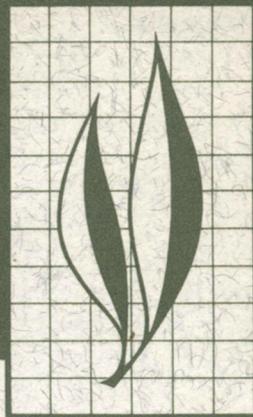


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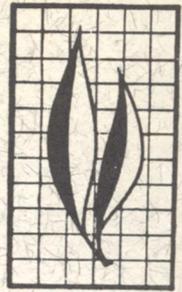
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Pathogen Strains and Leafhopper Species as Factors in the Transmission of Western X-Disease Agent under Varying Light and Temperature Conditions

Roger E. Gold and Edward S. Sylvester



The data base on transmission of western X-disease agent (WXDA) by *Colladonus montanus* leafhoppers was expanded in studies using celery, *Apium graveolens* as the host. Transmission was affected or limited by the availability threshold, instar efficiency, and acquisition and inoculation rates, along with vector biotype and pathogen strain. Transmission also was moderated by circadian rhythms. Serial passage by injection of the pathogen into a sequence of healthy insects was accomplished, and some evidence was obtained that tetracycline pressure during serial passage could result in selection of a tetracycline-resistant strain of the pathogen. Leafhoppers did not become infectious by membrane-feeding during trials made to concentrate or purify the WXDA.

Comparative tests using *C. geminatus*, *C. montanus*, and *Euscelidius variegatus* indicated *C. montanus* was the most, and *E. variegatus* the least, efficient experimental vector. Simultaneous feeding of the three species on a common disease source reduced vector longevity. Transmission efficiency was temperature sensitive. At 30 C, transmission by any species was rare. Comparisons at 20 and 25 C resulted in similar acquisition rates. The median latent period and longevity of both *Colladonus* species decreased at higher temperatures. *Euscelidius variegatus* however, apparently was not affected by the pathogen and survived longer at 25 C than at 20 C. When inoculated by injection, *C. geminatus* was a more efficient vector than *E. variegatus* under insectary conditions, but the reverse was true under conditions of constant light and temperature.

A fourth leafhopper species, *Fieberiella florii* was similar to *C. montanus* in acquisition efficiency and transmission of the WXDA, but it had a longer developmental time; thus, transmission could occur in the nymphal stage. The median latent period of the pathogen in *F. florii*, following inoculation by injection, was half of that when infectivity was established by feeding.

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Pathogen Strains and Leafhopper Species as Factors in the Transmission of Western X-Disease Agent under Varying Light and Temperature Conditions^{1,2}

INTRODUCTION AND REVIEW OF LITERATURE

The disease

Western X-disease (WXD) affects stone fruits in western North America and has a wide host range among both woody and herbaceous plants. In California, economic losses of peaches (*Prunus persicae* Batsch.) and sweet cherry (*P. avium* L.) occur as the result of general decline and eventual death of infected trees.

Originally described in cherry by Rawlins and Horne (1931) as "cherry buckskin," the disease has had several common names in various hosts, e.g., western X-disease (Reeves and Hutchins, 1941), red-leaf and little cherry (Richards, Wadley, and Cochran, 1948; Richards, Hutchins, and Reeves, 1949) wilt and decline (Richards, Reeves and Hutchins, 1946), leaf casting yellows (Thomas, Rawlins, and Parker, 1940), and small bitter cherry (Lott, 1947). X-disease of cherry and peach in the eastern U.S. may be the same as Western-X (Richards and Hutchins, 1941; Palmiter and Parker, 1948). The X-diseases have been extensively reviewed by several authors (Reeves, et al., 1951; Rawlins and Thomas, 1951; Stoddard et al., 1951; Gilmer and Blodgett, 1976).

"Western X-disease," the name currently most commonly used, refers to a complex of symptomologically distinct strains (Rawlins and Thomas, 1941, 1951; Zeller, 1942; Reeves et al., 1951; Nyland, 1955). Peach yellow leaf roll (PYLR), a relatively severe form (Nyland and Schlocker, 1951), was used for research from 1952 to 1972 by Jensen and co-workers at Berkeley, California.

Graft and dodder transmission

Buds, bark patches, or scions, are used in graft transmission of the western X-disease agent (WXDA), but it is not juice inoculable (Rawlins and Thomas, 1941; Richards and Hutchins, 1941; Reeves et al., 1951; Stoddard et al., 1951; Nyland, 1955). Dodder, *Cuscuta campestris* Yunck. was used to transmit the X-disease agent (XDA) from peach to tomato (*Lycopersicon esculentum* Mill.), *Nicotiana glutinosa* L. and carrot (*Daucus carota* L. var. *sativa* DC.) (Kunkel, 1944; Hildebrand, 1945), parsley (*Petroselinum crispum* Nym.), and Madagascar periwinkle (*Catharbinus rosea* (L.)) (Kunkel, 1944; Weathers and Cochran, 1950), and from choke cherry (*Prunus virginiana* L.) to milkweed (*Asclepias syriaca* L.) (Gilmer, 1960). Recovery of XDA from infected herbaceous plants and return to either peach or cherry using dodder, has not been reported.

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Vectors

Studies on insect transmission of WXDA began with reports by Wolfe, Anthon, and Jones (1950), and Wolfe et al. (1951) that geminate leafhoppers *Colladonus geminatus* (van Duzee) were economic vectors of WXDA in peach and cherry orchards. Other leafhopper species reported to be vectors include the mountain leafhopper *C. montanus* (van Duzee) (Wolfe, 1955; Jensen, 1956, 1957b), the sharpnosed leafhopper *Scaphytopius acutus* (Say), the furcate leafhopper *Fieberiella florii* (Stål), the willow sharpshooter *Koenoella confluens* (Ühler) (Anthon and Wolfe, 1951; Wolfe and Anthon, 1953; Nielson and Jones, 1954), the myrtle leafhopper *Osbornellus borealis* (DeLong and More) (Jensen, 1957a), the variegated leafhopper *Euscelidius variegatus* (Kirschbaum) (Jensen, 1969a), *S. nitridus* (DeLong) and the angulate alfalfa leafhopper *Acinopterus angulatus* Lawson (Purcell, 1979).

Vectors of eastern forms of the XDA include: the saddle-back leafhopper *Colladonus clitellarius* (Say) (Gilmer, 1954), the laminate leafhopper *Gyponana lamina* (DeLong), *Paraphlepsius irroratus* (Say), *Norvellina seminuda* (Say), as well as *Fieberiella florii* and *Scaphytopius acutus* (Gilmer et al., 1966; Thornberry, 1954).

All vectors of XDA, except *Koenoella confluens*, are in the subfamily Deltocephalinae, and are presumed phloem feeding leafhoppers. The vector status of *K. confluens*, a xylem-feeding sharpshooter, needs confirmation (Frazier, 1966).

Vector competency and strains of the disease agent

Colladonus geminatus was believed to be an economic vector of WXDA in both cherry and peach orchards (Wolfe and Anthon, 1952). Jensen, Frazier, and Thomas (1952) and Jensen and Thomas (1954, 1955) reported the Napa Valley and Green Valley strains of the WXDA in California were acquired and transmitted less efficiently from peach and cherry than was the PYLR strain. The latter strain was believed to be more severe in test trees and more readily available to feeding *C. geminatus* leafhoppers than were other strains. Jensen (1953b) also found that *C. montanus* lived but for a few days on peach, and so perhaps would not be an important field vector, whereas *C. geminatus* could complete its life cycle on peach and cherry and could acquire and transmit the WXDA among cherry and peach (Jensen, 1953a).

Vector transmission to herbaceous hosts

Four strains of WXDA, including the PYLR strain, have been experimentally transmitted to celery, *Apium graveolens* L., by *C. geminatus* (Jensen, 1956). Strains could not be differentiated by symptoms in celery, but this host was a better source of the agent than either peach or cherry. Acquisition of WXDA from celery by *C. geminatus* resulted in efficient transmission to peach. The XDA (eastern form) was reported to be reciprocally transmitted between chokecherry and periwinkle by *Scaphytopius acutus* (Gilmer et al., 1966).

Colladonus montanus was also an efficient vector of WXDA when celery was used as both the source and test plant (Jensen, 1957b), although this species had poor survival on peach and cherry. When infective *C. montanus* were transferred alternately to peach and celery, transmission occurred to 27% of the celery, but to only 2% of the peach test plants. Only one transmission of WXDA by *C. montanus* occurred out of 168 field trials of transmission from peach to peach.

In addition to celery, other proven herbaceous hosts inoculated by *C. montanus* are periwinkle, chrysanthemum (*Chrysanthemum carinatum* L.), aster (*Callistephus chinensis*

Nees), radish (*Raphanus sativus* L.), turnip (*Brassica rapa* L.), cauliflower (*B. oleracea* var. *botrytis* L.), filaree (*Erodium moschatum* L'Her.), strawberry (*Fragaria vesca* L.), carrot (*Daucus carota* L. var. *sativa* DC.), coriander (*Coriandrum sativum* L.), and *Gomphrena globosa* L. (Frazier and Jensen, 1970; Jensen, 1971a).

Pathogenicity of the WXDA to vectors

Jensen (1959), in the first report of a plant pathogen causing premature death of a vector, found *Colladonus montanus* that became infective after feeding on diseased celery died sooner than did noninfective insects. (See Jensen, 1963, 1969b; Maramorosch and Jensen, 1963, for reviews on the effects of plant pathogens on vectors). Fecundity of infected female leafhoppers also was reduced (Jensen, 1971b). However, when *C. montanus* were infected by injection of infectious extracts (Whitcomb, Jensen, and Richardson, 1966a), rather than by feeding on diseased celery, the impact on fecundity was comparatively less. Thus, an undetermined reduction in fecundity was associated with diseased celery plants (Jensen, 1971b).

A series of papers, reviewed by Gold (1979) described consequences of WXDA infection in *C. montanus*. Changes noted included crystalline inclusions in the gut (Lee and Jensen, 1963), lesions in salivary, neural, and adipose tissues, as well as in the 1st and 2nd ventriculus and Malpighian tubules (Whitcomb, Jensen, and Richardson, 1967, 1968a). Proliferative cytopathology was found in salivary glands, mycetome, urate cells, collateral glands, the corpus cardiacum, pericardial cells, and connective tissues. Also outgrowths were detected from the esophageal valve, spermatheca, and rectal pads (Whitcomb and Jensen, 1968).

Reduced oxygen consumption, lower body weight, reduced protein content, and a faster depletion rate of fat and triglycerides were reported (Amin, 1971; Amin and Jensen, 1971b), although Whitcomb, Jensen, and Richardson (1968) stated that infected insects used less stored fat.

This descriptive pathology, as well as the reports of a dosage-sensitive latent period and an increase in titer of WXDA following injection of *C. montanus* with infectious extracts, was convincing evidence that WXDA could multiply in both plants and insects (Whitcomb, Jensen, and Richardson, 1966a, 1966b).

Successful serial passage experiments (Gold, 1979), the details of which are reported in the present paper, provided additional evidence for propagation of WXDA in *C. montanus*. Earlier attempts at serial passage were frustrated by bacterial contamination (Whitcomb, Shapiro, and Richardson, 1966; Jensen, Whitcomb, and Richardson, 1967).

Effect of light and temperature on transmission

Temperature and light conditions affect the pathogenicity of the WXDA to *C. montanus*, as well as the inoculativity of the leafhoppers (Jensen, 1968, 1972). Insect longevity and the period over which insects would transmit were inversely proportional to the temperature at which the leafhoppers were held during testing. Fluctuating temperature and light conditions were reported to increase vector efficiency.

Partial inactivation of WXDA occurred when infective *C. montanus* were held at 38 C and 41 C for 7 to 20 days, and resumption of transmission would occur only after completion of a second latent period in treated insects. Longevity of heat-treated leafhoppers increased in comparison to untreated insects. The most favorable temperature for development of infectivity was 20 C.

Tissue culture

A cell culture of *C. montanus* tissue culture was developed in an attempt to find a rapid bioassay for the infectious agent of WXD (Richardson and Jensen, 1971). A cell line of large epithelial-like cells, was maintained as a monolayer in flask tissue culture. However, WXDA was not recovered from tissue cultures seeded with WXD-inoculum.

Etiology

Until 1970, WXD was classified with the "yellows" group of diseases and assumed to be caused by a virus. Investigations of properties of the WXDA by Whitcomb, Jensen, and Richardson (1968b) indicated concentrations of extractable agent were higher in infected *C. montanus* than in several species of diseased herbaceous plants. WXDA was sensitive to ultrasonication, butanol, chloroform, fluorocarbons, and to dialysis against normal saline. The agent survived freezing and dialysis against protective buffers, and infectivity was distributed throughout a column following rate zonal density gradient centrifugation for 20 to 25 min at 20,000 rpm. Highest concentrations were in the bottom one-third of the tube. A note added to the paper suggested a possible mycoplasma etiology of WXDA should be investigated in view of reports of mycoplasma-like bodies being found in several plants having "yellows" diseases (Doi et al., 1967). This led directly to electron microscopic studies of WX-infected insects and plants (Nasu, Jensen, and Richardson, 1970), and mycoplasma-like bodies, 200 to 400 nm in diameter, were found in tissues of infected leafhoppers and in infected phloem cells of celery. While direct evidence was not obtained that the observed bodies were, in fact, the causal agent of WXD, it was concluded the observations supported such a hypothesis.

Additional evidence of a mycoplasma-like etiology was provided by demonstration that tetracycline treatment (Ishii, et al., 1967) reduced the titer of the WXDA in both infected plants and leafhoppers (Amin and Jensen, 1971a). Fewer *C. montanus* acquired the agent from treated than from untreated source plants, and when the antibiotic was injected into infected vectors, the latent period increased over that found in control insects. Diseased leafhoppers fed on tetracycline laid more eggs and lived longer than did untreated, diseased insects.

Other workers (Huang and Nyland, 1970; Granett and Gilmer, 1971; MacBeath, et al., 1972; Jones et al., 1974) described mycoplasma-like bodies from tissues of cherry and peach trees, providing additional evidence for a mycoplasma etiology. Nyland (1971) and Nyland and Moller (1973) used field trials to demonstrate that infusion of tetracycline solutions into peach and cherry trees with WXD resulted in remission of symptoms. A detailed review of these early attempts to characterize the WXDA is given by Gold (1979).

Finally, there are reports of culturing helical, motile *Spiroplasma* (a genus of Mycoplasmatales) from X-infected plants (Thompson et al., 1979) and leafhoppers (Raju, Purcell, and Nyland, 1980), but the possibility that the pathogen involved is *S. citri* has yet to be settled. For purposes of this paper, the WXDA will not be further defined.

The following study was undertaken to increase the data base on pathogen-vector relationships among strains of the WXDA and *C. montanus* vectors and to expand comparative transmission work begun by Jensen (1957a, 1969a) using *C. geminatus*, *Euscelidius variegatus*, and *Fieberiella florii*.

MATERIALS AND METHODS

Disease agents

The PYLR strain of WXDA (Jensen 1956), used in all experiments unless otherwise specified, was maintained in celery in greenhouses at Berkeley, California, using bi-monthly transmissions by *C. montanus*. A second strain, presumably similar to the Green Valley (GV) strain (Jensen, 1956) was obtained in 1969 by confining *C. geminatus* and *C. montanus* leafhoppers in sleeve cages for 14–30 days on infected trees in the field before transferring the insects to celery plants in the greenhouse. The GV strain was subsequently maintained in celery.

Vectors

Colladonus geminatus, *C. montanus*, *E. variegatus* and *F. florii* were reared in the greenhouse insectary at Berkeley under ambient light and temperature conditions.

Colladonus geminatus was maintained by caging, on a bi-weekly schedule, ca. 150 noninfected females and ca. 50 noninfected males on healthy celery for 14 days for egg deposition. Adults then were removed and the celery recaged.

Colladonus montanus was used for WXDA transmission tests by Jensen in 1957 (Jensen, 1957b). Since that time, large numbers of this species have been continuously reared under greenhouse insectary conditions. Egg-laying colonies of noninoculative individuals were established on a weekly schedule to provide a continuous supply of all ages for experimental work. Additional colonies of leafhoppers infected with WXDA were maintained by transferring first- or second-stage nymphs to diseased celery for 30 days before being used as potential vectors.

Euscelidius variegatus, was reared on Sierra oats (*Avena sativa* L.), using a 14-day egg-laying schedule. All insects were preconditioned to celery for 7 days before being used in transmission tests for WXDA. Routinely, insect infection was induced by feeding young nymphs on infected celery.

Fieberiella florii used in these studies was collected on California privet (*Ligustrum ovalifolium* Hassk.) and reared on that host for two seasons. A biotype was isolated that reproduced on celery when held under constant light at 25 C. Under these conditions, generation time was ca. 75 days, resulting in several generations each year.

Transmission tests

Celery (var. Utah Green) was used for WXDA source and test plants. Seeds were sown in flats containing vermiculite, held until seedlings were at the three-leaf stage (1 month), and then transplanted into 7.0 cm plastic pots in enriched soil (U.C. Mix No. C, 50% fine sand and 50% peat moss) and kept in the greenhouse until used. Depending on the time of the year, celery seedlings were used as test plants usually within 4 days of transplanting.

Leafhoppers were confined to plants with 5 × 10 cm butyrate-acetate cylindrical cages, the tops of which were covered with organdy. For all tests, one leafhopper per test plant was used. Normally, test insects were transferred to fresh seedlings twice a week, but they could be held as long as 2 weeks on a single plant.

As insects were removed, test plants were fumigated with nicotine, moved to the greenhouse, and sprayed with Guthion® (active ingredient 0,0-diethyl S-[4-oxo-1,2,3-benzotriazin-3(4H)-methyl] -phosphoro-dithioate). Test plants were observed for a

maximum of 60 days for symptom development. Greenhouse temperatures fluctuated between 20 and 30 C.

Test plants were treated with insecticides (nicotine and pyrethrum) on a regular basis to control aphids and whiteflies. Additional fertilizer was used to promote growth during winter months along with the once-daily watering with one-half strength nutrient solution.

Transmission tests were conducted either in the insectary or in environmental chambers, using Sherer-Gillett models CEL 512-317 and CEL 25-7 programmed to fit specific needs of experiments. Attempts were made to standardize the illumination in the chambers at 6500 lux by either adjusting plant bed height or by removing lights. Temperature samples were recorded four times daily.

Insect extracts

Inoculum was prepared from extracts of whole insects and "heads" of *C. montanus* fed on diseased celery for 40 days or longer. "Whole insect extracts" were made by triturating 100 infected leafhoppers/1.5 cc of either 10% sucrose solution (w/w) or tissue culture media (Richardson and Jensen, 1971). The resulting slurry was clarified by centrifugation at 8,000 g for 10 min in a SW 39 rotor. The supernatant was removed and passed through a 0.45 μ Millipore® filter to eliminate bacteria. These procedures were carried out at 3 to 5 C to maintain infectivity (Whitcomb, Jensen and Richardson, 1966b).

With "head extracts" (Sylvester and Richardson, 1971), an infected leafhopper was surface-sterilized with 95% ethanol, and the head capsule teased off with an injection needle containing 5 μ l of 30% sucrose solution (w/w). Contents of the head capsule were triturated in the solution which then was redrawn into the needle. After five heads had been processed, extracts were pooled and mixed on a cold plate before being used for injection.

Infective insects

Feeding and injection were the two methods used to obtain infective insects.

a. **Plant feeding.**—Leafhoppers (2nd to 4th stage nymphs) were aspirated from a colony plant and caged on diseased celery. After an acquisition access period (AAP), diseased plants were cut off and nymphs were transferred to healthy celery.

Colonies were checked routinely for WXDA infection by caging on a large healthy celery for 5 days. Insects were then removed, the plant fumigated with nicotine, sprayed with Guthion® and held in the greenhouse for symptom observation. During experimental work, any abnormally short incubation periods among test insects were taken as a sign of possible colony contamination and triggered additional testing of control insects.

b. **Membrane feeding.**—Membrane feeding was done at room temperature under artificial light unless otherwise indicated. Acquisition was assayed by transferring insects to test celery plants. Membrane-feeding cages were constructed by covering the ends of perspex tubing (3.2 cm dia \times 1.5 cm high) with nylon gauze. A hole, plugged with a cork, in the side of each cage allowed access. Feeding rings, of perspex tubing of the same dimension, were prepared by stretching Parafilm® over the polished end of the ring. A feeding sachet (Mittler and Dadd, 1964) was formed by placing 0.2 ml of feeding solution on the membrane and spreading the droplet with another thinly stretched membrane through which leafhoppers fed.

Leafhoppers were aspirated from pathogen-free stock colonies, placed in each cage,

and the feeding-ring sachet positioned. To attract insects to the membrane, light transmitted through a celery leaf disc placed on the outer membrane was used.

When a perspex cold plate was used to lower the temperature of feeding solutions, the nylon gauze at one end of the cage was removed so that feeding solutions were in direct contact with the cold surface of the plate. The cold plate was constructed of sheets of perspex $37 \times 37 \times 0.6$ cm bonded to a frame to form a flat, enclosed chamber through which ice water was circulated with a low pressure pump. The temperature of feeding solutions could be maintained several degrees below ambient. The cold plate could be placed either above or below cages, and in all tests light transmitted through a green acetate filter placed on the plate was used to attract insects to the membrane.

Feeding solutions were made in a cold room (4 C) by triturating ca. 100 *C. montanus*, fed on WX-diseased celery for at least 40 days, in 1.5 cc of 10% sucrose solution (w/w), then centrifuging at 8,000 g in a SW 39 rotor for 10 min. Clarified supernatant was removed, centrifuged for 60 min at 100,000 g, and the pellet resuspended in 10% sucrose and filtered through 0.80 and 0.45 μ Millipore® filters.

Radioactive phosphorus ($\text{NaH}_2\text{P}^{32}\text{O}_4$), used to measure intake, was added to feeding solutions at a final concentration of 2.5×10^{-2} mc/ml. After access to labeled solutions, the entire cage containing the insects was read, both top and bottom, for radioactivity with an end-window Geiger-Muller tube attached to a scaling unit. The P^{32} activity was expressed in counts/min; all measurements were corrected for background and isotope decay. Amounts ingested were estimated ($\mu\text{l/hr}$) by comparing average counts/min/cage with a standard volume of labeled feeding-solution.

Infective WXDA in the feeding sachets was assayed by injecting leafhoppers. Sampling was done during preparation, as well as during feeding periods to determine the inactivation rate of WXDA.

In two experiments the pellet from the high speed centrifugation was resuspended in 5% sucrose, placed on top of a sucrose gradient (5 to 50%), and centrifuged 150 min at 65,000 g in a SW 25.1 rotor. Sixteen 2-cc samples of the gradient were collected, the sucrose concentration in each read with a refractometer and adjusted to a final sucrose concentration of 10% to make feeding sachets.

Injection

Inoculum was injected into last-stage nymphs with glass needles double-pulled from 3 mm OD soda glass tubing. Two injection techniques were used. In one, inoculum was drawn into needles with a vacuum generator. Once loaded, they were connected to a compressed air outlet with a T-system of tubing. Closure of the open end of the T-outlet forced inoculum out of the needle. During injection, the tip of the needle was inserted through the intersegmental membrane in the mesal surface between the thorax and the first abdominal segment of anesthetized (CO_2) insects. In this "continuous injection" one to 20 leafhoppers were injected from the original amount of inoculum in the needle. Needles, calibrated by weighing leafhoppers before and after injection, delivered an estimated $0.37 \pm 0.15 \mu\text{l}$ of inoculum/insect.

The other type of injection used a single dose of inoculum ($0.06 \pm 0.034 \mu\text{l/insect}$) consisting of the quantity drawn by capillarity into the needle tip when it was dipped into an inoculum droplet kept on a plate cooled with ice. The dosage delivered was ca. one-sixth of the amount delivered by the continuous injection method. The mean weight of an injected insect was 2.58 mg; thus single-dose inoculation resulted in a 40-fold dilution of original inoculum, compared to a six-fold dilution in the continuous injection method.

Serial passage of WXDA

Each passage of WXDA was done using single-dose injections of head extracts from five potentially infected leafhoppers into ca. 100 *C. montanus* nymphs. After 28 days, five injected leafhoppers were selected at random and used to make extract for the next passage. The procedure was repeated for seven passages over a 7-month period, resulting in an estimated 2×10^{-24} dilution of original inoculum.

Passages were "blind," because transmission results from one passage were not available until after the next passage was made.

"Tetracycline extraction medium" was made by dissolving 0.2 mg of hydrochloride tetracycline (100%) in 10 cc of 30% sucrose solution (w/w). The medium was used to control contaminating bacteria and to select for possible tetracycline-resistant strains of WXDA.

Excretion measurements

Excretion was used as an estimate of feeding (ingestion) activity. Honeydew droplets were caught on filter paper liners of 7.5 cm high cages made from 5 cm OD extruded butyrate-acetate tubing. Lined cages were prepared in advance of actual use to facilitate rapid changing. The bottom of the cage was enclosed by fitting two, notched filter-paper pieces around the stem of test celery. Cage tops were covered with 6 cm unlined plastic petri dishes to allow observations of leafhoppers during transfer.

Leafhoppers were transferred to fresh test plants at 6-hr intervals. All tests were done at 25 C and constant light. Exposed liners were developed in 1% ninhydrin-ETOH solution, drained, and dried in a warm oven. Purple excretion spots were counted as were the number of spot clusters (Smith, 1937; Auclair, 1958; Mittler, 1958; Banks and Nixon, 1959; Sylvester, 1967). The procedure gave data on excretion rates and changes in feeding sites.

Honeydew volume from *C. montanus* was measured by collecting droplets in light mineral oil contained in siliconized petri dishes. Leafhoppers were caged over the dishes with a porous screening, small enough to retain the insects but large enough to allow passage of the droplets. Droplets were measured with a calibrated ocular micrometer and the average volume/droplet calculated (Mittler and Sylvester, 1961). The product of mean droplet size (petri dishes) and mean number of droplets (filter paper) gave an estimate of the mean volume of liquid removed from test plants by leafhoppers in a 6-hr period.

Electrophoresis

Protein separation in WXDA extracts was attempted by electrophoresis. A slurry was made by triturating 1.8 g of 30- to 40-day infected *C. montanus* adults in 5 cc of phosphate-buffered (0.005 M) 10% sucrose solution (w/w), clarified at 2,000 g for 10 min in a SW 39 rotor, and the supernatant concentrated by centrifugation onto a 60% sucrose shelf at 100,000 g for 60 min in a SW 39 rotor. Samples were collected by puncturing the centrifuge tube wall with a hypodermic needle at the level of the shelf and withdrawing only pelleted material. The pellet was in 40% sucrose with a volume of 2 cc at pH 7.0. The procedures were done at 4 C.

A density-gradient electrophoresis apparatus was prepared in advance so that the WXDA concentrate could be processed rapidly. The procedure used followed van Regenmortel's (1964) except during the run the column was surrounded by an ice-water jacket to maintain a temperature of 7 C. The buffered 5 to 40% sucrose gradient was con-

tinuous and added hydrodynamic stability, opposing both thermal convection and sampling disturbances.

A 2 cc WXDA sample was injected into the base of the column (origin), and a current of 4.0 milliamp and 800 volts applied for 20 hr. Samples were removed from the column in 2 ml aliquots. For the bioassay of activity, 1 ml of every third aliquot was diluted 5:1 with tissue culture medium (Richardson and Jensen, 1971) and filtered through a 0.45 μ Millipore®. The remaining 1 ml portion of the aliquot was adjusted to 10% sucrose and made into sachets for membrane feeding.

For the bioassay, leafhoppers were fed on sachets or *C. montanus* nymphs were injected. In all, 14 groups were injected to test for the presence of WXDA up the column. Following injection or feeding, nymphs were caged as groups for 15 days on healthy celery. On the fifteenth day, 20 adults were individually caged on plants and tested for transmission.

Statistical tests

Data were analysed using standard statistical procedures, i.e., Chi square (Yates adjustment for continuity as needed), t , and F tests. Estimates of relative concentrations of WXDA in insect extracts were calculated by plotting a probit transformation of the cumulative number of first transmissions against time (normal or log transformed) to estimate the median latent period (LP_{50}) (Sylvester, 1965). Transmission was assumed to have occurred at the midpoint of a given inoculation access period (IAP).

Median survival period (SP_{50}) and the median acquisition access period (AAP_{50}) were estimated using a similar procedure, with appropriate transformations to achieve linearity being used as needed.

Transmissions were assumed to be separate and independent events with a constant probability of occurrence in test intervals used. Theoretical expected values for probability of transmission per unit of access time were calculated using binomial expansion $(P + Q)^N$ in which P = probability of transmission, $Q = 1 - P$, and N is the number (Storey, 1938).

RESULTS AND DISCUSSION

Availability threshold

The availability threshold period (Sylvester, 1948) (= minimum incubation period of Severin (1921) and Maramorosch (1953), of WXDA, i.e., the time after inoculation that a disease agent can be acquired by feeding noninfective vectors, has not been established.

Availability of WXDA to *C. montanus*, as a function of the age of the infection in celery, is given in Table 1. The proportion of insects transmitting WXDA increased, and the LP_{50} decreased, as the age of infection increased, with the exception in Trial II where there was a decrease in the proportion of insects acquiring, during the last plant age interval (43 to 50 days). But here, a near minimum LP_{50} value suggested that while these host plants may have been a relatively poor food source, due to wilting and general decline, titer of WXDA was not appreciably lower. No significant evidence was found of heterogeneity among multiple source plants within various age groups, but results indicated that celery plants of similar age should be used when doing comparative work.

Plant symptoms appeared at relatively the same age of infection in both trials, and the

TABLE 1.
 AVAILABILITY THRESHOLD OF WXD-AGENT IN CELERY (*APIUM GRAVEOLENS*)
 TO *COLLADONUS MONTANUS* LEAFHOPPER VECTORS. INDIVIDUAL
 LEAFHOPPERS WERE TESTED BY TRANSFERRING TO FRESH TEST PLANTS
 TWICE WEEKLY UNTIL DEAD.

| Trial | Age of plant infection | Number of insects | | | Median latent period (LP ₅₀) |
|-------|---------------------------|-------------------|-----------|---------|---|
| | | Tested | Infective | | |
| | days | number | number | percent | days |
| I | 34—41* | 48 | 27 | 56 | 30 |
| | 42—48 | 49 | 30 | 61 | 29 |
| | 49—55 | 50 | 46 | 92 | 27 |
| II | 22—29* | 60 | 8 | 13 | 32 |
| | 30—36 | 42 | 26 | 62 | 31 |
| | 37—43 | 60 | 51 | 85 | 28 |
| | 44—50 | 60 | 39 | 65 | 28 |

* Interval during which symptoms appeared on the virus source plant.

results in Trial II demonstrated that leafhoppers could acquire WXDA before completion of the incubation period (29 to 36 days). However, the best time to use inoculated celery for a source of WXDA appeared to be shortly after onset of symptoms, i.e., 30 to 40 days after inoculation. These plants would support high populations of leafhoppers for several days, and provided titers sufficiently high for at least 50 per cent of *C. montanus* leafhoppers to acquire WXDA during a 1-week acquisition access period (AAP).

Acquisition and transmission as a function of age

Variation in age-specific acquisition and transmission of plant pathogens has been reported frequently. The extreme example is where one stage lacks the capacity to acquire a pathogen, although it may transmit it if acquisition occurred in a prior stage and transstadial passage took place, such as in the case of spotted wilt virus transmission by thrips. Acquisition is restricted to larval stages, but adults are infective (Bald and Samuel, 1931; Sakimura, 1962).

Most transmission work on WXDA, whether infectivity was established by feeding or by injection, routinely involved nymphs (Jensen, 1957a; Whitcomb, Jensen, and Richardson, 1966a). Age-specific transmission rates, however, have not been determined.

TABLE 2.
 TRANSMISSION OF WXD-AGENT FROM AND TO CELERY BY, AND MEDIAN
 LATENT PERIOD (LP₅₀) IN, *COLLADONUS MONTANUS* AT 25 C AND CONSTANT LIGHT.
 INDIVIDUAL INSECTS WERE TESTED BY TRANSFERRING TO FRESH TEST PLANTS
 TWICE WEEKLY UNTIL DEAD.

| Acquisition access (AAP) | Instar stage | Number of insects | | | LP ₅₀ |
|--------------------------------|-----------------|-------------------|-----------|---------|------------------|
| | | Tested | Infective | | |
| days | | number | number | percent | days |
| 7 | 2—3 | 30 | 22 | 70 | — |
| | Adult | 30 | 29 | 96 | — |
| 18 | 1—2 | 30 | 19 | 63 | 29 |
| | 3—4 | 30 | 23 | 77 | 27 |
| | 5 | 30 | 26 | 87 | 25 |
| | Adult | 30 | 27 | 90 | 25 |

Table 2 shows acquisition and transmission of WXDA by various age groups of *C. montanus*. In Trial I, more adults became infective than did 2nd to 3rd stage nymphs (97% versus 73%, respectively, adj $\chi^2 = 4.71$, df 1, $p < 0.05$). A similar trend occurred in Trial II comparing four instar groups. Here the rate of transmission increased from 63 to 90 percent as a function of increasing age ($\chi^2 = 7.83$, df 3, $p < 0.05$), with a decreasing difference between successive instar groups. Decrease in the LP_{50} also corresponded with age groups. These differences perhaps reflected an increased feeding rate associated with growth, or the rate of multiplication of the WXDA increased with larval age.

Transmission by two biotypes of *C. montanus*

Efficiency of transmission of viruses by insect vectors has been demonstrated frequently to be under genetic control, ranging from inactive (nontransmitting) races (Storey, 1932) to selections or biotypes varying considerably in transmission efficiency (Bennett and Wallace, 1938; Black, 1943; Stubbs, 1955; Björling and Ossiannilsson, 1958; Rochow, 1963; Röchow and Eastop, 1966; Watson and Okusanya, 1967).

Jensen (1969a) compared transmission of WXDA by *C. geminatus*, *C. montanus* and *E. variegatus*. *Colladonus montanus* consistently was the most efficient vector to celery. He hypothesized continual selective pressure resulted in isolation of both a *C. montanus* biotype and a strain of WXDA that gave efficient transmission in laboratory tests. In the present work, two California biotypes of *C. montanus*—Berkeley (insectary reared) and Dixon (recently field-collected from alfalfa) were directly compared (Table 3).

TABLE 3.
INSECTARY STUDIES OF ACQUISITION AND TRANSMISSION OF WXD-AGENT FROM AND TO CELERY BY, AND LP_{50} IN, TWO BIOTYPES OF *COLLADONUS MONTANUS*. INDIVIDUAL LEAFHOPPERS WERE TESTED BY TRANSFERRING TO FRESH TEST PLANTS TWICE WEEKLY UNTIL DEAD.

| Time of 21-day trial | Biotype | Number of insects | | | LP_{50} |
|----------------------|----------|-------------------|--------|-----------|-----------|
| | | Tested | | Infective | |
| | | number | number | | |
| Sept. | Berkeley | 80 | 26 | 33 | 28 |
| | Dixon | 50 | 15 | 25 | 35 |
| Dec. | Berkeley | 90 | 28 | 31 | 29 |
| | Dixon | 90 | 14 | 16 | 38 |

In both trials, the Dixon biotype transmitted at ca. one-half the rate of the insectary-reared Berkeley biotype (adj $\chi^2 = 3.91$ and 5.25, with 1 df, and $p < 0.05$ in Trials I and II, respectively). The two biotypes also could be differentiated by the LP_{50} values, indicating that they either acquired different doses of WXDA, or the agent multiplied at a different rate in each biotype.

There was some evidence that WXDA was not as readily available to leafhoppers in the second, as in the first trial (23% versus 34% transmission, respectively, adj. $\chi^2 = 3.69$, df 1, $p < 0.10$). These insectary trials were conducted at different times of the year.

Again, as suggested by Jensen (1969a), continual rearing of *C. montanus* in the insectary at Berkeley may have resulted in selection of a biotype that more efficiently transmitted WXDA from celery to celery than insects collected in field situations. Although the Dixon biotype was reared on celery under insectary conditions for approximately 1 year, differences in vector competency between it and the Berkeley biotype were apparent.

Comparison of two isolates of WXDA

Jensen and Thomas (1954, 1955) observed that Napa Valley (NV) and GV strains of WXDA were less severe in infected peach trees and perhaps were acquired and transmitted less efficiently by leafhopper vectors than the PYLR strain. The PYLR and GV strains exhibited interference in grafting tests (Nyland, 1955). In the present work, acquisition, transmission and survival rates of vectors fed on celery infected with either PYLR or GV strains of WXDA were compared (Table 4 and Figs. 1 to 3).

TABLE 4.

ACQUISITION OF WXD-AGENT FROM INFECTED CELERY BY, AND MEDIAN SURVIVAL PERIOD (SP₅₀) IN, *COLLADONUS MONTANUS* FED UNDER INSECTARY CONDITIONS. INDIVIDUAL LEAFHOPPERS WERE TRANSFERRED DAILY TO FRESH CELERY PLANTS FOR 50 DAYS, THEN WEEKLY FOR 125 DAYS.

| AAP days | Number of insects | | | | SP ₅₀ | |
|-------------|-------------------|--------------------|---------|-----------|-------------------|----------------------|
| | Tested number | Infective | | Expected* | Infective days | Noninfective days |
| | | Observed number | percent | | | |
| 1 | 40 | 9 | 22.5 | 22.5 | 60.4 | 97.5 |
| 3 | 20 | 12 | 60.0 | 53.5 | 60.7 | 86.8 |
| 7 | 19 | 15 | 79.0 | 83.2 | 63.9 | 84.0 |
| 14 | 20 | 19 | 95.0 | 97.2 | 62.3 | 70.0 |
| 28 | 20 | 20 | 100.0 | 99.9 | 62.0 | — |

* Expected values were based upon a binomial assumption of an equal probability of acquisition per day.

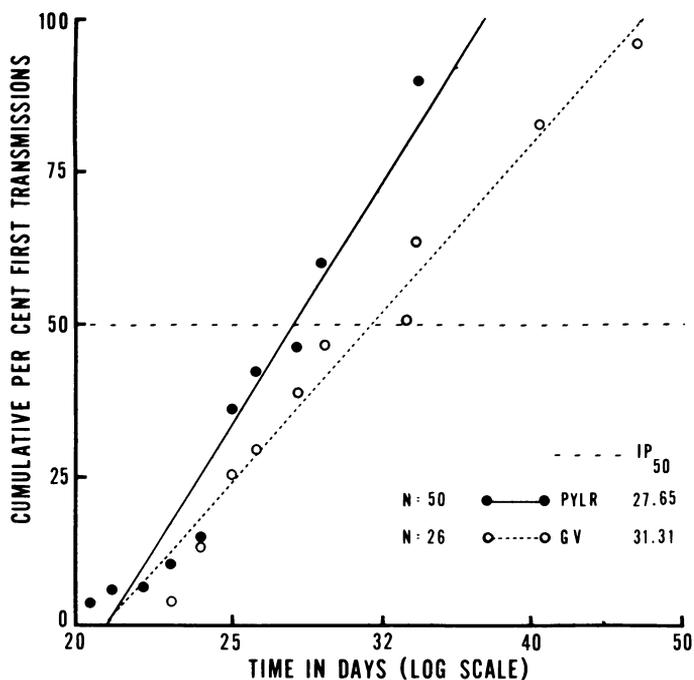


Fig. 1. Incubation period of peach yellow leaf roll (PYLR) and Green Valley (GV) strains of WXD-agent in *Colladonus montanus* leafhoppers.

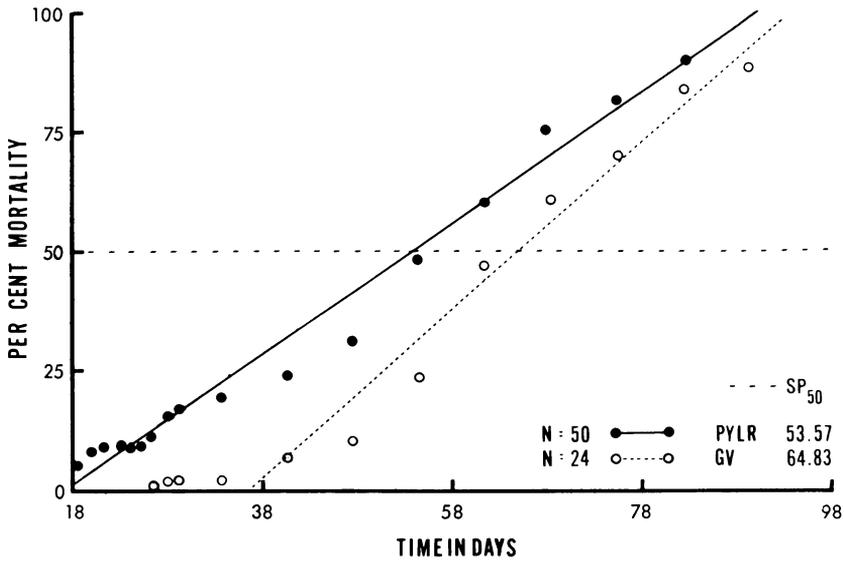


Fig. 2. Survival of *Colladonus montanus* leafhoppers infected with either peach yellow leaf roll (PYLR) or Green Valley (GV) strain of WXD-agent.

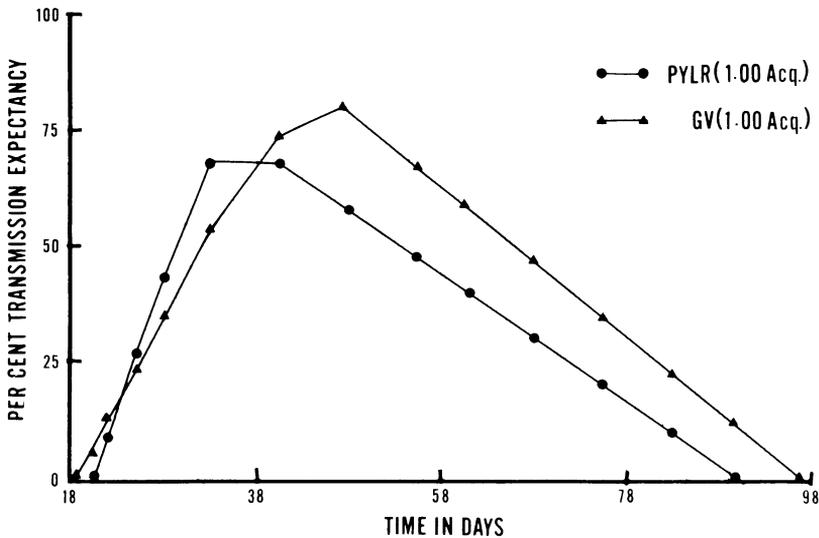


Fig. 3. Transmission expectancy curves for the transmission to celery of peach yellow leaf roll (PYLR) and Green Valley (GV) strains of WXD-agent by infective *Colladonus montanus* leafhoppers. Points were calculated as the product of the age-specific transmission and the age-specific survival rates.

The GV isolate was acquired and transmitted less efficiently by the biotype of *C. montanus* than was the PYLR isolate (24/90, 27% versus 50/90, 56%, adj $\chi^2 = 14.35$, df 1, $p < 0.01$). The GV isolate also had a longer LP₅₀ compared to that for the PYLR isolate (31 versus 28 days, respectively, Fig. 1), suggesting that lower titers were available in celery infected with the GV isolate.

Jensen (1959) reported the longevity of infected leafhoppers was reduced by infection with WXD. Insects fed on plants infected with the PYLR isolate did not live as long as those given an AAP on celery infected with the GV isolate (Fig. 2). The 3-day difference between SP₅₀ values for leafhoppers that actually transmitted either isolate was not as great as the 22-day difference in SP₅₀ values between subsets of leafhoppers that

did not transmit. One possible reason could be that a significant mortality among those leafhoppers fed on the PYLR isolate occurred before they became infective.

Transmission expectancy curves, a product of age-specific transmission and mortality rates (Sylvester and Richardson, 1966) for PYLR and GV isolates are shown in Figure 3. The transmission expectancy curve for PYLR isolate peaked earlier and began to decline sooner than that for GV isolate, again perhaps reflecting a greater pathogenicity of the PYLR isolate for the vector.

Daily acquisition rates of WXDA by *C. montanus*

It is generally assumed that probability of acquisition of plant disease agents by vectors increases as a function of length of the AAP, nonpersistent aphid-borne viruses perhaps excepted. While some viruses can be acquired by leafhoppers in brief periods, i.e., 15 sec to 1 min (Storey, 1938; Bennett and Wallace, 1938), acquisition of yellows disease agents is generally more prolonged. Jensen (1953a) reported only one out of 64 *C. geminatus* leafhoppers acquired WXDA from peach after a 1-day AAP, whereas nine out of 65 transmitted following a 10-day AAP.

Preliminary tests to estimate daily acquisition rates of WXDA by *C. montanus* have been given by Whitcomb, Jensen, and Richardson (1966a). In the present work, data given in Table 4 show transmission of WXDA by *C. montanus* increased with increasing length of the AAP on infected celery. Median acquisition access period (AAP₅₀), when 50% of the insects tested acquired the WXDA, was estimated to be approximately 2.5 days.

If probability of acquisition per day is constant, then binomial expansion can be used to estimate the expected number of vectors to be realized in any given AAP. This uses the relationship $P = 1 - Q^n$, in which P is the probability of transmission per unit of acquisition access time and n is the number of units of acquisition access time, in this case the length of the AAP in days. Table 4 indicates that approximately 22% of the insects acquired WXDA per day of acquisition access.

SP₅₀ values for inoculative insects that had a 1-day AAP was 60 days, compared to 98 days for those insects in the sample that failed to transmit. Again the data indicate that infection with WXDA reduces longevity of *C. montanus* (Jensen, 1959). Only one day was spent on diseased plants, and thus there was little opportunity for factors other than acquisition of WXDA to be responsible for reduced longevity of inoculative individuals.

As the AAP increased, a reduction occurred in the SP₅₀, even for nontransmitting insects (Table 4). With longer feeding periods on WXD-celery, there may be unfavorable nutritional effects, or again, some insects may have been infected, but died before becoming infective.

Inoculation threshold period of WXDA and *C. montanus*

Inoculation of pathogens by vectors, as contrasted to acquisition, generally is accomplished in relatively brief periods. Minimums reported in the literature vary from seconds (nonpersistent viruses) to minutes, and include viruses as well as yellows agents (Severin, 1931; Storey, 1938; Bennett and Wallace, 1938; Watson, 1940; Maramorosch, 1953; Sylvester, 1954).

Whitcomb, Jensen, and Richardson (1966a) believed transmission of WXDA by *C. montanus* followed a binomial expectancy, and transmission rates were calculated for inoculation access periods (IAP) varying from 1 to 4 days. However, no reports have been made for the inoculation threshold period of WXDA.

Results of tests to determine the IAP for WXDA in celery using *C. montanus* as vectors

are given in Table 5 and Figure 4. Increasing the IAP increased transmission rates. During short test-IAPs, transmission did not follow a binomial expectancy as did previously reported daily transmission rates (Whitcomb, Jensen, and Richardson, 1966a). Probability of transmission was higher on a per-hr basis during the first hour of inoculation access than in subsequent test intervals (Table 6). A theoretical inoculation curve, based upon the regression $Y = 40.44 X^{0.19}$, in which Y is per cent transmission and X is IAP in hours, is given in Figure 4. Extrapolation suggests inoculation could occur in one second, a value exceedingly difficult to evaluate experimentally. Rarely do insects begin to feed immediately after being transferred to a test plant. The IAP_{50} was estimated to be ca. three hours.

TABLE 5.
TRANSMISSION OF WXD-AGENT BY INFECTIVE *COLLADONUS MONTANUS* AND MEDIAN INCUBATION PERIODS (IP_{50}) IN CELERY USING VARYING INOCULATION ACCESS PERIODS (IAP), AT 25 C AND CONSTANT LIGHT. CANDIDATE VECTORS WERE REARED ON DISEASED CELERY AND PRETESTED FOR INFECTION.

| IAP hours | Number of insects | | | IP_{50} days |
|--------------|-------------------|---------------------|----------------------|-------------------|
| | Tested number | Infective number | Infective percent | |
| 1 | 15 | 6 | 40 | 42 |
| 2 | 17 | 8 | 47 | 40 |
| 4 | 17 | 9 | 53 | 39 |
| 8 | 17 | 10 | 59 | 40 |
| 16* | 17 | 7 | 41 | 40 |
| 32 | 18 | 12 | 67 | 40 |

* The 16-hr inoculation access period was begun at 1700 hours, the others were begun at 0900 to 1000 hours.

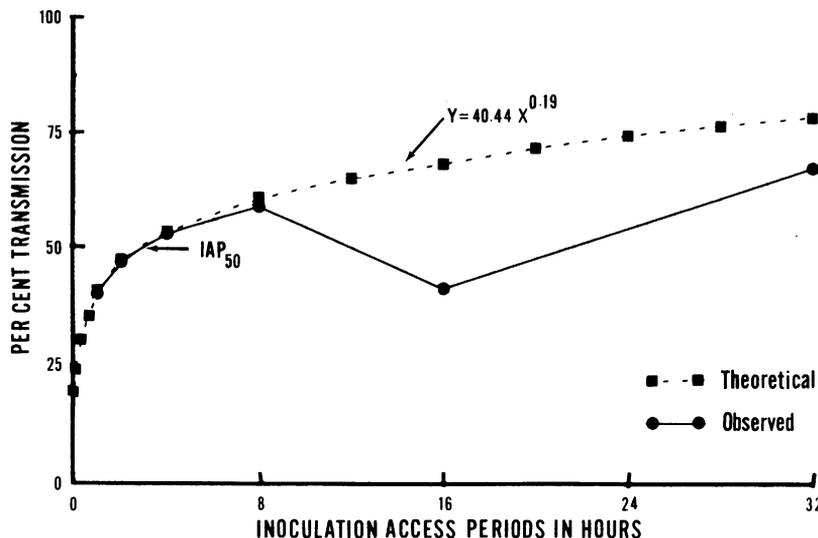


Fig. 4. Observed and theoretical inoculation curves for transmission of WXD-agent (WXDA) by infective *Colladonus montanus* leafhoppers to celery. The theoretical curve was calculated using the equation $Y = 40.44X^{0.19}$, in which Y = probability of transmission expressed as per cent, and X = the inoculation access period (IAP) in hr.

The regression of Figure 4 was based on transmission values realized from IAPs of 1 through 8-hr. There was an obvious drop from expected rates of transmission during periods of 16 and 32 hr. This depression was believed to have been due to circadian rhythm. The 16-hr IAP was begun at 1700 hr (5 P.M.) so all tests would end about the same time. Insects were reared in the insectary, and apparently maintained a normal circadian rhythm that was reflected in reduced feeding and transmission during evening and night hours. The expected transmission curve suggests an equivalent of 15 hr of effective inoculation time was lost during the night.

Endogenous rhythms and the transmission of WXDA

Reduced transmission in evening and night hours (Table 5) indicated operation of circadian rhythms in transmission of WXDA by *C. montanus*. To further test for such rhythms, infective leafhoppers were first tested for 3 days to establish their infectivity and then given the following sequence of IAPs: 12 hours in a growth chamber (constant light and 25 C), 24 hours in the insectary, 12 hours in a growth chamber, and finally 12 hours in the insectary. One lot of insects began the sequence in a growth chamber in the morning, while a second lot began in the evening.

The results are given in Table 6. Insects given a 12-hr IAP beginning in the evening (1800 hr) transmitted less frequently than insects tested in the morning (0600 hr) (47% versus 86%, adj. $\chi^2 = 17.61$, df 1, $p < 0.01$). Overall, insects reared and infected with WXDA in the insectary transmitted at reduced rates in evening hours, regardless of whether tests were conducted in the insectary under fluctuating light and temperature conditions, or in environmental chambers under constant temperature (25 C) and light conditions.

TABLE 6.

EFFECTS OF ENTRAINED RHYTHMS ON TRANSMISSION OF WXD-AGENT TO CELERY BY INFECTIVE *COLLADONUS MONTANUS* TESTED UNDER INSECTARY CONDITIONS OR AT 25 C AND CONSTANT LIGHT.

| Trial* | Feeding sequence | | | Number of insects | | |
|--------|------------------|-----------|----|-------------------|--------------|---------|
| | Location | Time | Hr | Tested | Transmitters | |
| | | | | number | number | percent |
| I | Chamber | 6 AM—6 PM | 12 | 21 | 17 | 81 |
| | Insectary | 6 PM—6 PM | 24 | 21 | 19 | 90 |
| | Chamber | 6 PM—6 AM | 12 | 21 | 8 | 38 |
| | Insectary | 6 AM—6 PM | 12 | 21 | 18 | 86 |
| II | Chamber | 6 PM—6 AM | 12 | 16 | 8 | 38 |
| | Insectary | 6 AM—6 AM | 24 | 16 | 16 | 100 |
| | Chamber | 6 AM—6 PM | 12 | 16 | 15 | 95 |
| | Insectary | 6 PM—6 AM | 12 | 19 | 9 | 56 |

* Reverse schedule trials were done at the same time. Infective insects were given four successive alternating inoculation access periods first in a growth chamber, at 25 C and constant light, and then in the insectary with varying light and temperature. The inoculation access period in the insectary following the initial period in the growth chamber was 24 hr, rather than 12 hr, to permit the insects to readjust to their normal daily rhythm.

Entrained circadian rhythms and the transmission of WXDA

Circadian rhythms are well documented in biological systems, being considered necessary for organisms to adapt to changing environmental conditions (Hamner and Hoshizaki, 1974). The role of such rhythms in virus transmission has received minor experimental emphasis, although seasonal and daily fluctuations in transmission efficiency by aphids had been noted (Watson and Roberts, 1939; Chalfant and Chapman, 1962; Cook and Sylvester, 1964). Reproducible peaks in efficiency have been noted in

transmission of aster yellows agent by *Macrosteles fascifrons* leafhoppers (Maramorosch, 1964).

The following experiments give additional evidence for rhythms in inoculation phases of WXDA transmission by *C. montanus*.

Initially, infective leafhoppers were entrained for 10 days to a 12-hr diurnal cycle in a growth chamber set at 25 C. Individual insects then were tested by transferring them to fresh test plants every 6 hr using a 12:12 hr light:dark cycle (four transfers) followed by continuous light for 48 hr (eight transfers). Excretion records were kept as a measure of feeding activity.

Results are given in Figure 5. Transmission and excretion followed similar patterns. Correlation between transmission and excretion activities ($r = 0.40$) was minor, indicating the former was not a simple function of the ingestion, a result noted in connection with virus transmission (Sylvester, 1948; Magyarosy and Sylvester, 1979).

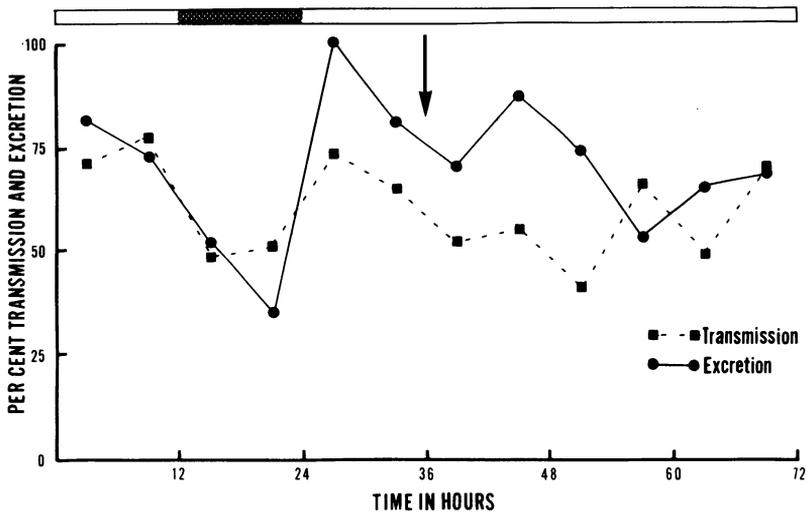


Fig. 5. Excretion (per cent of a maximum rate of 27 droplets/6-hr) of, and WXD-agent transmission by, 30 individual infective *Colladonus montanus* leafhoppers during successive 6-hr inoculation access periods on celery. Leafhoppers were preconditioned for a 12:12 hr light-dark (LD) for 10 days before testing. The L phase began at 0600 hr and the D phase at 1800 hr. The 12:12 LD cycle was maintained during testing until, at the arrow, a continuous light regime was begun (24 LL). The bar at the top of the figure indicates light conditions during the 6-hr test periods.

During the dark-period that followed entrainment, transmission and excretion dropped, and cycles of both events occurred thereafter—even though the insects were under constant light and temperature conditions. However, the period between oscillations (the rhythm) advanced.

Mean number of excrement droplets for leafhoppers that transmitted during the test period was 20.6 ± 11.5 ($N = 196$), compared to 17.8 ± 11.8 ($N = 128$) for those that did not transmit ($t = 2.09$, $df 322$, $p < 0.05$).

In a second experiment, test leafhoppers again were entrained with a reverse 12-hr diurnal cycle, i.e., photophase began at 1800 hr instead of 0600 hr. Insects were preconditioned with this reverse cycle for 40 days, then tested using a sequence identical to that used previously. However, no excretion record was kept.

As before, transmission was highest at the beginning of the first photophase and lowest during the 12-hr of dark (Fig. 6). The periodicity was repeated, again with evi-

dence of advancement in time. In the final 12-hr period of testing there were indications of the development of an arrhythmic "free running" pattern.

In a final test (Fig. 7), an arrhythmic condition was induced by entraining infective

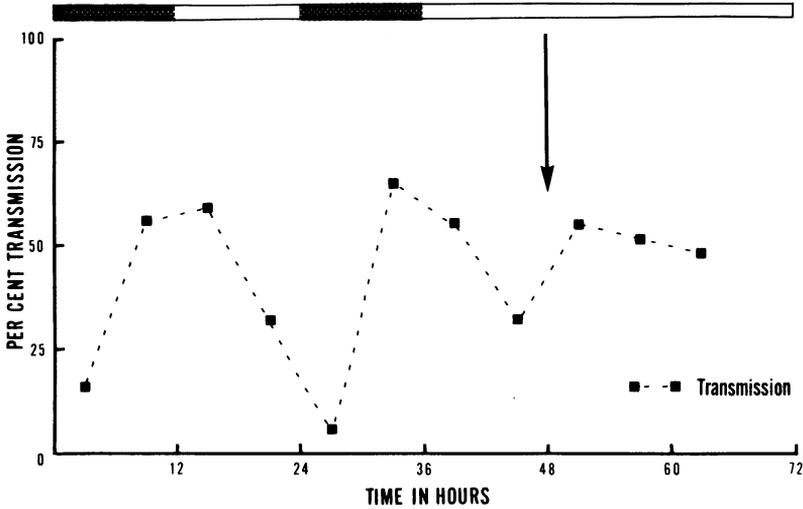


Fig. 6. Transmission of WXD-agent by 30 single infective *Colladonus montanus* leafhoppers during successive 6-hr inoculation access periods on celery. Leafhoppers were preconditioned for 40 days to a 12-hr dark: 12 hr light (12:12 DL) cycle prior to testing. The D cycle was begun at 0600 hr and the L cycle at 1800 hr (reverse of that used in Fig. 5). The 12:12 DL cycle was maintained for six test periods after which a continuous light regimen (24 LL) was begun (arrow). The bar at the top of the figure indicates light conditions during testing.

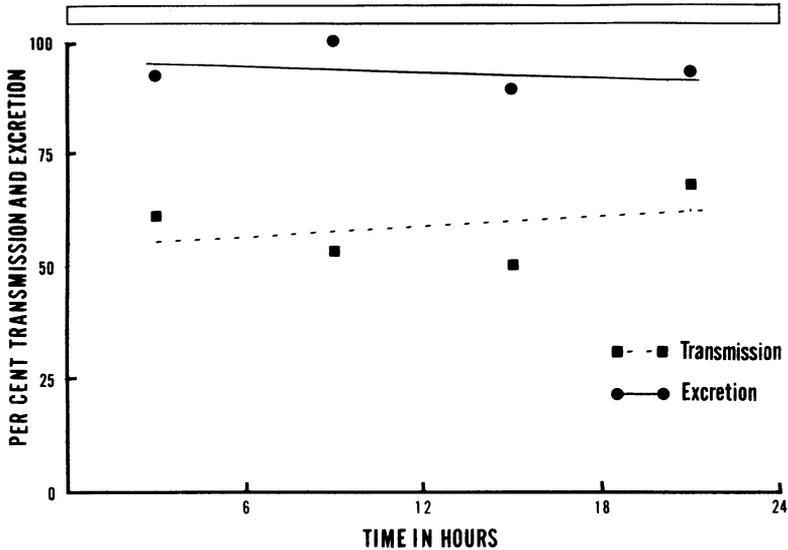


Fig. 7. Excretion (per cent of a maximum of 19 droplets/6-hr) and WXD-agent transmission rates by 36 single infective *Colladonus montanus* leafhoppers during successive 6-hr inoculation access periods on celery. Light conditions were constant (24 LL), as indicated by the bar at the top of the figure. Insects were preconditioned by a 4-day exposure to 24 LL prior to testing.

leafhoppers to constant light for 4 days then transferring each individual over a sequence of four test celery plants at 6-hr intervals at 25 C and constant light. Excretion was monitored. No significant evidence for heterogeneity occurred in transmission or excretion ($\chi^2 = 2.09$, $df\ 3$, $p > 0.50$, and $F = 0.23$, $df\ 3/150$, $F_{0.05} = 2.67$, respectively). Both activities remained relatively constant over the four test intervals. Mean excretion rates per test interval for transmitting and nontransmitting insects were 16 ± 11 and 18 ± 11 droplets, respectively. Evidence for a difference was not significant.

Overall, the data indicated a diurnal cycle affected both transmission of WXDA and excretion by *C. montanus*. The highest level of each activity occurred during light phases of the cycle. It was possible to entrain insects to the reverse (Fig. 6) of the normal (Fig. 5) light:dark cycle, as well as to an arrhythmic pattern. Entrained circadian rhythms were retained for a short period under constant light with an advance in periodicity and a tendency to become arrhythmic.

Correlation between excretion and transmission was low. Excretion is an indirect measure of ingestion; transmission, in these experiments, did not correlate with ingestion activity. Thus, number of penetrations made, rather than total time spent in feeding, probably would have been a more relevant measure. However, if one were interested in maximizing transmission efficiency during access periods of less than 24 hr, pre-conditioning and testing insects under conditions of constant light are indicated. The possible confounding role of plant and pathogen rhythms was not investigated.

Effect of fluctuating temperature on transmission

Jensen (1968, 1972) reported constant temperatures of 38 to 41 C inactivated WXDA in *C. montanus*. He also found the rate of transmission was greater if insects were tested in an environment whose mean temperature was not constant, but rather an average value of a temperature cycle (Messenger, 1964). In the present work, additional tests were done on the influence of fluctuating temperatures on the multiplication and transmission of WXDA in *C. montanus*.

Colladonus montanus nymphs, injected with WXDA, were divided into three groups. Groups were held in an environmental chamber programmed for a temperature regimen of 16 to 24 C, 12 to 28 C, or 20 C, respectively, all with an average of 20 C and constant light. Results of the trials are given in Table 7.

Transmission and LP_{50} values were similar for all groups, with no significant evidence of heterogeneity among treatments, or between trials ($adj\ \chi^2 = 0.002$, $df\ 1$,

TABLE 7.

EFFECT OF CONTROLLED, 12-HR TEMPERATURE CYCLES ON TRANSMISSION OF WXD-AGENT TO CELERY BY, AND LP_{50} IN, *COLLADONUS MONTANUS* INJECTED WITH WXDA EXTRACT. INDIVIDUAL LEAFHOPPERS WERE TESTED BY TRANSFERRING TWICE WEEKLY UNTIL DEAD TO FRESH TEST PLANTS.

| Trial | Temperature C | | | Number of insects | | | LP_{50} |
|-------|--------------------------|-------------------|------|-------------------|---------------|----------------|-------------|
| | Low | High | Ave. | Tested | Infected | | |
| | | <i>Centigrade</i> | | <i>number</i> | <i>number</i> | <i>percent</i> | <i>days</i> |
| I | 16.5 ± 0.9 | 24.3 ± 0.8 | 20.4 | 40 | 28 | 70 | 26 |
| | 12.1 ± 0.5 | 27.8 ± 0.6 | 20.0 | 40 | 31 | 78 | 29 |
| | Constant— 20.3 ± 1.0 | | 20.3 | 34 | 30 | 78 | 25 |
| II | 16.4 ± 0.6 | 23.7 ± 0.4 | 20.1 | 50 | 38 | 76 | 28 |
| | 11.7 ± 0.6 | 27.5 ± 0.5 | 19.6 | 50 | 37 | 74 | 30 |
| | Constant— 19.4 ± 0.4 | | 19.4 | 50 | 37 | 74 | 30 |

$p > 0.80$, and $\chi^2 = 0.03$, $df\ 2$, $p > 0.90$ respectively).

Fluctuating temperatures around a mean of 20 C did not change the rate of transmission, or consistently affect the LP_{50} (an indirect measure of the dose of inoculum or the rate of multiplication). The conclusion of Jensen (1972) viz., that "variable temperatures, even some that are unfavorable when held constant, may be more conducive to vector efficiency than are constant temperatures" was not supported. However, mean temperatures and upper limits used by Jensen (1972), i.e., 25 C and 30 C, respectively, were less than the 20 C and 28 C selected for the present work. Overall, rate of transmission (75% in the Jensen tests) was similar to that in the present study.

Sequential transmission of WXDA

Data on the IAP (Table 5) indicated efficient inoculation of WXDA could occur in an hour or less. A test was designed to determine if successive inoculation could be effected by moving infective insects over a sequence of test plants at hourly intervals.

Two different feeding sequences were used. First (Sequence I, Table 8) 30 infected leafhoppers were caged singly on a test plant for 8 hr, transferred to a second set of plants for 16 hr (overnight), and finally, on the following morning (at 0900 hours), to fresh celery plants every hour for 8 hours. Second (Sequence II, Table 8) infected individuals were transferred hourly to a succession of 8 test plants, then fed on fresh plants for 16 hr (overnight), and finally, the following morning, allowed a final IAP of 8 hr. Results are given in Table 8 and Fig. 8.

Leafhoppers removed from a WX-diseased plant on which they had spent a relatively prolonged AAP (40 days) had a low probability of transmitting WXDA to the first test plant to which they were given access. This response was independent of length of the IAP, i.e., the rate of transmission using an initial 8-hr IAP was 7% compared to 10% when the initial IAP was 1 hr (Sequence I and II, respectively, Table 8).

Maximum transmission (37%) occurred in Sequence I during the 16-hr IAP that followed the initial 8-hr IAP, in spite of the fact that the 16-hr period was at night (1700 to 0900 hours).

Without the 8-hr period of "adjustment" to the test conditions (Sequence II), transmission rate per hour (ca. 5%) was low, when compared to the hourly transmission rate of ca. 20% obtained when the insects had an initial adjustment sequence of an 8-hr IAP followed by a 16-hr IAP.

Number of insects dying before completion of the entire sequence was greater when the initial IAPs were in a sequence of eight 1-hr intervals than when the initial IAP was 8-hr in length (10 deaths versus 2, $adj\ \chi^2 = 5.01$, $df\ 1$, $p < 0.05$).

Finally, there was evidence, based on the results of both sets of 1-hr sequential transfers, of cyclic periods of high and low probability of transmission of WXDA during the day (Fig. 8). Highest probability of transmission occurred in the morning (0900 hours) on the first test plants, while a second peak occurred on the sixth series of plants (1500 hours). Rapid decline in transmission followed both peaks, until late afternoon when there were few transmissions. A similar phenomenon was reported by Maramorosch (1964) in transmission of aster yellows agent by *Macrostoteles fascifrons* leafhoppers.

Injection and serial passage of WXDA

Establishment of infectivity by injecting vectors was demonstrated by Storey in 1933.

Whitcomb, Jensen, and Richardson (1966a, 1966b) injected clarified insect extracts containing WXDA into *C. montanus* and demonstrated hemolymph from infected donors was infectious. Jensen et al. (1967) obtained three successive serial passages of

TABLE 8.
TRANSMISSION OF WXD-AGENT TO CELERY BY *COLLADONUS MONTANUS* DURING SEQUENTIAL 1-HR IAP IN THE INSECTARY. LEAFHOPPERS FED ON INFECTED CELERY 40 DAYS BEFORE USE.

| Transfer sequence | Frequency of transmission pattern | Results* | | | | | | | | | | |
|-------------------|---|------------------|---|---|---|---|---|---|---|--------------------|------------|---|
| | | Hourly transfers | | | | | | | | Continuous feeding | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 16 hr (night) | 8 hr (day) | |
| I. | 1 | + | + | + | + | - | + | + | - | + | + | |
| | 1 | + | + | + | - | - | - | - | - | + | - | |
| | 1 | + | + | - | + | - | - | - | - | + | - | |
| | 1 | + | + | - | - | - | + | + | + | + | - | |
| | 1 | + | + | - | - | - | + | - | + | - | - | |
| | 1 | + | - | - | - | - | - | - | - | + | - | |
| | 1 | + | - | + | - | - | + | - | - | - | - | |
| | 1 | + | - | + | - | - | - | - | - | - | - | |
| | 1 | + | - | - | - | - | + | - | - | - | - | |
| | 2 | + | - | - | - | - | - | - | - | + | - | |
| | 8-hr continuous access; 16-hr [night]; 8 successive 1-hr transfer | 1 | + | - | - | - | d | - | - | - | - | - |
| | | 1 | - | + | - | - | - | - | - | - | - | - |
| | | 1 | - | + | - | - | - | - | d | - | + | - |
| | | 2 | - | + | - | - | - | - | - | - | - | - |
| | | 1 | - | - | + | - | - | + | + | - | - | - |
| | | 1 | - | - | + | - | - | - | - | - | - | - |
| | | 1 | - | - | - | + | - | - | - | - | - | - |
| | | 1 | - | - | - | - | + | - | - | - | - | - |
| | | 1 | - | - | - | - | - | + | - | - | - | - |
| | | 1 | - | - | - | - | - | - | + | - | - | + |
| 3 | | - | - | - | - | - | - | - | - | + | - | |
| 4 | | - | - | - | - | - | - | - | - | - | - | |
| II. | 1 | + | - | - | - | - | - | d | - | - | - | |
| | 2 | + | - | - | - | - | - | - | - | - | - | |
| | 1 | - | + | - | - | - | - | - | - | d | - | |
| | 1 | - | - | - | + | - | - | - | - | d | - | |
| | 1 | - | - | - | - | + | - | - | - | + | - | |
| | 1 | - | - | - | - | + | - | - | - | + | - | |
| | 2 | - | - | - | - | - | + | - | - | - | - | |
| | 1 | - | - | - | - | - | - | + | - | - | - | |
| | 1 | - | - | - | - | d | - | - | - | - | - | |
| | 1 | - | - | - | - | - | - | - | d | - | - | |
| | 5 | - | - | - | - | - | - | - | - | d | - | |
| | 12 | - | - | - | - | - | - | - | - | - | - | |

+ = transmission, - = nontransmission, d = death of insect during the previous IAP.

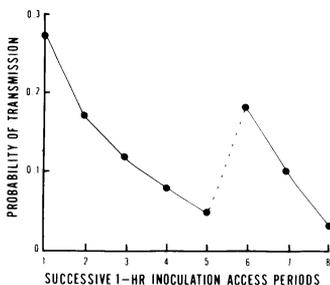


Fig. 8. Transmission of WXD-agent to celery by 60 single infective *Colladonus montanus* leafhoppers under insectary conditions. Each leafhopper had a 1-hr inoculation access period on each of 8 successive test plants. Tests were begun of 0900 hr and concluded at 1700 hr. Dotted line (----) indicates a rapid increase in the rate of transmission between the fifth and sixth hr of testing.

WXDA in *C. montanus* nymphs by injection before bacterial contamination ended the series. Amin and Jensen (1971a) reported tetracycline, introduced either by feeding or injection, reduced or eliminated WXDA from infected insects.

Before attempting serial passage of the WXDA by injection, trials were made to compare "whole insect extracts" versus "head extracts" as inoculum, and to measure the effect on infectivity of sucrose (Nasu et al., 1974), tissue culture media, and tetracycline as carriers. Results are given in Tables 9 and 10.

TABLE 9.
TRANSMISSION OF WXD-AGENT BY, AND LP₅₀ IN, *COLLADONUS MONTANUS*
FOLLOWING INJECTION WITH EXTRACTS PREPARED FROM WHOLE INSECTS
OR HEAD CAPSULES OF INFECTED LEAFHOPPERS. INSECTS WERE INDIVIDUALLY
TESTED BY TWICE-WEEKLY TRANSFERS TO FRESH CELERY TEST PLANTS
FOR 60 DAYS AT 25 C AND CONSTANT LIGHT.

| Treatments* | Number of insects | | | LP ₅₀ |
|-----------------------|-------------------|-----------|---------|------------------|
| | Tested | Infective | | |
| | number | number | percent | days |
| Whole-insect extract: | | | | |
| Undiluted | 20 | 16 | 80 | 24 |
| 1:10 | 20 | 7 | 35 | 26 |
| 1:100 | 20 | 1 | 5 | 29 |
| 1:1000 | 20 | 0 | 0 | — |
| Head extract: | | | | |
| Undiluted | 20 | 17 | 85 | 24 |

* "Whole-insect extract" was prepared by triturating 100 infected leafhoppers in a 1.5 cc of 30% sucrose (w/w), clarifying by centrifugation (10 min at 8,000 g, 4 C in a SW39 rotor), and passing the supernatant through 0.8 and 0.45 μ Millipore® filters. Dilutions were made with 30% sucrose.

"Head extract" was made by surface sterilizing five infected leafhoppers in 95% ethanol, removing head capsules and triturating in 25 μ l of 30% sucrose.

TABLE 10.
EFFECT OF EXTRACTION MEDIA ON SURVIVAL OF, TRANSMISSION OF WXD-AGENT
BY, AND LP₅₀ IN, INJECTED *COLLADONUS MONTANUS*. LEAFHOPPERS WERE
INDIVIDUALLY TESTED FOR 60 DAYS AT 25 C AND CONSTANT LIGHT
USING TWICE WEEKLY TRANSFERS TO CELERY TEST PLANTS.

| Treatment solution* | Number of insects | | | LP ₅₀ |
|----------------------|-------------------|----------|---------|------------------|
| | Tested | Infected | | |
| | number | number | percent | days |
| 30% sucrose (w/w) | 30 | 17 | 57 | 16 |
| Tissue culture media | 30 | 15 | 50 | 25 |
| 200 ppm tetracycline | 30 | 10 | 33 | 27 |

* Nymphs were injected with WXD-agent extracts prepared by triturating the contents of five head capsules from infected leafhoppers in 25 μ l of treatment solution. Tissue culture media was formulated as by Richardson and Jensen (1971), and the tetracycline hydrochloride was in 30% sucrose solution.

As expected (Whitcomb, Jensen, and Richardson, 1966a), the number of inoculative insects decreased and length of the LP₅₀ increased with dilution of whole insect extracts. More significant from a technique standpoint, was the finding that a head extract prepared from five leafhoppers in 25 μ l of 30% sucrose was equivalent to an extract from 100 insects in 1.5 cc of the same carrier.

The w/w dilution factors for head and whole insect extracts were estimated to be 77 and 6, respectively, indicating that the former provided a titer 15 times greater than the latter.

Thirty per cent sucrose was judged to be the best media for general use, based on number of inoculative insects obtained and length of the LP₅₀ (Table 10).

To avoid bacterial contamination (Jensen, Whitcomb, and Richardson, 1967), head extracts were prepared in 30% sucrose containing 200 ppm of tetracycline. Results are given in Table 11.

TABLE 11.
TRANSMISSION OF WXD-AGENT BY LP₅₀ IN *COLLADONUS MONTANUS* INJECTED WITH TETRACYCLINE-TREATED HEAD EXTRACTS FROM INFECTED LEAFHOPPERS IN SERIAL PASSAGE TRIALS. INSECTS WERE INDIVIDUALLY TESTED BY CAGING ON TEST CELERY PLANTS FOR 28 DAYS AT 25 C AND CONSTANT LIGHT.

| Treatment* | Passage | Number of insects | | | LP ₅₀ |
|--------------|---------|-------------------|--------|-----------|------------------|
| | | Tested | | Infective | |
| | | number | number | percent | days |
| Tetracycline | 1 | 20 | 7 | 33 | 27 |
| Controls:† | | | | | |
| Tetracycline | 1 | 30 | 10 | 33 | 27 |
| 30% sucrose | 1 | 30 | 17 | 57 | 16 |
| Tetracycline | 2 | 20 | 10 | 50 | —‡ |
| Tetracycline | 3 | 20 | 9 | 45 | —‡ |
| Tetracycline | 4 | 60 | 37 | 62 | 24 |
| Tetracycline | 5 | 30 | 27 | 90 | 24 |
| Tetracycline | 6 | 60 | 39 | 65 | 23 |
| Tetracycline | 7 | 30 | 18 | 60 | 20 |
| Controls: | | | | | |
| Tetracycline | 1 | 30 | 7 | 23 | 25 |
| 30% sucrose | 1 | 30 | 14 | 47 | 18 |

* Last instar nymphs were injected with extracts prepared by triturating five head capsules from infected insects in 25 μ l of 200 ppm tetracycline hydrochloride in 30% sucrose (w/w). Twenty-eight days after injection five individuals were selected at random from the surviving insects and used to prepare inoculum for the next passage.

† Controls consisted of stock colonies checked for response to tetracycline and to sucrose at the time of the first and seventh serial passages.

‡ Over 50% transmission occurred on the first test plant.

Seven serial passages of WXDA in *C. montanus* were obtained over a period of 7 months. Estimated dilution of the original inoculum was 2×10^{24} , if no multiplication occurred. There is little doubt that WXDA multiplied in *C. montanus*. There also was evidence for rapid selection of an isolate of tetracycline-resistant WXDA. By the fourth passage, the probability of obtaining an inoculative insect appeared to stabilize around 60%, and the LP₅₀ was steadily declining. At the time of the seventh passage, tetracycline-treated WXDA that had been serially passed was compared with original stock colony. The probability of obtaining an inoculative insect was greater with the serially passed, tetracycline exposed, inoculum (adj $\chi^2 = 6.86$, df 1, $p < 0.01$), and the LP₅₀ was 5 days shorter.

Evidence supports the hypothesis that continuous tetracycline pressure can result in the selection of resistance to the antibiotic in populations of WXDA.

Membrane feeding on WXDA extracts

Acquisition of plant pathogenic agents by insects fed on solutions using artificial membranes (Carter, 1927) has been reviewed by Duffus and Gold (1967). Membrane systems, coupled with radioactive tracers also have been used to determine feeding rates of suctorial insects (Hamilton; 1935, Day and McKinnon, 1951; Day and Irzykiewicz, 1953; Banks and Nixon, 1959; Duffus and Gold, 1967).

With plant-infecting mycoplasma-like organisms transmitted by arthropod vectors, membrane feeding has been used successfully only in the acquisition of culturable *Spiroplasma* (Chen and Liao, 1975; Rana, et al., 1975). Although *C. montanus* feeds on parafilm sachets, attempts to transmit WXDA have not been successful (Amin and Jensen, 1971a). Further attempts were made in the present work to bioassay WXDA using membrane-feeding systems.

Leafhoppers were fed on WXDA prepared in 5% and 10% sucrose solutions, but no transmission occurred in four trials made under various conditions (Table 12). Uptake was monitored by P³² in the feeding solutions, and injections of samples from these solutions demonstrated that the sachets contained infectious WXDA.

The coefficient of variation for the feeding rates ranged from 0.31 to 1.18, indicating uptake was highly variable. In comparative tests, insects fed more consistently on 10% sucrose than on WXDA extracts. Extracts in 5% sucrose were taken up more readily than were those in 10% sucrose, but both were infectious when assayed by injection.

Density gradient centrifugation, while yielding extracts that were readily fed upon, also failed to give inoculative insects. Table 12 shows that uptake of solution was greater in the morning (Trial IV) than in the evening (Trial III).

TABLE 12.
UPTAKE OF SOLUTIONS LABELED WITH P^{32} BY *COLLADONUS MONTANUS* FED THROUGH PARAFILM® SACHETS CONTAINING WXD-AGENT. INSECTS WERE TRANSFERRED WEEKLY TO FRESH CELERY PLANTS AND TESTED UNTIL DEATH INDIVIDUALLY.

| Trial | Test conditions | Test inoculum* | Counts per minute† | Feeding rate ($\mu\text{l/hr}$) | Infectivity assay‡ | |
|-------|--|-----------------------|--------------------|-----------------------------------|--------------------|-----------|
| | | | | | Feeding | Injection |
| I | Laboratory—27 C; 24-hr access beginning in the evening. | 10% WXDA extract | 2,770 | 0.15 | 0/32 | — |
| II | Laboratory—28 C; 8-hr access beginning in the evening. | 5% WXDA extract | 529 | 0.09 | 0/15 | 12/15 |
| | | 10% WXDA extract | 321 | 0.05 | 0/15 | 15/15 |
| | | 10% sucrose (control) | 795 | 0.13 | 0/5 | — |
| III | Environmental chamber—25 C; constant light; 14-hr access beginning in the evening. | Gradient samples | 30 | 0.03 | 0/80 | — |
| | | 10% sucrose (control) | 90 | 0.09 | 0/5 | — |
| IV | As in III, except an 8-hr access beginning in the morning. | gradient samples | 370 | 0.18 | 0/80 | — |
| | | 10% sucrose (control) | 397 | 0.19 | 0/5 | — |
| | | 10% WXDA extract | 110 | 0.05 | 0/5 | — |

* WXD-agent extracts were prepared by triturating 100 infected leafhoppers in 1.5 cc of 10% (w/w) sucrose. The slurry was clarified by centrifugation (8,000 g for 10 min 4 C, SW 39 rotor), and the supernatant then centrifuged at 100,000 g for 60 min. The pellet was resuspended in 5 or 10% sucrose and passed through an 0.45 μ Millipore® filter. Aliquots of 0.2 cc were used in feeding sachets with P^{32} added at a final concentration of 2.5×10^{-2} mc/ml.

† Following the acquisition access periods, insects and cages were read for P^{32} activity with an end-window Geiger-Muller tube attached to a scaling unit.

‡ Insects were fed on extracts in sucrose solutions. Injections were made of extracts removed from feeding sachets. In ratios listed, numerator is number of insects tested; denominator, number transmitting.

Frequency and volume measurements on excretion of leafhoppers fed on test plants indicated that an average of 21 droplets, each containing an estimated 8×10^{-3} μl , was excreted each 6-hr of access time, with at least 0.17 μl of fluid being removed from test plants. In a similar 6-hr period on the feeding sachets, as measured with P^{32} (Table 12, Trial I), 0.90 lambda/insect was removed, a feeding rate ca. 5 times that on plants. Rate of fluid uptake did not account for the apparent lack of acquisition of WXDA during membrane-feeding experiments.

Uptake increased with increasing temperature up to 30 C (Table 13). The greatest increase occurred between 25 to 30 C, and at 33.5 C the feeding rate tended to be equal to, or less than, that at 30 C. In all tests the 10% sucrose solution was preferred (as measured by uptake) to WXDA extract.

TABLE 13.
EFFECT OF TEMPERATURE AND MEDIA COMPOSITION ON UPTAKE OF SOLUTIONS
CONTAINING P³² BY *COLLADONUS MONTANUS* FED THROUGH PARAFILM®
SACHETS AT CONSTANT LIGHT AND TEMPERATURE.

| Temperature | Treatment* | Insects tested | Radioactivity | | Feeding rate |
|-------------|--------------|-------------------|---------------|------------|--------------|
| | | | Insects | Excretion | |
| | | | | <i>cpm</i> | <i>μl/hr</i> |
| 20 C | 10% sucrose | 20 | 31 | 255 | 0.19 |
| | WXDA extract | 5 | 54 | 193 | 0.16 |
| 25C | 10% sucrose | 20 | 42 | 321 | 0.24 |
| | WXDA extract | 5 | 49 | 179 | 0.15 |
| 30C | 10% sucrose | 20 | 59 | 410 | 0.31 |
| | WXDA extract | 5 | 91 | 276 | 0.24 |
| 33.5 C | 10% sucrose | 20 | 54 | 381 | 0.28 |
| | WXDA extract | 5 | 63 | 309 | 0.24 |

* Leafhoppers were held on feeding sachets for 24 hr before measuring specific P³² activity using an end-window Geiger-Muller tube. Feeding sachets contained 0.2 ml of 10% (w/w) sucrose solution made with distilled water or WXDA-agent extract. Extracts were prepared by triturating 100 diseased insects per 1.5 cc of 10% sucrose, and clarifying the slurry by centrifugation (8,000 g for 10 min 4 C, SW 39 rotor). The supernatant was then centrifuged for 60 min at 100,000 g, the pellet resuspended in 10% sucrose and passed through a 0.45 μ Millipore® filter. P³² was added to feeding sachets to give 64 cpm/μl of solution.

Jensen (1968) reported inactivation of WXDA in leafhoppers held at high temperatures. The activity of WXDA in feeding sachets was assayed at intervals following exposure to 25 and 30 C. Loss of infectivity occurred faster at the higher temperature (Table 14). Assuming linearity, the median survival period of WXDA agent in the sachets was 6.4 and 4.2 hr at 25 and 30 C, respectively.

TABLE 14.
EFFECT OF TEMPERATURE ON INFECTIVITY OF EXTRACTS* CONTAINING
WXDA-AGENT USED IN PARAFILM® SACHETS FOR MEMBRANE-FEEDING OF
COLLADONUS MONTANUS. SOLUTIONS WERE ASSAYED BY INJECTING NYMPHS
AND CAGING THEM ON CELERY TEST PLANTS FOR A SUITABLE IAP.

| Temperature | Infectivity of WXDA extract after storage (hours) | | | | | | |
|-------------|---|-------|-------|-------|------|------|------|
| | 0 | 1 | 2 | 4 | 6 | 8 | 24 |
| 25C | 10/10† | 13/15 | — | 10/15 | 9/15 | 5/15 | 0/20 |
| | (100) | (87) | — | (67) | (60) | (33) | (0) |
| 30C | 16/17 | 14/20 | 11/20 | 10/20 | 8/20 | 7/20 | 0/20 |
| | (94) | (70) | (55) | (50) | (40) | (35) | (0) |

* WXDA-agent extracts were prepared by triturating 100 diseased insects per 1.5 cc of 10% sucrose, clarifying the slurry by centrifugation (8,000 g for 10 min 4 C, SW 39 rotor). The supernatant was then centrifuged for 60 min at 100,000 g, the pellet resuspended in 10% sucrose and passed through a 0.45 μ Millipore® filter.

† Ratios show number of insects transmitting. Figures in parentheses are per cent transmitting.

Attempts were made to increase the longevity of the WXDA in the feeding sachets by using a cold plate, a technique successfully used by Long and Timian (1971) in work with the oat blue dwarf virus. Results of two trials are given in Table 15. Activity was retained for 30, but not for 48 hr. The test insects in Trial I (Table 15, 0 time) were given a 48-hr AAP on the membrane. Fifty-four out of 101 leafhoppers survived the AAP on sachets held on the cold plate, while all 50 insects fed at 29 C died.

In a second trial (Table 15, Trial II), the possible existence of a "helper factor" (Govier and Kassanis, 1974) was sought by preconditioning the leafhoppers on a WX-infected celery plant for 24 hr before feeding on the membrane. No evidence was obtained that such preconditioning resulted in infectious WXDA being acquired from the membrane-fed extract.

TABLE 15.

EFFECT OF COOLING ON INFECTIVITY OF EXTRACTS CONTAINING WXD-AGENT FED TO *COLLADONUS MONTANUS* THROUGH PARAFILM® SACHETS. INDIVIDUAL TEST LEAFHOPPERS WERE TRANSFERRED TWICE WEEKLY UNTIL DEAD TO FRESH TEST PLANTS.

| Initial vector status* | Test inoculum† | Assay method | Hr before assay was made | | | | |
|-------------------------------------|--|--------------|--------------------------|-------|-------|----|------|
| | | | 0 | 4 | 30 | 48 | |
| I. Healthy; AAP = 48 hr on sachet: | control | — | 0/20‡ | — | — | — | |
| | WXDA extract: | 14C | Feeding | 0/30 | — | — | — |
| | | | Injection | 19/20 | 16/20 | — | 0/20 |
| | | 29C | Feeding | 0/0 | — | — | — |
| | Injection | 19/20 | 9/20 | — | 0/20 | | |
| II. Healthy; AAP = 30 hr on sachet: | control | — | 0/20 | — | — | — | |
| | WXDA extract | Feeding | 0/40 | — | — | — | |
| | WXDA-exposed; AAP = 24 hr on diseased plant: | control | — | 9/40 | — | — | |
| | WXDA extract | Feeding | 10/40 | — | — | — | |
| | Injection | 16/40 | — | 19/20 | — | | |

* Leafhoppers were taken from colonies free of WXD-agent infection.

† WXD-agent extracts were prepared by triturating 100 diseased insects/1.5 cc of 10% sucrose, clarifying the slurry by centrifugation (8,000 g for 10 min 4 C, SW 39 rotor). The supernatant was then centrifuged for 60 min at 100,000 g, the pellet resuspended in 10% sucrose and passed through a 0.45 μ Millipore® filter. Feeding sachets containing extracts were placed either on a cold plate or held in an environmental chamber. In Trial I, when using the cold plate, the sachet membrane and cage temperatures were 14 and 18 C, respectively; in Trial II they were 15 and 19 C, respectively. Sachets in the environmental chamber had membrane and cage temperatures of 29 C.

‡ In ratios listed, numerator is number of insect transmitting; the denominator, the number tested.

In summary, none of 316 insects tested after feeding on sachets containing active WXDA transmitted the agent. Uptake occurred and the WXDA in the sachets was infectious, yet there was no evidence of acquisition. The reason for the consistent failure of the insects to develop infectivity has not been determined. It may be analogous to the presence of a "gut barrier threshold" as can be found in arbovirus transmission by mosquitoes (Chamberlain, 1968). A few trials were made to render membrane-fed insects infective by piercing the abdomen with an insect pin (Storey, 1933), but without success. Assuming that a gut threshold exists, a further attempt was made, using electrophoresis, to obtain a more concentrated preparation of WXDA. Fourteen successive 2-ml aliquots were removed from a electrophoresis column and assayed for infectivity by injection and by membrane feeding. No infective insects were obtained by feeding, but as shown in Figure 9, the injection assay demonstrated infectious material moved up the sucrose column. Infectivity occurred in sample 2 through 8, a spread of 10 cm (14 ml), although there was a tendency for infectivity to decrease with distance from the origin.

Samples with the highest WXDA activity also had the greatest rate of mortality for the vectors, a result consistent with those of Whitcomb, Jensen, and Richardson (1968b). However, sample 10, which lacked infectivity, was associated with the highest death rate during the initial 30-day testing period. The sample may have contained a component that was not infectious to plants, but yet was quite toxic to injected leafhoppers. It also could have contained an isolate of WXDA that caused death in the insect vectors before they could transmit to the test plants.

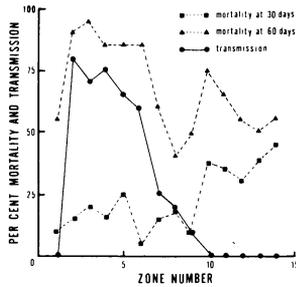


Fig. 9. Mortality and transmission following injection of *Colladonus montanus* leafhoppers with samples of clarified extract from infective insects run at a density gradient electrophoresis column in an attempt to isolate WXDA-agent.

The continuum of infectivity through much of the column was similar to that described by Whitcomb, Jensen, and Richardson (1968b) and Nasu, Jensen, and Richardson (1974) during attempts to purify WXDA by rate-density gradient zonal centrifugation. The collective evidence suggests that WXDA has infectious forms of varying size, mass and electrical charge. Indeed, Nasu, Jensen, and Richardson (1970) recognized six forms of WXDA in electron micrographs of thin sections of both vectors and plant hosts.

COMPARATIVE STUDIES ON LEAFHOPPER VECTORS OF WXDA

Colladonus montanus has been a convenient laboratory tool to study various vector-pathogen-host plant relationships of WXDA. Work with this vector made it possible to explore and develop necessary methodology for additional studies. WXDA is not vector-specific, and it is potentially important to compare vectoring ability of other leafhopper species, some of which may play a larger role in the field spread of WXDA than *C. montanus*. To this end, a limited comparative study was made using *C. montanus*, *C. geminatus*, *Euscelidius variegatus*, and *Fieberiella florii*.

Interactions among leafhopper vectors of WXDA

Jensen (1969a) reported preliminary studies on comparative transmission rates of *C. montanus* and *E. variegatus* in tests done in 1960. Similar comparisons made in 1968 included *C. geminatus*. Transmission rates of *E. variegatus* decreased from 75% in 1960 trials to 40% in 1968 tests. Jensen (1969a) suggested since *C. montanus* was used to maintain WXDA in celery over the years, a strain of the pathogen that was less efficiently transmitted by *E. variegatus*, may have been isolated during the 8-yr period between comparative trials. However, in the 1960 tests, only two species, i.e., *E. variegatus* and *C. montanus* were caged together on the same diseased celery, while in tests made in 1968, which included *C. geminatus*, all three species were caged together. The addition of another species to the group may have resulted in competition for feeding sites, or other behavior artifacts that reduced efficiency of WXDA transmission by *E. variegatus*. Tests for possible interactions among simultaneously fed species were made in the present work.

Colladonus geminatus, *C. montanus* and *E. variegatus* were fed simultaneously on one WX-diseased plant, and each species also was fed on a different WXDA source. Two trials were conducted in the greenhouse insectary at different times of the year (Table 16). No significant evidence of heterogeneity was found between the two trials (adj. $\chi^2 = 0.11$, df 1, $p > 0.70$), although insects in Trial I had an AAP of 34 days, as compared to a 21-day AAP in Trial II.

TABLE 16.

TRANSMISSION OF WXD-AGENT FROM AND TO CELERY BY, AND LP₅₀ IN, *COLLADONUS GEMINATUS*, *C. MONTANUS*, AND *EUSCELIDIUS VARIEGATUS* LEAFHOPPERS REARED (TRIAL I) OR FED FOR 21 DAYS (TRIAL II) SEPARATELY OR AS A GROUP ON DISEASED CELERY UNDER INSECTARY CONDITIONS. INDIVIDUAL TEST INSECTS WERE TRANSFERRED TWICE WEEKLY UNTIL DEAD TO FRESH TEST PLANTS.

| Trial | Acquisition conditions | <i>C. geminatus</i> | | | | <i>C. montanus</i> | | | | <i>E. variegatus</i> | | | |
|-------|------------------------|---------------------|-----------|------------------|------|--------------------|-----------|------------------|------|----------------------|-----------|------------------|------|
| | | Tested | Infective | LP ₅₀ | days | Tested | Infective | LP ₅₀ | days | Tested | Infective | LP ₅₀ | days |
| I | Single species | 30 | 18 | 60 | — | 30 | 25 | 90 | — | 30 | 6 | 10 | — |
| | Three species | 15 | 9 | 60 | — | 19 | 17 | 90 | — | 30 | 3 | 10 | — |
| II | Single species | 40 | 20 | 50 | 33 | 40 | 30 | 75 | 35 | 40 | 18 | 45 | 39 |
| | Three species | 40 | 15 | 38 | 39 | 40 | 24 | 60 | 37 | 40 | 10 | 25 | 3 |

Analysis of individual 2 × 2 cells (Table 16) indicated transmission in each trial was similar, whether leafhoppers fed on WXDA source plants as single species, or as a group of three species. However, when trials were combined, *E. variegatus* was a better vector when fed on WXDA source plants alone than when fed in a group (34% transmission versus 16%, adj $\chi^2 = 5.60$, df 1, $p < 0.02$). The three species each differed in overall transmission efficiency, with *C. montanus*, *C. geminatus*, and *E. variegatus* transmitting at rates of 74%, 50%, and 25%, respectively ($\chi^2 = 68.8$, df 2, $p < 0.001$).

In Trial II (Table 16) LP₅₀ values for the two *Colladonus* species integrated on the same source plants were somewhat longer than for those caged separately. If transmission rates and LP₅₀ values indicate relative titers of WXDA in insect vectors (Whitcomb, Jensen, and Richardson, 1966a), then the mixed species may have acquired lower doses of WXDA.

Comparative acquisition and transmission of WXDA by three leafhopper species

An additional test on differential acquisition and transmission of WXDA by the same three species was made in the greenhouse insectary by combining the species for a 6-day AAP on WX-diseased plants. Thereafter, individual insects were transferred at semi-weekly intervals until death to a series of healthy test plants.

Under varying light and temperature conditions of the insectary, again *C. montanus* acquired WXDA more efficiently, and had a shorter LP₅₀ than did *C. geminatus* or *E. variegatus*. The last named species was the poorest vector, with the longest LP₅₀, and *C. geminatus* was intermediate (Table 17).

TABLE 17.

TRANSMISSION OF WXD-AGENT TO AND FROM CELERY BY, LP₅₀ IN, AND SP₅₀ OF, *COLLADONUS GEMINATUS*, *C. MONTANUS*, AND *EUSCELIDIUS VARIEGATUS* UNDER INSECTARY CONDITIONS, USING A 6-DAY AAP AND TWICE WEEKLY TRANSFERS TO TEST PLANTS UNTIL DEAD.

| Vector species | Number of insects | | | LP ₅₀ | SP ₅₀ |
|----------------------|-------------------|-----------|---------|------------------|------------------|
| | Tested | Infective | | | |
| | number | number | percent | days | |
| <i>C. geminatus</i> | 30 | 16 | 53 | 30 | 44 |
| <i>C. montanus</i> | 30 | 26 | 87 | 28 | 41 |
| <i>E. variegatus</i> | 30 | 9 | 30 | 34 | 38 |

The two *Colladonus* species had comparable SP_{50} values, while that for *E. variegatus* was somewhat shorter. This may have been due to less favorable environmental conditions for *E. variegatus* rather than a difference in susceptibility to the WXDA.

As the three vector species were treated in the same manner, it is believed that differences in transmission rates, as well as the individual SP_{50} values, were inherent to species. A method of expressing these differences is a transmission expectancy curve (Fig. 10). Curves were calculated using the product of age-specific survival rate (L_x), and age-specific transmission rate (T_x). Both *C. geminatus* and *C. montanus* had similar transmission expectancy curves, with peak transmission occurring at 38 and 32 days, respectively, and a constant decrease thereafter due to mortality either from pathological effects of WXDA infection, or adverse environmental conditions (Fig. 10). The transmission expectancy curve for *E. variegatus* differed from that of the *Colladonus* species. WXDA appeared to multiply more slowly in this species, and this, coupled with poor survival, resulted in a relatively low maximum transmission expectancy (ca. 30%). However, *E. variegatus* maintained infectivity at near maximum levels for extended periods (35 to 45 days, Fig. 10).

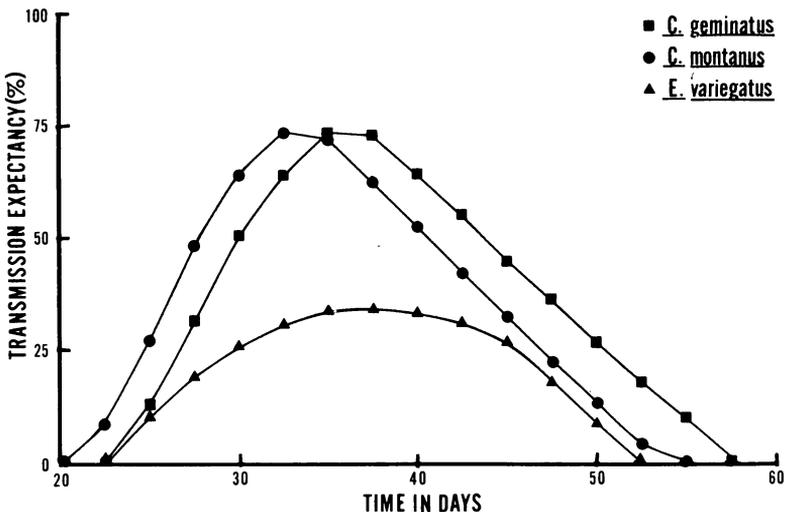


Fig. 10. Transmission expectancy curves under insectary conditions for transmission of peach yellow leaf roll strain of WXDA-agent from and to celery by infective *Colladonus geminatus*, *C. montanus*, and *Euscelidius variegatus*. The points were calculated as the product of age-specific transmission rates and the age-specific survival rates.

These comparisons of vectors of WXDA were made in the insectary under fluctuating light and temperature conditions. Additional comparisons were made in environmental chambers with three controlled-temperature regimes and constant light (Table 18).

The proportion of *C. montanus* and *E. variegatus* acquiring and transmitting at 20 C was similar. *Euscelidius variegatus* had increased (avg = 75%), while *C. geminatus* had reduced (ave = 24%), acquisition and transmission efficiencies in these trials as compared to those done in the insectary (30% and 54% inoculative insects obtained, respectively see Table 17). The LP_{50} for these insects tested at 20 C was similar, indicating that WXDA multiplied in the different species at similar rates.

In tests at 25 C, *C. montanus* and *E. variegatus* again were nearly equal in proportion of insects transmitting WXDA (Table 18). *Colladonus geminatus* once more was the

TABLE 18.

TRANSMISSION BY, AND LP_{50} OF WXD-AGENT IN, *COLLADONUS GEMINATUS*, *C. MONTANUS* AND *EUSCELIDIUS VARIEGATUS* FROM AND TO CELERY AT CONTROLLED TEMPERATURES AND CONSTANT LIGHT. LEAFHOPPERS WERE MOVED UNTIL DEATH TWICE WEEKLY TO FRESH TEST PLANTS.

| Trial | Acquisition | | <i>C. geminatus</i> | | | | <i>C. montanus</i> | | | | <i>E. variegatus</i> | | | |
|-------|-------------|-----------|---------------------|-----------|------------------|--------|--------------------|------------------|---------|-----------|----------------------|--------|-----------|------------------|
| | Days | Temp. (C) | Tested | Infective | LP ₅₀ | Tested | Infective | LP ₅₀ | Tested | Infective | LP ₅₀ | Tested | Infective | LP ₅₀ |
| | | | number | number | percent | days | number | number | percent | days | number | number | percent | days |
| I | 7 | 20 | 23 | 5 | 22 | 43 | 25 | 18 | 72 | 38 | 35 | 30 | 86 | 43 |
| II | 25 | 20 | 30 | 12 | 40 | 48 | 30 | 16 | 53 | 49 | 30 | 22 | 73 | 48 |
| III | 26 | 20 | 60 | 8 | 13 | 42 | 60 | 50 | 83 | 43 | 60 | 43 | 72 | 51 |
| IV | 25 | 25 | 18 | 4 | 22 | 27 | 25 | 18 | 72 | 27 | 30 | 22 | 73 | 34 |
| V | 25 | 25 | 20 | 7 | 35 | 29 | 20 | 14 | 70 | 27 | 20 | 14 | 70 | 38 |
| VI | 7 | 30 | 12 | 0 | 0 | — | 30 | 1 | 3 | — | 30 | 0 | 0 | — |
| VII | 26 | 30 | 12 | 0 | 0 | — | 22 | 1 | 5 | — | 30 | 0 | 0 | — |

poorest vector, acquiring at the reduced rate of 29% rather than 54% obtained in insectary tests (Table 17).

A decrease in LP_{50} values at 25 C indicated that WXDA multiplied faster at 25 C than at 20 C. At 25 C *E. variegatus*, while similar to *C. montanus* in acquisition of WXDA, had a longer LP_{50} than either *C. montanus* or *C. geminatus* (Table 18). The response of the LP_{50} to temperature regimes of 20 and 25 C was most evident with the two *Colladonus* species in which there was a 16-day difference as compared to an 11-day difference for *E. variegatus*.

At 30 C, both WXDA and the leafhoppers were adversely affected (Table 18). Only 2/52 *C. montanus* transmitted the WXDA when fed at this temperature. The survival of *C. geminatus* at 30 C was poor, and although *E. variegatus* survived well, neither of

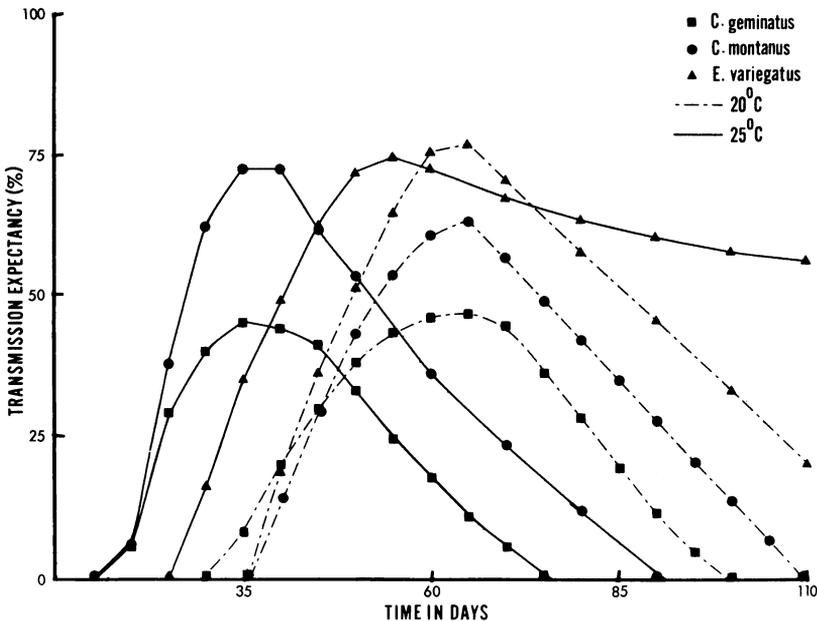


Fig. 11. Transmission expectancy curves for transmission at 20 or 25 C and constant light of peach yellow leaf roll strain of WXD-agent from and to celery by infective *Colladonus geminatus*, *C. montanus*, and *Euscelidius variegatus*. Points were calculated as the product of age-specific transmission rates and age-specific survival rates.

these latter species transmitted. As a result, 30 C was not included as a temperature for further comparisons.

The same three vector species also were compared using a varying AAP at constant temperature (20 and 25 C) and light conditions (Table 18 and Fig. 11).

Acquisition of WXDA was somewhat more frequent with increasing access time at both temperatures (Table 19). The AAP₅₀ was 11.2, 12.9 and 10.6 days at 20 C and 10.6, 9.8 and 10.6 days at 25 C for *C. geminatus*, *C. montanus*, and *E. variegatus*, respectively. *Euscelidius variegatus* had the same AAP₅₀ at both temperatures, while both *Colladonus* species had higher values at the lower temperature.

TABLE 19.

TRANSMISSION BY, LP₅₀ IN, AND SP₅₀ OF, *COLLADONUS GEMINATUS*, *C. MONTANUS*, AND *EUSCELIDIUS VARIEGATUS* ACQUIRING WXD-AGENT FROM DISEASED CELERY AT TWO TEMPERATURES AND CONSTANT LIGHT. TWENTY INDIVIDUALS OF EACH SPECIES WERE TESTED FROM EACH SPECIFIED AAP AND TEMPERATURE CONDITION BY TWICE WEEKLY TRANSFERS TO FRESH CELERY TEST PLANTS UNTIL DEATH.

| Vector and temperature | AAP (days) | | | | | AAP ₅₀ | LP ₅₀ | SP ₅₀ |
|------------------------|----------------|----|----|----|----|-------------------|------------------|------------------|
| | 1 | 3 | 7 | 14 | 21 | | | |
| <i>C. geminatus</i> | <i>percent</i> | | | | | | <i>days</i> | |
| 20 C | 0 | 10 | 10 | 20 | 30 | 11.2 | 51.0 | 67.0 |
| 25C | 5 | 10 | 15 | 25 | 35 | 10.6 | 29.4 | 41.1 |
| <i>C. montanus</i> | | | | | | | | |
| 20 C | 0 | 10 | 15 | 30 | 45 | 12.9 | 50.0 | 74.3 |
| 25 C | 15 | 35 | 40 | 80 | 80 | 9.8 | 27.2 | 51.2 |
| <i>E. variegatus</i> | | | | | | | | |
| 20 C | 10 | 15 | 35 | 50 | 65 | 10.6 | 48.7 | 86.3 |
| 25 C | 10 | 15 | 30 | 50 | 70 | 10.6 | 37.6 | 125.0 |

Mean daily probability of obtaining an infective insect was 0.0164, 0.0222, and 0.0598 at 20 C, and 0.0297, 0.1078, and 0.0615 at 25 C for *C. geminatus*, *C. montanus* and *E. variegatus*, respectively (Table 20). This probability tended to remain constant over the access periods tested (Table 20).

At 20 C, WXDA multiplied at a faster rate, as indicated by LP₅₀ values, in *E. variegatus* than in either *Colladonus* species. The LP₅₀ value for all species was less at 25 C than at 20 C. However, based upon comparative LP₅₀ values, *C. geminatus* and *C. montanus* were more sensitive to temperature changes than was *E. variegatus*.

The SP₅₀ decreased with increased temperature for both *Colladonus* species, but increased for *E. variegatus* (Table 19). It can be hypothesized that, as the rate of multiplication of WXDA in *C. geminatus* and *C. montanus* increased with temperature, the pathological effects of the agent on the vectors were more severe. Indications are, however, that *E. variegatus* was not adversely affected by the WX-pathogen, since increasing ambient temperature of 25 C prolonged its survival. This increased longevity under constant temperature and light conditions may increase the vector potential of this species.

Transmission expectancy curves for the three species at 20 and 25 C are given in Fig. 11. Curves for all species at 20 C were similar in shape with peak transmission occurring at 65 days. Curves for trials conducted in the greenhouse insectary (Fig. 10) had a similar shape, except the relationship among vectors changed. *Euscelidius variegatus* was the poorest vector of the three in insectary trials, but most efficient when tested at a constant temperature of 20 C. *Colladonus geminatus*, which was similar to *C. mon-*

TABLE 20.

OBSERVED AND EXPECTED NUMBER, OUT OF POSSIBLE TWENTY EACH OF *COLLADONUS GEMINATUS*, *C. MONTANUS*, AND *EUSCELIDIUS VARIEGATUS*, THAT TRANSMITTED WXD-AGENT FROM AND TO CELERY FOLLOWING SPECIFIED AAP AT 20 AND 25 C AND CONSTANT LIGHT. EXPECTED NUMBERS WERE BASED ON BINOMIAL EXPECTANCY, ASSUMING THAT THE PROBABILITY OF ACQUIRING AN INFECTIOUS DOSE EACH DAY WAS A CONSTANT AND INDEPENDENT EVENT.
DATA DERIVED FROM THOSE IN TABLE 19.

| Vector and temperature | Number of infective insects after AAP (days) | | | | | | | | | | χ^2 | Prob. | |
|------------------------|--|---------|------|---------|------|---------|------|---------|------|---------|----------|-------|--|
| | 1 | | 3 | | 7 | | 14 | | 21 | | | | |
| | Obs. | Expect. | Obs. | Expect. | Obs. | Expect. | Obs. | Expect. | Obs. | Expect. | | | |
| <i>C. geminatus</i> | | | | | | | | | | | | | |
| 20C | 0 | 0.3 | 2 | 1.0 | 2 | 2.2 | 4 | 4.1 | 6 | 5.9 | 2.41 | 0.30 | |
| 25 C | 1 | 0.6 | 2 | 1.7 | 3 | 3.8 | 5 | 6.8 | 7 | 9.3 | 1.95 | 0.50 | |
| <i>C. montanus</i> | | | | | | | | | | | | | |
| 20 C | 0 | 0.4 | 2 | 1.3 | 3 | 2.9 | 6 | 5.3 | 9 | 7.5 | 1.24 | 0.70 | |
| 25 C | 3 | 2.0 | 7 | 5.5 | 8 | 10.6 | 16 | 15.6 | 16 | 17.9 | 1.69 | 0.50 | |
| <i>E. variegatus</i> | | | | | | | | | | | | | |
| 20 C | 2 | 1.2 | 3 | 3.3 | 7 | 6.8 | 10 | 11.3 | 13 | 14.3 | 0.91 | 0.80 | |
| 25 C | 2 | 1.2 | 3 | 3.4 | 6 | 7.0 | 10 | 11.6 | 14 | 14.5 | 0.95 | 0.08 | |

* Calculated mean probability of obtaining infective *C. geminatus*, *C. montanus*, and *E. variegatus* during 1 day of feeding was 0.0164, 0.0222, respectively, at 20 C and 0.0297, 0.01078, and 0.0615 respectively, at 25 C.

tanus in insectary trials, was poorest among the three species at 20 C constant temperature and continuous light. Also, the maximum transmission peak shifted from 35 days in insectary trials to 65 days in texts at 20 C in environmental chambers.

Transmission expectancy curves for the three vectors at 25 C indicated that *C. montanus* and *E. variegatus* were similar, with *C. geminatus* the poorest of the vectors tested (Fig. 11). *Colladonus* species were shown to have the same shape curves at 25 C with both peaking at 35 days, as compared with a peak at 55 days for *E. variegatus*. Apparently this species was not as sensitive to temperature changes as were the *Colladonus* species.

As a whole, ambient conditions greatly influenced and, in some cases, regulated performance of these three leafhopper vector species. There was strong evidence temperature and light conditions affected acquisition, transmission, and longevity of vectors.

Influence of light conditions on the acquisition of WXDA

Jensen (1972) reported that WXDA-injected *C. montanus* held under fluctuating light and temperature conditions were better vectors than if held under constant conditions. However, in an earlier section of the present paper, a fluctuating temperature around a mean of 20 C, under constant light, did not appear to affect transmission by *C. montanus* (see page 19, Table 7). Those experiments were conducted primarily to evaluate the influence of light and temperature on changes in the LP₅₀ and transmission rates of infected vectors, but did not measure the effect on acquisition *per se*.

Tests on the effect of light cycles on acquisition of WXDA from infected celery by the three species discussed here (Table 21) indicated at 25 C, a cycle of 12:12 light:dark was better for acquisition by *C. montanus* and *E. variegatus* than was a constant light, while transmission by *C. geminatus* was too poor to permit differentiation. Results parallel those of Jensen (1972).

TABLE 21.

TRANSMISSION FROM AND TO CELERY OF WXD-AGENT BY, AND LP₅₀, IN *COLLADONUS GEMINATUS*, *C. MONTANUS*, AND *EUSCELIDIUS VARIEGATUS* UNDER DIFFERENT LIGHT AND TEMPERATURE REGIMES. INDIVIDUAL TEST INSECTS WERE TRANSFERRED TWICE WEEKLY TO TEST PLANTS UNTIL DEAD.

| Acquisition regimen | <i>C. geminatus</i> | | | | <i>C. montanus</i> | | | | <i>E. variegatus</i> | | | |
|------------------------|---------------------|--------|-----------|------|--------------------|--------|-----------|------|----------------------|--------|-----------|------|
| | Tested | | Infective | | Tested | | Infective | | Tested | | Infective | |
| | number | number | percent | days | number | number | percent | days | number | number | percent | days |
| 25 C, constant light | 40 | 2 | 5 | 31 | 40 | 13 | 33 | 34 | 40 | 14 | 35 | 46 |
| 25 C, 12-hr photophase | 40 | 1 | 3 | 31 | 40 | 23 | 58 | 29 | 40 | 26 | 65 | 40 |
| Insectary | 20 | 11 | 55 | 32 | 20 | 20 | 100 | 28 | 20 | 15 | 75 | 33 |

However, when the two *Colladonus* species and *E. variegatus* were given access to WX-infected celery in the insectary (Table 21, Treatment III—variable temperature and light), and then moved to healthy test plants in a 25 C chamber under constant light, acquisition of WXDA was enhanced. There appeared to be a synergistic effect between fluctuations in both light and temperature, one that promoted feeding and thus acquisition of WXDA.

LP₅₀ values appeared to be affected by test treatments (Table 21), being similar for *C. geminatus* under all conditions but variable for both *C. montanus* and *E. variegatus*. Factors affecting acquisition also apparently influenced the amount of WXDA taken in by the vectors and were reflected in the dosage-sensitive LP₅₀.

Again, when *C. geminatus* was given an AAP at 25 C (Treatments I and II, Table 21), acquisition, and thus transmission efficiency, was minimal (5% and 3%, respectively). But when the AAP was in the insectary, acquisition efficiency increased to 55% (Table 21). This species apparently is sensitive to its environment. *Euscelidius variegatus*, a species that did poorly in efficiency tests conducted in the insectary (Tables 16 and 17), was found to have increased acquisition of WXDA in tests done under controlled conditions (Table 21). Moving *E. variegatus* to the 25 C chamber, following an AAP period on infected celery in the insectary, again resulted in increased longevity of the vectors, an increase in the rate at which WXDA multiplied (shorter LP₅₀), and allowed more vectors to be realized.

Comparison of vector species injected with WXDA

The three species just discussed were further compared in their ability to transmit WXDA using injection of whole insect extracts (Table 22).

Infectivity rate was similar among the two groups of insects inoculated, i.e., 32% for those held in the insectary and 34% for those held at 25 C. A greater proportion of *C. montanus* became infective than either of the other two species ($\chi^2 = 11.94$, df 2, $p < 0.01$). There was no significant evidence that the environment following injections affected inoculativity. However, fewer *E. variegatus* became infective in the insectary than in an environment of 25 C and constant light (adj $\chi^2 = 3.15$, df 1, $p < 0.10$).

Assuming insects were injected with similar doses, then LP₅₀ values suggest the multiplication rate of WXDA varied with vector species as well as environment. Under similar conditions, *E. variegatus* had the highest, and *C. montanus* the lowest, LP₅₀ value (Table 22). LP₅₀ values following injection were lower for all three species than they were when WXDA was acquired by feeding (compare Tables 17, 18, and 19 to Table 22). This suggests the requirement of an initial gut infection may result in a measurable lengthening of the LP₅₀.

TABLE 22.
TRANSMISSION OF WXD-AGENT TO CELERY BY, AND LP₅₀ IN, *COLLADONUS GEMINATUS*, *C. MONTANUS* AND *EUSCELIDIUS VARIEGATUS* FOLLOWING INJECTION OF WHOLE INSECT EXTRACTS* UNDER TWO LABORATORY CONDITIONS. INDIVIDUAL LEAFHOPPERS WERE TESTED AT 25 C AND CONSTANT LIGHT BY TRANSFERRING AT TWICE WEEKLY INTERVALS TO FRESH CELERY TEST PLANTS UNTIL DEAD.

| Vector and test condition | Number of insects | | | LP ₅₀ |
|---------------------------|-------------------|-----------|---------|------------------|
| | Tested | Infective | | |
| | number | number | percent | days |
| <i>C. geminatus</i> | | | | |
| Insectary | 20 | 6 | 30 | 24 |
| 25 C chamber | 30 | 5 | 17 | 28 |
| <i>C. montanus</i> | | | | |
| Insectary | 20 | 11 | 55 | 15 |
| 25 C chamber | 30 | 15 | 50 | 19 |
| <i>E. variegatus</i> | | | | |
| Insectary | 20 | 2 | 10 | 28 |
| 25 C chamber | 30 | 11 | 33 | 33 |

* WXD-agent extracts were prepared by triturating 100 diseased insects/1.5 cc of 10% sucrose, clarifying the slurry by centrifugation (8,000 g for 10 min, 4 C, SW 39 rotor). Supernatant was then centrifuged for 60 min at 100,000 g, the pellet resuspended in 10% sucrose and passed through a 0.45 μ Millipore® filter.

Acquisition and transmission of WXDA by *C. geminatus*, *C. montanus*, and *Fieberiella florii*

Fieberiella florii was reported by Anthon and Wolfe (1951) to be a vector of WXDA in Washington. Jensen (1957a) believed this species was important in field spread of WXDA in "hot spots" of the "peach bowl" region of California. This species has not been extensively studied as a vector, because it is difficult to rear in the insectary. However, small numbers could be reared on celery at 25 C and constant light, and a limited number of insects became available for use in comparative tests. Because of the prolonged developmental time of this species, transmission of WXDA occurred, while many of the vectors were still in the nymphal stage.

Table 23 shows *F. florii* and *C. montanus* had similar acquisition rates of WXDA from infected celery. *Fieberiella florii* consistently had higher LP₅₀ values, suggesting a slower multiplication rate of WXDA in this species than in *C. montanus*.

Acquisition rates of *F. florii* were compared with those of *C. geminatus* and *C. montanus* by both feeding and injection (Table 23, Trial III). Injection, as compared to feeding, resulted in a greater proportion of all species transmitting WXDA. LP₅₀ values for injected vectors were one-half or less than those of leafhoppers that acquired WXDA by feeding.

TABLE 23.
TRANSMISSION OF WXD-AGENT TO CELERY BY, AND LP₅₀ IN, *COLLADONUS GEMINATUS*, *C. MONTANUS*, AND *FIEBERIELLA FLORII* AFTER ACQUISITION BY FEEDING OR BY INJECTION. LEAFHOPPERS WERE TRANSFERRED TO FRESH CELERY PLANTS AT TWICE WEEKLY INTERVALS-UNTIL DEAD.

| Trial & Acquisition regimen | Test condition | <i>C. geminatus</i> | | | | <i>C. montanus</i> | | | | <i>F. florii</i> | | | |
|-----------------------------|----------------|---------------------|--------|-----------|------|--------------------|--------|-----------|------|------------------|--------|-----------|------|
| | | Tested | | Infective | | Tested | | Infective | | Tested | | Infective | |
| | | number | number | percent | days | number | number | percent | days | number | number | percent | days |
| I: Fed 1 day | 25C | — | — | — | — | 30 | 6 | 20 | 26 | 30 | 7 | 23 | 44 |
| | Fed 3 days | — | — | — | — | 30 | 5 | 17 | 30 | 30 | 10 | 33 | 36 |
| II: Fed 21 days | Insectary | — | — | — | — | 30 | 20 | 67 | 34 | 20 | 21 | 70 | 41 |
| III: Fed 21 days | Insectary | 25 | 9 | 36 | 35 | 35 | 16 | 64 | 28 | 25 | 14 | 56 | 48 |
| | Injected* | Insectary | 30 | 25 | 83 | 15 | 30 | 28 | 93 | 14 | 30 | 23 | 77 |

* WXD-agent extracts for injection were prepared by triturating 100 diseased insects / 1.5 cc of 10% sucrose, clarifying the slurry by centrifugation (8,000 g for 10 min 4 C, SW 39 rotor). The supernatant was then centrifuged for 60 min at 100,000 g, the pellet resuspended in 10% sucrose and passed through a 0.45 μ Millipore® filter.

DISCUSSION

The etiological agent of western X-disease, once considered to be a virus in the "yellows virus complex," is currently presumed by many workers to be a plant mycoplasma, perhaps a *Spiroplasma* (Thompson et al., 1978; Raju, Purcell, and Nyland, 1980). Prokaryotic microorganisms found in vascular tissues of WX-infected celery (Nasu, Jensen, and Richardson, 1970) and peach (Huang and Nyland, 1970) are sensitive to tetracycline antibiotics (Amin and Jensen, 1971; Nyland, 1971; Nyland and Moller, 1973). Discovery and reclassification of the etiological agent of WXDA, however, does not diminish the importance of the contribution of earlier workers who emphasized the vector-pathogen relationships and epidemiology of the disease in field investigations. In the present work, attempts were made to further define characteristics of WXDA transmission by leafhopper vectors.

Among the more interesting and applicable aspects of these studies was the discovery that circadian rhythms impose severe limitations on transmission potential of inoculative insects. These endogenous rhythms have received little emphasis in pathogen-vector studies, yet they are extremely important, particularly in short-term experiments where accurate quantification of inoculation and acquisition threshold periods is attempted.

Maximum transmission efficiency occurred in morning hours, with a second peak in late afternoon, followed by a decline to a lower level of transmission that persisted through the night. These cyclic transmission patterns were retained, even when tests were conducted in environmental chambers with constant light conditions. Also, leafhoppers do not feed and inoculate continuously during given periods of access time; therefore, effective transmission can be reduced or nonexistent for long periods. It is impossible, therefore, to state what is the actual length of a feeding period, particularly if experiments are initiated in the afternoon or carried over into evening hours. Comparative experiments should be planned within comparable periods of transmission efficiency.

Inoculative insects conditioned to constant temperature and light, can become arrhythmic. Such conditioning can result in similar transmission levels during all test periods. Conditioning techniques allow transmission to be maximized during short periods and entrained rhythms can be retained even in the absence of external stimuli.

During experiments on conditioning of leafhopper species to constant light and temperature conditions, it became apparent that each vector species had its own competency that was influenced, or regulated, by ambient test conditions. No one could have predicted that *C. geminatus* would be an efficient vector of WXDA under ambient insectary conditions, but not under constant conditions of environmental chambers. Such phenomena merit more investigation.

A second vector species, *E. variegatus*, had been reported by Jensen (1969a) to be variable in transmission efficiency. Yet, in comparative tests done in environmental chambers, this species consistently exhibited unanticipated high levels of transmission. Perhaps of more importance, *E. variegatus* was extremely long-lived even when transmitting WXDA. Indications were that *E. variegatus* was not adversely affected by WXDA. This, along with its ability to survive unfavorable conditions, would appear to make this species of special interest in field spread of WXDA.

All attempts to infect *C. montanus* by feeding on sachets containing active WXDA failed, although it was repeatedly demonstrated that insects ingested P^{32} tracer from sachets and that it accumulated in the insect's tissues and was excreted. Also, sachets contained active WXDA, because injection of sample inoculum from the sachets resulted in transmission to a high proportion of test plants. Honeydew of insects feeding on WXDA in sachets was not sampled for infectivity; further tests are needed to

determine if WXDA was actually ingested. However, puncturing the gut of insects fed on infectious solutions did not result in transmission.

The first successful long-term serial passage of WXDA, prepared from head extracts, at monthly intervals for 7 months provided additional evidence that WXDA multiplies in the vector tissues and supported the conclusions of Nasu, Jensen, and Richardson (1970).

Since the report that tetracycline solution, infused into WX-infected peach and cherry trees, causes remission of symptoms (Nyland, 1970), there has been considerable interest in field use of such chemotherapeutic management of mycoplasma-caused disorders in commercially important woody hosts. Two obvious disadvantages are the relatively high cost of application, either as foliage sprays or by infusion into the vascular system, and symptom recurrence (Nyland and Moller, 1973).

A tetracycline-resistant isolate of WXDA was quickly selected during serial passage of inoculum treated with 200 ppm hydrochloride tetracycline to *C. montanus* nymphs held at 25 C under constant light. By the fourth passage, 60% of injected leafhoppers were transmitting the resistant isolate 28 days after injection. These limited tests were not designed to deter further field application of antibiotics for management of vectored mycoplasma-caused disorders, but it should be remembered that when both plants and insect vectors are capable of multiplying isolates of disease agents under tetracycline pressure, significant levels of resistance may rapidly appear.

Summary and Conclusions

Additional investigations on transmission of WXDA by *Colladonus montanus* indicated the following:

1. The availability threshold of WXDA in celery to feeding *Colladonus montanus* was less than 25 days. Low titers of WXDA were available to insects before symptoms developed in plants, but suitability of celery as a source plant for WXDA increased with age of infection.

2. All instars of *C. montanus* acquired infective doses of WXDA from diseased celery. The pathogen appeared to multiply at a somewhat higher rate in last-stage nymphs and young adults than in 1st and 2nd stage nymphs.

3. Continual selection and inbreeding of *C. montanus* in the greenhouse insectary apparently resulted in a biotype, (Berkeley) that acquired, multiplied, and transmitted WXDA more efficiently than the F₁ and F₂ progeny of a field population of the same species (Dixon biotype).

4. Peach yellow leaf roll strain (PYLR) of WXDA was more efficiently acquired, transmitted, and pathogenic to, *C. montanus* than Green Valley strain (GV).

5. Daily acquisition rates of WXDA by *C. montanus* followed a binomial expectancy with an estimated median acquisition access period (AAP₅₀) of 2.5 days in insectary trials.

6. Efficient transmission of WXDA to celery by *C. montanus* occurred in less than 1 hr. Estimated median inoculation access period (IAP₅₀) was 3 hr, and extrapolation of the theoretical curve suggested successful inoculation could occur in 1 second.

7. Endogenous circadian rhythms affected transmission of WXDA by *C. montanus*. A higher rate of transmission occurred in morning than in late afternoon and evening hours and persisted for short periods of time under conditions of constant light and temperature. *Colladonus montanus* could be conditioned to light and temperature rhythms that persisted for a short period in the absence of external stimuli. Effects of endogenous rhythms could be reduced by conditioning infective insects to constant light and temperature for 4 days before testing.

8. Fluctuating temperatures around a mean of 20 C did not affect transmission of

WXDA by *C. montanus* infected by injection, nor was the median LP₅₀ consistently correlated with any temperature regimen.

9. *Colladonus montanus*, moved from WX-diseased celery to which they had access for more than 40 days, had a low probability of inoculating the first test plant on which they were caged. This response was independent of the length of the IAP, up to 8 hr. Vectors given an adjustment period of 8 hr on the first test plant had an increased probability of inoculating the next plant in the series. Insects given a sequence of eight 1-hr transfers had a higher mortality rate than did leafhoppers given 8 hr of continuous access.

10. Contents of five head capsules from WXDA-infected *C. montanus* triturated in 5 μ l of 30% sucrose solution, was equivalent to 100 infected whole leafhoppers triturated in 1.5 cc of 30% sucrose solution. WXDA from head extracts was passed serially seven times at monthly intervals in *C. montanus*. The estimated dilution was 2×10^{-24} , providing strong evidence for multiplication of WXDA in this vector. Serial passage of inoculum treated with 200 ppm of tetracycline, resulted in rapid selection of a tetracycline-resistant strain of WXDA. After four passages of the treated isolate, 60% of injected vectors were consistently transmitting 28 days after injection. This compared with ca. 25% success rate using once-treated WXDA.

11. None of 316 *C. montanus*, fed on sachets containing WXDA, transmitted, although assays showed the sachets contained infectious material. In addition, the presence of P³² tracer in body tissues and excreta meant leafhoppers fed on infectious extracts.

Attempts to purify and concentrate WXDA by electrophoresis yielded active agent, but again no transmission occurred when aliquots were fed to *C. montanus* via membrane feeding. Why leafhoppers failed to acquire WXDA using membrane-feeding systems containing active WXDA remains unknown.

Comparative studies on transmission of WXDA by *C. geminatus*, *C. montanus*, and *Euscelidius variegatus* and *Fieberiella florii*, resulted in the following:

1. Caging three species on the same WX-diseased plant, as compared to each being caged alone, reduced longevity. Each species had a specific vector-competency, but group-feeding reduced the probability of obtaining an infective insect only with *E. variegatus*.

2. When given a 6-day AAP on WX-diseased celery in the insectary, *C. montanus* had the highest transmission rate (87%) and the shortest LP₅₀ (25 days), while *E. variegatus* had the lowest transmission rate (30%) and the longest LP₅₀ (34 days). *Colladonus geminatus* was intermediate, with a transmission rate of 50% and an LP₅₀ of 30 days. A comparison of median survival periods (SP₅₀) indicated low transmission efficiency of *E. variegatus* was due primarily to poor survival, rather than to any pathogenic effect of WXDA infection.

3. Transmission of WXDA by the three species was compared under three constant temperature regimes—20, 25, and 30 C—and constant light. *Colladonus geminatus* was the poorest vector at 20 C. LP₅₀ values for all species were similar at 20 C, but not at 25 C. At 30 C, both WXDA and leafhoppers were affected adversely. Only ca. 4 per cent of *C. montanus* transmitted. The survival of *C. geminatus* at 30 C was poor, and although *E. variegatus* survived well, neither species transmitted at this temperature.

4. Leafhoppers given an AAP on WX-diseased celery at 20 and 25 C had an AAP₅₀ of 11.2, 12.9, and 10.6 days at 20 C and 10.6, 9.8, and 10.6 days at 25 C for *C. geminatus*, *C. montanus*, and *E. variegatus*, respectively. The mean daily probability of acquisition of WXDA followed a binomial distribution for all three species. The LP₅₀ decreased when the temperature increased from 20 to 25 C. At the higher temperature, the SP₅₀ decreased for both *Colladonus* species, but increased for *E. variegatus*. *Euscelidius variegatus* was not affected by WXDA infection as were *C. geminatus* and *C. montanus*.

5. In insectary tests with insects infected by injection, *C. geminatus* was a better

vector than *E. variegatus*, while in controlled light and temperature conditions, the latter was more efficient. *C. montanus* consistently was the best vector under all test conditions.

6. Acquisition of WXDA by leafhopper vectors was influenced by photoperiod. Better acquisition from diseased celery by *C. geminatus*, *C. montanus* and *E. variegatus* occurred, if the AAP was during a 12-hr photoperiod than under constant light. When insectary conditions were included in the comparisons, apparently fluctuation in both light and temperature further increased acquisition efficiency of all species tested.

7. Comparisons of *C. geminatus*, *C. montanus*, and *F. florii* under insectary conditions indicated the last named species was similar in acquisition and transmission efficiency to *C. montanus*. LP₅₀ of WXDA in *F. florii* was longer than in either *Colladonus* species, suggesting a slower rate of pathogen multiplication in this leafhopper host. When injected with WXDA, the LP₅₀ in *F. florii* was one-half that following acquisition by feeding. *Fieberiella florii* also differed from the *Colladonus* species in that it transmitted WXDA while still in the nymphal stage. Also, it did not appear to be adversely affected by infection with WXDA.

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