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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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1Regular research article for Biological Control

2

3Short title: Plant feeding by *N. tenuis*

4

**5Plant feeding by *Nesidiocoris tenuis*: quantifying its behavioral and mechanical
6components**

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27ABSTRACT

28 Zoophytophagous predators play an important, though sometimes controversial, role in pest
29 management programs in different crops. In tomato crops, damage caused by phytophagy of the
30 mirid *Nesidiocoris tenuis* has mainly been reported at high predator population levels or when
31 prey is scarce. Previous research has focused on predator/prey ratios, stylet morphology and
32 saliva composition to explain plant damage by *N. tenuis*. In this study, we investigated the
33 behavioral and mechanical components of the phytophagy. For this, we compared the feeding
34 behaviors of males, females and fifth-instar nymphs of *N. tenuis*. Additionally, we investigated
35 the type of stylet activities performed by each stage while probing in plant tissue, using the
36 electrical penetration graph technique (EPG). Furthermore, stylectomy was performed and plant
37 histology studied with the aim to correlate the feeding activities observed in the EPG recordings
38 with stylet tip positions in specific tissues of the leaf petioles. Behavioral observations during a
39 30-min period showed that nymphs probed more frequently (38.6 ± 1.5 probes) than males and
40 females (25.3 ± 1.1 and 24.3 ± 1.1 probes, respectively). Similarly, nymphs spent a higher
41 proportion of time (656.0 ± 67.6 s) feeding on tomato apical sections compared to males and
42 females (403.0 ± 48.8 s and 356.0 ± 43.7 s, respectively). The EPG recordings during 5 h
43 indicated that cell-rupturing was the main stylet activity for all insect stages, and that fifth-instar
44 nymphs spent a higher proportion of time on cell-rupturing events compared to adults. The
45 histological studies revealed a trend of *N. tenuis* for the tissues within the vascular semi-ring.
46 The stylet tips were found both in the vascular bundles and in the parenchyma of the
47 interfascicular region. The findings of this study confirm an important role of fifth-instar
48 nymphs feeding behavior in the damage potential of *N. tenuis*. Moreover, the increased time
49 spent on cell rupturing behaviour suggests that stylet laceration and enzymatic maceration of the
50 saliva occurring during this event might greatly contribute to the inflicted damage. A
51 comprehensive understanding of the interactions of *N. tenuis* with the plant, at both the
52 behavioral and mechanical levels, might shed light on new approaches to minimize its damage
53 potential to tomato while maintaining its benefits as biocontrol agent.

54

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

57

581. INTRODUCTION

59The use of zoophytophagous predators for biological control of pests in agroecosystems has
60increased over the last decades (van Lenteren et al., 2018). *Nesidiocoris tenuis* (Reuter)
61(Hemiptera: Miridae) is one of these predators widely used in current biocontrol programs in
62Southern Europe, where it is occurring naturally and can spontaneously colonize vegetable
63crops (Arnó et al., 2010; Pérez-Hedo and Urbaneja, 2016). *Nesidiocoris tenuis* is commercially
64available and performs a crucial role in integrated pest management (IPM) programs in tomato
65(Albajes et al., 2006; Calvo and Urbaneja, 2004; Pérez-Hedo and Urbaneja, 2016; van Lenteren,
662012; van Lenteren et al., 2018). Advantages such as preying upon several key pest species,
67high predation efficiency and its capacity to stay in the crop under prey shortage conditions
68(Urbaneja et al., 2009, 2005) are some of the primary reasons this predator is considered a
69successful biocontrol agent in Southern Europe. Moreover, recent studies have demonstrated the
70benefits deriving from its phytophagy in terms of activation of plant defenses that enhance
71biological control (Bouagga et al., 2020, 2018a; Naselli et al., 2016; Pérez-Hedo et al., 2018,
722015b, 2015a). However, despite its services as biocontrol agents, under certain conditions
73damage caused by their phytophagy has also been reported. Plant damage ranges from necrotic
74rings in stems and petioles, to abortion of small fruits and flowers, reduced vegetative growth,
75and blemishes in fruits (Arnó et al., 2010; Calvo et al., 2009; Calvo and Urbaneja, 2004;
76Castañé et al., 2011; El-Dessouki et al., 1976; Pérez-Hedo and Urbaneja, 2016; Sánchez, 2008;
77Sánchez and Lacasa, 2008). The damage caused by *N. tenuis* can become very important in
78tomato crops cultivated in heated greenhouses and/or with low pest pressure. For instance, in
79northern Europe, where these conditions are common to tomato production, *N. tenuis* is
80considered a serious pest (Ferguson et al., 2020; Pérez-Hedo and Urbaneja, 2016).

81Regardless of its damage potential, the success and widespread use of *N. tenuis* as a biological
82control agent in cultivated systems have prompted researchers to investigate the mechanisms
83underlying its phytophagy, and ways to reduce its negative impacts. For instance, research first
84focused on predator-prey interactions. Several studies have demonstrated that damage occurs
85mainly at high predator population levels and its severity is prey density-dependent, with an
86increase in number of necrotic rings as prey populations decrease (Arnó et al., 2010; Calvo et
87al., 2009; Sánchez, 2008). The role of temperature has also been explored, and it was shown that
88the severity of the damage inflicted by *N. tenuis* increased at higher temperatures (Sánchez,
892008; Siscaro et al., 2019). Stylet morphology and saliva composition of important
90zoophytophagous species, including *N. tenuis*, have been studied aiming at finding the
91mechanisms underlying plant damage, but these factors alone did not explain *N. tenuis* damage
92potential (Castañé et al., 2011). Histological studies on stained tissues *N. tenuis* fed upon have
93also been carried out to characterize the damage (Raman and Sanjayan, 1984).

94 More recently, research trying to explain the mechanisms causing plant damage by
95 zoophytophagous predators has changed the focus from general to more specific approaches.
96 Hence, more attention has been given to biotic factors such as plant cultivar and plant
97 interaction with microorganisms (Cabello et al., 2013; Garantonakis et al., 2018; Siscaro et al.,
98 2019). For instance, mixed results have been reported regarding the influence of tomato cultivar
99 on damage incidence by *N. tenuis*, with significant differences between cultivars reported by
100 Cabello et al. (2013), whereas differences between cultivars found by Siscaro et al. (2019) were
101 not significant. Moreover, the role of microorganisms associated to the plants in damage caused
102 by zoophytophagous predators has been demonstrated for *N. tenuis* by Garantonakis et al.
103 (2018), who reported that tomato plants inoculated with the endophytic strain *Fusarium solani*
104 K had significantly less damage than non-inoculated plants. However, the behavioral aspects
105 and the stylet activities of *N. tenuis* while piercing the plant remain unexplored.

106 Direct behavioral observations are a practical approach that has been applied to
107 zoophytophagous species to study their phytophagous behavior (Bouagga et al., 2018a, 2018b).
108 For *N. tenuis*, its behavior on sweet pepper plants was recently described by Bouagga et al.
109 (2018a), however, its behavior on tomato has not been described yet. Additionally, the feeding
110 behavior of piercing-sucking insects can be studied with the electrical penetration graph (EPG)
111 technique. In brief, this technique consists of incorporating the plant and the insect as
112 components of an electrical circuit: one of the electrodes holds a wired insect (EPG probe) and
113 the other electrode is a copper post that is inserted in the soil of the potted plant. When the
114 insect pierces the plant, the circuit is closed and the different activities of the stylets in different
115 tissues are recorded as waveforms, hence allowing for an *a posteriori* biological interpretation
116 (Tjallingii 1978). Although EPG has been most often used to study feeding behavior of aphids
117 (Fereses and Collar, 2001; Garzo et al., 2016; Jiménez et al., 2019; ten Broeke et al., 2013;
118 Tjallingii, 1985, 1978) and other piercing-sucking insects (AB Ghaffar et al., 2011; Antolinez et
119 al., 2017; Guedes et al., 2018; Jin et al., 2012; Lucini and Panizzi, 2016), its application to other
120 Hemiptera, such as Miridae, is rather recent (Backus et al., 2007; Cervantes et al., 2016; Cline
121 and Backus, 2002).

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

130

1312. MATERIALS AND METHODS

132The experiments were performed in three different laboratories. The behavior observation
133experiment was carried out at the entomology laboratories of Instituto Valenciano de
134Investigaciones Agrarias (IVIA) in Valencia, Spain. The EPG recordings were performed in the
135entomology laboratories of Wageningen University in Wageningen, The Netherlands. The
136stylectomy experiment, the histological work and waveform characterization and identification
137was conducted at the entomology laboratories of Instituto de Ciencias Agrarias - Consejo
138Superior de Investigaciones Científicas (ICA-CSIC) in Madrid, Spain.

139 2.1. Behavioral observation

140 2.1.1. Plants and insects

141A rearing of *N. tenuis* was established in the laboratory in a plastic insect cage (60 x 60 x 60
142cm) (BugDorm-2 insect tents; MegaView Science Co., Ltd, Taichung, Taiwan). Green bean
143pods (*Phaseolus vulgaris* L.) and eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae)
144were provided twice a week as oviposition substrate and food source, respectively. Cohorts of
145similar age were obtained every week by placing three green bean pods in the rearing cage for
146females to oviposit during 3 days. After this time period, the green bean pods bearing mirid
147eggs were removed from the rearing cage and placed in plastic containers (14 x 14 x 8 cm), with
148an opening in the lid covered with fine mesh for ventilation. One fresh green bean pod and *E.*
149*kuehniella* eggs *ad libitum* were provided twice a week to the cohorts in each plastic container
150until they were at the developmental stage required for the experiments. Both the rearing and
151the cohorts were kept at 25 ± 2 °C, constant relative humidity of 50 ± 10 % RH and 14L:10D
152photo:scotoperiod. *N. tenuis* and *E. kuehniella* eggs were supplied by Koppert Biological
153Systems (Águilas, Murcia, Spain).

154Tomato plants *cv.* Raf Supermarmande (Mascarell Seeds, Spain) used in this experiment were in
155vegetative stages V6 to V7 (ca. 30-40 cm height). Plants were grown in plastic pots (8 x 8 x 8
156cm) and kept in pest-free climatic chambers until the start of the experiment, at the same
157experimental conditions previously described for the *N. tenuis* rearing.

158 2.1.2. Behavioral test

159Insects used were isolated in test tubes and starved during 24 h, with water supplied through
160moistened cotton plugs. Less than 3-day-old females (presumably mated) and males, and fifth-
161instar-nymphs (N5) were used. One individual with its respective tomato plant apical section
162was considered a replicate. A total of 20-22 replicates per developmental stage were recorded.

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

176 Apical bud (AB): apical bud

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

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

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

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

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

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

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

186 Resting (R): the predator stands motionless.

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

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

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

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

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

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

200 **2.2. Electrical Penetration Graph (EPG) recordings**

201 *2.2.1. Plants and insects*

202A *N. tenuis* rearing was established with individuals provided by Wageningen UR Greenhouse
203Horticulture (Bleiswijk, The Netherlands), which were originally sourced from Koppert
204Biological Systems (Águilas, Murcia, Spain). Green bean pods (*Phaseolus vulgaris* L.) and *E.*
205*kuehniella* eggs (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) were
206provided twice a week as oviposition substrate and food source *ad libitum*, respectively. One
207tomato plant *cv.* Moneymaker was supplied weekly to the rearing for the mirids to get
208experience with the plant tissues used during the EPG recordings. The rearing was kept in a
209muslin cage (25 x 25 x 25 cm) at the same environmental conditions as described above for the
210behavior experiment.

211Tomato plants *cv.* Moneymaker used in this experiment were in vegetative stages V5-V6 (ca. 30
212cm height). Plants were grown in plastic pots (6 x 6 x 6 cm) and kept in a greenhouse
213compartment at 19–21 °C, 60-70% RH and 16L:8D h photo:scotoperiod.

214 *2.2.2. EPG recordings*

215The EPG recordings were carried out inside Faraday cages to prevent electromagnetic
216interference. For wiring, the mirids were first anesthetized on ice for approximately one minute
217and then placed at the tip of a pipette tip (200 µl) connected to a vacuum under low suction.
218Then, a 2-3-cm-long gold wire (18 µm diameter) was attached to the dorsum of the insect using
219a small drop of water-based silver glue (EPG Systems, Wageningen, The Netherlands). Insects
220used in this experiment were individually starved during hanging on their respective wires for 5
221h. Water was not supplied during starvation since cotton plugs provided surface and traction for
222the insects to detach from their wires. To start the EPG recordings, the wired insect was
223carefully placed on the petiole of the second or third fully developed leaf from the apical bud of
224a potted tomato plant. We used less than 3-day-old females (presumably mated) and males, and
225N5 nymphs for the recordings. Fifteen replicates were recorded for males and females, and

226fourteen for N5-nymphs, for a total of 44 individuals. A new plant was used for each individual.
227EPG recordings were obtained with a Giga-8 DC-EPG device (EPG Systems) during an
228undisturbed 8-h period. EPG data acquisition and analysis were conducted using Stylet+
229Software for Windows (EPG Systems).

230The broad waveform classification into probing and non-probing behaviors for this experiment
231was done following Backus (2000), who defined as probing behaviors all behaviors from the
232start of the style insertion into the plant tissue until stylet withdrawal. The non-probing
233behaviors comprise all other behaviors that do not involve stylet penetration (Backus, 2000).
234Probing behaviors in cell rupture feeders are further classified into probing waveforms: cell
235rupturing (CR), transition (T) and ingestion (I) (Cervantes et al., 2016). The identification and
236classification of the probing waveforms for *N. tenuis* was based on the waveform library of the
237mirid species *Lygus lineolaris* (Palisot de Beauvois) and *Lygus hesperus* (Knight) (Hemiptera:
238Miridae) (Cervantes et al., 2016). Since waveforms T are suggested to be species dependent
239(Cervantes et al., 2016), and were scarce and not clearly distinguishable in our recordings,
240waveforms resembling T patterns were included as CR. For the purposes of this study, non-
241probing behaviors were not included in the analysis.

242 2.3. Stylectomy and plant tissue histology

243Histological thin-section analysis was performed to correlate the position of the stylet tips with
244the cell rupturing and active ingestion waveforms observed during the EPG recordings. For this
245study, additional adults of *N. tenuis* were monitored on tomato petioles with a Giga-4 DC-EPG
246device (EPG Systems) under conditions similar to those of the previous EPG recordings. When
247the respective waveform of interest was observed, the feeding activity was artificially
248terminated by stylet amputation with a tungsten needle of a Zapper RF micro-cautery unit
249(www.aphidzapper.com) following the methodology proposed by Downing and Unwin (1977).
250Petiole segments (ca. 0.5 cm) containing the severed stylets of *N. tenuis* were carefully removed
251from the plant with a scalpel. Then, the petiole segments (hereafter samples) were immersed in
252Karnovsky fixative at room temperature and placed under a low vacuum for 1 h to prevent air
253bubbles in the tissues. Afterwards, the samples were dehydrated in graded ethanol series (10-
254100%) and then infiltrated and embedded in paraffin. Serial transverse sections (15-20 μ m
255thick) were cut on a Leica 1512 microtome and stained in 0.05% toluidine blue solution for 10
256minutes. Permanent slides were prepared in mounting medium DePeX (SERVA Electrophoresis
257GmbH, Heidelberg, Germany) and examined using a Nikon Eclipse E800 microscope. Digital
258images were captured using the same microscope coupled with a Canon EOS 6D Mark II
259camera.

260 Mounted transverse-sections were examined for any indication of salivary sheath and to
 261 determine the position of the stylet tips inside the petiole tissues. Petiole tissues examined were:
 262 epidermis, ground tissue (i.e. parenchyma between epidermis and vascular tissue), vascular
 263 tissue (i.e. vascular bundles and interfascicular region [i.e. parenchyma between vascular
 264 bundles]). In tomato petioles the vascular tissue is arranged in a semi-ring shape (Maiti et al,
 265 2012).

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

270 2.4. Statistical analysis

271 Behaviors were analyzed with Generalized Lineal Models (GLM) with Poisson and
 272 quasipoisson error distributions, by using the function *glm* to assess differences in behaviors
 273 between developmental stages/sex (hereafter Stages/Sex). Stage/Sex was entered as independent
 274 variable for all behaviors except for oviposition. Significant differences between Stages/Sex
 275 were followed by multiple comparisons with Bonferroni correction ($\alpha = 0.05$), by applying the
 276 *emmeans* function. Differences in time spent on each location were analyzed with GLM with
 277 quasipoisson error distribution. In this model, Location and Stage were entered as independent
 278 variables. Multiple comparisons were applied with the *emmeans* function (Bonferroni correction
 279 $\alpha = 0.05$) for the variables with significant differences.

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

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

298

2993. RESULTS

300 3.1. Behavioral observation

301 Significant differences were found between Stages/Sex for number of probes, feeding, resting
 302 and searching (Table 1). In contrast, no significant differences were found between Stages/Sex
 303 for time allocation to walking-searching, cleaning, out of plant and out of sight (Table 1).

304 Multiple comparisons for significant Stages/Sex effect revealed that N5-nymphs spent longer
 305 time feeding than both males and females ($Z = -3.07$, $P < 0.05$ and $Z = -3.82$, $P < 0.05$,
 306 respectively). Similarly, nymphs probed more frequently on the plant tissues than both males
 307 and females ($Z = -7.16$, $P < 0.05$ and $Z = -7.98$, $P < 0.05$, respectively). Resting time was higher
 308 in males than females ($Z = -3.36$, $P < 0.05$), but similar to that of nymphs ($Z = 1.60$, $P = 0.329$).
 309 Time spent searching was higher in females than males ($Z = 2.79$, $P < 0.05$), but did not differ
 310 from nymphs ($Z = 0.61$, $P = 1.00$).

311 Time spent on each apical section varied across locations ($F_4 = 45.60$, $P < 0.001$) but no
 312 differences were found among Stages/Sex ($F_2 = 0.90$, $P = 0.390$) and no Stages/Sex \times location
 313 interaction was found ($F_8 = 1.50$, $P = 0.163$). All stages spent most of their time on Leaf 2 (L2)
 314 (56%) and the least Out of plant (OP) (4%) (Figure 1).

315 3.2. Electrical Penetration Graph (EPG) recordings

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

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

322 3.2.1. Probing behaviors: Cell Rupturing (CR)

323 Waveform duration per insect (WDI) was similar for N5-nymphs, females and males ($H = 0.19$;
 324 $P = 0.910$) (Figure 3A). The mean waveform duration per event per insect (WDEI) differed
 325 ($F_2 = 20$; $P < 0.0001$), with N5-nymphs displaying the highest mean, followed by males and with
 326 females showing the lowest mean value (Figure 3C). Significant differences were found in the

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

329 3.2.2. Probing behaviors: active Ingestion (I)

330 No significant differences were found for WDI across insect Stages/Sexes for I ($H = 2.0$; $P =$
 331 0.365) (Figure 3B). Similarly, the WDEI mean values were not significantly different across
 332 insect Stages/Sexes ($H = 3.1$; $P = 0.217$) (Figure 3D). Significant differences were observed for
 333 NWEI ($F_2= 19$; $P < 0.0001$), with females showing more ingestion periods, followed by males,
 334 and N5-nymphs showing the lowest number (Figure 3F).

335 3.3. Correlation between EPG waveforms and stylet tip positions in the plant tissue

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

342

343 3.4. DISCUSSION

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

351 Previous studies reported a higher damage potential of *N. tenuis* nymphs compared to that of the
 352 adults (Arnó et al., 2006; Calvo et al., 2009; Perdakis et al., 2009). For instance, Arnó et al.,
 353 (2006) demonstrated a two-fold difference in the number of necrotic rings caused by nymphs
 354 relative to adults in tomato side shoots. The present results revealed that N5-nymphs probe (i.e.
 355 insertion of the stylet) and feed for longer time in tomato than the adults, thus suggesting an
 356 important mechanical component in the damage potential of the different stages of *N. tenuis*.
 357 According to Hori (2000), the mechanical destruction is likely the primary cause of plant
 358 damage in heteropterans, with the rupture of cells by the stylets as the first step in the injury
 359 process. Contrary to salivary-sheath feeders (e.g. aphids, mealybugs), in which there is

360 minimum disruption of plant cells (Miles, 1968), *N. tenuis* is a cell rupture feeder. In this
361 common feeding strategy in mirids, the insect lacerates the plant tissue with the stylet
362 movements, and injects watery saliva in the surrounding cells, forming pockets of diluted cell
363 contents that will eventually be ingested (Backus et al., 2007, 2005; Cervantes et al., 2016; Hori,
364 2000). Therefore, the higher number of probes observed in N5-nymphs is likely among the main
365 causes that could explain its higher damage ability, due to the continuous piercing of the plant
366 tissues. However, although feeding time in N5-nymphs was found to be significantly higher
367 than that of adults in the behavior experiment, conclusions about the damage potential of *N.*
368 *tenuis* cannot be made on the basis of feeding time alone. This specific result is in conflict with
369 the total probing duration (TPD) in the EPG experiment (i.e. feeding time = TPD: time the
370 stylets remains inserted in the plant tissue), where no differences were found across insect
371 stages. The 24 h starvation to which all insects were subjected before the behavior experiment
372 could have affected N5-nymphs more severely than adults, thus likely explaining longer feeding
373 time observed in this stage during the first minutes of plant contact (30 min of behavior
374 experiment). In contrast, TPD results suggest that feeding time is similar across life stages when
375 time of plant contact increases (5 h of EPG recording). Moreover, the starvation time before the
376 EPG experiment was shorter (i.e. 5 h), which could also partially explain the similarities in TPD
377 due to less severe conditions experienced by the insects. The lack of differences in the time to
378 first probe suggest a similar acceptance of the host plant by all stages and sexes of *N. tenuis*
379 evaluated.

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

392 In the cell rupture feeders, the CR waveforms represent the probing behavior in which the plant
393 cells are lacerated and macerated by the action of the stylet movements, and injection of watery
394 saliva, respectively (Cervantes et al., 2016). The EPG results show that CR is performed about
395 77-89% of the total waveform duration (TWD) for the insect stages and sexes evaluated. This

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

412 During the ingestion (I) waveforms, the cell-rupture feeder uses its cibarial pump to swallow the
413 pre-digested cell contents mixed with watery saliva through the stylets (Cervantes et al., 2016).
414 Overall, I activity was numerically lower than CR as demonstrated by WDI, WDEI and NWEI
415 mean values. Moreover, ingestion was performed only about 11-23% of the TWD by all insect
416 stages and sexes evaluated in this study. Hence, the role of ingestion activity is presumably
417 minor in the plant feeding behavior of N5-nymphs and adults of *N. tenuis*, compared to CR
418 behavior. The low proportion of time spent on I activity found in this study are consistent with
419 those reported for different life stages of *Lygus* spp. (Cervantes et al., 2016; Cline and Backus,
420 2002). It is worth mentioning that although not all parameters for I activity showed significant
421 differences, there was a trend for N5-nymphs that these were numerically lower than for adults.
422 This could mean that N5-nymphs are less efficient at ingestion as a consequence of smaller size
423 and/or characteristics of the saliva, as suggested by Tuelher et al. (2020). Deficient ingestion in
424 nymphs could also mean more enzymatic saliva left in the plant tissue compared to more
425 efficient ingestion in adults, thus causing more damage over time due to maceration. The
426 decreased ingestion efficiency in N5-nymphs is further supported by its TWD, which is < 50%
427 of that observed in adults. Shorter stylets in immature stages have been suggested as limiting
428 factor for feeding (Cooper & Spurgeon, 2013), and it is likely an additional reason for this
429 decreased efficiency.

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

432either CR or I waveforms were all found in vascular bundles or in parenchyma cells of the
433interfascicular region. Similar results were reported in previous studies based on histological
434sections of stained tissues with damage inflicted by *N. tenuis* (Raman and Sanjayan, 1984).
435Different position of mandibular stylet tips relative to maxillary stylet tips was observed in
436some samples from both CR and I events, hence laceration is likely occurring during both
437probing activities, and in the different tissues reached by the stylets. Our results suggest that *N.*
438*tenuis* does not feed on a specific cell type within the vascular semi-ring. Instead, *N. tenuis*
439creates pre-digested pockets of mixed contents from cells in this region, which could vary in
440nutrient contents depending on its proximity to the phloem. This “unspecific” cell selection is
441further supported by the fine structure and polarity of the I waveforms observed during the EPG
442recordings. The peak-and-wave structure is common in active ingestion (contrary to passive
443ingestion typical of phloem feeders) where the regular pattern is attributed to the rhythmical
444pumping and swallowing produced by the cibarial muscle (Cervantes et al., 2016; Dugravot et
445al., 2008; Lucini and Panizzi, 2016). Additionally, the positive polarity of the probes observed
446in our recordings is contrary to the negative polarity expected from intracellular stylet
447penetrations (Walker, 2000). Further studies with more histological samples are necessary to
448confirm these results, and to determine whether other tissues are also targeted under other
449circumstances, such as prey availability.

450The findings of this study provide insights about the role of feeding and probing behaviors in
451the plant feeding by *N. tenuis*. CR probing events stand out as a primary mechanical component
452of the overall phytophagy of the insect stages evaluated. The increased number of probes and
453longer CR events observed in N5-nymphs could be the mechanisms underlying the higher
454damage potential of this life stage. Based on EPG results and the histological observations, most
455CR events are then expected to occur in the vascular region, thus probably comprising important
456damage to plant nutrient transport as well. Overall, this study broadens the understanding of the
457mechanical aspects underlying the phytophagy of *N. tenuis* on tomato. This could be useful in
458the development of new methods aimed at diminishing its negative impacts. For instance plant
459breeders could benefit from this knowledge to target specific plant tissues and develop varieties
460less susceptible to suffer from *N. tenuis* phytophagy.

461

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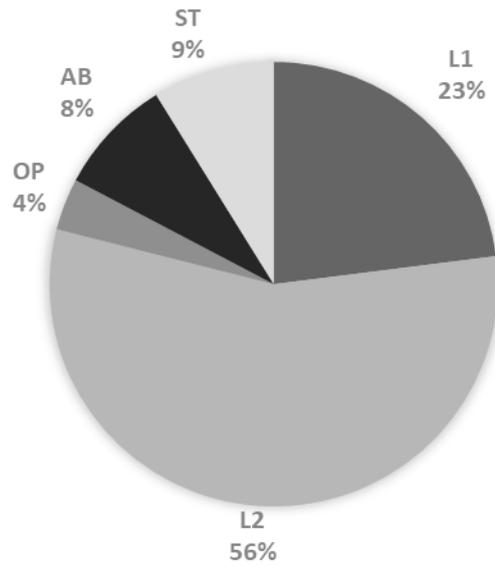
651 FIGURES AND TABLES

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

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

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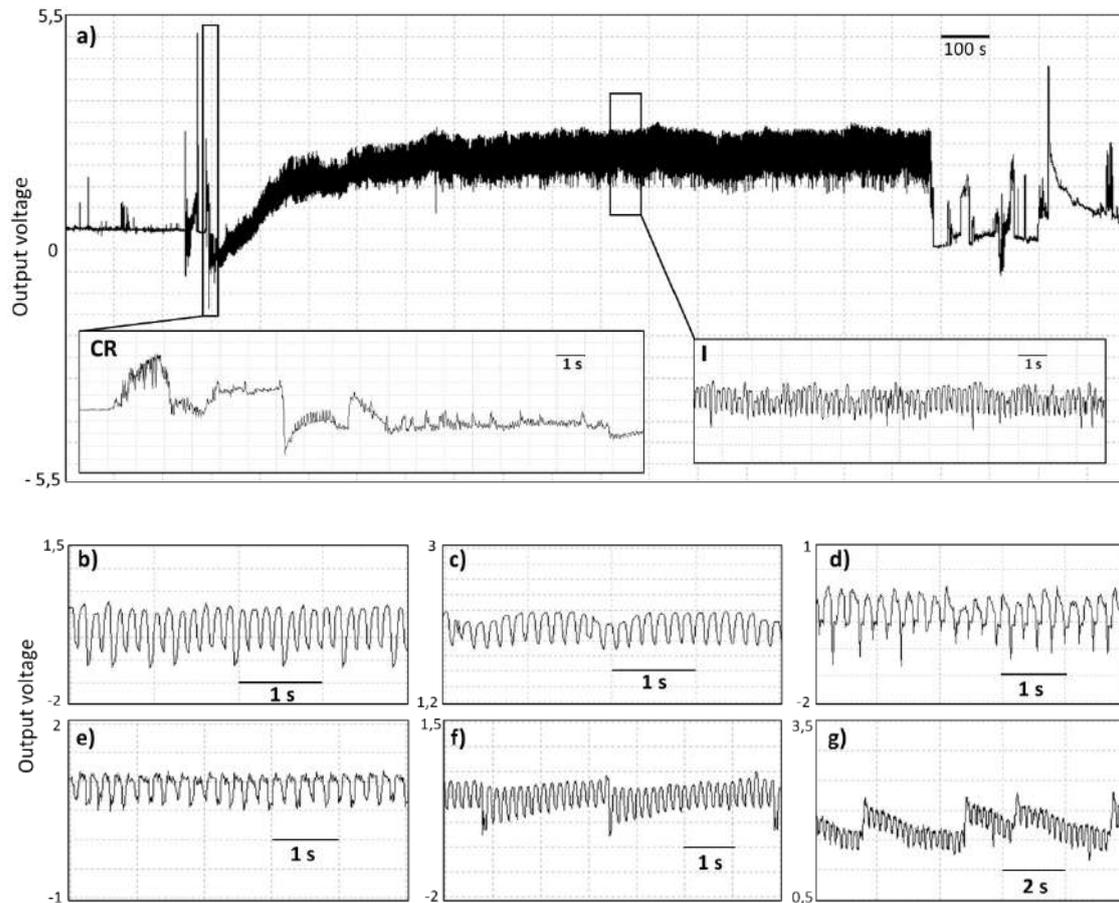


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659

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)
 665 with details of the coarse structure of Cell rupturing (CR) and Ingestion (I) waveforms in inset
 666 boxes. Details of the coarse structure of Ingestion (I) waveforms during different recordings (b-
 667 g).

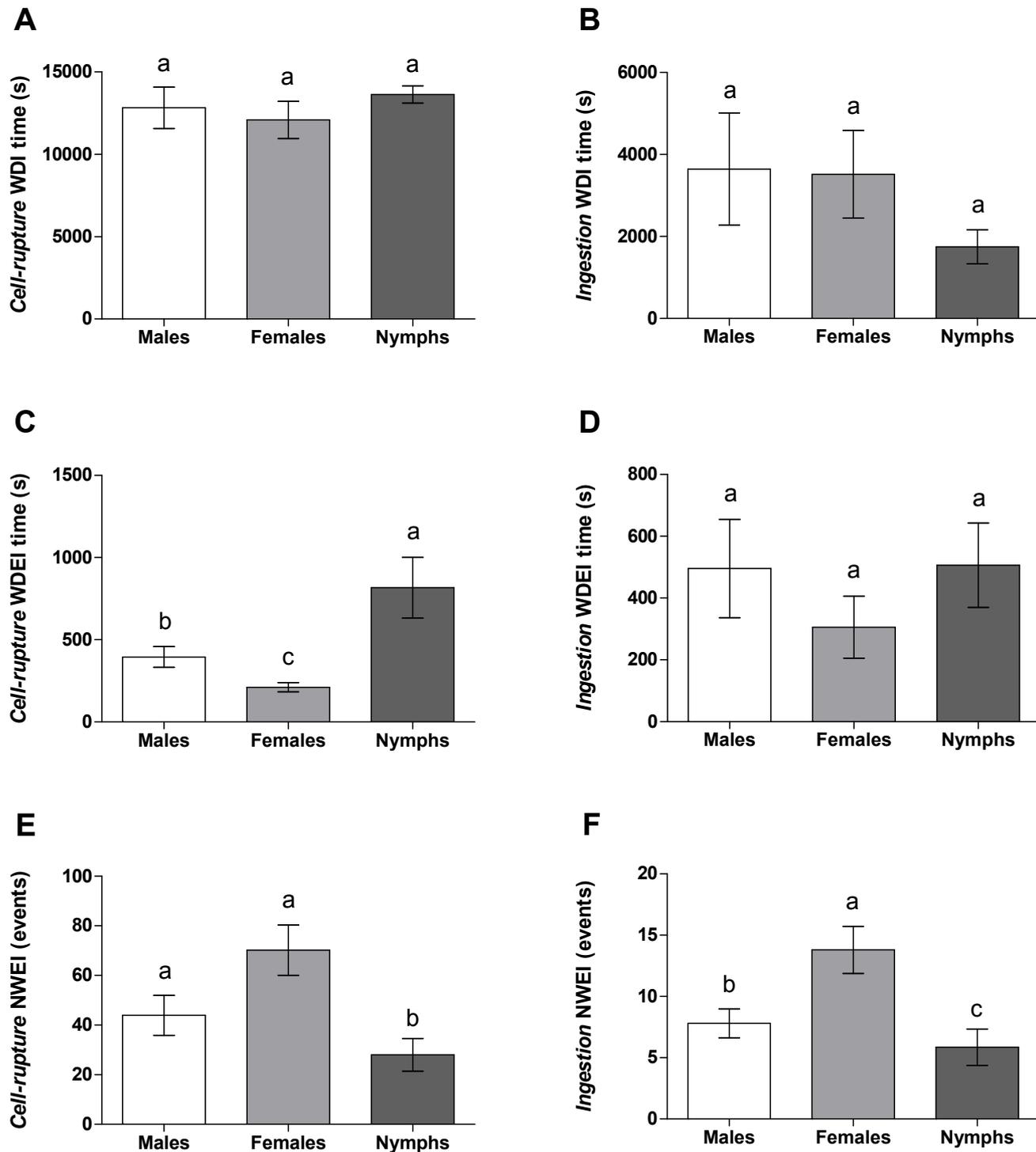
668

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*.

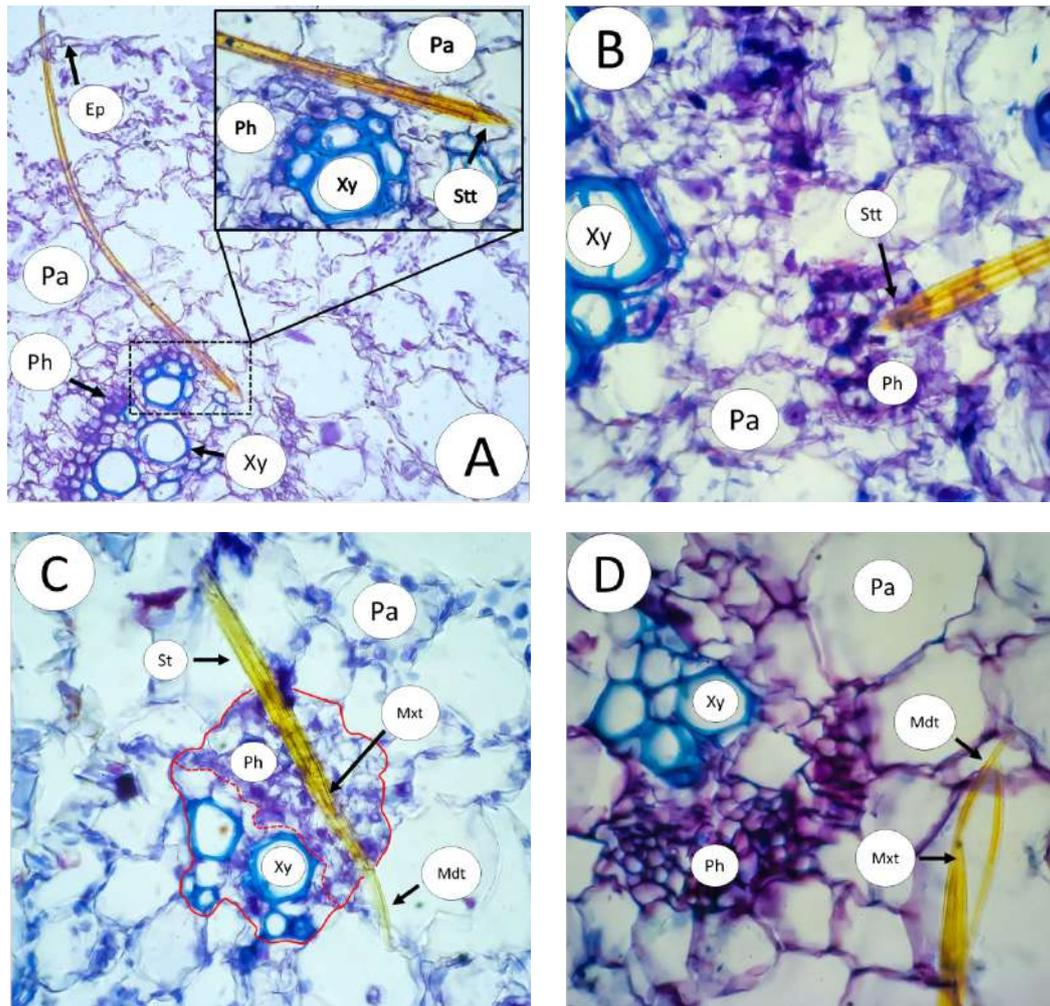
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Insect stage (n)	Cell rupturing		Ingestion	
	TWD (s)	%	TWD (s)	%
Male (15)	192,198.02	78	54,654.2	22
Female (15)	181,185.76	77	52,782.32	23
N5-nymph (14)	190,645.56	89	24,455	11

673



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 \pm 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.

687**Table S1.** Calculations of total probing duration (TPD) and time to first probe (TFP) in males,
 688females and N5-nymphs of *N. tenuis*. Values are expressed in seconds (mean \pm SE) (Means
 689compared with One-way ANOVA test for TPD and Kruskal-Wallis test for TFP).

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