






# DC-EPG assisted comparison of European spittlebugs and sharpshooters feeding behaviour on grapevine

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## Abstract

Xylem-feeding is apparently the only requirement making an insect a competent vector of the bacterium *Xylella fastidiosa*, an organism responsible for the devastation of the Southern Italian olive forest and nowadays considered one of the most feared threats to agriculture and landscape in Europe, including vineyards. Here, we used the direct current-electrical penetration graph (DC-EPG) technique to compare and describe the feeding behaviour on grapevine of four xylem-feeding species considered candidate vectors of *X. fastidiosa* widespread in Europe, namely two spittlebugs (the meadow spittlebug *Philaenus spumarius* and the spittlebug *Neophilaenus campestris*) and two sharpshooter leafhoppers (the rhododendron leafhopper *Graphocephala fennahi* and the green leafhopper *Cicadella viridis*). We created a standard for the analysis of EPG waveforms recorded with a DC-EPG device, describing feeding activities performed by these insects from stylet insertion into the plant to withdrawal. This standard, along with freely available software, has been developed to harmonize the calculation of feeding behavioural parameters in xylem-feeders. The most relevant differences between the two vector *taxa* were the probing frequency and the dynamics of xylem ingestion. Sharpshooters tended to perform significantly more probes than spittlebugs. In contrast, the latter spent longer times in low-frequency xylem ingestion, characterized by scattered contractions of the cibarial dilator muscle interspersed with periods of pump inactivity. *Cicadella viridis* was the species displaying the highest frequency of the electrical pattern found to be associated with *X. fastidiosa* inoculation in spittlebugs (Xe). Feeding behavioural data presented here represent an important step forward for deepening our knowledge of xylem-sap feeding insects' interaction with both the host plants and the bacterium they transmit.

## KEYWORDS

feeding behaviour, host-plant selection, Pierce's disease, plant preference, stylet penetration, xylem-sap feeders

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## 1 | INTRODUCTION

Probing, feeding and dispersal behaviours in response to plant cues by insects transmitting plant pathogens directly determine the probability of the pathogen itself being acquired, retained and subsequently inoculated. Therefore, the host preference and feeding behaviour of the insect vector directly govern insects' and hosts' exposure to pathogens (Jiménez et al., 2020; Rashed et al., 2011).

Phytophagous insects have evolved to feed on every part of a plant, including one of the hardest-to-extract component: xylem sap (Bergman, 2021). Xylem feeding originated in Auchenorrhyncha, a suborder of Hemiptera, and is a feature shared by insects belonging to the superfamily Cercopoidea (spittlebugs), Cicadoidea (cicadas) and the Cicadellidae sub-family Cicadellinae (sharpshooters) (Redak et al., 2004). Xylem sap is the least nutritional fluid present in plants in terms of its nitrogen and carbon content (Redak et al., 2004). Insects feeding predominantly from the xylem tend to be polyphagous, as a narrow host plant range would limit their choice of nutritious food sources (Novotny & Wilson, 1997; Ranieri et al., 2020). This could lead to a disadvantage regarding the need to process multiple sensory inputs for discriminating suitable host plants compared to species with a more limited host range (Smith & Chuang, 2014). In xylem feeders, tactile cues and substrate-borne intra-specific vibrational communication were suggested to be involved in the recognition and acceptance of a plant as a host upon landing (Avosani et al., 2020; Cornara, Morente, et al., 2019). Following the settlement, the insect proceeds through a sequence of brief, stereotypical behaviours: plant surface exploration, stylet probing, xylem-sap ingestion and stylet withdrawal. Labial dabbing prior to probing permits the insect to gather information about the plant surface through hairs, setae and pegs of presumed sensory function located at the tip of the labium (Backus, 1985; Leopold et al., 2003). However, the insertion of the stylets into the host plant tissues and uptake of xylem-sap are the pivotal steps in host-plant discrimination (Markheiser et al., 2020; Sandanayaka & Backus, 2008). Indeed, the chemosensilla lining the precibarium, the narrow corridor connecting the food canal to the cibarial pump, mediate gustatory discrimination of chemical compounds within the plant (Backus, 1985).

The vector-borne bacterium *Xylella fastidiosa* Wells (1987) depends on the completion of these steps by its insect vectors to escape the host plant and spread to healthy plants, thus surviving and evolving (Chatterjee et al., 2008; Retchless et al., 2014; Sicard et al., 2018). Only insects feeding predominantly on the xylem are competent vectors for the bacterium (Frazier, 1965). While sharpshooters are the main epidemiologically relevant vectors across the Western Hemisphere and Taiwan, evidence produced so far indicates spittlebugs are the main driver of *X. fastidiosa* spread around the Palearctic (Cornara, Morente, et al., 2019). Feeding behaviour is a fundamental aspect governing the tripartite interaction vector-host-bacterium, as is well understood by sharpshooters (Backus, 2016; Backus & Shih, 2020). In contrast, relatively little is known about spittlebugs' feeding behaviour. Achieving an equally detailed understanding of the feeding process of spittlebugs as sharpshooters will

shed light on poorly understood aspects of *X. fastidiosa* transmission dynamics and the bacterium-vector relationship in Europe.

Here, we used the Electrical Penetration Graph technique (also called electropenetrography, both abbreviated EPG) (Backus & Bennett, 2009; Mclean & Kinsey, 1964; Tjallingii, 1978) to compare and describe the feeding behaviour of grapevine of four xylem-sap feeding species widespread in Europe: the spittlebugs *Philaenus spumarius* L. (1758) and *Neophilaenus campestris* Fallén (1805) (Hemiptera: Aphrophoridae), and the sharpshooter leafhoppers *Graphocephala fennahi* Young (1977) and *Cicadella viridis* L. (1758) (Hemiptera: Cicadellidae: Cicadellinae). Grapevine was selected as the host for the feeding behaviour comparison since individuals of the four insect species were found on grapevine during independent surveys across European vineyards (Xf-ACTORS vector working group, 2020). We also present a simple standard for marking EPG recording outputs obtained with a direct current EPG (DC-EPG) device, along with a freely-available software, for the characterization and analysis of xylem-feeders feeding behaviour.

## 2 | MATERIALS AND METHODS

### 2.1 | Provision of plant material

Grapevines (*Vitis vinifera* cv. "Cabernet sauvignon") as host plants were propagated from *X. fastidiosa*-free dormant cuttings, cultured in 1 L pots in a glasshouse at Julius Kühn-Institut (JKI; Siebeldingen, Germany) filled with garden soil (Fruhstorfer Erde "Tray Substrat + Perlite," Hawita Gruppe GmbH, Vechta, Germany) and sand (4:1). Glasshouse conditions were  $25 \pm 5^\circ\text{C}$  temperature,  $30 \pm 10\%$  relative humidity, photoperiod of 16:8 h light/dark and 12,000 lx light intensity. Plants were fertilized three times a week with a 0.2% solution Hakaphos Soft Elite 24 + 6 + 12(+2) (Compo Expert, Krefeld, Germany). Plants reaching the phenological stages 14–15 according to the scale of BBCH (Biologische Bundesanstalt für Land- und Forstwirtschaft, Bundessortenamt und Chemische Industrie) (Lorenz et al., 1994) were used for the DC-EPG studies, while BBCH stages 15–19 were used for rearing insect nymphs.

### 2.2 | Insect collection and maintenance

Spittlebugs and sharpshooters were collected between May and July 2019 at nymphal stage from *X. fastidiosa*-free areas in Rhineland-Palatinate, Germany. *P. spumarius* and *C. viridis* were collected from various herbaceous plants in an untreated orchard in Göcklingen (N 49.156120°, E 8.027385°). *N. campestris* was collected in dry grassland in Albersweiler (N 49.225350°, E 8.034115°) from Poaceae. Nymphs of *G. fennahi* were gathered from *Rhododendron* spp. plants at JKI, Siebeldingen (N 49.218491°, E 8.046745°).

Nymphs were transferred to the laboratory and reared in groups of 15 individuals per plant on potted grapevines placed inside gauze

cages (33×33×60cm, Live Monarch, Blairsville, Georgia, USA) and kept in a walk-in climatic chamber “Fitotron type SGR233” (Weiss Technik UK Ltd, Loughborough, United Kingdom) until adults emergence. Climatic conditions were set up to 16:8 h light: dark (1 h each of dusk and dawn), 23:19 ± 2°C and 70 ± 5% relative humidity. Newly moulted adults were sexed and separately caged on new grapevine plants (therefore the individuals used for the feeding behaviour observation were unmated).

### 2.3 | EPG recording

The feeding behaviour of either male or female spittlebugs (*P. spumarius* and *N. campestris*) and sharpshooters (*C. viridis* and *G. fennahi*) (age: 1–14 days from adults emergence) on grapevine was explored by DC-EPG, following the method described by Cornara et al. (2018), Cornara et al. (2020) and Markheiser et al. (2020). An eight-channel DC-EPG amplifier (model Giga-8d, EPG-Systems, Wageningen, Netherlands) was used for the recordings. Data were acquired with “Stylet+d” software (EPG Systems, Wageningen, Netherlands).

Insects were starved for 1 h inside a 2 ml plastic tube, then anaesthetised with carbon dioxide for 5 s, tethered (18 µm gold wire; EPG-Systems, Wageningen, Netherlands) and connected to the EPG device following the protocol by Cornara et al. (2018). We opted for a wire narrower than the one reported in the literature for Western Hemisphere sharpshooters (50–60 µm; see for example Almeida & Backus, 2004) to reduce the possible influence of the tethering material on the EPG outcomes (Chesnais & Mauck, 2018).

Since the physical properties of the insects' pronotum differed between the insect *taxa*, we used Ted Pella, no. 16031; Pelco® Colloidal Silver (Ted Pella, Redding, CA, USA) for spittlebugs (whose pronotum is scantily hairy) and Ted Pella, no. 16062; Pelco® Conductive Silver Paint (Ted Pella, Redding, CA, USA) for sharpshooters (whose pronotum is covered by a thin wax layer inhibiting colloidal silver adhesion).

The feeding behaviour of the four xylem-feeding species was recorded for 6 h, with one EPG recording per day carried out between 9:00 AM and 3:00 PM. The experimental design was a randomized complete block with two blocks per day (two individuals per species per day randomly distributed inside the Faraday cage). For the recording, the insects were offered a plant portion where they had access to the stem, petiole and leaf. A single plant per insect per recording was used. Experiments were performed under semi-controlled conditions at 25 ± 5°C and artificial light of 1000 ± 200 lx. A total of 15 recordings per species and per sex were conducted.

### 2.4 | Processing of EPG data

For the comparative analysis of the feeding behaviour of the four species tested, we created a standardized protocol for marking EPG recording files with “Stylet+a” software (EPG-Systems, Wageningen, Netherlands), suitable for xylem-feeders. The protocol and the

definition of the waveform were generated by merging data reported by Cornara et al. (2018), Cornara et al. (2020) and Markheiser et al. (2020) for spittlebugs, and by Almeida and Backus (2004), Backus et al. (2005), Miranda et al. (2009), Backus (2016) and Backus and Shih (2020) for Western Hemisphere sharpshooters.

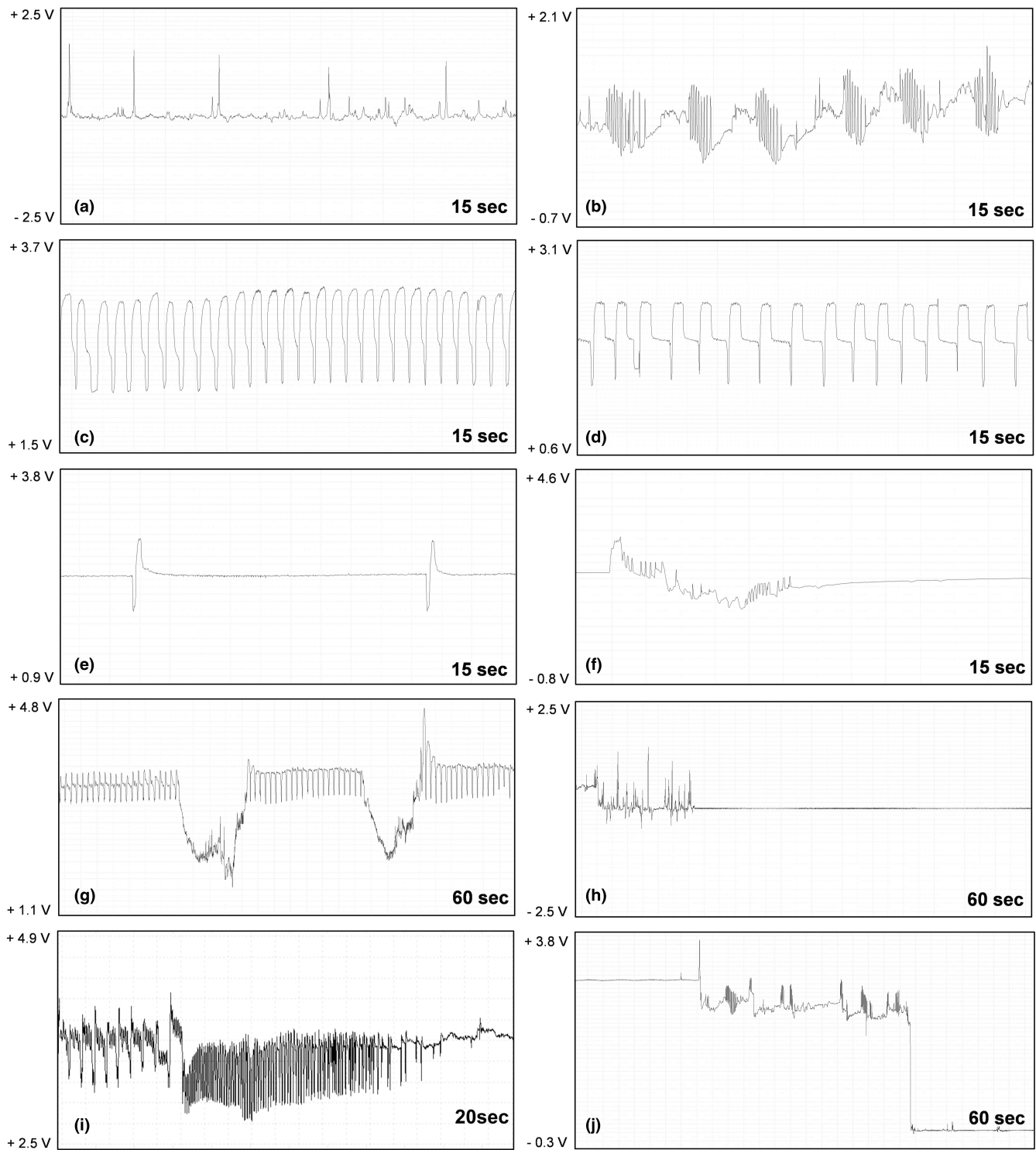
Briefly, we considered ten patterns representing the different behavioural steps performed by the insect, from the insertion of stylets into the plant to their withdrawal (Figure 1): (i) np, non-probing; (ii) C, pathway; (iii) Xc, xylem contact; (iv) Xi, xylem ingestion (frequency > 0.1 Hz); (v) LF, low-frequency xylem ingestion (frequency < 0.1 Hz); (vi) N, non-pathway interruption of xylem activity; (vii) R, resting; (viii) Xe, behaviour putatively associated with *X. fastidiosa* inoculation (Cornara et al., 2020); (ix) W, stylets withdrawal and (x) Esc, escaping of the individual from the host plant. For a more detailed explanation of waveforms, their hypothesized/correlated biological meaning, and coarse as well as fine structures, please refer to Supplementary Material 1 and Figure 1.

### 2.5 | Generating sequential and non-sequential parameters

A novel MS Excel macro (hereafter referred to as software “XylFeed”) was developed to analyse essential waveform variables for xylem-feeding insects (Markheiser et al., 2022; software available at <https://doi.org/10.5073/20221107-091816>). EPG waveforms were manually marked in “Stylet+a” software (EPG Systems, Wageningen, Netherlands) and analysed by “XylFeed.” The software enables the analysis of the probing and feeding activity among the four xylem-feeding species through a calculation of:

- (i) Number of probes: successful (with xylem ingestion), unsuccessful (without xylem ingestion), initial (without xylem ingestion, but with xylem contact) and total (successful + initial + unsuccessful);
- (ii) Sequential variables (np to first Xc; np to first Xi; np to first LF; first C to first Xc; first C to first Xi; first C to first LF; time to first probe; time to first probe with Xi and time to first probe with LF);
- (iii) Non-sequential variables (WDI, waveform duration per individual; NWEI, number of waveform events per individual; WDEImd, median duration of each waveform event per individual).

For a detailed description of the software usage see Supplementary Material 1 (output variables are defined within the software): “XylFeed” was developed for analysing the EPG recordings marked with “Stylet+a” and also returns variables related to “sustained” xylem sap ingestion (both Xi and LF). Five minutes was assumed as a critical threshold in accordance with previous reports on sharpshooters (Almeida & Backus, 2004; Backus et al., 2005; Miranda et al., 2009). Since the purpose of the present work was to develop a protocol suitable for all xylem feeders, we suggest adopting this threshold for all the species, although the software allows adaptations for future works.



**FIGURE 1** Spittlebugs and sharpshooters waveforms recorded by direct current-electrical penetration graph (DC-EPG): (a) np (non-probing), (b) C (pathway), (c) Xc (xylem contact), (d) Xi (xylem ingestion), (e) LF (low-frequency xylem ingestion), (f) R (resting) with spikelet burst drop, (g) two N (non-pathway interruptions) within xylem activity, (h) transition from np to Esc (escaped), (i) Xe (spikelet burst) and (j) W (withdrawal) with the transition to np

## 2.6 | Statistical analyses

Statistical analysis was carried out using R, version 4.1.0 (R Core Team, 2021). Feeding behavioural differences among the species tested (i.e., total probing time, WDI, NWEI, WDEImd and sequential

variables) were investigated with a linear model (LM). Discrepancies in the number of probes (successful, unsuccessful and total probes) were evaluated through generalized linear models (GLM) with a negative binomial distribution (link-function: log). The explanatory variables were species, sex and their interaction. In case of a statistically significant

effect ( $p < 0.05$ ) of model variables, pairwise comparisons were conducted by Tukey's HSD (honest significant differences) test using "emmeans" package (Lenth, 2021). Species-related differences in NWEI and WDI trends over the 6 h access to the host plant were calculated using linear mixed-effects models (LME, restricted maximum likelihood method). The explanatory variables were the species, the time of the recording (expressed in hours), and the interaction between these factors, while sex was included as a random factor. In the case of LM and LME, the data were transformed when necessary with  $\ln(x+1)$  or  $\sqrt{x}$  to reduce heteroscedasticity and improve normal distribution.

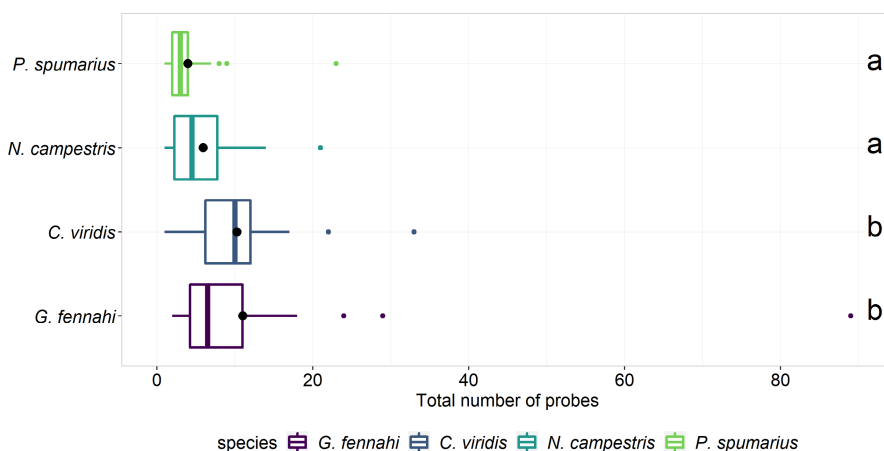
Models were run using the "MASS" and "lme4" packages (Bates et al., 2015; Venables & Ripley, 2002), while residual distribution was checked using the "car" package (Fox & Weisberg, 2019). There was no evidence of either spatial or temporal autocorrelation of model residuals according to analyses performed using the "ncf" and "acf" packages (Bjornstad, 2020), respectively. A summary of the raw dataset was prepared by "psych" package (Revelle, 2021) (Supplementary Material 2). Plots were generated using "ggplot2" package (Wickham, 2016). In boxplots, lines represent the median, bold dots the mean, boxes the interquartile range (IQR), whiskers  $1.5 \times$  IQR and dots outside boxes outliers.

### 3 | RESULTS

For simplicity, in the results section, we report WDI, NWEI and WDEImd as mean values and refer to the four species using acronyms (*Cicadella viridis*: CV; *Graphocephala fennahi*: GF; *Neophilaenus campestris*: NC; *Philaenus spumarius*: PS). Detailed data are reported in Supplementary Material 2 (descriptive statistics) and 3 (statistical analyses, model results and pairwise comparisons), respectively.

#### 3.1 | Total probing time

Over the 6 h of EPG recording, NC spent a significantly shorter time (226.5 min) with stylets inserted into grapevine plant tissues than CV (279.6 min), GF (313.3 min) and PS (341.4 min).



**FIGURE 2** Total number of probes performed by xylem-feeder species (*Philaenus spumarius*, *Neophilaenus campestris*, *Cicadella viridis* and *Graphocephala fennahi*) during the 6 h EPG-recordings (small letters indicate statistically significant differences between species according to Tukey's HSD test for pairwise comparisons;  $N = 30$ ) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

#### 3.2 | Number of probes

Overall, both sharpshooter species performed significantly more total probes (CV: 10.3, GF: 11.0) than either NC (6.0) or PS (4.0) (Figure 2). Both, NC (2.1) and PS (2.1), made significantly fewer successful probes than the sharpshooter species (GF: 5.2, CV: 4.5). Furthermore, the number of unsuccessful probes was significantly lower in PS (1.8) compared to the other species (CV: 5.7, GF: 5.4, NC: 3.8). Males tended to perform more unsuccessful probes than females (GLM:  $F_{4,119} = 3.8$ ,  $p < 0.001$ ). No species-related significant differences were found in the number of initial probes, while males performed more initial probes than females (GLM:  $F_{4,119} = 2.1$ ,  $p = 0.03$ ).

#### 3.3 | Escaping

The highest number of escaped insects (performing the Esc "waveform") was observed for NC, with 12 out of 30 individuals jumping off the plant during the 6 h recording period. The earliest escape was observed for a GF female that abandoned the plant it was placed on during the fifth hour of the EPG (at minute 255).

#### 3.4 | Sequential parameters

The time from the beginning of the recording to the first contact with a xylem vessel was significantly shorter for PS (11.2 min) and GF (13.1 min) compared to CV (32.3 min), while none of the species differed significantly from NC (30.1 min). PS (12.2 min) was the fastest species in commencing xylem ingestion from the beginning of the EPG record, with NC (32.1 min) and GF (15.0 min) showing intermediate values, and CV (41.0 min) being significantly slower than both PS and GF. Males required overall more time than females for performing the first Xi (LM:  $F_{7,110} = 3.1$ ,  $p = 0.003$ ). In males, GF (5.7 min) was significantly faster than CV (23.2 min) in performing the first Xc calculated from the first stylet insertion into the host plant tissue, while none of these species differed significantly from

PS (9.4 min) or NC (20.8 min). Exclusively in NC, males were significantly slower than females in finding a xylem vessel. PS (8.5 min) was significantly faster than CV (32.1 min) in beginning xylem ingestion after the insertion of the first stylet, while neither NC (22.8 min) nor GF (11.2 min) differed from the former two species. There were no species- or sex-related differences in the time required from the first probe to the first LF (LM:  $F_{4,81} = 0.7, p > 0.05$ ). Independently from sex (LM:  $F_{4,114} = 1.2, p > 0.05$ ), CV (11.1 min) required significantly more time than PS (3.1 min) to probe the host plant once given access to it, while GF (3.8 min.) and NC (9.0 min) did not differ significantly from the other species. PS (5.7 min) and GF (11.8 min) started a probe including Xi significantly earlier than NC (27.4 min) and CV (28.8 min); in all the species tested, females tended to insert the stylets into the host plant tissues faster than males (LM:  $F_{7,110} = 3.8, p < 0.001$ ).

### 3.5 | Non-sequential parameters

The four species significantly differed in their feeding behaviour in terms of the waveform duration per individual (WDI; npmv:  $F = 8.299, p < 0.001$ ), the number of waveform events per individual (NWEI; npmv:  $F = 13.175, p < .$ ), and the median waveform duration per event per individual (WDEImd; npmv:  $F = 4.179, p < 0.001$ ), were compared in terms of the waveform patterns np, C, Xc, Xi, LF, Xi+LF, Xe, N, R and W.

#### 3.5.1 | Non-probing

Non-probing duration (np-WDI) (LME:  $F_{2,720} = 3.8, p < 0.001$ ) and their number (np-NWEI) (LME:  $F_{2,720} = 5.1, p < 0.001$ ) showed a decreasing trend over the 6 h recordings in all the species tested. Males of PS (18.3 min) and GF (21.8 min) spent a significantly shorter time in non-probing than individuals of the same sex of CV (115.4 min) and NC (188.5 min). Independently of sex, PS showed the significantly shortest np-WDI (16.7 min), while NC was the longest (128.4 min). PS (4.2) displayed significantly lower np-NWEI than the sharpshooter species (GF: 11.3, CV: 10.6). The difference between PS and NC (6.7) was not significant. Males tended to show more np-NWEI than females (LM:  $F_{4,115} = 2.7, p = 0.007$ ). Independent of sex (LM:  $F_{4,111} = 1.7, p > 0.05$ ), the duration of single np events (np-WDEImd) was overall significantly higher in NC (17.2 min) than in GF (1.8 min), PS (3.3 min) and CV (3.8 min).

#### 3.5.2 | Pathway (C)

The number of pathway events (C-NWEI) (LME:  $F_{2,711} = 5.6, p < 0.001$ ) and their duration (C-WDI) (LME:  $F_{2,711} = 6.2, p < 0.001$ ) similarly decreased during the 6-h recordings in the four xylem-feeders (data not shown). C-WDI was significantly greater in CV (23.8 min) than in spittlebugs (PS: 9.3 min, NC: 12.0 min) and GF

(15.0 min), with no sex-related differences (LM:  $F_{4,115} = 1.0, p > 0.05$ ). Similarly to C-WDI, C-NWEI was significantly higher in sharpshooters (CV: 10.3, GF: 11.0) than in spittlebugs (PS: 4.0, NC: 6.0), and in males compared to females (LM:  $F_{4,115} = 2.8, p = 0.006$ ). The duration of a single C event per individual (C-WDEImd) was significantly longer in PS (2.3 min) than in GF (1.3 min) with no sex-related differences (LM:  $F_{4,115} = 2.7, p = 0.008$ ).

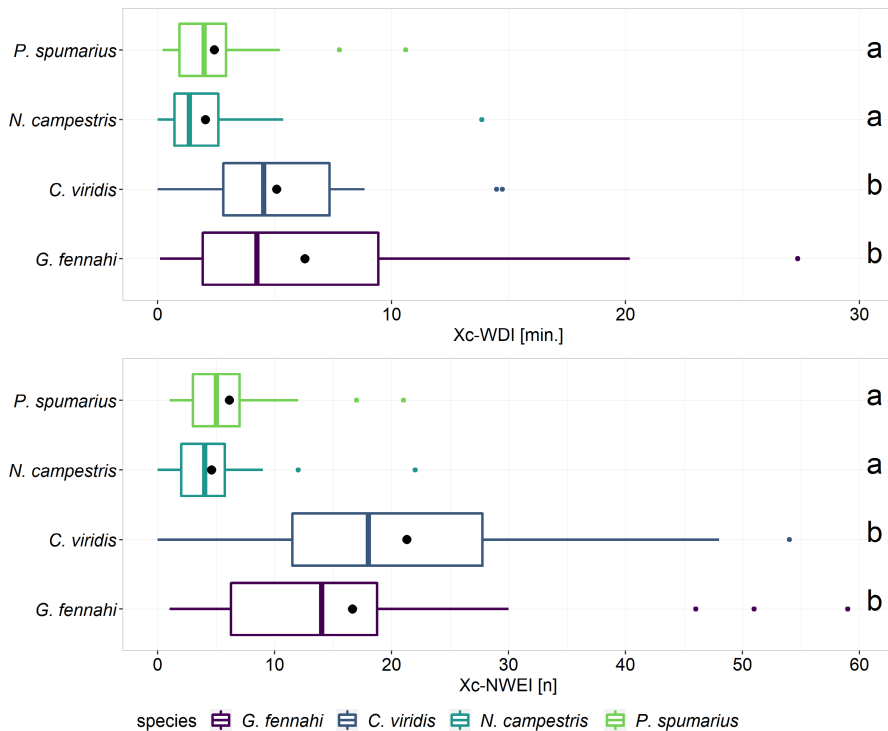
#### 3.5.3 | Xylem contact (Xc)

GF displayed a longer duration of xylem contact (Xc-WDI) at the beginning of the recording, followed by a sharp decrease over time; this decreasing trend over the recording was significantly smoother in the other species tested (LME:  $F_{2,711} = 3.2, p = 0.001$ ). In addition, the number of Xc events (Xc-NWEI) in all species showed a significant decrease over the 6 h EPG recording (LME:  $F_{2,711} = 2.5, p = 0.049$ ) (data not shown). Xc-WDI was overall significantly shorter in spittlebugs (NC: 2.0 min; PS: 2.4 min) compared to sharpshooters (CV: 5.1 min; GF: 6.3 min) (Figure 3, top) and longer in males compared to females (LM:  $F_{4,115} = 3.1, p = 0.002$ ). Spittlebugs (NC: 4.6, PS: 6.1) performed significantly less Xc-NWEI than sharpshooters (CV: 21.3, GF: 16.7) (Figure 3, bottom). In all the species tested, males exhibited more Xc events (Xc-NWEI) than females (LM:  $F_{4,115} = 2.8, p = 0.006$ ). CV (15.6 s) displayed a significantly shorter Xc-WDEImd than NC (29.4 s), while the other species showed intermediate durations (PS: 24.0 s, GF: 22.8 s).

#### 3.5.4 | Xylem ingestion (Xi)

The trend of xylem ingestion duration (Xi-WDI) over time significantly differed between NC and the other species (Figure 4, top). In particular, PS tended to perform a longer Xi-WDI at the beginning of the recording compared to the other three species, with ingestion remaining constant over the 6 h observation. In contrast, the sharpshooters showed an increase in Xi-WDI as the recording progressed. NC displayed a trend roughly similar to CV and GF, although with a lower Xi-WDI.

The number of xylem ingestion events (Xi-NWEI) sharply decreased over time in PS and GF; in NC this decreasing trend was less steep, while CV tended to perform a Xi-NWEI during all hours of the recording (data not shown). Xi-WDI was overall significantly lower in NC (146.3 min) than in GF (239.2 min) and PS (210.5 min), while none of the species differed significantly from CV (202.4 min). In all the species tested, Xi-WDI was shorter in males compared to females (LM:  $F_{4,115} = 3.9, p < 0.001$ ). No sex-related differences were observed in Xi-NWEI (LM:  $F_{4,115} = 1.6, p > 0.05$ ), while NC made significantly fewer Xi-NWEI (11.7) compared to PS (26.5), CV (26.3) and GF (26.6). The median duration of single Xi events (Xi-WDEImd) was similar among the species and between the sexes (LM:  $F_{4,113} = 1.6, p > 0.05$ ).



**FIGURE 3** Total waveform duration per individual of xylem contact (Xc-WDI, top) and total number of waveform events of xylem contact (Xc-NWEI, bottom) performed by spittlebugs (*Philaenus spumarius* and *Neophilaenus campestris*) and sharpshooters (*Cicadella viridis* and *Graphocephala fennahi*) during 6 h EPG-recordings (small letters indicate statistically significant differences between species according to Tukey's HSD test for pairwise comparisons;  $N = 30$ ) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

### 3.5.5 | Low-frequency xylem ingestion (LF)

No significant differences in the trend of the duration of LF (LF-WDI) (LME:  $F_{2,711} = 0.5, p > 0.05$ ) and their number (LF-NWEI) (LME:  $F_{2,711} = 0.2, p > 0.05$ ) were observed among the species over the recorded period of 6 h. However, due to the different levels of these trends, spittlebugs (PS: 61.2 min, NC: 53.9 min) spent significantly longer time in LF compared to sharpshooters (CV: 22.3 min, GF: 21.5 min) (Figure 5, top). LF-NWEI was significantly more numerous in PS (12.4) compared to CV (5.2) and GF (4.1) while none of the species differed significantly from NC (7.6) (Figure 5, bottom). No sex-related differences in LF-WDI (LM:  $F_{4,115} = 0.8, p > 0.05$ ) and LF-NWEI (LM:  $F_{4,115} = 0.1, p > 0.05$ ) were evident. No sex- or species-related differences in LF-WDEImd were evident (LM:  $F_{4,81} = 1.5, p > 0.05$ ).

### 3.5.6 | Total xylem ingestion (Xi+LF)

The trend of total xylem ingestion duration per individual (Xi+LF-WDI) in PS over the 6 h EPG (Figure 4, bottom) significantly differed from the tendencies observed in the other three xylem-feeders, and was overall similar to Xi-WDI (Figure 4, top). The shortest Xi+LF-WDI was observed in NC (202.2 min), which was significantly lower than both GF (260.7 min) and PS (281.7 min). For NC, Xi+LF-WDI was significantly higher in females (271.5 min) than in males (128.8 min). The number of ingestion events (Xi+LF-NWEI) was significantly lower in NC (19.3) compared to the other species (PS: 38.9, CV: 31.4, GF: 30.6) and was comparable among the sexes (LM:  $F_{4,115} = 1.7, p > 0.05$ ).

### 3.5.7 | Behaviour putatively associated with *X. fastidiosa* inoculation (Xe)

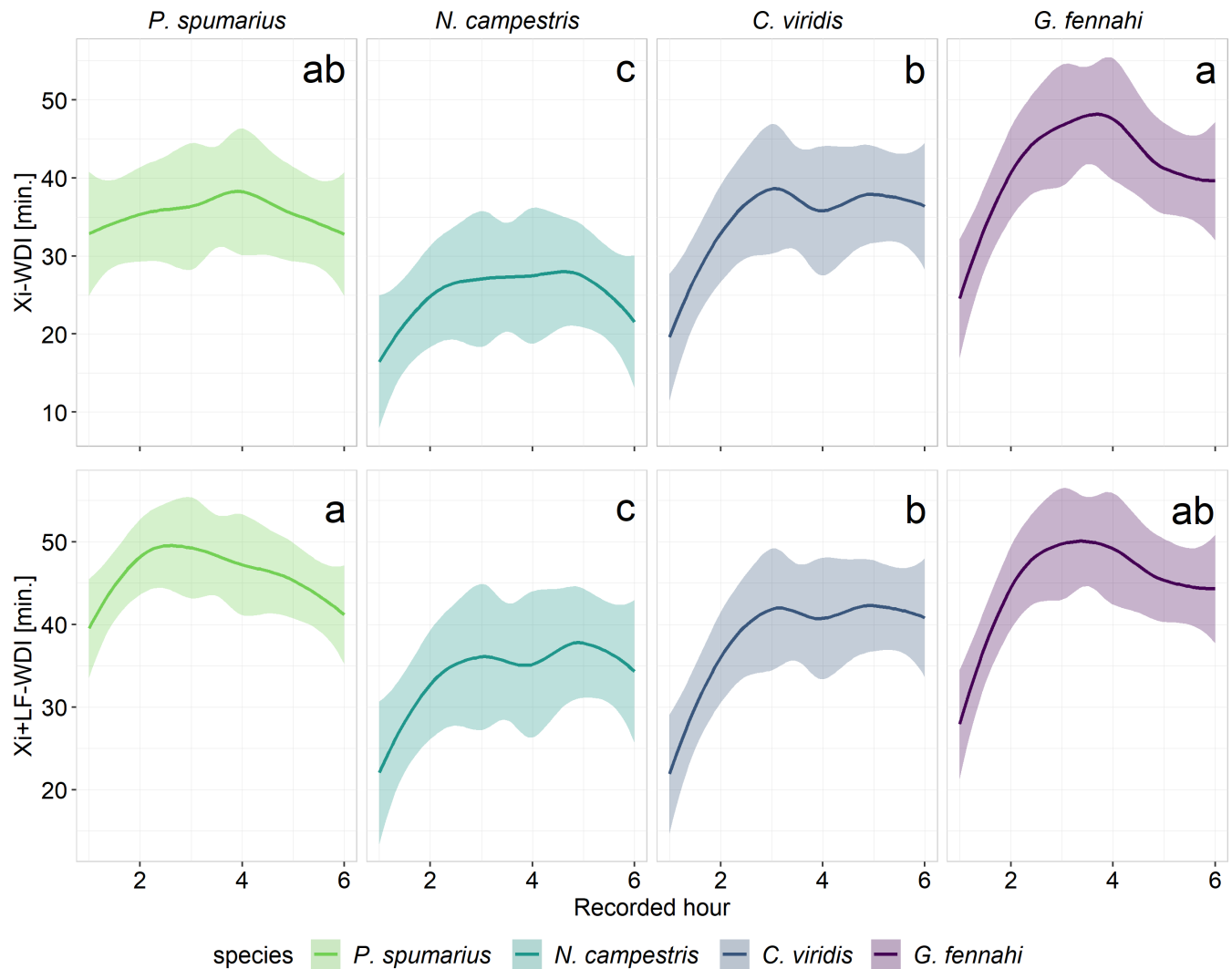
A waveform similar to that described for PS on oleander and olive by Cornara et al. (2020) (Xe, spikelet bursts) (Figure 1i) was observed in 12 CV (9 females and 3 males), 2 female GF, 2 NC (1 male and 1 female) and 3 PS (1 female and 2 males).

### 3.5.8 | Non-pathway interruption of the xylem activity (N)

Overall N-WDI and N-NWEI tended to decrease during the recording period in the four species. The total duration of N (N-WDI) was significantly lower in NC (1.5 min) compared to CV (4.9 min), GF (4.1 min) and PS (3.5 min), and in males compared to females (LM:  $F_{4,115} = 2.9, p > 0.003$ ), possibly as a reflection of the shorter time spent by the former species in xylem ingestion. Independently of sex, CV performed significantly more N-NWEI (27.5) than the two spittlebugs (NC: 6.0, PS: 15.9). Furthermore, NC showed significantly fewer events than GF (18.6) and PS. CV presented the significantly shortest N-WDEImd (8.4 s), while NC (15.6 s) was the longest.

### 3.5.9 | Resting (R)

In PS, R-WDI tended to increase over the EPG recording period (Figure 6). The overall duration of the resting behaviour was significantly longer in PS (49.7 min) compared to the other xylem-feeders



**FIGURE 4** Trends of total waveform duration per individual of xylem ingestion (Xi-WDI, top) and xylem ingestion + low-frequency xylem ingestion (Xi+LF-WDI, bottom) observed in spittlebugs (*Philaenus spumarius* and *Neophilaenus campestris*) and sharpshooters (*Cicadella viridis* and *Graphocephala fennahi*) during 6 h EPG-recordings (small letters indicate statistically significant differences between species according to Tukey's HSD test for pairwise comparisons;  $N = 30$ ) [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/terms-and-conditions)]

(NC: 8.6 min, CV: 19.2 min, GF: 15.3 min), with no sex-related differences (LM:  $F_{4,115} = 1.2$ ,  $p > 0.05$ ). Neither R-NWEI (LM:  $F_{4,115} = 0.5$ ,  $p > 0.05$ ) nor R-WDEImd (LM:  $F_{4,66} = 1.2$ ,  $p > 0.05$ ) did diverge among species or sexes.

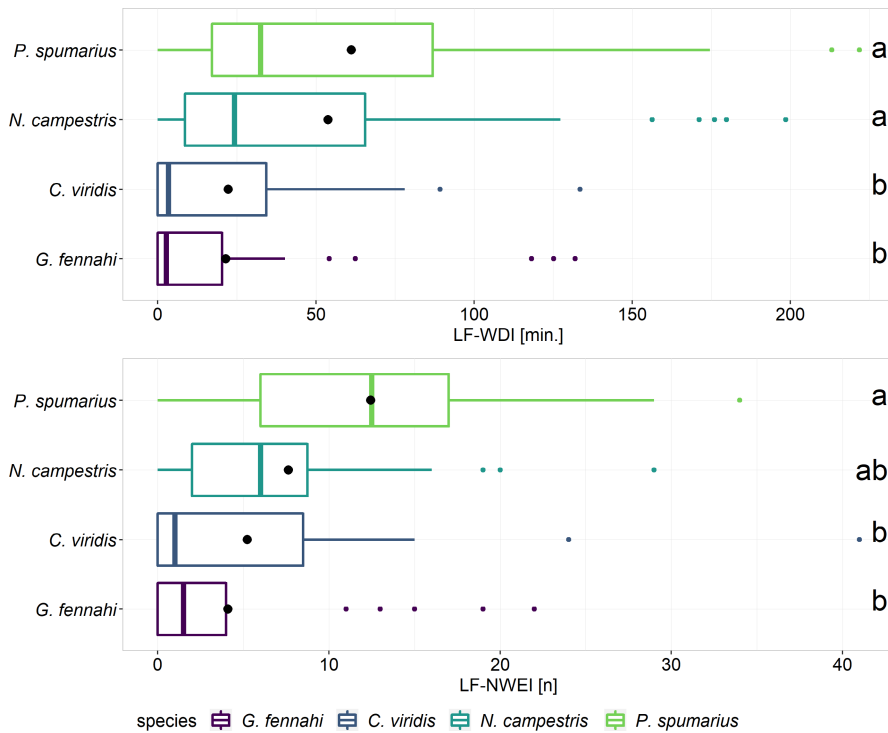
### 3.5.10 | Stylets withdrawal (W)

There was no species-related difference in the stylets withdrawal hourly trend either in terms WDI (LME:  $F_{2,711} = 0.5$ ,  $p > 0.05$ ) or NWEI (LME:  $F_{2,711} = 0.2$ ,  $p > 0.05$ ). Similarly, the overall duration of the stylet withdrawal (W-WDI) was not significantly different among the four species (PS: 39.6 s, NC: 46.2 s, CV: 46.8 s and GF: 118.8 s). On the contrary, W-NWEI was significantly higher in CV (3.7) than in the spittlebugs (NC: 1.6, PS: 1.3), and in GF (4.4) compared to PS. W-WDEImd was significantly lower in CV (7.2 s) compared to spittlebugs (NC: 31.2 s.; PS: 29.4).

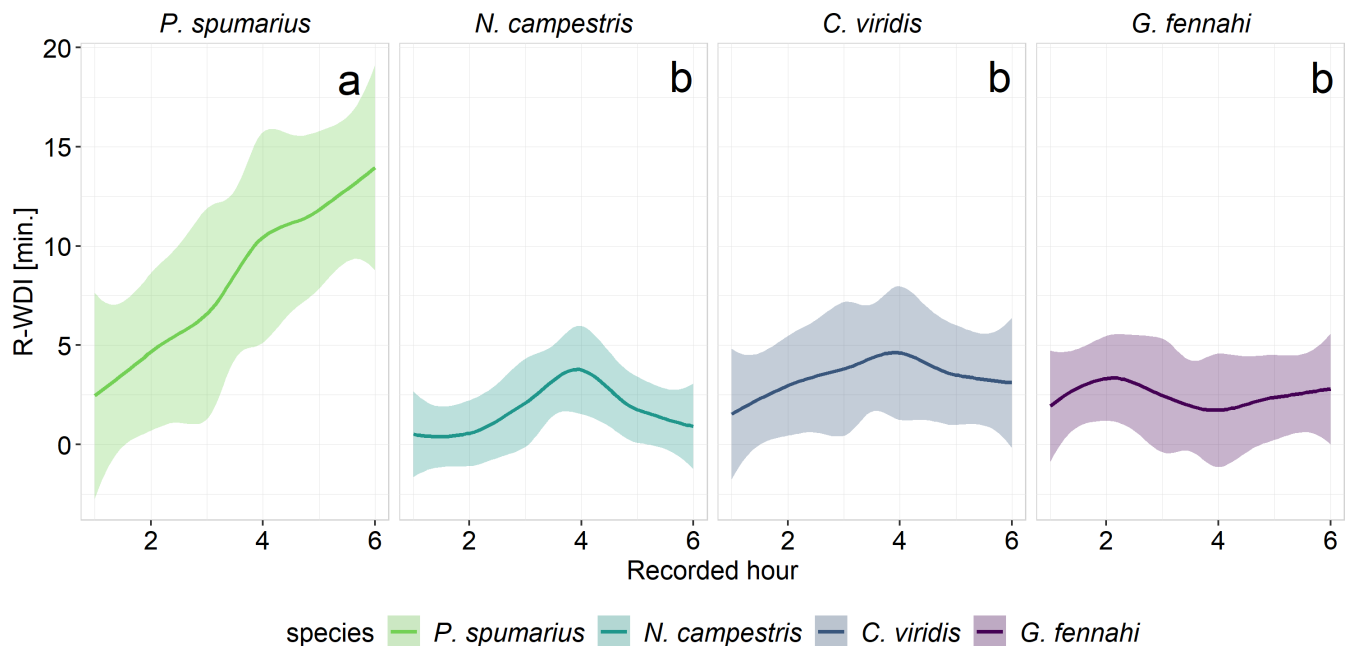
## 4 | DISCUSSION

Knowledge of the ecology and behaviour of European vectors of *X. fastidiosa* and the epidemiology of the bacterium considerably lags behind other lines of research developed since the first finding of the quarantine organism in olive trees in the Apulia Region, Southern Italy (Saponari et al., 2018, 2019). One of the main limitations to European research was the tendency to directly transfer the vast knowledge generated through decades of research on American sharpshooter leafhoppers to the meadow spittlebug, the only epidemiologically relevant vector described in the Palearctic so far (Bodino, Cavalieri, Dongiovanni, et al., 2021; Cornara et al., 2016). In the present work, the feeding behaviour on grapevine recorded with a DC-EPG device of two spittlebug and two sharpshooter species widespread in Europe, candidate or ascertained vectors of *X. fastidiosa*, was characterized and compared. Previous research (Cornara et al., 2020; Ranieri et al., 2020; Sandanayaka et al., 2013), together with data presented here, highlight





**FIGURE 5** Total waveform duration per individual of low-frequency xylem ingestion (LF-WDI, top) and total number of waveform events per individual of low-frequency xylem ingestion (LF-NWEI, bottom) observed in spittlebugs (*Philaenus spumarius* and *Neophilaenus campestris*) and sharpshooters (*Cicadella viridis* and *Graphocephala fennahi*) during 6 h EPG-recordings (small letters indicate statistically significant differences between species according to Tukey's HSD test for pairwise comparisons;  $N = 30$ ) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIGURE 6** Trend of waveform duration per individual of resting (R-WDI) in spittlebugs (*Philaenus spumarius* and *Neophilaenus campestris*) and sharpshooters (*Cicadella viridis* and *Graphocephala fennahi*) over the 6 h EPG-recordings (small letters indicate statistically significant differences between species according to Tukey's HSD test for pairwise comparisons;  $N = 30$ ) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

taxa-related feeding behavioural differences that might possibly impact the tri-partite interaction insect vector-host plant-bacterium.

*Philaenus spumarius* and *G. fennahi* tended to more readily contact xylem vessels and begin xylem sap ingestion than the other xylem-feeders tested. In addition, the same two species showed the longest duration of xylem ingestion, both in terms of Xi and Xi+LF. For *P. spumarius*, the rapid stylets insertion and onset of a long feeding activity are possible indicators of high host suitability.

Data from field surveys conducted in European vineyards reported the meadow spittlebug as the most abundant xylem-feeder present on grapevine canopies (Markheiser et al., 2018; Xf-ACTORS vector working group, 2020). In contrast, *G. fennahi* was collected on grapevine only in private gardens, where grapes were surrounded by ornamental plants such as *Rhododendron* sp. (their main breeding host), and never in commercial vineyards (Markheiser et al., 2018; Sergel, 1987; Xf-ACTORS vector working group, 2020).

The analysis of the temporal trend of xylem ingestion during the recording period could help illuminate this apparent discrepancy between feeding behavioural data and records on insect abundance in the host plant. Indeed, in the meadow spittlebug, xylem sap ingestion (both Xi and Xi+LF) is sustained from the very beginning of insect-plant contact and remains relatively steady over the recording. In contrast, in *G. fennahi*, similarly to *C. viridis*, xylem sap ingestion was lower than in the meadow spittlebug at the beginning of the recording, and sharply increased with access time. These trends could reflect host acceptability, with grapevine being a very suitable host for *P. spumarius*, while suitability could be relatively lower to both the sharpshooter species that were forced to feed on grapevine. Therefore, our EPG data coupled with results from field surveys (Markheiser et al., 2018; Sergel, 1987; Xf-ACTORS vector working group, 2020) suggest grapevine could serve as an alternative transient host for *G. fennahi* and *C. viridis* in vineyards in close proximity to their preferred breeding habitats (ornamentals for the former, humid areas for the latter). Grapevine could be a very suitable host for *P. spumarius*, although intensive management practices adopted in commercial vineyards may have a detrimental impact on the species abundance within this agro-ecosystem (Sanna et al., 2021; Santoemma et al., 2019).

Considering *N. campestris*, *X. fastidiosa* transmission to olive is considered negligible due to its only occasional presence on olive canopies (Bodino et al., 2020; Morente et al., 2018) and low preference for this plant (Cornara et al., 2021). On the contrary, the spittlebug has been consistently collected on vine shoots (Markheiser et al., 2018). Even if *N. campestris* displayed the shortest xylem sap ingestion among the species tested in the present study, its possible role in the epidemiology of *X. fastidiosa* in commercial vineyards should not be ruled out and deserves dedicated investigations.

We also observed differences between the two taxa with regard to the probing frequency. Sharpshooters tended to perform more probes and contact xylem vessels (Xc) more often than spittlebugs. This trend could be related to differences in host recognition and stylets insertion: spittlebugs need to grab a rounded surface with the forelegs and pull while pushing the stylets through the plant (Cornara, Ripamonti, et al., 2019), while sharpshooters have been suggested to probe even on flat surfaces (Joost et al., 2006; Killiny & Almeida, 2009). Therefore, stylets insertion, withdrawal and reinsertion could be performed more easily in the latter group, which could explain the more frequent probes observed in our study. On the other hand, more frequent probes could reflect distress and low acceptability of the host plant. Regardless of the reason underlying the phenomenon, more frequent probing could be associated with a higher *X. fastidiosa* transmission efficiency, considering that: (i) acquisition depends on the probability the stylets contact a xylem vessel colonized by the bacterium and (ii) the higher the probing frequency, the greater the chances the inoculation behaviour might be performed (Cornara et al., 2020; Daugherty & Almeida, 2009; Jackson et al., 2008). With regards to the Xe waveform, that is, the behaviour suggested to be associated with *X. fastidiosa* inoculation

in spittlebugs (Cornara et al., 2020), the pattern was displayed more frequently (12/30 individuals) by the sharpshooter *C. viridis* compared to the other species tested. Even if Bodino et al. (2019) observed in case of *C. viridis* a low transmission efficiency of *X. fastidiosa* subsp. *pauca* (ST53) to olive, the transmission ability of *X. fastidiosa* subsp. *fastidiosa* to grapevine was never experimentally investigated for this insect species. *C. viridis* is usually abundant in humid areas and temperate regions (Bodino, Cavalieri, Dongiovanni, et al. 2021; Hasbroucq et al., 2020; Markheiser et al., 2020; Pavan & Gambon, 2004); its host plants are generally monocots (mainly *Polygonum* sp., *Phragmites* sp., *Arundo* sp., *Juncus* spp., *Carex* spp. and Cyperaceae), not known hosts for any *X. fastidiosa* isolate (Cornara, Morente, et al., 2019; Delbianco et al., 2021; Kunz et al., 2010). However, *C. viridis* females oviposit on a variety of host plants, including grapevine (Cornara, Morente, et al., 2019). Overall, our results on *C. viridis* feeding behaviour on grapevine and high frequency of the putative inoculation behaviour Xe outline the importance of carefully addressing the sharpshooter epidemiological role in European and Asian outbreaks of *X. fastidiosa* strains causing Pierce's disease (EPPO, 2019; Olmo et al., 2021).

Besides the probing frequency and the occurrence of the Xe waveform, one of the most intriguing difference between spittlebugs and sharpshooters is the time spent in low-frequency xylem ingestion (LF), namely xylem sap ingestion occurring at a frequency lower than 0.1 Hz. We propose to mark as LF a behavioural pattern characterized by scattered contractions (one complete sequence of contraction and release of the cibarial pump permitting the insect to uptake xylem sap and swallow), or sequences of one to ten contractions, interspersed with a flat signal, during which the muscles are apparently motionless. In previous DC-EPG studies on spittlebugs, this pattern was marked as either Xi or R (Cornara et al., 2018; Markheiser et al., 2020), although it represented one of the major difficulties encountered by researchers when analysing the recordings from EPG-assisted studies on the meadow spittlebug (personal observation). Beside this practical reason affecting the transferability of the data, separating "low" and "high" frequency ingestion (LF and Xi) may provide relevant biological information about xylem-feeding species. Overall, spittlebugs on grapevine ingested xylem sap at "low-frequency" for far longer and more frequently than sharpshooters. It is tempting to point to the anatomy of the cibarium and the cibarial muscle as the key factor underlying the differences in feeding behaviour. Spittlebugs are characterized by a very prominent cibarium, hosting cibarial dilator muscles (CDM) so strong that they are supported by an additional chitinous plate on the vertex (Bergman et al., 2021; Ossianilsson, 1981). Continuous contraction and release of the CDM at a "high" constant rhythm in spittlebugs could be extremely energy-demanding, and should therefore be followed by a gradual slowdown and interspersed with periods of inactivity (resting). Such difference in the dynamic of xylem sap ingestion might explain why sharpshooters, at least the Nearctic species tested, were reported to host within their foregut larger *X. fastidiosa* populations compared to the meadow spittlebug (Bodino, Cavalieri, Pegoraro,

et al. 2021; Cornara et al., 2016; Ranieri et al., 2020). Indeed, at a theoretical level, the slower a muscle contract, the greater the internal tension, thus the greater the force it can generate (Malone et al., 1999; Sutton & Burrows, 2018). Therefore, longer periods with CDM contracting occasionally rather than constantly would expose bacterial colonies on the cuticle lining the vector foregut to longer intervals characterized by stronger turbulences, capable of dislodging the cells that would end up being ingested. Our hypothesis about the role of the frequency of CDM contraction and fluid dynamics in shaping *X. fastidiosa* within the vector's foregut is based so far only on a comparison among species for which *X. fastidiosa* transmission competence was never experimentally demonstrated, and on a single host. Further evidence could be gathered through dedicated studies with infective vectors. Moreover, additional studies are needed to characterize the relationship between the physiology and metabolic profile of the xylem sap and the ingestion dynamic.

Research on European xylem-feeders has accelerated considerably in recent years given their role as vectors of *X. fastidiosa*. However, given that feeding behaviour is a pivotal aspect of transmission for every vector-borne plant pathogen, we discussed our results considering their reflection on *X. fastidiosa*-vector relationship and bacterium epidemiology. Our data, and the hypothesis our data raise, constitute an important step forward for research on vectors of *X. fastidiosa*, and on the transmission biology and ecology in the different pathosystems present throughout Europe.

#### AUTHOR CONTRIBUTIONS

Anna Markheiser: Conceptualization, Formal analysis, Methodology, Software, Validation, Visualization, Writing—original draft. Giacomo Santoemma: Formal analyses, Writing—review and editing. Alberto Fereres: Validation, Writing: review and editing, Funding acquisition. Michael Maixner: Formal analyses, Software, Validation, Writing: review and editing, Funding acquisition. Daniele Cornara: Conceptualization, Formal analysis, Methodology, Software, Validation, Visualization, Writing—original draft, Funding acquisition.

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#### CONFLICT OF INTEREST

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript. We certify that the submission is original work and is not under review at any other publication.

#### DATA AVAILABILITY STATEMENT

Additional information and the raw data summary have been uploaded as Supplementary Materials 1–3. The software “XylFeed” can be downloaded at <https://doi.org/10.5073/20221107-091816>, while the raw dataset supporting the results can be accessed at <https://doi.org/10.5073/20221107-084158>.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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