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Research

Feeding behavior increases strength and frequency of vibrational communication signals of *Neoaliturus tenellus* (Hemiptera: Cicadellidae)

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Leafhoppers (Hemiptera: Cicadellidae) use substrate-borne vibrations to communicate, and characteristics of these vibrational signals help identify, locate, and assess suitability of potential mates. The strength and frequency composition of signals influence behavioral responses in conspecifics. Leafhoppers are known to produce these vibrational signals while probing, but it is unclear whether this dual activity degrades signal transmission, inducing a trade-off between nutrient acquisition and mate attraction. In contrast, we hypothesized that inserted mouthparts could provide an additional point of contact with the plant, improving signal transmission. Here, we combine simultaneous electropenetrography (EPG) and accelerometer recordings to assess how beet leafhopper [*Neoaliturus tenellus* (Baker)] vibrational signals vary with probing behavior, including xylem ingestion, phloem salivation/ingestion, and probing of epidermis and mesophyll cells. Signals documented during 6-h recordings of male leafhoppers had significantly higher amplitude and dominant frequency when leafhoppers were probing than when they were not probing. However, the dominant frequency of signals was similar when leafhoppers were engaged in pathway phase, phloem ingestion, and xylem ingestion. Of the different probing behaviors, xylem ingestion had the strongest positive effect on signal amplitude, but phloem ingestion did not influence signal amplitude. Additional contact between the leafhopper and the plant surface, provided by leafhopper mouthparts, may improve vibration transmission, potentially increasing signal active space. In light of our finding that the act of probing plant tissues influences information conveyed in leafhopper vibrational signals, we suggest further research to evaluate the impacts these signal changes have on the behavior of other mates and natural enemies.

Keywords: leafhopper, feeding behavior, electropenetrography (EPG), mating behavior, biotremology

Introduction

Vibrational communication is prevalent among insects, with an estimated 195,000 species using substrate-borne vibrations either exclusively or with other communication modality to convey information (Cocroft and Rodríguez 2005). Characteristics of these vibrations are highly species- and individual-specific, as they are under strong selection pressure from environmental conditions, natural enemy responses, and mate preference (Michelsen et al. 1982, Cocroft and Rodríguez 2005, Rodríguez et al. 2006, Cocroft et al. 2010, Sullivan-Beckers and Cocroft 2010, Virant-Doberlet et al. 2011, Rodríguez and Barbosa 2014, Mazzoni et al. 2015). Stronger signals have a greater effective transmission range and can be better perceived above background noise, thus increasing the chance of reaching the intended receiver and leading to successful communication (Cocroft and Rodríguez 2005, Eriksson et al. 2011, Mazzoni et al. 2014, Caorsi et al. 2021). However, stronger signals are not always beneficial in the context of eavesdropping natural enemies (Virant-Doberlet et al. 2011, 2019).

For leafhoppers (Hemiptera: Cicadellidae), vibrational communication plays an essential role in mate location and courtship (eg Claridge 1985). Males usually produce a calling song to identify a potential mate, and a successful male call is then followed by a duet for mate localization, and eventually copulation (eg Čokl and Virant-Doberlet 2003, de Groot et al. 2012, Krugner and Gordon 2021). Many leafhoppers use a specialized organ, the tymbal, to produce vibrations that

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propagate through a plant (Ossiannilsson 1949, Davranoglou et al. 2020) and which can be perceived by conspecifics using mechanoreceptors located in the legs or abdomen (Čokl and Virant-Doberlet 2003, Ehlers et al. 2022). Vibrations are thought to travel from the leafhopper tymbal to the plant through any points of contact between the insect and the plant surface (Claridge 1985, Čokl et al. 2005), after which they can be perceived up to several meters away on a continuous substrate (Čokl and Virant-Doberlet 2003, Mazzoni et al. 2014).

To maximize signaling space on a plant, male leafhoppers often use a "call-fly" strategy, in which the male produces a series of vibrational signals on a leaf, waits for a female reply, and if no reply is perceived, he moves to another leaf on the plant and repeats this process until a duet and subsequent copulation occurs (Hunt and Nault 1991, Kuhelj et al. 2015a). This strategy is widespread within the Cicadellidae (eg Hunt and Nault 1991, Mazzoni et al. 2009, de Groot et al. 2012, Nieri and Mazzoni 2018, Shan et al. 2023). Although, once sexually mature, male leafhoppers may be sexually active throughout the majority or entirety of their lifespan (Perkes 1970, Kuhelj et al. 2015b), suggesting that they must balance feeding and reproduction throughout their life. Indeed, males of multiple different leafhopper species have been observed to produce signals while probing plant tissues (Claridge 1985, Kuhelj et al. 2015b) and this has been hypothesized to represent a way to minimize the tradeoff between energy replacement/supplementation and mobility that leads to reproduction (Kuhelj et al. 2015b, Kuhelj and Virant-Doberlet 2017). However, it is not known if signal characteristics are affected by feeding. On one hand, the rostrum serves as an additional point of contact for the insect and could improve signal strength by increased connectivity with the plant. Furthermore, it is possible that higher frequencies (eg >1,000 Hz) would propagate more effectively while probing due to increased connectivity, as herbaceous plants often function as low-pass filters in which higher frequencies attenuate more rapidly with distance than lower frequencies, although this relationship is difficult to predict (eg Michelsen et al. 1982, Čokl et al. 2007, 2014). On the other hand, signaling is energetically demanding (Kuhelj et al. 2015b), and signals produced while probing could be lower in strength as a result of the additional energy expenditure. Therefore, we propose three mutually exclusive hypotheses: (i) a feeding-communication tradeoff, where probing reduces signal strength and frequency pattern, (ii) a feeding-communication facilitation where probing increases signal strength and frequency pattern, or (iii) a scenario where probing behavior has no influence on signal transmission.

Historically, leafhopper signaling while probing has only been visually observed, without clear determination of whether leafhoppers are actively feeding from the phloem or xylem, or if the stylets are located within other plant tissues from which they do not necessarily feed. Electropenetrography (EPG) is a powerful tool for quantifying probing behavior of piercing–sucking insects, allowing precise quantification of feeding durations within different plant tissues over time (e.g, Walker et al. 2024). EPG involves passing an electrical current through an insect and a plant and analyzing patterns of voltage changes over time which correspond to known feeding activities within different plant tissues (McLean and Kinsey 1964, Tjallingii 1988). While it is established that insect feeding activity is influenced by various types of vibrational stimuli (Avosani et al. 2021, Zippari et al. 2024), to our knowledge, EPG and substrate-borne vibrational recordings have not been applied simultaneously to assess interactions between feeding and communication in an individual insect. Pairing measurements of vibrational behavior with precise feeding observations would help identify impacts of feeding on vibrational communication.

The beet leafhopper, *Neoaliturus tenellus* (Baker) (Hemiptera: Cicadellidae), is an ideal model organism to assess impacts of feeding on communication. Due to its economic importance as a vector of beet curly top virus and potato purple top phytoplasma (e.g Horton et al. 2018), considerable research effort has been dedicated to characterizing *N. tenellus* behaviors. Vibrational signals and mating behaviors of *N. tenellus* have previously been recorded (Perkes 1970, Smith 1970), and the morphology of the vibration-producing tymbal organ has been characterized (Smith and Georghiou 1972). Recently, male beet leafhopper signals from these recordings were analyzed in detail to help delineate species in the *Neoaliturus* species complex (Sinaiko and Dietrich 2025). While these original recordings (ie Smith 1970) were conducted using current technology at the time, recording the beet leafhopper using updated recording and spectral analysis techniques could provide a useful resource for future work regarding the beet leafhopper or leafhopper vibrational duetting more broadly. For leafhoppers, EPG waveforms are species-specific and need to be characterized and correlated with probing behaviors using histological methods for each different species that is recorded (Backus et al. 2019). EPG waveforms have been characterized and correlated with probing behaviors of *N. tenellus*, allowing identification of when *N. tenellus* is engaged in pathway phase (stylets moving through the epidermis and mesophyll), phloem ingestion and salivation, and xylem ingestion (Stafford and Walker 2009, Stafford et al. 2009, Angelella et al. 2025). Simultaneously recording *N. tenellus* probing behaviors and substrate-borne vibrations allows for an opportunity to identify if communication differs when leafhoppers are engaged in different probing behaviors.

In the current study, we combine EPG and recording of substrate-borne vibrations to evaluate the potential for feeding-induced changes in vibrational signal transmission of a leafhopper. Specifically, we assess how spectral and temporal characteristics of male *N. tenellus* vibrational signals differ when leafhoppers are engaged in different probing behaviors on beet, *Beta vulgaris* (Caryophyllales: Amaranthaceae). We address the question of whether rostrum contact/insertion allows for improved signal transmission by quantifying metrics that capture the strength and frequency content of signals and comparing these values between non-probing and probing behaviors within individuals. We evaluate feeding in xylem and phloem separately, along with pathway probing behavior, and compare the number of signals while probing, and signal characteristics, to signal production when non-probing. In addition, to evaluate potential effects of EPG tethering on our observed behaviors, we record male vibrational signals in the absence of EPG to compare with signals made by isolated males. Lastly, we recorded the beet leafhopper male–female duet to illustrate the behavioral context in which male signals occur and to provide a base of comparison for future work on beet leafhopper vibrational communication.

Materials and Methods

Insects and Plants

N. tenellus colonies were maintained at the Tree Fruit Research and Extension Center in Wenatchee, WA, USA, initiated from colonies at the USDA Temperate Tree Fruit and Vegetable Research Unit (TTFVRU) in Wapato, WA, USA. These initial colonies were maintained from leafhoppers originally collected from radish (*Raphanus sativus*; Brassicales: Brassicaceae) and kochia (*Bassia scoparia*; Caryophyllales: Amaranthaceae) in Moxee, WA in *ca.* 2003. Leafhoppers were reared on beets (*B. vulgaris* cv. "Avalanche"; Johnny's Selected Seeds, Winslow, ME, USA) in a growth chamber (Model E41L2, Percival Scientific, Inc., Perry, IA, USA) at a daytime temperature of 24 ± 1°C, nighttime temperature of 20 ± 1°C, 40% relative humidity, and a photoperiod of 16:8 (L:D) h. Beets were grown in 10.8 × 10.8 × 9.5 cm plastic containers with Miracle-Gro potting mix (The Home Depot, Wenatchee, WA, USA). Unmated male and female leafhoppers were used for characterization of the mating duet, but the previous mating status of male leafhoppers used for simultaneous vibrational and EPG recordings, and for male vibrational recordings without EPG, was unknown. One- to two-month-old beets, at the 6–8 leaf stage, were used for all experiments.

Recording of Substrate-borne Vibrations

Vibrational recordings were conducted at a temperature of 24 ± 4.7 (mean ± SD)°C and 38.9 ± 10.2 (mean ± SD)% relative humidity at the Tree Fruit Research and Extension Center in Wenatchee, WA, USA. Recordings were conducted in a room equipped with Isolé Sound Barrier and Sound Absorption Sheets (Audimute, Bedford Heights, OH, USA) to minimize external noise and building vibrations. For each recording, a piezoelectric accelerometer (~0.8 g; Model 352A24, PCB Piezotronics, Depew, NY, USA) was attached to the petiole of a beet leaf, immediately basal to the leaf blade-petiole junction. Beet leaf blades were approximately 10–15 cm long. A small amount of Petro wax (Model 080A24, PCB Piezotronics) was placed on the face of the accelerometer, and another piece of Petro wax was attached to each side of the accelerometer and wrapped around the petiole to ensure the accelerometer maintained contact with the petiole (Fig. 1). A total of ~0.1–0.15 g of Petro wax was used for each recording. The accelerometer was connected to a signal conditioner that was connected to an audio interface and signals were recorded on a laptop in Audacity v. 3.4.2 (Muse Group, San Francisco, CA, USA) at a sampling rate of 44.1 kHz and 32-bit float format. For simultaneous EPG and substrate-borne vibration recordings of individual male *N. tenellus* a model 480E09 signal conditioner (PCB Piezotronics) and model U-Phoria UMC202HD audio interface (Behringer, Willich, Germany) were used. For recordings of the *N. tenellus* male-female duet a model 482C15 signal conditioner (PCB Piezotronics) and a model SSL12 audio interface (Solid State Logic, Oxford, England) were used. Signal conditioners were set to 10× gain, and audio interface gain was set to approximately 50%–75% but was consistent within each individual recording. Beet plants were placed on top of a noise–isolation stage, consisting of a 40 × 40 × 4.8 cm concrete block on top of a partially inflated bicycle inner tube, to help reduce noise in recordings. For simultaneous EPG-accelerometer recordings, the experimental set-up was housed inside a faraday cage (61 × 61 × 61 cm).

Simultaneous Recording of Vibrational Communication and Probing Behavior

Simultaneous EPG waveforms and substrate-borne vibrations were recorded for 6 hours from 15 individual male *C. tenellus*. All recordings were initiated between 1000 and 1300 hours. Vibrational and EPG recordings were initiated at the same time on the same laptop, with a temporal discrepancy of ~0–0.5 s between initiation of recordings. To correct for slight clock drift between the EPG and accelerometer recordings, accelerometer timepoints were multiplied by a linear correction factor (1.000162), calculated as the ratio of the EPG recording duration to the accelerometer recording duration, averaged across a subset of recordings from which precise starting and ending points were measured. To quantify the degree of clock drift and obtain this linear correction factor, a video (recorded at 30 frames per second) was taken of simultaneous EPG and Audacity recording windows on the laptop and analyzed frame by frame in a video editor to identify the precise moment that EPG recordings ended in relation to the time observed in Audacity. The linear correction factor was applied after all temporal and spectral characteristics of signals within each measurement type (ie biotremology and EPG) had already been calculated. In addition, to assess possible effects of EPG tethering on vibrational signaling behavior of males, vibrational recordings were conducted on 10 male beet leafhoppers that were not wired for EPG, and values of signal parameters from EPG-wired leafhoppers were compared to the range of variation observed in non-wired leafhoppers. For the experiment involving free moving males, a single male was released into a cage housing a beet plant, but the male's location on the plant or feeding status when signaling were not recorded. Due to logistical constraints, only one to two leafhoppers were able to be recorded at a time; when two leafhoppers were recorded, recordings were conducted with plants spaced at least ~25 cm apart to eliminate the possibility of air-borne vibration transmission that can occur at distances of ≤11 cm between partially overlapping leaves of two plants (Eriksson et al. 2011).

Recording and Analysis of Electropenetrography Waveforms

EPG recordings were conducted using a direct current Giga-8d monitor (EPG Systems, Wageningen, Netherlands) with 1 GΩ resistance. Insect electrodes consisted of a 2 cm piece of 18 µm diameter gold wire attached to a 2 cm piece of copper wire that was soldered to a small brass pin. For attachment of insect electrodes, leafhoppers were immobilized using a vacuum device (EPG Systems, Wageningen, The Netherlands). The suction device consisted of a 10 µL pipette tip connected to a flowmeter (Model VFA-24, Dwyer®, Michigan City, IN, USA) that was connected to a vacuum pump (Model 2534B-01, Welch®, Mt. Prospect, IL, USA) using plastic tubing. Airflow was adjusted to 2–4 L/min on the flowmeter, and leafhoppers were carefully placed on top of the pipette tip of the suction device under a stereo microscope (Model S9E, Leica Microsystems Inc., Deerfield, IL, USA), with the gold wire attached to leafhopper's pronotum using a small amount of silver glue. Leafhoppers were starved for 1–1.5 h prior to recording to standardize previous host plant contact and leafhopper physiological status. A plant electrode was inserted into the well-watered soil of a beet container. Insect electrodes were attached to leafhoppers, inserted into head stage amplifiers,

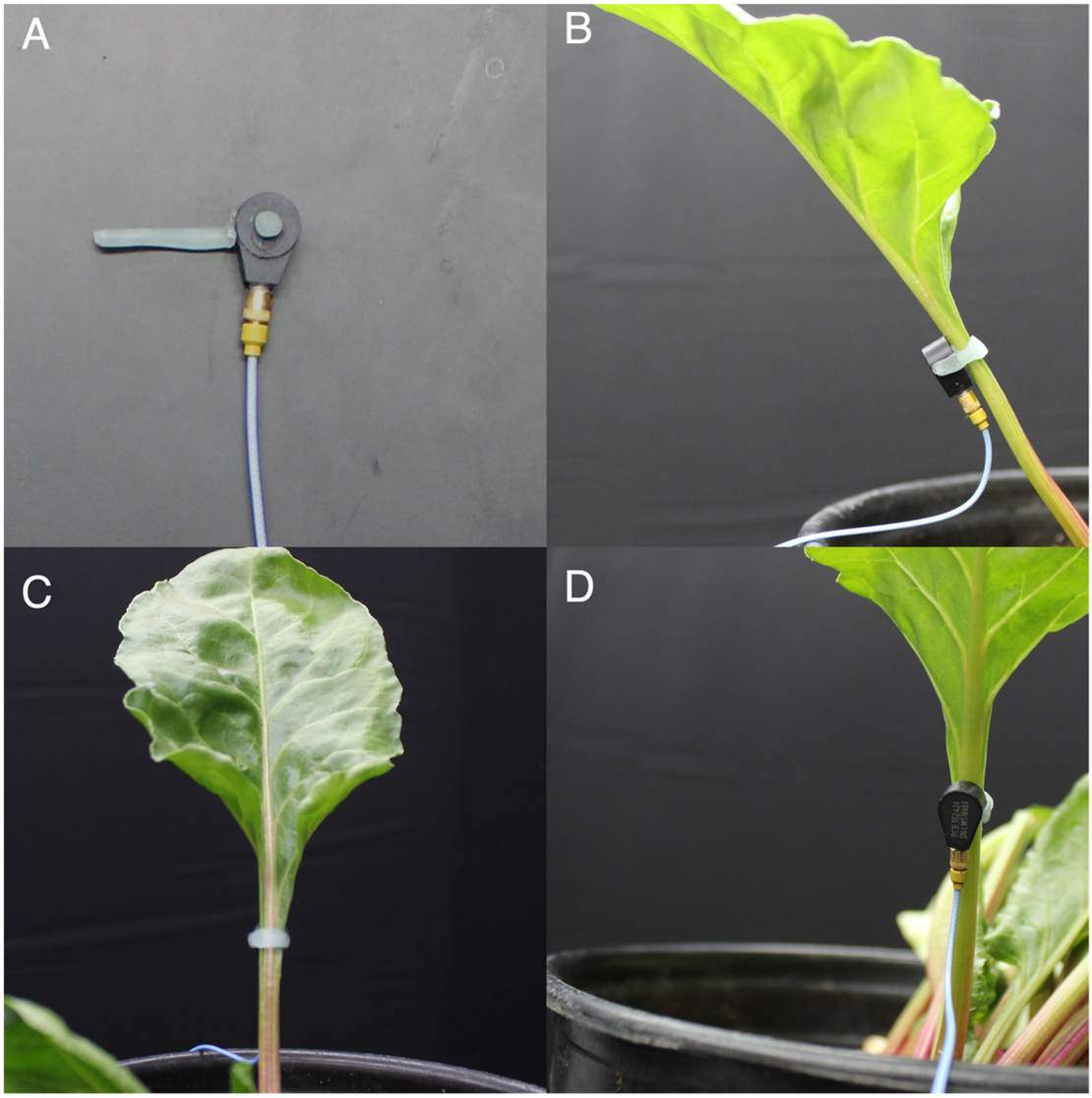

Fig. 1. Attachment of accelerometer to petiole of beet leaf. (A) Accelerometer with Petro wax; (B) lateral view; (C) adaxial view; (D) abaxial view.

and leafhoppers were lowered onto the adaxial surface of beet leaves immediately after the recording was initiated. Gain was set to the default 50× and applied voltage was adjusted so that the initial pathway phase waveform was at approximately 3–4 V, so that signals appeared within range (Trębicki et al. 2012, Tjallingii 2019). Because leafhoppers were tethered to 2 cm long wires, leafhoppers were restricted to the adaxial leaf surface, and the maximum variation in leafhopper location during a 6-h recording was 4 cm.

EPG waveforms were analyzed using Stylet+ software (EPG Systems). Waveforms were marked according to the *N. tenellus* waveform library developed by Stafford and Walker (2009). Waveforms that represent the same or similar biological activity were grouped and analyzed together: waveform Z as non-probing; waveforms A, B1, B2, and C grouped as pathway phase, which is when the stylets are moving through epidermis and mesophyll tissues; waveform D1 as phloem salivation; waveforms D2, D3, and D4 grouped as phloem ingestion and/or simultaneous phloem ingestion/salivation; waveform G as xylem ingestion. We refer to "non-probing" as representing no contact between the leafhopper's stylets and the plant, and "probing" as representing any of the four behaviors of pathway, phloem ingestion, phloem salivation, and xylem ingestion. We refer to "feeding" as representing phloem ingestion, phloem salivation, or xylem ingestion. A total of 18 leafhoppers were recorded, but three leafhoppers became disconnected from insect electrodes during the recording, resulting in a final sample size of 15. Waveforms were marked using Stylet+, and the total duration of each waveform or waveform grouping was calculated. Stylet+ grid files were exported as .csv files for analysis of which probing behaviors leafhoppers were engaged in while vibrational signals were produced.

Recording of *N. tenellus* Male–Female Duet

Substrate-borne vibrations were recorded simultaneously with video recordings to observe the male-female *N. tenellus* duet. Unmated male and female *N. tenellus* were obtained by rearing late-instar nymphs individually in clip cages (3.8 cm inside diameter and 2.5 cm height) attached to beets leaves until they reached adulthood. Unmated male and female *N. tenellus*

(4–8 days old) were placed on a beet plant inside of a mesh cage (30.5 × 30.5 × 30.5 cm; BioQuip Products Inc., Compton, CA, USA), that had a clear panel on one side to allow visual observation. Male and female leafhoppers were simultaneously released into the cage from separate collection vials and allowed to orient haphazardly on the plant, resulting in different starting positions and reciprocal distances between recordings. All leaves, except the leaf that the accelerometer was attached to, were removed from the plant to facilitate successful video recordings by restricting the amount of plant surface area that leafhoppers had access to. A plant grow light (Model FC-E1500 Bridgelux 150w LED, Mars Hydro, Shenzhen, China) was situated ~15–20 cm above the top of the cage. Video recordings were conducted using a digital single-lens reflex camera (Model Rebel EOS T7, Canon U.S.A, Inc, Melville, NY, USA) equipped with a macro-lens (Model EF-S 60 mm f/2.8 Macro USM, Canon U.S.A, Inc.) that was mounted on a tripod. Audio and video files were compiled and synchronized using Movavi© Video Editor v. 25.8.0 (Movavi Software Limited, Wildwood, MO, USA). Male and female signals were identified by observing abdomen movement (resulting from tymbal organ movement) that occurred during signal production and by comparing recordings to observations made by Smith (1970). Three simultaneous vibrational and video recordings of the male-female duet, and two recordings of the copulation song, were obtained.

Analysis of Substrate–Borne Vibrational Signals

Vibrational recordings obtained in Audacity were exported as WAV files (16-bit depth) and oscillograms and spectrograms were analyzed in Raven Pro v. 1.6 (K. Lisa Yang Center for Conservation Bioacoustics at the Cornell Lab of Ornithology 2024) and R software v. 4.5.1 (R Core Team 2022). For spectral analysis of unedited audio files in Raven Pro, selection boxes were created with a low frequency limit of 260 Hz and a high frequency limit of 5,000 Hz to prevent the inclusion of lower frequency energy generated by external noise, and to capture the energy of *N. tenellus* that is within this frequency range (Smith 1970, Sinaiko and Dietrich 2025, Fig. 2). For visualization, all spectrograms and oscillograms were subjected to a 250 Hz high-pass filter to remove low-frequency background noise. Spectrograms were analyzed using a fast Fourier transform with a Hann window size of 4,096 samples and 50% overlap.

Male *N. tenellus* vibrational signals have previously been characterized as a series of brief pulses (ie homogeneous parcels of sound not interrupted by silence; Broughton (1963) that collectively comprise a "pulse train" (Smith 1970, Sinaiko and Dietrich 2025). Following similar terminology in other leafhopper systems (Nieri et al. 2017, Krugner and Gordon 2021), a pulse train was treated as a "signal", and termed male signal type 1 (MS1). Multiple MS1 often occurred in succession and were termed "signaling bouts". MS1 that were separated by less than 3.62 s were considered a part of the same signaling bout. This threshold was determined by calculating the 100th percentile of male beet leafhopper pulse train latency values reported by Smith (1970) to ensure that pulse trains closely spaced in time were classified as part of the same signaling bout, while avoiding unnecessary categorization of closely spaced pulse trains into many separate signaling bouts. Two different subtypes of MS1 were produced (MS1a and MS1b) which were noticeably distinct in frequency composition of the last pulse in the pulse train (Fig. 2). Only MS1a was analyzed, as it was the most abundant signal type and was present in every recording (see the Results section). For each MS1a signal, the following strength and frequency characteristics were quantified in Raven Pro: filtered root mean square (RMS) amplitude (dimensionless sample units); peak power density (dB FS/Hz); and dominant frequency (Hz). Filtered RMS amplitude (hereafter, "amplitude") was chosen as a metric for signal strength, as this metric represents the average power over time contained within a frequency range of interest (Charif et al. 2010). In addition, peak power density was quantified, as this metric represents the maximum power produced at any given frequency within a signal. Dominant (ie peak) frequency represents the frequency containing the highest power/amplitude in a signal (ie the frequency at which the peak power density occurs). In addition, the following temporal features were quantified: MS1a duration (s); MS1a repetition time (the time from the beginning of one MS1a to the beginning of the next; s); number of MS1a per signaling bout. Individual male *N. tenellus* occasionally produced two other distinct types of signals, which were termed male signal types 2 and 3 (MS2 and MS3). MS2 and MS3 were rarely observed from isolated males, so spectral and temporal characteristics of these signal types were not quantified for simultaneous EPG-accelerometer recordings of individual male *N. tenellus*, but values were quantified for males from recordings of the male–female duet. Consistent with Smith (1970), we observed only one type of signal produced by female *N. tenellus*, and this was termed female signal type 1 (FS1). For recordings of the male–female duet, 30 representative examples of each signal type (MS1a and MS1b, MS2, MS3, FS1, and copulation signals), were randomly selected, when available, for quantification of spectral and temporal parameters. For one male–female duet, copulation was not achieved. For vibrational recordings of leafhoppers not wired for EPG, signals were analyzed for 1 h after the first signal occurred. For vibrational recordings of EPG-wired leafhoppers, all signals within the 6-h recording were analyzed.

Statistical Analysis

Statistical analyses and figure construction were conducted in R v. 4.5.1. (R Core Team 2022) using the packages "seewave," "lme4," "lmerTest," "tidyverse," "DHARMa," "tuneR," "MuMIn," and "ggpubr" (Sueur et al. 2008, Bates et al. 2015, Kuznetsova et al. 2017, Wickham et al. 2019, Hartig 2024, Ligges et al. 2024, Bartoń 2025, Kassambara 2025). General linear mixed-effects models were used to determine effects of probing behaviors on spectral and temporal values of MS1 produced by individual male *N. tenellus*. Using the "lmerTest" package in R, Satterthwaite's method was used to estimate denominator degrees of freedom and calculate *P*-values from corresponding t-tests for each fixed effect. The response variable for each model included mean values of MS1a amplitude, peak power density, dominant frequency, duration, repetition time, or number of signals per bout for each individual. For each signal parameter, two models were created, one with probing status ("non-probing" and "probing") and one with specific probing behavior ("non-probing," "pathway," "phloem ingestion," and "xylem ingestion") as fixed effects. No signals were produced while leafhoppers were engaged in phloem salivation (see the Results section), so this behavior was not included in models. In each model, the individual ID of

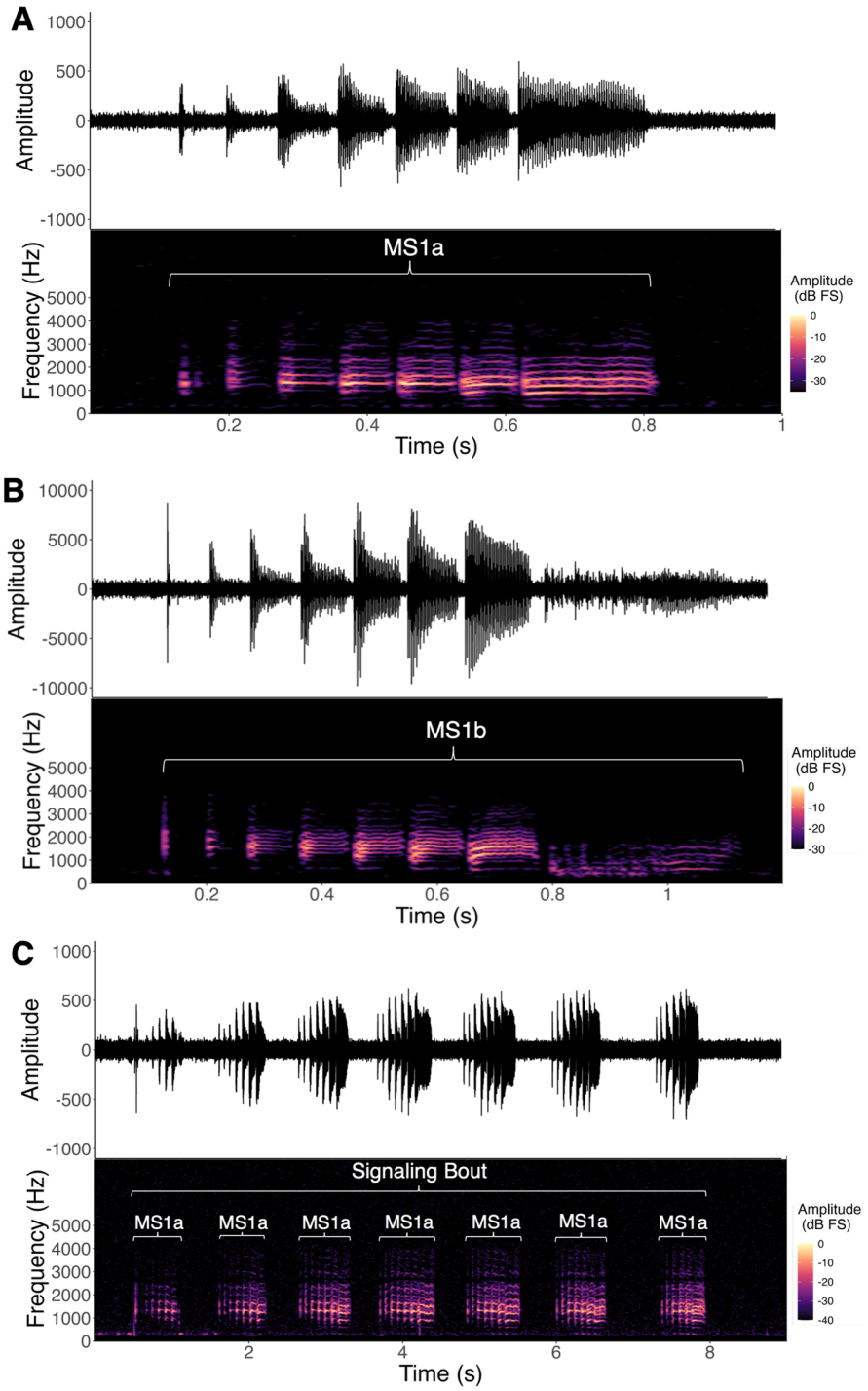

Fig. 2. *Neoaliturus tenellus* male vibrational communication signal type 1 (MS1). (A) Oscillogram (top) and spectrogram (bottom) of a single MS1a composed of multiple pulses; (B) Oscillogram (top) and spectrogram (bottom) of a single MS1b composed of multiple pulses with the final pulse distinguishing it from MS1a. (C) Oscillogram (top) and spectrogram (bottom) of a signaling bout of multiple MS1a. Audio files used for oscillograms and spectrograms were subjected to a 250 Hz high pass filter to remove low-frequency background noise. Hann window sizes and overlap were 1,024 samples and 90% for spectrograms in A and B, and 2048 samples and 75% overlap for the spectrogram in C.

leafhoppers was included as a random effect. This enabled modeling the effect of probing behaviors on MS1 parameters while accounting for potential variation arising from differences in individuals or the recording setup, such as slight differences in accelerometer placement on beet leaves, audio interface gain adjustment between recordings, or inherent differences in the number of signals produced between leafhoppers. For simultaneous EPG-accelerometer recordings of individual male *N. tenellus*, leafhoppers were recorded on individual plants in a randomized order. However, due to resource constraints, 11 beet plants were used, and two plants were used more than once in recordings. Thus, plant identity

for each assay was initially included in the model as a random effect to control for repeated sampling. However, plant ID did not improve the fit of any model, according to Akaike information criterion corrected for small sample sizes (AICc), suggesting that plant ID did not influence vibrational signals, so this random effect was removed in final models. Residuals from each final model were assessed to ensure they met assumptions of normality and homoscedasticity. Linear mixed-effects model structure can be found in Table S1.

In addition, because temperature can strongly impact signal dominant frequency (Jocson et al. 2019, Leith et al. 2021), a data logger (Model UX100-003, Onset Computer Corporation, Bourne, MA, USA) was used to monitor the temperature inside of the recording room for a subset ($n = 7$) of individual male beet leafhopper recordings. Preliminary analysis of a linear mixed-effects model with hourly mean MS1a dominant frequency per individual as a response variable, hourly mean temperature as a fixed effect, and individual ID as a random effect showed no significant influence of temperature on dominant frequency ($t = 1.5$; df = 8.2; $P = 0.18$), and was removed from the final model. In addition, we compared individual mean values of MS1a from 6-h recordings of 15 leafhoppers wired for EPG with individual mean values of MS1a measured during the first hour after signal onset in 10 leafhoppers not wired for EPG, using the Mann–Whitney U test. Parameter values for signals produced by duetting male and female *N. tenellus* were reported but not included in statistical analyses (Fig. S1; Table S2).

Results

Vibrational Signal Characterization

A total of 4,471 vibrational signals were recorded from 15 male *N. tenellus* during 6-hour EPG-accelerometer recordings. The majority (97.88%) of signals were categorized as MS1a (Fig. 2A and C; Files S1 and S2), while other signal types MS1b (Fig. 2B; File S3) and MS3 (Fig. S1B; File S4) occurred much less frequently (0.25% and 1.88% of signals, respectively). There was a high degree of variability between leafhoppers in the number of signals produced during 6-hour recordings. Male beet leafhoppers produced a median of 208 MS1a during 6-hour recordings (IQR: 96.0–302.5), and a median of 3.0 MS1a per signaling bout (IQR: 1–5). We observed that male MS1a signals elicited a female response, males moved to the position of the stationary female during the duet, and males engaged in a courtship dance prior to copulation. Signals observed from male-female recordings were distinct in spectral and temporal characteristics (Table S2; Fig. S1; Files S4–S6).

Influence of EPG Tethering on Male Vibrational Signals

The number of MS1a per signaling bout and MS1a dominant frequency were not significantly different between leafhoppers wired for EPG and leafhoppers not wired for EPG ($U = 54$ and $P = 0.261$, $U = 92$ and $P = 0.367$, respectively; Fig. S2C and F). However, MS1a duration was shorter and MS1a repetition rate was longer for EPG-wired leafhoppers compared to leafhoppers not wired for EPG ($U = 116$ and $P = 0.023$, $U = 118$ and $P = 0.016$, respectively; Fig. S2D and E). In addition, the strength metrics, amplitude and peak power density, were significantly higher for EPG-wired leafhoppers, which were restricted to the leaf the accelerometer was attached to, than non-wired leafhoppers that moved freely about the plant ($U = 28$ and $P = 0.008$, $U = 29$ and $P = 0.010$, respectively; Fig. S2A and B).

Probing Behaviors Occurring during Vibrational Signal Production

During non-probing behavior, leafhoppers produced a total of 1,403 MS1a. During probing behavior, leafhoppers produced a total of 2,973 MS1a. The most common probing behavior exhibited during signal production was pathway phase, followed by non-probing, xylem ingestion, and phloem ingestion (Table 1; Fig. 3A). All 15 leafhoppers produced signals while engaged in pathway phase and xylem ingestion, 13 produced signals while non-probing, and only 8 produced signals while engaged in phloem ingestion. No signals were recorded while leafhoppers were engaged in phloem salivation, although this probing behavior was only observed from 5 individuals in our recordings and constituted a very small percentage of the overall recording time (Table 1; Fig. 3B). Across our experimental leafhoppers, the relative time spent signaling in each probing behavior type (non-probing and each probing type) generally reflected the total duration of each probing behavior (Table 1).

Signals Are Higher Amplitude and Frequency When Probing

The amplitude of MS1a was significantly higher when leafhoppers were probing than when they were non-probing ($t = 3.1$; df = 12.8; $P = 0.009$; Fig. 4A). In addition, the dominant frequency of MS1a was significantly higher when leafhoppers were probing than when they were non-probing ($t = 6.3$; df = 12.6; $P < 0.0001$; Fig. 4C). The mean percent increase in amplitude and dominant frequency of MS1a produced while probing, compared to non-probing, was 37.1% and 41.2%, respectively. Brief simultaneous video-EPG-accelerometer recording, to obtain a visual representation of individual male *N. tenellus* calling behavior, showed an increase in signal strength and dominant frequency when the leafhopper was probing (Video S1; amplitude while probing ($n = 7$) and non-probing ($n = 3$) was 549.9 ± 98.3 and 284.8 ± 21.0, respectively [mean ± sd]; dominant frequency while probing and non-probing was 1488.9 ± 64.2 Hz and 861.3 ± 159.3 Hz, respectively). However, this video recording was intended for observational purposes and was not used for separate statistical analyses from the 15 simultaneous EPG-accelerometer recordings. MS1a amplitude and dominant frequency while probing and non-probing were variable between individual leafhoppers

Table 1. Total percentage of probing behavior durations and percentage of the number of male signal type 1a (MS1a) while engaged in each probing behavior from 6-h recordings ($n = 15$)

Probing behavior	Total recording time spent in behavior (%)	Total MS1a produced during behavior (%)
Non-probing	28.2	32.1
Probing	71.8	67.9
Pathway	32.1	37.5
Phloem ingestion	18.4	14.9
Phloem salivation	0.2	0.0
Xylem ingestion	21.2	15.5

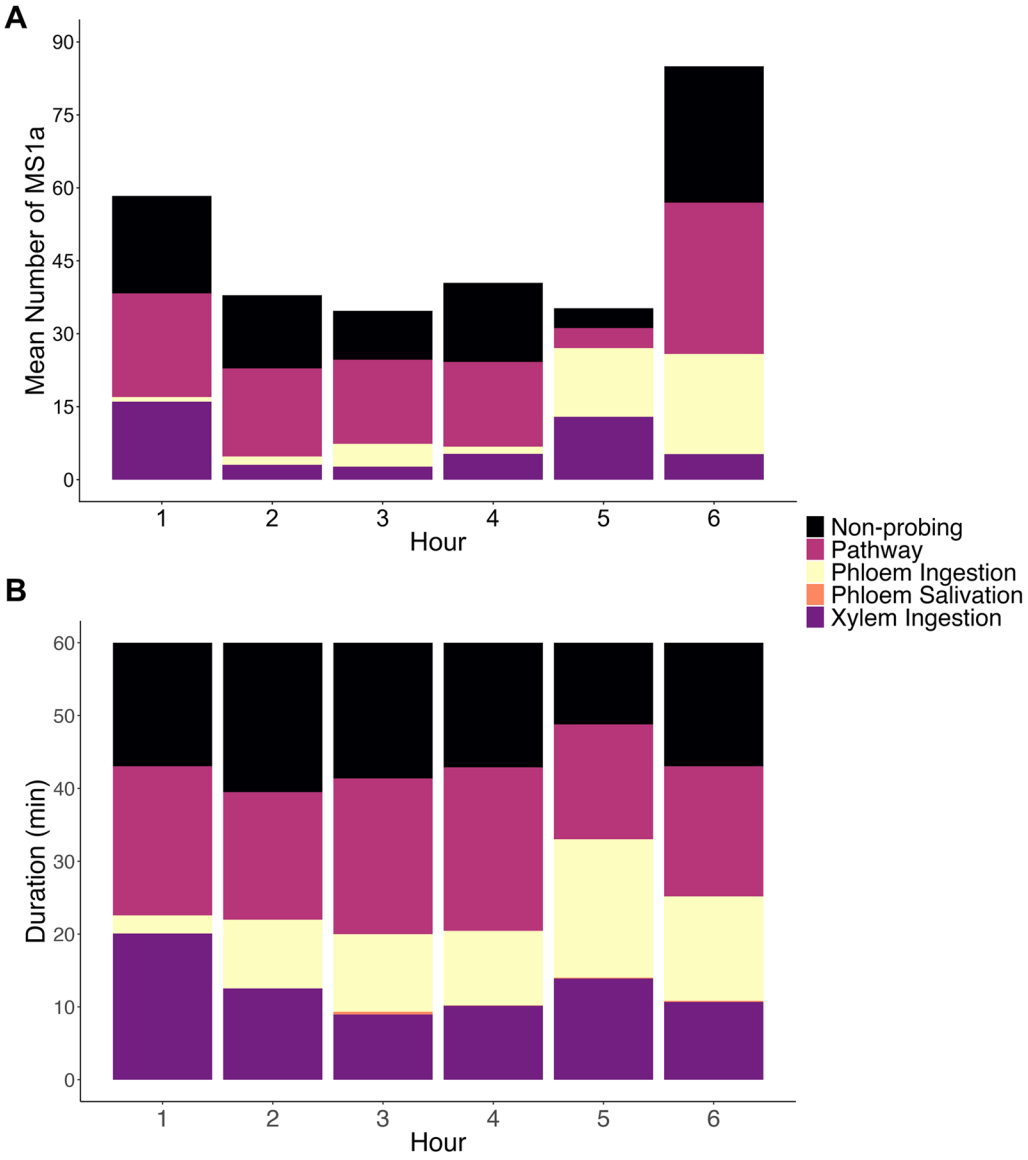

Fig. 3. (A) Mean number of male signal type 1a (MS1a) produced by 15 male *Neoaliturus tenellus* while engaged in each probing behavior by hour during 6-h recordings. (B) Mean duration of each probing behavior across leafhoppers by hour during 6-h recordings.

(Figs S3 and S4, respectively). There were no significant effects of probing status on MS1a peak power density, duration, repetition rate, or number of signals per signaling bout (Fig. 4B and D–F; Table S3).

Effects of Probing on Signal Characteristics Depend on Specific Probing Behavior

Different probing behaviors that leafhoppers were engaged in during signal production had significant effects on signal amplitude, peak power density, dominant frequency, and repetition rate (Table 2). Specifically, MS1a amplitude was significantly higher when leafhoppers were engaged in pathway phase ($t = 3.4$; df = 33.3; $P = 0.002$) and xylem ingestion ($t = 3.9$; df = 33.3; $P = 0.0005$) than when leafhoppers were non-probing. Similarly, compared to MS1a produced while non-probing, MS1a signals had higher peak power density when leafhoppers were engaged in pathway phase ($t = 2.1$; df = 33.6; $P = 0.040$) and xylem ingestion ($t = 2.1$; df = 33.6; $P = 0.040$). In addition, compared to signals produced while non-probing, signals had higher dominant frequency when leafhoppers were engaged in pathway phase ($t = 4.8$; df = 33.5; $P < 0.0001$), phloem ingestion ($t = 4.5$; df = 34.8; $P < 0.0001$), and xylem ingestion ($t = 4.7$; df = 33.5; $P < 0.0001$). MS1a repetition rate was significantly shorter when leafhoppers were engaged in phloem ingestion than when leafhoppers were non-probing ($t = -2.3$; df = 36.4; $P = 0.030$). Probing behavior did not have a statistically significant influence on MS1a duration or number of signals per signaling bout (Table 2).

Discussion

Vibrational communication signals that are stronger are generally more likely to be perceived (Cocroft and Rodríguez 2005, Eriksson et al. 2011, Caorsi et al. 2021). Further,

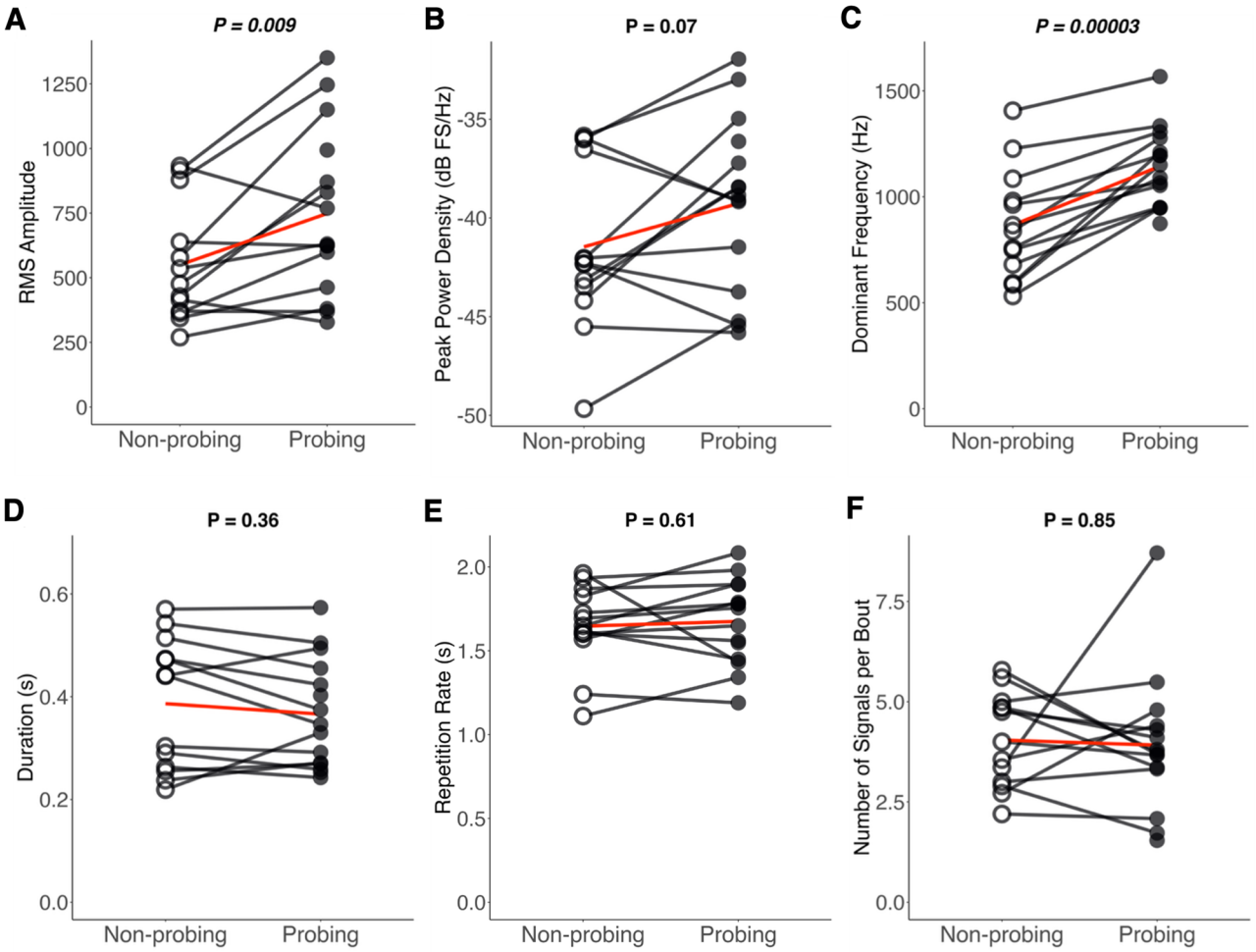

Fig. 4. Mean values of male signal type 1a (MS1a) root mean square (RMS) amplitude (A), peak power density (B), dominant frequency (C), duration (D), repetition rate (E), and number of signals per signaling bout (F) while leafhoppers were non-probing and probing. Each point represents the mean of values from a 6-h recording of an individual *Neoaliturus tenellus* ($n = 15$). Red lines represent mean change across individuals. *P* values are reported from linear mixed-effects models with signal parameter as a function of probing status, with individual ID as a random effect. Fixed effect summaries for each model can be found in Table S3.

Table 2. Fixed effects summaries from linear mixed-effects models of male signal type 1a (MS1a) spectral and temporal parameters as a function of probing behaviors ($N = 15$; $n = 51$)

	Pathway effect				Phloem ingestion effect[a]				Xylem ingestion effect			
	Estimate	df	*t*	*P*	Estimate	df	*t*	*P*	Estimate	df	*t*	*P*
Filtered RMS amplitude	*195.4*	*33.3*	*3.4*	*0.002*	52.8	33.8	0.8	0.50	*220.6*	*33.3*	*3.9*	*0.0005*
Peak power density (dB FS/Hz)	*2.3*	*33.6*	*2.1*	*0.04*	−0.14	34.3	−0.11	0.92	*2.3*	*33.6*	*2.1*	*0.04*
Dominant frequency (Hz)	*274.5*	*33.5*	*4.8*	*0.00004*	*318.7*	*34.8*	*4.5*	*0.00007*	*269.7*	*33.5*	*4.7*	*0.00005*
Duration (s)	−0.02	33.2	−1.0	0.30	−0.02	33.6	−0.7	0.50	−0.02	33.2	−0.86	0.40
Repetition rate (s)	0.09	34.6	1.0	0.34	*−0.24*	*36.4*	*−2.3*	*0.03*	−0.0005	34.6	−0.05	1.0
Number of signals per signaling bout	0.03	33.8	0.06	0.95	−0.15	35.0	−0.2	0.82	0.34	33.8	0.63	0.53

N represents the number of individuals; *n* represents the number of mean values from signals while individuals were engaged in different probing behaviors. Italicized values indicate a statistically significant effect (P<0.05).
[a]Only eight individuals produced MS1a while ingesting phloem.

frequency characteristics of signals can influence mate preference (Mazzoni et al. 2015). Here, by combining simultaneous EPG and accelerometer recordings of individual beet leafhoppers, we found that vibrational signals produced while leafhoppers were probing were on average 37.1% higher in amplitude and 41.2% higher in dominant frequency than

signals produced while non-probing. These data support our feeding-communication facilitation hypothesis, where rostrum contact/insertion allows for improved signal clarity by improving insect-plant connectivity. To our knowledge, this study represents the first simultaneous electronic monitoring of feeding and communication behaviors in an individual insect.

Consistent with findings from Perkes (1970) and Smith (1970), male–female duet recordings showed that the male calling signal MS1a elicited responses from females. The majority of signals of this type from 6-hour recordings of isolated males were produced while males were probing, reflecting the overall time spent feeding. In addition to potentially improved signal transmission, it is possible that leafhoppers produce signals while they are probing because of the need to quickly locate a mate in the context of rival males (Kuhelj and Virant-Doberlet 2017). Indeed, female beet leafhoppers only mate once in their lifetime, while males mate multiple times (Smith 1970), so the ability of males to quickly locate a female is presumably an important trait. The only probing behavior during which leafhoppers did not produce vibrational signals was phloem salivation, although, similar to findings from Angelella et al. (2025), this behavior was not as common as other probing behaviors in our recordings. Although the majority of signals from EPG-tethered leafhoppers were produced during probing behavior, it is important to recognize that leafhoppers were restricted to a single leaf and were not able to express typical call-fly behavior. To better understand the biological significance of calling-while-feeding, further work is needed to determine how male leafhoppers balance calling-while-feeding and call-fly behavior while sexually active. Yet it is also important to note that leafhoppers are known to frequently produce vibrational signals while probing or feeding (Claridge 1985, Kuhelj et al. 2015b), suggesting that calling-while-feeding is not a rare pattern of behavior.

Coupling of the animal and substrate is a key factor in propagation of vibrational signals (Mortimer 2017). The legs and mouthparts that couple insects and the plant surface presumably represent a bottleneck for signal transmission, as the strength of substrate-borne vibrations can be up to 10 dB greater when measured on the insect body than on the plant surface immediately below the insect (Amon and Čokl 1990). During probing, the labium of a leafhopper is in contact with the surface of the plant, while the mandibular and maxillary stylets move through plant tissues in an attempt to locate phloem or xylem. Interestingly, signal strength (ie amplitude and peak power density) was positively influenced by both pathway phase and xylem ingestion, but strength was the highest when leafhopper mouthparts were located in the xylem; signal strength was similar while nonprobing and ingesting phloem. Considering that xylem is under negative pressure in most cases (Scholander et al. 1965), and phloem is under high positive pressure (Münch 1930), it is possible that the suction resulting from negative pressure of xylem increased the contact force (ie coupling) between leafhoppers and the plant surface, leading to higher signal amplitude during xylem ingestion. However, the contact force between leafhoppers and the plant surface was not measured in the current study and this hypothesis would need to be specifically evaluated in future experiments. In addition, it is possible that signals produced while the beet leafhopper was probing were inherently stronger than signals produced while not probing, regardless of contact with the substrate. This could be tested using a similar method to that of Amon and Čokl (1990), in which a laser vibrometer would be used to measure signal strength of the leafhopper body directly while probing. Nonetheless, contact between the leafhopper and the plant substrate was measured indirectly using EPG in this study, and the fact that vibrational signals were higher in strength and frequency when probing supports the idea that the mouthpart insertion provides additional coupling between the leafhopper and the plant substrate, promoting signal propagation.

Detection of substrate-borne vibrations by leafhoppers is thought to be accomplished mainly through mechanoreceptors located within the legs and abdomen (Čokl and Virant-Doberlet 2003, Eriksson et al. 2011, Ehlers et al. 2022). These specialized mechanoreception systems consist of external and internal sensilla and chordotonal organs that perceive substrate-borne vibrations and transmit information to the central nervous system (Lakes-Harlan and Strauß 2014, Virant-Doberlet et al. 2023). Given the importance of mechanical coupling for vibration transmission within insect bodies (Strauß et al. 2024), it is possible that additional coupling between the leafhopper and plant substrate while probing improves vibration transmission from the substrate to the mechanoreceptive organs within the leafhopper body.

Because increased signal strength allows insects to extend the effective range that their signals can be perceived (ie active space; Mazzoni et al. 2014), it is possible that increased signal strength while probing is a way for leafhoppers to partially compensate for decreased active space incurred from the decreased mobility associated with probing. Indeed, male *Scaphoideus titanus* (Hemiptera: Cicadellidae) located females more when the relative amplitude of signals was higher (Eriksson et al. 2011). In addition, leafhoppers engage in eavesdropping behavior, where males "listen in" and attempt to intercept a female that is already engaged in a duet with a rival male (Mazzoni et al. 2009, Kuhelj and Virant-Doberlet 2017). Beet leafhopper males are known to produce signals in the presence of other males (Smith 1970), but we are not aware of any reports of rival male behavior in the beet leafhopper specifically. If the beet leafhopper expresses rival behavior similar to other leafhopper species, it is possible that the higher strength and frequency of signals produced while probing is a way for males to intercept a female already engaged in a duet. However, because comparisons of signal characteristics while probing and non-probing were conducted using individually recorded leafhoppers in this study, without presence of another male or female, increased signal strength and frequency as a result of rivalry behavior seems a less likely explanation than the function of extending active space.

Because we did not control for whether leafhoppers were signaling on veins or homogenous regions of lamina on the leaf, signals produced when leafhoppers were probing could have been influenced by an unidentified preference for probing on these tissues, which influence the strength and frequency of vibrations differently (Magal et al. 2000, McGinley et al. 2024, Shan et al. 2025). However, because leafhoppers were tethered for EPG using 2 cm wires, all signals produced by the same leafhopper during a recording could not be separated by more than 4 cm on the leaf, reducing variation in signal characteristics for an individual leafhopper. Further, supplemental video recording showed that an individual leafhopper produced stronger and higher frequency signals while probing as opposed to non-probing when at the same precise location on the leaf. The significant

influence of probing status on signal strength and frequency during 6-h recordings suggests that probing status is likely a source of variation for signal characteristics, a previously unrecognized factor that should be considered in future work.

Signals produced by free-moving leafhoppers were weaker but of longer duration than signals produced by leafhoppers wired for EPG. When interpreting this comparison, it is important to note that the location of free-moving males was not monitored, in contrast to males wired for EPG which were restricted to the same area (ie <4 cm diameter) on a leaf. This difference could greatly influence signal characteristics, as variations in substrate characteristics influence vibrations in ways that are difficult to predict (eg Magal et al. 2000). Despite this experimental limitation, the dominant frequency of signals was not significantly different, suggesting that the effect of probing on signal frequency was greater than the effect of signaling location in our recordings. In future work, the effects of EPG tethering on vibrational signals could be determined by using video recordings to monitor the location of free-moving leafhoppers and EPG-tethered leafhoppers during the entire recording period. However, effects of EPG tethering on vibrational behavior do not preclude comparisons of vibrational behavior within/between leafhoppers wired for EPG.

Substrate-borne vibrational communication does not occur in isolation, and natural enemies can exploit vibrational signals of prey, putting prey that signal with higher strength or longer periods of time at greater risk (Virant-Doberlet et al. 2019). For example, in a leafhopper-spider system, *Enoplognatha ovata* (Araneae: Theridiidae) altered its foraging behavior in response to higher amplitude signals of male *Aphrodes makarovi* (Hemiptera: Cicadellidae), but not in response to rarer, lower amplitude female signals or incidental signals from walking, resulting in higher predation rates of males (Virant-Doberlet et al. 2011). Common natural enemies of the beet leafhopper, such as spiders, big-eyed bugs, parasitic wasps, and twisted-wing parasites (Douglass and Cook 1954) could have a greater chance of detecting stronger signals produced while the beet leafhopper is probing. The immobility of leafhoppers while probing could also increase chances of predation. Future research should investigate natural enemy responses to beet leafhopper vibrational communication to better understand the significance of changes in vibrational signals produced while probing.

Simultaneous recording of feeding and communication behaviors of individual insects as an experimental approach yields potential to provide insight into insect behavioral ecology and management. This approach could provide insight into whether negative effects of experimental treatments on populations are due to reduced feeding or reduced reproduction, by means of impaired sexual communication. For example, simultaneous EPG and accelerometer (or laser vibrometer) recordings could be used to assess sublethal effects of insecticides by quantifying feeding behavior in addition to muscle use resulting from tymbal movement. In addition, various pathogens and symbionts are known to influence leafhopper feeding and reproductive behavior (eg Angelella et al. 2025, Han et al. 2024), so this approach could provide additional insight into vector manipulations. Overall, the significance of interactions between feeding and communication of individual leafhoppers is largely unexplored, and it is likely that novel interactions have yet to be uncovered.

Conclusions

In summary, we found that the act of probing plant tissues influences information contained in vibrational communication signals of a leafhopper in ways that may promote communication with conspecifics. Specifically, the higher strength and frequency of vibrational signals produced while probing supports a feeding-communication facilitation hypothesis, where probing improves signal transmission, potentially by increased coupling between the leafhopper and plant substrate. We evaluated probing and communication of the beet leafhopper on beet plants, but future work could determine whether this phenomenon extends to other leafhopper species. Combining simultaneous recording of feeding and communication behaviors has potential to provide insight into fundamental and applied questions related to leafhopper behavioral ecology and vector management.

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture. USDA is an equal opportunity provider and employer. William Jacob Pitt is a Washington Research Foundation postdoctoral fellow.

Author Contributions

William Jacob Pitt (Conceptualization [equal], Formal Analysis [equal], Investigation [lead], Project Administration [lead], Methodology [equal], Funding acquisition [equal], Writing—original draft [lead]), Monica Oropeza Rojas (Investigation [equal], Data curation [equal], Writing—original draft [supporting]), Valarie Manzaro (Investigation [equal], Data curation [equal], Writing–review & editing [equal]), Edith Chamberlain (Investigation [supporting], Data curation [equal], Writing–review & editing [equal]), Gina M. Angelella (Resources [equal], Validation [equal], Writing–review & editing [equal]), Adrian T. Marshall (Conceptualization [equal], Investigation [supporting], Methodology [equal], Writing–review & editing [equal]), Dowen Mae I. Jocson (Conceptualization [equal], Validation [equal], Writing–review & editing [equal]), W. Rodney Cooper (Conceptualization [equal], Funding acquisition [equal], Writing–review & editing [equal]), and Tobin D. Northfield (Conceptualization [equal], Supervision [lead], Formal Analysis [equal], Funding acquisition [equal], Writing—original draft [equal])

Supplementary Material

Supplementary material is available at *Annals of the Entomological Society of America* online.

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Conflicts of Interest

None declared.

Data Availability

Data from this study are available from the Dryad repository. DOI: 10.5061/dryad.5dv41nsm7. Pitt et al. (2026).

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