Highlights

- The behavioral and mechanical components of the phytophagy by *N. tenuis* were assessed.
- Fifth-instar nymphs, males and females of *N. tenuis* spend a high proportion of time on cell rupturing behaviors.
- Fifth-instar nymphs of *N. tenuis* probe more frequently on tomato apical sections than adults.
- Adults of *N. tenuis* tend to perform both cell rupturing and ingestion activities on the vascular region.

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Short title: Plant feeding by *N. tenuis*

Plant feeding by *Nesidiocoris tenuis*: quantifying its behavioral and mechanical components

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ABSTRACT

Zoophytophagous predators play an important, though sometimes controversial, role in pest management programs in different crops. In tomato crops, damage caused by phytophagy of the mirid *Nesidiocoris tenuis* has mainly been reported at high predator population levels or when prey is scarce. Previous research has focused on predator/prey ratios, stylet morphology and saliva composition to explain plant damage by *N. tenuis*. In this study, we investigated the behavioral and mechanical components of the phytophagy. For this, we compared the feeding behaviors of males, females and fifth-instar nymphs of *N. tenuis*. Additionally, we investigated the type of stylet activities performed by each stage while probing in plant tissue, using the electrical penetration graph technique (EPG). Furthermore, stylectomy was performed and plant histology studied with the aim to correlate the feeding activities observed in the EPG recordings with stylet tip positions in specific tissues of the leaf petioles. Behavioral observations during a 30-min period showed that nymphs probed more frequently (38.6 ± 1.5 probes) than males and females (25.3 ± 1.1 and 24.3 ± 1.1 probes, respectively). Similarly, nymphs spent a higher proportion of time (656.0 ± 67.6 s) feeding on tomato apical sections compared to males and females (403.0 ± 48.8 s and 356.0 ± 43.7 s, respectively). The EPG recordings during 5 h indicated that cell-rupturing was the main stylet activity for all insect stages, and that fifth-instar nymphs spent a higher proportion of time on cell-rupturing events compared to adults. The histological studies revealed a trend of *N. tenuis* for the tissues within the vascular semi-ring. The stylet tips were found both in the vascular bundles and in the parenchyma of the interfascicular region. The findings of this study confirm an important role of fifth-instar nymphs feeding behavior in the damage potential of *N. tenuis*. Moreover, the increased time spent on cell rupturing behaviour suggests that stylet laceration and enzymatic maceration of the saliva occurring during this event might greatly contribute to the inflicted damage. A comprehensive understanding of the interactions of *N. tenuis* with the plant, at both the behavioral and mechanical levels, might shed light on new approaches to minimize its damage potential to tomato while maintaining its benefits as biocontrol agent.

Key words: feeding behavior, zoophytophagous, tomato, electrical penetration graph, stylectomy, Hemiptera, Miridae.

1. INTRODUCTION

The use of zoophytophagous predators for biological control of pests in agroecosystems has increased over the last decades (van Lenteren et al., 2018). *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) is one of these predators widely used in current biocontrol programs in Southern Europe, where it is occurring naturally and can spontaneously colonize vegetable crops (Arnó et al., 2010; Pérez-Hedo and Urbaneja, 2016). *Nesidiocoris tenuis* is commercially available and performs a crucial role in integrated pest management (IPM) programs in tomato (Albajes et al., 2006; Calvo and Urbaneja, 2004; Pérez-Hedo and Urbaneja, 2016; van Lenteren, 2012; van Lenteren et al., 2018). Advantages such as preying upon several key pest species, high predation efficiency and its capacity to stay in the crop under prey shortage conditions (Urbaneja et al., 2009, 2005) are some of the primary reasons this predator is considered a successful biocontrol agent in Southern Europe. Moreover, recent studies have demonstrated the benefits deriving from its phytophagy in terms of activation of plant defenses that enhance biological control (Bouagga et al., 2020, 2018a; Naselli et al., 2016; Pérez-Hedo et al., 2018, 2015b, 2015a). However, despite its services as biocontrol agents, under certain conditions damage caused by their phytophagy has also been reported. Plant damage ranges from necrotic rings in stems and petioles, to abortion of small fruits and flowers, reduced vegetative growth, and blemishes in fruits (Arnó et al., 2010; Calvo et al., 2009; Calvo and Urbaneja, 2004; Castañé et al., 2011; El-Dessouki et al., 1976; Pérez-Hedo and Urbaneja, 2016; Sánchez, 2008; Sánchez and Lacasa, 2008). The damage caused by *N. tenuis* can become very important in tomato crops cultivated in heated greenhouses and/or with low pest pressure. For instance, in northern Europe, where these conditions are common to tomato production, *N. tenuis* is considered a serious pest (Ferguson et al., 2020; Pérez-Hedo and Urbaneja, 2016).

Regardless of its damage potential, the success and widespread use of *N. tenuis* as a biological control agent in cultivated systems have prompted researchers to investigate the mechanisms underlying its phytophagy, and ways to reduce its negative impacts. For instance, research first focused on predator-prey interactions. Several studies have demonstrated that damage occurs mainly at high predator population levels and its severity is prey density-dependent, with an increase in number of necrotic rings as prey populations decrease (Arnó et al., 2010; Calvo et al., 2009; Sánchez, 2008). The role of temperature has also been explored, and it was shown that the severity of the damage inflicted by *N. tenuis* increased at higher temperatures (Sánchez, 2008; Siscaro et al., 2019). Stylet morphology and saliva composition of important zoophytophagous species, including *N. tenuis*, have been studied aiming at finding the mechanisms underlying plant damage, but these factors alone did not explain *N. tenuis* damage potential (Castañé et al., 2011). Histological studies on stained tissues *N. tenuis* fed upon have also been carried out to characterize the damage (Raman and Sanjayan, 1984).

More recently, research trying to explain the mechanisms causing plant damage by zoophytophagous predators has changed the focus from general to more specific approaches. Hence, more attention has been given to biotic factors such as plant cultivar and plant interaction with microorganisms (Cabello et al., 2013; Garantonakis et al., 2018; Siscaro et al., 2019). For instance, mixed results have been reported regarding the influence of tomato cultivar on damage incidence by *N. tenuis*, with significant differences between cultivars reported by Cabello et al. (2013), whereas differences between cultivars found by Siscaro et al. (2019) were not significant. Moreover, the role of microorganisms associated to the plants in damage caused by zoophytophagous predators has been demonstrated for *N. tenuis* by Garantonakis et al. (2018), who reported that tomato plants inoculated with the endophytic strain *Fusarium solani* K had significantly less damage than non-inoculated plants. However, the behavioral aspects and the stylet activities of *N. tenuis* while piercing the plant remain unexplored.

Direct behavioral observations are a practical approach that has been applied to zoophytophagous species to study their phytophagous behavior (Bouagga et al., 2018a, 2018b). For *N. tenuis*, its behavior on sweet pepper plants was recently described by Bouagga et al. (2018a), however, its behavior on tomato has not been described yet. Additionally, the feeding behavior of piercing-sucking insects can be studied with the electrical penetration graph (EPG) technique. In brief, this technique consists of incorporating the plant and the insect as components of an electrical circuit: one of the electrodes holds a wired insect (EPG probe) and the other electrode is a copper post that is inserted in the soil of the potted plant. When the insect pierces the plant, the circuit is closed and the different activities of the stylets in different tissues are recorded as waveforms, hence allowing for an *a posteriori* biological interpretation (Tjallingii 1978). Although EPG has been most often used to study feeding behavior of aphids (Fereres and Collar, 2001; Garzo et al., 2016; Jiménez et al., 2019; ten Broeke et al., 2013; Tjallingii, 1985, 1978) and other piercing-sucking insects (AB Ghaffar et al., 2011; Antolinez et al., 2017; Guedes et al., 2018; Jin et al., 2012; Lucini and Panizzi, 2016), its application to other Hemiptera, such as Miridae, is rather recent (Backus et al., 2007; Cervantes et al., 2016; Cline and Backus, 2002).

In the present work, the behavior and stylet activities (i.e. cell rupturing and ingestion) of *N. tenuis* on tomato were investigated in order to determine their role in phytophagy. First, the feeding behavior of males, females and fifth-instar nymphs of *N. tenuis* on tomato apical sections was quantified and compared. Second, the stylet activities of males, females and fifth-instar nymphs of *N. tenuis* during probing events (i.e. the time the stylets remain inserted in the plant tissue) were evaluated with EPG. Finally, stylectomy and histological preparation of tomato petiole sections containing the inserted portion of the cut stylets of *N. tenuis* were performed to identify the plant tissues reached.

2. MATERIALS AND METHODS

The experiments were performed in three different laboratories. The behavior observation experiment was carried out at the entomology laboratories of Instituto Valenciano de Investigaciones Agrarias (IVIA) in Valencia, Spain. The EPG recordings were performed in the entomology laboratories of Wageningen University in Wageningen, The Netherlands. The stylectomy experiment, the histological work and waveform characterization and identification was conducted at the entomology laboratories of Instituto de Ciencias Agrarias - Consejo Superior de Investigaciones Científicas (ICA-CSIC) in Madrid, Spain.

2.1. Behavioral observation

2.1.1. Plants and insects

A rearing of *N. tenuis* was established in the laboratory in a plastic insect cage (60 x 60 x 60 cm) (BugDorm-2 insect tents; MegaView Science Co., Ltd, Taichung, Taiwan). Green bean pods (*Phaseolus vulgaris* L.) and eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) were provided twice a week as oviposition substrate and food source, respectively. Cohorts of similar age were obtained every week by placing three green bean pods in the rearing cage for females to oviposit during 3 days. After this time period, the green bean pods bearing mirid eggs were removed from the rearing cage and placed in plastic containers (14 x 14 x 8 cm), with an opening in the lid covered with fine mesh for ventilation. One fresh green bean pod and *E. kuehniella* eggs *ad libitum* were provided twice a week to the cohorts in each plastic container until they were at the developmental stage required for the experiments. Both the rearing and the cohorts were kept at 25 ± 2 °C, constant relative humidity of 50 ± 10 % RH and 14L:10D photo:scotoperiod. *N. tenuis* and *E. kuehniella* eggs were supplied by Koppert Biological Systems (Águilas, Murcia, Spain).

Tomato plants *cv.* Raf Supermarmande (Mascarell Seeds, Spain) used in this experiment were in vegetative stages V6 to V7 (ca. 30-40 cm height). Plants were grown in plastic pots (8 x 8 x 8 cm) and kept in pest-free climatic chambers until the start of the experiment, at the same experimental conditions previously described for the *N. tenuis* rearing.

2.1.2. Behavioral test

Insects used were isolated in test tubes and starved during 24 h, with water supplied through moistened cotton plugs. Less than 3-day-old females (presumably mated) and males, and fifth-instar-nymphs (N5) were used. One individual with its respective tomato plant apical section was considered a replicate. A total of 20-22 replicates per developmental stage were recorded.

Previous studies have demonstrated the preference of *N. tenuis* for the apical part of the tomato plant (Castañé et al., 2011; Perdikis et al., 2014); hence only apical sections (i.e. the apical bud and the two youngest fully developed leaves) were used for this experiment. The apical sections were excised and immediately placed inside a Petri dish (150 mm diameter) and covered with its lid. Then, one mirid was gently released inside the horizontally placed Petri dish at the base of the excised apical section. A piece of dry synthetic sponge was used to cover the excision point, to prevent the insects from feeding on the exudates produced by the cut or the water in the sponge. A new apical section was used for each replicate. Visual observation of feeding and trivial behaviors of the individuals started when the insect made the first contact with the plant tissue. Total observation time for each individual was 30 minutes. All behaviors exhibited by the insects and the time spent on each activity were documented. Observations were done under a Leica M165 C stereomicroscope with the Petri dishes in horizontal position. The time spent on each location inside the Petri dish was also documented. The locations were defined as follows:

Apical bud (AB): apical bud

Leaf 1 (L1): first fully developed leaf from the apical bud.

Leaf 2 (L2): second fully developed leaf from the apical bud.

Stem (ST): stem section to which the apical bud and the leaves were attached.

Out of plant (OP): the insect was in contact with the Petri dish but not with the plant tissues.

Behavior descriptions were adapted from Bouagga et al. (2018a) and defined as follows:

Feeding (F): the predator inserts its stylets into the plant tissue for more than two seconds. Stylets movements can be observed.

Probes (P): the predator inserts the stylets for less than two seconds.

Resting (R): the predator stands motionless.

Searching (S): the predator is at rest but moves its antennae and/or taps on the plant with the stylets/proboscis tip.

Walking-Searching (WS): the predator walks over the plant tissue, moves its antenna and taps on the plant with the stylets/proboscis.

Cleaning (C): the predator uses forelegs or hindlegs to clean mouthparts and/or other parts of the body

Out of plant (OP): the insect left the plant tissue and is in contact with the Petri dish only.

Out of sight (X): when the insect reached parts of the plant tissue that were out of the sight of the observer from any possible angle, even after adjusting the Petri dish position (without disturbing the insect).

Oviposition (O): The predator bends the abdomen and inserts the ovipositor into the plant tissue to lay an egg.

2.2. Electrical Penetration Graph (EPG) recordings

2.2.1. Plants and insects

A *N. tenuis* rearing was established with individuals provided by Wageningen UR Greenhouse Horticulture (Bleiswijk, The Netherlands), which were originally sourced from Koppert Biological Systems (Águilas, Murcia, Spain). Green bean pods (*Phaseolus vulgaris* L.) and *E. kuehniella* eggs (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) were provided twice a week as oviposition substrate and food source *ad libitum*, respectively. One tomato plant *cv.* Moneymaker was supplied weekly to the rearing for the mirids to get experience with the plant tissues used during the EPG recordings. The rearing was kept in a muslin cage (25 x 25 x 25 cm) at the same environmental conditions as described above for the behavior experiment.

Tomato plants *cv.* Moneymaker used in this experiment were in vegetative stages V5-V6 (ca. 30 cm height). Plants were grown in plastic pots (6 x 6 x 6 cm) and kept in a greenhouse compartment at 19–21 °C, 60-70% RH and 16L:8D h photo:scotoperiod.

2.2.2. EPG recordings

The EPG recordings were carried out inside Faraday cages to prevent electromagnetic interference. For wiring, the mirids were first anesthetized on ice for approximately one minute and then placed at the tip of a pipette tip (200 µl) connected to a vacuum under low suction. Then, a 2-3-cm-long gold wire (18 µm diameter) was attached to the dorsum of the insect using a small drop of water-based silver glue (EPG Systems, Wageningen, The Netherlands). Insects used in this experiment were individually starved during hanging on their respective wires for 5 h. Water was not supplied during starvation since cotton plugs provided surface and traction for the insects to detach from their wires. To start the EPG recordings, the wired insect was carefully placed on the petiole of the second or third fully developed leaf from the apical bud of a potted tomato plant. We used less than 3-day-old females (presumably mated) and males, and N5 nymphs for the recordings. Fifteen replicates were recorded for males and females, and

fourteen for N5-nymphs, for a total of 44 individuals. A new plant was used for each individual. EPG recordings were obtained with a Giga-8 DC-EPG device (EPG Systems) during an undisturbed 8-h period. EPG data acquisition and analysis were conducted using Stylet+ Software for Windows (EPG Systems).

The broad waveform classification into probing and non-probing behaviors for this experiment was done following Backus (2000), who defined as probing behaviors all behaviors from the start of the style insertion into the plant tissue until stylet withdrawal. The non-probing behaviors comprise all other behaviors that do not involve stylet penetration (Backus, 2000). Probing behaviors in cell rupture feeders are further classified into probing waveforms: cell rupturing (CR), transition (T) and ingestion (I) (Cervantes et al., 2016). The identification and classification of the probing waveforms for *N. tenuis* was based on the waveform library of the mirid species *Lygus lineolaris* (Palisot de Beauvois) and *Lygus hesperus* (Knight) (Hemiptera: Miridae) (Cervantes et al., 2016). Since waveforms T are suggested to be species dependent (Cervantes et al., 2016), and were scarce and not clearly distinguishable in our recordings, waveforms resembling T patterns were included as CR. For the purposes of this study, non-probing behaviors were not included in the analysis.

2.3. Stylectomy and plant tissue histology

Histological thin-section analysis was performed to correlate the position of the stylet tips with the cell rupturing and active ingestion waveforms observed during the EPG recordings. For this study, additional adults of *N. tenuis* were monitored on tomato petioles with a Giga-4 DC-EPG device (EPG Systems) under conditions similar to those of the previous EPG recordings. When the respective waveform of interest was observed, the feeding activity was artificially terminated by stylet amputation with a tungsten needle of a Zapper RF micro-cautery unit (www.aphidzapper.com) following the methodology proposed by Downing and Unwin (1977). Petiole segments (ca. 0.5 cm) containing the severed stylets of *N. tenuis* were carefully removed from the plant with a scalpel. Then, the petiole segments (hereafter samples) were immersed in Karnovsky fixative at room temperature and placed under a low vacuum for 1 h to prevent air bubbles in the tissues. Afterwards, the samples were dehydrated in graded ethanol series (10-100%) and then infiltrated and embedded in paraffin. Serial transverse sections (15-20 μm thick) were cut on a Leica 1512 microtome and stained in 0.05% toluidine blue solution for 10 minutes. Permanent slides were prepared in mounting medium DePeX (SERVA Electrophoresis GmbH, Heidelberg, Germany) and examined using a Nikon Eclipse E800 microscope. Digital images were captured using the same microscope coupled with a Canon EOS 6D Mark II camera.

Mounted transverse-sections were examined for any indication of salivary sheath and to determine the position of the stylet tips inside the petiole tissues. Petiole tissues examined were: epidermis, ground tissue (i.e. parenchyma between epidermis and vascular tissue), vascular tissue (i.e. vascular bundles and interfascicular region [i.e. parenchyma between vascular bundles]). In tomato petioles the vascular tissue is arranged in a semi-ring shape (Maiti et al, 2012).

Tomato plants *cv.* Moneymaker used in this experiment were in vegetative stages V3-V4 (ca. 15 cm height), smaller than those used for the EPG recordings of the previous experiment because of size restrictions of the stylectomy equipment. Plants were grown in pots (6 x 6 x 6 cm) in a climatic chamber at 14L:10D °C, 60-70% RH and 16L:8D h photo:scotoperiod.

2.4. Statistical analysis

Behaviors were analyzed with Generalized Lineal Models (GLM) with Poisson and quasipoisson error distributions, by using the function *glm* to assess differences in behaviors between developmental stages/sex (hereafter Stages/Sex). Stage/Sex was entered as independent variable for all behaviors except for oviposition. Significant differences between Stages/Sex were followed by multiple comparisons with Bonferroni correction ($\alpha = 0.05$), by applying the *emmeans* function. Differences in time spent on each location were analyzed with GLM with quasipoisson error distribution. In this model, Location and Stage were entered as independent variables. Multiple comparisons were applied with the *emmeans* function (Bonferroni correction $\alpha = 0.05$) for the variables with significant differences.

The EPG data analysis was conducted based on 5 h out of the 8 h of recording time due to mortality of experimental insects observed after the 5th hour. EPG parameters were calculated for every mirid tested using the EPG analysis worksheet created by Sarria et al. (2009). Description of *N. tenuis* feeding behaviors was performed based on the variables defined by Backus et al. (2007). These variables were calculated for each waveform type (CR and I) and each cohort (in this study, cohort = N5-nymphs, males or females and N = number of individuals of the same cohort tested): total probing duration (TPD = sum of probing time per cohort/N), total waveform duration (TWD = sum of time spent by all individuals of the same cohort performing one waveform), number of waveform events per insect (NWEI = sum of events of one waveform type per cohort/N), waveform duration per insect (WDI = TWD/N), waveform duration per event per insect (WDEI = mean time spent in one waveform type per cohort/N), and time to first probe from the start of the EPG recording. Comparison of variable means across insect Stages/Sex were performed with nonparametric Kruskal-Wallis test followed by Dunn's test for multiple comparisons when variables did not meet normality assumptions. One-way ANOVA followed by Tukey's test for multiple comparisons, and

Student's t-test were applied for variables following normality assumptions before or after transformation by ln(x), ln(x+1), sqrt(x), sin(x) or $1/x^2$. All statistical analyses were performed in R software (version 3.4.3).

3. RESULTS

3.1. Behavioral observation

Significant differences were found between Stages/Sex for number of probes, feeding, resting and searching (Table 1). In contrast, no significant differences were found between Stages/Sex for time allocation to walking-searching, cleaning, out of plant and out of sight (Table 1). Multiple comparisons for significant Stages/Sex effect revealed that N5-nymphs spent longer time feeding than both males and females (Z = -3.07, $P < 0.05$ and Z = -3.82, $P < 0.05$, respectively). Similarly, nymphs probed more frequently on the plant tissues than both males and females (Z = -7.16, $P < 0.05$ and Z = -7.98, $P < 0.05$, respectively). Resting time was higher in males than females (Z = -3.36, $P < 0.05$), but similar to that of nymphs (Z = 1.60, P = 0.329). Time spent searching was higher in females than males (Z = 2.79, $P < 0.05$), but did not differ from nymphs (Z = 0.61, P = 1.00).

Time spent on each apical section varied across locations (F_4 = 45.60, $P < 0.001$) but no differences were found among Stages/Sex (F_2 = 0.90, P = 0.390) and no Stages/Sex × location interaction was found (F_8 = 1.50, P = 0.163). All stages spent most of their time on Leaf 2 (L2) (56%) and the least Out of plant (OP) (4%) (Figure 1).

3.2. Electrical Penetration Graph (EPG) recordings

Probing events recorded for *N. tenuis* (Figure 2a) showed irregular patterns for cell rupturing (CR) (Figure 2b), and regular, peak-and-wave patterns for ingestion (I) (Figure 2c). Variability in the fine structure of I was also observed (Figure 2d-i).

Males, females and N5-nymphs spent proportionally more time on CR compared to I (Table 2). No significant differences were found across insect Stages/Sexes for total probing duration (TPD) and time-to-first probe since the start of the EPG recording (Table S1).

3.2.1.Probing behaviors: Cell Rupturing (CR)

Waveform duration per insect (WDI) was similar for N5-nymphs, females and males (H = 0.19; P = 0.910) (Figure 3A). The mean waveform duration per event per insect (WDEI) differed (F_2= 20; $P < 0.0001$), with N5-nymphs displaying the highest mean, followed by males and with females showing the lowest mean value (Figure 3C). Significant differences were found in the

mean number of waveform events per insect (NWEI) (F_2= 12; P = 0.029), with females and males showing higher means compared to that of N5-nymphs (Figure 3E).

3.2.2.Probing behaviors: active Ingestion (I)

No significant differences were found for WDI across insect Stages/Sexes for I (H = 2.0; P = 0.365) (Figure 3B). Similarly, the WDEI mean values were not significantly different across insect Stages/Sexes (H = 3.1; P = 0.217) (Figure 3D). Significant differences were observed for NWEI (F_2= 19; P < 0.0001), with females showing more ingestion periods, followed by males, and N5-nymphs showing the lowest number (Figure 3F).

3.3. Correlation between EPG waveforms and stylet tip positions in the plant tissue

Plant histological studies confirmed that *N. tenuis* does not generate a salivary sheath while performing CR waveform or I waveform in tomato petioles. The stylet tips during the CR waveform (n = 3) were located in the interfascicular region (i.e. parenchyma between vascular bundles, inside the vascular semi-ring) (n = 2) (Figure 4A), and in the vascular bundle (n = 1) (Figure 4B). For the I waveforms (n = 3) the stylet tips were located in the vascular bundle (n = 1) (Figure 4C) and in the interfascicular region (n = 2) (Figure 4D).

4. DISCUSSION

In this study, the feeding behavior and stylet activities of immature and adult stages of *N. tenuis* in tomato were quantified, and their role in the plant feeding was investigated. Our findings show that N5-nymphs perform significantly more plant feeding activities than adult stages. Moreover, results of EPG studies suggest a primary role of cell rupturing behavior in the plant feeding compared to ingestion behavior, in all insect stages analyzed in this study. Furthermore, histological studies revealed a trend of *N. tenuis* adults for probing and feeding from cells within the vascular ring region.

Previous studies reported a higher damage potential of *N. tenuis* nymphs compared to that of the adults (Arnó et al., 2006; Calvo et al., 2009; Perdikis et al., 2009). For instance, Arnó et al., (2006) demonstrated a two-fold difference in the number of necrotic rings caused by nymphs relative to adults in tomato side shoots. The present results revealed that N5-nymphs probe (i.e. insertion of the stylet) and feed for longer time in tomato than the adults, thus suggesting an important mechanical component in the damage potential of the different stages of *N. tenuis*. According to Hori (2000), the mechanical destruction is likely the primary cause of plant damage in heteropterans, with the rupture of cells by the stylets as the first step in the injury process. Contrary to salivary-sheath feeders (e.g. aphids, mealybugs), in which there is

minimum disruption of plant cells (Miles, 1968), *N. tenuis* is a cell rupture feeder. In this common feeding strategy in mirids, the insect lacerates the plant tissue with the stylet movements, and injects watery saliva in the surrounding cells, forming pockets of diluted cell contents that will eventually be ingested (Backus et al., 2007, 2005; Cervantes et al., 2016; Hori, 2000). Therefore, the higher number of probes observed in N5-nymphs is likely among the main causes that could explain its higher damage ability, due to the continuous piercing of the plant tissues. However, although feeding time in N5-nymphs was found to be significantly higher than that of adults in the behavior experiment, conclusions about the damage potential of *N. tenuis* cannot be made on the basis of feeding time alone. This specific result is in conflict with the total probing duration (TPD) in the EPG experiment (i.e. feeding time = TPD: time the stylets remains inserted in the plant tissue), where no differences were found across insect stages. The 24 h starvation to which all insects were subjected before the behavior experiment could have affected N5-nymphs more severely than adults, thus likely explaining longer feeding time observed in this stage during the first minutes of plant contact (30 min of behavior experiment). In contrast, TPD results suggest that feeding time is similar across life stages when time of plant contact increases (5 h of EPG recording). Moreover, the starvation time before the EPG experiment was shorter (i.e. 5 h), which could also partially explain the similarities in TPD due to less severe conditions experienced by the insects. The lack of differences in the time to first probe suggest a similar acceptance of the host plant by all stages and sexes of *N. tenuis* evaluated.

The findings of the present study also revealed a preference of both N5-nymphs and adults of *N. tenuis* for the L2 leaf (second fully developed leaf from the apical bud). Although a conclusion about the influence of trichomes on *N. tenuis* location preference cannot be made on the basis of the data collected for this study, it is important to highlight the faster and smoother mobility along the petiole/leaflets of L2 for all insect stages (M. Chinchilla-Ramírez, personal observation). One study about the biomechanics of the interaction between *Dicyphus errans* Wolff (Hemiptera: Miridae) and several plant species revealed that performance of this omnivorous species on hairy plant surfaces was positively influenced by trichome length and diameter (Voigt et al., 2007). Hence, trichome characteristics of the different plant locations in tomato cannot be discarded as a factor influencing location preference. Further research addressing *N. tenuis* feeding behavior on tomato cultivars with different trichome density/types could provide valuable information for a more precise prediction of the damage location.

In the cell rupture feeders, the CR waveforms represent the probing behavior in which the plant cells are lacerated and macerated by the action of the stylet movements, and injection of watery saliva, respectively (Cervantes et al., 2016). The EPG results show that CR is performed about 77-89% of the total waveform duration (TWD) for the insect stages and sexes evaluated. This

suggests a prominent role of CR behavior in the overall plant feeding of *N. tenuis*. These results are consistent with those from Tuelher et al. (2020), who noted that CR behavior in *L. lineolaris* were the primary reason for leaf damage in cotton. They argued a combination of probing-related wounding, and saliva-mediated solubilization over time, as the mechanisms underlying such damage. In our experiment, the remarkably longer CR events (WDEI) contrast with the low number of CR counts (NWEI) in N5-nymphs. This suggests that when plant tissues are exposed to N5-nymphs they endure fewer but longer periods of laceration and maceration than when exposed to adults, hence partially explaining the increased damage capacity of nymphs observed in previous studies (Arnó et al., 2006; Calvo et al., 2009; Perdikis et al., 2009). This also suggests that nymphs might be deploying a "quality over quantity" strategy, with longer CR events allowing for better enzymatic digestion of cell contents previous to I events, thus providing the nymphs with ingestion of more readily available nutrients. Cervantes et al. (2016) observed several periods of walking/waiting between single CR events and I events in *Lygus* spp, and argued the enabling of more salivary degradation on cell contents as a likely reason for this behavior. These longer CR events could also explain the increased feeding time observed in N5-nymphs relative to adults in the behavioral observation experiment.

During the ingestion (I) waveforms, the cell-rupture feeder uses its cibarial pump to swallow the pre-digested cell contents mixed with watery saliva through the stylets (Cervantes et al., 2016). Overall, I activity was numerically lower than CR as demonstrated by WDI, WDEI and NWEI mean values. Moreover, ingestion was performed only about 11-23% of the TWD by all insect stages and sexes evaluated in this study. Hence, the role of ingestion activity is presumably minor in the plant feeding behavior of N5-nymphs and adults of *N. tenuis*, compared to CR behavior. The low proportion of time spent on I activity found in this study are consistent with those reported for different life stages of *Lygus* spp. (Cervantes et al., 2016; Cline and Backus, 2002). It is worth mentioning that although not all parameters for I activity showed significant differences, there was a trend for N5-nymphs that these were numerically lower than for adults. This could mean that N5-nymphs are less efficient at ingestion as a consequence of smaller size and/or characteristics of the saliva, as suggested by Tuelher et al. (2020). Deficient ingestion in nymphs could also mean more enzymatic saliva left in the plant tissue compared to more efficient ingestion in adults, thus causing more damage over time due to maceration. The decreased ingestion efficiency in N5-nymphs is further supported by its TWD, which is < 50% of that observed in adults. Shorter stylets in immature stages have been suggested as limiting factor for feeding (Cooper & Spurgeon, 2013), and it is likely an additional reason for this decreased efficiency.

The histological studies revealed a trend of *N. tenuis* to perform both CR and I in the tissues comprised in the vascular semi-ring when piercing on the petiole. Stylet tips corresponding to

either CR or I waveforms were all found in vascular bundles or in parenchyma cells of the interfascicular region. Similar results were reported in previous studies based on histological sections of stained tissues with damage inflicted by *N. tenuis* (Raman and Sanjayan, 1984). Different position of mandibular stylet tips relative to maxillary stylet tips was observed in some samples from both CR and I events, hence laceration is likely occurring during both probing activities, and in the different tissues reached by the stylets. Our results suggest that *N. tenuis* does not feed on a specific cell type within the vascular semi-ring. Instead, *N. tenuis* creates pre-digested pockets of mixed contents from cells in this region, which could vary in nutrient contents depending on its proximity to the phloem. This "unspecific" cell selection is further supported by the fine structure and polarity of the I waveforms observed during the EPG recordings. The peak-and-wave structure is common in active ingestion (contrary to passive ingestion typical of phloem feeders) where the regular pattern is attributed to the rhythmical pumping and swallowing produced by the cibarial muscle (Cervantes et al., 2016; Dugravot et al., 2008; Lucini and Panizzi, 2016). Additionally, the positive polarity of the probes observed in our recordings is contrary to the negative polarity expected from intracellular stylet penetrations (Walker, 2000). Further studies with more histological samples are necessary to confirm these results, and to determine whether other tissues are also targeted under other circumstances, such as prey availability.

The findings of this study provide insights about the role of feeding and probing behaviors in the plant feeding by *N. tenuis*. CR probing events stand out as a primary mechanical component of the overall phytophagy of the insect stages evaluated. The increased number of probes and longer CR events observed in N5-nymphs could be the mechanisms underlying the higher damage potential of this life stage. Based on EPG results and the histological observations, most CR events are then expected to occur in the vascular region, thus probably comprising important damage to plant nutrient transport as well. Overall, this study broadens the understanding of the mechanical aspects underlying the phytophagy of *N. tenuis* on tomato. This could be useful in the development of new methods aimed at diminishing its negative impacts. For instance plant breeders could benefit from this knowledge to target specific plant tissues and develop varieties less susceptible to suffer from *N. tenuis* phytophagy.

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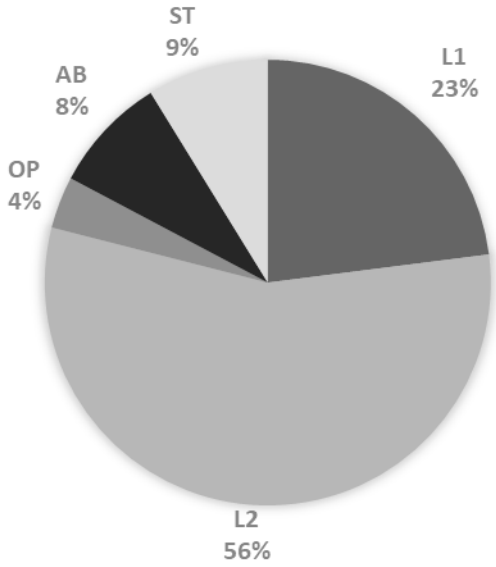
FIGURES AND TABLES

Table 1. Number observed or duration in seconds (mean ± SE) spent by females, males and fifth-instar nymphs of *N. tenuis* performing eight different types of behavior on tomato apical sections during 30-min observation periods. Significant differences between *Stages/Sex* are indicated by different letters (Bonferroni correction $\alpha = 0.05$).

Behavior	*Nesidiocoris tenuis*			Statistics		
	Females (n = 22)	**Males (n = 20)**	**Nymphs (n = 20)**	**df**	***F***	***P***
Number of probes	24.3 ± 1.1 b	25.3 ± 1.1 b	38.6 ± 1.5 a	2	5.84	0.004
Feeding	356.0 ± 43.7 b	403.0 ± 48.8 b	656.0 ± 67.6 a	2	8.21	< 0.001
Resting	30.6 ± 17.2 b	232.2 ± 49.8 a	126.1 ± 39.8 ab	2	9.19	< 0.001
Searching	228.0 ± 30.5 a	117.0 ± 23.0 b	200.0 ± 32.5 ab	2	4.01	0.023
Walking-searching	698.0 ± 63.4 ab	813.0 ± 71.8 a	569.0 ± 65.2 b	2	3.11	0.052
Cleaning	209.0 ± 26.9 a	184.0 ± 26.4 a	122.0 ± 23.4 a	2	2.7	0.076
Out of plant	42.6 ± 21.3 a	78.2 ± 30.2 a	90.6 ± 35.3 a	2	1.12	0.333
Out of sight	14.7 ± 8.16 a	39.2 ± 13.97 a	22.0 ± 11.35 a	2	1.73	0.187
Oviposition	193.7 ± 28.5	-	-	-	-	-

657



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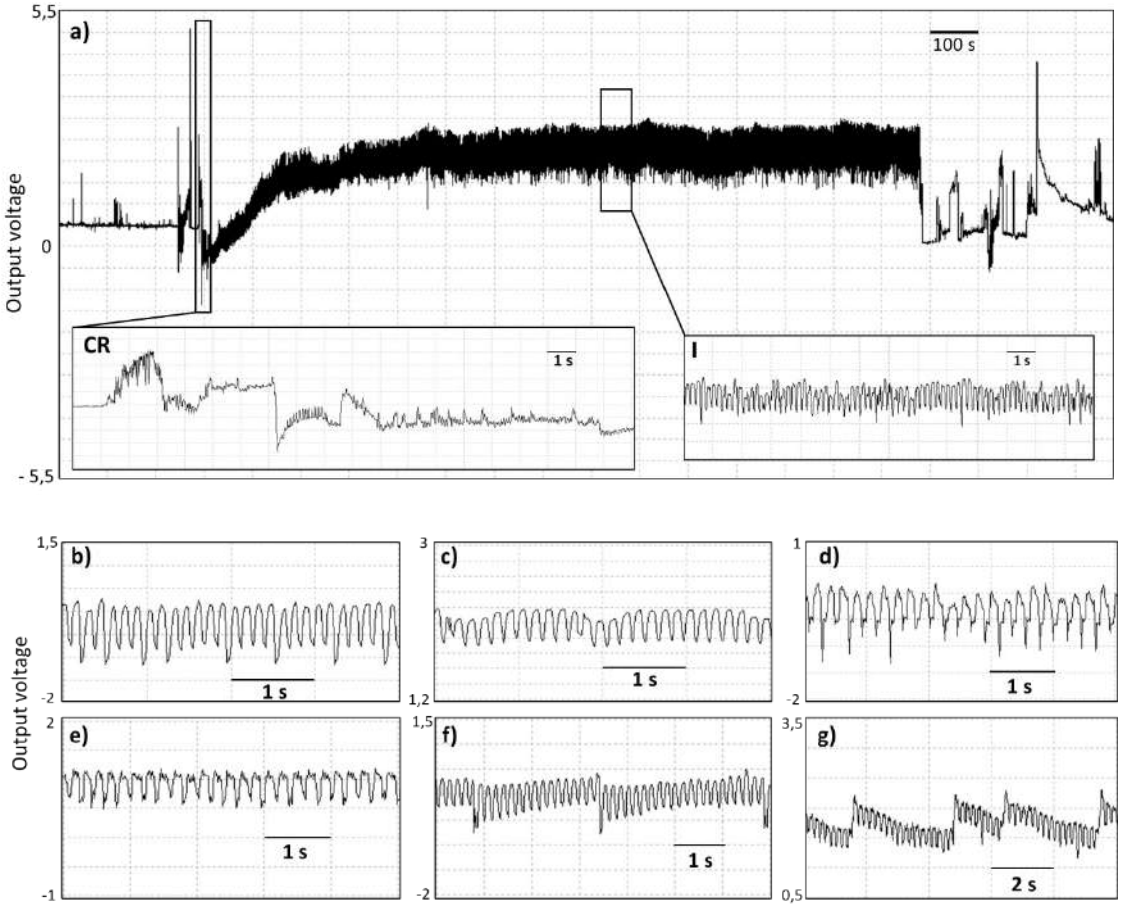
660**Figure 1.** Proportion of time (percentage) spent by *N. tenuis* on different apical sections of
661tomato in the behavior experiment. AB: apical bud, ST: stem, L1: leaf 1 from the apical bud,
662L2: leaf 2 from the apical bud, OP: out of plant (GLM quasipoisson, $F_4 = 45.60$, $P < 0.001$)

663



664**Figure 2.** Overview of a probing event by *N. tenuis* on tomato stems during EPG recordings **(a)**
665with details of the coarse structure of Cell rupturing (CR) and Ingestion (I) waveforms in inset
666boxes. Details of the coarse structure of Ingestion (I) waveforms during different recordings **(b-**
667**g)**.

668

669**Table 2.** Calculated total waveform duration (TWD) in seconds (s) and percentage (%) of time
670for Cell rupturing (CR) and Ingestion (I) waveforms in males, females and N5-nymphs of *N.*
671*tenuis*.

672

Insect stage (n)	Cell rupturing		Ingestion	
	TWD (s)	%	TWD (s)	%
Male (15)	192,198.02	78	54,654.2	22
Female (15)	181,185.76	77	52,782.32	23
N5-nymph (14)	190,645.56	89	24,455	11

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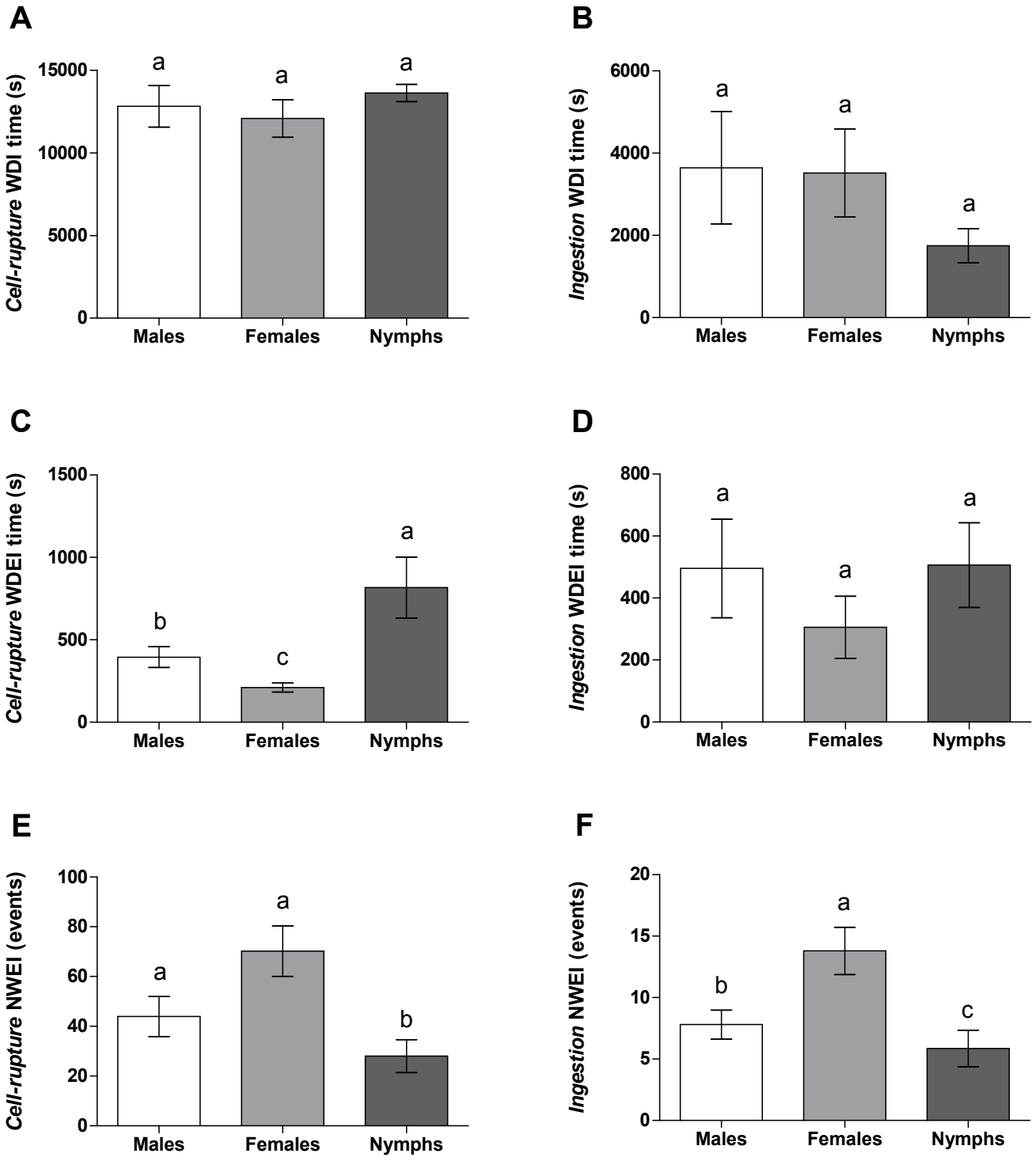
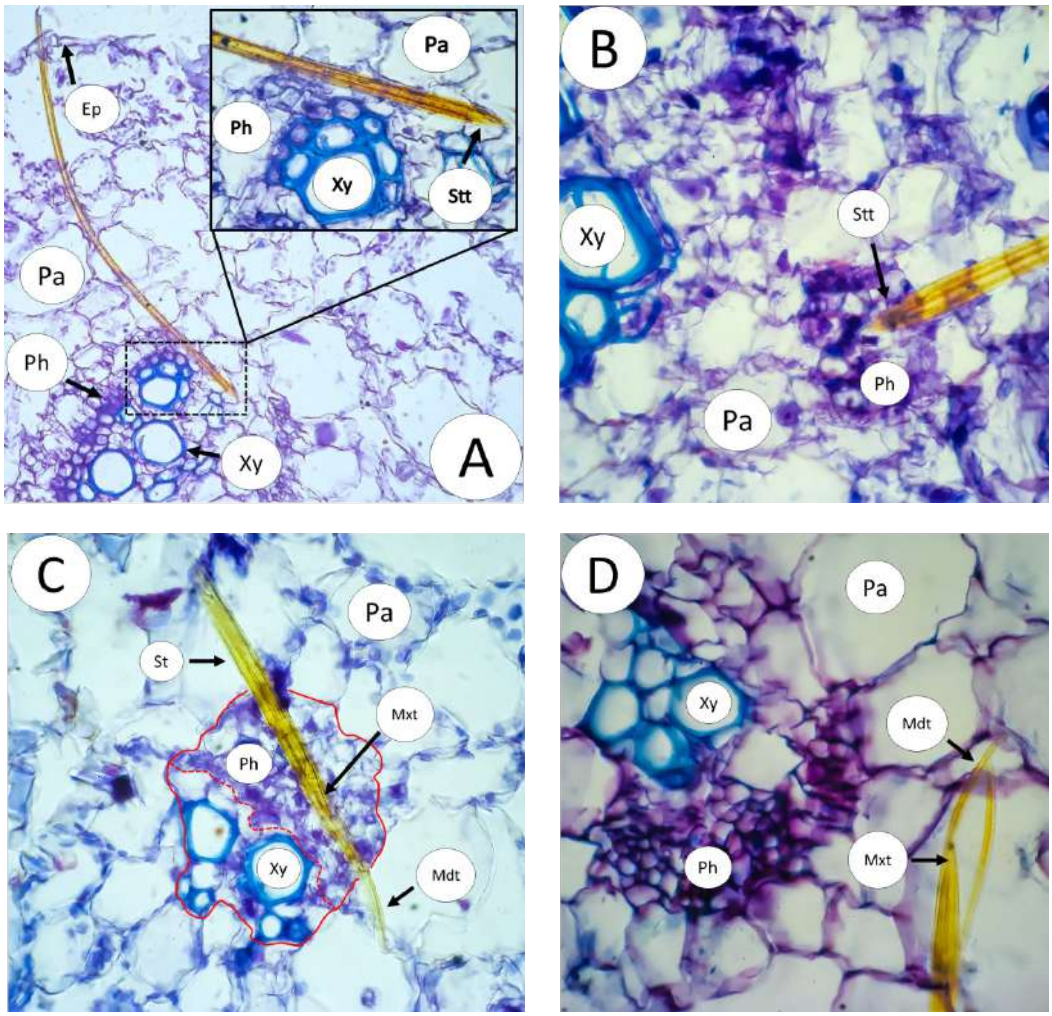
674



675**Figure 3.** Calculated waveform duration per insect (WDI) **(A-B)**, waveform duration per event
676per insect (WDEI) **(C-D)** and number of waveform events per insect (NWEI) **(E-F)** for cell
677rupturing (CR) and ingestion (I) waveforms (means ± SE). Different letters indicate significant
678differences (Tukey test or Dunn's test, $\alpha = 0.05$).



679

680**Figure 4.** Light micrographs of cross-sections of tomato petioles containing severed stylets of
681*N. tenuis*. Stylet tip in: **(A)** parenchyma tissue (200x, and 1000x in expanded image), and **(B)**
682vascular bundle (1000x) during CR waveform. Stylet tip in: **(C)** vascular bundle (1000x) and
683**(D)** parenchyma (1000x) during I waveform. **Pa**: parenchyma, **Xy**: xylem, **Ph**: phloem, **Ep**:
684epidermis, **St**: stylet, **Stt**: stylet tip, **Mdt**: mandibular stylet tip, **Mxt**: maxillary stylet tip. In (C)
685the red solid line surrounds a vascular bundle, and the dashed line indicates the separation
686between phloem and xylem.

Table S1. Calculations of total probing duration (TPD) and time to first probe (TFP) in males, females and N5-nymphs of *N. tenuis*. Values are expressed in seconds (mean ± SE) (Means compared with One-way ANOVA test for TPD and Kruskal-Wallis test for TFP).

Insect stage	TPD	TFP
Male	16,457.0 ± 743.0	72.0 ± 9.6
Female	15,598.0 ± 332.5	114.4 ± 22.2
N5-nymphs	15,364.0 ± 660.0	174.9 ± 45.1
	F_2= 0.91; P = 0.409	H = 4.5; P = 0.110