

Feeding behavior in relation to spittlebug transmission of *Xylella fastidiosa*

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Abstract

Here we provide the first insights into the transmission dynamics of the bacterium *Xylella fastidiosa* by the meadow spittlebug *Philaenus spumarius*, gathered through DC-EPG (Electrical Penetration Graph)-assisted transmission tests and comparative observations of the probing and feeding behavior of infective versus non-infective vectors on healthy olive plants. Bacterial cells binding to *P. spumarius*' foregut occurred at a very low rate and in a time as short as 15 minutes spent by the insect in xylem ingestion or activities interspersed with xylem ingestion (interruption during xylem ingestion and resting). *P. spumarius* inoculation of bacterial cells into the xylem was exclusively associated with an early (ca. 2 to 7 minutes after the onset of the first probe) and occasional behavior, provisionally termed waveform Xe, presumably related to egestion regulated by pre-cibarial valve fluttering. Infective spittlebugs compared to non-infective ones exhibited: i) longer non-probing and shorter xylem ingestion; ii) longer duration of single non-probing events; iii) fewer sustained ingestions (ingestion longer than 10min) and interruptions of xylem activity (N); iv) longer time required to perform the first probe. These observations suggest difficulties in feeding of infective *P. spumarius* probably caused by the presence of *X. fastidiosa* within the foregut. Overall, our data indicate that likely short-time -few minutes- is required for *X. fastidiosa* transmission by *P. spumarius*, thus vector control strategies should aim at preventing spittlebug access to the host plant. Furthermore, our findings represent an important contribution for further research on the disruption of spittlebug-bacterium interactions.

Keywords: *Philaenus spumarius*; EPG; olive; oleander; transmission dynamic; fastidious bacterium

Key message

- Here we provide the first insights into the transmission dynamics of the bacterium *Xylella fastidiosa* by its main European vector, the spittlebug *Philaenus spumarius*.
- Acquisition occurs at a very low rate during the first minutes the insect is ingesting the xylem sap. Inoculation is likely related to an occasional behavior that occurs early in the probe, possibly egestion regulated by pre-cibarial valve fluttering. Infective spittlebugs exhibited feeding difficulties possibly caused by the presence of the bacterium within the foregut.
- Given the short-time required for *X. fastidiosa* transmission, vector control strategies should aim at preventing spittlebug access to the host plant.

Introduction

Since the first report of a grapevine disease (Pierce 1892) later found to be caused by a vector-borne microorganism successively identified as a bacterium (Davis et al. 1978), named *Xylella fastidiosa* (Wells et al. 1987), research has clarified many aspects of bacterium-vector-host plant interactions. Nevertheless, an essential question still remains unanswered: what are the vector behaviors necessary for bacterial transmission to plants (Almeida 2016)? *X. fastidiosa* is a xylem-limited bacterium, whose natural spread relies on insects specialized for feeding on xylem sap (Houston et al. 1947; Frazier 1965). Therefore, it is assumed the vector should access xylem vessels to acquire the bacterium as well as to inoculate it (Houston et al. 1947); however, the exact behaviors, or sequence of behaviors resulting in transmission are unknown. Vector acquisition efficiency is a direct function of vector access period to the source plant, and of the bacterial population inside the infected tissue (Purcell and Finlay 1979; Hill and Purcell 1997). Following acquisition, *X. fastidiosa* cells bind to the vector foregut, putatively to the portion of the precibarium proximal to the cibarium, behind the precibarial valve (Almeida and Purcell 2006). The bacterium persists in its vectors during the entire insect life span but is shed with molting (Purcell and Finlay 1979; Purcell et al. 1979; Almeida and Purcell 2006). The loss of vector infectiousness with molting suggests that the foregut is the essential retention site of *X. fastidiosa*. Given the heterogeneous distribution of *X. fastidiosa* in the host plant, and the turbulent rapid flow of xylem-sap into the vector foregut upon uptake, bacterial cells' binding to the foregut is thought to be a rare event, with the majority of the bacterial cells swallowed rather than retained (Retchless et al. 2014). *X. fastidiosa* inoculation positively correlates with the access period to the recipient plant (Hill and Purcell 1995; Almeida and Purcell 2003), the number of infective vectors on the host plant (Daugherty and Almeida 2009), and the number of probes performed by the single infective vector (Jackson et al. 2008). Bacterial inoculation can occur as soon as one hour after acquisition, thus bacterial multiplication and biofilm formation are not required (Purcell and Finlay 1979). Backus et al. (2009) proposed that vectors introduce *X. fastidiosa* into plants through a mechanism defined as "salivation-ingestion-egestion": once stylets reach a xylem vessel, the insect might ingest a mixture of saliva (previously secreted during the formation of the salivary sheath) and xylem sap that is swished through the pre-cibarium and sensed by the pre-cibarial sensilla. This process could lead to an enzymatic (saliva) and mechanical (fluid turbulence) detachment of *X. fastidiosa* cells within the foregut. These loosened cells could be inoculated into the xylem vessel through egestion, the putative active expulsion of fluid from the food canal (Ramirez et al. 2008; Backus et al. 2012; Backus 2016). Although indirect evidences support this theory, a final correlation between the occurrence of such sequence of behaviors and *X. fastidiosa* inoculation to a recipient plant is missing (Almeida 2016). Identifying the inoculation mechanism of a plant pathogen by its vector involves the real-time observation of the probing behavior of an infective insect given access to a healthy plant; the

probe should be terminated when the putative inoculation behavior is performed (Backus 2016). The EPG (Electrical Penetration Graph) is a technique that permits the real-time monitoring of hemipterans' probing and feeding behavior (McLean and Kinsey 1964; Tjallingii 1978; Backus and Bennett 2009); the use of the EPG has been crucial in determining the behaviors associated with acquisition and inoculation of several vector-borne plant pathogens (Prado and Tjallingii 1994; Martin et al. 1997; Moreno et al. 2012; Antolinez et al. 2017; Jimenez et al. 2018). However, similar studies on *X. fastidiosa* have failed because of the very low inoculation efficiency per individual vector and per probe (Backus 2016). Regarding the *X. fastidiosa*-vector relationship, the idea of the insect as a mere carrier of the bacterium has been recently challenged by the finding of bacterial exploitation of vector cuticle as carbon source, with possible detrimental effects for the insect (Killiny et al. 2010; Labroussaa et al. 2017). However, to the best of our knowledge, no qualitative or quantitative data on bacterial-mediated effects on the behavior of infective vectors have been produced so far. Such effects of the bacterium on its vectors may have direct consequences on the epidemiology of *X. fastidiosa*-related diseases. Most of the background on *X. fastidiosa* transmission dynamics and bacterium-vector interactions exposed above come from studies on the *X. fastidiosa*-grapevine-sharpshooters pathosystem in California (USA) (Rapicavoli et al. 2018). However, vectors other than sharpshooters, i.e. spittlebugs, seem to play the key role in *X. fastidiosa* spread in Europe (Cornara et al. 2018a; Cornara et al. 2019). Indeed, the meadow spittlebug *Philaenus spumarius* L. (1758) (Hemiptera: Aphrophoridae) has been proven to be the main vector of *X. fastidiosa* to olive in South Italy, and is likely involved in bacterial spread in all the European outbreaks reported so far (Saponari et al. 2014; Cornara et al. 2017a; Cornara et al. 2017b; Cruaud et al. 2018; Morente et al. 2018; Cornara et al. 2019). *P. spumarius* has some different features with respect to its relationship with *X. fastidiosa* compared to sharpshooters. These differences may relate to spittlebug feeding behavior and the dynamics of fluids within the foregut (Cornara et al. 2016; Cornara et al. 2018b; Sicard et al. 2018; Ranieri et al. 2019). Such differences might have major implications on the spittlebug-mediated transmission of the bacterium that could so far differ in some extent to what has been described for sharpshooters. Therefore, *X. fastidiosa* transmission dynamics by *P. spumarius* and spittlebug-bacterium interactions must be investigated in detail. We began to explore the transmission dynamic of *X. fastidiosa* by *P. spumarius* by using EPG in experiments to study the relationship of vector feeding behavior to transmission. We addressed three main questions: i) what is the behavior/sequence of behaviors leading to bacterium acquisition by the meadow spittlebug?; ii) what is the *P. spumarius* behavior/sequence of behaviors leading to bacterium inoculation to the host plant?; iii) are there any differences in probing and feeding behavior between infective and non-infective *P. spumarius*? The data presented here constitute an essential step for research on spittlebugs transmission of *X. fastidiosa*.

Materials and methods

Collection and rearing of *Philaenus spumarius*

Philaenus spumarius individuals used to study the acquisition behavior and for comparison of the feeding behavior of infective versus non-infective spittlebugs were collected at the nymphal stage with a fine-tip brush on ground-vegetation in a *X. fastidiosa*-free olive orchard in Apulia region (Southern Italy) on March 2018. The nymphs were reared until adulthood on a mix of different suitable plant species (*Conyza* sp., alfalfa (*Medicago sativa*), oat (*Avena* sp.) sp., vetch (*Vicia sativa*)) inside a cage (2x1x1m) placed beneath an olive tree inside an experimental field in the premises of the Campus of the University of Bari (Apulia region, Southern Italy). Three to four weeks after emergence, adult spittlebugs (males and females) were collected using a mouth aspirator, transferred to a plastic aerated empty cylinder, and moved to an indoor facility located in the *X. fastidiosa* infected area (Racale (LE), South Italy) where the transmission experiments took place. Before the experiments, the spittlebugs were pre-screened for *X. fastidiosa* by caging them on periwinkle (*Catharanthus roseus*) plants, in groups of five per plant, for an IAP of four to seven days, inside an insect-proof air-conditioned chamber (25±2°C, 40% HR). The pre-screened periwinkles, tested for *X. fastidiosa* ca. 50 days after the IAP by qPCR (following the protocol by Loconsole et al. (2014)), were negative for the bacterium.

To assess the inoculation behavior, *P. spumarius* adults (males and females) were collected in the *X. fastidiosa* infected area (Salve (LE), Apulia region). Briefly, in August 2017 insects were collected by sweep net and mouth aspirator in an olive grove with a high disease prevalence (ca. 80% of the olives exhibiting clear symptoms of Olive Quick Decline Syndrome caused by *X. fastidiosa*), mainly on the bordering trees and shrubs (oak (*Quercus ilex*), lentisk (*Pistacia lentiscus*), persimmon (*Diospyros kaki*), pomegranate (*Punica granatum*), and cypress (*Cupressus sempervirens*)). The spittlebugs were then caged in groups of six per plant on 15-days old vetch plants inside an insect-proof air-conditioned chamber (25±2°C, 40% HR) until the transmission experiments were performed. In 2018, insects were collected in the same olive grove but during the months of June and July, and on olive plants (*Olea europaea*) in addition to the plants described above for the 2017 collection.

Experimental plants

Seedlings of *Conyza* sp., alfalfa, oat, and vetch were used to rear the juveniles, while plants of vetch were used to maintain the adult spittlebugs until the EPG experiments. Source plants for the acquisition of the bacterium (*X. fastidiosa* subsp. *pauca*, ST53) consisted of olive seedlings infected in 2014 (transmission experiments details described by Cornara et al. 2017b). The recipient plants used for the inoculation varied according to the EPG experiment and consisted

of (i) two-year old olive seedlings (20-30 cm height); (ii) 4-month old self-rooted oleander plants, and (iii) 3-month old periwinkle plants. The plants used for the EPG-assisted transmission experiments were grown in soil, sand and vermiculite (6:3:2).

***Philaenus spumarius* probing and feeding behavior: EPG waveforms**

The probing and feeding behavior of *P. spumarius* has been characterized through a combination of EPG, video-assisted observations and micro-computed tomography (Cornara et al. 2018b). Five distinct main EPG waveforms were described in that study, with each waveform corresponding to a different behavior during the probe: C (pathway waveform, corresponding to stylets penetration activities during the pathway phase, salivation and build-up of the salivary sheath, and tissue exploration while stylets move toward the xylem vessels); Xc (xylem contact/pre-ingestion, representing the first contact with a xylem vessel and possible pre-ingestion or trial ingestion); Xi (active xylem sap ingestion); R (a resting phase alternated with xylem ingestion); N (brief interruption during the xylem phase, either Xc or Xi, of unknown biological meaning. Waveform N is not considered a “proper” interruption of xylem activities, i.e. in case of occurrence of N, the activities preceding and following N are not calculated as separated xylem contact or ingestion bouts/events (Cornara et al. 2018b)). These patterns are always (or almost always) displayed during all *P. spumarius* probes, as observed on olive, grapevine, vetch and other plants (Cornara et al. 2018b; Markhaiser et al. 2019). Other occasional patterns not previously described represent exceptions to these stereotypically repeated events. Therefore in this work, for the nomenclature of the waveforms (thus the behavioral patterns), we adopted the one used by Cornara et al. (2018b) specifically for *P. spumarius*. Main waveforms produced by *P. spumarius* are reported in Fig. 1.

Acquisition behavior

After pre-screening, non-infective adult spittlebugs were transferred to two weeks old vetch plants (non-host of the ST53 strain used in this experiment), in groups of ten per plant, inside an insect-proof air conditioned chamber (same conditions described for pre-screening) until the EPG-assisted AAP (Acquisition Access Period) (one to 14 days). The probing and feeding behavior of pre-screened insects on *X. fastidiosa* olive source plants was monitored through EPG, in order to identify the behavior(s) associated with bacterial acquisition. There were four treatments, with at least 30 replicates per treatment: interruption of the probe during pathway (C); interruption of the probe during xylem contact (Xc); one hour AAP on an olive infected plant; three hours of AAP on an olive infected plant (Tab. 1). *X. fastidiosa* source plants for the EPG-assisted AAP were three infected olives showing approximately the same vegetative conditions. The plants were trimmed at 30 cm of height one week prior to the

beginning of the experiment, and pruned leaving non symptomatic green lateral shoots more suitable for meadow spittlebug settling and probing (Cornara et al. 2018a). For each of the infected-source plants, we selected a middle non-symptomatic shoot; one week before the EPG, half of the leaves of the selected shoots were tested by qPCR (following the protocol by Loconsole et al. (2014)) and found positive to the bacterium. Additionally, at the end of the acquisition experiment, the shoots offered to the spittlebugs were re-tested by qPCR (pooling together the leaves and the stems); all the tissues selected for the acquisition tests showed similar bacterial population (ranging from $8.34\text{E}+04$ to $3.38\text{E}+05$ CFU/ml). We EPG-recorded three spittlebugs per time, each on one EPG-channel and on one source plant, and all the three subjected to the same treatment (interruption of the probe during the waveform C, or Xc, or after 1h or 3h). Following the EPG-assisted AAP, each spittlebug was gently removed from the infected-source plant with a paint brush, and caged on a non-infected periwinkle plant for an IAP of 96 hours, inside an insect proof air-conditioned chamber ($25\pm 2^\circ\text{C}$, 40% HR). At the end of the IAP, the insects were collected and stored in ETOH 70% at -20°C ; the receptor plants were maintained in an insect proof air-conditioned chamber at $27\pm 2^\circ\text{C}$ 40% HR, and watered twice a week. Insect infectivity and periwinkle infection status (the latter assessed ca. 40 to 60 days after the IAP) were tested by qPCR, following the protocols by Harper et al. (2010) and Loconsole et al. (2014). We considered acquisition as having occurred if at least one of the two samples per replicate (the insect and the receptor plant) tested positive by qPCR.

Inoculation behavior

The spittlebugs were given a seven to ten-day AAP on five infected olive seedlings inside a Bugdorm-2 Insect tent (<https://shop.bugdorm.com>). Following the AAP on the infected olive plants, the spittlebugs were moved to healthy vetch plants until the EPG-assisted-inoculation tests began (the spittlebugs remained caged on the healthy vetch plants approximately from two to 20 days after the AAP on infected olive plants). After tethering and connection to the EPG probe, each *P. spumarius* was placed on a 5 cm portion of a healthy olive seedling stem, having access to at least one leaf. The probing and feeding behavior of *P. spumarius* on olive receptor plants was monitored through EPG, in order to identify the behavior(s) (EPG's waveform(s)) associated with bacterial inoculation. Each spittlebug was left probing until the occurrence of the waveform of interest; once the waveform occurred, the insect was removed from the receptor plant with a paint brush, and stored in ETOH 70% at -20°C until the assessment of its infectivity. In the 2017 EPG experiments, there were four treatments with termination of probing during different EPG waveforms: pathway (C); xylem contact (Xc); xylem ingestion (Xi; from five to 15 minutes); first interruption during xylem activity (N) (either during Xc or Xi) (Tab.2A). After each replicate, the probed olive portion (ca. 1cm) was marked

with tape; the plants were stored in an insect proof air-conditioned chamber at 27±2°C 40% HR, and watered once a week. The recipient plants were tested three months after the EPG-assisted IAP; inoculation of bacterial cells into the xylem was assessed by qPCR on either the probed part of the seedling (2cm portion, both stem and at least one leaf petiole), or a portion three to four cm distal to the former (2cm portion, both stem and at least one leaf petiole). Moreover, in 2017 we had an additional treatment: some of the insects were given a one-hour EPG-assisted IAP (insects tethered and connected to the EPG) on the receptor test plant without artificially interrupting the probe during a certain waveform (Tab. 2A). The plants were maintained at the same conditions described above for the recipient plants used for waveform interruption IAP; plant status was ascertained by qPCR three months after the IAP, testing a portion of ca. 10 cm including the five cm stem/leaves exposed to *P. spumarius* probing. The sample was not split in two (probed and distal parts) as for the waveform-interruption treatments, since the spittlebugs were allowed to make multiple probes. For the 2017 experiment, we performed 25 replicates for each waveform interruption treatment, and 30 for the 1 h IAP, which included both infective and non-infective *P. spumarius*. Tab.2A reports results only from replicates where spittlebugs were positive for *X. fastidiosa* by qPCR (with a Ct approximately ranging from 26 to 32). None of the recipient plants that were exposed to spittlebugs found to be non-infective according to qPCR results tested positive for *X. fastidiosa*.

In 2018, treatments in the inoculation tests were either interrupting the probe during the waveform of interest, or giving the spittlebug an IAP of three hours on the olive recipient plant. The waveform interruption treatments were: pathway (C); xylem contact (Xc); xylem ingestion (Xi; from five to 15 minutes), either with or without xylem activity interruptions (N); interruption during xylem activity (N); resting (R; from one to two minutes) (Tab.2B). For the 2018 inoculation experiment, there were 30 to 60 replicates for each of the waveform-interrupted treatments, and 90 replicates for the 3 h IAP. In Tab.2B we reported only the plants exposed to infective *P. spumarius* according to qPCR (with Ct values approximately ranging from 24 to 33). None of the plants exposed to non-infective spittlebugs (as determined by qPCR) tested positive for *X. fastidiosa*. In addition to olive, in 2018 we performed also EPG-assisted waveform interruption inoculation tests on 4-month old oleander plants. As shown by Cornara et al. (2017b), *X. fastidiosa* inoculation rate to oleander by *P. spumarius* is greater than to olive, despite oleander being a very poor host for the spittlebug. Furthermore, *P. spumarius* on oleander performs single or repeated unconventional and “occasional” EPG signals different from the stereotypically repeated patterns (C, Xc, Xi, R, N) far more frequently than in olive and other plants (vetch, grapevine, cherry) (Cornara et al. 2018b; Markheiser et al. 2019). These unconventional EPG patterns, occurring from seconds to few minutes after the insect has reached the xylem vessel, include a spikelet burst similar to the B1s waveform described for sharpshooters (Backus et al. 2009; Backus et al. 2005; Joost et al. 2006) (Fig.1f

and Fig.2a and b), and a voltage drop similar to N but occurring during an initial resting phase alternated with low frequency Xi (frequency $\leq$ 0.1Hz) (Fig.2c). Here we grouped these two EPG patterns under a single treatment, provisionally termed Xe. Therefore, for the waveform interruption inoculation tests on oleander, we added the treatment Xe to those described for olive (Tab.2C); Xe was not produced by spittlebugs on olive during the waveform interruption experiments carried out either during 2017 or 2018.

For the 2018 EPG-assisted inoculation tests, and insects and plant maintenance, we followed the same protocol described above for the 2017 experiments. The waveform-interruption recipient plants (both olive and oleander) were tested three months after the EPG-assisted inoculation tests; the presence of bacterial cells in the recipient plants was assessed by qPCR on either the probed part, or a portion three to four cm distal to the probed part. For the olive recipient plants where *P. spumarius* had an EPG-assisted 3-h IAP on, we tested a portion of ca. 10 cm including the 5 cm stem/leaves exposed to *P. spumarius* probing; as for the 1-h IAP of 2017, we did not split the sample in two.

For qPCR on insects and plants, we followed the protocols described by Harper et al. (2010) and Loconsole et al. (2014), respectively.

As explained above, during both 2017 and 2018, we added to the waveform-interruption experiments further treatments, namely 1h IAP (2017) and 3h IAP (2018), without interruption of the probe during specific waveforms. We decided to use relatively short IAPs for two main reasons: i) as remarked by Wayadande and Nault (1993), long feeding periods result in more switching from one behavior to another, making it difficult to know which behavior(s) is/are associated with pathogen inoculation; ii) several indirect evidences suggest that *X. fastidiosa* inoculation might occur during the initial steps of the probe (Jackson et al. 2008; Daugherty and Almeida 2009; Backus et al. 2009; Backus 2016).

Comparison of infective versus non-infective *Philaenus spumarius* probing behavior

To compare the feeding behavior of infective *versus* non-infective *Philaenus spumarius*, adult females were used. The insects were given a 10-day AAP on five infected olive seedlings inside a Bugdorm-2 Insect tent (<https://shop.bugdorm.com>). Following the AAP, the spittlebugs were moved to healthy vetch plants until the EPG-assisted IAP (from one to 7 days). After tethering and connection to the EPG, each spittlebug was given a 3h IAP on a 5 cm portion of a stem of a healthy olive seedling, having access to at least one leaf. We selected a 3-h IAP in order to be consistent with the protocol used for the 2018 inoculation behavior experiment. Following the EPG recording, each spittlebug was caged on a healthy periwinkle plant for an IAP of 96 hours, inside an insect proof air-conditioned chamber (T=25 $\pm$ 2°C, HR=40%). At the end of the IAP, the insects were collected and stored in ETOH 70% at -20°C; the plants (olives and periwinkles)

were maintained in an insect proof air-conditioned chamber at $27\pm 2^{\circ}\text{C}$ 40% HR, and watered twice a week for periwinkles, once a week for olives. Insect infectivity and plant infection status (the latter assessed ca. 50 days after the IAP for periwinkles, and three months for olives) were tested by qPCR, according to Harper et al. (2010) and Loconsole et al. (2014), respectively. For olive, we tested a portion of ca. 10 cm including the 5cm stem/leaves part exposed to *P. spumarius* probing. Each insect given the 10-days AAP on olive infected plants was considered infective if at least one of the two samples per each replicate (either the insect or the periwinkle recipient plant) tested positive for *X. fastidiosa* by qPCR.

EPG procedure and data analysis

For running the EPG tests, the insects were: 1) starved for one hour inside an aerated Petri dish; 2) slightly stunned by exposure to 4°C for ca. 30 sec.; 3) immobilized with a cased diaphragm pump (Dymax 5, Charles Austen Pumps Ltd, Byfleet, Surrey, England/UK); 4) tethered according to the protocol described by Cornara et al. (2018b). Briefly, the tip of an 18 μm -gold wire, 3 cm long, was placed on the insect pronotum, and glued with a double layer of silver conductive glue (Ted Pella, no. 16034; Pelco® Colloidal Silver, Ted Pella, Redding, CA, USA). The tip of the wire was bent in order to create a loop that enhanced the resistance of the connection. The other end of the wire had been attached previously with silver paint to a copper electrode measuring 3 cm in length $\times$ 1 mm in diameter. Thereafter, the electrode was plugged into the EPG probe, with the insect left hanging over the plant without touching it for ca. ten minutes before placing it on the plant. The soil copper electrode (10 cm long $\times$ 2 mm wide) of the EPG device was then inserted into the pot substrate. The system was assembled inside a Faraday cage, in an acclimatized room ($25 \pm 2^{\circ}\text{C}$), and under artificial light (20W, 1200 Lm (lumen)). Probing and feeding behavior was recorded with a Giga 4-DC EPG device (EPG-systems, Wageningen, The Netherlands) with 1 Giga Ohm input resistance. Output from the EPG at 100x gain was digitalized at a rate of 100 samples per sec. per channel, and recorded using Stylet+ software (EPG-systems, Wageningen, The Netherlands). Substrate voltage was adjusted following the calibration instructions of the DC EPG equipment so that EPG signals fit into the +5V to -5V window provided by the software Stylet+ (EPG-systems, Wageningen, The Netherlands). The EPG recordings were analyzed by Stylet+. For the acquisition experiment (Tab.1) and the comparison between infective and non infective *P. spumarius* (Tab.3), several sequential and non-sequential variables were calculated. The variables calculated and the abbreviations used in tables 1, 2, and 3 are described in Tab.4. EPG variables were calculated with an Excel Workbook developed purposely for *P. spumarius* waveforms by Antonio J. Alvarez (Universidad de Almeria, Spain) (Cornara et al. 2018b). Differences in probing and feeding behavior between infective and non-infective spittlebugs were assessed by Mann-

340 Whitney U-test. Statistical analysis was performed with the software R 3.5.2 (R Core Team,
341 2015).

Results

Feeding behavior associated with the acquisition/retention of *X. fastidiosa* by *P. spumarius*

Two *P. spumarius* individuals only, one given 1h AAP and the other given a 3h AAP tested positive for *X. fastidiosa* by qPCR (Ct=31.67 and 31.34) (Tab. 1); no transmission to periwinkle occurred. None of the spittlebugs whose probe was interrupted during pathway (C) or xylem contact (Xc) phases acquired and retained the bacterium. The extremely low acquisition rate did not permit any statistical inference; nevertheless, we analyzed the sequence of events and calculated non-sequential variables for the two replicates that acquired the bacterium, in order to have preliminary indications about the feeding activities associated with the acquisition and retention of *X. fastidiosa* (Tab.1). Considering the acquisition that occurred in the 1h treatment, the spittlebug performed a single probe of 31.2 min, of which 14.8 min spent in xylem ingestion and xylem interruption activities (2.3 of the 14.8 min of Xi were spent in N; 12 N waveforms were performed), and 1.2 min in resting. The spittlebugs that acquired the bacterium in the 3h treatment performed a long xylem ingestion phase (122.8 min), with a single xylem interruption, and a resting phase that lasted 0.8 min.

Feeding behavior associated with the inoculation of *X. fastidiosa* by *P. spumarius*

Considering the waveform-interruption experiments, no inoculation to olive was obtained in 2017 and in 2018 by interrupting the probes during the occurrence of the patterns C, Xc, Xi (whether or not containing from one to three interruptions N), N or R (Tab.2A and 2B). On oleander, five *P. spumarius* positive for *X. fastidiosa* to qPCR produced the pattern Xe (namely one of these five spittlebugs performed a voltage drop, and 4 spittlebugs performed each a spikelet bursts). Three of these five spittlebugs, i.e. one performing a drop and two producing spikelet bursts, inoculated *X. fastidiosa* to the receptor plant (Tab.2C). Both the probed and the distal parts of each of the inoculated oleanders were positive for the bacterium by qPCR, indicating that bacterial cells were released into the xylem. The voltage drop performed by the spittlebug that successfully inoculated the recipient plant occurred 7 minutes after the beginning of the probe, and 3 minutes after the first contact with xylem. The two spikelet bursts in the spittlebugs that inoculated the plants occurred two and two and a half minutes after the beginning of the probe, and 0.5 and 1 minutes after the xylem contact. No inoculation to oleander occurred with the other patterns tested (Tab.2C). Considering the 1-hour IAP on olive, the two spittlebugs (out of the 12 infective insects) that were able to infect the plants were the only ones that produced an Xe pattern (one voltage drop occurring 6 minutes after the onset of the probe, and 5 minutes after the contact with xylem; one spikelet burst performed 2.5 minutes after the beginning of the probe, and 2 minutes after the xylem contact). Finally, considering the 3-hours IAP, one inoculation out 49 infective *P. spumarius*

was obtained. The inoculative spittlebug was one of the only three insects (out of the 49 infective) producing Xe (a drop in R occurring 3 minutes after the onset of the probe, 1 minute after the first contact with xylem); the other two, producing spikelet bursts, did not transmit *X. fastidiosa* to the host plant.

Comparison of infective versus non-infective *Philaenus spumarius* probing behavior

We included in the analysis only clear recordings (without noise or unclear signals) performed by *P. spumarius* that: i) remained on the plant for the 3h of EPG without breaking the wire and escaping or falling off of the host; ii) were alive and active at the end of the IAP on periwinkle; iii) probed the tissue at least once during the recording. By these criteria, 49 *P. spumarius* females, 14 infective and 35 non-infective were selected for statistical analysis. Nine out of 14 *P. spumarius* positive to the bacterium by qPCR transmitted *X. fastidiosa* to the periwinkle recipient plants. Two out of the 14 infective spittlebugs inoculated the fastidious bacterium during the EPG-assisted three hours IAP to olive; the limited number of inoculations did not permit any statistical inference. Looking at the behavioral patterns displayed during the probes, the waveform Xe was performed only by the two insects that inoculated *X. fastidiosa* to olive (one spittlebug producing a drop and one a spikelet burst both occurring ca. 4 minutes after the beginning of the probe, and 3 minutes after the xylem contact). A third spittlebug performing a spikelet burst did not inoculate the bacterium. Sequential and non-sequential variables calculated for the infective and non-infective spittlebugs are reported in Tab.3. Infective *P. spumarius* spent significantly longer time in non-probing ($W=340$, $p=0.036$) and shorter time in xylem ingestion ($W=136$, $p=0.016$) activities compared to non-infective spittlebugs. Furthermore, we observed also that the average duration of the single non-probing events in infective insects was almost twice the value recorded for non-infective ones ($W=350$, $p=0.020$). Moreover, infective *P. spumarius* performed significantly fewer sustained xylem ingestion events, i.e. xylem ingestions longer than 10 minutes ($W=152.5$, $p=0.032$) and interruptions of the xylem activity (waveform N) ($W=146$, $p=0.027$) than non-infective. Finally, infective spittlebugs required longer time to perform the first probe compared to individuals not carrying the bacterium ($W=346.5$, $p=0.024$).

Discussion

The data presented here can guide further attempts to determine the vector feeding behaviors necessary for *P. spumarius* transmission of *X. fastidiosa* to plants. Our principal conclusions were that: i) spittlebug acquisition rate appeared to be extremely low, and bacterial cells binding to the foregut might occur in a time as short as 15 minutes spent by the insect performing xylem ingestion, or other activities interspersed with xylem ingestion (interruption or resting); ii) inoculation of bacterial cells into the host plant xylem by *P. spumarius* was associated with an early and very occasional waveform that we provisionally termed Xe (that occurred ca. 2 to 7 minutes after the onset of the probe). The common feeding behavioral patterns, i.e. C, Xc, Xi, N, R, that the spittlebugs stereotypically repeat during most of the probes, were not associated with bacterial cells delivery to the host plant. Our hypothesis is that Xe waveform likely represents egestion of fluids regulated by the precibarial valve fluttering following a possible lack of insect phagostimulation. However, the low inoculation rate displayed by *P. spumarius* during our experiments make it difficult to draw a definitive conclusion about the exact behavior associated with bacterial cells inoculation, and more research efforts are needed; iii) probing and feeding behavior of infective *P. spumarius* differed from the one of non-infective spittlebugs. The EPG analysis showed that infective *P. spumarius* had more difficulties than non-infective ones in feeding on a non-infected host plant.

Feeding behavior associated with acquisition/retention of *X. fastidiosa*

The interaction between two main factors makes *X. fastidiosa* acquisition and retention within the vector foregut a relatively rare event: first, the bacterium is unevenly distributed within the plant, thus for acquisition (uptake) to occur the insect should probe from one of the vessels colonized by the bacterium (Hopkins 1981; Newman et al. 2003; Cardinale et al. 2018); second, the xylem sap flows within the insect foregut at an extremely high velocity, generating turbulence, thus hindering the bacterial cells attachment (Purcell et al. 1979; Dugravot et al. 2008). Therefore, even if the insect lands on an infected plant, and probes a vessel containing *X. fastidiosa* cells, most of the cells up-taken would be swallowed without being retained in the precibarium (Retchless et al. 2014). However, it is expected that long access periods could increase the probability of vector-pathogen encounters, overall increasing the acquisition rate (Almeida 2016). Our data suggest that *X. fastidiosa* acquisition and retention by *P. spumarius* do not necessarily require very long probe, and likely occur during xyle ingestion (waveform Xi) from infected vessels; a xylem ingestion as short as 15 minutes is sufficient for successful binding. Therefore acquisition and successful retention might occur during the xylem ingestion, with cells binding during the simultaneous collapse of the cibarial diaphragm and closure of the precibarial valve sealed by the bell-like invagination (Ruschioni et al. 2019), or during activities interspersed with xylem ingestion, namely xylem interruption N (a single

interruption could be sufficient) or resting. The extremely low acquisition rate we observed for the meadow spittlebug is consistent also with previous data by Cornara et al. (2016). However, our experiments did not permit any statistical inference, or to draw conclusions about the precise behavior(s) or sequence of events leading to *X. fastidiosa* acquisition. Furthermore, the low acquisition rate could have been influenced by the relatively low bacterial population within our olive source plants, given the positive correlation between *X. fastidiosa* population within the infected plant and the transmission efficiency ((Hill and Purcell 1997). Nevertheless, high *X. fastidiosa* population lead to symptoms development, and the vectors tend to discriminate against symptomatic plants (Marucci et al. 2005; Miranda et al. 2013; Zeilinger and Daugherty 2014; Del Cid et al. 2018). Therefore, considering our scenario, consisting of infected but non-symptomatic plants bearing a bacterium population still too low to cause severe symptoms and consequent reduction of host plant attractiveness, the most epidemiologically realistic for inferences on acquisition dynamic. However, further experiments either with olive or with other host plants should be conducted to deepen our knowledge about the mechanism of acquisition of *X. fastidiosa* by *P. spumarius*, and about how and where bacterial cells do initially bind to the spittlebug foregut.

Feeding behavior associated with the inoculation of *X. fastidiosa*

X. fastidiosa cells delivery into the xylem vessels by *P. spumarius* was associated with the occurrence of a pattern that we provisionally called Xe, as demonstrated by: i) the successful inoculation to oleander plants only when the probe was interrupted in correspondence of this particular pattern; ii) the only inoculations to olive occurred during IAPs where the spittlebugs engaged in Xe; iii) the lack of inoculation with the other behavioral patterns tested either on olive or on oleander. In other words, whenever there was infection of test plants, spittlebugs always made at some point an Xe waveform on receptor test plants. The spittlebug performed this specific behavior 2 to 7 minutes after the beginning of the probe, and 0.5 to 5 minutes after the first contact with the xylem. Furthermore, in all the observed cases in olive, the pattern Xe was always preceded by waveforms C (pathway), Xc (xylem contact) and Xi (xylem ingestion activity); in oleander, Xe was preceded by the sequence of events C-Xc in two out of three inoculative probes, and by C-Xc-Xi in the other positive case. The pattern Xe was never preceded by the xylem interruption N waveform. No inoculation occurred when infective spittlebugs probe was interrupted during waveforms C, Xc, or Xi, neither in olive nor in oleander. Under the term Xe we grouped two apparently different EPG signals, a voltage drop occurring during a period where resting (R) alternates with low frequency Xi (frequency $\leq$ 0.1 Hz) (Figs.2c and f), and a “simple” spikelet burst (Fig.1 f and Figs.2a, b, d, e). The common element between the two signals is the presence of spikelet bursts (indicated with arrows in Figs. 2a, b and c), characterized by highly variable frequency (3 to 10 Hz) and amplitude (4 to

25%). During voltage drops, spikelet bursts were repetitive and no longer than 1-2 seconds, while the duration of the “simple” spikelet bursts ranged between 6 and 17 sec. This similarity suggests that the inoculation of *X. fastidiosa* cells into the plant by *P. spumarius* could be associated with the spikelet bursts occurring when the insect stylets are located in a xylem vessel, after having built the salivary sheath, penetrated through the plant tissues reaching a xylem vessel, and after a first tasting of the host plant suitability through the pre-cibarial chemosensilla. According to Joost et al. (2006) and Backus et al. (2009), spikelet burst (termed B1s in these and in further works on sharpshooters performed by Backus and colleagues) represents an insect internal activity, possibly streaming potentials (Walker 2000) caused by pre-cibarial valve movements defined as fluttering. This behavior is interspersed during the probe, and occurs frequently during the pathway phase before reaching the xylem vessel; its occurrence may therefore be associated with movements of the pre-cibarial valve during tasting of the host plant (Backus et al. 2009; Backus and McLean 1982; Backus 1985). For a thoroughly review of the different waveforms subtypes in sharpshooters refer to Backus (Backus 2016). As shown in our experiment, at least for *P. spumarius*, the occurrence of spikelet bursts when the insect stylets are located in a xylem vessel, thus putative pre-cibarial valve fluttering within the xylem vessel pushing bacterial cells out of the food canal possibly helped by the tension of the xylem fluid while the insect is feeding, may lead to *X. fastidiosa* inoculation. Pre-cibarial valve involvement in *X. fastidiosa* inoculation has also been proposed by other authors (Purcell et al. 1979; Almeida and Purcell 2006). Spikelet bursts are also major components of the X-waveform found to be associated with the inoculation of the Maize Chlorotic Dwarf Virus (MCDV, Waikavirus), a semi-persistent virus sharing with *X. fastidiosa* the characteristic of being foregut-borne ((Childress and Harris 1989; Ammar and Nault 1991; Wayadande and Nault 1993). Wayadande and Nault (1993) suggested that the biological meaning of the X-waveform is egestion (*sensu* Harris (1977); termed extravasation by McLean and Kinsey (1984)), the delivery of plant fluids present within the food canal anterior to the cibarial pump back to the stylets and then into the plant, occurring when the plant fluid itself fails to induce phagostimulation (McLean and Kinsey 1984).

Theoretically, valve fluttering occurring during the voltage drop inside a resting/low frequency Xi phase (when stylets are inside the xylem), even if shorter than “simple” fluttering (not occurring during a drop), would generate a force sufficient to egest bacterial cells from the foregut to the plant. Indeed, during the resting phase, insect cibarial (and pre-cibarial) muscles are either not contracting, or contracting at a very low frequency (<0.1Hz) (Cornara et al. 2018b). Theoretically, the slower a muscle contracts, the greater the internal tension, thus the greater the force it can generate (Malone et al. 1999; Sutton and Burrows 2018). Therefore, if the fluttering occurs after resting, the force generated (likely by the pre-cibarial valve) would be sufficient to propel bacterial cells toward the xylem vessels even if the behavior is performed for a short period. Therefore, the Xe waveform may represent the opening and

likely fluttering of the pre-cibarial valve, that propels the bacterial cells toward the xylem vessel possibly helped by the negative tension of the xylem sap.

Considering the spittlebugs that had 3h of IAP without interruption of the probe (both the 49 infective spittlebugs in the inoculation experiment, and the 14 individuals in the behavioral comparison; Tab.2B), this behavior (Xe) was performed by six out of the 63 infective individuals, leading to successful inoculation in three cases. Therefore, Xe represents a relatively occasional/relatively rare behavior (on olive), given that in our experiment, ca. 9.52% (six out of 63, data not shown in the table) of the infective individuals performed it, and only a half of these individuals (three out of 63, ca. 4.76% of the infective spittlebugs) inoculated the bacterium. This inoculation rate is consistent with data on *P. spumarius* bacterium inoculation to grape, with one out of 30 plants infected by single insects given an IAP of either 1.5 or 4.5 hours (Cornara et al. 2016). The association of *X. fastidiosa* inoculation by the meadow spittlebug with a relatively infrequent/occasional behavior is also consistent with the occasional transmissions to grapevine during spittlebugs sequential daily transfer to healthy recipient plants reported by Severin (1950) (infection rate ranging from 5 to 16%). In fact, transmission rate of *X. fastidiosa* by *P. spumarius* is much more inefficient than the rate of transmission of other foregut-borne plant-pathogens such as Beet yellows virus (which is close to 50% by a single aphid) (Jimenez et al. 2018). Therefore, data presented here, supported by the observations by other authors described above, suggest that *P. spumarius* likely inoculates *X. fastidiosa* during the pattern Xe occurring just few minutes after the beginning of the probe, and that, at least on suitable plants as olive, this behavior is a relatively rare event different from the patterns stereotypically repeated by the insect during most of the probes (namely C, Xc, Xi, N, R). Overall, considering not only the Xe behavior, but also the sequence of events preceding it, we propose that the behavior leading to *X. fastidiosa* inoculation into the host plant by *P. spumarius* is egestion driven by pre-cibarial valve fluttering resulting from a failure of insect phagostimulation following the tasting of the host-plant xylem sap, possibly helped by xylem fluid tension while the insect is feeding. Moreover, as discussed in the materials and methods section, such unusual behavior occurs more frequently in oleander than in olive (observed by Cornara et al. 2018b and Markheiser et al. 2019). Cornara et al. (2017b) reported that *P. spumarius* transmission rate to oleander is far greater than to olive, although all the insects on the former host died within 24 hours from caging. Therefore, transmission is apparently enhanced if the spittlebug is forced to feed on an unsuitable substrate, possibly because lack of phagostimulation (or feeding deterrence) and subsequent egestion would be more likely to occur on a less -or not- acceptable host. This hypothesis is also supported by increased rate of transmission by *Homalodisca vitripennis* Germar (1821) (Hemiptera: Cicadellidae) caged on grapevines treated with the insecticide pymetrozine (Bextine et al. 2004). Therefore, the behavior associated to *X. fastidiosa* inoculation could be triggered by conditions of the host plant unfavorable for the insect; the identifications of such factors,

whether related to the host plant, to the vector, or to the interactions between the two elements, deserve further investigation. Furthermore, the frequency of Xe may also increase because of the presence of the bacterium in the foregut, but this needs further investigation.

Comparison of infective versus non-infective *Philaenus spumarius* probing and feeding behavior

Plant pathogens influence the transmission process, i.e. the recruitment of the vector on the infected plants for acquisition and the successive dispersal for inoculation, via effects on plant or vector phenotypes that modify the nature and the frequency of the interactions between them (Mauck 2016; Mauck et al. 2018). To be categorized as parasite manipulation a documented effect of a plant pathogen on its vector should: 1) enhance, or create conditions expected to enhance transmission; 2) be under genetic control of the pathogen (Mauck et al. 2019). Vector transmission may be enhanced by the pathogen through indirect effects, i.e. effects on host derived sensory cues (Eigenbrode et al. 2002; Jimenez-Martinez et al. 2004; Mauck et al. 2010; Shapiro et al. 2012), or direct effects on insects behaviors such as probing and host-searching/dispersal (Stafford et al. 2011; Ingwell et al. 2012; Moreno-Delafuente et al. 2013; Martini et al. 2015). The same effects can be induced by highly divergent pathogens sharing the same mechanism of transmission (Mauck 2016; Stafford et al. 2011; Lefevre and Thomas 2008). According to Moreno-Delafuente et al. (2013), persistent circulative viruses are more likely to influence vector behavior given that the vector-pathogen relationship lasts for the entire insect life span, although semi-persistent viruses effects on vector behavior have been documented (Lu et al. 2017; Pereira et al. 2019). Mauck et al. (2019) suggest proteins encoded by pathogens to facilitate interaction with their vectors following acquisition may be co-opted to induce behavioral changes that enhance transmission. *X. fastidiosa* fulfill both the previously mentioned “requirements”, being persistent in its vectors (Severin 1950; Purcell and Finlay 1979), and encoding proteins necessary for interacting with the insect vector (Killiny and Almeida 2014). Additionally, the fact that *X. fastidiosa* exploits the cuticle of its vectors as a substrate for multiplication, suggests a parasitic relationship, with a negative impact of the bacterium on the insect (Labroussaa et al. 2017). As observed in our experiment, the probing and feeding behavior of infective *P. spumarius* females significantly differs from that of non-infective ones. The main affected behaviors were non-probing and xylem ingestion, with an overall longer time spent in non-probing and a shorter time spent in xylem ingestion by infective insects. Particularly, *P. spumarius* carrying *X. fastidiosa* showed evident difficulties in performing sustained xylem ingestion (ingestion longer than ten minutes), with fewer events compared to healthy insects. Furthermore, infective spittlebugs showed a duration of individual non-probing events twice that of non-infective insects, fewer xylem interruptions N, and longer time before probing the host plant for the first time compared to insects not

carrying the bacterium. Taken together, these observations suggest difficulties in feeding caused by the presence of *X. fastidiosa* within the foregut, similarly to what has been recently hypothesized by Ranieri et al. (2019), possibly caused by a mechanical obstruction of the food canal. However, a biological effect caused directly by *X. fastidiosa* on the insect aimed at creating a favorable environment for the bacterium within its vector cannot be ruled out. Indeed, longer non-probing alternated with short xylem ingestion, thus longer period with almost no muscle contraction, sap flow or turbulence, would represent a perfect condition for bacterial cells to bind, multiply, and colonize the foregut. Such manipulation could either affect vector fitness, or be conducive for transmission. Indeed, as observed for example in mosquitos bearing the malaria Plasmodium, the vector could respond to difficulties in feeding by increasing the number of probes (Lefevre and Thomas 2008). Since, as observed in this study, inoculation of bacterial cells by the meadow spittlebug can occur just a few minutes after the beginning of the probe and is possibly associated with an occasional event (Xe), an increased number of probes could theoretically increase the probability of the inoculation behavior to occur, thus the overall inoculation rate.

Conclusions and further perspectives.

Recent researches on vector-pathogen relationship disruption (Killiny et al. 2012; Labroussaa et al. 2016), bacterium biological control (Baccari et al. 2018), and sources of resistance (Giampetruzzi et al. 2016) offer promising perspectives for a sustainable and effective *X. fastidiosa*-diseases control. However, with regards to the European outbreaks of the bacterium, these perspectives are limited by our lack of knowledge about several pivotal aspects of the epidemics, especially concerning the spittlebugs-bacterium interaction and the spittlebugs-mediated transmission mechanism. Here we began to shed some light on *X. fastidiosa* transmission dynamics by *P. spumarius*, opening at the same time new challenging questions. For example, the identification of conditions triggering the putative egestion behavior associated with bacterial cells inoculation would have interesting implications on sustainable control strategies. The *X. fastidiosa* inoculation behavior should also be characterized on other vector-host plant-strain combinations. Furthermore, an in-deep characterization and description of the waveform Xe and its sub-patterns is absolutely needed.

Considering the relatively low acquisition and inoculation rates displayed by *P. spumarius*, an effective control of the meadow spittlebug populations could result in a significant reduction of the risk of *X. fastidiosa* spread. Indeed, according to Irwin and Ruesink (1986), vector intensity depends on vector propensity (innate ability of the vector to transmit a certain pathogen) and vector activity (number of insect vectors alighting on the host plant for a

certain period of time); therefore, a reduction of vector activity would lead to a decrease of vector intensity. However, several aspects related to vector ecology should be investigated in order to develop a sustainable long-term *X. fastidiosa* management strategy: i) vector population abundance within the orchard; ii) factors driving vector host selection and within-host plant preference; iii) vector aggregation and dispersal dynamics; iv) influence of landscape on vector population dynamics (Santoemma et al. 2018; Bodino et al. 2019).

Finally, other challenging questions come from our finding about differences in probing and feeding behavior between infective and non-infective *P. spumarius*; we discussed above how these differences could be beneficial to the bacterium and detrimental for the spittlebug. However, we recognize the limits of our experimental approach (we used only females, monitored for a relatively short period (three hours) and with the bacterium acquired from the infected plant). This does not permit drawing conclusions about pathogen manipulation exerted by the bacterium on the spittlebug. More research efforts should be put in place to thoroughly characterize the intimate *X. fastidiosa*-*P. spumarius* interaction. First, possible plant effects on the behavioral manipulation should be excluded by artificial acquisition of the bacterium; second, observations should be extended to males and to the entire adult life span, also increasing the duration of the IAP; third, it should be verified if such behavioral effect is under genetic control of *X. fastidiosa*

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Author Contribution

- DC and AF conceived research.
- DC and MoM conducted experiments.
- DC, MaM and EG analyzed the data.
- DC wrote the manuscript.
- MoM, MaM, EG, AM, MS and AF reviewed and edited the manuscript.
- AM, MS and AF secured funding.
- All authors read and approved the manuscript.

Data availability statement

Additional data will be furnished by the authors upon reasonable request.

Competing interests statement

The authors declare no competing interests.

References

- Almeida RP (2016) *Xylella fastidiosa* vector transmission biology. Vector-Mediated Transmission of Plant Pathogens Ed: APS Press St Paul, Minnesota, USA 165–174.
- Almeida RP, Purcell AH (2003) Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera: Cicadellidae). J Econ Entomol 96: 264–271.
- Almeida RP, Purcell AH (2006) Patterns of *Xylella fastidiosa* colonization on the precibarium of sharpshooter vectors relative to transmission to plants. Ann Entomol Soc Am 99: 884–890.
- Ammar ED, Nault LR (1991) Maize chlorotic dwarf viruslike particles associated with the foregut in vector and nonvector leafhopper species. Phytopathology 81: 444–448.
- Antolinez CA, Moreno A, Appezzato-da-Gloria B, Fereres A (2017) Characterization of the electrical penetration graphs of the psyllid *Bactericera trigonica* on carrots. Entomol Exp Appl 163: 127–139.
- Baccari C, Antonova E, Lindow S (2018) Biological control of Pierce's disease of grape by an endophytic bacterium. Phytopathology 109(2): 248-256.
- Backus EA, McLean DL (1982) The sensory systems and feeding behavior of leafhoppers. I. The aster leafhopper, *Macrostelus fascifrons* Stål (Homoptera, Cicadellidae). J Morphol 172: 361–379.
- Backus EA (1985) Anatomical and sensory mechanisms of planthopper and leafhopper feeding behavior. p. 163–194. In Nault LR and Rodriguez JG (ed.), The leafhoppers and planthoppers. John Wiley & Sons, Inc., New York, N.Y.
- Backus EA, Habibi J, Yan F, Ellersieck M (2005) Stylet penetration by adult *Homalodisca coagulata* on grape: electrical penetration graph waveform characterization, tissue correlation, and possible implications for transmission of *Xylella fastidiosa*. Ann Entomol Soc Am 98: 787–813.
- Backus EA, Bennett WH (2009) The AC–DC correlation monitor: new EPG design with flexible input resistors to detect both R and emf components for any piercing–sucking hemipteran. J Insect Physiol 55: 869–884.
- Backus EA, Holmes WJ, Schreiber F, Reardon BJ, Walker GP (2009) Sharpshooter X wave: correlation of an electrical penetration graph waveform with xylem penetration supports a hypothesized mechanism for *Xylella fastidiosa* inoculation. Ann Entomol Soc Am 102: 847–867.
- Backus EA, Andrews KB, Shugart HJ, Greve LC, Labavitch JM, Alhaddad H (2012) Salivary enzymes are injected into xylem by the glassy-winged sharpshooter, a vector of *Xylella fastidiosa*. J Insect Physiol 58: 949–959.

Backus EA (2016) Sharpshooter Feeding Behavior in Relation to Transmission of *Xylella fastidiosa*: A Model for Foregut-Borne Transmission Mechanisms. In Vector-Mediated Transmission of Plant Pathogen. Aps Symp Ser (pp. 173-195).

Bextine BR, Harshman D, Johnson MC, Miller TA (2004) Impact of pymetrozine on glassy-winged sharpshooter feeding behavior and rate of *Xylella fastidiosa* transmission. J Insect Sci 4:1-6.

Bodino N, Cavalieri V, Dongiovanni C, Plazio E, Saladini MA, Volani S, Simonetto S, Fumarola G, Di Carolo M, Porcelli F, Gilioli G, Bosco D (in press) Phenology, seasonal abundance and stage-structure of spittlebug (Hemiptera: Aphrophoridae) populations in olive groves in Italy. Sci Rep DOI: 10.1038/s41598-019-54279-8

Cardinale M, Luvisi A, Meyer JB, Sabella E, De Bellis L, Cruz AC, Ampatzidis Y, Cherubini P (2018) Specific fluorescence in situ hybridization (FISH) test to highlight colonization of xylem vessels by *Xylella fastidiosa* in naturally infected olive trees (*Olea europaea* L.). Front Plant Sci 9: 431.

Childress SA, Harris KF (1989) Localization of virus-like particles in the foreguts of viruliferous *Graminella nigrifrons* leafhoppers carrying the semi-persistent maize chlorotic dwarf virus. J Gen Virol 70: 247–251.

Cornara D, Sicard A, Zeilinger AR, Porcelli F, Purcell AH, Almeida RPP (2016) Transmission of *Xylella fastidiosa* to grapevine by the meadow spittlebug. Phytopathology 106: 1285–1290.

Cornara D, Saponari M, Zeilinger AR, de Stradis A, Boscia D, Loconsole G, Bosco D, Martelli GP, Almeida RP, Porcelli F (2017a) Spittlebugs as vectors of *Xylella fastidiosa* in olive orchards in Italy. J Pest Sci 90: 521–530.

Cornara D, Cavalieri V, Dongiovanni C, Altamura G, Palmisano F, Bosco D, Porcelli F, Almeida RPP, Saponari M (2017b) Transmission of *Xylella fastidiosa* by naturally infected *Philaenus spumarius* (Hemiptera, Aphrophoridae) to different host plants. J Appl Entomol 141: 80–87.

Cornara D, Bosco D, Fereres A (2018a) *Philaenus spumarius*: when an old acquaintance becomes a new threat to European agriculture. J Pest Sci 91: 957–972.

Cornara D, Garzo E, Morente M, Moreno A, Alba-Tercedor J, Fereres A (2018b) EPG combined with micro-CT and video recording reveals new insights on the feeding behavior of *Philaenus spumarius*. PloS One 13(7): e0199154.

Cornara D, Morente M, Markheiser A, Bodino N, Tsai CW, Fereres A, Redak RA, Perring T, Lopes JRS (2019) An overview on the worldwide vectors of *Xylella fastidiosa*. Entomol Gen doi: 10.1127/entomologia/2019/0811

Cruaud A, Gonzalez AA, Godefroid M, Nidelet S, Streito JC, Thuillier JM, Rossi JP, Santoni S, Rasplus JY (2018) Using insects to detect, monitor and predict the distribution of *Xylella fastidiosa*: a case study in Corsica. Sci Rep 8: 15628.

750 Daugherty MP, Almeida RPP (2009) Estimating *Xylella fastidiosa* transmission parameters:
 751 decoupling sharpshooter number and feeding period. Entomol Exp Appl 132: 84–92.

752 Davis MJ, Purcell AH, Thomson SV (1978) Pierce's disease of grapevines: isolation of the causal
 753 bacterium. Science 199: 75–77.

754 Del Cid C, Krugner R, Zeilinger AR, Daugherty MP, Almeida RP (2018) Plant Water Stress and
 755 Vector Feeding Preference Mediate Transmission Efficiency of a Plant Pathogen. Environ
 756 Entomol 47: 1471–1478.

757 Dugravot S, Backus EA, Reardon BJ, Miller TA (2008) Correlations of cibarial muscle activities of
 758 *Homalodisca* spp. sharpshooters (Hemiptera: Cicadellidae) with EPG ingestion waveform and
 759 excretion. J Insect Physiol 54: 1467–1478.

760 Eigenbrode SD, Ding H, Shiel P, Berger PH (2002) Volatiles from potato plants infected with
 761 potato leafroll virus attract and arrest the virus vector, *Myzus persicae* (Homoptera:
 762 Aphididae). Proc Royal Soc B 269: 455–460.

763 Frazier NW (1965) Xylem viruses and their insect vectors, in: Proceedings of the International
 764 Conference on Virus and Vectors on Perennial Hosts, with Special Reference to Vitis. pp. 91–
 765 99.

766 Giampetruzzi A, Morelli M, Saponari M, Loconsole G, Chiumenti M, Boscia D, Savino VN,
 767 Martelli GP, Saldarelli P (2016) Transcriptome profiling of two olive cultivars in response to
 768 infection by the CoDiRO strain of *Xylella fastidiosa* subsp. *pauca*. BMC genomics 17: 475.

769 Harper SJ, Ward LI, Clover GRG (2010) Development of LAMP and real-time PCR methods for
 770 the rapid detection of *Xylella fastidiosa* for quarantine and field applications. Phytopathology
 771 100: 1282–1288.

772 Harris KF (1977) An ingestion-egestion hypothesis of noncirculative virus transmission, in:
 773 Aphids as Virus Vectors. Elsevier, pp. 165–220.

774 Hill BL, Purcell AH (1995) Acquisition and retention of *Xylella fastidiosa* by an efficient vector,
 775 *Graphocephala atropunctata*. Phytopathology 85: 209–212.

776 Hill BL, Purcell AH (1997) Populations of *Xylella fastidiosa* in plants required for transmission
 777 by an efficient vector. Phytopathology 87: 1197–1201.

778 Hopkins DL (1981) Seasonal concentration of the Pierce's disease bacterium in grapevine
 779 stems, petioles, and leaf veins. Phytopathology 71: 415–418.

780 Houston BR, Esau K, Hewitt WB (1947) The mode of vector feeding and the tissues involved in
 781 the transmission of Pierce's disease virus in grape and alfalfa. Phytopathology 37: 247–253.

782 Ingwell LL, Eigenbrode SD, Bosque-Pérez NA (2012) Plant viruses alter insect behavior to
 783 enhance their spread. Sci Rep 2: 578.

784 Irwin ME, Ruesink WG (1986) Vector intensity: A product of propensity and activity. Pages 13-
785 33 in: Plant Virus Epidemics: Monitoring, Modelling and Predicting Outbreaks. GD McLean, RG
786 Garrett, and WG Ruesink, eds. Academic Press, Sydney, Australia.

787 Jackson BC, Blua MJ, Bextine B (2008) Impact of duration versus frequency of probing by
788 *Homalodisca vitripennis* (Hemiptera: Cicadellidae) on inoculation of *Xylella fastidiosa*. J Econ
789 Entomol 101: 1122–1126.

790 Jiménez J, Tjallingii WF, Moreno A, Fereres A (2018) Newly distinguished cell punctures
791 associated with transmission of the semipersistent phloem-limited Beet yellows virus. J
792 Virol 92(21), e01076-18.

793 Jiménez-Martínez ES, Bosque-Pérez NA, Berger PH, Zemetra RS, Ding H, Eigenbrode SD (2004)
794 Volatile cues influence the response of *Rhopalosiphum padi* (Homoptera: Aphididae) to Barley
795 yellow dwarf virus–infected transgenic and untransformed wheat. Environ Entomol 33: 1207–
796 1216.

797 Joost PH, Backus EA, Morgan D, Yan F (2006) Correlation of stylet activities by the glassy-
798 winged sharpshooter, *Homalodisca coagulata* (Say), with electrical penetration graph (EPG)
799 waveforms. J Insect Physiol 52: 327–337.

800 Killiny N, Almeida RP (2014) Factors affecting the initial adhesion and retention of the plant
801 pathogen *Xylella fastidiosa* in the foregut of an insect vector. Appl Environ Microbiol 80: 420–
802 426.

803 Killiny N, Prado SS, Almeida RP (2010) Chitin utilization by the insect-transmitted bacterium
804 *Xylella fastidiosa*. Appl Environ Microbiol 76: 6134–6140.

805 Killiny N, Rashed A, Almeida RP (2012) Disrupting the transmission of a vector-borne plant
806 pathogen. Appl Environ Microbiol 78: 638–643.

807 Labroussaa F, Zeilinger AR, Almeida RP (2016) Blocking the transmission of a noncirculative
808 vector-borne plant pathogenic bacterium. Mol Plant Microbe In 29: 535–544.

809 Labroussaa F, Ionescu M, Zeilinger AR, Lindow SE, Almeida RP (2017) A chitinase is required for
810 *Xylella fastidiosa* colonization of its insect and plant hosts. Microbiology 163: 502-509.

811 Lefevre T, Thomas F (2008) Behind the scene, something else is pulling the strings:
812 emphasizing parasitic manipulation in vector-borne diseases. Infect Genet Evol 8: 504–519.

813 Loconsole G, Potere O, Boscia D, Altamura G, Djelouah K, Elbeaino T, Frasheri D, Lorusso D,
814 Palmisano F, Pollastro P (2014) Detection of *Xylella fastidiosa* in olive trees by molecular and
815 serological methods. J Plant Pathol 96: 7–14.

816 Lu S, Li J, Wang X, Song D, Bai R, Shi Y, Gu Q, Kuo YW, Falk B, Yan F (2017) A semipersistent
817 plant virus differentially manipulates feeding behaviors of different sexes and biotypes of its
818 whitefly vector. Viruses 9: 4.

819 Malone M, Watson R, Pritchard J (1999) The spittlebug *Philaenus spumarius* feeds from
820 mature xylem at the full hydraulic tension of the transpiration stream. *New Phytol* 143: 261–
821 271.

822 Markheiser A, Cornara D, Fereres A, Maixner M (2019) Analysis of vector behavior as a tool to
823 predict *Xylella fastidiosa* patterns of spread. *Entomolol Gen* doi:
824 10.1127/entomologia/2019/0841

825 Martin B, Collar JL, Tjallingii WF, Fereres A (1997) Intracellular ingestion and salivation by
826 aphids may cause the acquisition and inoculation of non-persistently transmitted plant viruses.
827 *J Gen Virol* 78: 2701–2705.

828 Martini X, Hoffmann M, Coy MR, Stelinski LL, Pelz-Stelinski KS (2015) Infection of an insect
829 vector with a bacterial plant pathogen increases its propensity for dispersal. *PLoS One* 10:
830 e0129373.

831 Marucci RC, Lopes JRS, Vendramim JD, Corrente JE (2005) Influence of *Xylella fastidiosa*
832 infection of citrus on host selection by leafhopper vectors. *Entomol Exp Appl* 117: 95–103.

833 Mauck, K.E., De Moraes, C.M., Mescher, M.C., 2010. Deceptive chemical signals induced by a
834 plant virus attract insect vectors to inferior hosts. *Proc Natl Acad Sci* 107: 3600–3605.

835 Mauck KE (2016) Variation in virus effects on host plant phenotypes and insect vector
836 behavior: what can it teach us about virus evolution? *Curr Opin Virol* 21: 114–123.

837 Mauck KE, Chesnais Q, Shapiro LR (2018) Evolutionary determinants of host and vector
838 manipulation by plant viruses. In *Advances in virus research* 101: 189-250. Academic Press,
839 Cambridge MA USA.

840 Mauck KE, Kenney J, Chesnais Q (2019) Progress and challenges in identifying molecular
841 mechanisms underlying host and vector manipulation by plant viruses. *Curr Opin Insect Sci* 33:
842 7-18.

843 McLean DL, Kinsey MG (1964) A technique for electronically recording aphid feeding and
844 salivation. *Nature* 202: 1358.

845 McLean DL, Kinsey MG (1984) The Precibarial Valve and Its Role in the Feeding Behavior of the
846 Pea Aphid, *Acyrtosiphon pisum*. *Bull Entomol Soc Am* 30: 26–31.

847 Miranda MP, Villada ES, Lopes SA, Fereres A, Lopes JRS (2013) Influence of citrus plants
848 infected with *Xylella fastidiosa* on stylet penetration activities of *Bucephalogonia xanthophis*
849 (Hemiptera: Cicadellidae). *Ann Entomol Soc Am* 106: 610–618.

850 Moreno A, Tjallingii WF, Fernandez-Mata G, Fereres A (2012) Differences in the mechanism of
851 inoculation between a semi-persistent and a non-persistent aphid-transmitted plant virus. *J*
852 *Gen Virol* 93: 662–667.

Moreno-Delafuente A, Garzo E, Moreno A, Fereres A (2013) A plant virus manipulates the behavior of its whitefly vector to enhance its transmission efficiency and spread. *PLoS One* 8: e61543.

Morente M, Cornara D, Plaza M, Durán J, Capiscol C, Trillo R, Ruiz M, Ruz C, Sanjuan S, Pereira J, Moreno A, Fereres A (2018) Distribution and Relative Abundance of Insect Vectors of *Xylella fastidiosa* in Olive Groves of the Iberian Peninsula. *Insects* 9(4), 175.

Newman KL, Almeida RP, Purcell AH, Lindow SE (2003) Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. *Appl Environ Microbiol* 69: 7319–7327.

Pereira LS, Lourenção AL, Salas FJS, Bento JMS, Rezende JAM, Peñaflor M (2019) Infection by the semi-persistently transmitted Tomato chlorosis virus alters the biology and behaviour of *Bemisia tabaci* on two potato clones. *Bull Entomol Res* 1–8.

Pierce NB (1892) The California vine disease: a preliminary report of investigations. US Government Printing Office.

Prado E, Tjallingii WF (1994) Aphid activities during sieve element punctures. *Entomol Exp Appl* 72: 157–165.

Purcell AH, Finlay AH (1979) Evidence for noncirculative transmission of Pierce's disease bacterium by sharpshooter leafhoppers. *Phytopathology* 69: 393–395.

Purcell AH, Finlay AH, McLean DL (1979) Pierce's disease bacterium: Mechanism of transmission by leafhopper vectors. *Science* 206: 839–841.

R Core Team (2015). R Foundation for Statistical Computing; Vienna, Austria: 2014. R: A language and environment for statistical computing, 2013.

Ramirez JL, Lacava PT, Miller TA (2008) Detection of the bacterium, *Xylella fastidiosa*, in saliva of glassy-winged sharpshooter, *Homalodisca vitripennis*. *J Insect Sci* 8: 1-7.

Rapicavoli J, Ingel B, Blanco-Ulate B, Cantu D, Roper C (2018) *Xylella fastidiosa*: an examination of a re-emerging plant pathogen. *Mol Plant Pathol* 19: 786–800.

Retchless AC, Labroussaa F, Shapiro L, Stenger DC, Lindow SE, Almeida RP (2014) Genomic insights into *Xylella fastidiosa* interactions with plant and insect hosts, in: *Genomics of Plant-Associated Bacteria*. Springer, pp. 177–202.

Ranieri E, Zitti G, Riolo P, Isidoro N, Ruschioni S, Brocchini M, Almeida RP (2019) Fluid dynamics in the functional foregut of xylem-sap feeding insects: a comparative study of two *Xylella fastidiosa* vectors. *J Insect Physiol* 120: 103995.

Ruschioni S, Ranieri E, Riolo P, Romani R, Almeida RP, Isidoro N (2019) Functional anatomy of the precibarial valve in *Philaenus spumarius* (L.). *PloS One* 14: e0213318.

887 Santoiemma G, Tamburini G, Sanna F, Mori N, Marini L (2019) Landscape composition predicts
888 the distribution of *Philaenus spumarius*, vector of *Xylella fastidiosa*, in olive groves. J Pest Sci
889 92(3) : 1101-1109.

890 Saponari M, Loconsole G, Cornara D, Yokomi RK, De Stradis A, Boscia D, Bosco D, Martelli GP,
891 Krugner R, Porcelli F (2014) Infectivity and transmission of *Xylella fastidiosa* by *Philaenus*
892 *spumarius* (Hemiptera: Aphrophoridae) in Apulia, Italy. J Econ Entomol 107: 1316–1319.

893 Severin H (1950) Spittle-insect vectors of Pierce's disease virus. II. Life history and virus
894 transmission. Hilgardia 19: 357-382.

895 Shapiro L, De Moraes CM, Stephenson AG, Mescher MC (2012) Pathogen effects on vegetative
896 and floral odours mediate vector attraction and host exposure in a complex pathosystem. Ecol
897 Lett 15: 1430–1438.

898 Sicard A, Zeilinger AR, Vanhove M, Schartel TE, Beal DJ, Daugherty MP, Almeida RP (2018)
899 *Xylella fastidiosa*: Insights into an emerging plant pathogen. Annu Rev Phytopathol 56: 181–
900 202.

901 Stafford CA, Walker GP, Ullman DE (2011) Infection with a plant virus modifies vector feeding
902 behavior. Proc Natl Acad Sci 108: 9350–9355.

903 Sutton GP, Burrows M (2018) Insect jumping springs. Curr Biol 28: 142–143.

904 Tjallingii WF (1978) Electronic recording of penetration behaviour by aphids. Entomol Exp Appl
905 24: 721–730.

906 Walker GP (2000) A beginner's guide to electronic monitoring of homopteran probing
907 behavior. Principles and applications of electronic monitoring and other techniques in the
908 study of homopteran feeding behavior. Thomas Say Publications in Entomology, Entomological
909 Society of America, Lanham, MD 14–40.

910 Wayadande AC, Nault LR (1993) Leafhopper probing behavior associated with maize chlorotic
911 dwarf virus transmission to maize. Phytopathology 83: 522–526.

912 Wells JM, Raju BC, Hung HY, Weisburg WG, Mandelco-Paul L, Brenner DJ (1987) *Xylella*
913 *fastidiosa* gen. nov., sp. nov: gram-negative, xylem-limited, fastidious plant bacteria related to
914 *Xanthomonas* spp. Int J Syst Evol Micr 37: 136–143.

915 Zeilinger AR, Daugherty MP (2014) Vector preference and host defense against infection
916 interact to determine disease dynamics. Oikos 123: 613–622.

920 **Tab.1 Acquisition behavior.** Table “Acquisition” (on the left) summarizes the experimental design, the treatments, and the
 921 number of replicates, together with the number of spittlebugs that acquired the bacterium for each treatment. The tables on the
 922 right, report three EPG non-sequential variables (WDI, WDEI and NWEI) calculated for the two spittlebugs that acquired *X.*
 923 *fastidiosa*. WDI: waveform duration per individual. WDEI: waveform duration per event per individual. NWEI: number of waveform
 924 events per individual. The variables are described in Tab. 4. Time is expressed as minutes.

Acquisition		
Treatment	n Replicates [§]	Ps positive ^{§§}
C	30	0
Xc	34	0
1h	30	1
3h	37	1

}

WDI ^{§§§}								
AAP	Succ pr	Unsucc pr	np	C	Xc	Xi	N	R
1h	1	0	28.5	13.7	1.5	14.8	2.3	1.2
3h	1	3	24.2	27.6	4.4	122.8	0.2	0.8

WDEI ^{§§§}								
AAP	Succ pr	Unsucc pr	np	C	Xc	Xi	N	R
1h	1	0	14.5	4.56	1.5	4.93	0.19	1.2
3h	1	3	6.05	3.94	1.1	61.4	0.2	0.8

NWEI ^{§§§}								
AAP	Succ pr	Unsucc pr	np	C	Xc	Xi	N	R
1h	1	0	2	3	1	3	12	1
3h	1	3	4	7	4	2	1	1

§=number of replicates per each treatment; §§=number of spittlebugs that acquired *Xylella fastidiosa*;
 §§§=WDI, WDEI and NWEI calculated only for the spittlebugs that acquired (and retained) the bacterium.

Tab.2 Inoculation behavior. “Sequence of events” stands for the sequence of behaviors performed by the insect before interrupting the probe (not shown for the 1-hour and 3-hours IAP). “Ps positive” stands for the number of replicates carried out with infective spittlebugs (as determined by qPCR) for each treatment. “Inoculation” indicates the number of plants inoculated with *X. fastidiosa* by the qPCR positive *P. spumarius* per each treatment. NA (not applicable) is used for the sequence of events of the treatments 1h and 3h, since the insects were given access to the plant without interrupting the probe after a precise event/sequence of events.

A)

Inoculation 2017 (olive)				
Treatment		Sequence of events	Ps positive	Inoculation
Waveform interruption	C	C	3	0
	Xc	C-Xc	9	0
	Xi	C-Xc-Xi	6	0
	N	C-Xc-N or C-Xc-Xi-N [§]	12	0
1h		NA	12	2

B)

Inoculation 2018 (olive)				
Treatment		Sequence of events	Ps positive	Inoculation
Waveform interruption	C	C	17	0
	Xc	C-Xc	16	0
	Xi	C-Xc-Xi	21	0
		C-Xc-Xi-N-Xi or C-Xc-N-Xc-Xi ^{§§}	15	0
	N	C-Xc-N or C-Xc-Xi-N [§]	30	0
	R	C-Xc-Xi-R	19	0
3h IAP ^{§§§}		NA	63 ^{§§§}	3 ^{§§§}

C)

Inoculation 2018 (oleander)				
Treatment		Sequence of events	Ps positive	Inoculation
Waveform interruption	C	C	8	0
	Xc	C-Xc	1	0
	Xi	C-Xc-Xi	8	0
	N	C-Xc-Xi-N	7	0
	R	C-Xc-Xi-R	1	0
	Xe	C-Xc-Xe or C-Xc-Xi-Xe	5	3

[§]=probe interrupted after the first N occurred; ^{§§}=the spittlebugs performed from 1 to 3 xylem interruptions N; ^{§§§}=calculated by pooling together the inoculation results from the 3h IAP inoculation experiment (49 infective spittlebugs) and the comparison infective vs non-infective (14 infective spittlebugs)

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Tab.3 Comparison of infective versus non-infective *P. spumarius* probing behavior. WDI: waveform duration per individual. WDEI: average waveform duration per event per individual. NWEI: number of waveform events per individual. Sequential variables are variables related to a succession of events/behaviors. The EPG variables are explained in Tab. 4. Time is expressed as minutes.

	WDI									
	Infective (n=14)					Non infective (n=35)				Mann-Whitney
	min	max	mean	se (±)		min	max	mean	se	W p
np*	12.3	79.4	47.36	6.15		0.7	114.8	32.06	5.19	340 0.036
C	1.1	47.2	11.16	4		1.1	33.7	8.26	1.29	228 0.707
Xc	0.1	7.3	2.914	0.51		0.4	11.9	2.72	0.4	281 0.425
Xi*	32.9	163.2	89.88	10.01		47.6	163.7	116	4.94	136 0.016
N	0	2.5	0.63	0.21		0	6.4	1.25	0.24	171 0.100
R	0	99.3	28.68	8.48		0	74.6	20.97	3.5	260.5 0.731

	WDEI									
	Infective (n=14)					Non infective (n=35)				Mann-Whitney
	min	max	mean	se (±)		min	max	mean	se	W p
np*	5.9	79.4	16.82	5.02		0.7	29.06	8.28	1.18	350 0.020
C	0.52	5.721	1.84	0.47		0.5	11.23	1.81	0.35	229.5 0.731
Xc	0.1	7.3	1.64	0.48		0.3	11.9	1.57	0.37	283 0.400
Xi	1.73	81.6	22.79	6.89		2.57	155.1	22.28	4.91	229 0.723
Xi<10min	1.73	6.13	3.91	0.42		0	11.22	3.43	0.37	294 0.278
Xi>10min	0	157.9	45.82	13.33		0	155.1	45.72	7.48	227 0.690
N	0	0.62	0.22	0.05		0	0.4	0.2137	0.018	241 0.928
R	0	14.8	3.55	1.04		0	11.4	2.47	0.4	282 0.412

	NWEI									
	Infective (n=14)					Non infective (n=35)				Mann-Whitney
	min	max	mean	se (±)		min	max	mean	se	W p
np	1	9	4.00	0.65		1	11	4.17	0.49	245.5 0.991
C	1	10	5.57	0.86		1	14	5.82	0.63	241 0.929
Xc	0	5	2.07	0.35		1	5	2.17	0.19	231 0.747
Xi	2	19	8.21	1.52		1	36	11.03	1.53	204 0.363
Xi<10min	1	19	6.50	1.48		0	36	8.43	1.58	234.5 0.815
Xi>10min*	0	4	1.71	0.28		0	6	2.6	0.22	152.5 0.032
N*	0	8	1.86	0.60		0	21	4.8	0.88	146 0.027
R	0	18	6.36	1.47		0	34	9.03	1.54	215 0.505

	SEQUENTIAL VARIABLES									
	Infective (n=14)					Non infective (n=35)				Mann-Whitney
	min	max	mean	se (±)		min	max	mean	se	W p
np to Xc	2	84	16.15	6.02		1.2	45.4	9.91	1.57	253.5 0.546
np to Xi	2.4	85.3	17.08	5.89		1.5	46.5	11.87	1.6	253.5 0.851
C to Xc	0.5	8.2	3.18	0.76		0.6	22.6	5.27	0.91	165.5 0.150
C to Xi	1	12.7	4.99	0.98		1.1	24.4	7.22	1.01	187 0.199
np to Xi>10	6.6	68	25.13	4.42		1.5	118	31.46	5.49	209.5 0.891
C to Xi>10	2.2	63.8	17.79	4.49		1.1	117.4	26.68	5.38	187 0.671
Time to the 1st probe*	0.5	79.4	12.08	5.60		0.05	43.7	4.65	1.47	346.5 0.024
Time to the 1st probe with Xi	1.11	79.4	13.98	5.61		0.4	44.66	8.08	1.58	209.5 0.314
Time to the 1st probe with Xi>10	1.11	66.2	17.13	5.06		0.4	110.3	16.52	4.13	238 0.395

	OTHERS VARIABLES									
	Infective (n=14)					Non infective (n=35)				Mann-Whitney
	min	max	mean	se (±)		min	max	mean	se	W p
Succ pr	1	5	2.00	0.28		1	5	2.05	0.18	238.5 0.879
Unsucc pr	0	7	1.78	0.57		0	10	2.03	0.41	234 0.799
Tot pr	1	8	3.78	0.64		1	11	4.08	0.48	239.5 0.902
Frequency Xi	2.78	6.68	4.40	0.30		0.23	0.65	0.43	0.01	247.5 0.956

*= variables significantly different between infective and non-infective spittlebugs as indicated by Mann-Whitney U-test

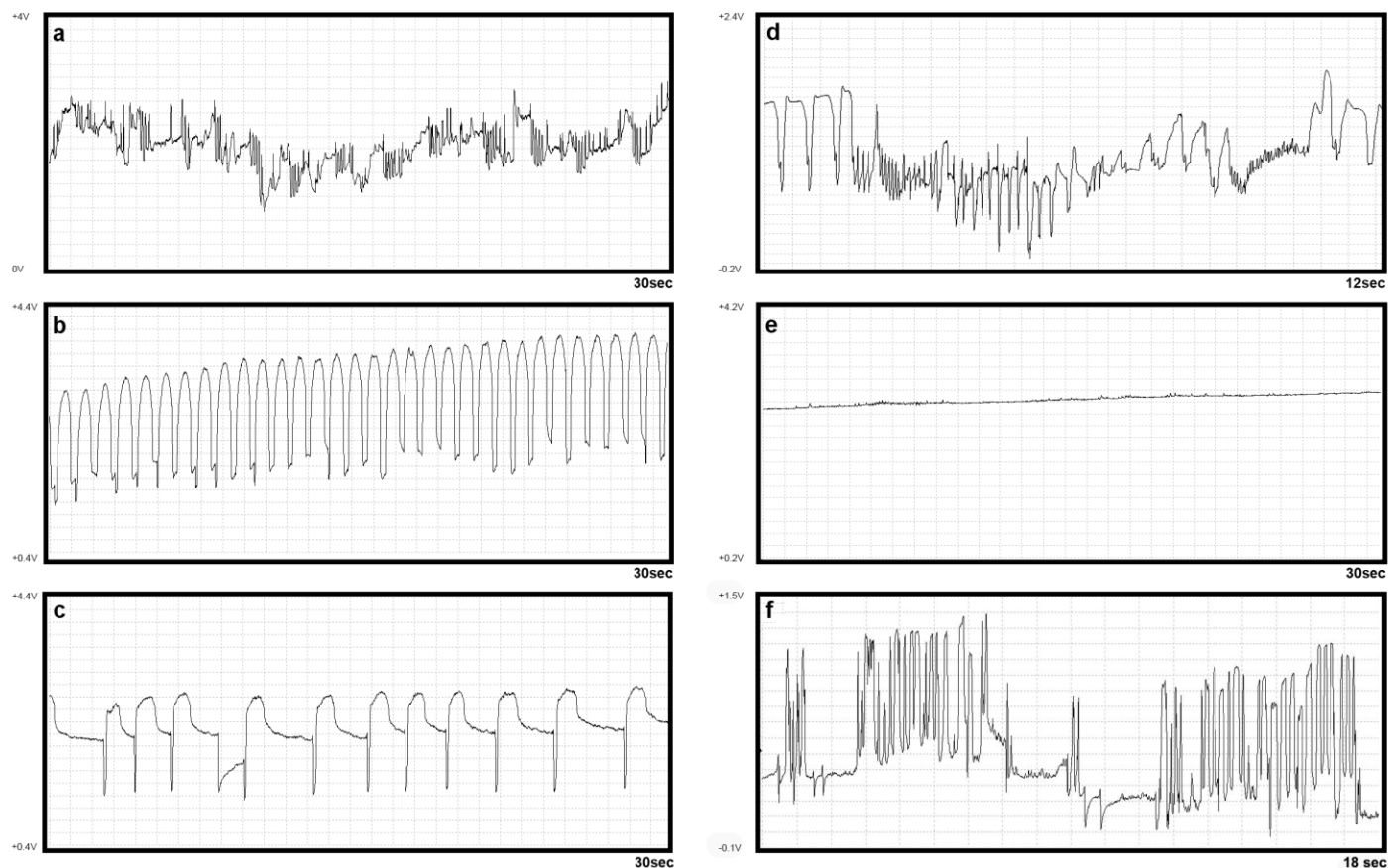
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939 **Tab.4 Explanation of the meaning of the EPG variables calculated in the different experiments.** Frequency of the xylem ingestion
 940 waveform (Xi) was calculated manually on intervals of 10 seconds; intervals were randomly selected every five minutes. The values
 941 reported here are averages of all the intervals per each monitored spittlebug.

Variable abbreviation	Variable definition
Succ pr	number of successful probes (probes where the insect reaches the xylem)
Unsucc pr	number of unsuccessful probes (probes where the insect does not reach the xylem)
Tot pr	number of total probes (successful + unsuccessful)
np WDI	non-probing total duration per individual
C WDI	pathway total duration per individual
Xc WDI	xylem contact total duration per individual
Xi WDI	xylem ingestion total duration per individual
N WDI	xylem interruption total duration per individual
R WDI	resting total duration per individual
np NWEI	non probing total number of events per individual
C NWEI	pathway total number of events per individual
Xc NWEI	xylem contact total number of events per individual
Xi NWEI	xylem ingestion total number of events per individual
Xi<10min NWEI	xylem ingestion shorter than 10 minutes total number of events per individual
Xi>10min NWEI	xylem ingestion longer than 10 minutes total number of events per individual
N NWEI	xylem interruption total number of events per individual
R NWEI	resting total number of events per individual
np WDEI	average non probing duration of single events per individual
C WDEI	average pathway duration of single events per individual
Xc WDEI	average xylem contact duration of single events per individual
Xi WDEI	average xylem ingestion duration of single events per individual
Xi<10min WDEI	average xylem ingestion shorter than 10 minutes duration of single events per individual
Xi>10min WDEI	average xylem ingestion longer than 10 minutes duration of single events per individual
N WDEI	average xylem interruption duration of single events per individual
R WDEI	average resting duration of single events per individual
np to Xc	time from the beginning of the recording to the first xylem contact
np to Xi	time from the beginning of the recording to the first xylem ingestion
C to Xc	time from the first probe to the first xylem contact
C to Xi	time from the first probe to the first xylem ingestion
np to Xi10	time from the beginning of the recording to the start of the first xylem ingestion longer than 10 minutes
C to Xi10	time from the first absolute probe to the start of the first xylem ingestion longer than 10 minutes
Time to the 1st probe	time from the beginning of the recording to the first probe
Time to the 1st probe with Xi	time from the beginning of the recording to the first probe with a xylem ingestion
Time to the 1st probe with xi>10	time from the beginning of the recording to the first probe with a xylem ingestion longer than 10 minutes
Frequency Xi	average frequency of the peaks of the xylem ingestion waveform (Hz)

943 **Figures**

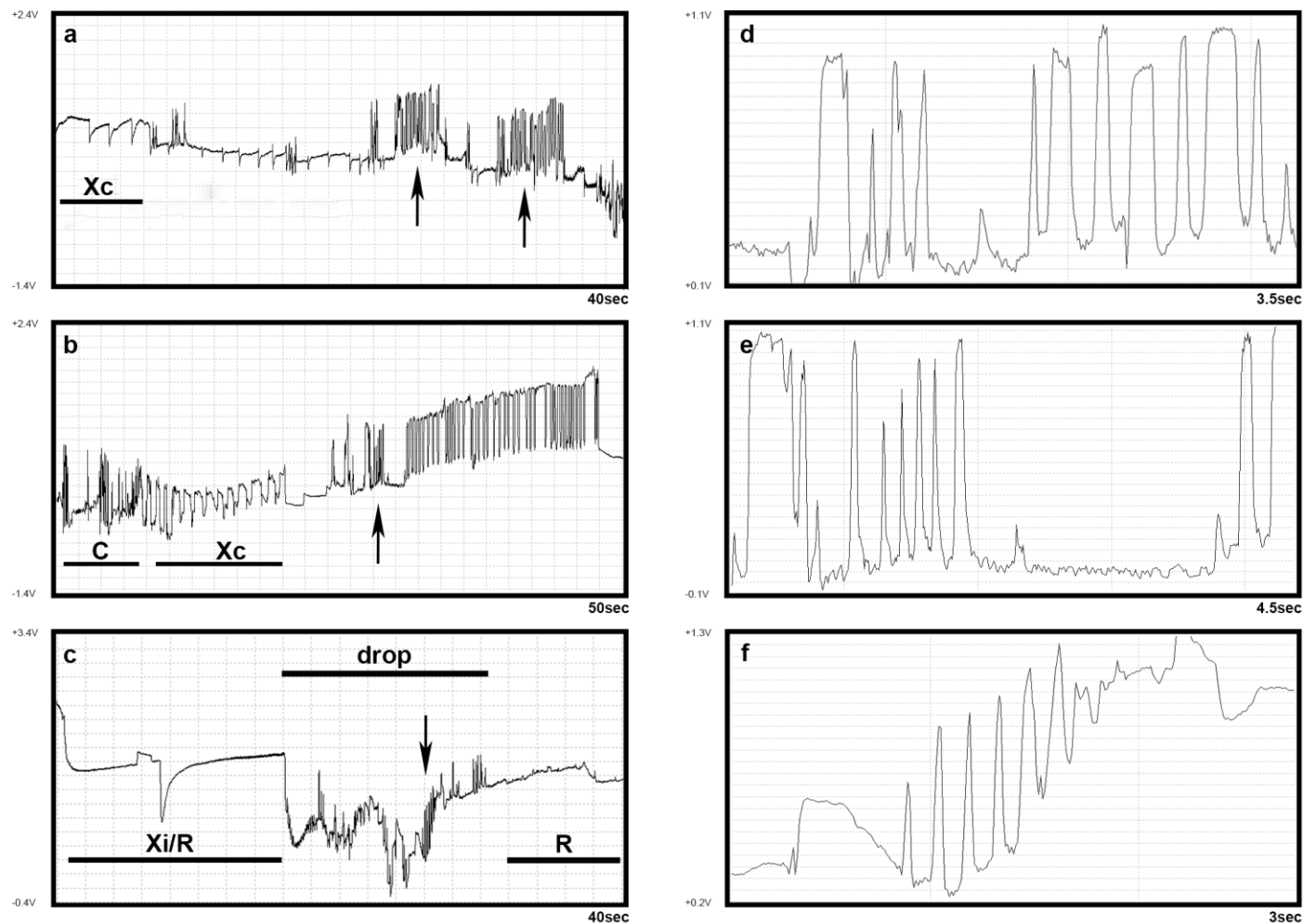
944 **Fig.1 EPG waveforms (behavioral patterns) displayed by *Philaenus spumarius*.** a) waveform C (pathway); b) waveform Xc (xylem
 945 contact/trial ingestion); c) waveform Xi (xylem ingestion); d) waveform N (interruption during the xylem activity); e) waveform R
 946 (resting phase); f) waveform Xe (spikelet burst). Time (sec) is reported on the x-axis; Voltage (V) is reported on the y-axis. Images a to
 947 e are derived from EPG recordings made with *P. spumarius* on olive plants; image f is derived from a recording made with *P.*
 948 *spumarius* on oleander.



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Fig.2 Xe waveform. a and b) coarse structure of Xe (simple spikelet burst) in oleander following pathway C and xylem contact Xc, coarse structure; c) coarse structure Xe (voltage drop) in olive following a resting phase alternated with very low frequency xylem ingestion (≤ 0.1 Hz) Xi/R; d) fine structure of the spikelet burst in figure 2.a; e) fine structure of the spikelet burst in figure 2.b; f) fine structure of the drop in figure 2.c. Spikelet bursts in figures 2.a, 2.b, and 2.c are indicated with black arrows. Time (sec) is reported on the x-axis; Voltage (V) is reported on the y-axis.



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