Temperature and Transmission of the Western X-Disease Agent by Colladonus montanus

D. D. Jensen

Professor, Division of Entomology, University of California, Berkeley 94720.

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ABSTRACT

Previously noninfective *Colladonus montanus* leafhoppers were injected with extract from leafhoppers infected with the Western X-disease agent (WXA). The leafhoppers were then caged singly on small celery plants used as disease indicators, and kept at constant temperatures ranging from 10 to 30 C. Another group of insects was maintained in a plant growth chamber programmed to run alternately at 15 and 30 C for 12 hr during each 24-hr period. Test insects were transferred to new plants weekly or twice weekly. The median incubation period of WXA in leafhoppers kept at constant temperatures was shortest (26 days) at 25 C, and longer at either higher (38 days at 30 C) or lower (125 days at 15 C) temperatures. Only one of 83 insects held at 10 C transmitted WXA. The most favorable constant temperature for the development of infectivity in injected insects was 20 C. Only 4 and 24% of the insects injected as nymphs became infective at 30 and 15 C, respectively. However, 75% of the insects maintained alternately at 15 and 30 C became infective. Therefore, variable temperatures, even some that are unfavorable when held constant, may be more conducive to vector efficiency than are constant temperatures.

Retention of WXA by *C. montanus* and insect longevity were, in general, inversely proportional to the temperatures at which the insects were held.

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The Western X-disease agent (WXA), earlier thought to be a virus, is now considered to be a mycoplasmalike organism (1, 14). This agent is of unusual interest because it is pathogenic to its leafhopper vector, *Colladonus montanus* (Van Duzee), as well as to its plant hosts, which include stone fruits in the genus *Prunus* and also a number of herbaceous plants (7). In *C. montanus*, WXA causes premature death (3, 8), reduced fecundity (4, 6), needlelike crystals in the alimentary tract of leafhoppers feeding on diseased celery plants (10), cytopathological effects in several organ systems (20), and impaired oxygen utilization (2).

Exposure of infective vectors to high temperatures (38-41 C) for 4 to 20 days inactivated WXA in some insects and reduced its concentration in all others (5). In the latter instance, a second incubation period of as long as 4 weeks was required before the insects again became capable of inoculating test plants with WXA. The longevity of infective insects receiving heat treatment was also greater than that of unheated insects, which suggested that the pathological effects

of WXA in the vector were arrested or retarded by these temperatures, presumably by destroying most of the mycoplasma.

In earlier work (17, 18), we showed that the concentration of WXA in the extract injected into *C. montanus* affected both the percentage of leafhoppers that became infective and the incubation period of WXA in the vector. The lower the concentration of WXA, the fewer the insects that became infective and the longer the incubation period.

This paper reports the results of studies on the effects of temperature on the relation of WXA to its vector, *C. montanus*, as indicated by the percentage of injected insects that became infective, the length of the incubation period, transmission efficiency, and retention of WXA by the vector. Temperature effects on insect longevity are also reported.

MATERIALS AND METHODS.—The yellow leaf roll strain of WXA used in these studies was obtained originally from peach near Marysville, Calif., and is maintained in celery in the greenhouse at Berkeley. The test insects were reared in the greenhouse on celery, *Apium graveolens* L. 'Utah Green', which was also used as the indicator plant in bioassays for the presence of WXA.

Healthy *C. montanus* leafhoppers were infected with the WXA by injecting them with extracts made from leafhoppers that had fed on diseased celery plants for 30-40 days. All insects were in the last nymphal instar when they were injected except those in Experiment I, of which some were young adults (Table 1). The insect extracts were prepared at ca. 4 C by grinding 100 infective adults in 1.5 ml of 0.85% NaCl which contained the antibiotics streptomycin, penicillin, and Chloromycetin, each at 0.1% (w/v) to control bacterial contaminants (20), The extract was then clarified by centrifugation at 8,000 *g* for 10 min, and the resulting supernatant solution received the same treatment. The extract was kept in ice water until all injections had been made. The injection procedure and equipment were described previously (17).

In a given experiment, all insects were injected with the same inoculum. They were then caged together on a large celery plant to insure randomization, and held overnight in a growth chamber at a constant temperature of 20 C which was favorable for their recovery from the wounding due to injection. The injected insects were then divided into groups of equal size, caged on healthy celery plants, and placed in plant growth chambers at different temperatures. After 7-14 days, depending upon the temperature and the experiment, surviving insects were caged singly on small celery plants for transmission feedings at the respective temperatures. The insects at most temperatures were transferred twice weekly to new plants. Plants exposed to a transmission feeding were kept in a greenhouse for symptom development. The cages used to confine the insects on the plants were plastic tubes (2-inch diam, 4-inches tall); the upper end of the tube was covered with organdy cloth for ventilation, and the lower end was lowered over the plant and pressed into the soil.

TABLE 1. The effect of temperature on the establishment and the incubation period of the Western X-disease agent after its injection into the vector, *Colladonus montanus*, and on postinjection longevity of the vector

Exp	Temp	Vectors		Incubation period		Avg insect longevity
				Range	IP_{50}[a]	
No.	*C*	*no.*	*%*	*days*	*days*	*days*
I	10	0/25[b,c]	0			39
		1/59[d]	1.7	93		134
	20	55/65[c]	85	32-60	39	76
		30/44[d]	55	28-60	35	64
	30	4/96[c]	4	34-62	38	57
		5/54[d]	9	29-46	38	49
II	15	14/55	24	40-172	125	145
	20	35/59	59	16-73	44	96
	25	33/55	60	16-65	34	88
III	17	31/60	51	49-105	64	87
	20	39/58	67	30-70	40	60
	25	29/60	48	16-38	26	49
	28	9/60	15	23-45	32	44
	15-30[e]	44/59	75	18-115[f]	31	53

[a] IP_{50} = median incubation period; i.e., time after injection required for 50% of the insects that will ultimately transmit WXA to transmit at least once.

[b] Numerator = number of injected leafhoppers transmitting WXA; denominator = number of injected leafhoppers tested.

[c] Leafhoppers, when injected, were in the last nymphal stage.

[d] Leafhoppers, when injected, were in the adult stage.

[e] Insects were maintained in a growth chamber set to run 12 hr at 15 C and 12 hr at 30 C during each 24-hr period.

[f] If the single insect that required 115 days is omitted from consideration, the range for the other 43 infective vectors was 18-49 days.

The plant growth chambers were adjusted to provide desired temperatures inside the individual insect cages; monitoring thermocouples were located in the cages near the plants on which the leafhoppers fed. Temperatures were recorded daily for each chamber and fluctuated ± 1-1.4 C from the desired temperatures.

All growth chambers except one were run at constant temperatures and with continuous light (880-890 ft-c). In Experiment III (Table 1), one chamber was programmed to run 12 hr at 15 C in darkness and 12 hr at 30 C in light (600-620 ft-c) during each 24-hr period. However, ca. 3.5 hr/day were required for the temperature in the insect cages to change from one level to the other. The time required to effect the change from 15 to 30 C was 1 hr and 55 min (to change from 15 to 27 C averaged 66 min, but an additional 49 min were needed for the rise from 27 to 30 C).

The time required to reduce the temperature from 30 to 15 C was 1 hr and 39 min (44 min to drop from 30 to 18 C, and 55 min to drop from 18 to 15 C).

RESULTS.—Temperatures from 10 to 30 C were involved in three different experiments. The number

and percentage of insects that transmitted at the different temperatures, the ranges in incubation period of the WXA in *C. montanus* at the various temperatures, and the median incubation period (IP_{50}) are given in Table 1. IP_{50} (15) represents the time, after acquisition of the WXA, required for 50% of the insects that will ultimately transmit WXA to transmit it at least once. The concept embodied in IP_{50} has advantages over those reflected by minimum incubation period and average incubation period, which have been used commonly, since minimum incubation period is based on only one or a few insects that represent an atypical extreme and average incubation period may be influenced unduly by a few exceptionally long incubation periods.

Experiment I.—The temperatures 10, 20, and 30 C were used. Some of the insects when injected were adults; some were nymphs (Table 1). Only one of the insects held at 10 C transmitted WXA, and it had been injected while in the adult stage; the incubation period of WXA in this vector, as might be expected, was long (93 days).

All 25 nymphs injected and held at 10 C died prior to completion of the incubation period. Their mean longevity after injection was only 39 days. The adults, in contrast, had a mean longevity of 134 days. Nevertheless, only one became infective. These results suggest that a temperature of 10 C either destroyed the WXA in the leafhoppers or extended the incubation period so long that all but one of the vectors died before becoming infective.

Of the insects held at 20 C, 85% of the nymphs and 55% of the adults transmitted WXA. At 30 C, however, the number of insects that became infective dropped substantially, and amounted to only 4 and 9% for nymphs and adults, respectively. There were no significant differences among the IP_{50} values for the four groups held at 20 and 30 C.

Experiment II.—Though temperatures (15, 20, 25 C) varied by only 5 C, there was a conspicuous effect of temperature. At 15 C, only 24% of the insects became infective as compared to 59 and 60% for those held at 20 and 25 C, respectively. The range in incubation period was more than twice as long at 15 C as at 20 and 25 C, and the IP_{50} at 15 C was 3 times longer than that at each of the higher temperatures.

Experiment III.—In this experiment, injected insects were held at a constant 17, 20, 25, or 28 C. In addition, a fifth group was held for 12 hr at 15 C (without lights), and 12 hr at 30 C (with lights) during each 24-hr period.

Percentages of insects that became infective at the respective temperatures are shown in Table 1. The fewest insects (15%) became infective at 28 C, whereas at 25 C (i.e., only 3 C cooler), infectivity increased more than 3-fold to involve 48% of the insects, and at 20 and 17 C amounted to 67 and 51%, respectively.

The most surprising results were obtained with the insects maintained alternately at 15 and 30 C, both temperatures which, if held constant, were relatively unfavorable for the survival and multiplication of WXA in the insects. Thus, when temperatures were a constant 30 or 15 C, only 4 and 24%, respectively, of the nymphs became infective. In contrast, 75% of the insects transmitted when they were kept in the growth chamber in which the temperature alternated between 15 and 30 C.

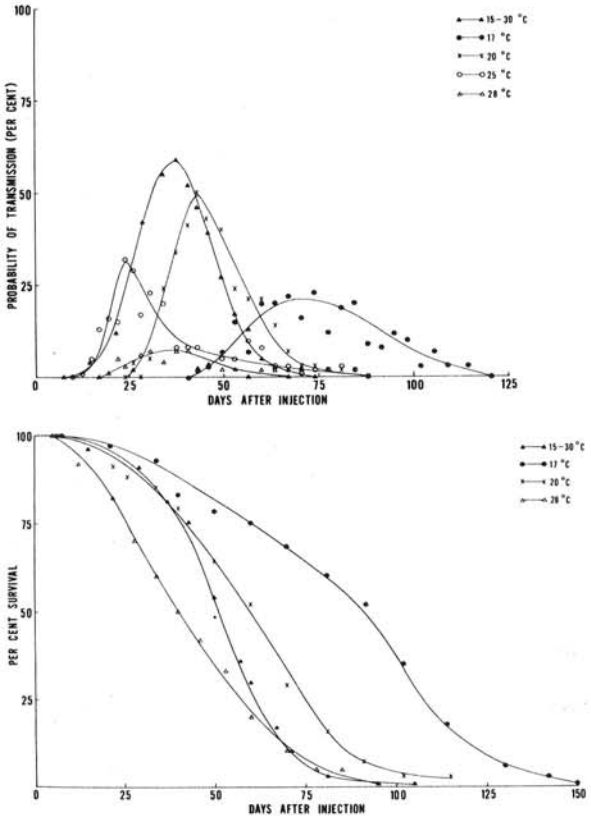

Fig. 1. (Above) Transmission of Western-X disease agent (WXA) by *Colladonus montanus* leafhoppers under five different temperature conditions. The curves are based on the probability of transmission (expressed as per cent) by surviving leafhoppers during the respective test periods and were obtained using the formula, LX X TX = transmission probability, where LX = age specific survival probability (number of insects surviving on any given week [X] divided by the original number tested) and where TX = age specific transmission rate (number of transmitters divided by the number of surviving insects at interval X). The insects were held at constant temperatures and under continuous light except those designated 15-30 C which were tested in a growth chamber programmed to run 12 hr at 15 C (in darkness) and 12 hr at 30 C (in light) during each 24-hr period. (Below) Survival curves for *C. montanus* leafhoppers held at the indicated temperatures. Temperatures and light were constant except for insects in the 15-30 C group, which were tested in a growth chamber programmed to run 12 hr at 15 C (in darkness) and 12 hr at 30 C (in light) during each 24-hr period. The curve for insects held at 25 C was not included because it coincided closely with that of insects held at alternating temperatures of 15 and 30 C.

The WXA transmission curves obtained for the five different temperature conditions used in Experiment III are shown in Fig. 1, above. The curves depict the probability of transmission expressed as

per cent according to the formula LX X TX = transmission probability, where LX = age specific survival probability (number of insects surviving on any given week [X] divided by the original number tested) and where TX = age specific transmission rate (number of transmitters divided by the number of surviving insects at interval X).

Maximum transmission at a given constant temperature was achieved earliest for insects at 25 C (Fig. 1, above), but the level of transmission was much lower than that which occurred at 20 C or when the temperature alternated between 15 and 30 C. The maximum level of transmission recorded for insects at 28 C occurred later than that observed for insects at 25 C, was less sharply associated with a given number of days after injection, and was the lowest of any recorded for the five temperatures tested in Experiment III. Transmission by insects at 17 C did not reach a maximum level until 75 days after injection of the insects, but was nearly maximum over a broad interval and was much more frequent than was observed for insects at 28 C.

Effect of temperature on leafhopper survival.—The longevity of leafhoppers held at constant temperatures after injection with WXA was, in general, inversely proportional to the temperature. However, 10 C was too cold for nymphs; their mean longevity, after injection, was only 39 days. In contrast, injected adults held at 10 C lived an average of 134 days (Table 1, Experiment I). At 15 C (Experiment II), injected nymphs survived well, underwent transformation into adults, and had a mean longevity of 145 days, the longest of any group tested. The other insects tested in Experiment II also had exceptional longevity when compared to those held at the same temperatures in Experiments I and III (Table 1).

Survival curves for the test insects in Experiment III are given in Fig. 1, below, except that the curve for insects held at 25 C was deleted because it coincided closely with that for insects held at the alternating temperatures of 15 and 30 C.

The longevity values (Table 1) are based on the survival of both transmitting and nontransmitting insects at the respective temperatures. It has been well established (3, 8) that WXA causes the premature death of the vector *C. montanus*. The present study further confirmed this observation at temperatures that permit adequate samples of transmitting and nontransmitting insects.

DISCUSSION.—Previous work had demonstrated that high temperatures inactivated viruses and mycoplasmalike organisms in vectors that were infective before they were given a heat treatment. Kunkel (9) showed that temperatures of 31 to 32 C eliminated the aster yellows agent or reduced its titer temporarily to a subinoculative level in colonies of aster leafhoppers, *Macrosteles fascifrons* (Stål) Sigma virus titer was reduced in *Drosophila* held at 30 C (11). The WXA in *C. montanus* was inactivated or reduced in titer below that effective for transmission if held at 38 and 41 C for 7 and 20 days, respectively (5). Tanada (16) has reviewed the brief history of heat therapy of insect viruses and the theories advanced to explain the heat-induced resistance of insects to virus infection and injury.

In the present work, when *C. montanus* leafhoppers were injected with WXA extract and held at constant temperatures during the incubation and test feeding periods, there was a consistent relationship between the temperature level and (i) the percentage of insects that became infective; (ii) the length of the incubation period; and (iii) the longevity of the leafhoppers. Neither low nor high constant temperatures were conducive to a high percentage of infectivity among injected insects (Table 1). At very low temperatures, the WXA apparently had difficulty in multiplying to an infective level in the vectors and may actually have been inactivated. The data suggest that at 30 C, WXA in *C. montanus* is undergoing multiplication and inactivation simultaneously, with the inactivation process predominating in most insects. The optimum constant temperature for development of infectivity in the highest percentage of injected insects appeared to be between 20 and 25 C. At constant temperatures that were either higher or lower than this range, a difference of a few degrees was reflected in a relatively profound effect on the percentage of insects that became infective (Table 1).

Although constant temperatures of either 15 or 30 C were unfavorable for WXA transmission, leafhopper vectors subjected within a 24-hr period to both these temperatures on a 12-hr alternating basis produced a higher level of transmission (75%) than usually occurred at any of the constant temperatures. These results suggest that constant temperatures and/or light per se may be less favorable for WXA multiplication and transmission than are variable temperatures and/or light. This possibility is supported by the results of many other injection experiments in which the test insects were held under fluctuating temperature and light conditions in the greenhouse. For example, in 11 such experiments made during the same general period in which the study reported herein was under way, 397 of 410 insects (97%) injected with WXA and tested for infectivity transmitted WXA. In contrast, under conditions of constant temperature and light, the highest level of infectivity achieved was 85% which occurred in only one of three experiments at 20 C (Table 1). In two other experiments at 20 C, and at all other temperatures, the level of transmission never exceeded 73%. Thus, fluctuating temperatures, even those cycling between limits that individually were not conducive to good WXA transmission, usually resulted in better transmission than did the most favorable of the constant temperatures.

The basic factors that determine these responses are not yet known. Multiplication of WXA in the leafhopper may not be the only factor involved. Feeding habits may also be changed by constant temperature and light, probably as a result of the disturbance of the insects' diurnal rhythm. Moreover, in the greenhouse environment there are more factors that might interrupt the feeding of the test insects

more frequently than is the case in a growth chamber.

Although an enhancing of insect transmission of viruses or mycoplasma by fluctuating temperatures apparently has not been reported previously, it is consistent with the results obtained in other experiments on physiological responses in insects. For example, Messenger (13) reported that fluctuating temperatures were conducive to increased growth and fecundity in aphids.

The length of the incubation period of WXA in the leafhopper vector was also a function of temperature, and appeared to be consistently shorter for leafhoppers held in the greenhouse, under fluctuating temperatures, than for leafhoppers maintained at the most favorable constant temperatures in controlled chambers. Thus, the IP_{50} value for 397 transmitting leafhoppers in 11 greenhouse experiments was 22 days or less, whereas it was 31 days or more in all groups held under controlled temperatures except for one experiment at 25 C (Table 1, Experiment III), when it was 26 days.

A constant temperature of 25 C resulted in the shortest incubation periods of WXA in the vectors (Table 1). At temperatures higher or lower than 25 C, the incubation period (IP_{50}) was lengthened ca. 30% by lowering the temperature from 25 to 20 C, and an additional 150% for a decrease from 20 to 15 C.

Maramorosch (12) reported that the minimum incubation period of the aster yellows agent in the aster leafhopper was 11 days at 30 C, 12 days at 25 C, and 16 days at 20 C. His results were based on tests on 10 groups of 10 insects each for each of the above temperatures rather than on insects tested individually. Five, ten, and six groups effected transmission at 30, 25, and 20 C, respectively.

The mean retention period of WXA in the vectors was, in general, also a function of temperature. The lower the temperature, the longer infectivity was retained. The shortest retentions were at 25 and 28 C, with no significant difference between these temperatures.

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