Journal of Insect Physiology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

First finding of a dual-meaning X wave for phloem and xylem fluid ingestion: Characterization of *Scaphoideus titanus* (Hemiptera: Cicadellidae) EPG waveforms

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ARTICLE INFO

Article history:
Received 4 August 2016
Received in revised form 10 January 2017
Accepted 10 January 2017
Available online xxxx

Keywords:
Cicadomorpha
Cicadellidae
Electropenetrography
Electrical penetration graph
Sap-feeder
Flavescence dorée

ABSTRACT

The leafhopper *Scaphoideus titanus* (Hemiptera: Cicadomorpha: Cicadellidae), an invasive deltocephaline species introduced into Europe from North America, is the vector of the most important phytoplasma disease in European viticulture, flavescence dorée. In this first electropenetrography (EPG) study of *S. titanus*, we characterized its feeding waveforms and defined their biological meanings. Four typical waveform phases (pathway, X wave, sustained ingestion, and interruption) and four families within those phases (A, B, C, and N) were characterized using DC EPG technology. We proposed biological meanings for these waveforms based on excreta pH-ingestion correlations, presence of X waves, and comparison with previous AC, DC, and AC-DC EPG waveforms conducted on Cicadomorpha. We observed that sustained (i.e., >10 min) ingestion by a deltocephaline leafhopper can occur from both xylem and phloem vascular cells. Waveform C2x represented ingestion of xylem fluid, and two waveforms represented behaviors when stylets were inserted into phloem sieve elements: C2p variant 1 (C2p-1), which may represent salivation (perhaps simultaneous with ingestion), and C2p variant 2 (C2p-2), which represented active ingestion. Furthermore, we found that the EPG-recorded X wave has a dual meaning by occurring prior to sustained ingestion from either phloem or xylem. This X wave was very similar in appearance to the model X wave of sharpshooters, an entirely different leafhopper subfamily, Cicadellinae. All cicadellines are obligate xylem-ingesters. Such a "dual-meaning X wave" will provide insights into how the feeding tactics of *S. titanus* relate to other sheath-feeding hemipterans, and will provide support for future research to clarify the role of this leafhopper as a vector of plant pathogens.

1. Introduction

Studying the feeding behavior of insects is useful, because it provides information on their biology, ecology (Backus et al., 2005a; Behmer, 2009), and evolution, and cause of damage to agricultural crops. However, the ability to observe feeding behavior is strongly dependent on the insects' habits. Hemipterans are difficult to study by direct observation because most of their feeding behavioral sequences occur inside plant tissues. The electropenetrography (EPG) technique is therefore indispensable for the study of the sap-feeding process. EPG was first invented by McLean and Kinsey (alternating current or AC EPG) for aphids (McLean and Kinsey, 1964); whose design was then improved by Tjallingii (1978) in his development of direct current (DC) EPG. By connecting an insect and its host plant to an electrical circuit, EPG allows variations in biopotentials (electromotive force or emf component) and electrical resistance (R component) to be recorded and related to different feeding behaviors. These variations produce different electrical waveforms that correspond to specific behaviors that reflect both the position of the stylets in the plant tissue (cell type) and the actual feeding activities, such as stylet movements, cibarial pumping (ingestion), and salivation (Sauvion and Rahbé, 1999; Backus, 2016).

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http://dx.doi.org/10.1016/j.jinsphys.2017.01.013
0022-1910/

Despite the differences between DC and AC monitors, a number of similarities actually exist (Backus, 1994; Reese et al., 2000; Tjallingii, 1990), as finally identified by recent development of a new EPG monitor (AC-DC) with selectable amplifier sensitivities (input resistor or Ri levels) that also allows recordings of insects with either AC or DC applied signal (Backus and Bennett, 2009). This new development provides comparability of AC and DC waveforms to achieve reinterpretation of most previous studies, leading to better biological meanings of the waveforms (Backus et al., 2013).

In 1967, McLean and Kinsey established the concept of the X wave in EPG science. This term was first named for phloem sap-sucking (also termed phloem-feeding) hemipterans, e.g. aphids (Sternorrhyncha: Aphididae) (McLean and Kinsey, 1967) and deltocephaline leafhoppers (Cicadomorpha: Cidadellidae) (Rapusas and Heinrichs, 1990; Triplehorn et al., 1984; Wayadande and Nault, 1993). Presence of X waves was important in AC EPG studies because it separated phloem and xylem ingestion (Wayadande, 1984). In some aphid species, long potential drops immediately preceding sieve element phase (termed R-pds in Tjallingii and Gabryś, 1999) are thought to represent intracellular punctures into phloem cells and can be interpreted as the first part of aphid X waves (Reese et al., 2000). However, aphid researchers generally do not use the term X wave anymore. Backus et al. (2009) advocated restored use of the X wave concept, and using a sharpshooter leafhopper (Cicadellidae: Cicadellinae) model, they formally characterized the component waveform types of an X wave. The sharpshooter X wave is composed of X wave-specific variants of waveform sub-types B1w (representing salivation), B1s (hypothesized to represent precibarial valve or cibarial diaphragm movement for tasting/rinsing egestion), types C1 (representing discharging egestion, i.e. fluid outflow from the stylet tips) and C2 (representing ingestion/cibarial pumping/swallowing). X waves can be seen as a "visual portrayal of the behavioral acceptance process for an ingestion site" (Backus, 2016). Thus, X waves represent a series of behaviors that: "1) sensorially test and judge the acceptability of a phloem sieve element or xylem vessel element (depending on the preference of the species), 2) secure a firm attachment to that cell, and 3) begin overcoming any challenges or defenses presented in the cell by the plant" (Backus et al., 2009). For these reasons, X waves generated in the EPG signal bear great significance in the study of feeding behavior by sheath-feeders.

Flavescence dorée (FD) is one of the most serious phytoplasma diseases that impact European viticulture (Chuche and Thiéry, 2014). The causative agent of FD, '*Candidatus* Phytoplasma viti' (Angelini et al., 2003) is transmitted from one grapevine to another by the leafhopper *Scaphoideus titanus* Ball 1932 (Hemiptera: Cicadellidae: Deltocephalinae), which is a specialist of *Vitis* spp. (Vitales: Vitaceae) in Europe (Chuche and Thiéry, 2014). FD phytoplasma has a high degree of specificity to its vector, and movements made by the insect from one plant to another constitute its only natural transmission path. Acquisition of the phytoplasma from one infected grapevine occurs during phloem ingestion, and phytoplasma inoculation to healthy grapevine by the vector takes place with salivation into phloem. These two steps of the FD biological cycle are decisive and, therefore, there is a great need to better understand these two feeding behaviors in order to develop new control methods for FD.

The objectives of this study were to identify and characterize the EPG waveforms of adults of *S. titanus* on grapevine by using the DC EPG technique, and interpret the recordings via excretory droplet pH measurement and correlation with appearance of putative ingestion waveforms, combined with waveform-behavior correlations established in EPG studies on other leafhoppers in the literature. Sap ingestion is always accompanied by excretory droplet production and the pH of excreta is a good indicator that may help to discriminate between sources of ingestion for salivary-sheath feeders. Excreta pH resulting from xylem ingestion is slightly acidic, while pH from phloem-derived excreta is slightly alkaline (Wayadande and Nault, 1993; Stafford and Walker, 2009). Our work demonstrates for the first time, in AC, DC or AC-DC EPG, that an X wave similar in appearance to the model X wave of sharpshooters occurs prior to sustained ingestion by a leafhopper from either phloem or xylem. Such a "dual-meaning X wave" will provide insights into how the feeding strategy of *S. titanus* relates to other sheath-feeding hemipterans, and will provide support for future research to determine how '*Candidatus* Phytoplasma vitis' is acquired and inoculated.

2. Materials and methods

2.1. Biological materials

Grapevine (*Vitis vinifera* cv. Cabernet Sauvignon) cuttings used for insect rearing and bioassays were grown in a potting compost mix (Substrate 5; Klasmann-Deilmann, Geeste, Germany) and irrigated twice a week. No pesticide was used on the plants and all plants used in the study were at the same phenological stage (10 leaves).

A laboratory colony of *S. titanus* was initiated from wild populations, as previously described in the literature (Caudwell et al., 1970; Chuche and Thiéry, 2009). Two-year-old woody grapevine canes carrying eggs were collected in an organic vineyard in Burgundy (46°45′16″ N; 4°41′14″ E), where sizable populations of *S. titanus* have occurred for successive years, including during the summer preceding our study.

After collection, the woody canes were checked to see if they were bearing eggs, and then were kept in a cold room at 5 ± 1 °C and 85–90% relative humidity until use. Egg hatching was achieved by placing the wood pieces (20–25 cm long) inside plastic hatching cages (50 × 38 × 36 cm) in a climate-controlled chamber under a 16:8 (L:D) photoperiod at 23 ± 1 °C, and 65–70% relative humidity. To avoid desiccation of the eggs, the wood pieces were placed on a 1-cm layer of vermiculite (Efisol, Strasbourg, France), which was sprayed with distilled water every 7 days. After 20 days, six healthy grapevine leaves put in glass tubes containing water were placed in each cage to collect neonates. Every day, nymphs were gently removed from the lower side of the leaves with an aspirator and placed into breeding cages (same as hatching cages) with FD-free Cabernet-Sauvignon cuttings until they reached the adult stage. Leaves were replaced as soon as they began to wither.

2.2. EPG system and wiring leafhoppers

A Giga-8 DC-EPG device (EPG-Systems, Wageningen, The Netherlands) was used to monitor the stylet penetration/probing and ingestion activities of *S. titanus* adults (30 males and 30 females) on grapevine cuttings during 4-h recording periods. Insects, plants, and the electrodes were isolated from background noise by a Faraday cage. The electrical signals picked up by the electrodes were converted into digital signals via the Di710-UL (DATAQ, Akron, OH, USA) analog-to-digital board. The digital signals were then visualized and recorded on a computer using Probe 3.5 software (EPG Systems, Wageningen, The Netherlands). The recordings were made in an air-conditioned room under constant temperature (23 ± 1 °C) and artificial light.

Phytoplasma is mainly transmitted by adult insects whose dispersal is usually linked to reproduction. We used 7- to 14-day-old adults in this study because female insects begin to be sexually active 6 days after emergence (Mazzoni et al., 2009). Leafhoppers

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were collected in a breeding cage and then placed individually in Petri dishes (8.5 cm in diameter) and stored at 4 °C for 15 min to facilitate handling. The Petri dishes were then placed on a bed of ice until used. Each adult insect was held stationary at the end of a plastic pipette tip with a slight suction so that the gold wire could be affixed as follows: under a dissecting microscope (M 7; Wild Heerbrugg, Gays, Switzerland), an approximately 5-cm long gold wire (Ø 18.5 μm) was connected to the pronotum with a drop of silver glue (EPG Systems, Wageningen, The Netherlands). Once the insect was attached, the other end of the gold wire was connected to a copper electrode; also using silver glue. The electrode was then inserted into the EPG head stage amplifier and the insect was placed onto the plant. Finally, the copper substrate electrode (5 cm long, 2 mm in diameter) was put in the potting compost mix in which the cutting was growing. Once all the leafhoppers were connected to the recording apparatus, the Probe 3.5 software was run. Each insect's recording signal was adjusted individually according the 4 steps of the procedure described in the Giga-4/8 Manual to provide optimal resolution. In particular, each recording signal was adjusted individually in order that baseline was 0 V and extracellular stylet location waveform was 3–4 V.

Recordings were made simultaneously on four well-irrigated cuttings, each hosting one adult and used only once, for a period of 4 h. After each recording, cuttings were replaced by new ones. Visual observation of the insects was carried out at the same time as the recordings so that the signals could be better correlated with the insects' behavior.

2.3. Excretory droplet correlations with waveforms

To further define the meanings of waveforms C2x and C2p (respectively, putative xylem and phloem sap ingestion), pH of excreta produced during pathway and sustained ingestion was measured. A piece of pH paper sensitive to pH range 5–9 (Rotilabo AX03.1, Roth, Karlsruhe, Germany) was curved and put around each leafhopper in order to receive the excretory droplets ejected during putative ingestion waveforms. When a droplet was expelled to the pH paper, the time was scored, the pH of the excreta was measured (by color) and a new pH paper was put around the leafhopper. At the end of the 4 h recordings, the pH of the excretory droplets and the waveforms occurring during the droplet emissions were compared. To avoid any perturbation in the *S. titanus* feeding behavior, these tests were made, with both males and females, during 15 extra recordings not used for EPG signal analysis.

2.4. Interpretation of electropenetrograph waveforms

Signals were analyzed using the software Stylet + a (EPG Systems). Signals that were recorded for several insects, and several times in the same individual, were considered characteristic signals and were retained for analysis. In their study on the beet leafhopper, *Circulifer tenellus* Baker, 1896, Stafford and Walker (2009) showed that many of this insect's characteristic waveforms are similar to waveforms produced by other hemipterans studied with the DC-EPG system (at 10^9 Ω). In particular, waveforms associated with sap ingestion are very similar in appearance to waveforms produced by other leafhoppers. In her recent review, Backus (2016) provides an up-to-date list and description of the most important waveforms identified for sharpshooter leafhopper feeding, including all published waveform names and known biological meanings for all sharpshooter studied species. Thus, in the current study, interpretations of the waveforms were based primarily on waveform behavior correlations established for two Deltocephalinae species, *Cicadulina mbila* Naudé 1924 (Lett et al., 2001) and *C. tenellus* (Stafford and Walker, 2009) feeding, respectively, on maize and sugar beet plants, plus five sharpshooters on grape, *i.e.*, *Graphocephala atropunctata* Signoret 1854 (Almeida and Backus, 2004), *Homalodisca vitripennis* Germar 1821, *Homalodisca liturata* Ball 1901, and *Homalodisca coagulata* Say 1832 (Backus et al., 2005b, 2009; Backus and Morgan, 2011; Dugravot et al., 2008; Joost et al., 2006; Sandanayaka and Backus, 2008), and *Bucephalogonia xanthophis* Berg 1879 (Miranda et al., 2009).

2.5. Electrical characterization of waveforms

The frequency of repetition for each type of regularly repeating waveform (primarily C2) was measured according to the method used by Stafford and Walker (2009). Electrical signal characteristics of each waveform were measured from individual segments from numerous recordings, each recording from a different insect and plant. The frequency was measured using two randomly selected 1-s segments of each waveform, per insect. The amplitude of the fluctuations in voltage was measured on the same segments as the frequency. Each segment had relatively constant voltage amplitude. Some recordings did not show a specific waveform, or showed one only once. Therefore, the number of waveform events used for the characterization is below 120 (60 insects × 2 segments).

To be consistent in terminology and avoid confusion as much as possible, we used the hierarchical naming convention and similar names for waveforms introduced by Almeida and Backus (2004) and Backus et al. (2005b), then echoed by Joost et al. (2006), Backus et al. (2009), and Backus (2016). Although the above papers studied sharpshooter leafhoppers, our waveforms were remarkably similar in appearance, therefore justifying use of the same names.

3. Results

We identified six typical stylet probing-related EPG waveforms of *S. titanus* on grapevines associated with: stylet movements and likely secretion of the salivary sheath in the mesophyll tissues (A and B), acceptance of a vascular cell for ingestion (X wave), ingestion of xylem fluid (C2x), ingestion of phloem sap (C2p), and nonpathway-like interruption during ingestion (N). These waveforms were organized into four phases (pathway, X-wave, sustained ingestion, and interruption) and also certain waveforms were subdivided into families, types, sub-types or variants. The main characteristics of the waveforms are summarized in Fig. 1 and Table 1. At the beginning of most probes, the voltage level of waveforms fell sharply below 0 V as the stylets first penetrated the plant, then ascended gradually, after one to a few minutes, above 0 V. This "potential drop" is clearly evident on the enlarged views of the Figs. 2A, 3A, and 4A. The most negative waveforms were those associated with pathway (i.e. waveforms A and B1), but also sometimes waveform C2 (e.g. Fig. 1F, and H; Fig. 2A, and D). However, most C2 waveforms (both C2p and C2x) were located at a positive voltage level.

3.1. Pathway phase

After a period of non-probing phase (np), stylet insertion always progressed directly into pathway phase. Two broad families of waveforms were performed, A and B.

3.1.1. Waveform family A

Waveform A occurred first during stylet probing/penetration into the plant. This waveform family consisted of irregular peaks and valleys of highly variable amplitude (Fig. 2A–C and E). Performance of this waveform was intermittent; in some stylet penetra-

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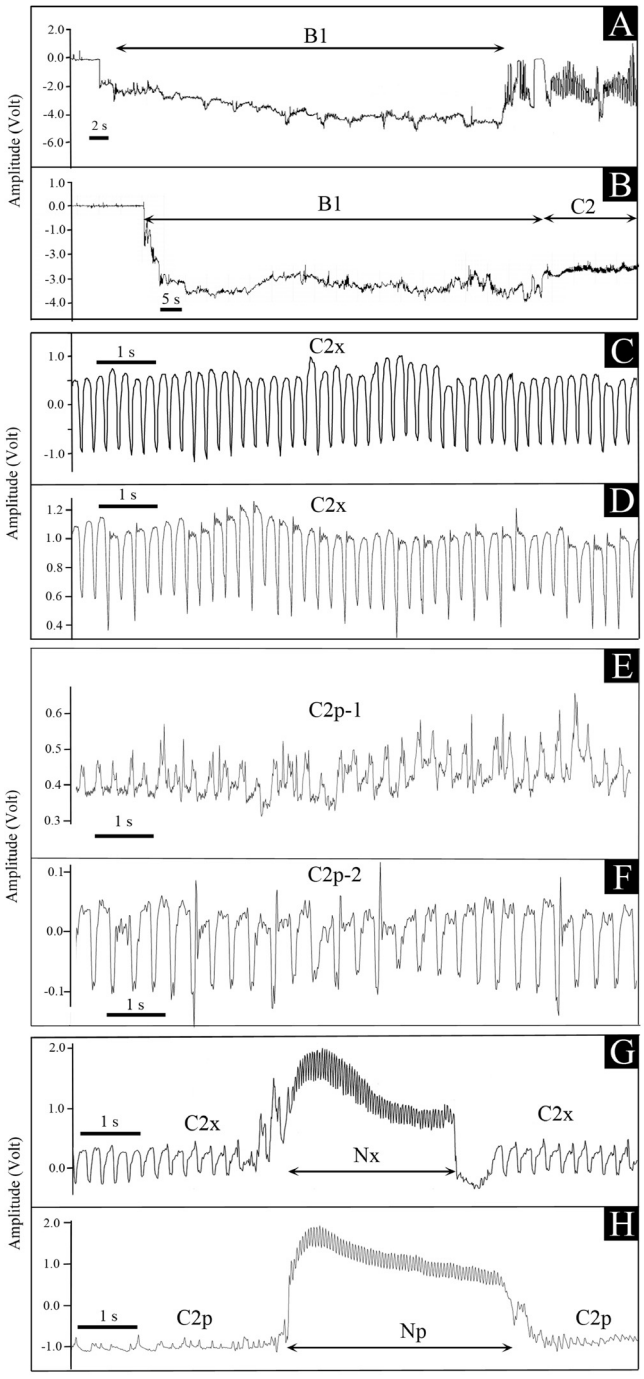

Fig. 1. EPG recordings of *Scaphoideus titanus* on grapevine. (A, B) waveform B1, tentatively correlated with stylet pathway phase in the epidermis and mesophyll; (C,D) waveform C2x, ingestion of xylem sap; (E, F) waveform C2x, ingestion from phloem sieve elements; (G, H) waveform N, nonpathway-like brief interruption within sap ingestion in xylem (G) or phloem (H), hypothesized to represent salivation in vascular cells.

tions it was omitted entirely, in others it was quite long. It was not possible to subdivide this family into types, such as A1 or A2.

3.1.2. Waveform family B, type B1

Waveform B1 often occurred first during stylet penetration into the plant (Figs. 1A and B, 2). This waveform was characterized by a potential drop, followed by a gradual increase to a higher relative amplitude signal (Figs. 2A, 3A). Its amplitude was highly variable and it fluctuated rapidly up or down in voltage level; rising, falling or stable (Fig. 2A, C and D). It was the most frequently recorded waveform, was observed for all the insects tested and was, on average, of short event duration (1.6 ± 10.1 min, Table 1). Waveform B1 could be followed by either np (termination of stylet penetration) or XC (see Section 3.2.2).

Unlike A, the structure of B1 was highly uniform, consisting of two sub-types. Sub-type B1s (for B1 spikes) consisted of rapid, very high frequency, low amplitude "spikelet bursts" (Fig. 2C and D). These spikelet bursts alternated with the second sub-type, B1w, composed of relatively flat or wave-like, irregular events (Fig. 2A, C and D). B1 (especially B1s) was the only waveform that was interspersed in all pathway events. No other waveform types (e.g., B2 of sharpshooters) were seen during *S. titanus* pathway.

3.2. X Wave phase

The X wave is a special, transitional phase that occurs between pathway and sustained ingestion. In *S. titanus* EPG recordings, it was composed of an alternating sequence of waveform types or sub-types (Figs. 3A and 4A). These sub-types were always consistently present, some of which were uniquely found only in X waves, not elsewhere in recordings. There were two, alternating waveform families in X waves, named XN and XC. The overall duration of all X waves was rarely higher than 5 min.

3.2.1. Waveform family XN

XN sometimes followed pathway, or (most often) was an interruption of sustained ingestion (see Section 3.3.1). The duration of XN events were generally short (10–60 s). XN was always composed of three components, B1s, fB1w, and C1 (Fig. 3B–D).

3.2.1.1. Waveform sub-type B1s. Appearance of the special B1s found in XN is very similar to B1s during pathway, except that amplitude of B1 spikes was usually higher, making these events more distinctive in recordings (Figs. 3B–E, 4B).

3.2.1.2. Waveform sub-type fB1w. Appearance of B1w in XN was diagnostic for an X wave, being quite different from B1w during pathway (Figs. 2A and D *vs.* 3B–E and 4D, E). We termed this special type fB1w, because it often (especially when preceding C2x during XC) was not flat like pathway B1w, but instead had a high-frequency, very low amplitude sine wave superimposed on it (therefore, it was termed "fuzzy" or fB1w). In addition (unique to *S. titanus* fB1w), there were intermittent, sharp, brief potential drops at irregular frequencies, throughout fB1w (Figs. 3B and C; 4D and E).

3.2.1.3. Waveform type C1. Sometimes at the beginning of XN, but always at the end of XN just before XC, a tall peak or series of peaks occurred (Figs. 2A, D and E, 3B–D). This peak resembled C2 (see next Section, 3.2.2.2), but was always strongly triangular/pointed, taller than C2 and always shorter in duration. This peak was termed C1.

3.2.2. Waveform family XC

Immediately following the C1 peaks at the end of XN, there occurred a continuous series of regular-frequency plateaus with various adornments in fine structure. Despite differences in fine structure, all of these plateaus were classified as C2 because they had a consistent frequency of 3–6 Hz. (Table 1; Figs. 1C–F; 2A, D and E; 3A and E; 4A–F).

3.2.2.1. Waveform sub-type C2x. Waveform C2x was present in more than 80% of the recordings (50/60) and occurred after XN (Fig. 3A and E) or B (i.e., briefly during pathway without a preceding XN; Fig. 3A; see Section 3.2.2.3, below). C2x was a stereotypical

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Table 1
Summary of the main characteristics of DC-EPG waveforms for *Scaphoideus titanus* on grapevine.

Waveform	N_w[a]	Amplitude[b] (%)	Amplitude range (%)	Frequency (Hz)	N_t[c]	Mean duration ± SE of individual events	Putative tissue[d]
A	94	Variable	Variable	2–12	382	3.5 ± 1.2 (min)	Epidermis and mesophyll
B	60	100	1.91–13.31	Mixed	2476	1.6 ± 0.1 (min)	Epidermis and mesophyll
C2x	103	16.6	5.1–50.9	3–6	66	25.9 ± 7.0 (min)	Xylem
C2p	84	3.3	0.9–8.7	3–6	299	28.6 ± 6.2 (min)	Phloem
N	86	7.6	0.6–23.3	15–34	188	12.8 ± 6.6 (s)	All tissues

A, **B**, stylet pathway in the epidermis and mesophyll; **C2x**, xylem-related activities; **C2p**, phloem-related activities; **N**, nonpathway-like interruption in Xylem or phloem.
[a] Number of waveform records (for C2x, C2p, and N) used for the amplitude and frequency calculations; see Section 2 for details.
[b] Relative amplitude in proportion to the waveform with maximum amplitude (B = 100%).
[c] Total number of waveform events recorded from 60 insects monitored for 4 h.
[d] Tissue location is deduced from previous studies (Backus, 2016).

pattern of rounded plateaus (i.e., stereotypically-sized, usually rounded or slightly-peaked rectangles) and valleys repeating at a very constant rate (3–6 Hz, depending on individual insect) (Figs. 1C and D; 3A). Plateau average amplitude was 16.6% of the highest amplitude (B) (Table 1). The shape of waveform C2x was highly constant even if its amplitude sometimes decreased within the same event, and changed into a form with lower amplitudes (Fig. 1D).

3.2.2.2. Waveform sub-type C2p. Waveform C2p occurred after XN (Figs. 2A, 4A–E) or B (again, briefly during pathway without preceding XN; Fig. 4A) and was observed in 65% (39/60) of the recordings (Figs. 1E and F; 4). This waveform, like C2x, had a 3–6 Hz frequency (depending on individual insect), and was usually preceded by a XN portion of typical waveform sub-types B1s, B1w, or C1 (Figs. 2A; 4B–E). However, contrary to C2x waveform, C2p presented varying appearances: lower average amplitudes (3.3% of A, Table 1) and a fairly regular succession of the variable signal shapes (Fig. 1E and F). We called the irregularly shaped C2p with variable amplitudes C2 variant 1, or C2p-1 for short (Fig. 1E). We termed C2 variant 2, or C2p-2 for short, the most regularly shaped C2p, with stereotypical, rounded plateaus resembling C2x but with "ruffles" (like spikelets) on the top of the plateaus and also occasional spikes on the leading edges of plateaus (Fig. 1F). Over time within a probe, as waveforms progressed from one XC event to another, the appearance of C2p evolved in appearance from variant 1 to variant 2. C2p event durations (for either variant) varied from some seconds to many minutes; eventually, after several X waves, we observed the longest (sustained) C2p (see Section 3.3, below).

3.2.2.3. Pathway XC. Most *S. titanus* produced a lone XC (C2) event during pathway that was not preceded by an XN event (Fig. 3A). We termed this lone pathway XC a "partial X wave." Only full XN + XC sequences were considered "true X waves." Similarly, it was common with *S. titanus* that the first XC in a series of true X waves was not preceded by an end-of-pathway XN; thus, a partial X wave was often performed before a series of true X waves.

Excretory droplets were collected from individual insects during performance of both pathway XC and sustained ingestion. C2p (both variants 1 and 2) from XC events and sustained ingestion phase were correlated with slightly alkaline excretory droplets (pH range [7.5–8] for 38 measurements made on seven individuals), supporting that both C2p-1 and C2p-2 represented ingestion from phloem sieve elements. C2x from XC events and sustained ingestion phase had an acidic pH (pH range [6–6.5] for 24 measurements made on five individuals), supporting that C2x ingestion was from xylem tracheary elements. Thus, excretory droplet pH correlation corresponded to C2x or C2p appearance, regardless of whether each was or was not preceded by XN (i.e., pH was a correlate for vascular tissue type, regardless of partial or true X wave).

3.3. Sustained ingestion phase

3.3.1. Waveform sub-type C2x

Eventually, after several repeated X waves, longer (few minutes to more than 2 h, see Section 3.2) C2x events were observed without interruption by XN. Termed sustained ingestion, these C2x events looked the same but were longer-duration than C2x in XC, with gradually decreasing plateau amplitude until it reached a stable level. Mean event duration of C2x in sustained ingestion was >25 min (Table 1).

3.3.2. Waveform sub-type C2p

Sustained ingestion in phloem was similar to C2p in X waves (Fig. 4A, fourth inset box), but again, these events were longer-duration events, with gradually decreasing amplitude. C2p-2 dominated sustained ingestion events, while C2p-1 dominated XC events. Mean event duration of C2p in sustained ingestion was >28 min (Table 1).

3.4. Interruption phase (not X wave)

3.4.1. Family N, waveform types Nx and Np

As described under X wave phase, short events of C2 waveforms were interspersed with XN waveforms early in a stylet probe, and together these XN and XC events were considered X waves. However, unique to *S. titanus*, longer C2 events of sustained ingestion also were sometimes interspersed with another type of waveform, nonpathway-like interruption (N). Waveform N was characterized by a rapid increase in voltage level, following by a highly regular and constant event of low amplitude signal (7.6% of A) with very high frequency (15–34 Hz, Table 1). N events had a mean duration of <13s (Fig. 1G and H). The end of the N waveform was characterized by a rapid decrease of potential and the beginning of a new C2x or C2p waveform, depending on the previously-interrupted waveform (respectively, C2x and C2p). Waveform N occurred in about 68% (41/60) of the recordings. N waveforms were named Np or Nx, depending on whether they interrupt sustained C2p or C2x, respectively.

4. Discussion

This is the first EPG study dedicated to the vector of the most important phytoplasma disease in European viticulture, *Scaphoideus titanus*, an invasive species introduced from North America. We characterized four typical waveform phases (pathway, X wave, sustained ingestion, and interruption) and four families within those phases (A, B, C, and N) using DC EPG technology. Below we propose biological meanings for these waveforms based on honeydew production, existence of X waves, and comparison with previous AC, DC and AC-DC EPG waveform analyses conducted on Cicadomorpha (*sensus* Bourgoin and Campbell, 2002).

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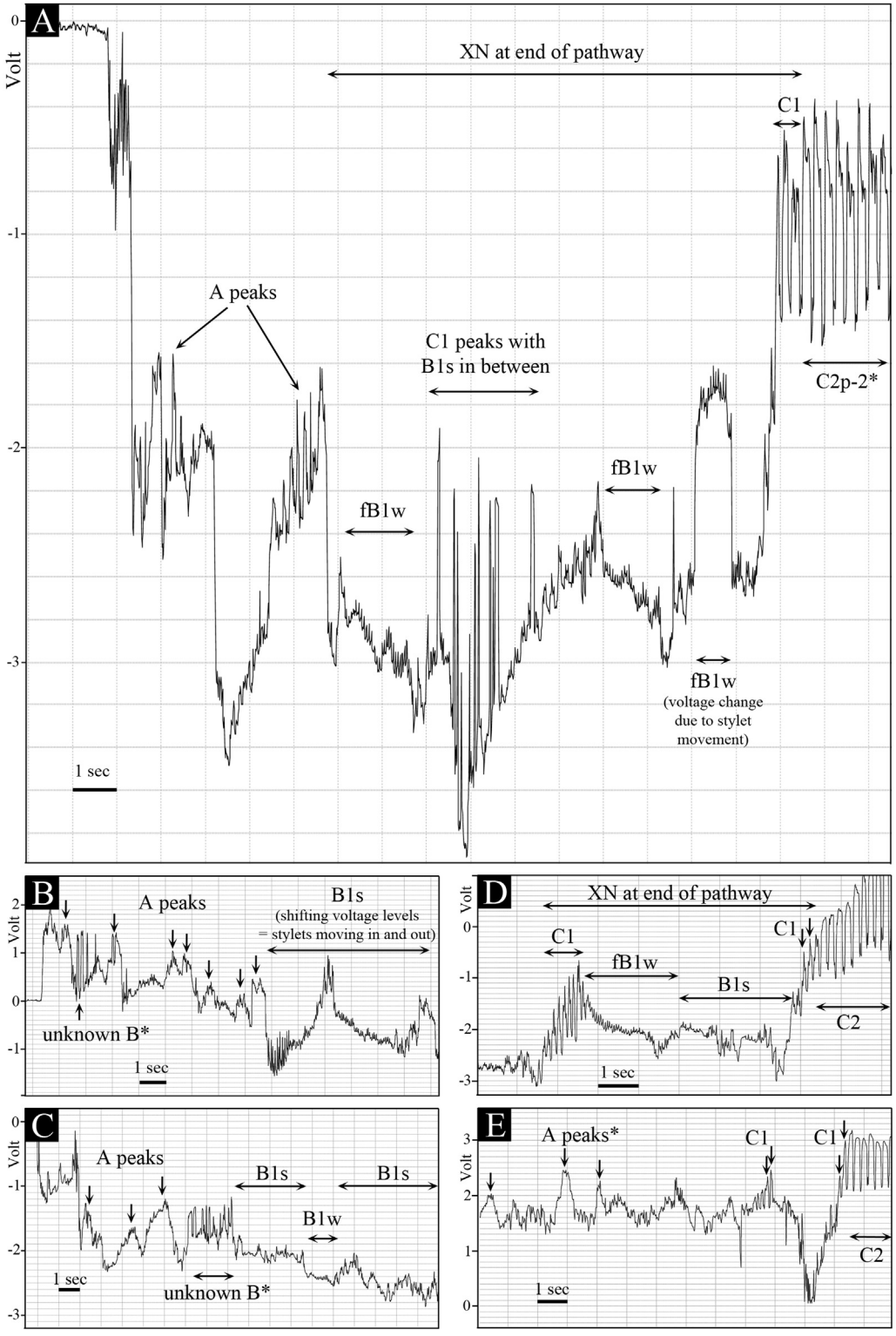

Fig. 2. First example of EPG waveforms representing stylet penetration by adult *S. titanus* on grapevine. (A) Fine structure views of a 16s recording in pathway phase, showing waveforms A (tentatively correlated with formation of the solid trunk of the salivary sheath), B1w (possibly salivation), B1s (possibly rinsing egestion [fluid outflow from the stylet tips] via precibarial valve fluttering or cibarial quivering), and C1 (possibly representing discharging egestion). C2p-2* (ingestion of phloem fluid); (B, C) Fine structure views of 15–20 s recordings at the beginning of a pathway phase showing A peaks, B1s and B1w; (D, E) Fine structure views of 8–12 s recordings at the end of a pathway phase showing A peaks, B1s, B1w, and C1 just before a ingestion phase (C2). * waveforms in this specific view that are tentatively named.

Our work demonstrated that, as predicted by Backus and Bennett (2009), understanding the amplifier sensitivity (input resistor or Ri value) of a particular version of EPG monitor unlocks comparative understanding of EPG waveforms recorded by different versions of monitor. The AC-DC monitor has been used as a bridge to promote inter-monitor waveform characterization. The present work builds on that new process in constructive ways,

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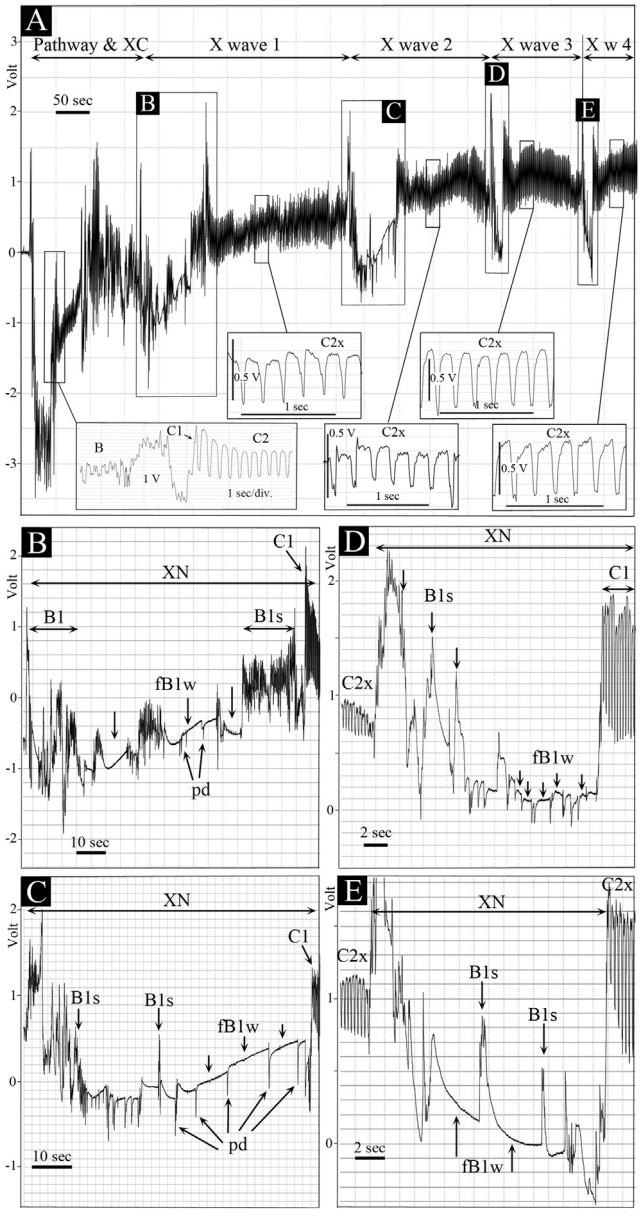

Fig. 3. Second example of EPG waveforms representing stylet penetration by adult *S. titanus* on grapevine. (A) Overview of a 13 min recording, showing four X waves in xylem preceded by pathway phase. Insets show amplified (y-axis) and expanded (x-axis) views to reveal the fine structure of waveforms during a pathway phase or during the ingestion in xylem (C2x); (B to E) Fine structure views of ingestion interruptions in xylem, named XN, showing waveforms fB1w (possibly salivation), B1s (possibly rinsing egestion via precibarial valve fluttering or cibarial quivering), pd (potential drop), and C1 (representing discharging egestion).

by backwards-applying new insights to waveforms recorded via DC EPG.

4.1. Pathway phase

Pathway phase is ubiquitous in deltocephaline leafhopper EPG, because these insects are obligate producers of a salivary sheath, and pathway has been consistently found to represent salivation (both watery and gelling saliva), formation of the salivary sheath, and stylet movements as they progress from outer, epidermal layers of the plant into internal layers.

4.1.1. Waveform family A

In our recordings, stylet probing/penetration began most of the time with a rapid drop in voltage. This is a common occurrence in the literature, observed for DC EPG (Miranda et al., 2009) and AC-DC EPG (Backus et al., 2012) leafhopper recordings when the Ri (input resistor) level (or amplifier sensitivity) is $10^9\ \Omega$ (which emphasizes emf [electromotive force]/biopotential components of the waveforms). DC monitors have a fixed Ri of $10^9\ \Omega$; AC-DC has variable Ri. Negative-going voltages recorded at those high Ri levels indicate intracellular penetration, as is well-known to occur in Cicadomorpha.

Following the initial potential drop, waveform A sometimes (but not always) occurred in *S. titanus* EPG. Waveform A has been primarily characterized from the sharpshooter (cicadelline) leafhopper recordings (several species) (Backus, 2016; Backus et al., 2005b, 2009; Joost et al., 2006), and is best visualized at lower Ri levels like 10^6 (AC monitor) or $10^7\ \Omega$ (AC-DC) because it is a strongly R (resistance)-dominated signal. At those Ri levels (lower than the $10^9\ \Omega$ level of the DC system used herein), A is composed of very tall, broad peaks that are consistently the highest amplitude signals in the recording, and can be separated into A1 (tallest) and A2 (much shorter) peaks (Backus et al., 2005b). When sharpshooter A waveforms are recorded at $10^9\ \Omega$ they become strongly distorted due to loss of R components at low Ri (E.A.B., unpub. data). Distortion causes either attenuation of signal amplitude or complete loss of A waveforms; as described further by Backus (2016) this distortion explains the lack of A in the B-dominated pathway recordings of Miranda et al. (2009). In similar fashion, the present *S. titanus* recordings showed attenuated, presumably distorted A peaks of variable amplitude, due to recording at $10^9\ \Omega$ by a DC monitor.

Sharpshooter A peaks have been histologically correlated with formation of the solid trunk of the salivary sheath, in which the outer, mandibular stylets are braced (Backus et al., 2005b, 2009). We propose a similar meaning for the A waveform of *S. titanus*.

4.1.2. Waveform family B

S. titanus waveform B shows very strong similarity to the waveforms described from other Cicadomorpha, for example, at Ri $10^9\ \Omega$ with DC EPG system: waveform w1-w3 complex, described for the planthopper *C. mbila* (Lett et al., 2001), waveform A-B-B1-B2-C complex described for *C. tenellus* (Stafford and Walker, 2009; Stafford et al., 2009), and waveform S described for the sharpshooter *B. xanthophis* (Miranda et al., 2009). At Ri $10^9\ \Omega$ (and lower Ri levels) with the AC-DC EPG system, extensive recordings have shown B waveforms nearly identical in appearance. Similar waveforms have also been seen using Ri $10^6\ \Omega$ using the AC EPG system: e.g., waveform 1 described for the leafhopper *Dalbulus maidis* DeLong 1923 (Carpane et al., 2011). In all cases, B-like waveforms have been correlated with stylet pathway phase in the epidermis and mesophyll; characterized by salivary sheath production, mechanical advancement of the stylets, and other activities not directly involved in ingestion. The sharpshooter correlations (Backus et al., 2005b, Backus, 2016) further characterized B as representing extension of salivary sheath branches using only the maxillary stylets, as is typical of deep penetration in Cicadomorpha.

4.2. X wave phase

The existence of X waves has been well documented in leafhopper EPG for many years, starting with the work of Wayadande and Nault (1993) and Triplehorn et al. (1984), and continuing most recently with extensive recordings of deltocephaline leafhoppers (Stafford and Walker, 2009; Carpane et al., 2011) and cicadelline (sharpshooter) leafhoppers (Backus, 2016 and others). X waves

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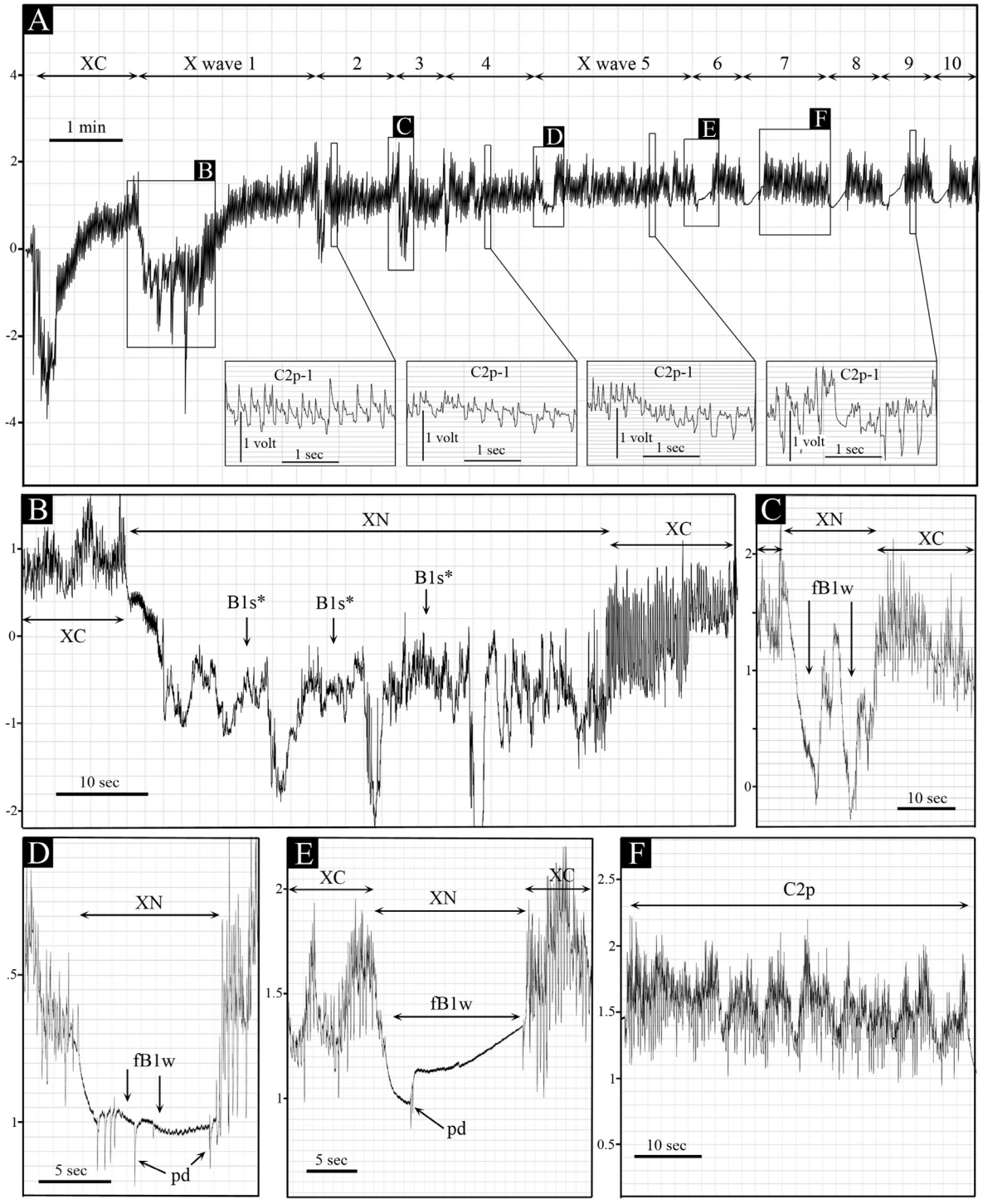

Fig. 4. Third example of EPG waveforms representing stylet penetration by adult *S. titanus* on grapevine. (A) Overview of a 14 min recording, showing ten X waves in phloem. Insets show amplified (y-axis) and expanded (x-axis) views to reveal the fine structure of ingestion waveforms in phloem, C2p-1; (B-E) Fine structure views of ingestion interruptions in phloem, named XN, showing waveforms B1s (possibly rinsing egestion), fB1w (possibly salivation), and pd (potential drop); (F) Overview of a 50 s recording during a XC event (second part of an X wave), showing waveforms C2p-1 (ingestion in phloem). * = waveform in this view only that are tentatively named.

are important in hemipteran EPG research because they are thought to represent the behavioral process of acceptance of a preferred plant tissue for sustained ingestion (Backus et al., 2009; Backus, 2016; Carpane et al., 2011; McLean and Kinsey, 1967; Wayadande and Nault, 1993). They represent sensory testing of a (primarily) vascular cell and the process of overcoming the defensive mechanisms of the plant to achieve sustained ingestion. Thus, finding of an X wave in an EPG recording of leafhopper feeding has meant the unequivocal sign of sustained ingestion from (in the case of leafhoppers) a vascular cell. By definition (Backus et al., 2009 and references therein), an X wave must be species-specific in appearance and must precede sustained ingestion.

Our EPG study is the first to identify a deltocephaline leafhopper X wave that is very similar in appearance to that of cicadelline (sharpshooter) leafhoppers; hence our use of the same waveform names as those first proposed by Backus et al. (2005b).

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In the Wayadande and Nault (1993) study of deltocephaline X waves using AC EPG (therefore, Ri of $10^6\ \Omega$), the appearances of X waves from 10 different species of deltocephaline leafhoppers varied greatly by species, and also were different from the later-characterized X waves of sharpshooter (cicadelline) leafhoppers (Backus, 2016). We propose that these differences were due to the greater amount of R component present in AC EPG (due to lower Ri level; Backus, 2016). Therefore, there may be greater similarities in X wave appearance among species when recorded at higher Ri levels, as with a DC and AC-DC EPG system, due to dominant recording of emf component. Accordingly, X waves may be less species-specific when recorded at high Ri than when recorded at low Ri.

4.2.1. Waveform family XN

The first section of the *S. titanus* X wave, XN, bears a striking resemblance to the XN of sharpshooters (Backus, 2016), with three waveform components, B1s, fB1w, and C1. B1s and C1 are nearly identical in appearance to those of sharpshooters, however, fB1w is quite different. XN preceding C2p lacks the high-frequency "fuzziness" (the "f" of fB1w) (being nearly flat), whereas the high-frequency "fuzz" for XN preceding C2x is much higher amplitude than the fB1w of sharpshooters. In addition, *S. titanus* fB1w has intermittent, frequent, sharp potential drops of unknown meaning. Thus, despite the similarity of *S. titanus* and sharpshooter XN, there are also species-specific characteristics as well.

According to hypotheses supported by sharpshooter studies (Backus, 2016), B1s is thought to represent fluttering of the precibarial valve and/or quivering of the cibarial diaphragm sufficient to take up tiny amounts of plant fluid into the precibarium for tasting by the precibarial chemosensilla (Backus, 1988). B1w has been correlated with salivation (Joost et al., 2006). Thus, the "fuzzy" version of B1w (fB1w) is thought to represent simultaneous secretion of saliva (perhaps watery saliva) and fluttering/quivering uptake of plant fluid. C1 is thought to represent rapid, discharging egestion of fluid from the precibarium (Backus, 2016). Therefore, XN as a whole may likely represent sequential uptake/tasting/testing/expulsion of fluid in a vascular cell, prior to trial ingestion. We propose that the same behaviors occur during *S. titanus* XN, because the histological and other evidence underlying this interpretation of XN now applies to *S. titanus*.

4.2.2. Waveform family XC

XC events are very short, followed immediately by another iteration of XN. This alternating XN-XC-XN-XC series is similar to a sharpshooter X wave; thus, we propose that XC represents short events of trial ingestion (Almeida and Backus, 2004) for the purpose of testing the mechanical strength of the salivary sheath connection into a vascular cell, prior to onset of sustained ingestion (Backus, 2016). For the first time in any leafhopper EPG study, we have identified two types of trial (and sustained) ingestion during X waves, from both phloem and xylem.

4.2.2.1. Waveform sub-type C2x. Waveform C2x is identical in appearance to the 'active xylem ingestion' waveforms recorded in previous DC EPG studies of both Cicadomorpha and Sternorrhyncha, e.g. produced by aphids (waveform G: Prado and Tjallingii, 1994), whiteflies (waveform G: Lei et al., 1999), the mealybug *Planococcus citri* (Risso, 1813) (waveform G: Cid and Fereres, 2010), the planthopper *Peregrinus maidis* (Ashmead, 1890) (waveform class 2: Lei et al., 1999), the sharpshooter *B. xanthophis* (waveform Xi: Miranda et al., 2009), and the leafhoppers *C. mbila* and *C. tenellus* (respectively waveforms w2 and G: Lett et al., 2001; Stafford and Walker, 2009). It is also nearly identical to the highly regular, squared valley-and-plateau C (later renamed C2) waveform of sharpshooters, recorded with both AC EPG at low Ri (Almeida and Backus, 2004; Backus et al., 2005b) and AC-DC EPG at higher Ri (Joost et al., 2006 Backus et al., 2009). Most of the above studies performed extensive histological correlations showing salivary sheath termini in xylem cells. In our work, the production of acidic pH excretory droplets during *S. titanus* C2x also supported xylem ingestion because sap-feeding insects produce excreta while they ingest, and slightly acidic pH is typical to xylem ingestion (Walker, 2000). Therefore, the preponderance of evidence strongly supports that *S. titanus* C2x represents ingestion of xylem fluid.

4.2.2.2. Waveform sub-type C2p, variants 1 and 2. The *S. titanus* waveform C2p variant 2 (C2p-2) is very similar in appearance to phloem-ingestion waveforms recorded in previous DC EPG studies of both Cicadomorpha and Sternorrhyncha, e.g. waveform E2 produced by aphids (Prado and Tjallingii, 1994), waveform E(pd)2 produced by whiteflies (Lei et al., 1999), waveform 5a produced by the leafhopper *C. mbila* (Lett et al., 2001), and waveform D2 produced by leafhopper *C. tenellus* (Stafford and Walker, 2009). In addition, it is similar to AC-DC EPG recordings of phloem ingestion by *D. maidis* (Carpane et al., 2011). The production of slightly basic honeydew during C2p (both variants 1 and 2) supports that a phloem ingestion is correlated with this waveform (Walker, 2000). Low signal amplitude for C2p-2 also supports (at least partially) passive ingestion. Thus, we propose that C2p-2 represents ingestion from phloem sieve elements.

The shape variation in the observed C2p signals may be partly explained by the different activities performed inside the phloem. Thus, the waveform C2p variant 1 (C2p-1) could correspond to the waveform 5b described by Lett et al. (2001) for *C. mbila*, and waveform D3 to that described by Stafford and Walker (2009) for *C. tenellus*. The similarity of these waveforms to the phloem salivation waveforms of aphids and whiteflies (respectively E1: Prado and Tjallingii, 1994, and E(pd)1: Lei et al., 1999) supports (as first suggested by Lett et al., 2001) the possibility of concurrent ingestion and salivation during waveforms D3 and 5b of these two leafhoppers, as well as C2p-1 in *S. titanus*.

4.3. Sustained ingestion phase

Ours is the first DC EPG study to demonstrate that sustained ingestion by a deltocephaline leafhopper can occur from both xylem and phloem vascular cells, although this was a frequent finding of earlier AC EPG studies (reviewed in Wayadande, 1984). Our evidence for this finding is as follows: 1) Unequivocal evidence of an X wave in *S. titanus* recordings that is both species-specific (in part, even when recorded at high Ri level), and always precedes sustained ingestion, 2) correlation of C2p-derived excreta with acidic pH, therefore phloem, and C2x-correlated excreta with neutral-to-alkaline pH, therefore xylem, and 3) comparison with the appearances of other xylem- and phloem-ingestion waveforms from hemipterans whose waveforms have been histologically correlated.

We find the *S. titanus* X wave appearance of particular interest because it resembles the X wave of leafhopper species from an entirely different subfamily, Cicadellinae, which are known to be obligate xylem-ingesters. This similarity in appearance further supports that sustained xylem ingestion occurs in *S. titanus*, and also supports a further search for Cicadomorphan X waves that resemble those of sharpshooters. There may be greater similarity among Cicadomorphan X waves than was originally thought, at least when recorded at high Ri levels. Although our study is the first to identify a dual-purpose, phloem- and xylem-indicating X wave, it is unlikely to be last such finding.

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4.4. Voltage level reversal

Our leafhopper EPG waveforms demonstrate a reversal of voltage level for pathway versus phloem activity waveforms, an interesting finding compared with EPG waveforms from aphids and other sternorrhyncha. Indeed, to our knowledge, a potential drop at the beginning of stylet penetration into pathway activities, seen in most of our recordings, has never been described for sternorrhynchans. As explained in more depth in Backus (2016), aphid waveforms are stereotypical for voltage level. Using DC EPG (with fixed input resistance [Ri] of $10^9\ \Omega$), pathway waveforms begin with a rise in potential and continue above 0 V. Based on the principle that the voltage level reflects the electrical charge surrounding the stylets tips at high Ri (Backus, 2016), such a positive voltage level has been termed "extracellular" because aphid stylets are located in the apoplastic space between cell walls during their intercellular-type stylet probing. Once the stylets penetrate through the cell membrane into a living cell, the voltage level falls to below 0 V to a negative, "intracellular" voltage level, because the symplastic interior of the cell is negatively charged (Walker, 2000). Thus, extracellular pathway for aphids "rides" at a positive voltage level, while intracellular phloem activities ride at a negative voltage level.

Several authors have observed a reversal of voltage levels with both cicadomorphan and heteropteran EPG waveforms, identical to what we observe with *S. titanus*. Like our study, these recent papers have performed correlations using both histology and excretory droplet analysis, supporting the strength of the biological meanings of their waveforms. For example, in their study of the sharpshooter *B. xanthophis*, Miranda et al. (2009) describe a waveform S (pictured in their Fig. 1A and B), that was always the first during stylet penetration for all recorded individuals, and always occurred at negative voltage levels (in most recordings) lower than those observed for the other waveforms. This S waveform (identical to our and other sharpshooters' B1 waveform [Backus, 2016]) was histologically correlated with intracellular stylet penetration during pathway, similar to that in other species of cicadomorphans (Backus, 1988). More recently, Backus (2016) describes a sharpshooter C2 waveform that starts below 0 V (pictured in her Fig. 4A), then gradually rises in voltage level, and later crosses above 0 V after 3.4 h. In addition, short potential drops were also observed at the onset of stylet penetration by the delphacid planthopper *P. maidis* (Buduca et al., 1996) with negative pathway waveforms. Indeed, a larger review of the EPG literature shows that the stylet probes of cicadamorphan and most pentatomomorphan heteropteran species recorded at Ri of $10^9\ \Omega$, regardless of whether the studies used DC EPG or AC-DC EPG, usually begin with a potential drop and subsequent pathway is at a negative voltage (e.g., Backus et al., 2013; Lucini and Panizzi, 2016a; Lucini et al., 2016). In contrast, cimicomorphan heteropterans like *Lygus* spp. and some pentatomomorphans show the typical sternorrhynchan-type of voltage levels, with positive-level pathway at Ri $10^9\ \Omega$ (Lucini and Panizzi, 2016b; Cervantes et al., 2016).

Several of the above studies show images of salivary sheaths. According to these observations, there appears to be a relationship between thickness of the salivary sheath and EPG waveform voltage level. Voltage reversal seems to occur for insects that make robust salivary sheaths (cicadomorphans [like sharpshooters] and certain pentatomomorphan heteropterans). Aphid-like voltage levels (with pathway positive rather than negative) are associated with the non-sternorrhynchan insects that make very thin or non-existent salivary sheaths (certain pentatomophoran and cimicomorphan heteropterans, respectively). We hypothesize that this variability in EPG waveform voltage levels may be due to the electrical properties of intracellular stylet penetration (with and without salivary sheaths), as is performed by all cicadomorphans and heteropterans (Backus, 1988), compared with those of intercellular penetration by aphids (Walker, 2000).

Shifts in voltage level at high Ri settings occur because highly sensitive amplifiers detect charge separations (i.e., biopotentials) very near the stylet tips. During intercellular stylet penetration by aphids, the delicate aphid stylets and their thin salivary sheath are shielded from the negative charge of the plant cell interior by the cell wall within which the stylets and sheath are embedded. In contrast, during intracellular stylet penetration, the wider, blunter stylets of cicadamorphans and heteropterans come into direct electrical contact with the negatively charged interior of a living, symplastic plant cell (although not in the case of a xylem cell because it is non-living, apoplastic). If the salivary sheath is very thick and robust, it is possible that it can seal the cell membrane breakages caused during intracellular stylet penetration, retaining charge separation and the interior-negativity of a living cell. Thus, because the stylets are directly inside symplastic space (rather than inside the positively-charged apoplastic space like those of an aphid), the pathway activities are negatively charged if the salivary sheath is robust. Likewise, xylem ingestion waveforms gradually rise above 0 V into the positive voltage level over time, as shown in the present study and sharpshooter EPG, because the salivary sheath solidifies and charge separation may be restored after puncturing. However, an intracellular-probing insect that produces no salivary sheath (like *Lygus* spp., Cervantes et al., 2016), or a thin sheath (like *Edessa meditabunda*, Lucini and Panizzi, 2016b) would shred the cell membrane when it probes a living cell, destroying charge separation and thus causing all cellular spaces, apoplastic and symplastic, to become positively charged. Thus, voltage levels would not indicate extracellular versus intracellular stylet position in these non-aphid insects. It seems likely that stylet position cannot be reliably determined exclusively by electrical means for non-sternorrhynchan insects.

4.5. Interruption phase (not X wave)

Waveform N consisted of brief (< 15s) interruptions within sap ingestion in xylem (C2x, therefore termed Nx) or phloem (C2p, therefore termed Np). Contrary to XN that was also an interrupting sap ingestion waveform, N did not resemble any pathway waveform. A similar waveform is observed during *C. tenellus* ingestion of sugar beet phloem (waveform D1: Stafford and Walker, 2009). That study suggested that N represented watery salivation during ingestion, and we agree with this suggestion.

4.6. Implications for S. titanus transmission of plant pathogens

Scaphoideus titanus is the vector of flavescence dorée (FD), the most important phytoplasma pathogen of grape in Europe. As phloem-limited pathogens that are propagative, and persistent in their vectors, phytoplasmas are thought to be acquired during ingestion, especially sustained ingestion, from phloem (Weintraub and Beanland, 2006). As circulative pathogens, phytoplasmas are also thought to be inoculated into healthy plants via salivation into phloem from salivary glands infected during the latent period, when phytoplasmas become resident in most organs of the vector body. Thus, our work is important for future, continuing research on FD, because we propose that we have now identified the EPG waveforms responsible for acquisition (C2p variant 2; C2p-2) and inoculation (C2p variant 1; C2p-1) of the FD pathogen.

The finding of sustained xylem ingestion by *S. titanus*, represented by waveform C2x, especially that it is preceded by a sharpshooter-style X wave, also allows us to speculate about a second, potential role as a vector for *S. titanus*. *Xylella fastidiosa* is a bacterial agent, some of whose sub-species can cause the lethal

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Pierce's disease of grape in North America. Transmitted by sharpshooter leafhoppers, *X. fastidiosa* is proposed to be acquired during the xylem ingestion waveform, C2 in sharpshooters, and inoculated during the sharpshooter X wave, especially during egestion represented by B1 and C1 (Backus et al., 2015; Backus, 2016); the same references have discussed the possibility that any hemipteran that produces a sharpshooter-style X wave prior to xylem ingestion might have the potential to become a vector of *X. fastidiosa*. Because *S. titanus* is an introduced species that evolved in North America, the native home also of *X. fastidiosa* (a highly ubiquitous epiphyte in North American plants), it is possible that *S. titanus* and *X. fastidiosa* co-evolved on that continent. *X. fastidiosa* ssp. pauca and ssp. multiplex were recently introduced into Italy and France, respectively (European Food Safety Authority, 2013; Jacques et al., 2016). Although neither of these subspecies are presently documented to cause Pierce's disease in grape, future introductions of additional subspecies/strains of *X. fastidiosa* are possible. Should a grape xylellae pathogen be introduced into European vineyards, *S. titanus* might be a potential vector, already present in the vineyards. Continuing study of the feeding behaviors of *S. titanus*, like all vector insects, is therefore highly warranted.

Acknowledgments

This study was supported by the French National Institute for Agricultural Research (Inra, http://www.inra.fr), the Conseil Interprofessionnel du Vin de Bordeaux (CIVB, http://www.bordeaux.com) and the Région Aquitaine (http://www.aquitaine.fr). Efforts by E. A. Backus were supported by in-house funding from the Crop Diseases, Pests, and Genetics Research Unit of the USDA Agricultural Research Service.

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Please cite this article in press as: Chuche, J., et al. First finding of a dual-meaning X wave for phloem and xylem fluid ingestion: Characterization of *Scaphoideus titanus* (Hemiptera: Cicadellidae) EPG waveforms. Journal of Insect Physiology (2017), http://dx.doi.org/10.1016/j.jinsphys.2017.01.013