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Mixed xylem and phloem sap ingestion in sheath-feeders as normal dietary behavior: Evidence from the leafhopper *Scaphoideus titanus*

Julien Chuche [a,b], Nicolas Sauvion [c], Denis Thiéry [a,*]

[a] INRA, UMR 1065 Santé et Agroécologie du Vignoble, Bordeaux Sciences Agro, ISVV, 33882 Villenave d'Ornon Cedex, France
[b] Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland
[c] INRA, UMR 0385 Biologie et Génétique des Interactions Plantes-Parasites, Campus International de Baillarguet, Montpellier, France

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ABSTRACT

In phytophagous piercing-sucking insects, salivary sheath-feeding species are often described as xylem- or phloem-sap feeding specialists. Because these two food sources have very different characteristics, two feeding tactics are often associated with this supposed specialization. Studying the feeding behavior of insects provides substantial information on their biology, ecology, and evolution. Furthermore, study of feeding behavior is of primary importance to elucidate the transmission ability of insects that act as vectors of plant pathogens. In this study, we compared the durations of ingestion performed in xylem *versus* phloem by a leafhopper species, *Scaphoideus titanus* Ball, 1932. This was done by characterizing and statistically analyzing electrical signals recorded using the electropenetrography technique, derived from the feeding behaviors of males and females. We identified three groups of *S. titanus* based on their feeding behavior: 1) a group that reached the phloem quickly and probed for a longer time in phloem tissue than the other groups, 2) a group that reached the xylem quickly and probed for a longer time in xylem tissue than the other groups, and 3) a group where individuals did not ingest much sap. In addition, the numbers and durations of waveforms representing ingestion of xylem and phloem saps differed significantly depending on the sex of the leafhopper, indicating that the two sexes exhibit different feeding behaviors. Males had longer phloem ingestion events than did females, which indicates that males are greater phloem feeders than females. These differences are discussed, specifically in relation to hypotheses about evolution of sap feeding and phytoplasma transmission from plant to plant.

1. Introduction

Since at least fifty years and the Miles' seminal work (1972), it was recognized that all hemipterans (i.e., piercing-sucking insects) use one of two different strategies: sheath feeding and cell rupture feeding (*sensus* Backus et al., 2005). The latter strategy concerns mesophyll feeders (i.e., insects essentially feeding in mesophyll), and have been described in the Typhlocybinae, a Cicadellidae (leafhoppers) subfamily (Marion-Poll et al., 1987; Hunter and Backus, 1989; Backus et al., 2005; Saguez et al., 2015). Most phytophagous hemipterans use the salivary sheath feeding strategy, and ingest sap as their main or unique source of nutrients (Backus, 1988; Press and Whittaker, 1993). According to the targeted cell compartment, two feeding categories are usually distinguished for sheath-feeders: 1) phloem sap-sucking insects (termed phloem-feeders), such as aphids, whiteflies, mealybugs, scale insects, psyllids, and many planthoppers and leafhoppers; and 2) xylem sap-sucking insects, including cicadas, spittlebugs, and sharpshooter leafhoppers (Almeida et al., 2005; Dolling, 1991).

Because plant defence secondary metabolites to sap-feeders are mostly located in phloem, phloem feeders confronted to resistant varieties decrease their phloem intake but increase their xylem ingestion. As an example, the total duration of xylem related waveform recording is three time greater for the aphid *Aphis glycines* Matsumura, 1917 (Hemiptera: Aphididae) feeding on the Dowling resistant wheat cultivar rather on the susceptible Glenwood (Crompton and Ode, 2010). Mixed phloem-xylem sap ingestion has been especially observed in Deltocephalinae, a leafhopper subfamily of great economic importance including many vectors of plant pathogens (Khan and Saxena, 1984a; Lett et al., 2001; Mesfin and Bosque-Perez, 1998; Stafford and Walker, 2009). These leafhoppers can probe frequently in the xylem without really feeding xylem sap. As an example, EPG recordings of the leafhopper *Cicadulina mbila* Naudé, 1924 (Hemiptera: Cicadellidae) showed

* Corresponding author.
E-mail addresses: Julien.Chuche@nuim.ie (J. Chuche), nicolas.sauvion@inra.fr (N. Sauvion), denis.thiery@inra.fr (D. Thiéry).

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708 xylem probing periods (22 insects) against 37 phloem probing periods (16 insects), but the mean duration of each event were respectively 2.4 and 30.4 min (Lett et al., 2001). In this case, there was no correlation between the number of probing, *i.e.* when the stylets of the leafhopper were in the xylem, and feeding, *i.e.* sap ingestion.

Because xylem and phloem have very different characteristics, hemipteran insects often specialize on one or the other. Indeed, phloem is nutrient-rich and flows from the leaves to other organs, while xylem, which is drawn up from the roots to the upper parts of plants, consists mainly of water, mineral elements, and little organic nutrients. Xylem is thus less nutritional than phloem and contains few carbon, and particularly few nitrogen, compounds (Mattson, 1980). Feeding on xylem, therefore, has to be compensated for by higher ingestion rates to achieve nutritional values equivalent to phloem (Andersen et al., 1989; Brodbeck et al., 1993). Additionally, xylem sap is under negative pressure, making it energetically costly for insects to extract. On the other hand, phloem sap is under positive pressure and requires less effort to ingest (Raven, 1983). As a consequence of the relatively high metabolic costs for extracting xylem sap, xylem-feeders are generally larger-bodied than phloem-feeders (Novotny and Wilson, 1997; Redak et al., 2004). Indeed, the suction pressure required to ingest xylem fluid decreases as the food canal width increases, which is correlated to body size (Novotny and Wilson, 1997). The evolutionary process leading to xylem and phloem specialization is not yet well known; some authors have postulated a transition from phloem- to xylem-feeding (Novotny and Wilson, 1997), or vice versa (Dietrich, 2003). Therefore, both the evolutionary history and the mechanisms of specialization for feeding on phloem or xylem need further investigations.

European viticulture is confronted with two serious phytoplasma diseases, *i.e.*, Flavescence dorée (FD) and bois noir (Angelini et al., 2003). FD transmission is highly vector-specific and is transmitted by the American grapevine leafhopper, *Scaphoideus titanus* Ball, 1932 (Hemiptera: Cicadomorpha: 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 the only natural transmission paths. Thus, the feeding behavior of this insect is the determining variable involved in FD transmission (Chuche and Thiéry, 2014).

Because *S. titanus* is a competent vector of FD phytoplasmas restricted to phloem, this leafhopper is widely considered as a phloem-feeder. Electropenetrography (EPG) can be used to acquire direct temporal information on the feeding behavior. This tool was first introduced by McLean and Kinsey (AC-EPG) for aphids (McLean and Kinsey, 1964, 1967), and DC-EPG was then developed by Tjallingii (1978). By connecting an insect and its food plant to an electrical circuit, variations in biopotentials and electrical resistance can be recorded and related to different feeding activity patterns. These variations produce different electrical waveforms that correspond to specific behaviors that reflect both the position of the stylets in the plant tissue and the actual feeding activity, such as stylet movements, cibarial pumping (ingestion), and salivation (Sauvion and Rahbé, 1999; Backus, 2016). We have recently characterized and defined five *S. titanus* EPG waveforms linked to non-probing, pathway, X-wave, sustained ingestion and interruption phases (Chuche et al., 2016a).

This work question on the evolution of feeding behavior depending on the sex of *S. titanus*, specifically the relative importance of mixed xylem and phloem sap ingestion, by recording and analyzing their feeding behavior using EPG according to the waveform-behavior correlations established in our previous study (Chuche et al., 2016a). Because several pathogens including FD phytoplasmas are acquired and transmitted during sap ingestion, the vectorial capacities of *S. titanus* are discussed depending on the feeding behavior of each sex.

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, larvae 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 turned adults. Leaves were replaced as soon as they began to wither.

Phytoplasma is mainly transmitted by adult insects whose dispersal is usually linked to reproduction. To be sure to have sexually mature adults, we used 7–14 days old adults, females being mature at 6 days and males at emergence (Mazzoni et al., 2009).

2.2. EPG system

A Giga-8 DC-EPG device (EPG-Systems, Wageningen, The Netherlands) was used to monitor the 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).

Leafhoppers were collected in a breeding cage, placed individually in Petri dishes (8.5 cm in diameter), sexed 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 was held stationary at the end of a plastic 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

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inserted into the EPG probe 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 on which grow the cutting.

Recordings were made simultaneously on four well irrigated cuttings, each hosting one adult and was only used 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.

Signals were analyzed using the software Stylet+a (EPG Systems, Wageningen, The Netherlands). Because this work emphasized the behavioral sequences essential to phytoplasma transmission, we focused on the waveforms correlated with the stylet location in the xylem or the phloem (Table 1). Electrical penetration graph waveforms were interpreted from the correlation study reported by Chuche et al., (2016a).

2.3. Statistical analysis

All statistical analyses were conducted with the R software (v3.0.2) (R Development Core Team, 2007).

Non-sequential and sequential EPG response variables related to stylet location out of the plant tissues (non-probing, waveform np), in the mesophyll (waveforms B), in the xylem (waveforms XNx, C2x, Nx), or in the phloem (waveforms XNp, C2p, Np) were calculated for each EPG recording using a Microsoft Excel workbook. A total of 27 non-sequential variables were calculated using the parameters defined by Backus et al., (2007). Thus, we calculated 3 variables associated to the probing: PDi, the Probing (or Penetration) Duration by Insect (sum of durations of all probes made by an individual insect = Backus's variable 11); NPi, the Number of Probes by Insect (total number of probes made by each insect in a cohort = Backus's variable 4); PDPi, the Probing Duration per Probe (by Insect) (average duration per probe made by an individual insect = Backus's variable 13); and 24 variables corresponding to the 3 following parameters estimated for the 8 waveforms of our study (Table 2): WDi, the Waveform Duration by Insect (sum of duration of all events of one waveform type made by each individual insect that produced that waveform = Backus's variable 20); NWEi, the Number of Waveform Events by Insect (number of events of the same waveforms performed by an individual insect across all its probes = Backus's variable 6); WDEi, the Waveform Duration per Event by Insect (duration of the average event of a specific waveform type across all probes of each individual insect = Backus's variable 15). We also calculated TWD, the Total Waveform Duration (=Backus's variable 22) for each event.

In addition, five sequential variables were calculated using the method of Sarria et al. (2009), as listed in Table 3.

In a first step, we have used statistical analyses without *a priori* distinguishing between males and females. Multivariate and clustering analyses were used to identify and summarize joint relationships among the 60 individuals and the 27 non-sequential variables. One approach would have been to apply a multivariate method, such as a principal component analysis (PCA) (Chessel et al., 2004; Dray and Dufour, 2007); however, we were not only interested in the diversity among individuals, but also in the diversity between groups of individuals. Global diversity can be decomposed using a standard, multivariate, analysis of variance model as: total variance = (variance between groups) + (variance within groups). The traditional multivariate methods focus on the total variance amongst individuals, and may overlook differences between groups. We used the discriminant analysis of principal components (DAPC) (Jombart et al., 2010), which, in contrast, optimizes the variance between groups while minimizing variance within groups. This method aims to identify and describe genetic clusters, but it can be applied to any quantitative data. The analysis was conducted using the adegenet package (v1.3-1) (Jombart, 2008). As a first step, DAPC requires groups to be pre-defined. We then used the K-means procedure to infer K – the optimal number of clusters – by implementing the function "find.cluster". This function first transforms the data using a PCA. To avoid a loss of information, all the principal components were retained to run the K-means algorithm. Finally, we used the Bayesian information criterion to determine that K = 3; the most efficient summary of our data. In a second step, the DAPC was conducted using the "dapc" function, which first transforms the data using PCA, and then performs a Discriminant Analysis. Scatterplots were created with the generic function "scatter" – with a dedicated method for objects produced by DAPC. For each of the three groups defined by the K-means procedure, the variables PDi, NPi, and PDPi were averaged by dividing the sum of values obtained for each individual insect by the number of insects of each group (N); and the variables WDi, NWEi, and WDEi were averaged for each particular waveform by dividing the sum of values obtained for each individual insect by the number of insects that performed that particular waveform as previously described (Table 1 in Backus et al., 2007). These means values, respectively PDI (amount of time an average insect had stylets inserted = Backus's variable 12), NPI (total number of probes made by each insect in a group averaged over N = Backus's variable 5), PDPI (average per insect of the average

Table 1
Summary of EPG-waveform names representing the behaviors most important for the *S. titanus* vector comparing to the Backus model of *X. fastidiosa*.

Phase	Backus' 2015 waveform names			*S. titanus* waveform names	
	Family	Type	Sub-type	Chuche et al. (2016a,b)	Present study
np				np	np
Pathway	A	A1		–	–
	A	A2		–	–
	B	B1	B1s	B1s	B
			B1w	B1w	B
	B	B2	B2m	–	–
X wave	XN	B1s		B1s	B
		fB1w		fB1w	B
	XC	C1		C1	C2x, C2p
		C2		C2x, C2p	C2x, C2p
Sustained ingestion	C	C2		C2x, C2p	C2x, C2p
Interruption	N	NA	NA1, NA2	Nx, Np	Nx, Np
		NB	NB1, NB2	–	–
Withdrawal	W			–	–

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Table 2
Mean (±standard error) non-sequential DC-EPG variable values (ranges in parenthesis) for the probing behavior of *Scaphoideus titanus* adults on grapevine during a 4-h recording, based on data of 60 individuals.

Tissue	Waveform[a]	Group[b]	PPW[c]	WDI[a]		P[d]	NWEI[a]		P[d]	WDEI[a]		P[d]
–	np (min)	I	12/13	27.2±9.7	(0.8–108.7)	a	5.8 ± 1.0	(1–12)	a	6.0 ± 1.8	(0.1–21.7)	
		III	19/19	54.7 ± 7.8	(3.8–115.8)	b	8.8 ± 0.9	(2–18)	a	7.8 ± 1.0	(1.3–17.7)	
		II	28/28	102.1 ± 9.8	(22.1–229.4)	c	16.7 ± 2.3	(3–48)	b	10.6 ± 1.8	(1.7–40.0)	
Mesophyll	B (min)	I	13/13	37.3 ± 11.7	(4.3–136.8)	a	29.2 ± 9.1	(5–110)	a	2.2 ± 0.2	(0.4–3.4)	
		II	28/28	44.7 ± 5.6	(8.4–97.4)	a	33.6 ± 3.7	(9–78)	a	2.4 ± 0.2	(0.4–6.5)	
		III	19/19	97.6 ± 8.3	(49.1–174.2)	b	57.6 ± 4.4	(31–113)	b	2.6 ± 0.1	(1.2–3.2)	
Xylem	XNx (s)	I	2/13	4.4 ± 2.2	(3.5–7.8)	a	1.7 ± 0.5	(1–2)	a	3.1 ± 0.2	(3.5–3.9)	a
		II	27/28	225.5 ± 61.5	(8.7–1566.3)	b	30.0 ± 4.0	(1–82)	b	11.8 ± 0.9	(1.3–19.8)	b
		III	17/19	445.0 ± 198.9	(21.8–2818.7)	b	25.6 ± 10.8	(2–145)	b	19.3 ± 4.8	(7.0–85.9)	b
	C2x (min)	I	5/13	9.1 ± 1.4	(6.7–13.2)	a	7.3 ± 4.4	(2–25)	a	3.3 ± 1.0	(0.5–5.8)	a
		III	19/19	17.4 ± 4.5	(0.8–83.8)	a	38.1 ± 12.5	(4–188)	b	1.5 ± 0.1	(0.1–1.4)	b
		II	27/28	78.0 ± 11.3	(1.3–188.3)	b	43.5 ± 4.6	(3–95)	b	2.9 ± 0.4	(0.4–9.9)	a
	Nx (s)	II	15/28	31.6 ± 11.2	(0.6–158.1)	a	9.0 ± 2.1	(1–29)	a	3.7 ± 0.4	(0.6–6.0)	
		I	12/13	259.9 ± 107.3	(2.4–1010.3)	ab	38.2 ± 14.3	(1–118)	ab	6.0 ± 0.9	(1.7–10.4)	
		III	14/19	409.6 ± 67.0	(9.0–981.8)	b	61.6 ± 8.8	(6–129)	b	7.6 ± 1.0	(1.5–17.9)	
Phloem	XNp (s)	III	13/19	2.7 ± 0.7	(0.1–9.1)		10.2 ± 3.5	(1–44)	a	1.1 ± 0.0	(0.1–0.3)	
		II	9/28	4.7 ± 3.0	(0.1–28.1)		7.1 ± 2.5	(1–21)	a	1.7 ± 0.5	(0.1–3.9)	
		I	12/13	8.8 ± 3.7	(1.1–39.9)		40.0 ± 13.9	(8–168)	b	1.1 ± 0.0	(0.1–0.3)	
	C2p (min)	II	13/28	12.5 ± 3.8	(0.2–40.4)	a	10.6 ± 3.8	(1–43)	a	2.7 ± 0.9	(0.2–11.9)	a
		III	17/19	52.5 ± 10.2	(0.4–141.4)	b	44.8 ± 8.6	(1–104)	b	2.3 ± 0.3	(0.4–4.4)	a
		I	13/13	146.2 ± 15.3	(57.6–227.0)	c	53.4 ± 12.9	(13–171)	b	6.2 ± 1.3	(0.6–14.4)	b
	Np (s)	III	3/19	14.2 ± 10.8	(2.4–35.7)	a	5.7 ± 4.2	(1–14)	a	2.4 ± 0.1	(2.3–2.6)	a
		I	11/13	118.9 ± 61.5	(2.4–603.9)	b	20.9 ± 9.2	(1–76)	b	4.4 ± 0.6	(1.7–9.3)	b
		II	13/28	220.9 ± 63.5	(12.1–722.3)	b	39.6 ± 9.2	(4–94)	b	5.1 ± 0.6	(2.9–8.6)	b

[a] See Materials and Methods for waveforms and parameters definition.
[b] The 60 individuals have been separated into three groups using a K-means procedure as described in the Materials and Methods. For each waveform, the groups have been ordered according the mean value of WDI.
[c] The proportion of individuals that produced a specific waveform type.
[d] See Materials and Methods for statistical test used for comparison of the means; significant differences within columns are marked with different letters.

Table 3
Mean (±standard error) sequential DC-EPG variable values (ranges in parenthesis) for the probing behavior of *Scaphoideus titanus* adults on grapevine during a 4-h recording, based on data of 60 individuals.

Sequential variables		Group[a]	N	WDI (min)		P[b]
Time from start of EPG to 1st probe[c]	t>1stPr	II	28	3.5 ± 0.9	(0–17.8)	a
		III	19	6.5 ± 2.0	(0–35.1)	a
		I	13	11.1 ± 7.6	(0–101.4)	b
Recording time to 1st waveform	t-rec>1stC2x	II	27	16.1 ± 3.2	(0.3–65.3)	a
		III	19	22.9 ± 6.8	(0.6–134.3)	b
		I	4	53.6 ± 25.5	(3.3–127.5)	b
	t-rec>1stC2p	I	13	31.8 ± 15.9	(1.7–199.6)	
		III	17	58.1 ± 15.8	(5.2–227.9)	
		II	4	52.5 ± 25.7	(2.5–127.5)	
Time to first waveform from 1st probe	t>1stC2x	II	27	13.5 ± 3.0	(0.3–48.3)	a
		III	19	17.3 ± 7.1	(0.6–134.3)	a
		I	4	52.5 ± 25.7	(2.5–127.5)	b
	t>1stC2p	I	13	21.6 ± 14.7	(0–194.2)	a
		III	17	52.2 ± 15.6	(2.5–222.8)	b
		II	13	58.0 ± 20.6	(0.1–183.9)	b

[a] The 60 individuals have been separated into three groups using a K-means procedure as described in the Materials and Methods. For each waveform, the groups have been ordered according the mean value of WDI.
[b] See Materials and Methods for statistical test used for comparison of the means; significant differences within columns are marked with different letters.
[c] Several individuals began to insert their stylets into the tissues before the start of the recording; in this case t>1stPr = 0.

duration per probe made by an individual insect = Backus's variable 14), WDI (waveform duration per insect = Backus's variable 21), NWEI (number of waveform events per insect = Backus's variable 7) and WDEI (waveform duration per event per insect = Backus's variable 16), were compared using the Kruskal–Wallis rank sum test (function "krustal.test" of the package "stats") (Table 2). Significant results were then tested using the Steel-Dwass-Critchlow-Fligner pairwise ranking nonparametric method to compare the means of all pairs of groups (function "pSDCFlig" of the package "NSM3").

In a second step, we compared the mean behavior of the males and the females. Then, the mean values PDI, NPI, PDPI, WDI, NWEI and WDEI were compared using the Wilcoxon rank sum test (function "wilcox.test" of the package "stats") (Fig. 3).

3. Results

3.1. Variability in probing and ingestion behavior among groups of insects

Fig. 1A describes the probing behavior of the leafhoppers as a whole. Results show that the leafhoppers reached phloem sieve elements (and remained there for 27.0% of the 4-h recorded period)

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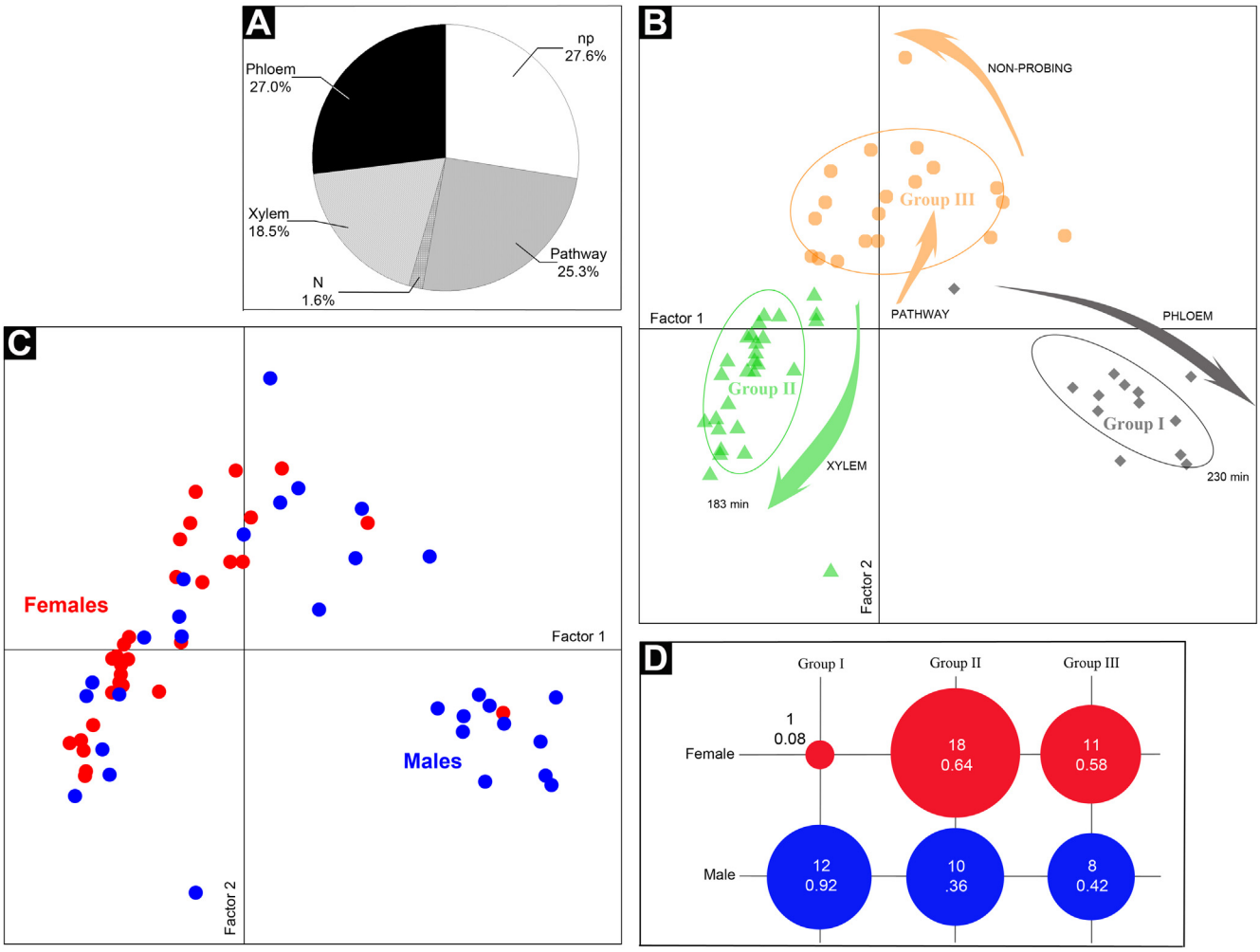

Fig. 1. Descriptive statistical analysis of the *Scaphoideus titanus* feeding behavior on grapevine. (A) Mean proportion of the Total Waveform Duration (TWD) of the different events observed by DC-EPG during 4 h recordings of 60 individuals. (B-D) Discriminant analysis of principal components (DAPC) on 27 non-sequential variables showing the variability in probing and ingestion behavior of 30 males and 30 females: (B) Coordinates of the individuals on the first two axes of the discriminant analysis. Samples are labeled according to the three groups defined by the K-mean procedure and illustrated by inertia ellipses; (C) Maps exactly the same as (B), samples are labeled in blue for males and in red for females; (D) Number and proportion of male and females belonging to the three groups, inferred using DAPC. **np**, non-probing activity; **Pathway**, stylet pathway in the mesophyll; **Xylem**, xylem-related activities; **Phloem**, phloem-related activities; **N**, nonpathway-like interruption of ingestion.

as well as xylem (18.5% of recorded time). On average, they were in a non-sap-ingesting phase for half their recording time, as follows: 27.6% in a non-probing phase and 26.9% in the epidermis and mesophyll (25.3% in pathway phase [B] and 1.6% in nonpathway-like interruption [N] phase).

We attempted to show diversity between groups of individuals, without knowing *a priori* which groups would form. Table 2 shows non-sequential EPG variables that describe the probing behavior of the three groups of leafhoppers defined by a K-mean procedure. The information in Table 2 is summarized in Fig. 1, and Fig. 2 shows the correlation between the variables WDI_{C2x} (duration of xylem sap ingestion per insect) and WDI_{C2p} (duration of phloem sap ingestion per insect), separated by sex. Table 3 details the precise sequence of waveforms that correspond to ingestion in the xylem or the phloem during the 4 h of EPG recording.

Group I consisted of 13 insects (12 males and 1 female; Fig. 1B, C & D). On average, their stylets remained significantly longer (3–12 times more) inserted in the phloem (Table 2: WDI_{C2p} ~2 h 26 min) in comparison to groups II and III (WDI_{C2p} ~12 min and 52 min respectively). In particular, 10 insects (9 males *vs.* 1 females) stayed for >2 h in the phloem (of which 3 males stayed >3 h, Fig. 1C). The insects in group I only took an average of ~22 min to reach the phloem (Table 3: waveform t >1stC2p) against ~52—~58 min for the two other groups; they also remained there for significantly longer duration than the insects in the other groups (Table 2: $WDEI_{C2p}$ ~6 min *vs.* ~2–3 min). Repeated and brief interruptions of the phloem sap ingestion by B1 type waveforms (XN events) were (on average) as long for the insects from the three groups ($WDEI_{XNp}$). Fewer insects from group I than the two other groups ingested in the xylem (Table 2: PPW_{C2x} = 5/13, only males, *versus* 27/28, and 19/19 for respectively group II and group III), but this tendency was not significant (Fisher's Exact Test, P = 0.26). The mean duration per insect of xylem sap ingestion was very short (Table 2: WDI_{C2x} ~9 min) compared to group II (~1 h 18 min), but similar to group III (~17 min). Additionally, the mean duration per insect associated with salivation in the xylem (Nx) differed only between groups II and III (Table 2: WDI_{Nx} ~32 s *vs.* ~410 s), and was not significantly different for the duration per event ($WDEI_{Nx}$) between the three groups. In the epidermis and mesophyll, group I characteristically presented a mean duration, per insect, of stylet pathway almost thrice as short as

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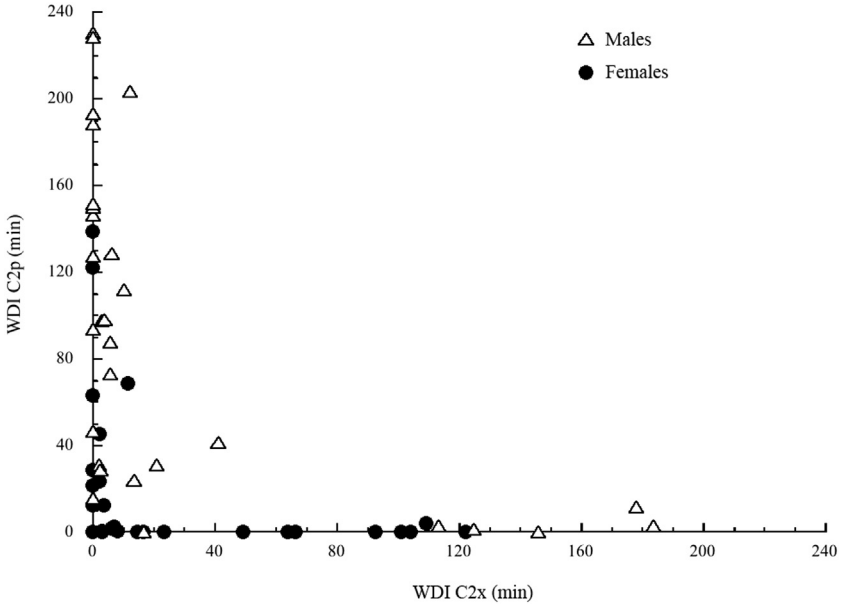

Fig. 2. Correlation of the duration of sap ingestion by the insects likely in xylem (WDI_{C2x}) and in phloem (WDI_{C2p}).

group III (Table 2: WDI_B ~1 h 38 min *vs.* ~37 min), but similar to group II (~45 min).

Group II consisted of 28 insects (10 males and 18 females; Fig. 1D; Fisher's Exact Test, P = 1.00). On average, their stylets remained inserted in the xylem much longer (5–9 times more) (Table 2: WDI_{C2x} ~1 h 18 min) than groups I and III (Table 2: WDI_{C2x} ~9–17 min). In particular 4 males against only 1 female showed a WDI_{C2x} >2 h (Fig. 2). On average, group II reached the xylem in only ~13 min (Table 3: waveform t >1stC2x) in comparison to ~52 min for the group I. The events of xylem sap ingestion were twice as long as those from group III (Table 2: $WDEI_{C2x}$ ~3 min *vs.*~1.5 min). Xylem sap ingestion often alternated with XN waveforms for insects from group II and III (XNx ~30, and ~25), and on average the duration per event was much shorter for insects from group I ($WDEI_{XNp}$). Xylem sap ingestion was also often interrupted by brief salivation phases ($NWEI_{Nx}$), but no significant difference between the three groups was observed for the duration per event ($WDEI_{Nx}$). It seems that fewer insects from group II ingested from the phloem (Table 2: PPW_{C2p} = 13/28) in comparison with the two other groups (group I: 13/13, group III: 17/19) but this tendency was not significant (Fisher's Exact Test, P = 0.246). The total durations of phloem sap ingestion were very short (Table 2: WDI_{C2p} ~12 min *vs.*~2 h 26 min for group I). In the mesophyll, group II was characterized by having a mean duration, per insect, of the stylet pathway being almost thrice as short as group III (WDI_B ~37 min *vs.*~1 h 38 min).

Group III consisted of 19 insects (8 males and 11 females) and comprised the individuals that stayed the longest duration in pathway phase (Table 2: WDI_B ~1 h 38 min), in comparison with the insects of the two other groups (~37 min–45 min; Fig. 1B). Their ingestion behavior in the xylem was almost identical to those of group I (WDI_{C2x} no significant difference; $NWEI_{C2x}$: mean duration per event twice shorter), and their behavior in the phloem was intermediate between groups I and II (Table 2: WDI_{C2p}: group III, ~52 min *vs.* group II, ~12 min and group I, ~2 h 26 min).

Two categories of individuals can be differentiated based on their phloem ingestion: group I (21.6% of the individuals, including 13 males and 1 female; Fisher's Exact Test, P = 0.03) and groups II and III (78.3% of the individuals, including 18 males and 29 females; Fisher's Exact Test, P = 0.30). Xylem ingestion can be used to distinguish between the group II (63.3% of the individuals, including 10 males and 18 females; Fisher's Exact Test, P = 0.42) from the groups I + III (36.7% of the individuals, including 20 males and 12 females; Fisher's Exact Test, P = 0.45).

3.2. Variability in probing and ingestion behavior between males and females

A second approach of our analyses was to compare the mean behavior of males and females (Fig. 3). Clearly, the males stayed for a longer duration in probing activity during the 4 h of the recordings (Fig. 3 "Probing *vs.* np": PDI: males = 183.5 ± 9.4 min *vs.* females = 141.8 ± 8.5 min; P < 0.01), and when they inserted their stylets into tissue, they probed for three times longer than females, before ending their probes (PDPI: males = 49.2 ± 10.9 min *vs.* females = 17.3 ± 3.7 min). The behaviors associated with xylem were very similar for males and females. They took an average of 2 h to reach xylem (Fig. 3 "Xylem": t-rec>1stC2x: males = 28.3 ± 8.7 min *vs.* females = 18.5 ± 2.9 min); males remained there for 3.1 ± 0.5 min compared with 2.0 ± 0.2 min for females ($WDEI_{C2x}$; P > 0.05). The mean duration per insect associated with xylem ingestion was relatively long for a species described as a phloem-feeder, but not significantly different between males and females (WDI_{C2x}: males = 44.7 ± 8.1 min *vs.* females = 54.1 ± 14.3 - min). On the contrary, the behaviors associated with phloem were very contrasted between males and females. Males reached the phloem quickly while females took an average of almost 3 h to reach it (Fig. 3 "Phloem": t-rec>1stC2p: males = 32.6 ± 9.3 min *vs.* females = 86.6 ± 20.5 min; P < 0.01); when the males found this tissue compartment, they remained there two times longer than did females ($WDEI_{C2p}$: males = 4.6 ± 0.8 min *vs.* females = 2.1 ± 0.2 - min; P < 0.01). Finally, the mean duration of phloem ingestion per insect was almost three times as long for males as for females (WDI_{C2p}: males = 90.8 ± 14.2 min *vs.* females = 33.3 ± 11.0 min; P < 0.01). Thus, males spent 38% of their time (*i.e.* the duration of recording) in phloem ingestion phase, while females did this only 14% of their time.

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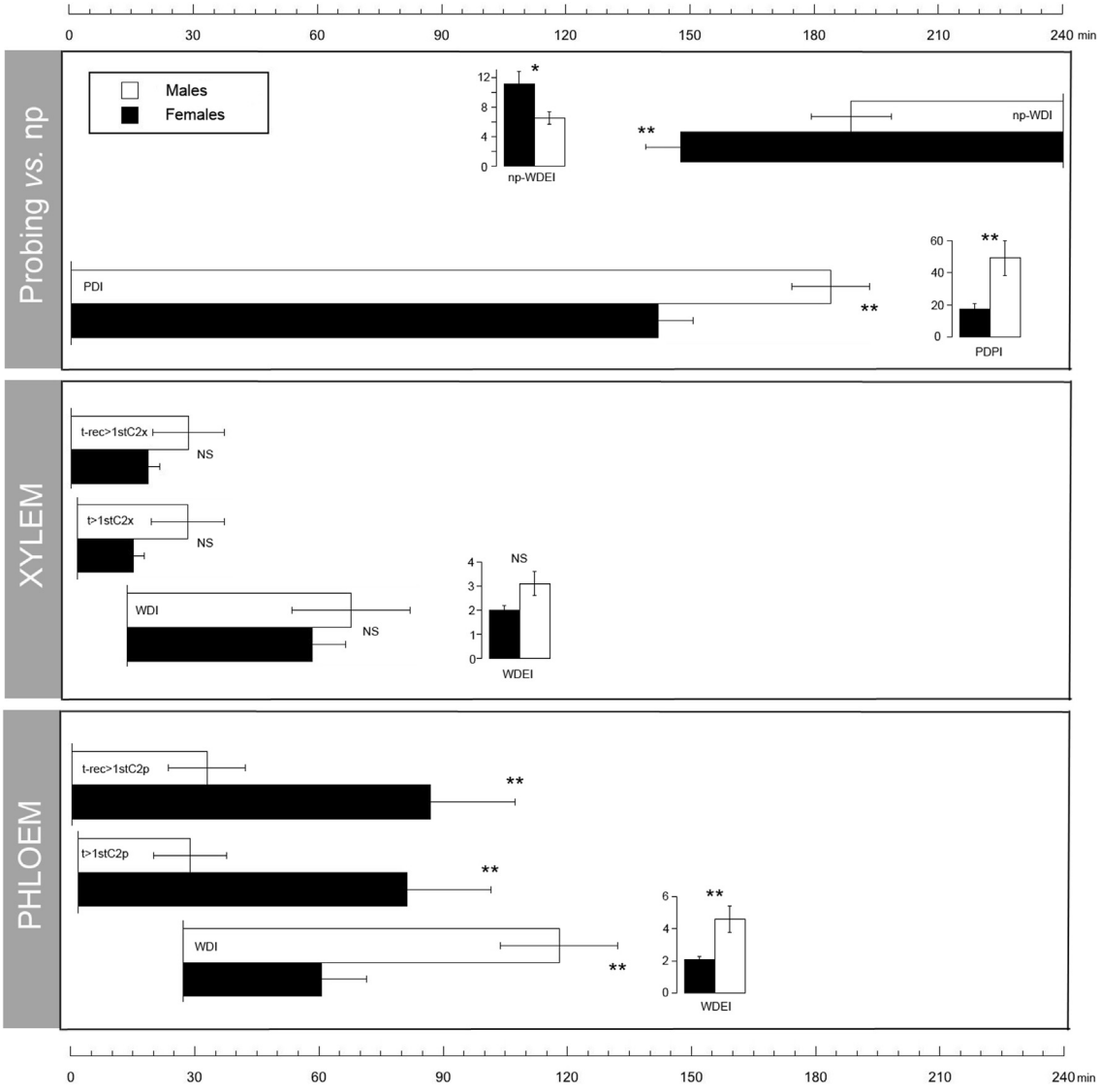

Fig. 3. Comparison of the probing behaviors of 30 males and 30 females of *Scaphoideus titanus* on grapevine during DC-EPG 4-h recordings. np, non-probing phase; Probing, probing phase; C2x, xylem-related activities; C2p, phloem-related activities; WDI, waveform duration (min) per insect; WDEI, waveform duration (min) per event per insect; t-rec>1stX: time from start of EPG to first waveform X; t>1stX: time to first waveform X from first probe. All mean values (±standard error) are expressed in minutes, and have been compared using the Wilcoxon rank sum test. Significant differences are marked by * for p-value <0.05 and by ** for p-value <0.01; NS, no-significant difference.

4. Discussion

The present study illustrates the evidence of a mixed xylem and phloem sap ingestion as normal dietary behavior in *S. titanus* and questions the advantages that feeding in two such different substrates could represent.

This is the first work providing quantitative information on the probing of this leafhopper known as the vector of the main phytoplasma disease in European viticulture.

4.1. S. Titanus is a true mixed xylem/phloem-feeder

Scaphoideus titanus has so far been described as a phloem-ingesting insect (Alma et al., 2009; Stacconi and Romani, 2012); however, this attribute was based on its role in the transmission of phytoplasmas and not on feeding observations. Early histological studies providing evidence of xylem-feeding in this species were so far neglected (Carle and Moutous, 1965; Schvester et al., 1962; Vidano, 1964). These older microscopic observations of cut petioles showed that the salivary sheath terminations reached xylem and phloem with a similar frequency. This suggests a mixed diet of both phloem and xylem sap in *S. titanus*. Furthermore, an anatomical study of this species revealed the existence of a highly differentiated filter chamber (Le Caherec et al., 1997) that is characteristic of xylem sap feeders (Noble-Nesbiti, 1990). Our behavioral data show that *S. titanus* also ingests significantly from xylem, which is consistent with early studies of salivary sheaths in grapevine petioles and the presence of a well-developed filter chamber (Carle and Moutous, 1965; Le Caherec et al., 1997). This true mixed feeding behavior by healthy insects on suitable and healthy host-plants is uncommon and leads to questions about the existence of a real segregation between xylem and phloem ingestion, as Fig. 2 seems to demonstrate.

4.2. A potential evolution and specialization depending on the sex

The evolutionary process leading to xylem and phloem specialization are controversial with hypotheses arguing a transition from

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phloem- to xylem-feeding (Novotny and Wilson, 1997), or vice versa (Dietrich, 2003). So, *S. titanus* could be the result of an incomplete transition from one sap-feeding to another one. *Scaphoideus titanus* could be an "ancestral" xylem-feeder that is feeding on phloem or an "ancestral" phloem-feeder feeding on xylem. In both assumptions *S. titanus* is still feeding on its "ancestral" sap. In this leafhopper species, males and females seem to have differently responded to selection pressure leading to different feeding behavior. If males and females have the same behavior regarding the xylem, their activity in phloem is very different. Males reach the phloem more quickly, probe more frequently, ingest longer, and exhibit more and longer salivation events in this tissue than do females.

Xylem composition varies more greatly between the growing seasons than does phloem, but also as a function of plant vigour, weather conditions, and circadian rhythm. These factors can lead xylem-feeders to change host plants to provide for their needs (Bi et al., 2005; Mizell et al., 2008; Novotny and Wilson, 1997). Xylem-feeding species have a great variety of hosts (Boudon-Padieu, 2000), with some species having extremely broad host ranges. An example is the glassy-winged sharpshooter, *Homalodisca vitripennis* Germar, 1821 (Hemiptera; Cicadellidae: Cicadellinae), which feeds on hundreds of different host plants (Redak et al., 2004). Conversely, phloem-feeders generally have narrower host ranges, and many species appear to only use a single plant family, genus, or species (Dietrich, 2003). In Europe, *S. titanus* lives most of the time in agricultural systems composed of only one species, *V. vinifera*. Additionally, the diversity within the genus *Vitis* is lower in Europe than in North America, the original area of *S. titanus*, where *V. labrusca* and *V. riparia* have been reported as the preferred host plants (Chuche and Thiéry, 2014). Thus, very few alternative host plants exist in Europe. If we suppose that *S. titanus* is mainly a xylem-feeder that also ingests phloem sap, the phloem consumption could be a response to its inability to feed on different host plant species to compensate xylem quality fluctuations. Comparisons with American individuals would clarify this point.

Considering the costs of xylemophagy (Raven, 1983), xylem must provide sufficient advantages to be maintained as a food source in a mixed diet. Essential nutrients for *S. titanus* probably occur in the xylem and not in the phloem. Symplastic transport in phloem results in an inability to transport some elements or to transport them with low mobility, in particular, cations (e.g., calcium, sodium) or boron. Others compounds are mainly found in xylem, such as silicon, nickel, iron, phosphate, and some growth regulators. These micronutrients can have positive effects on the development of aphids (Auclair and Srivastava, 1972) and on the fertility of phytophagous females in general (Awmack and Leather, 2002). We hypothesize that availability of micronutrients could explain the more balanced consumption of xylem and phloem by *S. titanus* females. The observation of relatively long xylem-ingestion per insect (for a putative phloem-feeder), as observed in this study, was already described with Hemiptera feeding on an artificial diet (Trębicki et al., 2012), on non-preferred host plants (Lei et al., 2001) or that are a resistant variety of the preferred species (Crompton and Ode, 2010; Hu et al., 2008; Khan and Saxena, 1984b). Our study, however, was conducted on the main host plant of *S. titanus* and on Cabernet-Sauvignon variety, which is very suitable for these insects that grow well on it (Chuche, 2010). Thus, long xylem-ingestion durations caused by an unusual behavior due to feeding on an unsuitable plant can be excluded.

Because sap is nutritionally deficient, sap-feeders must ingest large quantities to achieve sufficient nutrition and should regulate their osmotic pressure by excreting water (Douglas, 2006). Thus, water supply is not critical for sap-feeders except at unique times when phloemophagous insects can ingest xylem sap to rehydrate (Cid and Fereres, 2010; Powell and Hardie, 2002; Ramírez and Niemeyer, 2000). Ingestion of xylem sap can also be a response to osmotic stress induced by hemolymph dehydration (Pompon et al., 2010). Xylem sap consumption in insects that are considered phloem-feeders is often perceived as an unusual occurence that can normally be explained as a result of a specific event, such as a response to osmotic stress cause by dehydration or feeding on high-osmolarity medium (Pompon et al., 2010, 2011). In this way, it was shown that the mealybug *Planococcus citri* Risso, 1813 (Hemiptera: Pseudococcidae), described as a phloem-feeder, exhibited more xylem- than phloem-ingestion activities (Cid and Fereres, 2010). This result, similar to what we observed in *S. titanus*, was only explained as a consequence of dehydration induced by the removal of wax from the mealybug to improve wire attachment and was not considered to be as the insect's typical feeding tactic. Because there is no experiment without removing wax, we cannot conclude whether it is a common behavior or not. The insects used were not starved prior to the experiment; they were fed on well-irrigated cuttings and should, therefore, not have been particularly dehydrated. Xylem consumption has also been observed in hydrated insects and thus could have other functions (Pompon et al., 2010). We propose that higher expression of this behavior in *S. titanus* females is because they seek specific compounds present in the xylem, which could be useful for reproduction.

Interestingly, xylem-feeders are generally bigger than phloem-feeders. This has been attributed to the energy required to ingest from xylem, because the metabolic cost of extracting xylem sap decreases with insect size (because the suction force needed decreases as the radius of the food canal increases) (Novotny and Wilson, 1997; Redak et al., 2004). Moreover, xylem feeders need well-developed cibarial dilator muscles to pump sap. This is a reason why phloem-feeding insects have a flatter frontoclypeus (i.e., the face) (Dietrich, 2003). *Scaphoideus titanus* does not have the size and the cibarial dilator muscles of an efficient xylem-feeder and extraction of xylem fluid should be very costly in terms of the required energy (Novotny and Wilson, 1997). Indeed, xylem is very poor in sugars and carbon is an important limiting factor for xylem-feeding leafhoppers (Redak et al., 2004). Ingesting phloem sap, which is rich in sugars and amino acids, can be a solution to the energy costs caused by the extraction of xylem by a small leafhopper. This assumption implies that *S. titanus*, which is a small leafhopper, is a xylem-feeder that uses phloem sap as an energy source to compensate for the suction force need to ingest xylem. Males are smaller than females and according to the cost of extracting xylem sap in small-sized insects, males would need more energy than females to feed on xylem. This is exactly what our result show, males ingesting more often and for a longer duration in phloem than females. Similar observations on other leafhopper species would confirm such a hypothesis.

Sap-feeders harbor symbiotic micro-organisms that synthesize and provide essential amino acids lacking in the sap and consequently have positive effects on host fitness (Douglas, 2006). As an example, symbionts can confer the ability of their host to develop on a usually non-host plant (Chuche et al., 2016b; Giron et al., 2017). Thus, it can be assumed that symbionts are involved in the mix diet of *S. titanus* by allowing their host to efficiently exploit both phloem and xylem sap consumption. It could be interesting to study the effects of symbionts harbored by *S. titanus* on its feeding behavior.

4.3. Phytoplasma transmission efficiency depends on the sex

From an epidemiological point of view, differences in feeding behavior observed between insect vectors of the same species may have important consequences in terms of plant pathogen

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transmission (Bosco et al., 1997). For instance, the frequency of stylet probes by a vector, and the rapidity of finding the tissue where the pathogen is localized are essential factors in the probability of pathogen acquisition and inoculation (Brown, 2016). Acquisition of the pathogens by the leafhoppers occur during the ingestion of phloem sap (Herrbach et al., 2013), thus the time to reach the sieve elements and the duration of phloem sap ingestion phase are important, because their duration has been correlated to acquisition efficiency in another vector pathosystem (Prado and Tjallingii, 1994). Moreover, a minimal acquisition access period is needed to achieve minimum pathogen titer for inoculation (Schvester et al., 1969). The inoculation rate also increases with the contact duration between infectious insect and plant (Schvester et al., 1969). Because *S. titanus* males probe/ingest more in phloem, it is likely that they may be more efficient vectors of FD. These results are supported by a previous study (Carle and Moutous, 1965) showing that the percentage of salivary sheath termini in phloem was higher for males than for females. This finding should have the consequence that the males should be more efficient vectors than the females, which is exactly what has been observed in laboratory conditions (Schvester et al., 1969). The same phenomenon was observed for the transmission, by *S. titanus,* of grapevine yellows (related to FD) in the New York State (USA) (Maixner et al., 1993) and also with the leafhopper vector *Euscelidius variegatus* Kirschbaum, 1858 (Boudon-Padieu et al., 1989; Kuszala, 1986). Additionally, the proportion of *S. titanus* males carrying phytoplasmas causing FD is always higher in vineyards than the proportion of females. Because more males carry phytoplasmas than females in the field (Lessio et al., 2009; Maixner et al., 1993) and are more efficient vectors, males should be more important in the transmission of the FD in vineyards than females.

4.4. S. Titanus is a potential vector of Xylella fastidiosa

Scaphoideus titanus is a potential vector of pathogens inhabiting the xylem, such as the bacterium *Xylella fastidiosa*. This pathogen is the causal agent of Pierce's disease (PD) of grapevines and other diseases in crops such citrus, almond, alfalfa, stone fruits, landscape ornamentals, and native hardwoods (Almeida et al., 2005). Recently introduced into Italy, *X. fastidiosa* is currently considered a danger for European viticulture (European Food Safety Authority, 2013; Janse, 2012; Saponari et al., 2013). Bacteria are inoculated directly into a plant from retention sites in the precibarium (a narrow channel conveying fluid from the stylets to the cibarium [sucking pump]; Backus et al., 2015). Pathogens retained in these sites are termed non-circulative foregut-borne (Nault, 1997). Egestion (outward fluid flow from the precibarium into stylets and out into the plant) is the most important mechanism of inoculation of foregut-borne plant pathogens, such as *X. fastidiosa*, by expelling bacteria from the stylets (Backus et al., 2009, 2015; Fereres and Moreno, 2007). Up until now, no species of the subfamily Deltocephalinae has been proven to be a vector of *X. fastidiosa*. However, the behavior of *S. titanus* that ingest/egest for a long time in xylem and the foregut-borne transmission of *X. fastidiosa* may make it possible for *S. titanus* to transmit these bacteria to grapes. Moreover, the combination of: 1) the recent discovery that deltocephaline *Euscelis lineolatus* naturally carryies *X. fastidiosa* in Italy (Elbeaino et al., 2014), a species that is also described as a phloem-feeder, 2) the possibility of a nonpersistent *X. fastidiosa* transmission mechanism (Backus et al., 2015), and 3) our observations of X wave preceding xylem in a deltocephaline, suggest the need to investigate the vectoring ability of *S. titanus*.

Scaphoideus titanus has a mixed phloem-xylem diet that could result from an incomplete transition from xylem- to phloem-feeding, or the contrary. Selection pressures involved in this evolution acted differently on males and females that currently have different strategies of sap exploitation. The females have a more imbalanced diet in favor of xylem compared to males and the difference of size between the two sexes could be involved in these different strategies. The greater consumption of phloem sap by males could be an explanation of their higher phytoplasma transmission efficiency compared to females. However, the significant ingestion of xylem found in our work questions on the potential role of *S. titanus* as vector of pathogens inhabiting the xylem, such as *X. fastidiosa*.

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