sustained release methoprene, Altosid® XR briquet and Altosid® pellet formulations, reduced midge emergence from the ponds by 38 to 98% and 64 to 98% for seven weeks at rates of 0.82 kg Al/ha (briquet) and 0.22 kg Al/ha (pellets), respectively (Ali 1991b). A 3% sand granule formulation of pyriproxyfen (Nylar®) applied to the ponds at 50 g Al/ha gave long-term (nine weeks) midge control ranging from 81 to 100% (Ali et al. 1993). In a wetland in western Australia, 0.5% G pyriproxyfen at 50 g Al/ha inhibited >80% emergence of chironomids (predominantly P. nubifer) for three weeks (Trayler et al. 1994). In Japan, diflubenzuron and methoprene applied at 1 ppm maintained for 60 minutes for midge control in two rivers resulted in satisfactory control of C. yoshimatsui, with the former IGR being more effective (Tabaru 1985c). In the gutters and discharge channels of sewage treatment plants, methoprene concentrations of 0.13 and 4 ppm controlled C. yoshimatsui only at the higher concentration (Tsumuraya et al. 1982).

Laboratory and field studies of IGRs on chironomids have shown that most of these compounds were effective at very low concentrations (ppb range) against a variety of chironomid species. Some of these compounds induced midge control in the field for several weeks at rates <0.25 kg Al/ha. Due to their unique mode of action, the IGRs would be desirable for midge control because they may not decimate midge larval biomass (a vital component of the aquatic food chain) to the degree that OP and other larvicides do.

The use of OP insecticides and IGRs for chironomid control in aquatic environments has been shown to have temporary or chronic effects on nontarget biota coexisting with midge larvae (Ali and Mulla 1978b, 1978c; Ali and Stanley 1981). Therefore, the use of these compounds in aquatic environments needs cautious evaluation of their environmental implications and cost benefits. However, adverse effects of chemical control agents on nontarget biota would be of minimal concern in sewage ponds and polluted rivers. In some situations, only partial areas of a habitat that support large populations of midge larvae could be treated (Ali and Mulla 1977b, Ali et al. 1992). This practice would reduce some midge nuisance and simultaneously would be conducive to quicker restoration of the lost nontarget organisms from areas of a habitat left untreated. Toxic effects of the chemical used in such a practice would also diminish sooner due to dilution.

At present, temephos (G formulations) is the only chemical registered by the United States Environmental Protection Agency for use against chironomid larvae in standing waters in the USA, and diflubenzuron has a special local need registration for midge control in the State of California. Repeated and prolonged use of only one insecticide, such as temephos as a midge larvicide could result in build-up of resistance in midge larvae as already evidenced (Pelsue and McFarland 1971, Ohno and Okamoto 1980, Johnson and Mulla 1981). Therefore, chemical control of midges in a habitat requires a specific strategy of avoiding frequent and indiscriminate use of a chemical and promoting rotational use of alternate effective materials where possible. To facilitate this, registration of methoprene, pyriproxyfen, and a broader registration of diflubenzuron for midge control in the USA is warranted.

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An Ecological Survey of *Anopheles albimanus* Larval Habitats in Colombia

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**ABSTRACT:** The flora and fauna of 69 aquatic sites in Colombia were surveyed to identify ecological conditions that favor production of *Anopheles albimanus*. *Anopheles albimanus* larvae were most numerous at sun-exposed sites with abundant *Culex* larvae and grass at the edge of the water. Only 29% of the sites with *An. albimanus* larvae contained pupae, suggesting that poor larval survival prevented the production of adult mosquitoes at many sites. In the Atlantic region, *An. albimanus* production was highest from large ponds with an abundant and varied aquatic insect fauna, including many kinds of predators of *An. albimanus* larvae. Although productive sites were often covered with water hyacinth, aquatic vegetation was generally not a reliable indicator of *An. albimanus* production. In the Pacific region, *An. albimanus* production was highest from small water bodies with few aquatic macrophytes and an abundance of cladocera, reflecting an abundance of microalgal food for mosquito larvae. In both regions, *An. albimanus* production was negatively associated with a complete cover of *Lemna*, fish, hydrometrid nymphs, large species of cyclopoid copepods, and dragonfly or mayfly nymphs. *Anopheles albimanus* production was also negatively associated with dytiscid beetle larvae in the Pacific region.

**Keyword Index:** Mosquito, ecology, habitat, larvae, biological community.

**INTRODUCTION**

*Anopheles albimanus* Wiedemann is a common and widely-distributed neotropical mosquito species that breeds in a variety of aquatic habitats (Breeland 1972): ditches, temporary pools, ponds of all sizes, lakes, streams, and estuaries. Source reduction for such extensive larval habitat would appear to be an overwhelming task.

The prospects for source reduction might be improved if ecological common denominators could be identified that cut across the apparent diversity of habitats. Such information might provide ecological indicators for recognizing sites that are most important for *An. albimanus* production, so that larviciding or other forms of source reduction could be focused on those sites. Ecological information might also help to identify key characteristics of *An. albimanus* larval habitats that could be modified to render the habitats unsuitable for *An. albimanus* production.

*Anopheles albimanus* larvae are generally found at sites that are well exposed to sunlight (Breeland 1972). One way to expand and refine this characterization is to look for discernible communities of aquatic flora and fauna. Plants and animals that share aquatic ecosystems with *An. albimanus* larvae should have profound impacts on the larvae as food, shelter, predators, or competitors.

Savage et al. (1990), Rejmankova et al. (1991, 1992), and Rodríguez et al. (1993) surveyed aquatic habitats on the Pacific coastal plain of southern Mexico, where *An. albimanus* larvae were associated with emergent aquatic plants, planktonic algae, pasture grasses, or water hyacinth. In Belize, Rejmankova et al. (1993) observed *An. albimanus* larvae to be associated with cyanobacterial mats and submerged plants covered with periphyton.

*Anopheles albimanus* is common throughout the coastal zone of Colombia (Quiñones et al. 1987) (Fig. 1). We conducted a field survey of aquatic habitats in Colombia to identify ecological conditions that support
the production of *An. albimanus*. We particularly wanted to know if the habitat associations were the same in Colombia’s Pacific and Atlantic regions. The survey emphasized aquatic flora and fauna, their organization into biotic communities, and their associations with *An. albimanus* larvae and pupae.

**MATERIALS AND METHODS**

The survey was designed to cover the full range of aquatic habitats that might produce *An. albimanus* on the Pacific and Atlantic coasts of Colombia. Twenty-seven sites were sampled on the Pacific coast from October 1986 to April 1987 in the vicinity of Tumaco (Fig. 1) and extending 50 km inland along the highway east of Tumaco. Mangroves dominate the coastal part of the area surveyed, while farther inland the landscape is dominated by small-scale agriculture (coconut and palm plantations, sugar cane, and subsistence crops). The rainy season on the Pacific coast extends from September to June with peaks in January, February, and May. Small bodies of water are numerous because much of the land is only slightly above sea level, rainfall is plentiful, and the water table is high. Sample sites on the Pacific coast included borrow pits, stream impoundments, roadside ditches, and temporary pools.

Forty-two sites were sampled on the Atlantic coast (from May 1987 to October 1987) in the vicinity of Las Flores, Santa Catalina, and Carmen de Bolívar (Fig. 1). Cattle ranches dominate the Atlantic landscape along with small-scale agriculture. There are two rainy seasons on the Atlantic coast—September to November and May to June—with distinct dry seasons between them. Small bodies of water are unusual on the Atlantic coast. Large cattle ponds and impoundments to store water for household use are common.

A total of seven physical/chemical factors, ten categories of terrestrial or aquatic plants, and 39 categories of aquatic animals (five stages of *An. albimanus* plus 34 other kinds of animals) were assessed at each site (TABLE 1, TABLE 2). Physical and chemical properties of the water at each site were measured between 10 AM and 2 PM. The pH was measured with a colorimeter paper. Temperature, salinity, and conductivity were measured with a YSI meter (Yellow Springs Instrument Co., Yellow Springs, Ohio). Oxygen was measured by the Winkler method (Rutten 1963).

Terrestrial vegetation was assessed visually as percent ground cover of trees, bushes, flowering plants, or grasses within one meter of the water’s edge. Aquatic vegetation was assessed visually as percent cover of submersed, emergent, or floating macrophytes over the entire body of water.

Aquatic fauna, including mosquito larvae and pupae, were collected with a plankton net (120 μm mesh). The mouth of the net was attached to a square frame, 20 cm on a side, which was dragged with a pole through water up to 50 cm in depth. The total distance sampled by the net at each site varied from 10 m to 50 m, depending on the size of the body of water. Wherever possible, the net was dragged along a transect from one side of the water to the other, but it was necessary to drag the net only near the shore if the water was too deep in the middle. Sampling at the largest sites was at intervals along the shore.

Animals collected in the plankton net were preserved in formalin for identification and counting in the laboratory. Counts were by taxonomic groups (TABLE 2); subsamples were counted when animal numbers were large. Mosquito larvae were counted by instar. The large numbers of animals collected by the plankton net provided quantitatively reliable samples of most kinds of animals present at each site, including substantial numbers of mosquito pupae (if present), which we considered to reflect the production of adult mosquitoes. All animal counts, including mosquito larvae and pupae, were expressed as numbers per meter dragged by the plankton net. A log (X+1) transformation was applied to animal counts to bring them closer to a normal distribution before including them in the statistical analysis.

Gut contents of *An. albimanus* larvae were examined under the microscope to assess the quantity of microalgae, bacteria, detritus, and mineral particles. Gut content data were not included in the statistical analyses.

Two kinds of correlations were calculated between the 56 quantitative variables (physical, chemical, floral, and faunal): conventional linear correlation coefficients and nonparametric Spearman rank-order correlation coefficients. A positive correlation between two kinds of plants or animals reflected a tendency for both to be abundant at the same site, as well as scarce or absent at the same site. A negative correlation reflected a tendency to be present or abundant at different sites. The rank correlations were helpful for identifying associations that were not apparent from conventional correlations because the associations were not linear.

Factor analysis with varimax rotation was applied to the 1,540 conventional correlation coefficients between all physical/chemical, floral, and faunal variables to identify intercorrelated groups of variables. Factors with eigenvalues >2 were considered significant. A variable was considered to be part of the intercorrelated group represented by a particular factor if its factor loading exceeded 0.3.

The factor analysis was repeated with a single juvenile stage (larval instar or pupae) of *An. albimanus*
Figure 1. Geographic distribution of *Anopheles albimanus* in Colombia. Sampled sites were at locations 1-3 in the Atlantic region and locations 7-10 in the Pacific region.
TABLE 1. Physical/chemical characteristics evaluated at each sample site.

<table>
<thead>
<tr>
<th></th>
<th>Atlantic</th>
<th></th>
<th>Pacific</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Range</td>
<td>Mean ± SE</td>
<td>Range</td>
</tr>
<tr>
<td>Area (m²)¹</td>
<td>1500±272</td>
<td>144 - 4500</td>
<td>27±6</td>
<td>1 - 150</td>
</tr>
<tr>
<td>Depth (m)¹</td>
<td>1.54±0.10</td>
<td>0.4 - 4.0</td>
<td>0.34±0.04</td>
<td>0.15 - 1.0</td>
</tr>
<tr>
<td>Temperature (°C)²</td>
<td>29.3±0.3</td>
<td>27.5 - 32</td>
<td>27.7±0.4</td>
<td>24 - 33</td>
</tr>
<tr>
<td>Conductivity (mhos)²</td>
<td>566±106</td>
<td>80 - 4000</td>
<td>558±252</td>
<td>110 - 7000</td>
</tr>
<tr>
<td>Salinity (ppt)²</td>
<td>0.10±0.04</td>
<td>0 - 1.5</td>
<td>0.25±0.15</td>
<td>0 - 4.0</td>
</tr>
<tr>
<td>Oxygen (ppm)²</td>
<td>4.29±0.30</td>
<td>0.5 - 8.0</td>
<td>3.98±0.50</td>
<td>1.0 - 8.5</td>
</tr>
<tr>
<td>pH²</td>
<td>6.28±0.04</td>
<td>6.0 - 7.0</td>
<td>6.10±0.04</td>
<td>5.8 - 6.4</td>
</tr>
</tbody>
</table>

¹Area and depth of the sampled water body.
²Measured between 10 AM and 2 PM.

TABLE 2. Flora and fauna evaluated at each sample site.

FLORA

Terrestrial vegetation at edge of water. Trees, bushes, flowering plants (Compositae, Verbinaceae), grasses (Gramineae).

Aquatic plants. Submersed plants (e.g., Chara, Elodea), emergent plants (e.g., reeds), floating-leaved plants (e.g., Nuphar, Nymphaea, Brasenia), water hyacinth (Eichornia), duckweed (Lemma), water lettuce (Pistia).

FAUNA

Crustaceans. Cladocera, shrimp (malacostraca), ostracods, large cyclopoid copepods, small cyclopoid copepods, calanoid copepods.

Aquatic bugs (nymphs and adults). Hebrids, naucorids, hydrometrids, notonectids, nepids, belostomatids, gerrids, veliids, mesoveliids, corixids, plaeids.

Aquatic beetles (larvae and adults). Dytiscids, hydrophilids, hydraenids, miscellaneous Coleoptera.

Aquatic diptera larvae. An. albimanus (each instar and pupae), other Anopheles, chironomids, stratiomyids, Culex (I/II instars, III/IV instars), miscellaneous Diptera (e.g., ceratopogonids, tipulids)

Odonata nymphs. Mayfly, damselfly, dragonfly.

Other aquatic insects. Collembola.

Aquatic mites. Hydracarina.

Aquatic vertebrates. Fish, tadpoles.

RESULTS

Atlantic Region

Anopheles albimanus larvae were present at 78% of the sites that were sampled in the Atlantic region. The number of first instars per meter dragged by the plankton net ranged from 0.3 to 55; second instars ranged from 0.1 to 40, third instars ranged from 0.03 to 25, and fourth instars ranged from 0.05 to 12. Only 31% of the sampled sites had An. albimanus pupae, ranging from 0.1 to 3.5 pupae per net meter.

The factor analysis did not reveal discrete floral/faunal communities, but it did identify five significant
groups of associated flora and fauna (TABLE 3). One (or sometimes two) of the groups was prominently represented at every site we sampled. Anopheles albimanus larvae or pupae had a significant positive association with every group except one.

The most distinct floral/faunal group in the Atlantic region (Atlantic Group #1, TABLE 3) was associated with water hyacinth (Eichhornia sp.). Mid-day water temperatures were relatively low (range: 27.5°C-30°C); water temperatures at sites with a heavy cover of hyacinth averaged 2°C less than sites with few or no floating plants. Group #1 contained a diverse and abundant fauna, including An. albimanus, Anopheles pseudopunctipennis Theobald, Anopheles triannulatus (Neiva and Pinto), and Culex spp. Anopheles albimanus pupae and higher instar larvae were particularly abundant at sites where other animals in Group #1 were also abundant.

Atlantic Group #2 (TABLE 3) consisted of plants and animals associated with sites that had a minimum of bushes or trees at the edge of the water, so the water was fully exposed to the sun. Duckweed (Lemna sp.) was often the dominant plant. If the pond was not heavily covered with duckweed, first instar An. albimanus larvae were more abundant in association with this floral/faunal group than any other group in the Atlantic region. Other larval instars of An. albimanus, as well as pupae, were common as well. However, there were virtually no An. albimanus larvae or pupae if the duckweed cover was greater than 85%.

Anopheles albimanus larvae and pupae were a significant part of Atlantic Group #3, which included submersed vegetation and large numbers of aquatic Heteroptera. First instar An. albimanus larvae were positively associated with Atlantic Group #4, which included grass at the water’s edge and crustaceans such as shrimp and small species of cyclopoid copepods.

<table>
<thead>
<tr>
<th>TABLE 3.</th>
<th>Groups of associated flora and fauna in the Atlantic region, based on factor analysis of all variables in TABLE 1 and TABLE 2.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (15.8%).</td>
<td>Dytiids (.87), plaicids (.82), Anopheles triannulatus and Anopheles pseudopunctipennis (.80), damselflies (.78), stratiomyids (.74), hydrophilids (.73), Culex (all instars) (.70), naucorids (.58), chironomids (.56), water hyacinth (.55), grass at edge of water (.53), dragonfly nymphs (.52), An. albimanus pupae (.51), large cyclopoids (.50), temperature (.50), cladocera (.49), ostracods (.47), fourth instar An. albimanus (.46), belostomatids (.44), notonectids (.37), third instar An. albimanus (.36), nepids (.35), mesovelids (.34).</td>
</tr>
<tr>
<td>Group 2 (7.9%).</td>
<td>Veliids (.70), tadpoles (.70), duckweed (.67), first instar An. albimanus (.63), mesovelids (.63), calanoids (.53), second instar An. albimanus (.52), fourth instar An. albimanus (.49), third instar An. albimanus (.47), An. albimanus pupae (.41), hydrophilids (.39), belostomatids (.38), large cyclopoids (.36), shrimp (.33), collombola (.33), corixids (.32), trees and bushes (.30), fish (.30).</td>
</tr>
<tr>
<td>Group 3 (6.9%).</td>
<td>Hebrids (.84), nepids (.72), third instar An. albimanus (.54), An. albimanus pupae (.53), dragonfly nymphs (.53), second instar An. albimanus (.51), belostomatids (.50), fourth instar An. albimanus (.43), naucorids (.38), submersed plants (.38), chironomids (.38), mayfly nymphs (.34), first instar An. albimanus (.33), damselfly nymphs (.30).</td>
</tr>
<tr>
<td>Group 4 (6.1%).</td>
<td>Shrimp (.83), small cyclopoids (.71), mayfly nymphs (.67), notonectids (.64), pH (.41), grass at edge of water (.36), flowering plants (.34), plaicids (.32), first instar An. albimanus (.30).</td>
</tr>
<tr>
<td>Group 5 (5.9%).</td>
<td>Small cyclopoids (.72), misc. diptera (.67), fish (.56), mites (.56), misc. coleoptera (.52), ostracods (.51), cladocera (.45), grass at edge of water (.36).</td>
</tr>
</tbody>
</table>

1Percentage of total variation explained by each group is in parentheses after the group number. Factor loadings are in parentheses after each variable. A negative factor loading indicates negative association with the group.
Atlantic Group #5 was characterized by the presence of fish, the presence of small species of cyclopoid copepods (instead of large species), and the absence of ostracods and cladocera (Table 3). *Anopheles albimanus* larvae and pupae were not a significant part of Atlantic Group #5.

Results from stepwise multiple regressions (Table 4) reflected many of the associations identified by the factor analysis. Each of the positive regression coefficients in Table 4 represents a group of intercorrelated variables in Table 3. (i.e., most of the variables listed in Table 4 are also in Table 3, where they have the same relation with *An. albimanus*. The variables selected by multiple regression for Table 4 are the best predictors of juvenile *An. albimanus* abundance.)

Faunal variables predominate in Table 4; no physical/chemical or floral variables had significant regression coefficients, except for grass at the water’s edge. Third/fourth instar *Culex* larvae, velidi or nepid bugs, and grass at the edge of the water were the best predictors of sites with large numbers of *An. albimanus* larvae or pupae. Hydrometrid bugs were a strong negative predictor for all juvenile stages of *An. albimanus*.

All variables with strong negative regression coefficients also had strong negative rank correlations, typically in the range of -0.3 to -0.5. Although fish did not have regression coefficients strong enough to appear in Table 4, there was a strong negative rank correlation between fish and *An. albimanus* larvae (r = -0.45, P < 0.01) and pupae (r = -0.51, P < 0.001).

### Pacific Region

There were *An. albimanus* larvae at 81% of the sites sampled in the Pacific region. Where larvae were present, the number of first instars per meter dragged by the plankton net ranged from 0.1 to 127, second instars ranged from 0.1 to 31, third instars ranged from 0.03 to 8, and fourth instars ranged from 0.05 to 52. Only 26% of the sampled sites had *An. albimanus* pupae, ranging from 0.1 to 5 pupae per net meter.

The factor analysis revealed five groups of fauna and flora in the Pacific region (Table 5). *Anopheles albimanus* larvae or pupae were positively or negatively associated with each of the groups except one.

The faunal composition of Pacific Group #1 (Table 5) was similar to Atlantic Group #1 (Table 3), except Pacific Group #1 had lower diversity of animals than did Atlantic Group #1. First instar *An. albimanus* larvae were negatively associated with Pacific Group #1, in part because Pacific Group #1 included duckweed, and *An. albimanus* larvae were absent if the

### Table 4. Results of stepwise multiple regression analysis for the Atlantic region.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Significant independent variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st instar</td>
<td>Positive: <em>Culex</em> (50±10)</td>
</tr>
<tr>
<td></td>
<td>(R² = .74)³</td>
</tr>
<tr>
<td></td>
<td>velidi (.34±.09),</td>
</tr>
<tr>
<td></td>
<td>water depth (.32±.09)</td>
</tr>
<tr>
<td></td>
<td>tadpoles (.26±.10)</td>
</tr>
<tr>
<td></td>
<td>small cyclopoids (.20±.09)</td>
</tr>
<tr>
<td></td>
<td>Negative: large cyclopoids (.39±.11)</td>
</tr>
<tr>
<td></td>
<td>hydrometrids (.25±.09)</td>
</tr>
<tr>
<td>2nd instar</td>
<td>Positive: velidi (.35±.11)</td>
</tr>
<tr>
<td></td>
<td>(R² = .75)³</td>
</tr>
<tr>
<td></td>
<td>nepids (.35±.09)</td>
</tr>
<tr>
<td></td>
<td><em>Culex</em> (32±.09)</td>
</tr>
<tr>
<td></td>
<td>area of water body (.35±.07)</td>
</tr>
<tr>
<td></td>
<td>small cyclopoids (.23±.06)</td>
</tr>
<tr>
<td></td>
<td>grass at edge of water (.19±.07)</td>
</tr>
<tr>
<td></td>
<td>Negative: salinity (-.27±.07)</td>
</tr>
<tr>
<td></td>
<td>hydrometrids (-.26±.05)</td>
</tr>
<tr>
<td>3rd instar</td>
<td>Positive: velidi (.45±.07)</td>
</tr>
<tr>
<td></td>
<td>(R² = .92)³</td>
</tr>
<tr>
<td></td>
<td>nepids (.43±.06)</td>
</tr>
<tr>
<td></td>
<td><em>Culex</em> (4.1±.06)</td>
</tr>
<tr>
<td></td>
<td>area of water body (.35±.07)</td>
</tr>
<tr>
<td></td>
<td>small cyclopoids (.23±.06)</td>
</tr>
<tr>
<td></td>
<td>grass at edge of water (.19±.07)</td>
</tr>
<tr>
<td></td>
<td>Negative: salinity (-.47±.06)</td>
</tr>
<tr>
<td></td>
<td>hydrometrids (-.34±.07)</td>
</tr>
<tr>
<td></td>
<td>misc. beetles (-.27±.05)</td>
</tr>
<tr>
<td>4th instar</td>
<td>Positive: velidi (.44±.05)</td>
</tr>
<tr>
<td></td>
<td>(R² = .95)³</td>
</tr>
<tr>
<td></td>
<td>nepids (.42±.05)</td>
</tr>
<tr>
<td></td>
<td><em>Culex</em> (4.1±.06)</td>
</tr>
<tr>
<td></td>
<td>dytiscids (.32±.07)</td>
</tr>
<tr>
<td></td>
<td>grass at edge of water (.30±.06)</td>
</tr>
<tr>
<td></td>
<td>Negative: salinity (-.47±.06)</td>
</tr>
<tr>
<td></td>
<td>hydrometrids (-.34±.07)</td>
</tr>
<tr>
<td></td>
<td>misc. beetles (-.27±.05)</td>
</tr>
<tr>
<td>Pupae</td>
<td>Positive: nepids (.45±.09)</td>
</tr>
<tr>
<td></td>
<td>(R² = .77)³</td>
</tr>
<tr>
<td></td>
<td><em>Culex</em> (4.3±.09)</td>
</tr>
<tr>
<td></td>
<td>small cyclopoids (.41±.09)</td>
</tr>
<tr>
<td></td>
<td>grass at edge of water (.27±.09)</td>
</tr>
<tr>
<td></td>
<td>Negative: hydrometrids (-.28±.09)</td>
</tr>
<tr>
<td></td>
<td>mayfly nymphs (-.27±.10)</td>
</tr>
</tbody>
</table>

¹Dependent variables are each juvenile stage of *An. albimanus*.

²Significant independent variables (P < 0.05). Normalized partial regression coefficients and their standard errors are shown in parentheses.

³R² = percent of total variation in the dependent variable explained by the listed independent variables.

⁴III/IV instars.
cover of duckweed was complete. Mid-day oxygen was relatively low (0.5-1.7 ppm), and pH was low (5.8-5.9). Water hyacinth was not part of Pacific Group #1. No hyacinth was found at the sites sampled in the Pacific region.

Pacific Group #2 (TABLE 5) was associated with small, shallow bodies of water fully exposed to the sun. Mid-day water temperatures (27°-30°C) and dissolved oxygen (3.5-8.5 ppm) were higher than at other sites. There were seldom fish, and there were large numbers of Culex larvae. Early instar An. albimanus larvae were more positively associated with Pacific Group #2 than any other group.

Pacific Group #3 (TABLE 5) was associated with floating-leaved plants, as well as flowering plants along the shore. Tadpoles were usually abundant. Anopheles albimanus larvae and pupae were neither positively nor negatively associated with Pacific Group #3.

Pacific Group #4 (TABLE 5) was associated with bushes or trees at the edge of the water. The water was shaded, so mid-day water temperatures (24°-26°C) and oxygen (0.8-2.5 ppm) were relatively low. First instar An. albimanus larvae were conspicuously absent from Group #4, but Anopheles punctimacula Dyar and Knab larvae were a major part of this group.

Pacific Group #5 (TABLE 5) was associated with small water bodies that lacked aquatic plants such as reeds and floating-leaved plants. Group #5 had an abundance of zooplankton (cladocerans and small species of cyclopoid copepods), which were not a prominent part of the other floral/faunal group associated with small water bodies (Pacific Group #2). Anopheles albimanus pupae and higher instar larvae were more abundant in association with Group #5 than any other floral/faunal group in the Pacific region.

In stepwise multiple regressions for the Pacific region (TABLE 6), third/fourth instar Culex larvae and cladocera were the best predictors of the abundance of late-instar An. albimanus larvae and pupae. Dytiscid beetle larvae were the best negative predictors of An. albimanus larvae and pupae.

As in the Atlantic region, rank correlations for the Pacific region were in agreement with negative regression coefficients. In addition, malacostracan shrimp had significant negative rank correlations with An. albimanus larvae ($r = -0.42, P < 0.01$) and pupae ($r = -0.32, P < 0.01$) in the Pacific region.

TABLE 5. Groups of associated flora and fauna in the Pacific region, based on factor analysis of all variables in TABLE 1 and TABLE 2.1

<table>
<thead>
<tr>
<th>Group 1 (13.0%)</th>
<th>Misc. coleoptera (.93), placids (.87), misc. diptera (.86), duckweed (.79), hydrometrids (.55), mayfly nymphs (.52), shrimp (.51), pH (.45), notonectids (.45), chironomids (.45), dytiscids (.40), velliids (.36), first instar An. albimanus (.30).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 2 (9.9%)</td>
<td>Culex (all instars) (.85), dragonfly nymphs (.77), first instar An. albimanus (.76), second instar An. albimanus (.67), water temperature (.66), chironomids (.66), oxygen (.52), water depth (.49), fish (.33), area (.30).</td>
</tr>
<tr>
<td>Group 3 (9.4%)</td>
<td>Belostomatids (.81), tadpoles (.80), mesonelliids (.76), damselfly nymphs (.62), floating-leaved plants (.59), flowering plants (.42), pH (.37).</td>
</tr>
<tr>
<td>Group 4 (9.3%)</td>
<td>Anopheles punctimacula (.91), hydraenids (.81), grass at edge of water (.74), dytiscids (.68), oxygen (.50), trees and bushes (.50), first instar An. albimanus (.30), water temperature (.30).</td>
</tr>
<tr>
<td>Group 5 (6.7%)</td>
<td>Emergent plants (.74), cladocera (.67), third instar An. albimanus (.66), fourth instar An. albimanus (.63), An. albimanus pupae (.56), area of water body (.63), small cyclopoids (.56), mites (.50), pH (.34), oxygen (.33), floating-leaved plants (.32), collembola (.30).</td>
</tr>
</tbody>
</table>

1Percentage of total variation explained by each group is in parentheses after the group number. Factor loadings are in parentheses after each variable. A negative factor loading indicates negative association with the group.
TABLE 6. Results of stepwise multiple regression analysis for the Pacific region.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Significant independent variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st instar</td>
<td>Positive: temperature (.51±.15)</td>
</tr>
<tr>
<td>(R² = .45)³</td>
<td>collembo (0.50±.15)</td>
</tr>
<tr>
<td></td>
<td>Negative: (no significant variables)</td>
</tr>
<tr>
<td>2nd instar</td>
<td>Positive: Culex⁴ (1.67±.17)</td>
</tr>
<tr>
<td>(R² = .44)³</td>
<td>dytiscids (-.51±.17)</td>
</tr>
<tr>
<td>3rd instar</td>
<td>Positive: Culex⁴ (9.4±.11)</td>
</tr>
<tr>
<td>(R² = .88)³</td>
<td>cladocera (.50±.09)</td>
</tr>
<tr>
<td></td>
<td>grass at edge of water (.24±.09)</td>
</tr>
<tr>
<td></td>
<td>small cyclopoids (.20±.09)</td>
</tr>
<tr>
<td></td>
<td>Negative: dytiscids (-.41±.11)</td>
</tr>
<tr>
<td></td>
<td>dragonfly nymphs (-.50±.15)</td>
</tr>
<tr>
<td></td>
<td>hydraenids (-.31±.10)</td>
</tr>
<tr>
<td>4th instar</td>
<td>Positive: Culex⁴ (1.15±.11)</td>
</tr>
<tr>
<td>(R² = .89)³</td>
<td>cladocera (.72±.10)</td>
</tr>
<tr>
<td></td>
<td>belostomatids (.24±.09)</td>
</tr>
<tr>
<td></td>
<td>Negative: dytiscids (-.69±.11)</td>
</tr>
<tr>
<td></td>
<td>dragonfly nymphs (-.37±.10)</td>
</tr>
<tr>
<td>Pupae</td>
<td>Positive: cladocera (.87±.10)</td>
</tr>
<tr>
<td>(R² = .89)³</td>
<td>Culex⁴ (.72±.10)</td>
</tr>
<tr>
<td></td>
<td>duckweed (.72±.10)</td>
</tr>
<tr>
<td></td>
<td>stratiomyids (.31±.10)</td>
</tr>
<tr>
<td></td>
<td>belostomatids (.27±.09)</td>
</tr>
<tr>
<td></td>
<td>Negative: dytiscids (-.75±.12)</td>
</tr>
<tr>
<td></td>
<td>large cyclopoids (-.45±.11)</td>
</tr>
<tr>
<td></td>
<td>hydrometrids (-.35±.09)</td>
</tr>
</tbody>
</table>

1Dependent variables are each juvenile stage of An. albimanus.
2Significant independent variables (P<0.05). Normalized partial regression coefficients and their standard errors are shown in parentheses.
3R² = percent of total variation in the dependent variable explained by the listed independent variables.
4III/IV instars.

Although we did not find biological communities that were completely distinct from one another, we did find consistent associations among many of the aquatic plants and animals. Anopheles albimanus larvae and pupae had a discernable relation (positive or negative) with most of these floral/faunal groups, the connection generally being stronger with the fauna than with the flora. Among the physical/chemical factors that we measured, only salinity appeared to be of consequence to the distribution of An. albimanus larvae.

Although there were similarities between the floral/faunal groups in the Pacific and Atlantic regions (particularly Group #1 in each region), the floral/faunal groups in the two regions were far from identical. This is not surprising, considering the physical differences between aquatic habitats of the two regions. We found a greater diversity of flora and fauna in the Atlantic region, apparently because many of the water bodies that served as larval habitat for An. albimanus in the Atlantic region were larger than those in the Pacific region.

One of the most important findings of the survey was that some sites had large numbers of all larval instars of An. albimanus as well as pupae, while other sites had large numbers of early instar larvae but no pupae. Most sites without pupae also lacked fourth instar larvae. Sites that have large numbers of larvae because they are attractive to oviposition are not necessarily the best sites for larval survival and the production of adult mosquitoes.

Despite differences between the floral/faunal groups of the Pacific and Atlantic regions, the relation of An. albimanus larvae and pupae to floral/faunal groups was similar in both regions. In both regions first instar An. albimanus larvae (which we consider to reflect oviposition) were associated with sun-exposed sites, particularly sites with grass at the edge of the water. Sites that were shaded by trees or bushes at the edge of the water, or completely covered with floating plants such as duckweed, were least favored for oviposition.

Anopheles albimanus pupae (and presumably the production of adult mosquitoes) were associated with two ecological factors. First was food supply, as indicated by the abundance of An. albimanus pupae at sites with an abundance of animals (e.g., Culex larvae or cladocera) that feed on algae. Sites with large numbers of these animals had abundant phytoplankton (or submersed vegetation covered with periphyton), and the guts of An. albimanus larvae at these sites contained large quantities of microalgae. The hypothesis that microalgae are a key resource for An. albimanus production is compatible with the observation of Savage et al. (1990) and Rejmankova et al. (1993) that An. albimanus larvae

DISCUSSION

Ideally, it would be desirable to identify discrete floral/faunal communities, some of which include An. albimanus and others of which do not. Dominant vegetation or other floral/faunal indicators in each community could facilitate prediction of the magnitude of Anopheles production.
were associated with planktonic algae and periphyton in Mexico and cyanobacterial mats in Belize.

Predation was the second factor of importance to the abundance of An. albimanus pupae in our survey. Curiously, most predators of mosquito larvae were positively associated with An. albimanus larvae and pupae, apparently reflecting a positive response of all fauna, whether predator or prey, to sites with a high level of biological productivity. Only two kinds of predators—fish and hydrometrid nymphs—had a consistent negative association with An. albimanus larvae and pupae in both Atlantic and Pacific regions. The negative association with hydrometrids was most striking. No An. albimanus pupae were observed at any sites in the Pacific or Atlantic regions where hydrometrids were present, though An. albimanus pupae were found at 44% of the sites without hydrometrids (contingency table chi-square=7.07, df=1, P<.01). Dytsicid larvae and dragonfly nymphs were negatively associated with An. albimanus in the Pacific region.

Marten et al. (1989) reported a strong negative association between large cyclopoid copepods and juvenile An. albimanus from the first 42 sites sampled in this study. After all 69 sites were sampled, large cyclopoids had negative rank correlations with An. albimanus larvae and pupae ranging from -.24 to -.28. It appears the full magnitude of negative association between the most effective cyclopoid predators and An. albimanus was obscured by grouping all larger cyclopoid species for the statistical analyses reported here; large cyclopoids included Mesocyclops longisetus (a more effective predator) and Mesocyclops venezolanus (a less effective predator).

What are the implications of this study’s findings for control of An. albimanus? While water hyacinth was identified as an indicator of An. albimanus production, the study did not identify other macrophytes to signal production at sites without water hyacinth. However, some plants appear to be reliable as indicators of sites that do not produce An. albimanus. Production was low from sites that were completely shaded by trees, and a complete cover of small floating plants such as duckweed excluded An. albimanus larvae from a site. It might be practical to plant shade trees around small water bodies that would otherwise produce An. albimanus. Small floating plants (e.g., duckweed or Salvinia) might be used to render breeding sites unsuitable (Hobbs and Molina 1983; Margaret Dix, personal communication).

Planktonic, epiphytic, and benthic microalgae appear to be the most reliable indicators of a site’s capacity to produce An. albimanus. The practical significance of microalgae for An. albimanus control requires further study, which should be specific with regard to the kind of algae, because some algae are nutritious for An. albimanus larvae and others are not (Marten 1986). It is possible that An. albimanus production could be reduced if microalgae were suppressed by chemical or biological means or if nutritious algae were replaced by algae that are not nutritious.

Results from the survey point to specific predators of possible use for biological control: fish, hydrometrid nymphs, large cyclopoid copepods, and dytiscid larvae. While fish are in common use, copepods have been used for Anopheles control only in field trials (Marten et al. 1994). Dytiscids and hydrometrids are known to prey on mosquito larvae (Mijares and Broche 1985; G. G. Marten, personal observation), but they have not been used for operational mosquito control.

Acknowledgments

Guillermo Berruecos, Aquileo Bornacelli, Flavio Castillo, Parmenides Churta, Moises Cortes, Jose Vicente Manotas, Cesar Monje, and Jose V. Santander assisted with field and laboratory work. Financial support (Grant MVR-CO-2-85-46) was provided by the National Research Council, U.S. National Academy of Sciences, through a grant from the U.S. Agency for International Development. The New Orleans Mosquito Control Board and Tulane University provided computer facilities for statistical analyses. We thank Eliska Rejmankova for comments on the manuscript.

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Reanalysis of the C. G. Macnay Mosquito Repellent Data

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ABSTRACT: Data reported by C. G. MacNay on the protection periods of 38 repellents tested in the field against Aedes sticticus, Aedes stimulans, Aedes vexans, and Aedes trichuris in 1937 were reanalyzed by current methods. Protection periods decreased by 7.6 min. for each 1°C increase in air temperature over the range 17-27°C. Treatment means were adjusted to the 22.2°C level to permit comparison of the treatments on an equal basis. Pyrethrum extract, pine tar oil distillate, thyme oil, thymol, and geraniol provided 3 or more hr. protection. Geranium oil, citronella oil, cedar oil, American pennyroyal oil, and camphor provided relatively short protection. Confidence limits and multiple comparison data are provided for the adjusted treatment means.

Keyword index: Mosquitoes, repellents, mosquito repellents.

INTRODUCTION

In the 1992 keynote address to the annual conference of the Society for Vector Ecology in San Francisco, A. R. Barr stated that “one of our highest priorities should be the systematizing of the older literature in such a fashion that we can more easily determine what has been done” (Barr 1993). The work of Focks et al. (1993) is a good example of the effective use of data dating back as far as 1901. Focks et al. (1994) have shown that reanalysis of previously published data may be justified, even if it is very recent.

The mosquito repellent data of MacNay (1939) are unique in that they represent tests of mostly botanical repellents against a natural association of northern mosquitoes. Interest in botanical repellents has increased in recent years due to public concern for safety of synthetic chemicals. Although the northern mosquitoes include several important pest species, they have not had a high priority in repellent testing programs.

However, since the data bases for most computer searches of the literature do not go back to 1939, the MacNay data are little known and little used today. In addition, statistical analysis had not yet become the norm for scientific papers in 1939, and statistical data were not included in the report when it was published.

Duplication of tests conducted by MacNay would be costly today, because of modern requirements for use of human subjects in research. The cost of toxicology testing, alone, could easily exceed $100,000 for a single test material. The present report was prepared to restore the MacNay data to currency by republication of the original data with addition of confidence limits and multiple comparison data based on current methods of statistical analysis.

MATERIALS AND METHODS

Test Materials

Active ingredients included pine tar oil and pine tar oil distillates, essential oils, organic compounds, and an unspecified material, Pamph. 55 (8). Materials containing more than 1 active ingredient were pyrethrum-thyme (thyme oil and pyrethrum extract, 1:2), citronella-spice (citronella oil and spike lavender oil, 1:1), Pamph. 55 (5) (camphor and salol, 1:1), and Pamph. 55 (7) (cedar oil, citronella oil, and spirits of camphor 1:2:2).

Most materials were diluted 1:1 in olive oil for testing. Thymol was diluted 1:2 in olive oil. Pyrethrum extract (1 test only), camphor, and paradichlorobenzene were diluted 1:3 in olive oil. Pyrethrum-thyme was diluted 1:1 in castor oil. Citronella-spice was diluted 2:1:1 in beeswax and lanolin. Pamph. 55 (5) was diluted 3:2 in petrolatum. Phenyl salicylate was tested as the
saturated solution in olive oil. Pamph. 55 (7) was tested in the undiluted form.

Test Species
Repellents were tested against a natural association of Aedes sticticus (Meigen), Aedes stimulans (Walker), Aedes vexans (Meigen), and Aedes tricharvis (Dyar) in a wooded area in the vicinity of Ottawa, Ontario. Biting rates determined prior to the tests ranged from 2.3 to 17.3 bites/min. Tests were conducted during the period 25 May-1 July 1937.

Test Method
All tests were conducted by MacNay himself, working alone. Two ml of the test material were applied to each forearm, and the time until 4 or 5 bites were received was subsequently recorded. Air temperature, relative humidity, wind speed, cloud cover, biting rate, and time of day were recorded as concomitant variables.

Data were reported for 62 tests of 41 materials, but 5 tests were terminated prior to completion because of rain or other reasons. The reduced data set includes 57 tests of 38 materials. Within this set, 21 materials were tested one time only and 17 materials were tested 2 or more times.

Data Analysis
MacNay (1939) compared the test materials by rank, based on the means of the observed protection periods. The present analysis used the Statistical Analysis System for Microcomputers, Version II.0. 1984 (Statistical Consultants, Inc., Lexington, Kentucky) for a more precise analysis.

Preliminary analysis indicated that the effects of relative humidity, wind speed, cloud cover, mosquito biting rate, and time of day on protection period were not statistically significant. The model fitted in the final analysis included the response variable, protection period (quantitative), and 2 explanatory variables, test material (qualitative), and air temperature (quantitative). Reported air temperatures were converted to °C and rounded to the nearest whole number for analysis. Pyrethrum extract in olive oil (1:1) and pyrethrum extract in olive oil (1:3) were treated as different test materials.

RESULTS
Results of the analysis of covariance are shown in TABLE 1. Effects of the treatments (test materials) and the covariable (air temperature) on protection period were significant at the 5% level. The coefficient of regression of protection period on air temperature was -7.6 min./°C, indicating that for each increase of 1° in air temperature there was a corresponding decrease of 7.6 min. of protection.

To permit comparison of the treatments on an equal basis, mean protection periods were adjusted for air temperature (TABLE 2). The adjusted treatment means shown are the means expected at 22.2°C, the average air temperature recorded in the study.

Pyrethrum extract (1:1) provided 265.1 min. (4.4 hr) protection at 22.2°C (TABLE 2). This period was significantly longer than those of all other test materials except tar oil distillate II, pyrethrum extract (1:3), thymol, thyme oil, and geraniol.

The term “tar oil (rectified)” (TABLE 2) is a synonym of pine tar oil, a dark, reddish-brown liquid derived from pine tar. MacNay tested pine tar oil and 3 distillates prepared by F.A. Herman. The intermediate-boiling fraction (distillate II) provided 261.3 min. (4.4 hr) protection at 22.2°C.

Thyme oil (Labiateae: Thymus vulgaris) provided 199.4 min. (3.3 hr) protection (TABLE 2), and thymol (2-hydroxy-1-isopropyl-4-methylbenzene), a principal constituent of thyme oil, provided 212.3 min. (3.5 hr) protection. Geranium oil ("geranium bourbon" of TABLE 2) provided 140.5 min. (2.3 hr) protection, and geraniol (3,7-dimethyl-trans-2,6-octadien-1-ol), a principal constituent of geranium oil, provided 181 min. (3.0 hr) protection.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test material</td>
<td>37</td>
<td>329,451</td>
<td>8,904</td>
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</tr>
<tr>
<td>Error</td>
<td>18</td>
<td>22,257</td>
<td>1,236</td>
<td></td>
<td></td>
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<tr>
<td>Total</td>
<td>56</td>
<td>357,751</td>
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</table>
TABLE 2. Test materials, numbers of replicates (N), mean protection periods (min), 95% confidence intervals, and significance of differences between means at the 5% level.a

<table>
<thead>
<tr>
<th>Test material</th>
<th>N</th>
<th>Protection Period</th>
<th>Confidence Interval</th>
<th>Significance</th>
</tr>
</thead>
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<tr>
<td>Pyrethrum extract 1:1</td>
<td>4</td>
<td>265.1</td>
<td>225.6 - 304.6</td>
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</tr>
<tr>
<td>Tar oil distil. II</td>
<td>1</td>
<td>261.3</td>
<td>179.8 - 342.8</td>
<td>ab</td>
</tr>
<tr>
<td>Pyrethrum extract 1:3</td>
<td>1</td>
<td>260.8</td>
<td>186.4 - 335.2</td>
<td>ab</td>
</tr>
<tr>
<td>Thymol</td>
<td>2</td>
<td>212.3</td>
<td>157.5 - 267.1</td>
<td>abc</td>
</tr>
<tr>
<td>Thyme</td>
<td>2</td>
<td>199.4</td>
<td>140.7 - 258.1</td>
<td>abcd</td>
</tr>
<tr>
<td>Geraniol</td>
<td>1</td>
<td>181.0</td>
<td>106.9 - 255.1</td>
<td>abcde</td>
</tr>
<tr>
<td>Pyrethrum-thyme</td>
<td>2</td>
<td>162.0</td>
<td>106.2 - 217.8</td>
<td>bcde</td>
</tr>
<tr>
<td>Phenyl salicylate</td>
<td>2</td>
<td>153.6</td>
<td>99.8 - 207.4</td>
<td>bcedf</td>
</tr>
<tr>
<td>Bay laurel</td>
<td>1</td>
<td>148.6</td>
<td>73.6 - 223.6</td>
<td>bcedfg</td>
</tr>
<tr>
<td>Geraniol bourbon</td>
<td>1</td>
<td>140.5</td>
<td>57.6 - 223.4</td>
<td>bcdefghi</td>
</tr>
<tr>
<td>Tar oil distil. III</td>
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<td>128.4</td>
<td>54.5 - 202.3</td>
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</tr>
<tr>
<td>Cresol</td>
<td>1</td>
<td>125.8</td>
<td>51.4 - 200.2</td>
<td>bcdefghi</td>
</tr>
<tr>
<td>Tar oil distil. I</td>
<td>1</td>
<td>123.6</td>
<td>48.6 - 198.6</td>
<td>bcdefghi</td>
</tr>
<tr>
<td>Pine oil</td>
<td>2</td>
<td>123.5</td>
<td>71.0 - 176.0</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Tar oil (rectified)</td>
<td>2</td>
<td>117.4</td>
<td>60.0 - 174.8</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Citronella</td>
<td>2</td>
<td>117.3</td>
<td>64.2 - 170.4</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Pamph. 55 (5)</td>
<td>1</td>
<td>113.3</td>
<td>37.7 - 188.9</td>
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</tr>
<tr>
<td>Geraniol acetate</td>
<td>2</td>
<td>103.6</td>
<td>47.6 - 159.6</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Citral</td>
<td>2</td>
<td>102.3</td>
<td>47.5 - 157.1</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Sweet almond</td>
<td>1</td>
<td>96.3</td>
<td>14.8 - 177.8</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Citronella-spike</td>
<td>1</td>
<td>93.3</td>
<td>17.7 - 168.9</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Bitter orange</td>
<td>1</td>
<td>91.0</td>
<td>16.9 - 165.1</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Cedar</td>
<td>1</td>
<td>91.0</td>
<td>16.9 - 165.1</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>2</td>
<td>78.6</td>
<td>24.8 - 132.4</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Pamph. 55 (7)</td>
<td>1</td>
<td>75.8</td>
<td>1.4 - 150.2</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Hemlock</td>
<td>2</td>
<td>63.7</td>
<td>4.8 - 122.6</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Hedeoma</td>
<td>2</td>
<td>55.8</td>
<td>1.2 - 110.4</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Anethole</td>
<td>2</td>
<td>52.1</td>
<td>0.0 - 104.6</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Caprylic alcohol</td>
<td>1</td>
<td>48.7</td>
<td>0.0 - 127.5</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Camphor</td>
<td>2</td>
<td>42.0</td>
<td>0.0 - 95.7</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Spike</td>
<td>1</td>
<td>38.4</td>
<td>0.0 - 112.3</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Terebinth</td>
<td>1</td>
<td>33.4</td>
<td>0.0 - 107.3</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Eucalyptus</td>
<td>1</td>
<td>31.1</td>
<td>0.0 - 107.7</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Pamph. 55 (8)</td>
<td>1</td>
<td>23.1</td>
<td>0.0 - 103.0</td>
<td>cdefghi</td>
</tr>
<tr>
<td>Nutmeg</td>
<td>2</td>
<td>18.3</td>
<td>0.0 - 72.9</td>
<td>hi</td>
</tr>
<tr>
<td>Amyl salicylate</td>
<td>2</td>
<td>14.5</td>
<td>0.0 - 70.3</td>
<td>i</td>
</tr>
<tr>
<td>Paradichlorobenzene</td>
<td>1</td>
<td>5.8</td>
<td>0.0 - 80.2</td>
<td>ghi</td>
</tr>
<tr>
<td>Olive oil (check)</td>
<td>1</td>
<td>0.0</td>
<td>0.0 - 68.1</td>
<td>i</td>
</tr>
</tbody>
</table>

aMean protection periods and confidence intervals adjusted for comparison at 22.2°C.

bTerminology is that of MacNay (1939). The spelling of "anethole" has been corrected. The terms "tar oil" and "tar oil (rectified)" refer to pine tar oil. The term "bay laurel" usually refers to the sweet bay or Grecian laurel (Lauraceae: Laurus nobilis). The material designated as "geranium bourbon" is now known as "oil of geranium Reunion". Bourbon being the former name of Reunion Island. The identity of the plant source is not known to the authors. The term "spike" probably refers to the spike lavender, Lavandula latifolia (Labiatae). The bitter orange is Citrus aurantium (Rutaceae). "Hedeoma" refers to the American pennyroyal Hedeoma pulegioides (Labiatae). The term "sweet almond" distinguishes edible varieties of the almond, Prunus amygdalus (Rosaceae), from inedible varieties, known as "bitter almond". The term "eucalyptus" is generic; eucalyptus oil is obtained from several species, notably the blue gum, Eucalyptus globulus (Myrtaceae). The term "hemlock" usually refers to the poison hemlock, Conium maculatum (Umbelliferae).

cMeans followed by the same letter do not differ significantly at the 5% level.
Several well-known botanical repellents provided shorter periods of protection: Citronella oil (Gramineae: *Cymbopogon nardus*) provided 117.3 min. (2.0 hr) protection. Cedar oil (Cupressaceae: *Thuja occidentalis*) provided 91.0 min. (1.5 hr) protection. American pennyroyal oil (Labiatae: *Hedeoma pulegioides*) ("Hedeoma" of TABLE 2) provided 55.8 min. (0.9 hr) of protection. Camphor (Laureaceae: *Cinnamomum camphora*) provided 42.0 min. (0.7 hr) protection.

**DISCUSSION**

The present paper quantifies the relation of protection period and air temperature in the field for the first time. This relationship was previously evaluated in the laboratory by Khan et al. (1973), using deet (N,N-diethyl-1,3-methylbenzamide) against *Ae. aegypti* (L.). As calculated from TABLE 1 of Khan et al. (1973), the coefficient of regression of protection period on air temperature was -2.4 min./°C in that study, compared with -7.6 min./°C in the present study. The magnitude of the difference in values obtained reflects the difficulty of extrapolating field results from limited laboratory data.

Variation of air temperature over the range recorded in the study (17-27°C) could account for as much as 76 min. (1.2 hr) of the observed variation in protection periods. Accordingly, treatment means adjusted for comparison at 22.2°C (TABLE 2) differed substantially from those of the original report (MacNay 1939). Only 2 treatments retained the same rank in the reanalysis: Pyrethrum extract (1:1) at no. 1 and pine oil at no. 14. On the other hand, 7 treatments shifted up or down in rank by 5 or more places: Citronella-spikc, geranyl acetate, sweet almond, Pamph. 55 (8), hemlock, caprylic acid, and eucalyptus.

However, confidence limits obtained in the analysis of covariance (TABLE 2) provide more precise comparisons of the adjusted treatment means than rank alone. TABLE 2 shows that 134 differences among adjusted treatment means were significant at the 5% level. Adjusted treatment means and their associated confidence limits may be preferable to unadjusted treatment means alone as product performance data submitted to the Environmental Protection Agency for registration of products and approval of product labels.

Prior to the advent of synthetic repellents, pyrethrum and citronella oil were widely used in repellent lotions, sprays, smokes, and candles. Pyrethrum is still used in "mosquito coils" to produce repellent smoke, and citronella oil is still available in repellent lotions and candles. In the present study, the protection period of pyrethrum extract (1:1 and 1:3 in olive oil) was significantly longer than that of citronella oil (1:1 in olive oil) (TABLE 2).

Pine tar oil is also a traditional repellent that has persisted in use to recent times (Allen 1986). MacNay (1939) reported that the intermediate boiling fraction provided longer protection than the oil itself, without its objectionable color and staining properties. The present study shows that the observed difference was statistically significant (TABLE 2). The protection period of the intermediate boiling fraction was also significantly longer than that of citronella oil.

Although thymol and geraniol have had comparatively little use as repellents, their protection periods did not differ significantly from those of pyrethrum, pine tar oil, and citronella oil in the present study. In addition, the protection periods of thymol and geraniol did not differ significantly from those of the source materials, thyme oil, and geranium oil ("geranium bourbon" of TABLE 2). Osmani et al. (1972) reported that geranium oil provided 230 min. (3.8 hr) protection against *Culex pipiens* Linn. (as *Culex fatigans* Wiedemann) in India.

Results of this study therefore suggest that pine tar oil, thyme oil, and/or geranium oil, or their derivatives, could be commercially developed to be competitive with pyrethrum and citronella oil. This conclusion was not apparent prior to the present analysis.

**REFERENCES CITED**


Life-Table Characteristics of Toxorhynchites splendens (Diptera: Culicidae) Cohorts Reared Under Controlled Food Regimens

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ABSTRACT: Larval and adult life table characteristics of Toxorhynchites splendens were studied under different food regimens. The survivorship from first instar to adult ranged from zero to 0.27 and was greatest in food regimens of >6 mg (dry) prey/predator/day. Median developmental time from first instar to adult (E_{f,a}) ranged from 15.83 to 64.63 days but was only 15.83 to 19.8 days in food regimens of >7 mg (dry) prey/predator/day. Except at the lowest feeding rate, the proportion of the offspring that were female did not differ significantly among treatments. Life expectancy (e_{f}) ranged from 1.93 to 50.23 days for males and from 11.64 to 47.84 days for females. Daily survivorship of adults reared under food regimens of >2.8 mg (dry) prey/predator/day was >0.94. The net reproductive rate (R_{0}) ranged from zero to 518.33 living female offspring/female/generation and was related linearly to the amount of food offered. The generation times (G) ranged from 40.18 to 81.99 days and the instantaneous rate of increase in living female/female (r_{m}) ranged from zero to 0.14. Female survivorship patterns approximated Slobodkin’s Type-II curve and males exhibited either Type-II or Type-III curve. The number of eggs laid was greatest in the second week of adult life and oviposition continued for six to seven weeks. The suitability of this species for the biological control of container breeding mosquitoes is discussed in relation to its relatively high R_{0}, r_{m}, and long adult life span.

Keyword Index: Life-table, Toxorhynchites splendens, food regimens.

INTRODUCTION

The usefulness of life tables as tools for studying insect population dynamics has been recognized as early as 1954 (Morris and Miller 1954). Age-specific horizontal life tables present a succinct tabular summary of mortality and reproductive schedules. It measures the fate of a real cohort, such as a number of individuals of a single population (Reisen and Mahmood 1980). The advantage of the laboratory analysis of life tables of mosquito predators of that life table characteristics can be studied by manipulating biological factors which are known to influence the life structure of an organism. Several studies indicated that larval stress, primarily caused by food limitation within habitats, not only produced small adults but adversely affected larval survival, development rate, and adult fitness (Fish and Carpenter 1982, Mogi 1984, Hawley 1985). When determined under insectary conditions with different rates of feeding but keeping the other variables constant, the life table of a predator will express a species-specific genetic potential and may be used to study the effect of factors on the survivorship and reproductive strategies. Recently, the life table approach has been applied to studies of the survivorship and reproductive strategies of colonized culicines (Crovello and Hacker 1972, Walter and Hacker 1974, Reisen et al. 1979) and anophelines (Reisen and Mahmood 1980). However, complete survivorship and fecundity-fertility schedules have not been compiled for the genus Toxorhynchites.

A review of the control of vector mosquitoes using predators indicated that species of the genus Toxorhynchites have the greatest potential to effectively
control container breeding mosquitoes (Holck 1988) including Aedes aegypti, the major urban vector of yellow fever, dengue, and dengue hemorrhagic fever (DHF) (Rudnick 1967). Horsfall (1955) first reviewed the biology of Toxorhynchites and it was followed by the review of systematics, biology, and biological control potential of the members belonging to this genus (Steffan 1975, Steffan and Evenhuis 1981). Several workers studied the biology and life table characteristics of Toxorhynchites splendens in the laboratory (Paine 1934, Newkirk 1947, Muspratt 1951, Chan 1968, Furumizo and Rudnick 1978, Chowanadisai et al. 1984). These studies showed that adults of Tx. splendens survived longer when compared to prey species. Other desirable attributes of these biological control agents are high daily consumption, ability of the larvae to survive long periods of starvation when prey are not available (Trpis 1972, 1981), the phenomena of compulsive killing (Corbet 1963, Trpis 1972, Crans and Slaff 1977) and a high success rate in locating artificial oviposition sites (Focks et al. 1979, 1983a, 1983b). However, earlier attempts to keep the vector population below the threshold level by releasing Toxorhynchites were ineffective mainly due to difficulties in mass-rearing and releasing a sufficient number (Steffan 1975). This could be attributed to the poor understanding of the factors affecting the survivorship and reproductive strategies of the predator. On the Indian subcontinent, only Tx. splendens has been reported to breed in coastal and forest areas (Barraud 1934, Nagpal and Sharma 1987). Therefore, we carried out this study to assess the effect of food availability on survival of immatures and adults and the reproductive schedule of Tx. splendens. The data collected would help in mass-producing this predator at the optimum food level for field releases and should be an important background for evaluating future field predator-prey interactions during control attempts.

MATERIALS AND METHODS

A colony of Tx. splendens was initiated from fourth instar larvae collected from tire dumping yards in 1977 and has been continuously maintained at the Vector Control Research Centre. Aedes aegypti mosquitoes were used as the prey. Cohorts of Tx. splendens were reared from egg to adult by offering 0.35, 0.85, 1.4, 2.8, 3.4, 4.95, 6.8, 7.15, 19.8, 28.6, and 39.11 mg (dry wt.) early fourth instar Ae. aegypti/predator/day. The amount of food consumed/predator/day was not estimated. To determine their dry weight, 200 early fourth instar Ae. aegypti were placed in an oven at 50°C for 24 h. The dried larvae were allowed to cool in a desiccator and weighed with 0.1 mg of precision. The dry weight of an individual prey was then calculated. The number of prey offered per predator per day was multiplied by the dry weight of prey to calculate the amount of food offered (Amalraj and Das 1994). Early instar Tx. splendens had great difficulty killing and consuming Ae. aegypti, and because their development time was found to be very short, more growth and prey consumption occurred in the late instars (Chowanadisai et al. 1984). Minimum food required for completion of the fourth instar larval development was found to be between 60-70% of the total consumption during the entire larval stage (Vongtangswad and Trpis 1980). Therefore offering early fourth instar Ae. aegypti to young Tx. splendens would not have affected the overall consumption rate. Each cohort consisted of 200 eggs in enamel trays measuring 0.45 x 0.4 x 0.05 m and filled with 3 l tap-water. Each food regimen was replicated three times (n = 3). The proportion of egg hatch was calculated from the number of first instars emerging from these eggs. Old prey were replaced daily with fresh ones.

Numbers of larvae pupating each day were counted and the pupae were kept separately in 500 ml beakers containing fresh tap water. Water in the trays was replaced daily. Cannibalism and compulsive killing took place mostly before pupation. The generally accepted theory to explain the compulsive killing behavior in Toxorhynchites is protection of the relatively vulnerable pupae from cannibalism (Corbet and Griffiths 1963). Therefore cannibalism is not considered the major source of pupal mortality. Hence separating the pupae for adult emergence would not affect the life table characteristics of the predator.

Although there are many reports of cannibalism among larvae of Toxorhynchites (Newkirk 1947, Trpis 1973, Trimble and Carbet 1975, Furumizo and Rudnick 1978, Focks and Boston 1979, Loumibos 1979, Steffan et al. 1980, Steffan and Evenhuis 1981, Annis and Rusmiarto 1988), a recent study showed the relationship between the rate of cannibalism among different instars and prey density (Amalraj and Das 1992). In their study it was found that first instar Tx. splendens consumed larvae of its own species higher than the other instars in the absence of prey. However, when prey were provided, there was a significant fall in the rate of cannibalism. Because cannibalism is the function of prey density and the objective of the present study was to examine the life characteristics of the predator in relation to food availability, predator larvae were not protected from cannibalism by their density in the rearing containers. Dates on which adults of each sex emerged were noted.
The time taken for 50% of the first instar larvae to become adults (E₅₀) was calculated by fitting regressions of the form Probit (P) = a + b lnx, where P = proportion emerging on each day (x) transformed to probits. The E₅₀ values were then calculated by solving the equation for P = probit 50% (Reisen and Mahmood, 1980).

Females and males emerged from each set of experiments were released into 0.3 m³ mosquito cages and offered honey and glucose pads and oviposition trays with water. The honey pads were changed biweekly, whereas the glucose pads were changed on alternate days. The oviposition containers were changed daily and the number of eggs counted. Each morning all dead adults were recorded according to sex and then discarded. All experiments were conducted in controlled temperature (25° ± 2°C) and relative humidity (60-70%). Adult life-table characteristics were calculated following the methods of Reisen et al. (1979) and Reisen and Mahmood (1980).

Analysis of variance (ANOVA) was performed on the life table parameters to determine significant differences among food regimens. Comparison among life table statistics was made by correlation analysis from mean values for each food regimen. The functional relationship between food regimens and the net reproductive rate per cohort (Rₒ) was described by regression analysis following the method of Sokal and Rohlf (1981).

### RESULTS

#### Larval Life Table Characteristics

Mean proportions of eggs hatched for different cohorts ranged from 0.81 to 0.96. One way analysis of variance showed no significant difference in the egg hatch among cohorts of different batches (P > 0.05).

The food regimens clearly had a significant effect on the survival and development of immatures. Survivorship from first instar to adult was significantly (P < 0.001) less in cohorts that were reared under the food regimens of < 6 mg (dry) prey/predator/day than those reared on larger quantities of food (TABLE 1). Median developmental time (E₅₀) was influenced significantly (P < 0.001) by the food regimens. When the cohorts were offered > 7 mg (dry) prey/predator/day, the E₅₀ was only 15.83-19.8 days, whereas, predators reared under food regimens of < 3 mg (dry) prey/predator/day required as long as 39.73-64.63 days for 50% adult emergence (TABLE 1). There was no adult emergence when Tx. splendens was offered < 0.35 mg (dry) prey/predator/day. Except at the lowest feeding rate, the proportion of the offspring that were female did not differ significantly (P > 0.05) among food regimens (TABLE 1).

#### Adult Life Table Characteristics

The different food regimen treatments also had a

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### TABLE 1  Immature developmental attributes of Toxorhynchites splendens as functions of food regimens.

<table>
<thead>
<tr>
<th>Food regimens (mg. dry wt./ predator/day)</th>
<th>Immature developmental attributes of Tx. splendens # (S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survivorship (1st instar adult)</td>
</tr>
<tr>
<td></td>
<td>Male*** Female***</td>
</tr>
<tr>
<td>0.35</td>
<td>- -</td>
</tr>
<tr>
<td>0.85</td>
<td>0.007(0.003) 0.003(0.002)</td>
</tr>
<tr>
<td>1.40</td>
<td>0.02 (0.01) 0.02 (0.00)</td>
</tr>
<tr>
<td>2.80</td>
<td>0.06 (0.05) 0.05 (0.01)</td>
</tr>
<tr>
<td>3.40</td>
<td>0.08 (0.03) 0.07 (0.003)</td>
</tr>
<tr>
<td>4.95</td>
<td>0.08 (0.01) 0.09 (0.01)</td>
</tr>
<tr>
<td>6.80</td>
<td>0.19 (0.02) 0.16 (0.01)</td>
</tr>
<tr>
<td>7.15</td>
<td>0.10 (0.01) 0.09 (0.01)</td>
</tr>
<tr>
<td>19.80</td>
<td>0.27 (0.01) 0.20 (0.00)</td>
</tr>
<tr>
<td>28.60</td>
<td>0.20 (0.08) 0.17 (0.06)</td>
</tr>
<tr>
<td>39.11</td>
<td>0.24 (0.00) 0.25 (0.04)</td>
</tr>
</tbody>
</table>

# (S.E) = Mean (Standard error); n = 3; attributes marked with asterisks denote significant difference among the treatments using 1-way ANOVA. df = 10, 22. * = P 0.01 < 0.05; *** = P < 0.001.
significant (P < 0.001) effect on the adult life table parameters of *Tx. splendens* (TABLE 2). Among the eleven larval food regimens, the range of adult life expectancy (e) was 1.93-50.23 days for males and 11.64-47.84 days for females. Adult daily survivorship (s) was lower among adults that emerged from larvae reared under food regimens of < 2.83 mg (dry) prey/predator/day.

There was a linear relationship between the amount of food offered and the net reproductive rate (R) (b = 12.34, t = 17.61, P < 0.001) (Fig. 1). Highest R was obtained in the 39.11 mg (dry) prey/predator/day food regimen. R was not significantly correlated with female life expectancy at emergence (r = 0.16, P > 0.05).

Age in days at mean cohort reproduction (T) was significantly (P < 0.001) lower (occurred earliest in life) in females from cohorts fed < 2 mg (dry) prey/predator/day food regimen. Conversely, T was high for females from > 2 mg (dry) prey/predator/day food regimens. T was not significantly correlated with R (r = 0.17, P > 0.05). The capacity for increase, r, was significantly greater at high food regimens. It should be

TABLE 2. Adult life-table attributes of *Toxorhynchites splendens* as functions of food regimens.

<table>
<thead>
<tr>
<th>Food regimens (mg, dry wt./predator/day)</th>
<th>Adult-life-table characteristics of <em>Tx. splendens</em> # (S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>e&lt;sub&gt;1&lt;/sub&gt; (male)***</td>
</tr>
<tr>
<td>0.35</td>
<td>-</td>
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<td>45.39 (2.21)</td>
<td>1.18 (0.006)</td>
<td>1.04 (0.006)</td>
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e<sub>1</sub> = mean life expectancy from emergence in days; s = constant daily survivorship assuming a type II survivorship curve; T<sub>0</sub> = age in days at mean cohort reproduction; r<sub>m</sub> = instantaneous rate of increase in living female; female; r<sub>c</sub> = capacity for increase; G = mean generation time in days; b = instantaneous birth and d = death rate, assuming a stable age distribution.

# (S.E.) = Mean (Standard error); n = 3; attributes marked with asterisks denote significant difference among the treatments using ANOVA. *** = P < 0.001.
pointed out that $r_e$ utilized $T_o$ in its calculation, and thus was based only on the adult age.

The instantaneous rate of increase ($r_m$) was found to increase significantly ($P<0.001$) with increases in the amount of food. $r_m$ was significantly correlated with $r_e$ ($r=0.94$, $P<0.001$), and $R_o$ ($r=0.76$, $P<0.001$). The mean generation time ($G$) of *Tx. splendens* was significantly ($P<0.001$) lower in > 2.83 mg (dry) prey/predator/day. $G$ was significantly correlated with $T_o$ ($r=0.41$, $P<0.05$). As seen in $r_m$, the ratio $r_m/b$ increased significantly ($P<0.001$) with increase in amount of food offered. The instantaneous birth ($b$) and death ($d$) rates and the $b/d$ ratio were significantly ($P<0.001$) higher in > 2 mg (dry) prey/predator/day. The birth rate ($b$) and the death rate ($d$) estimated from the stable age distribution were found to be closely correlated with $r_m$ ($r=0.74, 0.68$ respectively, $P<0.001$). Lower birth and death rates accompanied by a lower $r_m$ would suggest a trend toward greater population stability (Reisen et al. 1979).

Age specific survivorship curves for males and females and fecundity curves for females have been depicted graphically in Figures 2 and 3. In general, female survivorship patterns approximated Slobodkins' (1962) Type II curve with little increase in mortality during early age intervals, whereas male curves exhibited either Type II or III curve (Fig. 2).

The number of eggs laid was high in the second week of adult life and egg laying activity was observed for six to seven weeks from adult emergence. Females reared from higher food regimens laid more eggs than the female reared from low food regimens (Fig. 3).

**DISCUSSION**

The present study made it easy to compute the per capita rate of increase of *Tx. splendens* population under different food regimens so as to produce the best possible quantification of population performance. High egg hatching rate (range 81 to 96%) reported in the present study suggests an effective fertilization and is in agreement with Furumizo and Rudnick (1978) who reported egg hatching rate of 77 to 91% in *Tx. splendens*. However, egg viability was reported to range from 25 to 100% in other species of the genus Toxorhynchites (Trimble 1979, Steffan et al. 1980). Greater immature survival was obtained when the predator was reared under food regimens of > 19.8 mg (dry) prey/predator/day. This clearly shows that both food availability and cannibalism are interrelated and are the contributing factors for the survival of the immatures (Amalraj and Das, 1992). The median developmental time ($E_{50}$) from
Figure 2. Age-specific survivorship in individuals/individual/day ($l_x$) plotted as a function of age in weeks under different food regimens. Each point represents the mean of three replicates. (A1) 0.85, (A2) 2.80, (B) 3.40, (C) 4.95, (D) 6.80, (E) 7.15, (F) 19.80, (G) 28.60, (H) 39.60 mg. (dry wt.) food/predator/day.
Figure 3. The number of female offspring produced/living female/week ($m_x$), plotted as a function of age in weeks under different food regimens. Each point represents the mean of three replicates. (A) 2.80, (B) 3.40, (C) 4.95, (D) 6.80, (E) 7.15, (F) 19.80, (G) 28.60, (H) 39.60 mg. (dry wt.) food/predator/day.
first instar to adult ranged from 15.83 to 64.63 when the highest and lowest food regimens were provided. Paine (1934) similarly reported a mean development time of 23 days for well fed larvae and 110 days for starved larvae. Chan (1968) reported an immature duration of 39 days in *Toxorhynchites splendens*. Furumizo and Rudnick (1978) reared *Toxorhynchites splendens* at high prey density and observed the immature developmental duration to be 21.5 days. By delaying pupation when food is scarce, *Toxorhynchites splendens* maintains its food ingested above the basal metabolism rate to accrue sufficient food reserves in the form of lipids and glycogen to initiate pupation. Further the total energy stored in adults provides a measure of the energy reserves built up during the larval stage. The massive larval abdominal muscles, which are histolysed during the first days of adult life must be a valuable source of energy for young adults (Briegel 1990a, 1990b).

Males survived slightly longer than did females. This is presumed to be a behavioral requirement for minimal swarm size. Focks et al. (1977) reported that females of *Toxorhynchites rutilus rutilus* survived an average of 49 days and laid an average of one egg/day. Furumizo and Rudnick (1978) estimated an adult life span of 28 to 35 days for *Toxorhynchites splendens*. However, a maximum survival of 120 days was reported by Steffan et al. (1980). In the present study, the highest male and female daily survivorship was 0.98. The laboratory estimates of survivorship and longevity were presumed to depict the genetic potentiality of this species. The contribution of individual males and females to the population increase would be directly related to life expectancy at emergence and the number of fertile eggs produced. The highest \( R_0 \) value of 518.33 was obtained by offering *Toxorhynchites splendens* 39.11 mg (dry) prey/day and was found to be many times higher than 16.3 reported by Chowanadisai et al. (1984). However, Hu (1955) reported female fecundity of 400 eggs in laboratory colonies of *Toxorhynchites amboinensis*. The present study indicated that the oviposition period was extended up to 50 days for females reared on high food regimens. Steffan et al. (1980) reported an oviposition period of 85 to 95 days in *Toxorhynchites amboinensis*. This is much longer than the 27 days reported for *Toxorhynchites splendens* (Furumizo and Rudnick 1978). A comparatively high \( r_m \) of 0.14 was estimated when the predator was reared under the food regimen of 39.11 mg (dry) prey/predator/day. Higher values of \( r_m \) generally were considered to be an evolutionary adaptation for existing in or colonizing variable environments (Hirston et al. 1970, Pianka 1972). Hence, with relatively high potential value of \( r_m \) *Toxorhynchites splendens* would be able to exploit favorable conditions such as the availability of prey in large numbers and rapidly increase its population size. However, the \( r_m/b \) and \( b/d \) ratios, an indication of theoretical colonizing ability (MacArthur and Wilson 1967), were relatively low. Thus, this species would be considered "r" strategist, but a relatively poor colonizer. Another adaptive character found in this species is elongation of G when food is scarce. High \( R_0 \) and \( r_m \) values at high food level as well as longer adult lifetime than those of the prey species give hope for exploitation of the species against container breeding mosquitoes such as *Ae. aegypti*.

**Acknowledgments**

We are grateful to Dr. Vijai Dhanda, Director, Vector Control Research Centre, Pondicherry, for his critical comments on the manuscript that facilitated great improvement in the quality of the manuscript. The technical assistance of Messrs K. Balarajan, G. Meganathan, and G. Sathianathan is gratefully acknowledged.

**REFERENCES CITED**


Laboratory and Field Trials of Fenthion and Cyfluthrin Against *Mansonia uniformis* Larvae

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**ABSTRACT:** A laboratory bioassay of insecticides against laboratory-reared late third/early fourth-instar larvae of *Mansonia uniformis* indicated that the synthetic pyrethroids tested (cyfluthrin and permethrin) were more active than the organophosphate compounds tested (chlorpyrifos, fenthion, and temephos). The LC₅₀ values ranged from 0.09 (cyfluthrin) to 7.68 µg/l (temephos). Field trials of fenthion (Baytex GR2) and cyfluthrin (Baythroid H10WP) against laboratory-cultured late third/early fourth-instar larvae of *Mansonia uniformis* in floating screened cages were conducted in small plots on Penang Island, Malaysia. At respective doses of 0.50 and 0.30 g/m² (5.0 and 3.0 kg/ha), both fenthion and cyfluthrin caused more than 90% mortality of the *Mansonia* larvae exposed at 0 hour post-treatment. The use of these larvicides for the control of *Mansonia* larvae in open field environments is discussed.

**Keyword Index:** *Mansonia uniformis*, larvicide, fenthion, cyfluthrin.

**INTRODUCTION**

*Mansonia uniformis* is one of the major vectors of Brugian filariasis in Southeast Asia (Mak 1981, Yap 1985). Approximately one billion people lived in endemic areas with approximately 5.8 million cases of infection in this region (World Health Organization 1994). Currently, Brugian filariasis is controlled through mass drug treatment using diethylcarbamazine citrate, and new drugs such as ivermectin are still under experimentation (Mak 1981, 1990, Ramachandran 1993, World Health Organization 1994).

In comparison with other mosquito genera, there have been few larvicidal tests against *Mansonia* (Yap 1985, Yap et al. 1995a). Chapman (1955) conducted laboratory and field tests with 15 insecticides against *Mansonia dubitans* and concluded that parathion and EPN were most effective. Further laboratory studies on *Mansoniaperturbans* (Yap et al. 1968) and *Ma. uniformis* (Yap and Salimain 1976) indicated good efficacy of temephos and chlorpyrifos. Field studies further confirmed the efficacy of temephos against *Mansonia* larvae (Gass et al. 1985). More recently, etofenprox, a relatively new insecticide with low mammalian toxicity, was also found to provide good larvicidal activity in the laboratory (Yap et al. 1995b).

We report here the efficacy of two synthetic insecticides, namely fenthion and cyfluthrin, as tested in the laboratory (compared with chlorpyrifos, temephos and permethrin) and field against *Ma. uniformis* larvae.

**MATERIALS AND METHODS**

The *Ma. uniformis* larvae used in both laboratory and field assessments were from well-established laboratory colonies at the Vector Control Research Unit, Universiti Sains Malaysia. The colony was initiated from collections in Permatang Damar Laut, Penang Island in 1990. The mosquito used in this study were late third and early fourth-instar larvae.

Technical grade fenthion (o,o-dimethyl-o-[4-(methylthio)-m-tolyl] phosphorothioate, temephos (o,o-(thiodi-4-1-phenylene) o,o,o,o-tetramethyl phosphorothioate), chlorpyrifos (o,o-diethyl o-3,5,6-trichloro-2-pyridyl phosphorothioate), cyfluthrin (alpha-cyano (4-fluoro-3-phenoxyphenyl) methyl 3-(2,2-dichloroethenyl)-2,2-dimethyl-cyclopropanecarboxylate) and permethrin [3-phenoxybenzyl-3-2,2-dichlorovinyl-2,2-dimethylyclopropane carboxylate] were used in the laboratory bioassay. The organophosphates chosen for
this study are currently being used as mosquito larvicides in Malaysia. Temephos is used for clear water breeders, such as the *Aedes* species, whereas fenthion and chlorpyrifos are used for the polluted water breeding *Culex* species. The choice of the two residual pyrethroids (permethrin and cyfluthrin) takes into consideration the greater use of pyrethroids for household and public health insect control in recent years based on their selective toxicity.

The bioassay method was essentially that established by the World Health Organization for larvicidal susceptibility (World Health Organization 1981) with the following modifications: (1) technical grades of test insecticides were diluted to test concentrations using analytical grade acetone as solvent; (2) twenty late third/early fourth-instar larvae in three replicates were used for each concentration of insecticides per test. The experiment was then repeated three times using different batches of larvae and insecticide preparations; (3) total volume of test solution was 100 ml instead of 250 ml per beaker and; (4) a small piece of styrofoam was placed in each beaker for attachment of *Mansonina* larvae after the addition of insecticide.

For field efficacy tests, only fenthion granule (Baytex GR2) and cyfluthrin wettability powder (Baythroid H10WP) formulations were used. The field trials were conducted by exposing mosquito larvae to insecticide applications in floating screened cages in small plots (15 - 18 m²). Test plots had a vegetative cover of water hyacinth (*Eichhornia crassipes* Solm), which is the natural host plant of *Mansonina*. Six treated and three untreated control plots were used. The nine plots totaled 145 m². The field plots were located in a swampy ditch of an abandoned coconut plantation in Permatang Damar Laut on the southern coastal alluvial plain of Penang Island, Malaysia. The detailed protocols for tests in small plot trials followed essentially those of Yap et al. (1991). Efficacy and residual effects of the insecticides against *Mansonina* larvae were determined by introducing batches of larvae into additional cages at intervals of 0, 24, 48, 72, and 168 hours post-treatment. The mortalities readings were conducted 24 hours after the introduction of each batch of larvae at the above designated intervals.

For both formulations tested, application rates ranged from 0.01 to 2.00 g/m². The fenthion granule formulation (Baytex GR2) was mixed thoroughly with fine sand and dispersed by hand. The cyfluthrin wettable powder formulation (Baythroid H10WP) was mixed with seasoned tap water (local tap water kept for more than 72 hours) and applied using a Geizhal ES10 pressurized knapsack sprayer (Dr Stahl and Sohm GmH and Co, Uberlinger, Germany).

Rainfall, temperature, pH, dissolved oxygen, and water conductivity at the field sites were recorded daily. Measurements were made using portable meters including a membrane pH meter (Hanna HI 8314, Italy), dissolved oxygen meter (Yellow Spring Instrument, YS IM67, USA) and conductivity meter (WTW LF 91 with probe KLEI/T, USA). Data were subjected to probit analysis (Finney 1971) using a computer program by Daum (1970).

RESULTS AND DISCUSSION

Based on the laboratory bioassays, the *Ma. uniformis* larvae appeared to be more susceptible to the two pyrethroids (cyfluthrin and permethrin) than to the organophosphates (chlorpyrifos, temephos, and fenthion) (TABLE 1). The LC₅₀ values for these insecticides ranged from 0.09 to 7.68 µg/l. Cyfluthrin seemed to be the most effective of all the insecticides tested. Among the organophosphates, chlorpyrifos and

### TABLE 1. Activity of five insecticides against late third/early fourth-instar *Mansonina uniformis* larvae in the laboratory.¹

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>LC (95% fiducial limit) [µg/l]</th>
<th>Slope ± SE</th>
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<tr>
<td></td>
<td>50</td>
<td>90</td>
</tr>
<tr>
<td>fenthion</td>
<td>(4.81-5.10)</td>
<td>7.54 (7.21-7.95)</td>
</tr>
<tr>
<td>temephos</td>
<td>(7.32-8.05)</td>
<td>18.54 (17.17-20.22)</td>
</tr>
<tr>
<td>chlorpyrifos</td>
<td>(3.36-4.27)</td>
<td>6.70 (5.87-8.31)</td>
</tr>
<tr>
<td>cyfluthrin</td>
<td>(0.08-0.10)</td>
<td>0.31 (0.26-0.37)</td>
</tr>
<tr>
<td>permethrin</td>
<td>(1.63-1.84)</td>
<td>3.93 (3.61-4.36)</td>
</tr>
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</table>

¹Based on pooled data of three experiments.
fenthion were more effective than temephos. Dose-response values of some of the insecticides tested here agree with those from earlier publications (Yap et al. 1968, Yap and Sulaiman 1976). Some minor discrepancies occurred due to species differences (Yap et al. 1968) and the use of laboratory-cultured versus field collected mosquito larvae (Yap and Sulaiman 1976).

Both fenthion and cyfluthrin proved to be effective in the simulated field control of *Ma. uniformis*. The larvicidal efficacy of fenthion and cyfluthrin against sentinel *Ma. uniformis* in small plots in swampy ditches showed that the latter had a higher efficacy than fenthion at the initial introduction of *Mansonia* larvae (immediately after insecticidal application) (TABLE 2). When new *Mansonia* larvae were introduced at 24 and 48 hours post-treatment, some significant mortalities of larvae occurred at the higher dosages used. For the cyfluthrin formulation, introduction of new batches of larvae at 24, 48, 72, and 168 hours post-treatment indicated significant residual effects of up to seven days at the two highest dosages used (1.00 and 2.00 g/m², TABLE 2). The effective dosages for both insecticides for *Mansonia* control in such habitats appeared to be lower than those of microbial insecticides such as *Bacillus thuringiensis* H-14 (Foo and Yap 1983) and *Bacillus sphaericus* (Yap 1990, Yap et al. 1991) tested under the same environmental conditions.

The meteorological and water quality conditions for the field site in Permatang Damar Laut, Penang Island were similar to those recorded in the same plots in earlier publication (Yap et al. 1991). The mean values (± S.E.M) for temperature, pH, dissolved oxygen, and conductivity of the field water were 27.4 ± 0.3°C, 6.7 ± 0.1, 1.8 ± 0.1 mg/l, and 10.1 ± 2.1 mmho/cm, respectively.

Field trials indicated that at respective application rates of 0.50 and 0.30 g/m² (equivalent to 5.0 and 3.0 kg/ha), fenthion and cyfluthrin caused more than 90% mortality of the *Mansonia* larvae at 0 hour post-treatment in natural larval habitats with high organic and ion contents. At subsequent intervals (24, 48, 72, and 168 hours post-treatment), there was a decrease in efficacy of insecticides against the introduced larvae (TABLE 2). Thus, both fenthion and cyfluthrin showed only slight residual effects against *Mansonia* larvae in the natural polluted habitats except at considerably higher dosages. However, such lack of persistence at lower dosages should still provide reasonable effective control if the application routine was conducted monthly. This is because *Mansonia* mosquitoes in general need a much longer time (20-25 days) to complete their larval development (Wharton 1962) as compared with other mosquito species.

Results from the present field studies indicated that

### TABLE 2. Small-plot field trials on fenthion and cyfluthrin against *Mansonia uniformis* larvae placed in floating cages.

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Dosage (g/m²)</th>
<th>No. live larvae over 50 introduced at following intervals after 24 hours exposure</th>
<th>0 hr</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
<th>168 hr</th>
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<td>fenthion</td>
<td>control</td>
<td>44.0 ± 2.6</td>
<td>44.3 ± 2.1</td>
<td>41.6 ± 1.5</td>
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<td>-</td>
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<tr>
<td></td>
<td>0.01</td>
<td>43.6 ± 1.2</td>
<td>41.0 ± 2.0</td>
<td>38.3 ± 7.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>26.0 ± 10.5</td>
<td>43.7 ± 4.6</td>
<td>37.3 ± 1.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>16.3 ± 18.0</td>
<td>28.3 ± 16.1</td>
<td>42.0 ± 2.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>2.3 ± 3.2</td>
<td>18.7 ± 12.7</td>
<td>42.3 ± 6.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>0.7 ± 0.6</td>
<td>7.3 ± 0.6</td>
<td>7.3 ± 3.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0</td>
<td>5.0 ± 4.6</td>
<td>30.6 ± 16.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cyfluthrin</td>
<td>control</td>
<td>46.0 ± 1.0</td>
<td>41.3 ± 1.2</td>
<td>43.7 ± 0.6</td>
<td>44.7 ± 1.5</td>
<td>46.3 ± 2.5</td>
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<tr>
<td></td>
<td>0.01</td>
<td>37.3 ± 1.2</td>
<td>38.0 ± 1.7</td>
<td>37.7 ± 0.6</td>
<td>42.7 ± 4.0</td>
<td>41.7 ± 2.9</td>
<td>-</td>
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<tr>
<td></td>
<td>0.10</td>
<td>8.3 ± 4.6</td>
<td>32.6 ± 2.1</td>
<td>35.7 ± 2.9</td>
<td>42.0 ± 1.0</td>
<td>45.3 ± 1.5</td>
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<tr>
<td></td>
<td>0.30</td>
<td>3.3 ± 4.0</td>
<td>19.3 ± 0.6</td>
<td>25.7 ± 6.0</td>
<td>36.0 ± 5.0</td>
<td>42.3 ± 3.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>1.3 ± 1.2</td>
<td>7.0 ± 5.6</td>
<td>18.7 ± 8.1</td>
<td>31.7 ± 4.2</td>
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<tr>
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<td>1.00</td>
<td>1.0 ± 1.7</td>
<td>6.7 ± 3.1</td>
<td>13.0 ± 6.9</td>
<td>26.3 ± 1.2</td>
<td>31.0 ± 3.5</td>
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<tr>
<td></td>
<td>2.00</td>
<td>0.3 ± 0.6</td>
<td>0</td>
<td>0</td>
<td>2.0 ± 1.7</td>
<td>6.7 ± 3.1</td>
<td>-</td>
</tr>
</tbody>
</table>

1 All values are mean ± S.E of three experiments of live larvae recovered after 24 hours exposure.
both fenthion and cyfluthrin have the potential to be effective larvicides for \textit{Mansonia} control in natural breeding habitats. However, the choice of any control agents, whether chemical or biological, should take into consideration the environmental impacts of such agents under operational usage.

\textbf{Acknowledgments}

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Natural Variation in Blood-Feeding Kinetics of Four Mosquito Vectors

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ABSTRACT. The blood-engorgement kinetics of Anopheles aquasalis, Aedes aegypti, Haemagogus janthinomys, and Culex quinquefasciatus were determined under laboratory conditions using females collected from three field sites in Trinidad. Most An. aquasalis, Ae. aegypti, and Hg. janthinomys completed probing within 70 s but Cx. quinquefasciatus averaged >180 s. Anopheles aquasalis (67.0 s) had the shortest gut filling time while Cx. quinquefasciatus had the longest gut filling time (222.8 s). Aedes aegypti and Hg. janthinomys had similar gut filling durations, 87.7 s and 90.4 s, respectively. More than 70% of An. aquasalis, Ae. aegypti, Cx. quinquefasciatus, and Hg. janthinomys showed prediuresis. Anopheles aquasalis exhibited the shortest mean duration of prediuresis (77.3 s) and Cx. quinquefasciatus the longest (526.9 s). Most individual mosquitoes spent less time filling their guts than in prediuresis. Overall, An. aquasalis exhibited the shortest total feeding time (135.7 s) and Cx. quinquefasciatus the longest (661.9 s). Individual An. aquasalis mosquitoes showed a high degree of variability in their ability to agglutinate human erythrocytes immediately upon blood engorgement, with individuals agglutinating <25% of the ingested erythrocytes and others ≥90%. Among Ae. aegypti and Cx. quinquefasciatus, the proportion showing agglutination varied from 0% (67% and 69% respectively) to <50%. The parity of the field collected mosquito vectors had no significant effect on probing, duration of blood-feeding, duration of prediuresis, or erythrocyte agglutination. The inter- and intra-specific variation in blood-feeding and erythrocyte processing observed for the four vector species collected from the field in Trinidad are described in terms of vector competence and evolution of these behaviors.

Keyword Index: Mosquito, blood-feeding, excretion, prediuresis.

INTRODUCTION

In the Americas, four significant vector mosquitoes include Anopheles aquasalis Curry, the coastal vector of malaria from Venezuela to Brazil and including Trinidad and Tobago (Faran 1980); Aedes aegypti (L.), the cosmopolitan vector of dengue and urban yellow fever (Christophers 1960); Haemagogus janthinomys Dyar, the sylvan vector of yellow fever in the Latin American and Caribbean region (Arnell 1973, Chadee et al. 1992); and Culex quinquefasciatus Say, the vector of bancroftian filariasis worldwide (Nathan 1981, Macdonald 1991).

Much information is available on the host-seeking activity of these four vectors but little is known about the duration of blood engorgement which includes probing time, gut-filling time, and duration of prediuresis. Indeed, information on this aspect of the biology of each species is long overdue and vital to the understanding of the transmission dynamics of yellow-fever, dengue, malaria, and filariasis.

Although Trinidad and Tobago are currently free of malaria, yellow-fever, and bancroftian filariasis, the presence of large numbers of potential vectors means that the risk of disease outbreaks remains high. Recently, Chadee and Beier (1995) developed methodology for evaluating the blood-feeding behavior of four anopheline mosquitoes from Trinidad and Tobago. Here we present the results of the duration and kinetics of blood-feeding of field-collected, An. aquasalis, Ae. aegypti, Hg. janthinomys, and Cx. quinquefasciatus.
MATERIALS AND METHODS

Field collections of *Ae. aegypti* and *Cx. quinquefasciatus* were made at St. Joseph (10° 38' N; 61° 25' W), a small town, 16 km east of Port of Spain, Trinidad. The study area, meteorology, topography, vegetation, and two mosquito populations were described by Chadee and Corbet (1987) and Chadee (1994). *Anopheles aquasalis* collections were carried out in the rice-fields of Frederick Settlement (10° 36' N; 61° 25' W), a village 5 km west of Piarco International Airport. The anopheline populations, meteorology, topography, and vegetation of this site were previously described by Chadee (1992) and Chadee and Beier (1995). Field collections of *Hg. janthinomys* were conducted at the Pt. Gourde Forest (10° 40' N; 61° 40' W), a site approximately 16 km west of Port of Spain, the capital of Trinidad. The mosquito populations, meteorology, vegetation, and topography of this area were described by Chadee and Tikasingh (1989).

Host-seeking mosquitoes were captured using human bait and standard procedures (Haddow 1954) during the hours of peak landing for each species; 16:00-18:00 hours for *Ae. aegypti* (Chadee 1988), 22:00-02:00 hrs. for *Cx. quinquefasciatus* (Nathan 1981), 18:30-19:00 hrs. for *An. aquasalis* (Senior-White 1953), and 10:00-14:00 hrs. for *Hg. janthinomys* (Chadee et al. 1992). Mosquito collectors were stationed at ground level with flashlights, collecting nets, and aspirator. The mosquito collection methodology, handling, storage, and transportation from the field to the laboratory were described by Chadee and Beier (1995). At the Insect Vector Control Division laboratory, the mosquitoes were anaesthetized lightly with chloroform, examined under a microscope at 40 x magnification, identified, and counted. Mosquitoes with any trace of blood were rejected. Mosquitoes were segregated by species and placed into 450 ml, net-screened, cylindrical ice-cream containers (18 cm in height x 16.5 cm in diameter). Mosquitoes were held for 24 hours and tested during their peak biting times.

Duration of Enorgement

To study blood-feeding, each mosquito was placed in a 5 ml glass tube covered with mosquito netting and allowed to engorge on a human forearm. Mosquitoes were allowed to probe into the skin (number of times recorded) and the duration of probing was timed with a stopwatch. Another stopwatch was activated at the first signs of engorgement (red color and abdominal swelling). A third stopwatch was activated at the first sign of prediuresis (anal excretion). This process was observed under a dissecting microscope (60 x) so that the number of probes, duration of probing, duration of the gut-filling phase, duration of prediuresis, proportion of females exhibiting prediuresis, and total feeding time could be calculated (Vaughan et al. 1991, Chadee and Beier 1995). After blood-feeding, the mosquitoes were immediately killed and dissected. Both ovaries of each female mosquito were examined and scored as nulliparous and parous using Detinova's method, according to the presence of tracheolar skeins (Detinova 1962).

Patterns of Prediuresis

To determine whether these mosquitoes excreted tubular fluid (prediuresis) during feeding, 20 to 60 mosquitoes were placed into an ice-cream cylinder cage containing filter paper on which the insects could prediurese and were offered a blood meal on a human forearm. Once the mosquitoes had fed to repletion, the filter paper was removed from the cage, examined, and the pattern of prediuresis (as indicated by spots of blood excreted) recorded (Vaughan et al. 1991).

Haemagglutination of Ingested Blood

The degree of erythrocyte aggregation was determined at 250 or 400 x in wet mount preparations of blood meals dissected from freshly fed mosquitoes into physiological saline. This was scored 0, 1, 2, 3, 4, or 5, for 0, 1%-25%, 26%-50%, 51%-70%, 71%-90%, and >90% of red cells agglutinated, respectively.

Statistical analysis

Data were subjected to a Duncan multiple range test (P=0.05) to compare means within each category, that is, within probing times, gut filling times, prediuresis times, and total blood-feeding times (Sokal and Rohlf 1980). In addition, the data were transformed into contingency tables and subjected to a G-test to determine the inter and intra specific differences in erythrocytic agglutination levels and differences in patterns among nulliparous and parous mosquitoes (Sokal and Rohlf 1980).

**RESULTS**

**TABLE 1** summarizes the probing behavior of the field-collected *An. aquasalis*, *Ae. aegypti*, *Hg. janthinomys*, and *Cx. quinquefasciatus* under laboratory conditions. No significant (P=0.09) differences in the mean number of probes was found among the four mosquito species belonging to four genera. The duration of probing varied significantly (P<0.0003) between species (**TABLE 1**), with *An. aquasalis* (19.6 s) being much faster than *Cx. quinquefasciatus* (148.3 s).
The times it took *Ae. aegypti* (87.7 s) and *Hg. janthinomys* (90.4 s) to fill their guts were not significantly different (P<0.001), but *Cx. quinquefasciatus* (222.8 s) was slower (P<0.01). In fact, *An. aquasalis* (P<0.001), *Ae. aegypti* (P<0.001), and *Hg. janthinomys* (P<0.001) were significantly faster than *Cx. quinquefasciatus* (TABLE 1).

TABLE 1 shows the mean prediuresis times for all four mosquito species. *Aedes aegypti* (205.8 s) and *Hg. janthinomys* (177.1 s) were similar (P<0.01). *Culex quinquefasciatus*, however, took longer over prediuresing (526.9 s) and was significantly (P<0.006) slower than *An. aquasalis* (77.3 s).

The total feeding time for the four mosquito species, of which prediures times were a large component, are summarized in TABLE 1. All species exhibited prediuresis but the proportion of individuals that did so varied with the mosquito species; *An. aquasalis* (86.1%), *Ae. aegypti* (86.7%), *Cx. quinquefasciatus* (86.1%), and *Hg. janthinomys* (70.0%) (TABLE 2). The lowest level of prediuresis was observed among *Hg. janthinomys* (70.0%) but this difference was not significant (P=0.9). There were no significant differences in the incidence and duration of prediuresis between parous and nulliparous females of any of the four mosquito species. In addition, within each mosquito species, blood-feeding times were similar for parous and nulliparous females.

The duration of feeding among individual mosquitoes varied significantly (P<0.001) according to species and genera, with the range of feeding times being 133.0 s to 650.0 s, 135.0 s to 650.0 s, 66.0 s to 505.0 s, and 263.0 s to 1287.0 s for *An. aquasalis, Ae. aegypti, Hg. janthinomys, and Cx. quinquefasciatus*, respectively.

There were both inter (P<0.001) and intra-specific differences among the four species of different genera in the level of erythrocyte agglutination in the blood meal. The inter-specific variation in *An. aquasalis*, with 72% (G=15.5; df = 6; P<0.01) being scored 2 and 3. In contrast, *Ae. aegypti* (67%) (G=14.9; df = 6; P<0.01), *Hg. janthinomys* (67%) (G=14.8 df= 6 P<0.01), and *Cx. quinquefasciatus* (66%; G=12.0 df = 6; P<0.05) were scored 0 (see TABLE 3). Within each species, there were no significant differences in agglutination by nulliparous and parous mosquitoes.

TABLE 2. Patterns of prediuresis exhibited by the four species of mosquitoes

<table>
<thead>
<tr>
<th>Species</th>
<th>No. feeding</th>
<th>No. and (%) prediures</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. aquasalis</em></td>
<td>30</td>
<td>26 (86.7%)</td>
</tr>
<tr>
<td><em>Ae. aegypti</em></td>
<td>36</td>
<td>31 (86.1%)</td>
</tr>
<tr>
<td><em>Hg. janthinomys</em></td>
<td>30</td>
<td>21 (70.0%)</td>
</tr>
<tr>
<td><em>Cx. quinquefasciatus</em></td>
<td>30</td>
<td>25 (83.3%)</td>
</tr>
</tbody>
</table>
DISCUSSION

Partitioning the blood-feeding process for mosquitoes into components, such as the number of probes, duration of probing, duration of the gut-filling phase, duration of prediuresis, proportion of females exhibiting prediuresis, and total feeding time provides a functional basis for interspecific comparisons of mosquito species (Vaughan et al. 1991, Chadee and Beier 1995). We employed this strategy for evaluating four vector species of mosquitoes from Trinidad: An. aquasalis, Ae. aegypti, Hg. janthinomys, and Cx. quinquefasciatus. In contrast to laboratory-based studies using colonized mosquito species (Vaughan et al. 1991), we used field-collected females captured coming to feed on humans within their ecological niche in Trinidad. The use of field-collected females for studies of blood-feeding represents an ideal way to obtain relevant information on natural behaviors in the absence of selection biases associated with laboratory colonization. Notably, if blood-feeding behavior has a genetic basis, then slow-feeding individuals would be selected against and lost very quickly in colonization efforts.

Significantly different patterns of blood-feeding were observed for the four vector species from Trinidad. Except for the number of probes per feeding event, the four species showed significant differences with respect to duration of probing, duration of gut-filling, duration of prediuresis, and total feeding time. Overall, mean total feeding time varied almost five-fold among the four species. The malaria vector An. aquasalis was the quickest feeder, with total feeding time averaging just over 2 min. per feeding event. The two arbovirus vectors, Ae. aegypti and Hg. janthinomys, were intermediate in duration, totaling 3-4 min. per feeding. Most surprisingly, the filariasis vector Cx. quinquefasciatus averaged 11 min. per feeding time. These results are probably representative of natural feeding events, as >80% of the field-collected females fed readily on volunteers when tested under laboratory conditions.

Without exception, the four vector species showed tremendous intra-specific differences for each of the parameters of the blood-feeding process. That is, individual mosquitoes varied significantly in their timing with respect to probing, gut-filling, prediuresis, and total feeding time. For example, female Cx. quinquefasciatus varied eight-fold in their duration of probing, from 40 to 320 s. For each species, the degree of intra-specific variation is expressed in TABLE 1 as the standard deviation for each mean value per blood-feeding parameter.

In groups of species tested in our studies, the duration of prediuresis usually accounted for most of the interspecific differences in total feeding times; duration of gut-filling tended to be less variable among species (Vaughan et al. 1991, Chadee and Beier 1995). This was not true in our current evaluation, as Cx. quinquefasciatus spent an average of 3.7 min. in the gut-filling stage, more than twice as long as the other three species. This species spent an even longer time undergoing prediuresis. Unlike some mosquito species that fail to exhibit prediuresis (Vaughan et al. 1991, Chadee and Beier 1995), from 70-86% of all the vector species tested in the current study exhibited prediuresis. Notably, the timing of prediuresis as a proportion of the total feeding time differed among the species, with An. aquasalis having the shortest period of prediuresis and Cx. quinquefasciatus the most pronounced.

Erythrocyte agglutination is a trait whereby substances in the saliva promote red blood cell

<table>
<thead>
<tr>
<th>Agglutination score</th>
<th>An. aquasalis</th>
<th>Ae. aegypti</th>
<th>Hg. janthinomys</th>
<th>Cx. quinquefasciatus</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>20</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>9</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>
agglutination in the midgut. Interestingly, a tremendous degree of inter-specific variation in agglutination patterns was seen for the vector species from Trinidad. Agglutination was observed for all 30 of the An. aquasalis tested. In contrast, for the other three mosquito species, only 33-40% exhibited agglutination. Inter-specifically, An. aquasalis had the most pronounced variation in agglutination patterns, with 10% of the females agglutinating 1-25% of the red cells and 29% agglutinating 71-90% of the erythrocytes ingested. Less intra-specific variation is normally found in laboratory colonized species (Vaughan et al. 1991).

The process of blood-feeding behavior does not appear to change significantly as mosquitoes become physiologically or chronologically older. None of the species tested showed any signs of behavioral differences between nulliparous and parous females. As the chronological ages of the parous mosquitoes were not known in this study because all were field-collected, there was no possibility for testing directly for age-related changes in the various parameters of blood-feeding behavior.

In conclusion, the parameters of blood-feeding measured in this study are extremely relevant to understanding the dynamics of pathogen transmission by mosquitoes and other insects. Under the standard conditions of our test system, the field-collected mosquitoes evaluated showed tremendous inter- and intra-specific differences in the timing of various parameters of the blood-feeding process. Presumably, a pronounced genetic component governs the degree of variation seen in the study. Functionally, pathogens, such as arboviruses, protozoa, and nematodes have unique requirements for transmission that depend closely on the characteristic parameters of the blood-feeding process. There are a number of consequences of having a long feeding time and contact with the host (Molyneux and Jefferies 1986). For example, it is not hard to see why Cx. quinquefasciatus is a successful vector of filariasis, as females average 11 min. feeding contact with humans (e.g., ample time for infective L3 stages of the parasite to escape the thorax and navigate to host tissues through the mouth parts of the mosquito). Further evaluations of mosquito blood-feeding behavior are needed, especially with respect to establishing a better understanding of pathogen transmission under field conditions.

Acknowledgments

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Effects of Age and Mating on the Host-Seeking Behavior of *Aedes aegypti* Mosquitoes

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**ABSTRACT:** Chronologically older female *Aedes aegypti* mosquitoes that had previously undergone a gonotrophic cycle were more likely to respond to host stimuli when gravid than those that had not blood-fed previously. Mating or injection with male accessory gland (MAG) homogenates decreased the percentage responding to host stimuli in both groups of old females and there was a greater reduction by those that had previously ingested blood. Older females that were mated late in life were less likely to respond to host stimuli than were old females mated early. Injection of MAG homogenates into older females that mated early in life caused a significant decline in host-seeking behavior. This difference in the age-related change in host-seeking behavior during oogenesis appeared to be due to the decline in the activity of MAG substances within the females.

**Keyword Index:** Mosquito, male accessory gland, host-seeking behavior

**INTRODUCTION**

The host-seeking behavior of *Aedes aegypti* mosquitoes is often inhibited during egg development until after oviposition occurs, when new attempts to locate a host define the beginning of the next gonotrophic cycle. Compared to females in their first gonotrophic cycle, older females of this species show less of an inhibition of host-seeking behavior and are more likely to approach a host when gravid. This effect is accentuated if they have undergone several gonotrophic cycles and are gonotrophically as well as chronologically old (Klowden and Lea 1984). Other differences in mosquito behavior have also been associated with aging (Gillies 1957, Hitchcock 1968, Crans et al. 1976).

We recently demonstrated that male accessory gland (MAG) substances that are transferred during mating modulate the host-seeking behavior of gravid *Ae. aegypti*, making them less likely to express host-seeking behaviors compared to unmated gravid females that were developing eggs (Fernandez and Klowden, 1995). Because females usually mate early in their lives and generally do not remate as a result of the inhibition of subsequent mating by MAG substances (Craig 1967), we tested the hypothesis that the inactivation of MAG substances with age is responsible for the increase in the host-seeking behavior of older mosquitoes when they are gravid.

**MATERIALS AND METHODS**

The UGAL strain of *Ae. aegypti* (L.) was used in all experiments. Larvae were reared at 27°C on a standard diet of rat chow, brewer's yeast, and lactalbumin hydrolysate (1:1:1 by weight). Adults were maintained at 27°C and 80% RH under a 14:10 (L:D) photoperiod and had access to 10% sucrose available from cotton wicks, except for two days prior to olfactometer testing. Males and females were separated by sex in the pupal stage using the criterion of the presence of the projecting plate of the ninth sternite in the female and its absence in the male (Christophers 1960). Blood meals were usually obtained from human hosts, but in some experiments measured volumes of rat blood were also administered by enema (Briegel and Lea 1975).

Newly emerged females were initially maintained without males. In the early mating group, males were introduced into their cage three days after female emergence. In the late mating group, the males were not introduced until day 22. The females were blood-fed on day 22 and their host-seeking behavior was evaluated in an olfactometer (Klowden and Lea 1978) three days later. In another experiment, mating occurred on day 3
and additionally, 0.2 μl of a 4 MAG/μl homogenate (0.8 MAG equivalents) was injected shortly before 1 μl of blood was introduced as an enema on day 22. The host-seeking behavior of these females was tested on day 25. Other mosquitoes were treated identically, but additionally blood-fed on days 5 and 10 post-emergence. Control groups were injected with saline or heat-inactivated MAGs. To compare responses between groups, we used the z-test based on the percentages responding (SigmaStat Statistical Software, ver. 1.0. Jandel Scientific Software, San Rafael, CA).

RESULTS

Effects of Age and Natural Mating on Host-Seeking Behavior

Females were mated either at three days post-emergence or on day 22, ingested blood on day 22; and their host-seeking behavior was measured on day 25 before oviposition occurred. As shown in TABLE 1, the time that the mating occurred determined the degree to which host-seeking behavior was affected. About half of the unmated females responded, and those that were mated on day 3 showed a significantly reduced response. Those females that did not mate until day 22, however, showed an even greater inhibition that was typical of that displayed by younger mosquitoes.

Effects of MAG Homogenates and Repeated Blood-Feeding on the Host-Seeking Behavior of Older Females

Females mated on day 3 were either given two blood meals on days 5 and 10 after emergence or were not allowed to feed, and on day 22, were all given 1 μl of blood by enema. Other groups were additionally injected with a MAG homogenate on day 22 or a homogenate first heated at 100°C for 15 minutes.

Controls were injected with the same volume of saline alone. Three days later when their host-seeking behavior was evaluated, controls that had mated on day 3 and that received blood for the first time on day 22 were less than half as likely to respond than were females of the same age that had blood-fed twice previously (TABLE 2), demonstrating that gonotrophic aging reduced the inhibition of host-seeking behavior during oogenesis. Within each group, those injected with the MAG homogenate were less likely to respond to host stimuli, and the one group additionally injected with heat-inactivated MAG substances responded no differently than did controls.

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Effects of physiological and gonotrophic age and MAG injection on the host-seeking behavior of 25-day-old females mated on day 3.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment of Females</td>
<td>MAG Injected</td>
</tr>
<tr>
<td>1 μl of blood on day 22 only</td>
<td>-</td>
</tr>
<tr>
<td>Mated on day 3</td>
<td>+</td>
</tr>
<tr>
<td>Mated on day 22</td>
<td>(heat inactivated)</td>
</tr>
<tr>
<td>Blood meals on days 5 and 10; 1 μl blood on day 22</td>
<td>-</td>
</tr>
</tbody>
</table>

* signifies significant differences from non-injected controls (P<0.05).

DISCUSSION

Aging and mating both modulate the host-seeking behavior of gravid Ae. aegypti mosquitoes. Our data suggest that the increased host-seeking behavior that is characteristic of the unmated and older gravid females appears to result from the absence or inactivation of MAG substances after they are introduced into the female during mating. When females mated early, the inhibition of host-seeking behavior after gonotrophic aging occurred was less than if they mated later in life (TABLE 1). Injections of MAG substances into already mated females increased the degree of behavioral inhibition that was expressed when the mosquitoes were older (TABLE 2). The absence of any effects from the injection of heat-treated homogenates suggests that this response was not a result of non-specific trauma from

TABLE 1. Host-seeking behavior of 25-day-old mosquitoes that were mated either early or late in life.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Responding to Host Stimuli (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmated</td>
<td>56.0±6.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mated on day 3</td>
<td>25.6±5.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mated on day 22</td>
<td>1.4±1.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values followed by the same letter do not differ significantly (P<0.05).
the presence of large amounts of protein in the hemolymph.

Previous studies identified age-related behavioral and physiological changes in *Ae. aegypti* (Klowden and Lea 1980, 1984). Chronologically older females that were mated soon after emergence were more likely to seek a host while gravid than when they were younger; but when they had undergone several gonotrophic cycles, there was an even greater probability of engaging in host-seeking (Klowden and Lea 1984). Male accessory gland substances also prevent the female from engaging in subsequent remating (Craig 1967), but these substances no longer provide as effective a barrier to mating after several gonotrophic cycles (Williams and Berger 1980, Young and Downe 1982). However, polyandry, when reported from field populations, is infrequent (Mahmood and Reisen 1980, Reisen et al. 1984, Gomulski 1990, Villarreal et al. 1994, Yuval and Fritz 1994); and there is little evidence that multiple mating would be likely to enhance the effects of an early receipt of MAG substances in natural populations. The increased tendency to seek a host by chronologically older, more epidemiologically important mosquitoes, appears to result from an age-related inactivation of MAG substances.

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Vertical Distribution of Adult Mosquitoes
(Diptera: Culicidae) in Southern and Central Sweden

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ABSTRACT: The vertical distribution of adult mosquitoes was studied in southern (Norra Åsum) and central Sweden (Tärnsjö) during August 1995, with an emphasis on the ornithophilic species Culiseta morsitans (Theobald), Culex pipiens L., and Culex torrentium Martini. A modified slingshot method was used to suspend Centers for Disease Control (CDC) miniature light traps, in combination with CO2, as additional attractant, into the canopy of deciduous trees. Altogether 15,186 mosquitoes, including 93 Cs. morsitans and 182 Cx. pipiens/torrentium, were collected at various heights above ground during night-time. The numbers of Cs. morsitans and Cx. pipiens/torrentium per trap-night were not significantly influenced by height, but the number of total mosquitoes per trap-night decreased with increasing height. Significantly larger proportions ($\alpha$ < 0.001, chi-square test) of both Cs. morsitans and Cx. pipiens/torrentium, to the total of other mosquitoes, were collected in the canopy than at chest height. The proportions of Cs. morsitans were 18% at 12 to 15.5 m and 2.2% at 1.5 m in Norra Åsum, and 49% at 14 to 18 m and 0.6% at 1.5 m in Tärnsjö. The proportions of Cx. pipiens/torrentium were 36% at 12 to 15.5 m and 6.9% at 1.5 m in Norra Åsum, and 5.1% at 6 to 9 m and 0.2% at 1.5 m in Tärnsjö. Our method of suspending traps in the canopy and use of a simplified protocol to quickly distinguish the relevant species was efficient for placing the traps and for sampling adult females of the ornithophilic species Cx. pipiens/torrentium and Cs. morsitans in Sweden. We suggest that this procedure might also be of use to investigate these and other ornithophilic mosquitoes for bird-associated arboviruses in woodlands in the European region.

Keyword Index: Ornithophilic mosquitoes, Culex, Culiseta, vertical distribution, arbovirus.

INTRODUCTION

Several human pathogenic arboviruses of the genera Alphavirus (Sindbis, eastern equine encephalomyelitis, western equine encephalomyelitis) and Flavivirus (Saint Louis encephalitis, Murray Valley encephalitis, West Nile) exploit birds as reservoir hosts and specialized ornithophilic mosquitoes of the genera Culex and Culiseta as enzootic vectors in their natural transmission cycles (Niklasson 1988, Reisen and Monath 1988, Morris 1988, Tsai and Mitchell 1988, Marshall 1988, Hayes, 1988). Ockelbo virus, a north European subtype of Sindbis virus (Lundström et al. 1993), is maintained in the passerine bird population by Culex torrentium Martini which is the main enzootic vector, and by a potential second enzootic vector, Culiseta morsitans (Theobald) (Lundström 1993). Transmission to humans is probably accomplished by Aedes cinereus Meigen (Francy et al. 1989, Turell et al. 1990) that feed predominantly on mammals but also on birds (Service 1971a, Jaenson and Niklasson 1986). Francy et al. (1989) found that Culex pipiens L./torrentium had a 28-fold higher Ockelbo virus field infection rate, and that Cs. morsitans had a 10-fold higher rate, than had Ae. cinereus. None of the other 17 species investigated hosted Ockelbo virus. Thus, specific sampling of Cx. pipiens/torrentium and Cs. morsitans, species specialized in feeding on birds in northern Europe (Service 1969, 1971a, Jaenson and Niklasson 1986), would greatly improve our ability to detect Ockelbo virus activity. These ornithophilic
mosquitoes are, however, difficult to collect as adults.

Most of the mosquito species that have been examined are active at lower levels, usually because little or no sampling is done at higher levels. In the USA, the ornithophilic Culiseta melanura (Coquillett) was found to be evenly distributed up to 7.6 metres in Massachusetts (Nasci and Edman 1981), and Culex salinarus Coquillett was evenly distributed up to 25 m in Tennessee (Snow 1955). The British Cs. morsitans and Cx. pipiens/torrentium were more active at 5.5 m than at ground level (Service 1971b). The sampling of mosquitoes well above ground level in the canopy may therefore provide more specific samples of ornithophilic mosquito species.

The aims of the study were to investigate the vertical distribution of adult mosquitoes in wooded biotopes in southern and central Sweden during the latter part of summer, and to establish a protocol for efficient sampling of ornithophilic mosquitoes with emphasis on the enzootic vectors of Ockelbo virus.

MATERIALS AND METHODS

Study Sites

Two study sites were chosen. The first was situated in Norra Äsüm (55°59'N, 14°10'E) at the western shore of the Lake Hammarsjön, Province of Skåne, in southern Sweden. The shore of this shallow lake was covered by extensive reed (Phragmites communis) belts and of periodically flooded open areas mainly covered by Carex spp. Outside the Carex belt the area is dominated by extensive farmland and patches of deciduous forest. The sampling of mosquitoes was conducted in one of these deciduous forest patches (approx. 30 hectares), at two study sites approximately 50 m apart. Alder trees (Alnus glutinosa), 15 to 20 m tall, dominate the forest. The ground was covered with dense herb vegetation and willow bushes (Salix spp.) up to 2 m high. One site was marshy, with extensive areas of water of less than 10 cm deep.

The second study site, in central Sweden, was near the south shore of the Lake Nordmyrasjön, Tärnsjö (60°10'N, 16°54'E), Province of Västmanland, in central Sweden. This lake has been almost completely overgrown with reed, Carex spp., willow, etc., and is now a wetland periodically flooded by the river Dalälven. Mosquitoes were collected in the borderland between the wetland and a mixed deciduous/coniferous forest. Aspen (Populus tremula) and Norwegian spruce (Picea abies), 25 to 30 m tall, are the most common trees on the study site; and the ground is covered by dense vegetation up to 1 m tall.

Collection and Identification of Mosquitoes

In order to count the absolute numbers of ornithophilic mosquitoes in relation to other species, we used the Centers for Disease Control (CDC) miniature light trap with carbon dioxide as additional attractant (Sudia and Chamberlain 1962), placed at different heights. Each CDC trap was baited with 1-2 kg of dry ice, kept in an insulated envelope to allow a slow release of carbon dioxide. In Norra Äsüm, two CDC traps were suspended in alder trees at 12 and 15 m, and one was suspended in a willow tree at 15.5 m. Ten traps were placed at 1.5 m, six of these traps were positioned above the water surface in the marshy biotope, while the remaining four traps were set in a similar biotope but without a visible water surface. All traps were operated on three consecutive nights between July 31 and August 3, 1995.

In Tärnsjö, two CDC traps were suspended in beech trees (Betula pubescens) at 8 m and at 9 m, and one trap was suspended in a dead Norwegian spruce at 6 m. Two traps were placed at 1.5 m. In Tärnsjö the traps were operated on two consecutive nights, between August 9 and 11, 1995. A second trial was conducted in Tärnsjö with six traps suspended at 14, 15.5, 16.5, 17, 17.5, and 18 m in aspen, and two traps at 1.5 m. These traps were operated the night between August 22 and 23, 1995. All mosquito collections were performed between 6 p.m. and 7 a.m.

To place the traps in tree canopies at the required height, the modified slingshot method (Novak et al. 1981) was used. A 90 g pellet of lead or tin was fitted to the nylon monofilament line of a spinning reel mounted on a spinning rod attached to the side of a 230 cm wooden plank that also served as the holder of a slingshot (Fig. 1). By placing the lower end of the plank against the ground, both hands could be used to stretch the elastic band of the slingshot, and the pellet with attached line could thereby be catapulted with great precision over a selected robust branch at considerable height (at least 25 m), and the mass of the pellet was sufficient to pull the line up over the branch and back to the ground. Once the line had been placed over the selected branch, it was used to draw a 50-meter 1.0 mm diameter interwoven multifilament nylon rope (rope A), over the branch. A metal loop was attached to one end of rope A, and a slightly shorter rope (B) was inserted through the loop, which was then suspended from the canopy so that both ends of rope B reached the ground. The trap was then attached to one end of rope B so that it could easily be raised and lowered for collection (Fig. 2). For each trap, the total length of rope B was measured and divided by two, to determine the height of the trap.

Collected mosquitoes were anaesthetized with
Figure 1. The design of the slingshot used for catapulting the 90 g lead pellet with attached line over a branch in dense canopies of deciduous trees.
Figure 2  The method for suspending and rapid lowering of CDC miniature light traps and the envelope with dry ice in the canopy of deciduous trees.
carbon dioxide, dispensed in 8 ml plastic vials (approximately 350 mosquitoes/vial), and transported on dry ice to the laboratory at the Swedish Institute for Infectious Disease Control in Stockholm for identification. Mosquitoes were identified according to the species descriptions in Mohrig (1969) under a stereomicroscope. Specimens of *Cx. morsitans*, *Culiseta annulata* (Schrank), *Ae. cinereus*, *Coquillettidia richiardii* (Ficalbi), and *Anopheles claviger* (Meigen) were identified to species. Females of *Cx. pipiens* and *Cx. torrentium* could not be reliably distinguished on morphological criteria and were therefore grouped together as *Cx. pipiens/torrentium*. Similarly, *Anopheles* spp., other than *An. claviger*, were identified as *An. maculipennis* sensu latu. To increase the speed of processing specimens for identification, and because *Aedes* spp. other than *Ae. cinereus* have not been considered vectors of Ockelbo virus in Sweden, other species in this genus were grouped together as *Aedes* spp.

The chi-square test, corrected for continuity (Siegel and Castellan 1989), was used for statistical evaluation of the eventual difference between the proportions of certain mosquito species collected in the canopy versus at chest height.

**RESULTS**

Altogether 15,186 mosquitoes, including 93 *Cs. morsitans* and 182 *Cx. p. torrentium*, were collected at various heights above ground on 43 trap-nights in the two study areas during three study periods (TABLE 1). The proportions of these species to the total number of mosquitoes collected were significantly higher in the canopy than at chest height. However, the numbers of *Cs. morsitans* and *Cx. p. torrentium* per trap-night were similar at all heights because the average number of total mosquitoes per trap-night decreased with increasing height.

*Culiseta morsitans* constituted significantly higher proportions of the total number of mosquitoes collected at canopy level than at chest height in both study areas during all three study periods. Traps at 12 to 15.5 m in Norra Åsum collected 18% *Cs. morsitans*, while traps at

**TABLE 1.** Vertical distribution of mosquitoes collected during July and August 1995 in southern Sweden (Norra Åsum) and in central Sweden (Tärnsjö) using CDC miniature light traps with carbon dioxide as attractant.

<table>
<thead>
<tr>
<th>Species</th>
<th>Norra Åsum July 31 to August 3</th>
<th>Tärnsjö August 9 to 11</th>
<th>Tärnsjö August 22 to 23</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12-15.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Trap-nights (no.)</td>
<td>9</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td><strong>Species</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Culex pipiens/torrentium</em>²</td>
<td>40</td>
<td>54</td>
<td>85</td>
</tr>
<tr>
<td><em>Culiseta morsitans</em></td>
<td>20</td>
<td>17</td>
<td>27</td>
</tr>
<tr>
<td><em>Culiseta annulata</em></td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>Anopheles claviger</em></td>
<td>4</td>
<td>46</td>
<td>18</td>
</tr>
<tr>
<td><em>Anopheles maculipennis</em> sl.</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Coquillettidia richardi</em></td>
<td>15</td>
<td>216</td>
<td>188</td>
</tr>
<tr>
<td><em>Aedes cinereus</em></td>
<td>0</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td><em>Aedes</em> spp.³</td>
<td>32</td>
<td>435</td>
<td>204</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>111</td>
<td>782</td>
<td>526</td>
</tr>
<tr>
<td><strong>Total/trap-night</strong></td>
<td>12.3</td>
<td>65.2</td>
<td>29.2</td>
</tr>
</tbody>
</table>

¹These traps were operated over shallow water pools in the forest.
²Female *Cx. p. pipiens* and *Cx. torrentium* both occur in the study areas but could not be distinguished reliably to species.
³Numbers pooled of all *Aedes* other than *Ae. cinereus*.
1.5 m collected 2.2% in the drier biotope (different from canopy $\chi^2=57.5, df=1, \alpha<0.001$) and 5.1% in the marshy biotope (different from canopy, $\chi^2=24.2, df=1, \alpha<0.001$). The traps in Tärnsjö collected 0.4% Cs. moritans at 6 to 9 m versus 0.02% at 1.5 m during August 9 to 11 ($\chi^2=30.4, df=1, \alpha<0.001$), and 49% at 14 to 18 m versus 0.6% at 1.5 m during August 22 to 23 ($\chi^2=267, df=1, \alpha<0.001$). However, the numbers of Cs. moritans collected per trap-night were not different in the canopy than at chest-height.

Similarly to Cs. moritans, the numbers of Cs. pipiens/torrentium per trap-night were not influenced by height, but the proportions of these species to the total number of mosquitoes collected were significantly larger in the canopy than at chest height at all comparisons. Traps at 12 to 15.5 m in Norra Åsum collected 36% Cx. pipiens/torrentium, while traps at 1.5 m in the marshy biotope collected 16% (different from canopy, $\chi^2=24.2, df=1, \alpha<0.001$), and traps at 1.5 m in the drier biotope collected 6.9% of these species (different from canopy, $\chi^2=84.5, df=1, \alpha<0.001$). The mosquito collection during August 22 to 23 in Tärnsjö gave 5.1% Cx. pipiens/torrentium at 14 to 18 m, and 0.2% of these species at 1.5 m ($\chi^2=32.5, df=1, \alpha<0.001$).

*Cq. richiardii* was collected at all heights in both Norra Åsum and Tärnsjö (TABLE 1). In Norra Åsum this species made up 14% of mosquitoes collected at 14 to 18 m and 28% of mosquitoes collected at 1.5 m (not different, $\chi^2=0.2, df=1, \alpha<0.90$). However, *Cq. richiardii* was significantly more prevalent at 14 to 18 m (15%) than at 1.5 m (1.4%) during August 22 to 23 ($\chi^2=38.7, df=1, \alpha<0.001$), and at 6 to 9 m (7.2%) versus 1.5 m (0.4%) during August 9 to 11 in Tärnsjö ($\chi^2=440, df=1, \alpha<0.001$).

*Aedes cinereus* was mainly collected at 1.5 m in both study sites (TABLE 1). However, in Tärnsjö during August 9 to 11 it constituted 25% of the catch at 6 to 9 m versus 16% at 1.5 m, and two *Ae. cinereus* were collected at 14 to 18 m in Tärnsjö during August 22 to 23.

**DISCUSSION**

We investigated the vertical distribution of adult mosquitoes in woodlots of south and central Sweden, and found a significantly larger proportion of the ornithophilic species Cs. moritans and Cx. pipiens/torrentium in the canopy than at chest height during all three study periods in both study sites. Studies in North America have also shown increased proportions of ornithophilic Culex and Culiseta species in the mosquito fauna at canopy level (Snow 1955, Nasci and Edman 1981, Novak et al. 1981). Service (1971b) reported larger numbers of Cx. pipiens/torrentium and Cs. moritans at 5.5 m than at ground in Britain. The activity of ornithophilic mosquitoes in the canopy is probably a reflection of the spatial distribution of their bird hosts. The energy demands of sustained search flight at various heights and the problems associated with locating small birds in the canopy should decrease the proportion of non-ornithophilic species in the canopy, which is in line with our results. Thus, mosquito collection at canopy level was shown to be efficient for sampling ornithophilic mosquitoes.

The mosquito fauna of both south and central Sweden includes Cs. pipiens as well as Cx. torrentium (Dahl 1977, Francy et al. 1989). The males could be identified to species based on the morphology of the genitalia, while reliable morphological methods to distinguish between the females of these species are not available (Dahl 1988). Thus, we are presently unable to report these species separately and have decided to report them as Cx. pipiens/torrentium, although we are aware of the differences in their experimental vector competence for Ockelbo virus (Lundström 1994). Future studies may also show differences in the behaviour of these two species.

The greater number of Cx. pipiens/torrentium collected in Norra Åsum than in Tärnsjö during August (late summer) was probably not caused by a major difference in the overall population size because very large numbers of Cx. pipiens/torrentium larvae were observed in Tärnsjö one month prior to the study (Lundström, unpublished information). More likely, the paucity of Cx. pipiens/torrentium in the Tärnsjö samples was caused by replacement of the host-seeking summer generation by the strictly nectar-feeding prehibernating generation that are not attracted by CO2. The shift between summer and pre-hibernating generations occur during the first half of August in central Sweden (Jaenson et al. 1986).

Similar numbers of Cs. moritans were collected in both study areas, which is in line with the phenology of this species in Sweden, with a single summer generation that takes multiple blood meals during June to October (Jaenson et al. 1986).

The frequent host-seeking of *Cq. richiardii* at canopy level, both in Norra Åsum and in Tärnsjö, indicates that it searches for bird hosts because birds are the most common vertebrates in the canopy. Also, search flight to find mammals in the canopy as compared to at ground level is a waste of energy because ground dwelling mammals of several species are common while only one species of squirrel occur in the canopy in the study areas. Observations in Africa and in North America have shown that mosquitoes of the genus *Cq. richiardii* feed readily in the canopy and that
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birds generally are the preferred hosts (Edman 1971, McIntosh et al. 1972, Rickenbach et al. 1974, Chandler et al. 1976). Further studies on the host preference of *Cq. richiardii* and assay of the species for bird-associated arboviruses could therefore be interesting.

We found that *Ae. cinereus* was active both at ground level and in the canopy, and our collection at 6 to 9 m and at 14 to 18 m in Tärnö is the first evidence that the species is active well above ground level. Mohrig (1969) and Jaenson and Niklasson (1986) reported that *Ae. cinereus* appears only close to the ground and that the species feeds mainly on small rodents. When taking blood from larger mammals, including humans, the blood-feeding attacks were directed preferably at the lower extremities. Our observation that *Ae. cinereus* also seeks hosts in the canopy, which provides increased opportunities to feed on roosting birds, is consistent with the reported feeding on birds (Service 1971a, Jaenson and Niklasson 1986) and with the reported isolation of Ockelbo virus from specimens caught in the wild (Francy et al. 1989).

We achieved efficient collecting of ornithophilic mosquito species by placing traps at canopy level in deciduous trees. The modified slingshot method was very successful for suspending the traps at various heights in tree canopies. One person was able to suspend a trap in 30 to 50 minutes, including locating a trap-site and selecting a branch, securing the rope to the branch, and suspending the activated trap to the desired height. The total cost of the equipment for suspending the six traps was US$ 140, half of which was for the interwoven multifilament ropes (approximately 2 x 50 m per trap). A less expensive 1.0 mm rope, spun from three nylon filaments, was also tested; but it caused rotation of the metal loop in the canopy and resulted in tangling of the line used for suspending the trap. Although ornithophilic mosquitoes could be collected together with large numbers of mosquitoes of other species in CDC traps placed at chest height (Francy et al. 1989), our method of suspending traps in the canopy and the use of a simplified protocol to distinguish the relevant species, further helped us to rapidly detect ornithophilic mosquitoes in the environment. The method could also be useful for comparative sampling of ornithophilic mosquitoes to study their biotope preference and to define areas with enzootic circulation of bird-associated and mosquito-borne arboviruses. For example, the European mosquito fauna includes several ornithophilic mosquito species, *viz.* *C. morsitans*, *Culiseta fumipennis* (Stephens), *Culiseta litorea* (Shute), *Culiseta ochroptera* (Peus), *C. pipiens*, *C. torren†i*, and other *Culex* spp. (Service 1969, 1971a; Dahl and White 1978; Jaenson and Niklasson 1986). These species have rarely been investigated for arboviruses although two bird-associated and mosquito-borne viruses that cause disease in humans have been identified in Europe (Lundström 1994).

Acknowledgments

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Use of the Fly Grill for Assessment of House Fly Populations: 
An Example of Sampling Techniques That Create Rough Fuzzy Sets

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ABSTRACT: Widely used since its initial development in 1944, the "Scudder Fly Grill" has been a successfully employed sampling system without any theoretical statistical validation. Yet, this unconventional method has been shown to be empirically sensitive for guiding fly control programs, and has shown a good correlation of fly population levels with fly-borne disease. This report provides the first workable explanation that grill sampling is really the use of "rough fuzzy sets" of data, part of a new computational system now becoming widely used in industry. Parallels of fly population sampling are made to other biological sampling problems where the use of fuzzy logic might also be useful.

**Keyword Index:** Fly, grill index, contagious distribution, sampling method, fuzzy logic.

**HISTORY OF THE SAMPLING METHOD**

When the U. S. Public Health Service reassigned the author to the Henry Rose Carter Laboratory in Savannah, Georgia in October 1944, his task was to determine the efficacy of that very new insecticide, DDT, for the control of adult flies. At the outset, in the author's analysis, the first and most difficult step in the research was to be the requirement for the formulation and design of a dependable and sensitive method for assessing fly populations in order that all treatments undertaken could be evaluated by quantitative measurements.

The study required that the census method be feasible for determining all naturally occurring fly population levels prior to and after treatments, and therefore be able to validly reflect any difference in dosage levels of the insecticide, duration and degradation of effect, difference in treatment techniques, and physical differences in situations treated. The data must be reproducible for validity of comparison.

Study of house fly populations revealed that they were very mobile, very gregarious, sensitive to light, wind, and temperature levels, and very responsive to a variety of attractants. No sampling method in a fixed position could be valid. No attractant (bait) could be used because there would be no way of evaluating it against those attractants already present in the environment being sampled, nor was there any practical way of quantitatively evaluating competing baits.

Extended field observations of fly behavior, such as their mobility, socializing, and in particular, their selection of edges for resting, finally led the author to design a replacement uniform resting surface, the fly grill (Fig. 1), on which one could very rapidly count the resting flies with minimal disturbance of the general population (Scudder 1947, McGuire and Lindsay 1950, West 1951, Schoof 1955, Ehlers and Steel 1965).

The grill proved to be an easy, quick, and reproducible way of measuring fly populations, and was adopted as the standard for studies of DDT- fly control in the Savannah area in 1945; and in the following year in the control of flies in nine Lower Rio Grande towns in Texas in a large scale experiment to determine the role of flies in the transmission of endemic (bacillary) dysentery (Shigella) (Watt and Lindsay 1948). This was followed next year by a large scale study in Thomasville, GA (Lindsay et al. 1953).

The Savannah study was successful, the use of the grill method was validated in many studies, and became the standard for measurement of fly populations in many control programs throughout the world (Holway et al. 1951, Ricciardi and Paulini 1955, Dhillon and Challet 1985).

Extensive experience in using the grill successfully to guide and evaluate fly control programs led in 1978 to incorporation of the method into the California Code of Regulations (Title 14 - §17682 California Integrated...
Figure 1. The fly grill (3' square) in use, placed over a fly population point, and showing about 469 houseflies resting on it.
Waste Management Board) as the required procedure for determining the effectiveness of landfill cover for the prevention of fly infestations (Barclay's 1992).

Submission of the original paper on the fly grill method for publication (Scudder 1947) went through the official channels of the U.S. Public Health Service, where at NIH, an authority on conventional statistical techniques stripped the author's text of all interpretative statements. This "correction" by the then "conventional wisdom" reduced the paper to only a "cook book" technique, which is where it has stood until this present paper, which proposes that its appropriate status should be as a sampling concept within the new computational method, "fuzzy logic."

In all the years since the original formulation of grill sampling, no analytical explanation has ever appeared to explain why the method has proved to be empirically sound, has been accepted, and is widely used.

The chance now to explain the grill sampling system in light of the newer concepts of fuzzy logic has been most compelling. The mystery can now be clarified, fifty years after the development of its methodology. The author has come to this conclusion with all the confidence of a "Eureka" event, born of his experience in cognition studies during the last twelve years, and more directly to the point, in coming to a recent clearer understanding of "fuzzy logic" that provides a very good fit for the sampling enigma of the ever-changing fly population. Pawlak (1992) states:

"The basic idea of rough set theory consists in replacing vague concepts with a pair of precise concepts (so that classical set theory can be applied. This is called lower and upper approximation... With each vague concept a boundary region is associated, which consists of all objects that cannot be placed clearly within the concept... The "size" of the boundary region can be used as a measure of vagueness of the vague concept. (The greater the boundary region, the more vague is the concept; precise concepts do not have boundary regions at all.) Obviously the boundary region is the difference between the upper and lower approximation of the concept."

THE SAMPLING PROCEDURE

For each premise or localized situation to be evaluated, the resident fly population, consisting of several groups of differing sizes and numbers of flies, is first carefully located and its several aggregations are noted throughout its range, which centers around all the major feeding and resting places available in the local situation under study. Brief note is taken of those locations, the attractants present, their exposure to wind and sun, as well as time of day. By this procedure one becomes familiar with the local extent and situations of greatest activity of the population about to be evaluated.

Next, the grill counts are made only of the most dense aggregations of flies, by momentarily placing the grill, with as little disturbance as possible, in the approximate center of each group of flies within the principal area in which the mobile population is circulating at the time of sampling. Only the highest three counts are used, but for the first inspection more should be taken and the lower numbers discarded, with retention of the highest three. This would appear arbitrary, but experience shows that it is sufficient to produce a valid index average.

Perhaps for greater clarity in explaining the procedure, one might more easily compare it with sampling a human population in a given community. The procedure would be to count the number of people at each of their principal points of aggregation in town within a short period of time, with careful consideration of weather, and at a time when the population is free of constraints, such the workday imposes. Selected for counting would be the number of persons at the most popular athletic game in town, or the number seated in the largest theater for a leading performance, or the dense crowd listening to an important speaker in the public park, or the number in a train or bus station at train or bus time. The purpose would be to find at least the highest three concurrent population aggregations of a limited or definable population, such as one town. The physical size of the locale counted should not be the limiting factor; in other words the numbers should not be constrained by the site, but rather by the choice of the persons attending.

Only the highest three numbers are used to produce the index. Whatever averaging is chosen should be consistently used for the same class of situation. The central idea is to determine the average largest number of individuals the population delivers to its most choice places of aggregation at peak times, and only at those peaks. This is what the index number determined by grill sampling is, an approximate number, the upper fuzzy set boundary, derived as a function of the size of the uncounted base population, sampled at optimal points in its most gregarious or contagious behavior.

CHARACTER OF THE GRILL INDEX

To speak figuratively, one is trying to determine the elevation of a mountain top by taking several
measurements of what are conceived as minor variations in configuration at its peak. Exactness of the figures is not the critical point, but rather the consistency in the manner of securing the average or index datum. A parallel assumption might be one that the mountain has a given slope of repose, and therefore its volume is derivable solely from the rounded-off peak figure.

If one again uses the example of human populations as an illustration, then consider that a city with maximum local residential densities of 25 houses (say 60 persons) per block, must be a small village, while one with 40 or more story condominiums, like Victoria in Hong Kong, must have very many thousands. Though this is not an activity index, it is still reflective of gregariousness, and shows the lack of any immediate relationship of the linearity of physical space to density of occurrence of the life form.

One arrives at a reasonably valid assumption that the maxima in the aggregation patterns of a gregarious population are collectively a function of the total population which produces them, up to discernible physical limitations of the immediate environment.

INDEX INTERPRETATION

The next step is one of interpreting the significance and utility of the fly grill index for each situation, or community being sampled, for the purpose of a sensitive assessment and management of a fly control program.

In this step a very broad range of significance is determined for fly grill indices in diverse situations. One establishes an acceptable upper level for the presence of flies in each human contact situation, and is able with grill indices to fine-tune control measures to meet the desired standards, whether one is the level of nuisance within a food or disposal industry, a public social gathering indoors or outdoors, or a disease transmission situation, actual or potential. This is accordingly the "upper fuzzy set boundary," above which active control measures are immediately invoked.

The diversity of acceptance of flies in the environment would run from none for a microbiologist in a laboratory or a couple dining in a good restaurant, to very few for a family at home depending on their social level, to progressively more in each situation for a family on a picnic, a shopper in an open public market, a farmer on his ranch, an employee in a food-processing plant, or an employee in a garbage dump. Grill indices have proved very constant over a few days time when taken under comparable conditions of weather. They have proved dependable, and are accepted as essential for evaluation and maintenance of fly control programs at whatever level.

A very broad range of significance has been determined for grill indices relative to very diverse situations, depending upon a variety of public acceptance and public health expectations. For each class of situation the grill readings found satisfactory for control are the following upper approximations, above which is the boundary region considered as exemplifying inadequate control. The lower approximation becomes the level below which control activity is either discontinued or put on a standby status. Each of these paired levels is now proposed as the upper and lower levels of a rough fuzzy set as defined by Pawlak (1992). Some sample upper level indices which have proved useful for maintenance of fly control are:

- Restaurant kitchen .................................. 2 flies
- Residential back yard ............................... 2-3
- City block ........................................... 5
- Milking parlor ...................................... 15
- General farm ...................................... 20

When the index rises above the standard given above for a specified area, field inspections and control measures should respond. Field use has verified that this is far more sensitive than ordinary visual inspection, so that by using effective control measures guided by grill indices, one may manage a control program very accurately to the particular standard selected, avoiding any perceptible outbreaks, if the sampling covers a large enough control area, provided some unusual event does not intervene, such as wind blowing an outside population into the control area. This event is uncommon, as the house fly is unusually very domiciliary, and tends to stay within its small universe unless its population overflows, or winds become unusual; in strong contrast to the green and blue-bottle flies that are scavengers ranging over large territories and are not gregarious.

THE ROUGH FUZZY SET

The fly grill indices are now being proposed in this paper as the parameters of "rough fuzzy sets," interpreted as good control even if the upper approximate level of control connotes rather different but very practical concepts of tolerance for a fly population. Used for the past fifty years as a derivative of a very unorthodox algorithm, it has functioned effectively in evaluating the significance of maximum gregariousness of given house fly populations at points in time, in each localized area of the ecology in which that population moves freely about, whether it is a home, dairy, chicken ranch, restaurant, or city block. This datum, a simple average of population maxima, represents at one point in time
the highest level of discernible gregarious activity which the locally resident fly population produces. This figure has been found so reliable as a measure of population level and so sensitive to change that it has been used successfully to guide fly control programs. In 1946 in the lower Rio Grande Valley, the transmission epidemiology of endemic bacillary dysentery was found to show a good correlation with the community fly grill indices (Watt and Lindsay 1948, Lindsay et al. 1953). This verification, that the level of fly-vector populations was in fact being accurately measured, supported the author’s search for basic factors which would explain the empirical. A general discussion of flies and disease is given in Lindsay and Scudder (1956).

AN ALIEN PARADIGM

In general the gregarious behavior of populations of many kinds was qualified as contagious by Neyman (1939) and more extensively by Neyman and Scott (1959). Flies, human beings, and cosmic matter all exhibit “contagious distributions,” which commonly disregard linear factors of the substrate upon, or medium in which, they are distributed, and cannot therefore be sampled by conventional systems. Very commonly, such populations are counted in full, instead of counted by sampling some aliquot.

With respect to a plague research project, in which the investigator wished to make counts of the number of fleas on domestic rats, the difference in flea infestation levels between individual rats in the same colony was found to be most extreme, to the point of very major differences between counts on cohabiting animals. The investigator quite properly felt that derivation of an average number of fleas per rat was meaningless and could not be a usable parameter. A contagious distribution analysis on the fly model was offered, but turned down by statisticians.

Quite comparable to the flea/rat picture is one easily observed on individual cattle in a pasture. One animal may have dozens of horn flies while an adjacent animal may have them by the thousands. One may easily conclude that the hosts have comparatively little ecological equivalence, one to another, so that any sampling validity must be based upon factors more consistent with representative ecological values than the simple assumption that all animals are so equal that random sampling of them is valid.

Another project was the study of red spider mites on roses in a greenhouse, where the investigator wished to evaluate populations and control measures. The finding was also one of major host difference, like the rat or the cow just mentioned, since one plant might be very heavily infested, while an adjacent one might be nearly free of mites. I suggest that for contagious distributions, the preoccupation with linear equivalence of the substrate for the event or for the life form creates a parameter of little or no value in analysis. Consider a computation of the average number of persons per square mile, as a randomized nonvalue. For example, using California, how can one rationally contemplate a per square mile calculation which would combine 20,000 or more persons in a square mile of downtown Los Angeles with vast areas of zero in a California desert, yielding a very low and meaningless figure for the entire state?

Another window of understanding for this problem can be illustrated by the following: a measure of a contagious distribution is nothing new to hunter-gatherers and the hunted, whether man or other animals, whose survival has long been tied to searching carefully and successfully for the prime locations of food/prey/ enemies, and calculating the search/avoidance effort needed on the basis of a conservative comparison of the searched area to past experience. Is this not in each such case, a rough fuzzy set?

I offer fly grill data collection and interpretation as a very real candidate for inclusion in rough fuzzy logic sets, essentially the same as survival calculations. Variations in even the same perceived information means a variety of valid conclusions to different perceivers, interpretable in each instance by the experience/need of the hunter/hunted. The innate hunter-gatherer/hunted instincts have long been validated by successful survival levels because they are successful for species after species. Is any other basis of explanation needed as a workable theory of what actually operates?

Some years after the beginning of extensive fly grill sampling, those doing fly control studies at the Savannah Technical Development Laboratories of CDC were able to show that an experienced field team was capable of visually estimating fly population data without using the grill. They had learned their observational discipline so well that their field estimates could be verified by another team using the grill (Welch and Schoof 1953). This successful experiment in data gathering was conducted after the team had two years of experience in grill sampling. Certainly this experience supports the analogy of the “hunter-gatherer/hunted” concept, and would justify the rough fuzzy logic nature of the data.

In conclusion, this discussion proposes that the history and acceptance of the fly grill for determining fly population indices may be a valid interpretation of contagious distributions by their analyses as rough fuzzy logic sets. A corollary would be that classical
statistical methods are not applicable to such data. A prospect would seem to exist that many additional data complexes in natural history would be better analyzed by methods comparable to grill sampling, which deliberately samples the most populated points in a distribution.

The author would invite comment on this interpretation, inclusive of a wide range of questions and ideas. So far this may be the first basic explanation to appear in all these years of successful use of the grill method.

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Bacterial Abundance in Larval Habitats of *Aedes albopictus* (Diptera: Culicidae) in a Florida Cemetery

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ABSTRACT: Bacterial abundance in bronze and non-bronze containers was studied in relation to distribution of immature *Aedes albopictus* (Skuse) mosquitoes in a cemetery in northern Florida. In non-bronze containers, immature *Ae. albopictus* occurred at higher density and greater frequency than in bronze containers, yet bacteria were more abundant in water from bronze containers (range, 2.59 x 10⁶/ml to 2.25 x 10⁷/ml) than in water from non-bronze containers (range, 8.01 x 10⁵/ml to 5.57 x 10⁶/ml). Thus, the hypothesis that chemicals such as copper would leach from the bronze, kill bacteria, and thereby eliminate the larval food supply was not supported. Other factors affecting skewed distribution of larvae away from bronze containers need to be studied.

*Keyword Index:* *Aedes albopictus*, larvae, bacteria, container habitats

INTRODUCTION

Intra- and interspecific competition for food among mosquito larvae in container habitats is an important process affecting larval growth, developmental success, and population regulation (see, for example, relevant papers in Frank and Lounibos 1983, and Lounibos et al. 1985). Consequently, studies that incorporate analysis of the nature, distribution, and abundance of larval food can offer insights into the patterns of larval mosquito abundance observed in natural settings. For example, supplementation of larval habitats with plant detritus improved growth of *Aedes triseriatus* (Say) mosquitoes in container habitats in south Florida (Lounibos et al. 1993), and total mass of organic material in tree holes was correlated with total numbers of *Aedes sierrensis* Ludlow emerging from tree holes in northern California (Colwell et al. 1995).

The invasion and establishment of *Aedes albopictus* (Skuse) in Florida has attracted considerable attention because it apparently has displaced *Aedes aegypti* (L.) in certain landscapes with various types of artificial containers, particularly in northern Florida (O’Meara et al. 1995). The mechanisms by which *Ae. albopictus* invades and displaces *Ae. aegypti* are not clear, but they may involve superior competitive interactions with *Ae. aegypti* and *Ae. triseriatus* larvae in larval habitats (Livdahl and Willey 1991, Juliano 1994). However, it is not known how food resource abundance and distribution affects these interactions, and the resulting patterns of distribution of *Ae. albopictus*. Recently, O’Meara et al. (1992a, 1992b) observed that *Ae. albopictus* and *Ae. aegypti* distributions in water-filled containers in cemeteries in Florida were skewed away from bronze containers. These authors hypothesized that the copper in the bronze vases may be directly toxic to larvae, or to microorganisms that serve as larval food (Merritt et al. 1992). Therefore, in this study we investigated the abundance of bacteria, one component of larval food (Walker et al. 1988, Merritt et al. 1992), in relation to distribution and abundance of *Ae. albopictus* and other mosquitoes in bronze and other types of containers in a cemetery in Florida, to determine if bacterial densities were lower in the bronze containers.

MATERIALS AND METHODS

The study site was a cemetery in Jacksonville, Florida, that has been described elsewhere (O’Meara et al. 1992a). Many of the grave sites have water-holding containers including those made of plastic, stone, and
bronze. Thirty bronze containers and thirty containers of one of the other materials ('non-bronze') were sampled as follows, on August 5, 1992. A water sample was drawn with a sterile syringe and preserved as 4% formalin to a final volume of 5 ml. Then, the entire contents of the container were emptied with a siphon, the volume of water from each container measured, and Aedes mosquito larvae and pupae retained for counting. On four previous occasions in 1990 and in 1991, containers in this cemetery were sampled for immature Aedes. At those times, among the Aedes-positive containers, nearly all (209/211) had immature Ae. albopictus, while Ae. aegypti was found in less than 5% of the samples (6/211). In the current study, a smaller portion of the total collection (21 immatures from 6 bronze containers and 98 immatures from 11 non-bronze containers) were identified to species.

Bacteria in water samples were enumerated using a direct count method after staining of bacterial DNA with 4′,6-diamidino-2-phenylindole (DAPI) (Porter and Feig 1980, Walker et al. 1988). Subsamples of the original water sample were exposed to DAPI in solution (final concentration of 2-4 μg/ml) and held for 15-30 min. on ice in the dark. The sample was then transferred to the glass chimney (17 mm diam.) of a filtering apparatus, and the sample drawn by low vacuum pressure (< 30 cm Hg) onto a black, polycarbonate Nuclepore™ filter (0.22 μm pore size, 25 mm diam.), thus leaving particulate material of bacterial size and larger on the filter surface. The Nuclepore filter was backed with a 0.45 μm pore size, HA-type Millipore filter to provide even pressure to eliminate clamping of material. After filtration, Nuclepore filters were removed from the holder and placed on slides previously prepared with a thin film of type B Cargille immersion oil. A drop of oil was added to the top of the filter and a cover slip applied. Slides were stored in the dark at 4°C until examined.

Bacteria were enumerated by exciting the DAPI-bacterial DNA complex with 365 nm wavelength of ultraviolet light using a JenLab A/D fluorescence microscope in the epifluorescent mode. Individual bacteria appear bright blue against a black background in such preparations. Counts of bacteria were performed at 1,000 times magnification using an ocular grid, until a minimum of 200 bacteria were counted among at least 15 different randomly-chosen fields on the filter preparation, to satisfy statistical assumptions of estimation of bacterial numbers (Kirchman et al. 1982). Bacterial density (i.e., numbers of bacteria per ml of original water sample) were calculated from the counts using a standard formula that accounts for sample volume, dilution owing to formalin, number of fields examined, number of bacteria counted, filter area, and field area.

The number of Aedes larvae per container, number of larvae per ml of water in containers (i.e., larval density), container volume, and bacterial density (number of bacteria per ml) were compared among bronze and non-bronze containers using either the unpaired t-test or the nonparametric Mann-Whitney U-test. Bacterial density data were transformed with log_{10} prior to statistical analysis. The frequency of containers with or without larvae, between the two container types, was compared with a 2 x 2 contingency table for test of independence with the G statistic. Correlation of larval density and log_{10} (bacterial density) was examined with the correlation coefficient (Sokal and Rohlf 1969).

RESULTS

Nine of 30 (30%) of bronze containers held Aedes larvae, while 30 of 30 (100%) of non-bronze containers held Aedes larvae. All specimens identified to species were Ae. albopictus. There were significantly fewer bronze containers with larvae compared to non-bronze containers (G-test on 2 x 2 contingency table, G = 41.0, P < 0.001).

TABLE 1 shows summarized data by container type, of water volume, numbers of larvae recovered per container, larval density, bacterial density in containers, and the results of t-tests or Mann-Whitney U-tests. Bronze containers held more water on average than did non-bronze containers, but harbored fewer larvae per container and had a much lower larval density than did non-bronze containers. Bacterial density was significantly higher (t-test on log_{10} transformed data; see TABLE 1) in water of bronze containers (range, 2.59 x 10^4/ml to 2.25 x 10^5/ml) than in water of non-bronze containers (range, 8.01 x 10^2/ml to 5.57 x 10^3/ml). There was no correlation between larval density and log_{10} (bacterial density) for bronze (r = 0.08, P > 0.05) or non-bronze (r = 0.149, P > 0.05) containers.

DISCUSSION

In this study, Aedes larvae were more common in non-bronze containers. These observations confirm the earlier finding by O'Meara et al. (1992a, 1992b) that mosquito larvae were less common and less abundant in bronze vessels compared to non-bronze vessels. Although the reason for this difference is not known, one possibility (discussed by O'Meara et al. [1992a, 1992b]) is that copper ions from the bronze metal leach into the water and accumulate to a sufficient concentration that is toxic to mosquito larvae.
An alternative hypothesis is that copper ions are toxic to the microorganisms, such as bacteria, that form the larval food (Walker et al. 1988, Merritt et al. 1992, Sota and Kato 1994). Copper compounds, such as copper sulfate, are often used to control unwanted aquatic, vascular plants and algae in recreational lakes and other bodies of water (Ware 1989). However, the effect of copper ions on bacteria in containerized habitats of mosquito larvae is not known. Here, we found that bacteria were more abundant in bronze than in non-bronze containers, thus we must reject our hypothesis that the skewed distribution of Aedes larvae in bronze and non-bronze containers is mediated through a toxic effect of copper on the microorganisms forming the larval food. Although the ranges of bacterial densities in larval habitats of the two container types overlapped, yet the higher densities of bacteria observed in water of bronze containers could be related to the absence, or much lower densities, of mosquito larvae in those containers compared to non-bronze ones. Although there was no correlation between larval density and bacterial density in non-bronze containers here, yet we did not take into account variation in larval stage in the samples which could account for this result. In controlled studies, we have demonstrated that larval feeding by Ae. triseriatus reduces microbial densities in field and laboratory microcosms (Walker et al. 1991), whereas selective removal of Ae. triseriatus larvae from tree holes and tires is followed rapidly by increases in microbial densities (Morgan and Merritt 1992; R. Merritt, W. Morgan, and E. Walker, unpublished data). Copper may also be toxic to bacteria adhering to the inner surface of the bronze containers, or to larvae that ingest copper as they feed. Aedes larvae browse underwater surfaces as part of their normal feeding behavior (Khawale et al. 1988, Walker and Merritt 1991, Juliano and Remminger 1992).

O’Meara et al. (1992a) noted that organic material, such as leaves and flower parts, accumulate in containers in cemeteries, particularly after holidays, and that such material may form a food supply for larval growth (cf. Barrera-Rodriguez et al. 1979, Lounibos et al. 1993). Such detritus aggregates cannot, in general, be used directly by mosquito larvae, but must be mineralized and transformed through decomposition processes by microorganisms (Cummins and Klug 1979, Bengtsson 1992) which then become food items directly (Walker et al. 1988). Whether detritus decomposition is inhibited

### TABLE 1. Water volume, number of Aedes larvae, larval density per ml, and bacterial density per ml in bronze and non-bronze containers at the Evergreen Cemetery, Jacksonville, Florida, August 5, 1992. N = 30 containers for each group. ***, P < 0.01, ***, P < 0.001.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Container Type</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Bronze</td>
<td>Non-bronze</td>
<td>Statistical test</td>
<td></td>
</tr>
<tr>
<td>Water volume (ml)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
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<td>382.8</td>
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<td>$t = 4.77^{***}$</td>
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<tr>
<td>Range</td>
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<tr>
<td>SEM</td>
<td>5.9</td>
<td>7.6</td>
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<td></td>
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<tr>
<td>No. of larvae</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Mean</td>
<td>8.8</td>
<td>95.5</td>
<td></td>
<td>$U = 57.5^{***}$</td>
</tr>
<tr>
<td>Range</td>
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<td>5 - 462</td>
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</tr>
<tr>
<td>SEM</td>
<td>3.8</td>
<td>17.7</td>
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<tr>
<td>Larval density (per ml)</td>
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<td></td>
</tr>
<tr>
<td>Mean</td>
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<td>0.35</td>
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<td>$U = 33.5^{***}$</td>
</tr>
<tr>
<td>Range</td>
<td>0.00 - 0.18</td>
<td>0.02 - 1.55</td>
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</tr>
<tr>
<td>SEM</td>
<td>0.007</td>
<td>0.070</td>
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<tr>
<td>Bacterial density (per ml)</td>
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<td></td>
</tr>
<tr>
<td>Mean</td>
<td>$2.66 \times 10^7$</td>
<td>$1.13 \times 10^7$</td>
<td></td>
<td>$t = 2.87^{**}$</td>
</tr>
<tr>
<td>Range</td>
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<td>$8.01 \times 10^6 - 5.57 \times 10^7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>$7.70 \times 10^6$</td>
<td>$2.51 \times 10^6$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
or altered in bronze containers compared to other ones is not known, however, larval mosquito growth was inhibited in bronze containers even when they were supplemented with a lab chow (O’Meara et al. 1992b).

Our study provides information on the variation in abundance of larval mosquito food in the setting where displacement of *Ae. aegypti* by *Ae. albopictus* has apparently occurred. One hypothesis regarding this species displacement phenomenon would suggest that *Ae. albopictus* larvae out compete *Ae. aegypti* larvae for food resources, or are more efficient in utilizing available food resources (Juliano 1994). Investigations of larval food of *Ae. aegypti* and *Ae. albopictus* are scant. We note that most of the published experimental studies (e.g., Black et al. 1989, Chan et al. 1971, Ho et al. 1989) that have examined competitive outcomes have utilized laboratory mosquito chow as food, despite recent evidence that *Ae. albopictus* and *Ae. aegypti* larvae grow when provided cultures of bacteria (Sota and Kato 1994). Thus, further studies on the nature of larval mosquito food are needed to clarify the role of larval nutrition and efficiency of utilization of food by larvae of different species in these competitive interactions.

**Acknowledgments**

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Interrelationships between Different *Borrelia* Genospecies and Their Principal Vectors

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ABSTRACT: Under conditions of similar prevalence of *Borrelia* infection in tick populations, a generalized infection with the presence of spirochetes in the salivary glands develops much more frequently in the unfed adult *Ixodes persulcatus* ticks, infected by *Borrelia afzelii* and *Borrelia garinii* in Russian natural foci, than in *I. scapularis* ticks from the northeastern United States, infected with *Borrelia burgdorferi* sensu stricto. During the first two to three days after infected *I. persulcatus* begin to engorge, the proportion of ticks with spirochetes in the salivary glands did not increase. In *I. persulcatus*, therefore, *Borrelia* migration from the gut into the salivary glands at the beginning of the blood-sucking phase is not a necessary or even important condition for *Borrelia* transmission with saliva. These data provide evidence for different interrelationships between particular *Borrelia* genospecies and their principal tick vectors.

**Keyword Index:** *Borrelia burgdorferi* sensu lato, vectors, ticks, generalized infection.

INTRODUCTION

*Borrelia* causing Lyme borreliosis are transmitted to vertebrates, including humans, in the saliva of tick vectors (Ribet et al. 1987, Zang et al. 1989). However, the salivary glands of unfed adult *Ixodes scapularis* ticks, the main vectors of *Borrelia burgdorferi* sensu stricto, rarely contain spirochetes; the latter are usually found in the gut of infected ticks (Burgdorfer et al. 1984, 1989a, 1992; Burgdorfer et al. 1988). Hence, some researchers questioned the very possibility of inoculation of the Lyme borreliosis pathogen with saliva of Ixodid ticks (Benach et al. 1987). Although this mode of *Borrelia* transmission is now beyond question, experiments have demonstrated that the tick must be attached to the host for at least 48 h for successful inoculation to occur. During this period, spirochetes migrate from the gut into the salivary glands where they accumulate (Piesman et al. 1987, 1991; Ribeiro et al. 1987; Burgdorfer 1989b; Piesman 1993, 1994). Therefore, the transmission of *B. burgdorferi* sensu stricto during the first two days after the *I. scapularis* tick attachment to the host body is unlikely, and the prompt removal of attached ticks reduces risk of Lyme borreliosis (Piesman et al. 1987, 1991).

On the other hand, in Russia, where *Borrelia garinii* and *Borrelia afzelii* spirochetes are transmitted by adult *Ixodes persulcatus* ticks, the disease is clinically manifested in almost 90% of cases even though the period of tick attachment to the patient's body is usually less than one day (Korenberg et al. 1994). The facts described above allowed us to suggest that interrelationships between particular *Borrelia* genospecies and their main vectors are characterized by a certain individuality. The latter may account for differences in the frequency of generalized infection (accompanied by the presence of *Borrelia* in the salivary glands of unfed ticks), in the fate of *Borrelia* in the tick gut after the initiation of blood-feeding, and in the probability of their penetration into the salivary glands during this period (Korenberg 1994). Consequently, different pathogens of Ixodid tick-borne borrelioses (ITB Bs), which we regard as a group of etiologically individual infectious diseases (Korenberg 1993a,b, 1994), should have certain peculiarities of their horizontal and vertical transmission. In this context, as noted by Gern (1994), it is important to demonstrate whether the development of different *Borrelia* genospecies in *I. persulcatus* and *I. ricinus* ticks is similar to that of *B. burgdorferi* sensu stricto in *I. scapularis* ticks.

In this paper, we describe the results of our studies.
aimed at revealing generalized infection in unfed adult *I. persulcatus* ticks from Russian natural foci. These results are compared with corresponding data on *I. scapularis* from the USA. In addition, we analyzed parameters of infection in *I. persulcatus* ticks during the first days after the initiation of blood-feeding.

**MATERIALS AND METHODS**

Unfed adult ticks were collected from vegetation by flagging in two ITBB foci located in the European part of Russia and in one focus located in the northeastern United States.

In May and June of 1992, *I. persulcatus* ticks were collected in the natural focus located in the Kirovskii district (Leningrad region of Russia). In this focus, described in detail previously (Korenberg et al. 1991a,b), the average prevalence of infection in *I. persulcatus* is about 34% (Kovalevskii et al. 1993). Spirochetes isolated from ticks were identified as *B. afzelii* and *B. garinii* (Baranton et al. 1992, Canica et al. 1993).

Ticks of the same species were also collected in May and June of 1992-1993 in the suburbs of Perm’ (near the Ural Mountains), where *B. garinii* and *B. afzelii* circulate endzootically (Korenberg et al. 1994), and the prevalence of infection in ticks is about 38%.

*Ixodes scapularis* ticks were collected in October of 1993, in the Lyme borreliosis focus located near Ipswich (eastern Massachusetts), which has been documented in previous publications (Spelman et al. 1985, Lastavica et al. 1989). *Borrelia burgdorferi sensu stricto* circulates there, as it does throughout the northeastern states (Baranton et al. 1992), and the prevalence of infection in adult ticks reaches 38% (Piesman et al. 1986).

Some *I. persulcatus* ticks from the Leningrad region were pooled into groups with approximately equal proportions of males and females (to stimulate blood-feeding) and fed on white mice placed under individual "hoods," according to the approved procedure. Partially fed ticks were removed one to three days after they had attached to mice, and subsequent analysis was performed using only females.

In 1992-1994, we collected *I. persulcatus* ticks removed from patients at Perm’ medical institutions. As these patients entered into contact with ticks largely on weekends while working on their garden plots or visiting suburban forests, the time of tick attachment and removal was easy to determine. In each case, the period of tick attachment was documented.

Live unfed ticks and some partially fed ticks were individually analyzed for the presence of *Borrelia* in internal organs using the technique of Sidorov et al. (1967). Briefly, ticks were half-embedded into a paraffin wax-rosin mixture, washed with ethanol and saline, and dissected under a binocular microscope by cutting the cuticle at the sides of the body. Isolated gut, salivary glands, ganglia, and gonads were used for preparing smears on glass slides. We made two preparations of each organ and used the second (reserve) preparation for better resolving any ambiguous cases. Preparations were air-dried and fixed in flame, stained with the Romanovsky-Giemsa mixture and crystal violet (Kovalevskii et al. 1988), and analyzed under a light microscope with oil immersion at magnification of 1125x. After detecting spirochetes in the salivary glands, we always analyzed preparations of ganglia and reproductive organs of the same ticks to confirm generalized infection.

In another group of partially fed ticks, screening for *Borrelia* was performed using only the gut contents. Standard live preparations were made as described by Kovalevskii et al. (1991) and analyzed under a dark-field microscope with water immersion at a magnification of 600x (40x1.5x10). Both methods for revealing *Borrelia* produce similar results (Kovalevskii et al. 1988).

In smears and live preparations, all *Borrelia* in 250 microscopic fields were counted. The concentration of spirochetes was expressed as counts per 100 microscopic fields. These values were recalculated with respect to differences in magnification and assessed using the following grades of *Borrelia* abundance (concentration in a particular organ), which were proposed previously for fixed preparations analyzed at a magnification of 700x: low, 0.1-5.0; medium, 5.1-25.0; high, 25.1-125.0; and very high, more than 125 spirochetes per 100 microscopic fields (Levin et al., 1993). In live preparations, corresponding values were 0.1-10, 10.1-50.0, 50.1-250.0, and more than 250 spirochetes per 100 microscopic fields, respectively (Kovalevskii et al. 1991).

Calculated mean values and percentages were analyzed statistically using a confidence interval based on a double error of mean or sampling error. Significance of differences between mean values (at p<0.05) was determined using Student’s t-test. Coefficient of correlation, designated r, was calculated by the usual formula.

A total of 1962 *I. persulcatus* and 156 *I. scapularis* ticks was analyzed individually using some of the methods described above.

**RESULTS**

According to the preliminary data (see above),
zones of the Leningrad and Perm' regions of Russia where ticks were sampled are characterized by similar prevalence of infection in *I. persulcatus* ticks. This allowed us to pool data on unfed ticks of this species and analyze them as a single, more representative sample. Thus, 740 *I. persulcatus* were analyzed, and *Borrelia* was detected in 194 of them. In 169 ticks, spirochetes were detected in the gut only, whereas the remaining 25 ticks also carried them in the salivary glands and other organs. In the case of *I. scapularis*, 41 out of 156 ticks were infected, but only one tick contained spirochetes both in the gut and salivary glands. These results were used for calculating indices of *Borrelia* infection in ticks (TABLE 1). On the whole, the proportions of infected ticks and of ticks containing *Borrelia* only in the gut were similar in both species. However, the proportion of ticks with generalized infection among all infected ticks in *I. persulcatus* greatly exceeded that in *I. scapularis*, and this difference was highly significant (*t* = 3.1).

Ticks with low and medium *Borrelia* populations in their guts prevailed among infected ticks of both species: 81% in *I. persulcatus* and 83% in *I. scapularis*. In *I. persulcatus*, we recorded a similar proportion (86%) of ticks with low and medium concentration of spirochetes in the salivary glands. A single *Borrelia*-positive preparation of *I. scapularis* salivary glands contained spirochetes at low concentration of 0.4 per 100 microscopic fields.

In *I. persulcatus*, the presence of higher *Borrelia* populations in the gut did not correlate with an increased percentage of ticks with spirochetes in their salivary glands. The difference between the minimum and maximum percentages registered in this study was not statistically significant (*t* = 1.3). We also compared the series of *Borrelia* counts (per 100 microscopic fields) in the gut and salivary glands of 251 *I. persulcatus* ticks with generalized infection and found no correlation between them (*r* = -0.23).

We used data on unfed ticks from the Perm' region only (i.e., from the same region where partially engorged ticks were collected) to compare the parameters of infection in unfed and partially engorged *I. persulcatus* more accurately. Out of 359 unfed ticks collected from the vegetation 110 were infected; 92 of them with *Borrelia* in the gut and 18 with *Borrelia* both in the gut and salivary glands. A total of 1,481 ticks was removed from human bodies after different periods of attachment. *Borrelia* was found in 364 of these ticks (infection prevalence 24.6%), but individual organs were analyzed in only 301 of them. In the latter sample, 62 ticks (20.6%) were infected: 39 with spirochetes in the gut only and 23 with spirochetes both in the gut and salivary glands. Parameters of tick infection calculated from these data are shown in TABLE 2. General indices of infection and proportions of ticks with spirochetes in the salivary glands among unfed and partially engorged ticks removed from people were similar. The prevalence of infection in unfed ticks was slightly higher than in partially engorged specimens (*t* = 2.2), and the difference in the proportion of ticks with spirochetes in the salivary glands was not statistically significant (*t* = 1.4). However, incidence of *Borrelia* in salivary gland preparations of partially engorged infected ticks was significantly higher than in preparations of unfed infected ticks (*t* = 3.3).

TABLE 3 shows the results of screening for *Borrelia* in the gut and salivary glands of all *I. persulcatus* ticks, both fed on mice and removed from humans, after tick organs were analyzed at different periods of blood-feeding. A total of 423 such ticks were studied; 76 of them were infected and 27 had *Borrelia* both in the gut and salivary glands.

It appeared that 24, 48, 72, and more hours after the initiation of blood-feeding, the proportion of *I. persulcatus* infected with *Borrelia* in the salivary glands remained virtually unchanged. Although this should be verified by other methods of spirochete detection, our data indicate that *Borrelia* concentration in the salivary glands of unfed and partially engorged *I. persulcatus* remained at the same level (10-14 spirochetes per 100 microscopic fields). On the other hand, the number of *Borrelia* in preparations from the gut of ticks fed for 24

---

**TABLE 1. Indices of *Borrelia* infection in unfed tick vectors.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Proportion of ticks with <em>Borrelia</em>, %</th>
<th>Prevalence of generalized infection in infected ticks, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
<td>in the gut only</td>
</tr>
<tr>
<td><em>I. persulcatus</em></td>
<td>26.2±3.2</td>
<td>22.8±3.1</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>26.3±7.0</td>
<td>25.6±7.0</td>
</tr>
</tbody>
</table>
hours was approximately the same as in preparations of unfed ticks (on average, 22-25 per 100 microscopic fields). After 25-48 hours of blood-feeding, this number decreased to 9-16; and after 49-72 hours, to 2-9 spirochetes per 100 microscopic fields.

TABLE 4 shows more detailed data on the proportions of ticks with different concentrations of *Borrelia* in the gut and salivary glands among unfed and partially engorged *I. persulcatus*. These proportions, as well as the average number of *Borrelia* in the gut, remained virtually unchanged during the first days after the beginning of blood-feeding. It is particularly noteworthy that we did not find any tendency toward an increase in the incidence and concentration of *Borrelia* in the salivary glands of ticks during the first days of blood-feeding.

**DISCUSSION**

The results of this study show that, at a similar prevalence of *B. garinii* or *B. afzelii* infection in *I. persulcatus* and *B. burgdorferi* sensu stricto infection in *I. scapularis* and even at similar proportions of ticks with different concentrations of spirochetes in the gut in corresponding natural foci, generalized infection in *I. persulcatus* ticks develops much more frequently (TABLE 1). Our results with *I. scapularis* agree with data obtained previously. Thus, 77 infected adult ticks of this species, collected in New York state, contained spirochetes only in the gut (Burgdorfer et al., 1982). According to our estimates, the proportion of ticks with generalized infection among 179 infected *I. scapularis* analyzed in subsequent studies (Burgdorfer et al. 1988,

**TABLE 2.** Indices of *Borrelia* infection in unfed and partially engorged *Ixodes persulcatus* ticks from the Perm' region of Russia.

<table>
<thead>
<tr>
<th>Analyzed Material</th>
<th>Proportion of ticks with <em>Borrelia</em>, %</th>
<th>Prevalence of generalized infection in infected ticks, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>in the gut only</td>
</tr>
<tr>
<td>Unfed ticks collected from vegetation</td>
<td>30.6±4.9</td>
<td>25.6±4.6</td>
</tr>
<tr>
<td>Partially engorged ticks removed from people: total</td>
<td>24.6±2.2</td>
<td>no data available</td>
</tr>
<tr>
<td>analyzed for <em>Borrelia</em> in individual organs</td>
<td>20.6±5.2</td>
<td>13.0±3.9</td>
</tr>
</tbody>
</table>

**TABLE 3.** Proportions of *Ixodes persulcatus* ticks with *Borrelia* in the salivary glands at different time after initiation of blood-feeding.

<table>
<thead>
<tr>
<th>Period of blood-feeding</th>
<th>Number of ticks studied</th>
<th>Proportion of infected ticks, %</th>
<th>Prevalence of generalized infection in infected ticks, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>up to 24 hours</td>
<td>245</td>
<td>24.5±5.5</td>
<td>35.0±12.4</td>
</tr>
<tr>
<td>25-48 hours</td>
<td>80</td>
<td>8.7±6.3</td>
<td>42.9±40.1</td>
</tr>
<tr>
<td>49-72 hours and more</td>
<td>98</td>
<td>9.2±5.9</td>
<td>33.3±33.3</td>
</tr>
</tbody>
</table>
Burgdorfer 1989a) was only 2.2±2.2%.

This figure contrasts sharply with the data on *I. pacificus* ticks from California, which can transmit not only *B. burgdorferi* sensu stricto but also *Borrelia* of the original DN 127 group (Postic and Baranton 1994). Only 25 of 1687 adult *I. pacificus* were infected, but a generalized infection was recorded in eight of them (32±19%) (Burgdorfer et al. 1985).

In Central Europe, the proportion of *I. ricinus* ticks with systemic infection can be estimated at 5.5±5.4% (see: Burgdorfer et al. 1989) or at 5.3±4.2% (see: Burgdorfer et al. 1983). Gern (1994) suggested that systemic infection (including the presence of spirochetes in the salivary glands) in *I. ricinus* is actually more frequent than indicated by the previous studies. In any case, the values given above exceed those determined for *I. scapularis* but are far lower than those for *I. persulcatus*. If confirmed in subsequent studies, this fact, in combination with other factors, would explain the lesser vector efficiency of *I. ricinus*, compared with *I. persulcatus*, in ITBB foci (Korenberg et al. 1991a,b, Korenberg 1993b).

The results described here suggest that direct microscopic analysis of fixed and live preparations is a reliable method for detecting *Borrelia* in the internal organs of partially engorged ticks as well as of unfed ticks. However, the *Borrelia* concentration in the tick gut decreased with an increase in the amount of fluid consumed during blood-feeding. Consequently, the number of spirochetes in preparations of this material also decreased, which reduced the probability of identifying infected ticks. We believe that these "technical" inconsistencies account for lower indices of infection in partially engorged *I. persulcatus* as compared with unfed ticks from the same focus. In blood-feeding ticks of this species, *Borrelia* is found in the salivary glands with the same frequency, and in the gut, with a lesser frequency than in unfed ticks; consequently, the proportion of ticks with spirochetes in the salivary glands among all infected ticks is also greater. This is a purely "arithmetic" effect, and a more detailed analysis (see TABLES 3 and 4) shows that it should not be regarded as evidence for an actual increase in the number of ticks containing spirochetes in the salivary glands or for higher concentrations of spirochetes in them. A similar situation was described

<table>
<thead>
<tr>
<th>TABLE 4. Proportions of ticks with different concentrations of <em>Borrelia</em> in the gut and salivary glands among infected <em>Ixodes persulcatus</em>.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyzed Material</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Unfed ticks collected from vegetation:</td>
</tr>
<tr>
<td>gut</td>
</tr>
<tr>
<td>salivary glands</td>
</tr>
<tr>
<td>Partially engorged ticks removed from people:</td>
</tr>
<tr>
<td>gut</td>
</tr>
<tr>
<td>salivary glands</td>
</tr>
<tr>
<td>Partially engorged ticks fed on laboratory animals:</td>
</tr>
<tr>
<td>gut</td>
</tr>
<tr>
<td>salivary glands</td>
</tr>
</tbody>
</table>
for adult I. ricinus collected in a natural focus: the prevalence of infection in partially engorged females decreased from 36.3±5.6% (characteristic of unfed ticks) to 21.6±6.1%, whereas the prevalence of generalized infection in infected ticks remained unchanged (Burgdorfer et al., 1983).

We regard the peculiarities of the relationship of B. garinii and B. afzelii spirochetes with I. persulcatus ticks to be important. These spirochetes are frequently detected in the salivary glands as well as in the gut of unfed ticks. As infected ticks engorge, the proportion of ticks with spirochetes in the salivary glands remains virtually unchanged for at least two to three days (TABLES 2 and 3), as concentration of spirochetes in this organ does (TABLE 4). In other words, Borrelia migration from the gut into the salivary glands in I. persulcatus ticks during blood-feeding is not a necessary or even important condition for the transmission of ITBB pathogens with their saliva. The frequency of this transmission, which occurs within the first 24 hours after the tick attachment to the human body (Korenberg et al. 1994), depends largely on the initial proportion of unfed ticks containing Borrelia in their salivary glands.

Based on our data, we cannot exclude the possibility of Borrelia migration from the gut into the salivary glands in adult I. persulcatus on the second or third day of blood-feeding. The role of such a phenomenon (if it exists) in the epizootic process remains unknown. However, it is apparently insignificant from an epidemiological standpoint, because adult ticks are usually physically removed at the onset of blood-feeding, and it is mainly adult I. persulcatus ticks that attack humans.

Data obtained in the laboratory and in the field showed that I. scapularis nymphs and adult ticks usually transmit B. burgdorferi sensu stricto only after the latter appear in the salivary glands into which they migrate from the gut during blood-feeding (Piesman et al. 1987, 1991, Burgdorfer et al. 1988). This limitation in the horizontal transmission of the pathogen, associated with peculiarities of its relationships with the vector, is responsible for certain specific features of Lyme borreliosis epizootiology and epidemiology in the northeastern and midwestern USA. Our data show, however, that this limitation is not necessarily essential for all ITBB foci. Certain practically important criteria pertaining to the American foci cannot be extrapolated to the foci located in other regions with different species of tick vectors. In particular, a strategy in prevention of ITBBs transmitted by I. persulcatus should not be based on the assumption that risk of human infection during the first two days after tick attachment is insignificant owing to low probability of pathogen transmission at the beginning of blood-feeding, as in the case of I. scapularis (Piesman et al. 1987; Ribeiro et al. 1987, Burgdorfer 1998b). In I. persulcatus, Borrelia are often found in salivary glands of the unfed ticks, i.e., the essential condition for pathogen transmission during the first hours of blood-feeding is satisfied. Thus, we took into account indices of spontaneous infection and the frequency of Borrelia presence in the salivary glands of I. persulcatus prior to blood-feeding and, on this basis, estimated that B. garinii and B. afzelii transmission by recently attached ticks occurs in four to five cases per 100 tick bites (data from the Perm' and Leningrad regions of Russia). Our experience shows that people do become infected in such a situation, although the duration of tick attachment is usually less than one day (Korenberg et al. 1994).

The results of our studies confirm that each of the ITBBs is characterized by a certain frequency of generalized infection in unfed ticks, its principal vectors. This phenomenon is based on the obscure specificity of relationships established between each of Borrelia species and its vectors. It agrees well with a more general principle formulated by Balashov (1995): the extent of generalization of infection is apparently a species-specific feature of the microorganism and its vector.

Acknowledgments

We are grateful to Professor Andrew Spielman of the Harvard School of Public Health (Boston, MA) and personnel of his laboratory for their help in collecting I. scapularis ticks. This comparative study would be impossible without their kind cooperation.

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Identification of Larval Instars of *Aedes albopictus* (Skuse) and *Aedes triseriatus* (Say) (Diptera: Culicidae) Based on Head Capsule Size

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ABSTRACT: Effects of constant temperature (15, 23, 31°C) on the head capsule widths of the larval instars of *Aedes albopictus* (Skuse) and *Aedes triseriatus* (Say) were determined in the laboratory. For both species, head capsule widths were significantly different between instars (*P*<0.001). Temperature significantly affected the size of head capsules of larvae of both species. For *Ae. albopictus*, except for the 1st instar, mean head capsule widths of 2nd-4th instars tended to be larger at 23°C than at 15 or 31°C; while for *Ae. triseriatus*, mean head capsule size was largest only at 23°C for 3rd and 4th instars. However, for both species, mean head capsules widths were significantly (*P*<0.05) larger at 23°C than at the other two temperatures only for 4th instars. The head capsule widths of all instars of *Ae. triseriatus* were larger than for the corresponding instars of *Ae. albopictus*. Using classification criteria developed through discriminant analysis, >98% of larvae of both species were identified to instar regardless of the temperature in which the larvae were reared.

**Keyword Index:** *Aedes albopictus*, *Aedes triseriatus*, head capsule width, discriminant analysis.

INTRODUCTION

Investigations of the population dynamics of mosquitoes often require estimates of age-specific mortality in larval populations (Hawley 1985). For mosquito larvae, instar provides an estimate of the relative age of mosquitoes. Head capsule width and body length have both been used to differentiate the instars of mosquitoes, such as *Culex quinquefasciatus* Say, *Aedes aegypti* (L.) (Rueda et al. 1990), and *Aedes vigilax* (Skuse) (Shinkarenko et al. 1986). Shinkarenko et al. (1986) found that head capsule width was a more reliable measure of instar than was body length for *Ae. vigilax*. Rueda et al. (1990) and Shinkarenko et al. (1986) reported temperature significantly affected the head capsule width of mosquito instars. Thus, cohort structure could potentially be altered seasonally or geographically as a result of the temperature of breeding sites.

Accordingly, we determined effects of temperature on head capsule width of *Aedes albopictus* (Skuse) and *Aedes triseriatus* (Say) in the laboratory, and used discriminant analysis to develop classification criteria for identification of the instars of both species.

MATERIALS AND METHODS

Mosquito Strains and Rearing Conditions

A colony of *Ae. albopictus* (ALBO-ROC) was established from eggs that were obtained from the late Dr. George Craig (Department of Biological Sciences, Notre Dame University, Notre Dame, IN). This mosquito strain was originally collected in Rockingham County, NC in June 1987. Eggs of a Walton strain of *Ae. triseriatus* (TRIS-WAL) were also obtained from Dr. Craig. This colony has been maintained in his laboratory for an unspecified number of years.

Mosquito colonies were maintained in an insectary
at 28°C, 85% RH, and a 16:8 h light:dark cycle. Females were fed citrated pig blood (Benzon and Apperson 1987) and provided with a 10% sucrose solution. Occasionally, mosquitoes were allowed to feed on the forearm of a human. Eggs were collected on white filter paper placed inside a black cup that was partially filled with water. Eggs were stored between moistened paper towels in plastic bags in an incubator at 21°C and a 16:8 h light:dark cycle until needed for experiments.

Size of Instars

To obtain first instars, eggs of both species (ALBOROC and TRIS-WAL) were separately immersed in a hatching medium of 0.1% (wt:vol) Bacto Nutrient Broth (Difco Laboratories, Detroit, MI) in distilled water (Novak and Shroyer 1978). After six hours, first instars were placed individually in shell glass vials (1.5 cm dia. x 6 cm ht.) and 2 ml of a 0.1% (wt/vol) food suspension in distilled water was added per vial. The food consisted of a 1:1:1 mixture (v:v:v) of brewer’s yeast, lactalbumin, and ground Purina rabbit chow (Chambers 1985). Groups of vials were placed in each of three reach-in environmental chambers (maintained at 15, 23, and 31°C) in the S. E. Plant Environmental Laboratory at N.C. State University. Larvae were observed daily at 10x to determine if they had molted. Groups of 15 to 40 larvae for each instar at each temperature were preserved in 70% ethanol after larval head capsules had darkened. When head capsule measurements were taken, larvae were placed on a glass microscope slide, and excess moisture was removed with a paper towel. Care was taken to assure that each larva was positioned such that its head capsule was perpendicular to the plane of view. The head capsule of each larva was measured at the widest point across the developing compound eyes at 30-60x using a calibrated ocular micrometer contained in a Wild M5 microscope.

Statistical Analyses

Effects of instar and temperature on head capsule widths for each species were analyzed by two-way analysis of variance (ANOVA) (Neter et al. 1985), using a general linear model procedure (SAS 1985). To determine if differences in head capsule size for each instar were statistically significant between temperatures ($P\leq0.05$), probability of difference values were calculated in least significant difference tests for least square mean (LSM) head capsule widths (SAS 1985) under the hypothesis $H_0: \text{LSM}_i=\text{LSM}_j$. Discriminant analysis was used to estimate probabilities of correct classification and to set up a classification criterion (Johnson and Wichern 1988, SAS 1985) to identify larvae of each species to instar.

RESULTS

Head Capsule Widths

Frequency distributions of head capsule widths for larval instars of each species were constructed (Fig. 1). Generally, head capsule sizes for each instar of each species were discretely distributed. However, there was some overlap in the size of head capsules for 3rd and 4th instars of Ae. albopictus, and the 1st and 2nd, and 2nd and 3rd instars of Ae. triseriatus (Fig. 1).

Head capsule width varied significantly ($P<0.005$) between instars and temperatures (TABLE 1) for each species. A significant temperature*instar interaction was found for both species (TABLE 1). Probability of difference values calculated (SAS 1985) for least square means of each instar within each temperature revealed that only 4th instars reared at 23°C had significantly larger head capsule sizes (TABLE 2). The

<table>
<thead>
<tr>
<th>Table 1</th>
<th>ANOVA of effects of temperature and instar on head capsule widths of Ae. albopictus and Ae. triseriatus larvae.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sources</td>
<td>Ae. albopictus</td>
</tr>
<tr>
<td></td>
<td>DF</td>
</tr>
<tr>
<td>Temperature</td>
<td>2</td>
</tr>
<tr>
<td>Instar</td>
<td>3</td>
</tr>
<tr>
<td>Temperature*Instar</td>
<td>6</td>
</tr>
<tr>
<td>Error</td>
<td>408</td>
</tr>
</tbody>
</table>

*Significant at $P=0.05$; **Significant at $P=0.0005$; ***Significant at $P=0.0001$.

$^a$Temperature was not replicated at the chamber level.
Figure 1. Head capsule widths (mm) of *Aedes albopictus* and *Ae. triseriatus* larvae that were reared at three different temperatures.

Head capsule widths of all instars of *Ae. triseriatus* were larger than for the corresponding instars of *Ae. albopictus*; however, differences between species for larval head capsule sizes could not be analysed statistically because effects of temperature on instars were not determined concurrently for both species. Mean head capsule widths ranged between 0.271-0.934 mm for 1st-4th instars of *Ae. albopictus*, and between 0.342-1.046 mm for corresponding instars of *Ae. triseriatus*. Coefficient of variations (CV's) ± SEcv (± standard errors of the CV) of 1st instars were larger than for other instars, indicating that variations in
### Table 2. Head capsule width of larvae of *Aedes albopictus* and *Aedes triseriatus* reared at three different temperatures.

<table>
<thead>
<tr>
<th>Temp. (° C)</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{x}$</td>
<td>CV</td>
<td>SE$_{CV}$</td>
<td>n</td>
</tr>
<tr>
<td>15</td>
<td>0.27a</td>
<td>12.50</td>
<td>1.40</td>
<td>40</td>
</tr>
<tr>
<td>23</td>
<td>0.27a</td>
<td>11.98</td>
<td>1.34</td>
<td>40</td>
</tr>
<tr>
<td>31</td>
<td>0.27a</td>
<td>11.07</td>
<td>1.24</td>
<td>40</td>
</tr>
</tbody>
</table>

**Aedes albopictus**

**Aedes triseriatus**

- Standard error of the coefficient of variation (CV): SE$_{CV}$ = CV/$\sqrt{2n}$.
- Head capsule widths were calculated as least square means (LSM), and tested for significant difference by LSD under the hypothesis $H_0$: LSM$_{(j)}$ = LSM$_{(i)}$. Within an instar, mean head capsule widths followed by the same letter are not significantly different ($P>0.05$).
Identification of Instars

In the ANOVA, type III SS for the main effect of temperature were relatively small compared to instar width for each temperature (Fig. 1), indicating that instar size in head capsule size were not strongly affected by temperature. Consequently, discriminant analyses were not conducted for each species, without regard to temp-

head capsule widths for this larval growth stage were significantly larger than for other instars. The trend in head capsule size variations generally, were not larger than for other instars, indicating that variation of head capsule size within an instar did not vary significantly between instars.

TABLE 3. Estimated probabilities of classification from discriminant analysis of head capsule widths for instars of *Aedes albopictus* and *Aedes triseriatus*.

<table>
<thead>
<tr>
<th>Actual Instar</th>
<th>Number Examined</th>
<th>Predicted Instar</th>
<th>Number Examined</th>
<th>Predicted Instar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>I</td>
<td>120</td>
<td>120 (100%)</td>
<td></td>
<td>120 (100%)</td>
</tr>
<tr>
<td>II</td>
<td>97</td>
<td>1 (10%)</td>
<td>96 (96%)</td>
<td>91 (91%)</td>
</tr>
<tr>
<td>III</td>
<td>100</td>
<td>100 (100%)</td>
<td></td>
<td>90 (90%)</td>
</tr>
<tr>
<td>IV</td>
<td>103</td>
<td>1 (0.97%)</td>
<td>102 (99.03%)</td>
<td>70 (100%)</td>
</tr>
</tbody>
</table>

*The probability of correct identification (expressed as a percentage) for each instar.
eration, to estimate the probabilities of correct classification of larvae for each instar. For *Ae. albopictus*, no misclassification would occur for the 1st instar and 3rd instar (TABLE 3). However, for the 2nd or the 4th instar, one out of 100 larvae might be misclassified. For *Ae. triseriatus*, there was a 1 in 100 chance that a 2nd, 3rd, or 4th instar might be misclassified.

**DISCUSSION**

As in our investigation, Shinkarenko et al. (1986) and Rueda et al. (1990) found that temperature significantly affected the head capsule width of instars of some *Aedes* and *Culex* mosquitoes. In contrast to our results, Rueda et al. (1990) found that head capsules for *Cx. quinquefasciatus* and *Ae. aegypti* were wider for all instars at 15°C relative to 25 and 30°C.

Our results for *Ae. albopictus* and *Ae. triseriatus* indicate that head capsule width is a relatively fixed morphometric character regardless of the temperature at which larvae are reared. A high level of precision in instar identification was achieved despite the significant effects of temperature on head capsule widths of both species. Furthermore, our results indicate that the classification criterion developed can be used to age grade field-collected larvae of *Ae. albopictus* and *Ae. triseriatus*. In their study of *Ae. vigilax*, Shinkarenko et al. (1986) found that head capsule width was a more reliable indicator of instar than was body length. Using discriminant analysis, Shinkarenko et al. (1986) correctly identified >99% of larvae to instar based on head capsule width despite the effects of temperature and salinity on head capsule size. However, it should be noted that other environmental factors such as larval density and food ration also affect larval growth rates (Hard et al. 1989, Moore and Fisher 1969). Therefore, under field conditions, the size of larvae could potentially be affected by the interactions of a number of environmental factors.

Larval instar is a suitable measure of relative age, and an appropriate parameter to include in life table models of stage-specific mortality. However, since the rate of larval development is temperature dependent (Rueda et al. 1990), larval instar conveys little information about the physiological age of mosquitoes. Consequently, instar would not be an appropriate parameter to include in mathematical models of temperature-dependent development.

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From Mouse to Sequence and Back to Mouse: Peregrinations of an Arbovirologist

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ABSTRACT: When the albino (laboratory) mouse was found to be useful for the production of high titering reagents, improvements in diagnostic methods soon followed. Dalldorf, Bugher, Casals, Lennette, Koprowski, Theiler, and Webster each made contributions that were central to the generation of assays allowing much more precise measurements of virus than had been possible. With the discovery of hemagglutination by Hirst and its application to both hemagglutination-inhibition tests for antibody to viruses and the study of virus-cell attachment mechanisms, a relatively simple and inexpensive tool became available. Subsequent efforts by Sabin and Buescher and by Clarke and Casals applied this method as well as complement-fixation and neutralization to epidemiological and laboratory studies of arboviruses and arboviral diseases. Worldwide studies of arboviruses, supported by the Rockefeller Foundation, led to the discovery of newly recognized viruses and their geographic distributions. Later, electron microscopic studies by Holmes and Murphy corroborated the antigenic studies of Casals, Shope, and others and the cascade of information regarding the molecular characteristics and genomic sequences of viruses subsequently provided powerful other analytical tools. In 1993 a previously unrecognized hantavirus, Sin Nombre virus, was shown to be the etiologic agent of Hantavirus Pulmonary Syndrome in the U.S., and various techniques were used to determine the epidemiology and natural history of this virus. Longitudinal studies of hantaviruses in the southwestern U.S. are yielding information useful for understanding the fundamentals of transseasonal transmission, epizooogy, epidemiology, evolution, epidemic potential, prevention, and control of hantaviruses, here and elsewhere. Mice of various species, sizes, shapes, and colors have been central, if involuntary, participants in these hantavirus studies and advances. We have, in a way, come full circle.

Keyword index: Bunyaviridae, hantavirus, Sin Nombre, mouse, history, arboviruses.

The laboratory mouse had not been used as a test animal in virology until 1930, when Max Theiler reported that newly born mice inoculated intraperitoneally with yellow fever virus died of encephalitis (Theiler 1930). In 1935 it was reported that the laboratory mouse was suitable for use as a test animal for the diagnosis of rabies (Webster and Dawson 1935). Then, in 1941, John Bugher reported that when mice were inoculated when they were younger than 10 days of age, they sickened and died after being subcutaneously inoculated with yellow fever virus (Bugher 1941) and, in 1944, Edwin Lennette and Hilary Koprowski reported taking advantage of the high susceptibility of three-day old mice to develop neutralization tests, particularly those to detect antibody or to identify western equine encephalitis, eastern equine encephalitis, and Venezuelan equine encephalitis viruses (Lennette and Koprowski 1944).

When Jordi Casals was beginning his scientific career in 1936, he went to work with Leslie Webster of the Rockefeller Institute in New York. Two years later an outbreak of encephalitis occurred in Massachusetts and Casals and Webster became interested in the etiologic agent, eastern equine encephalitis virus, and soon were involved in studies of antigenic comparisons of the viruses that were known at that time to cause encephalitis: rabies, eastern equine encephalitis (EEE), western equine

encephalitis (WEE), Russian spring-summer encephalitis, louping ill, and poliomyelitis (Casals 1944). Applying the meticulous methods needed to properly perform the complement-fixation test taught him by Jules Freund working on tuberculosis, Casals found relationships between the viruses of Russian spring-summer encephalitis and louping ill and no relationship between rabies, poliomyelitis, and other viruses (Casals and Webster 1944). Casals also found that St. Louis, Japanese, and Murray Valley encephalitis viruses were antigenically related to Russian spring-summer encephalitis and louping ill viruses but that antigenic proximity varied; that is, whereas all were related, some were more distantly and some more closely related one to another (Casals 1957).

The hemagglutination technique, developed at the Rockefeller laboratories by George Hirst working with influenza A virus in embryonated hens' eggs (Hirst 1941), useful in early studies of virus-cell attachment mechanisms, was shown by Casals and Brown to be suitable for work with arboviruses (Casals and Brown 1954). Then, using antigens produced in the brains of mice, Albert Sabin found that yellow fever and dengue viruses are antigenically related and, with Edward Buescher, demonstrated that the hemagglutination-inhibition (HI) technique could be applied to other arboviruses (Sabin and Buescher 1950). When Max Theiler suggested using HI as a serologic test for evidence of infection with arboviruses, a formidable, yet exquisitely simple, tool became available to show antigenic similarities rather than differences.

High titering viruses, antigens, and antibodies are essential for detecting distant antigenic relationships between viruses, so that the finding by Gilbert Dalldorf that coxsackieviruses replicate to high titer in newborn mice (Dalldorf and Melnick 1965) led Casals and others to replace adult mice with newborn mice for reagent production and for other purposes. By 1951, Casals and coworkers had adapted human poliomyelitis type 2 virus to replicate in newborn mice and developed a complement-fixation test to detect it (Casals et al. 1951a; Casals et al. 1951b). When they began using suckling mice for the study of arthropod-borne viruses, the results were shorter incubation periods, higher titers, and better polyclonal antibodies.

At that time the Rockefeller Institute was supporting laboratories studying yellow fever and other diseases in Africa, South America, and elsewhere, and the New York laboratory was receiving scores of virus isolates from arthropods and vertebrates (Theiler and Downs 1973). Casals, Robert Shope, Loring Whitman, and their coworkers were in the unique position of having available not only a profusion of viruses, but excellent reagents, marvelous collegiality, and appropriate financial and administrative support. Thus armed, they began the classic investigations that were so integral to our knowledge of the interrelationships among arboviruses (Theiler and Downs 1973).

In 1943, during studies of yellow fever in Uganda, Kenneth Smithburn and his colleagues of the Rockefeller Institute had made the initial isolation of Bunyamwera virus from Aedes species mosquitoes (Smithburn et al. 1946); the isolation was made using mice. A few years later, Richard M. Taylor, Telford H. Work (Fig. 1) a former member of SOVE who died early this year, and others, isolated Sindbis virus from mosquitoes, birds, and humans in Egypt (Taylor et al. 1955); the isolations were made using mice. Smithburn scarcely could have imagined that Bunyamwera virus would become the type species of both the genus *Bunyavirus* and of the family *Bunyaviridae*, nor could Taylor and Work have imagined that Sindbis virus would become the prototype virus of the genus *Alphavirus* and of the family *Togaviridae*.

The classical grouping fluids produced by Shope and others; the application of complement-fixation (Casals 1949), HI, and neutralization tests (Casals 1963a); the use of suckling mice, of acetone extraction of antigens and sucrose in antigen preparations (Clarke and Casals 1958)—all were used to define antigenic groups and to determine antigenic differences.

Casals formulated three dicta, which can be paraphrased as follows: (1) "No virus can belong to two antigenic groups," (2) "If two viruses cross-react, they are related," and (3) "If two viruses of two different groups cross-react, they do not belong to two different groups." These simple but effective premises have never been disproved. Perhaps their significance can best be understood if one thinks of them in terms of phenotypic expression of genotypes and in terms of phylogeny.

By 1960, it was known that EEE and WEE and certain other viruses were related. These were placed in what was termed the "Group A arboviruses" (now known as the genus *Alphavirus* in the family *Togaviridae*). Japanese encephalitis, Murray Valley encephalitis, and St. Louis encephalitis viruses, West Nile, yellow fever, Russian spring-summer encephalitis, louping ill, and others were known to be related to each other and were placed in what was termed the "Group B arboviruses" (now known as the genus *Flavivirus* in the family *Flaviviridae*). The laboratory mouse was central to all this work and to these findings.

In the following decades many more newly recognized arboviruses were isolated and worked at the Rockefeller Foundation laboratories and elsewhere used
meticulous testing to detect relationships between and among many of them and to establish serogroups in which to place them (Theiler and Downs 1973). Within about a ten-year period, not only had the Bunyamwera serogroup been recognized (Casals and Whitman 1960), but serogroups C (Casals and Whitman 1961), Guama, California, Capim, Anopheles A, Simbu, Bwamba, Patois, Koongol, Tete, and others had been distinguished (Hammon and Reeves 1952; Whitman and Casals 1961; Theiler and Downs 1973; Whitman and Shope 1962). Because at least one virus in each of these groups reacted serologically with at least one virus (or antibody to it) of another group, Casals suggested that these viruses all were interrelated, albeit distantly and enigmatically. These cross-reactions between one or another member of the various serogroups often were weak and some appeared tenuous but all were repeatable. Therefore, Casals suggested placing them in a "Bunyamwera Supergroup" (Casals 1963b).

In the late 1960s, sophisticated electron microscopic studies by Ian Holmes in Australia (Holmes 1971) and Fred Murphy at the Centers for Disease Control (CDC) in Atlanta (Murphy et al. 1973) recognized the morphological similarities of Bunyamwera Supergroup viruses and supported and extended the Supergroup concept in that they were able to show that these viruses were not

Figure 1. Telford H. Work, photo taken about 1964 while he was Chief, Virology Section and Arbovirus Unit, National Communicable Disease Center (now Centers for Disease Control and Prevention), Atlanta, Georgia.
clearly distinguishable by size, morphology, or morphogenesis in infected cells. Some of these viruses were later shown to belong to separate genera within the family Bunyaviridae (Murphy et al. 1995).

Subsequent studies of the phenotypic characteristics of these viruses and of viral structure-function relationships, replicative mechanisms, biology, and ecologies has led us first to classification, then to taxonomy, now to phylogeny. Whether or not one considers classification to be simply the obsessive collecting of things, it follows that taxonomy and phylogeny are the twin goals of such a compulsion.

In the 1960s the Virology Section at the CDC was headed by Telford Work. That was a time when virus research was not prioritized as a Disease-Of-The-Week Club. The methodical characterization of viruses, newly recognized or not, associated with disease or not, along with the emphasis placed on field studies, and the epidemiologic brilliance of Work, ran together to actually discover things rather than to solve only acute problems. On the staff at that time were Roy Chamberlain, Philip Coleman, Bernard Fields, Martin Hirsch, Brian Henderson, Blaine Hollinger, Rexford Lord, Fred Murphy, Daniel Sudia, and others, many if not all of whom are familiar to the readers of this journal. I was fortunate enough to have been added to Tel Work to that select group. In relatively short order, Murphy’s laboratory showed that the alphaviruses and flaviviruses differed from what were being called rhabdoviruses, reoviruses, arenaviruses, and filoviruses. Nearly as quickly as viruses could be amplified in mice, they were passaged in cell cultures, and had their photos taken. Still, while results of such biological and morphological analyses were instructive, they also were incomplete. Fortunately, there has been a cascade of information about viral nucleic acid sequences.

Arboviruses have the advantage of passing through at least two taxonomically distinct hosts during their life cycles. One host, the vertebrate, lives in a relatively constrained ecosystem, a defined ecological niche, produces antibody, and has other mechanisms that protect it from the potentially deleterious effects of the virus, while at the same time providing selection pressures that lead to diversity of virus genotypes. The mouse is a useful model for much of this. The arthropod seems to be mostly unaffected by infection with a virus and does not have immune mechanisms, in effect serving only as an incubator for amplification, maintenance, and transport of a virus population. Viruses, such as hantaviruses, which are transmitted from rodent to rodent, have no such protection, being constantly assaulted by antibodies that may provide a selective mechanism. Further, dual infections with closely related hantaviruses (Bunyaviridae in general) provide a milieu in which reassortment of RNA segments can occur.

Recent advances in molecular genetics, molecular epidemiology, and molecular evolution have been the result of a combination of developments, including access to computer-assisted analyses, availability of techniques for rapid and accurate sequencing of nucleic acids, and most importantly the appearance of highly skilled molecular biologists who can apply these techniques with imagination and who can articulate their findings. It has been only since these occurrences coincided that we have been able to scrutinize viral genomes. Viral classification, previously based solely on phenotypic characters, became gene-based and has evolved, and is evolving, into a taxonomy parallel to but very different from that of cellular organisms. Still, when attempting to identify a virus, it is useful to inoculate it into suckling mice and see what happens. If one knows the terrain and the species of arthropod, one can obtain a rough, but fairly reliable, idea as to which virus an isolate might be simply by determining how long it takes to kill the mouse.

I find it extraordinary that the serologic and antigenic studies done many years ago, using relatively crude techniques that never were more than an indication of phenotype, have been so predictive of genotype. Clearly, viruses with similar gene sequences produce similar proteins; it usually follows that the more dissimilar the gene sequence, the more dissimilar the antigens produced. Therefore the early tests and conclusions of Casals, Shope, and others provided a reliable set of indicators of genetic relationships although they did not think of them in this way at that time. The original intent, of course, was not to improve taxonomy but to improve laboratory diagnosis of arboviral and other viral infections; this they did, mostly with the help of untold numbers of laboratory mice.

In mid-May 1993, an outbreak of fatalities in adult humans with acute respiratory failure was recognized in New Mexico and, shortly thereafter, in Colorado and Arizona. The first evidence that these infections were caused by a hantavirus (genus Hantavirus, family Bunyaviridae) was that case-patients and rodents trapped near the homes of these patients had antibody to one or more hantavirus not known to occur in this country. It was serologic evidence that was first used to classify the hantaviruses. Soon thereafter, a newly recognized virus, Sin Nombre virus (SNV), was shown to be associated with this disease, now called hantavirus pulmonary syndrome (HPS) (Elliott et al. 1994). Elegant and innovative molecular epidemiologic studies of specimens from HPS patients and from deer mice, the putative natural vertebrate hosts of this virus, captured in case
residences, showed that SNV is distinct from other
known hantaviruses. Analyses and comparisons of
nucleic acid sequences of this and other hantaviruses
indicated that SNV is widely distributed in the U.S.
and that this virus naturally infects rodents, principally
Peromyscus maniculatus, throughout that rodent's range.

Since 1993, hantaviruses have been implicated in
illnesses in 124 people in the U.S., most of whom have
been residents of the area adjoining New Mexico,
Colorado, Arizona, and Utah; six of these cases have
been in Colorado and five of these six died. The
mortality rate has been slightly less than 50%.
Fortunately, not many people get this disease.

Most of the first cases in 1993 were detected in
Native Americans because the outbreak first was
recognized in an area with predominant populations of
Native Americans. In more recent studies, however, the
proportions of cases have been: Hispanic (8%), non-
Hispanic Caucasian (56%), Native American (34%),
and African-American (2%). Ages of patients have
ranged from 12 to 69 years, with a median of 32 years;
55% have been males.

Because hantaviruses in other parts of the world
had been associated only with rodents and not with
arthropods, rodents immediately became suspect
reservoirs. Indeed, epidemiologic evidence suggests
that the natural history of SNV involves cycling in
rodents, principally P. maniculatus, the deer mouse
(Childs et al. 1994). The virus appears to be transmitted
between rodents, in subclinical infections, and from
rodents to humans, in whom it may cause severe disease.

Hantavirus pulmonary syndrome now is known
to occur throughout most of the U.S., in Canada, and in
Brazil and Argentina. Retrospective evidence indicates
the occurrence of previously undiagnosed cases of HPS
in the U.S. well prior to the 1993 outbreak, making this
an emerging but not a new disease. Patients with HPS
have acute pulmonary edema and shock. Pathogenesis
appears to be related to the presence of viral antigens in
pulmonary capillaries (Zaki et al. 1995).

The story of the determination of the etiologic
agent is a classic in epidemiology and a tribute to the
knowledge, intellect, and organizational abilities of the
CDC, the U.S. Army, and many others. Because much
of this work has been published, there is little reason to
repeat the details of this remarkable story. However, I
will stay with the general theme of this paper by focusing
on the vertebrate host of SNV.

When the first serologic tests indicated that the
New Mexican patients might have had infections with a
hantavirus, the scientific scurrying began. The prototype
hantavirus, Hantaan virus, causes a disease known
principally as hemorrhagic fever with renal syndrome
(also called Epidemic hemorrhagic fever and Korean
hemorrhagic fever). Thus, patients with pulmonary
disorders were not what one would expect if one were
predicting an American disease caused by a hantavirus.
That inability to predict is one of the many reasons many
of us studied biology in the first place. Other hantaviruses
in other parts of the world can cause renal failure and
hemorrhagic illnesses (TABLE 1). The hantaviruses in
the U.S., when they are associated with human illness,
are associated with pulmonary disease.

One fascinating aspect of the hantaviruses is their
apparent specific association with specific rodent species
(Lee et al. 1991); Hantaan virus with Apodemus agrarius
(striped field mouse); Seoul virus (Kariwa et al. 1994)
with Rattus norvegicus and Rattus rattus; Puumala
virus (Brummer-Korvenkontio et al. 1980) with Clethrionomys
glareolus (bank vole); Prospect Hill
virus (Lee et al. 1985) with Microtus pennsylvanicus
(meadow vole); SNV and Convict Creek (Schmaljohn
et al. 1995) virus with Peromyscus maniculatus
(deer mouse); El Moro Canyon virus (Hjelle et al. 1994) with
Reithrodontomys megalotis (western harvest mouse);
Thottapalayam virus (Zeller et al. 1989) with Suncus
murinus (shrew); Black Creek Canal virus (Rollin et al.
1995) from Sigmodon hispidus (hispid cotton rat); Tula
virus (Plyusnin et al. 1994) from Microtus arvalis
and Microtus rossiaemerdigionalis (voles); and Bayou
virus (Morzunov et al. 1995) from Oryzomys palustris
(rice rat). A hantavirus associated with a human infection in
the northeastern U.S. has been isolated from Peromyscus
leucopus (white-footed mouse; Song et al. 1994) and
other, apparently heretofore unrecognized, hantaviruses
have been isolated from Neotoma mexicana in the
southwest and west (B. Hjelle, personal communication,
1995) and from harvest mice in Costa Rica (Hjelle et al.
1995). It is likely that, as more tests are done with
different rodent species in different locations, and even
in the same species in different ecological and
environmental circumstances (altitude, latitude, mean
temperature, rain fall, etc.), additional unique
hantaviruses will be discovered. Whether the gene
sequences of these viruses are sufficiently divergent to
warrant considering them distinct from SNV (i.e., Do the
viruses cross-protect and therefore have epidemiologic significance?) remains to be determined,
but it appears they do.

Two bonuses from all this have been the focus
required by the scientific community on the definition
of "virus" and whether a virus that has not been iso-
lated, but whose nucleotide sequence is entirely known
can legitimately be called "a virus" and registered as
such. Keep in mind that the work of Stuart Nichol and
coworkers allowed not only the sequencing of SNV and
TABLE 1. Viruses of the family Bunyaviridae, genus Hantavirus (Hantaan group; hantaviruses) by geographic distribution and whether they cause human illness.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Virus</th>
<th>Geographic distribution</th>
<th>Human illness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hantaan</td>
<td>Hantaan</td>
<td>Asia, Europe</td>
<td>KHF&lt;sup&gt;a&lt;/sup&gt;, HFRS&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Seoul</td>
<td>Asia, Europe, N. America, S. America</td>
<td>mild HFRS</td>
</tr>
<tr>
<td></td>
<td>Dobrava (Belgrade)</td>
<td>Europe</td>
<td>HFRS</td>
</tr>
<tr>
<td></td>
<td>Thailand</td>
<td>Asia</td>
<td>?</td>
</tr>
<tr>
<td>Puumala</td>
<td>Puumala</td>
<td>Europe, Asia</td>
<td>NE&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Prospect Hill</td>
<td>N. America</td>
<td>HPS&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Sin Nombre</td>
<td>Western U. S.</td>
<td>none known</td>
</tr>
<tr>
<td></td>
<td>Convict Creek</td>
<td>California</td>
<td>?</td>
</tr>
<tr>
<td>(unplaced)</td>
<td>Thottapalayam</td>
<td>India</td>
<td>none known</td>
</tr>
<tr>
<td></td>
<td>Bayou</td>
<td>Louisiana</td>
<td>HPS</td>
</tr>
<tr>
<td></td>
<td>El Moro Canyon</td>
<td>N. America, S. America</td>
<td>none known</td>
</tr>
<tr>
<td></td>
<td>Black Creek Canyon</td>
<td>Florida</td>
<td>HPS</td>
</tr>
<tr>
<td></td>
<td>Tula</td>
<td>Europe</td>
<td>none known</td>
</tr>
<tr>
<td></td>
<td>Rio Mamore</td>
<td>Bolivia</td>
<td>HPS</td>
</tr>
</tbody>
</table>

<sup>a</sup>KHF= Korean hemorrhagic fever  
<sup>b</sup>HFRS= hemorrhagic fever with renal syndrome  
<sup>c</sup>NE= Nephropathia Epidemica  
<sup>d</sup>HPS= hantavirus pulmonary syndrome

the production of antigens and antibodies against it but had the potential for producing a vaccine against a virus that had not been isolated. This situation is becoming quite common in virology.

Hantaviruses are notoriously difficult to coax into replicating in laboratory systems. Blind passages, long incubation periods, indirect detection systems, and significant biohazards are some of these difficulties. At the CDC in Atlanta, a team led by C. J. Peters approached this diagnostic problem on a variety of fronts. When serologic tests provided evidence that a hantavirus was the etiologic agent of HPS, virus isolation became a priority for diagnosis and for research; however, it was not until six months after the first case was detected that the etiologic agent was isolated (Elliott et al. 1994). Deoxyoligonucleotide primers (from Puumala and Prospect Hill hantaviruses) were constructed and used in a PCR assay to detect and define the gene sequences of the responsible hantavirus. Utilizing PCR practically as an art form, the molecular biology team, directed by Nichol, was able to retrieve hantavirus sequences from the lungs of HPS patients and, later, from rodent tissues. Comparisons of nucleotide sequences from case-patients and from infected rodents collected near the homes of those patients showed that the viruses from the patients were essentially identical to the viruses from the rodents. Sequences of a hantavirus from a patient who died in Arizona were the same as those of hantaviruses from people and rodents in the southwest corner of Colorado, which was puzzling. Additional, epidemiologic investigations determined that the patient had come from the southwest corner of Colorado; thus his infection likely had been acquired there and not in Arizona. This was a slick bit of molecular epidemiology.

Availability of sophisticated molecular tools notwithstanding, “the mouse” continues to play a major role in diagnostic studies. Although laboratory diagnosis of HPS has been made more rapid by inserting SNV gene sequences into Escherichia coli and allowing these bacteria to express nucleocapsid proteins useful as antigens in serologic tests, including IgM antibody capture and IgG ELISAs; E. coli-expressed nucleocapsid proteins of SNV have been used to immunize laboratory mice for production of reference hyperimmune mouse ascites fluids. However, detection of hantaviral RNA continues to be the assay of choice for both detection and definition of hantaviruses from clinical and field-collected specimens.
The emergence of SNV caused enormous concern in local Native American populations, in other residents of the Four Corners area and of the respective states, and in potential tourists and visitors to the area. For a while, lack of information concerning geographic distribution of the virus, its transseasonality in rodent hosts, and the risk factors for its transmission aggravated the situation and led to baseless associations of the virus with Native Americans.

Because there is little information about the ability of SNV to be maintained transseasonally in endemic areas, there is no reliable means of determining risks of infection in human populations in ensuing years. For example, little is known about the origin of the 1993 epidemic. Among many possible causes are: emergence of a new virus, evolution of established viruses, genetic changes within vertebrate populations or genetic selection of vertebrate hosts, and other epidemiologic factors as yet unrecognized. SNV appears to be a newly recognized but not a new virus, fitting the definition of "emerging."

Beginning in June 1994, we established study sites in southeastern, southwestern, and, west central Colorado with Barry Beaty, Director of the Arthropod-Borne and Infectious Diseases Laboratory, Colorado State University. Currently, we are performing longitudinal studies of the hantaviruses we find in those places, trapping rodents at each site every six weeks. The results can be summarized briefly, as follows: (1) The principal vertebrate host of the hantavirus in southeastern Colorado is the western harvest mouse, *Reithrodontomys megalotis*. Accessory hosts appear to be deer mice (*Peromyscus maniculatus*) and pion mices (*Peromyscus truei*). The hantavirus at this site may be what is being called El Moro Canyon virus. (2) The principal vertebrate host of the hantavirus in western Colorado is the deer mouse (*P. maniculatus*). The hantavirus at these sites may be SNV. (3) Rodent populations fluctuate dramatically but may fluctuate only by species. (4) The prevalence rate of antibody to SNV is much higher in male than in female rodents at all sites. (5) Transmission of these viruses appears to occur throughout the year.

Studies of the social behavior of the rodent hosts and of their familial genetics will be of great value in understanding the epidemiology and epizology and the evolutionary and epidemic potential of these viruses. Studies of coevolution of rodent host and virus will be instructional, not only in increasing our understanding of the general equilibrium between them, but possibly for understanding the underlying pathophysiological mechanisms whereby humans become ill when infected by these viruses.

Personally, this has been a great opportunity for me. I get out of the house a bit and have a chance to meet mice other than white ones. The work has helped me creep up the learning curve; until I began working with Barry Beaty and doing this field work I thought polyestrus was a fabric. We put four kids through college with the help of lab mice; I hope now to work with a different set of mice and buy a rocking chair. But before I sit down one last time, I would like to know all there is to know about these fascinating viruses. This may take some time.

**Acknowledgments**

I thank Jordi Casals, Telford Work, Robert E. Shope, Thomas P. Monath, Frederick Murphy, and many, many others for having taken the time, over many years, to discuss with me how people, viruses, institutions, and epidemics fit together.

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Scientific Note

Breeding of *Anopheles plumbeus* in Tires in France

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Keyword Index: *Anopheles plumbeus*, mosquito, ecology, FRANCE.

*Anopheles plumbeus* Stephens, 1828 is widespread in Europe, Western and Central Asia, and North Africa (Knight and Stone 1977). It is one of the few species of anophelines that can breed in tree holes. It overwinters in the egg stage and has two generations per year (Snow 1986). We found this species breeding in used and old tires in a garbage dump near Paris in the summers of 1994 and 1995. More than 50% of the tires filled with water harbored *An. plumbeus* larvae. The garbage dump was surrounded by small trees.

*Anopheles plumbeus* commonly feeds on humans at night along the edges of the Seine river, but it is relatively scare. Most likely, the colonization of tires is a secondary biotope for a mosquito population that breeds mainly in tree holes. However, these new breeding sites are now well established and around them adult anophelines were biting humans in large numbers until the tires were treated with temephos (Abate®).

Abnormal breeding sites of *An. plumbeus* have already been reported in flower vases in cemeteries in Algeria (Senevet et al. 1955). It was thought that leaves falling in the vases increased the content of the organic matter, creating conditions similar to tree holes. In Sardinia, Logan (1953) observed that 20% of *An. plumbeus* breeding did not occur in tree holes, including springs, river pools, and containers.

This anopheline is not considered as an efficient vector of malaria but in certain circumstances it can transmit *Plasmodium vivax*. Due to its low biting density it was never considered as a dangerous mosquito in France. However, its adaptations to tires can greatly increase its populations in areas where the number of immigrants is large. This phenomenon requires further surveillance.

REFERENCES CITED


