Tomato Spotted Wilt Tospovirus Ingestion by First Instar Larvae of *Frankliniella occidentalis* Is a Prerequisite for Transmission

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This research was supported by fellowship ABI22.2704 of the Foundation of Dutch Technology (STW) of the Netherlands Organization for Scientific Research (NWO).
We thank S&G Seeds, Enkhuizen, the Netherlands, for supplying us with *Petunia* × *hybrida* 'Polo Blauw' seeds. We also thank P. Krell for critical reading of the manuscript.
Accepted for publication 10 June 1996.

ABSTRACT

Van de Wetering, F., Goldbach, R., and Peters, D. 1996. Tomato spotted wilt tospovirus ingestion by first instar larvae of *Frankliniella occidentalis* is a prerequisite for transmission. Phytopathology 86:900-905.

Tomato spotted wilt tospovirus (TSWV) is, after ingestion by first instar larvae, efficiently transmitted by second instar larvae and adults of the thrips *Frankliniella occidentalis* in a propagative manner. The developmental stage at which thrips larvae acquire an infectious dose, resulting in adults that can transmit the virus, is further defined. TSWV accumulation and transmission occurred after ingestion by first instar larvae. Second instar larvae failed to acquire and retain TSWV upon ingestion and did not develop into transmitters. No correlation was found between the quantity of TSWV ingested by thrips and their ability to acquire TSWV. Instead, first instar larvae gradually lost their acquisition ability with increasing age, suggesting the development of a barrier preventing TSWV acquisition and, consequently, replication needed for transmission. The finding that TSWV is acquired exclusively by first instar larvae of *F. occidentalis* is of crucial importance for understanding the epidemiology of this devastating virus.

Additional keywords: Thysanoptera, virus-vector relations, western flower thrips.

Tomato spotted wilt tospovirus (TSWV) ranks among the most devastating plant viruses worldwide (19). The recent resurgence of TSWV, and other tospoviruses (27), is due, in part, to two major factors. First, TSWV has a very broad host range, spanning 650 plant species, including many important vegetable and horticultural crops, as well as numerous weeds, which provides a broad array of ecological niches (9,17,19). Second, the different thrips species that transmit TSWV infest a range of host plants. Currently, seven thrips species have been reported to transmit TSWV (6,29,45), of which *Frankliniella occidentalis* (Pergande) (Thysanoptera:Thripidae), the western flower thrips, is a very efficient vector (45). This species has expanded its geographic range to almost all of the subtropical and temperate climate zones of most of the continents (7,13,17,24,28,30,41). During the past few years, control of *F. occidentalis* became more difficult due in part to its resistance to many chemical insecticides. In addition, incidence of TSWV cannot be controlled by insecticides applied to incoming thrips (20,37). Host-plant resistance and biological control could possibly provide alternatives to control thrips and satisfy consumers' demands for a decrease in the use of pesticides (4,14,25). To develop sustainable management strategies for control of thrips and TSWV epidemics, a more detailed understanding of the TSWV-infection cycle and virus-vector interactions are essential (9).

It is generally accepted that only thrips larvae acquire TSWV (33). After ingestion and subsequent virus multiplication (39,48), TSWV can be transmitted by second instar (L2) larvae as well as adults (47). Once the vector becomes viruliferous, it remains so throughout its lifespan, but the rate at which each individual transmits may vary considerably (33,46).

In this report, the developmental stages at which thrips larvae acquire and transmit TSWV were analyzed and defined. The results show that only first instar larvae of *F. occidentalis* can acquire TSWV after ingestion and that these thrips become transmitters later in their development. Possible explanations for the exclusive capacity of first instar larvae to acquire TSWV are discussed.

MATERIALS AND METHODS

Thrips. The *F. occidentalis* used in our studies were obtained from infested bean plants in a greenhouse in the Netherlands and were reared as virus-free colonies on bean pods (*Phaseolus vulgaris* L.) at 27 ± 0.5°C with a 16-h photoperiod each day.

TSWV isolate and plant material. In all experiments, the Brazilian TSWV isolate BR-01 (13) was used. The isolate was maintained by thrips inoculation on *Datura stramonium* L. plants. Plants used in acquisition experiments were mechanically inoculated on the first two leaves with extracts from thrips-inoculated plants. These plants were kept in a greenhouse at approximately 22°C (16/8 h of light/dark).

Preparation of antiserum and conjugate for enzyme-linked immunosorbent assay (ELISA). Polyclonal antiserum raised against the nucleocapsid protein (N protein) of TSWV isolate BR-01 was used in ELISA (12). The immunoglobulin G (IgG) fraction was partially purified by ammonium sulfate precipitation (10). IgG was conjugated at a concentration of 1 mg/ml with 2,000 units of alkaline phosphatase (grade I, Boehringer GmbH, Mannheim, Germany) in phosphate-buffered saline (PBS; 0.14 M NaCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.5 mM KCl), pH 7.4 (3). The IgG and conjugate were stored with 0.05% sodium azide at 4°C.

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Publication no. P-1996-0710-02R

TSWV-N protein detection by ELISA. The presence of TSWV-N protein in individual thrips was detected by a cocktail ELISA with enzyme amplification (31,42). Wells of Nunc-Immunoplate Maxisorp F96 plates (Nunc, Roskilde, Denmark) were coated with 150 µl of 0.5 µg of IgG per ml in coating buffer (0.05 M sodium carbonate, pH 9.6). After incubation overnight at 4°C, the plates were rinsed three times with PBS-Tween (PBS with 0.05% Tween 20). Individual thrips were placed in Eppendorf tubes and ground with a micropestle in 100 µl of sample buffer (2% polyvinylpyrrolidone [M_r about 44,000] and 0.2% ovalbumin in PBS-Tween). The samples were added to the wells and mixed with 25 µl of 1 µg of TSWV anti-N protein conjugate per ml in sample buffer. Virus-free thrips and a dilution series of a nucleocapsid preparation purified from TSWV-infected *Nicotiana rustica* plants were used as the control and standard, respectively. The cocktail was incubated overnight at 4°C and, afterward, was rinsed three times with PBS-Tween.

The enzyme amplification reaction was performed as described by Van den Heuvel and Peters (42). Initially, 100 µl of 0.2 mM NADP monosodium salt in 0.05 M diethanolamine buffer, pH 9.5, was added to each well. After incubation at room temperature for 45 min, 140 µl of the amplification mixture was added per well. The stock amplification mixture consisted of 700 units of alcohol dehydrogenase and 100 µl of 1 mM *p*-iodonitrotetrazolium violet in 1 ml of 0.025 M phosphate buffer, pH 7.0. The reaction was allowed to proceed at room temperature. Absorbance values were determined with a EL 312 ELISA-reader (Bio-Teck Instruments, Greiner BV, Alphen aan de Rijn, the Netherlands) at 490 nm. The values were corrected for blank values read for wells that contained only sample buffer in the sample incubation step. The threshold value was calculated as the average of at least six virus-free thrips plus 3 times the standard deviation. All the readings above the threshold value were considered positive. The amount of TSWV-N protein found in each thrips was calculated by the values of the dilution series of nucleocapsid preparations.

Handling thrips in transmission experiments. Systemically infected leaves of *D. stramonium* plants that showed equally high TSWV-N protein titers in a dilution series, using double-antibody sandwich-ELISA (10,31), were used as sources for acquisition. The infected leaves were cut in pieces and placed randomly in Tashiro cages (36) in which *F. occidentalis* larvae were confined. Separate groups of instar larvae were caged on virus-free plant material as control treatments. After the acquisition access period (AAP), the thrips were transferred to virus-free *D. stramonium* plant material and allowed to complete their development until adult emergence. All the experiments were carried out at 25 ± 0.5°C. Adult-stage thrips were individually tested for TSWV transmission on leaf disks (13 mm in diameter) of *Petunia* × *hybrida* 'Polo Blauw,' as previously described (47), for three successive inoculation access periods (IAP) of 48 h. After each IAP, the leaf disks were floated on water for 2 days at 27 ± 0.5°C in 24-well plates (Costar Europe Ltd., Badhoevedorp, the Netherlands) for symptom development. Transmission efficiency was calculated as the percentage of leaf disks that developed local lesions (43).

TSWV transmission experiments. Newly hatched and 1-, 2-, 3-, 4-, and 5-day-old thrips larvae were placed on TSWV-infected material for 24 h, using in total 58, 71, 51, 40, 51, and 50 insects, respectively, in two replications. Before and after AAPs, the thrips were kept on virus-free *D. stramonium* plant material. Transmission efficiency was expressed as the number of adults that transmitted TSWV, using the petunia local lesion assay. Adult thrips were subsequently stored at –70°C prior to ELISA. Samples of thrips that fed on noninfected plant material were used as controls. The ELISA values for thrips were analyzed by Duncan's multiple range test with a STATGRAPHICS 6.0 PLUS computer program (32,34).

In another experiment, 0-, 12-, 20-, 28-, 36-, 44-, 52-, and 56-h-old larvae were given a 24-h AAP, with 30 insects per treatment per replication. Prior to AAP, the ratio of first instar (L1) and second instar (L2) larvae was determined by counting the molting skins found in each treatment. Transmission efficiency was determined for adults. This experiment was repeated three times. Thrips grown on virus-free material were used as controls.

Ingestion and transmission by the L1 and L2 larval stages. Newly hatched, 0-h-old first instar larvae (n = 84), 60-h-old second instar larvae (n = 102), and 40-h-old (n = 138) larvae were placed for 4 h on infected material. Before and after the AAP, the larval stage of the 40-h-old larvae was determined based on the number of molting skins and gut color. The gut has a green color during feeding on *D. stramonium* and turns transparent during the molting stage. Using this qualitative technique together with the molting skins found, the larvae were divided into four categories, i.e., first instar larvae (n = 26), second instar larvae (n = 21), larvae in the molting stage (n = 45), and a mixture of first and second instar larvae (n = 46). After the AAP of 4 h, larvae from each group were collected randomly, frozen at –70°C, and later assayed by ELISA. Transmission efficiency was determined for adults by the petunia local lesion assay.

TSWV accumulation in maturing thrips. TSWV accumulation was analyzed in maturing thrips after they ingested virus as newly hatched larvae (L1) and 2.5-day-old larvae (L2). The thrips were given a 4-h AAP on TSWV-infected *D. stramonium* plant material. Respective groups of 25 individuals were collected randomly immediately after AAP every day in the larval, the pupal, and the adult stages. These thrips were frozen immediately at –70°C and later tested individually for the presence of N protein. Thrips that were confined for 4 h on noninfected *D. stramonium* plant material were used as controls.

Determination of the amount of TSWV ingested. Larvae ranging in age from 0 to 5 days old were given an 8-h AAP on TSWV-infected *D. stramonium* plant material. Before the AAP, the thrips were kept on virus-free *D. stramonium* plant material. Immediately after the AAP, the larvae (approximately 25 per treatment) of each age group were collected and stored at –70°C until tested by ELISA. Samples of thrips confined for 8 h on virus-free *D. stramonium* plant material were used as controls.

RESULTS

Relationship between ingestion of TSWV by larvae and transmission by adults. We allowed different developmental stages of *F. occidentalis* larvae, ranging between 0 and 5 days old, to ingest TSWV to compare the ability of the adult-stage thrips to transmit TSWV. Our data showed that TSWV acquisition by 0- to 2-day-old larvae resulted in adults that were capable of transmitting TSWV, whereas when 3- to 5-day-old larvae fed on TSWV-infected plant material the adults failed to transmit TSWV (Fig. 1). The highest adult transmission rate was obtained when newly hatched larvae had ingested TSWV. Transmission efficiency decreased sharply with increase in the age at which first instar larvae fed on infected plant material. Also, when thrips were tested for TSWV-N protein by ELISA, the number of ELISA-positive adults decreased with increasing age at which TSWV was ingested by first instar larvae and paralleled the decrease in adult transmission efficiency.

Ninety percent of the adults that fed as newly hatched larvae were ELISA-positive, and 48% transmitted TSWV. No adult thrips that ingested TSWV as 3- to 5-day-old larvae were ELISA-positive. The results also showed that when ELISA values for adult thrips (TSWV transmitters versus nontransmitters) were compared, all thrips transmitters showed a significantly higher ELISA value than the nontransmitters ($P < 0.05$), hence a certain threshold amount of TSWV might need to be present for successful transmission (Table 1). Furthermore, thrips that were nontransmitters could be divided into adults that were ELISA-positive and -negative. ELISA-positive thrips were found only when TSWV was fed to 0- to 2-day-old larvae.

In the next experiment, we studied acquisition of TSWV, using groups of larvae ranging in age from 0 to 56 h to define more precisely the moment at which the larvae lost their ability to acquire TSWV. The L1/L2 ratio of larvae, age of larvae when fed on TSWV-infected plant material, and ability to transmit TSWV in the adult stage were determined. The highest transmission efficiency (42%), again, was observed for adults that were fed as newly hatched larvae on TSWV-infected plant material. Larvae by the age of 56 h (L2 larvae) could not acquire TSWV, because when they were adults they did not transmit the virus. These results, again, show that the ability to acquire TSWV decreased with the age of the L1 larvae and that the potential to acquire TSWV was significantly reduced for L2 larvae. The data, however, cannot exclude the possibility that some early L2 larvae acquired TSWV, because some were present in the 36- and 44-h-old populations analyzed (Fig. 2).

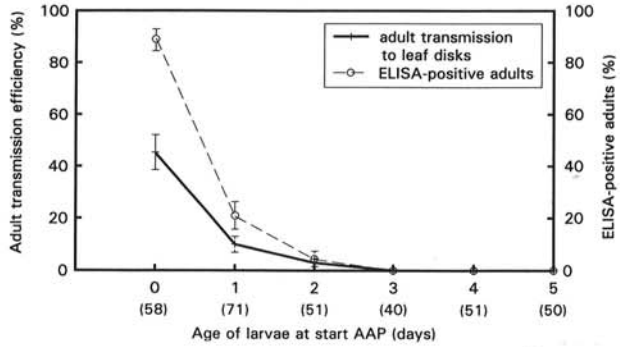

Fig. 1. Tomato spotted wilt virus (TSWV) transmission efficiency (±SE) and enzyme-linked immunosorbent assay (ELISA)-positive adult thrips after a 24-h acquisition access period (AAP) on TSWV-infected *Datura stramonium* plant material by 0- to 5-day-old larvae. Values below x-axis represent the number of thrips tested and are the sum of two replications.

TABLE 1. Average enzyme-linked immunosorbent assay (ELISA) values (±SE), using antiserum against tomato spotted wilt virus nucleocapsid protein (TSWV-N protein), for adult thrips after TSWV ingestion by larvae of different ages

Age of acquisition (days)	ELISA value[z]		
		Nontransmitter	
	Transmitter	ELISA-positive	ELISA-negative
0	0.785 ± 0.024 a (22)	0.390 ± 0.014 b (31)	0.001 ± 0.005 d (5)
1	0.723 ± 0.111 a (2)	0.219 ± 0.103 bc (9)	0.002 ± 0.001 d (60)
2	0.757a (5)	0.046 cd (1)	0.001 ± 0.002 d (45)
3	...	...	0.004 ± 0.002 d (40)
4	...	...	0.002 ± 0.002 d (51)
5	...	...	0.003 ± 0.001 d (50)

[z] Transmitter: thrips that transmitted TSWV to petunia. All were ELISA-positive. Nontransmitter: nontransmitting thrips were divided into two categories based on their readings in ELISA. The threshold value was calculated as the average of virus-free thrips + 3 times the standard deviation. All readings above this threshold, ranging from 0.009 to 0.025, were considered positive; readings below this value were considered negative. Values followed by the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$). The number of insects tested per combination is indicated in parentheses and is the sum of two replications.

TSWV acquisition by thrips larvae. Apparently, only L1 larvae are able to acquire TSWV. To more definitively determine whether early L2 larvae could acquire and retain TSWV, 0-h-old larvae (L1), 60-h-old larvae (L2), and 40-h-old larvae (L1-L2) were allowed a 4-h AAP on TSWV-infected plant material. TSWV ingestion by individual larvae, using 26 L1, 51 L1-L2, and 25 L2 larvae, was monitored by ELISA. The adult transmission efficiency of the remaining thrips was determined on petunia leaf disks (Table 2). The developmental stage of 40-h-old larvae was determined by counting molting skins and using gut color before and after AAP as an indicator for feeding. Four groups were distinguished by assessing gut color. One group ($n = 26$) consisted of larvae that had a green gut before and a transparent gut after the AAP. Because they may have molted during AAP, they were considered L1 larvae before the AAP. A second group ($n = 45$) consisted of larvae in which the gut was transparent before and after the AAP. They were evidently in the L1-L2 molting stage. The third group ($n = 21$) contained L2 larvae that had a transparent gut before and a green gut after AAP. Larvae in the fourth group ($n = 46$) had a green gut before and after the AAP and represented a mixture of L1 and L2 larvae (Table 2).

Newly hatched larvae ingested, on average, 3.6 ng of TSWV-N protein, as measured immediately after a 4-h AAP, and 53% of the

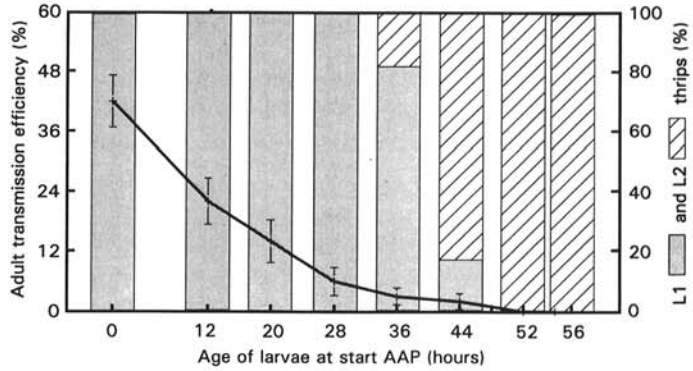

Fig. 2. Relationship between L1 (first instar)/L2 (second instar) ratio of thrips larvae at acquisition and the subsequent transmission efficiency of the resulting adults. Larvae between 0 and 56 h old were given a 24-h acquisition access period (AAP) on tomato spotted wilt virus-infected *Datura stramonium* plant material. Averages ± SE are presented for three replications, with 30 insects per treatment per replication.

TABLE 2. Tomato spotted wilt virus (TSWV) ingestion by 0-, 40-, and 60-h-old thrips larvae and subsequent transmission by the corresponding adults

Age of larvae (h)	Larvae gut color before and after AAP[r]		Larvae after AAP (ELISA)[s]		
	Before	After	Positive larvae[t]/ total tested	Amount of N protein ingested (ng)[u]	Transmitting adults[v]/ total tested
0	Transparent	Green[w]	23/26	3.6 ± 0.8	31/58
40	Green	Transparent[w]	8/13	0.6 ± 0.1	1/13
	Transparent	Transparent[x]	0/13	0.0	0/32
	Transparent	Green[y]	11/11	1.3 ± 0.4	0/10
	Green	Green[z]	11/14	5.4 ± 0.9	0/32
60	Green	Green[y]	23/25	10.8 ± 4.7	0/77

[r] Gut color was used, in combination with counting molting, to identify larval-stage of larvae that had been given a 4-h acquisition access period (AAP).
[s] Enzyme-linked immunosorbent assay.
[t] Larvae that contained nucleocapsid protein.
[u] Average ± SE.
[v] TSWV transmission to petunia leaf disks.
[w] L1 stage (first instar).
[x] Molting stage of L1 to L2 (second instar).
[y] L2 stage.
[z] Mixture of L1 and L2 stages.

thrips in this population transmitted TSWV after becoming adults. Second instar larvae that were 60 h old contained, on average, 10.8 ng of N protein immediately after the AAP but failed to transmit TSWV in the adult stage. At the age of 40 h, the late first instar larvae after AAP contained, on average, less TSWV-N protein than newly hatched larvae, and only one adult was able to transmit TSWV. After AAP, no N protein was detected in larvae that were in the process of molting. Adults emerging from these larvae failed to transmit TSWV. The thrips from the third group, consisting of early second instar larvae, contained, on average, lower amounts of N protein (1.3 ng) than 60-h-old larvae (10.8 ng) but more than late first instar larvae (0.6 ng) (Table 2). Although the early second instar larvae did ingest virus, the adults that developed from these larvae did not become transmitters. An average of 5.4 ng of N protein was detected in the larvae from the fourth group. It is possible that they might represent a mixture of first and second instar larvae. The level of TSWV-N protein was higher in these larvae than in 40-h-old first instar larvae. Therefore, it seems likely that this group of 40-h-old larvae consisted mainly of second instar larvae. The results obtained in these analyses show that only first instar larvae acquire TSWV.

TSWV accumulation in maturing thrips. TSWV accumulation was analyzed in thrips after they ingested TSWV as newly hatched (L1) and 2.5-day-old larvae (L2) to explain the exclusive capacity of L1 larvae to acquire TSWV. The L1 and L2 larvae were given a 4-h AAP on TSWV-infected plant material. Samples of 25 individuals were collected randomly at intervals during their development into adults and analyzed individually in ELISA for the presence of TSWV-N protein (Fig. 3). On average, 3.6 ng of N protein was detected in L1 larvae when sampled directly after the AAP. Four-day-old thrips contained an average of 13.1 ng of N protein. A decrease in the level of N protein was detected in the pupal stage (1.5 ng), and again, an increase of N protein was detected in the adults (16.3 ng). The L2 larvae, however, ingested more virus (10.8 ng of N protein on average), but no increase in the amount of this protein could be detected during their development (Fig. 3).

Influence of amount of virus ingested on ability to acquire TSWV. The loss of acquisition capacity with age may be explained by the relative amount of TSWV-N protein ingested by larvae during development. To study TSWV ingestion, larvae ranging in age from 0 to 5 days were placed on TSWV-infected plant material for 8 h. Immediately after AAP, the thrips were frozen at –70°C and later tested for their N protein content (Fig. 4A). The results show that the amount of N protein after AAP increased with the age of the larvae, and, hence, reflects an increasing consumption rate with age. The amount of N protein ingested by larvae, therefore, does not appear to determine or significantly influence the resulting potential of adults to become transmitters. Finally, at all ages, some individuals showed no evidence of having ingested TSWV, as determined by ELISA done immediately after AAP (Fig. 4B).

DISCUSSION

The results presented in this paper clearly show that TSWV ingestion by first instar larvae of *F. occidentalis* is a prerequisite for transmission by thrips at later stages (Figs. 1 and 2; Tables 1 and 2). The percentage of adults that transmitted TSWV decreased sharply when older first instar larvae were used in TSWV-acquisition experiments (Fig. 2). The exclusive capacity of first instar larvae to acquire TSWV was explained by the ability to accumulate TSWV in later stages after ingestion, whereas second instar larvae failed to do so (Fig. 3). No correlation was found between the amount of TSWV ingested and the ability of thrips to acquire TSWV (Fig. 4).

A novel technique, in combination with counting molting skins, was used to discriminate between late first instar and early second instar larvae, because they are difficult to differentiate by any morphological characteristic (8,23,44). Previously described methods (8,44) were based on counting molting skins (Fig. 2), which alone is not a highly accurate technique, because larvae also feed on molting skins. The new technique presented here is based on qualitatively scoring gut color. During the molting stages, thrips have a transparent gut, whereas it is green during feeding. The combination of assessing gut color and counting molting skins can very accurately distinguish between first and second instar larvae (Table 2).

The fact that only first instar larvae could acquire TSWV suggests that a barrier exists in second instar larvae and at later stages that prevents acquisition and subsequent TSWV replication needed for transmission. To date, four types of barriers to propagative plant viruses in insects are known, i.e., the midgut barrier, dissemination barrier, salivary gland escape barrier, and transovarial and venereal transmission barriers (1,17). The failure to infect the midgut in second instar larvae, and even in later stages, might be explained by an inactivation of TSWV by digestive or proteolytic enzymes in the gut lumen, encapsulation of the virus in the peritrophic membrane (18), or blocking of possible TSWV receptors (5,20,21,22).

The differences between adults and larvae in the ability to acquire TSWV have already been investigated histologically by Ullman et al. (38), who suggested that a midgut barrier, preventing TSWV dissemination into the hemocoel (40), exists in adults but not in larvae. In the future, similar experiments need to be executed to determine whether this barrier also is present in second instar larvae. Further research also might provide insight into a possible third barrier: the salivary gland escape barrier. The existence of

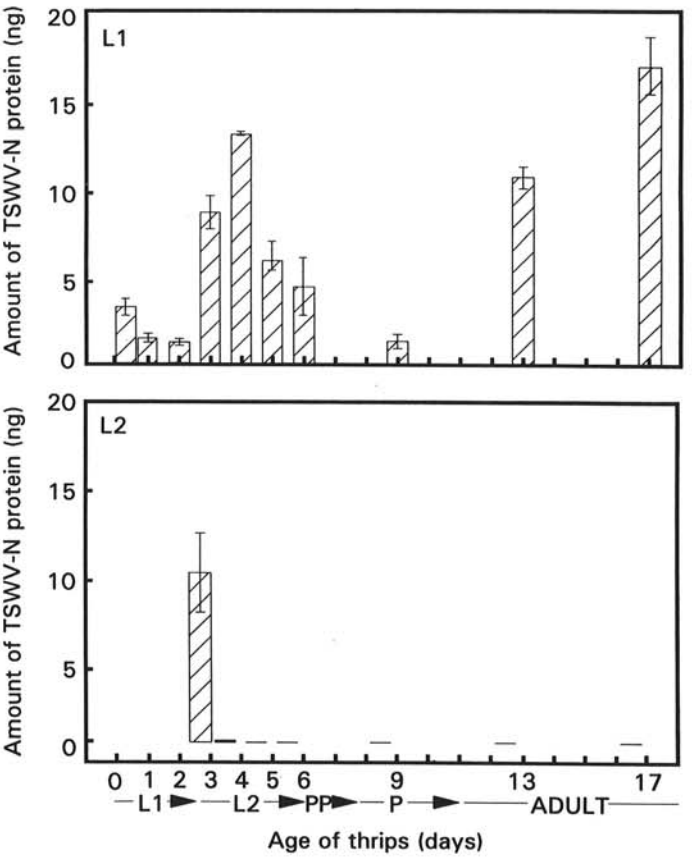

Fig. 3. Detection of tomato spotted wilt virus-nucleocapsid protein (TSWV-N protein) in maturing thrips ($n = 25$ per treatment; newly hatched larvae [L1] and 2.5-day-old larvae [L2]) after a 4-h acquisition access period on TSWV-infected *Datura stramonium* plant material. Amounts of TSWV-N protein higher than the threshold value, ranging from 0.005 to 0.050 ng, were considered positive. The average amount of N protein, determined by enzyme-linked immunosorbent assay values ± SE, is shown.

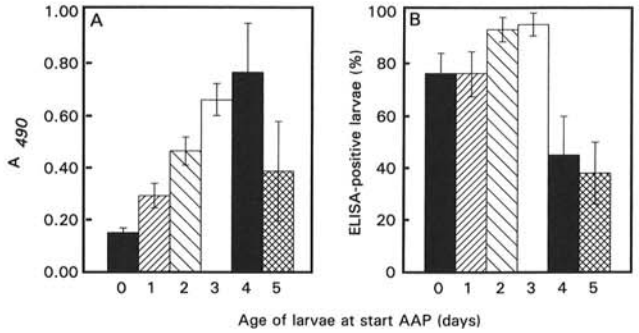

Fig. 4. A, The average enzyme-linked immunosorbent assay (ELISA) values ± SE, using antiserum against tomato spotted wilt virus nucleocapsid protein (TSWV-N protein), of individual larvae and **B,** the percentage of larvae that were ELISA-positive for TSWV. ELISA values higher than the threshold value, ranging from 0.006 to 0.011, were considered positive. Newly hatched to 5-day-old larvae were given an 8-h acquisition access period (AAP) to TSWV-infected *Datura stramonium* plant material ($n = 25$ per treatment).

transovarial and venereal barriers has been demonstrated already, because female thrips cannot transmit TSWV vertically (46).

Larval instars of aphids, leafhoppers, and planthoppers acquire circulative and propagative viruses more efficiently than adults (1, 35). However, a principal difference between larvae and adults and even between larval stages in the ability to acquire virus has been described only for Fiji disease reovirus (FDV), which is transmitted by the planthoppers *Perkinsiella saccharicida*, *P. vitiensis*, and *P. vastatrix*. This virus can be acquired only by the L1, L2, and L3 larvae, not by L4 and L5 larvae and adults. As with TSWV, the ability to acquire an infectious dose of FDV also decreases with increasing larval age (2,11,15,16,26,27). Ammar (1) suggested the occurrence of a midgut (infection or escape) barrier or other dissemination barriers for FDV in the later instar and adult stages.

The finding that only L1 larvae of *F. occidentalis* can acquire and retain TSWV and that only these individuals develop into transmitters is crucial for understanding TSWV epidemiology. Our data allow us to describe the TSWV-thrips transmission cycle more accurately. TSWV must be ingested by L1 larvae, and after a latent period and virus replication, TSWV subsequently can be transmitted by late L2 larvae and adults (39,47,48). In defining the TSWV-thrips transmission relationship more accurately, our findings are relevant for designing optimal TSWV control measures. Control of TSWV, at the moment, is focused on the transmitters, whereas control possibly could be more effective if aimed at the beginning of the transmission cycle, i.e., targeting L1 larvae.

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