The toxicity of flonicamid to cotton leafhopper, *Amrasca biguttula* (Ishida), is by disruption of ingestion: an electropenetrography study

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Abstract

BACKGROUND: The cotton leafhopper, *Amrasca biguttula*, is one of the most destructive pests of cotton in Asia. This species is thought to cause damage by injecting enzymatic saliva into various, presently unknown, cotton tissues and ingesting the resulting macerate. Flonicamid is a novel systemic insecticide used to control the cotton leafhopper; however, its mode of action is unknown.

RESULTS: The mechanism of action of flonicamid on cotton leafhopper was investigated using electropenetrography (EPG). EPG recordings revealed six waveforms, i.e. Np (non-probing), A1 (channel cutting), A2, A3, A4, A5 and A6. Waveforms A2 and A3 probably represent active ingestion with (A2) and without (A3) simultaneous watery salivation. The meanings of A4, A5 and A6 are presently unknown, but minor in duration. Flonicamid significantly increased the mean duration of non-probing events and strongly inhibited ingestion by treated insects, which resulted in the slow death of leafhoppers. Inhibition of ingestion was dose dependent, and near-complete suppression was observed when the flonicamid concentration was increased to 10 000 mg L^{-1}.

CONCLUSIONS: We propose that starvation caused by inhibition of active ingestion is the mechanism of toxicity for flonicamid. This knowledge could aid in applicability and use of this new insecticide for field management of leafhopper populations.
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Keywords: flonicamid; *Amrasca biguttula*; electrical penetration graph; electropenetrography; feeding behaviour

1 INTRODUCTION

Cotton (*Gossypium* spp.) is a major commercial crop, designated as the 'king of fibres', that has global importance in the production of fibre and seed, raw material for the textile industry.[1] China is one of the largest cotton-producing countries in the world and meets almost 20% of the annual worldwide cotton demand.[2]

inhibition of feeding activity.[10] Flonicamid inhibits the feeding activity of insects immediately after treatment and is an effective insecticide against sap-sucking insects such as whiteflies, aphids, leafhoppers (jassids), thrips, scales and mealybugs.[11] Flonicamid inhibited the feeding activity of aphids within 0.5 h of treatment, without showing symptoms of neuronal intoxication, and feeding activity was inhibited until death.[12] Ghelani *et al.*[13] reported that the application of flonicamid in cotton fields caused significant mortality of *A. biguttula* and was statistically as effective as dinotefuran and imidacloprid.

Although laboratory and field experiments provide useful information on mortality caused by the application of an insecticide, comprehensive information on how an insecticide affects the feeding behaviour of insects requires other methods.[14] Among those methods, electropenetrography (EPG) is the most rigorous method for examining the effects of an insecticide on the feeding behaviour of piercing-sucking insect pests and on the damage to plant tissues.[15–17] In EPG, an insect and feeding substrate are incorporated into an electrical circuit. Changes in voltage occur when the insect begins to feed and completes the circuit. The fluctuations in biopotentials (voltage inside the plant) and resistance produce waveforms that correspond to the feeding behaviour of the insect, such as cell puncturing, stylet insertion, salivation, ingestion and stylet location within plant tissue.[18]

Several previous studies have been conducted to evaluate the effects of insecticides on the feeding behaviour of sap-sucking insects,[15,19,20] but the effect of flonicamid on the feeding behaviour of *A. biguttula* has not yet been examined. This study characterised the effects of flonicamid on the feeding behaviour of *A. biguttula* on cotton plants treated with the systemic insecticide. The results of this research will help the scientific community interpret the toxicity mechanism of flonicamid to *A. biguttula* and may provide valuable information for field application of flonicamid.

2 MATERIALS AND METHODS

2.1 Insects
Colonies of *A. biguttula* used in this experiment were collected from a cotton field at Huazhong Agricultural University. The cultures were maintained in the laboratory without exposure to insecticides at $30 \pm 4\,°C$ with a 14:10 h light:dark photoperiod and 60–65% RH. The 25–30-day-old potted cotton plants (variety Zhong 206) were infested with insects from the field. Insects were confined on plants in cages; the leafhoppers were cultured for four generations.

2.2 Insecticide
The insecticide flonicamid 96% TC (Hangzhou Tialong Biotechnology Co., Ltd, Hangzhou, China) was diluted with distilled water to make a series of concentrations: 0, 20, 200, 1000, 5000 and 10 000 mg AI L^{-1}. To minimise any chemical decomposition, the solutions were used immediately after preparation.

2.3 Bioassay methods with *A. biguttula*
As developed and recommended by the Insecticide Resistance Action Committee (IRAC method No. 8), and previously described by Preetha *et al.*,[21] a leaf dip bioassay was conducted with *A. biguttula*. In the bioassay, two plastic cups were used, one as the inner test chamber and the other as the outer water reservoir. Fresh, uncontaminated cotton leaves were collected from the cotton field and were cleaned with a wet cotton swab. The leaf

petiole was cut to a length of approximately 5 cm. The leaves were dipped into freshly prepared insecticide solutions for 5 s and hung vertically for 5–10 min in the open shade to dry. The leaf petiole was passed through a small hole on the side of the inner cup bottom. The outer cup containing water was placed inside to prevent overdrying and to keep the leaf in an upright position.

Third-instar nymphs of *A. biguttula* were removed from the culture with a fine camel hair brush, and ten nymphs were released into the test cup. The cup was covered with a perforated lid. A total of 50 nymphs (five cups) were tested for each concentration. The control treatment contained a cotton leaf dipped in distilled water. Each treatment was replicated 3 times, including the control. The cups were maintained in laboratory conditions as described above, and mortality was assessed daily.

Insect starvation treatment was given as described by Yueping *et al.*[19] Briefly, ten third-instar nymphs of *A. biguttula* were confined inside a plastic cup containing a water-saturated cotton swab (no leaf). A total of 50 nymphs (five cups) were tested for starvation treatment. The experiment was repeated 3 times, and the rest of the data on mortality were recorded as described above. The bioassay experiment was conducted for 7 days.

2.4 Electropenetrography of *A. biguttula* feeding behaviour
The objective of this study was to determine the effects of flonicamid on the probing behaviour of *A. biguttula* compared with water-treated control plants. EPG of *A. biguttula* on cotton was recorded using one Giga-4 DC EPG amplifier with a $10^9\ \Omega$ input resistance and less than 1 pA input bias current (EPG Systems, Wageningen, The Netherlands). Treatments were five concentrations of flonicamid (20, 200, 1000, 5000 and 10 000 mg AI L^{-1}) and distilled water as the control. The leaves of 40–50-day-old cotton seedlings were treated by dipping in the different concentrations of flonicamid. After being starved for 2 h, third-instar nymphs of *A. biguttula* were chilled on ice for 2–3 min. The immobilised insects were individually connected, via the thoracic notum, to a gold wire (2 cm length, 18 µm diameter) with silver conductive glue (Pelco colloidal silver paint; Ted Pella, Inc., Redding, CA). The ground electrode was placed into the soil near the stems of treated cotton plants to provide the voltage, and the insect was connected to the electrical circuit by gluing the gold wire to a copper wire (0.5 mm diameter) with conducting silver paint, which was then soldered to a brass nail inserted into the input lead of the head stage amplifier. The insect attached to the gold wire was then carefully placed on the leaf of the treated cotton plant.

Voltage fluctuations representing feeding behaviours of nymphs of *A. biguttula* on insecticide-treated and control leaves were recorded onto a computer hard disk through an analog-to-digital conversion for further analysis. To reduce interference from external electrical noise, the entire experimental set-up was performed inside a large Faraday cage ($2 \times 2 \times 4$ ft, aluminium frame with a steel base) at $30 \pm 2\,°C$ and 60–65% RH under continuous light for 8 h using PROBE 3.4 software (Wageningen Agricultural University, Wageningen, The Netherlands) operating in Windows-XP. New, freshly treated cotton plants and insects were used for each replication. A total of ten 8 h EPG readings were recorded for each concentration of flonicamid, which were used for final data analysis.

2.5 Statistical analyses
Six primary types of waveform produced by cotton leafhoppers probing cotton leaves were determined (Figs 1A to D), based on their similarities to those described by Jin *et al.*[22] for the related
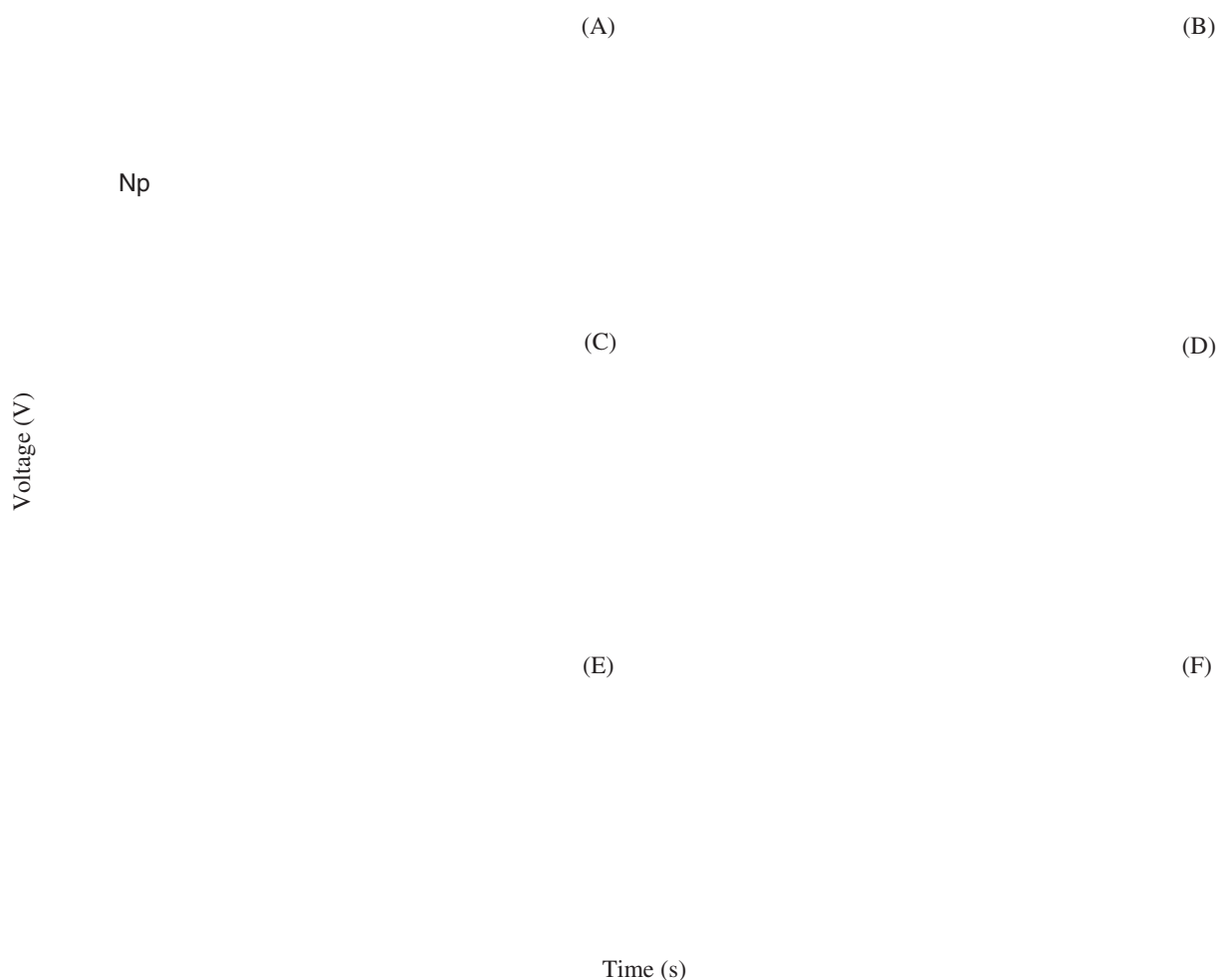
Figure 1. Characteristic EPG waveforms recorded from *A. biguttula* on cotton leaves. (A) non-probing (Np) and waveform A1 (including subtypes) early in a probe (only a portion of A1 is shown; A1-C continued after this excerpt); (B) A2, performed after A1; (C) A3 after A2; (D) A4, interspersed with A2 and A3; (E) A5; (F) A6. The *y*-axis indicates the amplitude (voltage) at standardised gain for all examples. The *x*-axis indicates time in seconds. Note that the *y*-axis indicates that waveforms have very different amplitudes despite being portrayed as similar heights.

leafhopper species *Empoasca vitis*. Biological meanings of these waveforms were hypothesised, again on the basis of Jin *et al.*[22] Each EPG waveform was summarised using four non-sequential variables: the number of events and the percentage, total duration and average duration of each waveform during the entire recording time. These variables were calculated similarly to those in Backus *et al.*[23] The number of events for each waveform was the number of times that a waveform appeared during the recording time. The percentage of each waveform was calculated by dividing the total duration of each waveform over the whole recording by the total recording time. The total waveform duration for each EPG waveform was calculated by summing the durations of all waveform events of all insects that occurred during the observation time. The average duration of each EPG waveform represented the mean duration of each waveform per insect. Sequential variables of EPG waveforms were also analysed similarly to Sarria *et al.*,[24] including the following: the duration of non-probing (A) from the start of the EPG recording to the first probe, the duration between the first and second probes of A1, the number of probes before the first probe containing A3, the duration of the first probe containing A3 and the interval from the start of the first probe to the first A3 event. Furthermore, the percentages of the insects that reached

A3, after being treated with different concentrations of flonicamid and water (CK), were also calculated.

EPG recording data were analysed with SAS v.9.2 for Windows (SAS Institute, Cary, NC) statistical software. The least significant difference (LSD) test was used to evaluate the differences in the EPG variables between control (CK) and treatments at various concentrations of flonicamid. Regression analyses were conducted to determine the relationship between EPG variables and concentrations of flonicamid (log transformed). The mortality data from dose–response bioassays and the calculations of LC_{50} values were determined using PoloPlus software (PoloPlus: probit and logit).

3 RESULTS

3.1 Characterisation of waveforms from *A. biguttula* nymphs feeding on cotton leaves

When *A. biguttula* probed on cotton leaves, six different types of waveform (Fig. 1 and Table 1) were identified in the EPG out. The characterisation of identified waveforms is as follows.

3.1.1 Np

When insects were not probing, the recording always showed a flat baseline (0 V reference level) (Fig. 1A). The output voltage of
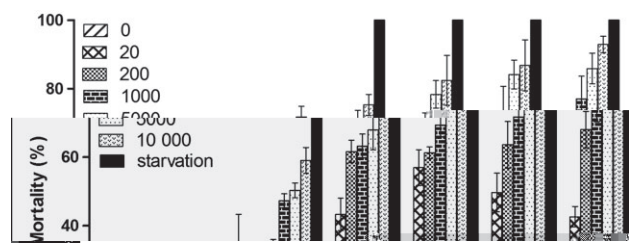
Table 1.

Figure 2. Mortality of third-instar nymphs of *A. biguttula* treated with flonicamid or starvation. * indicates 0%.

A5 generally followed an A4 event, but occurred only occasionally. Events of the A5 waveform often had shorter duration (up to 2–3 min) than events of A1, A2 and A3. The positive level of voltage indicated an extracellular position of stylets during A5.

3.1.7 A6

The A6 waveform was characterised by upward spikes with a highly variable amplitude and frequency (1–6 Hz) (Fig. 1F). This waveform was very stereotypical, appearing with the same shape which was easily distinguished from other waveforms. A6 was not detected in all tested leafhoppers, but when it happened, it was of relatively short duration (1.5–2 min). Also, A6 was always preceded by A1, and usually followed by A2.

3.2 Activity of flonicamid against *A. biguttula*

The toxicity of flonicamid 96% TC to third-instar nymphs of *A. biguttula* was compared with the control (CK: 0 mg L^{-1} of flonicamid) and starvation (water only, no cotton leaf for feeding) treatments (Fig. 2 and Table 2). After 24 h, the mortality of third-instar nymphs to flonicamid at different concentrations was less than 10%, whereas mortality of 0 and 13.16% was observed in the CK and starvation treatment respectively. After 48 h, mortality for all tested concentrations of flonicamid was less than 30%, whereas mortality was zero and 31.7% in the CK and starvation treatment respectively (Fig. 2). After 72 h, mortality was 5.6 and 71.7% in the CK and starvation treatment respectively. The LC$_{50}$ values of flonicamid to *A. biguttula* at 24, 48 and 72 h were 7510.94, 974.10 and 595.71 mg L^{-1} respectively (Table 2). Mortality in the starvation

treatment reached 100%, whereas in the CK the mortality was 7.3% after 96 h of flonicamid treatment. After 96 h of treatment, mortality for the highest concentration (10 000 mg L^{-1}) of flonicamid was 75.2%, and the LC$_{50}$ value was 358.11 mg L^{-1} (Fig. 2 and Table 2). After 120 h, less than 10% mortality was observed in the CK, and the LC$_{50}$ value (239.22 mg L^{-1}) of flonicamid was approximately half the value at 72 h. At 168 h after treatment, 8.8% mortality occurred in the CK, whereas mortality of 86.8% was recorded at the highest concentration (10 000 mg L^{-1}) of flonicamid. The LC$_{50}$ value at this time point was 155.27 mg L^{-1}, which was 3.8-fold lower than the value at 72 h. After 216 h of treatment, mortality in the CK was 10.7%, whereas mortality at 10 000 mg L^{-1} of flonicamid reached 92.9%, with an LC$_{50}$ value of 153.96 mg L^{-1} (Fig. 2 and Table 2). Bioassays demonstrated that flonicamid was slow acting and was significantly slower than starvation in causing mortality of *A. biguttula*.

3.3 EPG and feeding behaviour in flonicamid-treated *A. biguttula*

During the 8 h recording period, the average and total duration of the Np waveform of the CK were 8.2 ± 2.4 min and 46.2 ± 17.07 min respectively. The average and total durations of the Np waveform of the CK were significantly shorter than those on plants that were treated with flonicamid ($P < 0.05$) (Fig. 3A and supporting information Fig. S1A). Regression analysis showed a significant linear relationship between flonicamid concentrations and the average and total duration of the Np waveform ($P < 0.0001$, $R^2 = 0.85$; $P < 0.002$, $R^2 = 0.88$ respectively). The duration of Np waveforms increased as the concentration of flonicamid increased (Fig. 3A and supporting information Fig. S1A). The number of events of Np waveforms were not significantly different between the CK and the flonicamid treatments ($P = 0.35$) (supporting information Fig. S2A). Analysis of the average duration revealed no significant correlation between the CK and flonicamid treatment for A1 and A4 waveforms, and the fit to either a linear or an exponential model was poor (linear: $P = 0.15$, $R^2 = 0.046$ and $P = 0.035$, $R^2 = 0.77$; exponential decay: $P = 0.21$, $R^2 = 0.069$ and $P = 0.035$, $R^2 = 0.76$ respectively) (Figs 3B and E). However, a significant difference was observed in the average duration of the A2 and A3 waveforms among the treatments ($P = 0.0001$, $R^2 = 0.91$ and $P = 0.0001$, $R^2 = 0.96$ respectively) (Figs 3C and D). No significant difference was observed in the total duration of the A1 and A4 waveforms among the treatments ($P = 0.35$) (supporting information Figs S1B and E). A significant difference in the total duration of the A2 and A3 waveforms was found among the treatments ($P = 0.0001$, $R^2 = 0.99$ and $P = 0.0001$, $R^2 = 0.87$ respectively) (supporting information Figs S1C and D).

Table 2. Toxicity of flonicamid to *A. biguttula* after different time periods

Time after treatment (h)	Slope (± SE)	LC$_{50}$ (95% CL)(mg L^{-1})	N[a]	χ^{2} [b]	df
24	0.39(±0.055)	7510.94 (3764.78–20125.65)	150	2.12	2
48	0.31(±0.049)	974.10 (478.50–2057.10)	150	0.86	2
72	0.33(±0.050)	595.71 (283.71–1181.8)	150	2.08	2
96	0.36(±0.050)	358.11 (166.54–677.48)	150	3.71	2
120	0.37(±0.051)	239.22 (108.08–446.78)	150	5.22	2
168	0.35(±0.051)	155.27 (60.16–309.85)	150	5.02	2
216	0.38(±0.052)	153.96 (63.47–279.20)	150	7.30	2

[a] *N*: number of *A. biguttula* tested.
[b] Chi-square testing linearity of dose–mortality response: $P < 0.05$.

(A)

(B)

$y = 12.588x + 0.0928$
$R^2 = 0.853$

(D)

(C)

$y = 37.215e^{-0.34x}$
$R^2 = 0.9163$
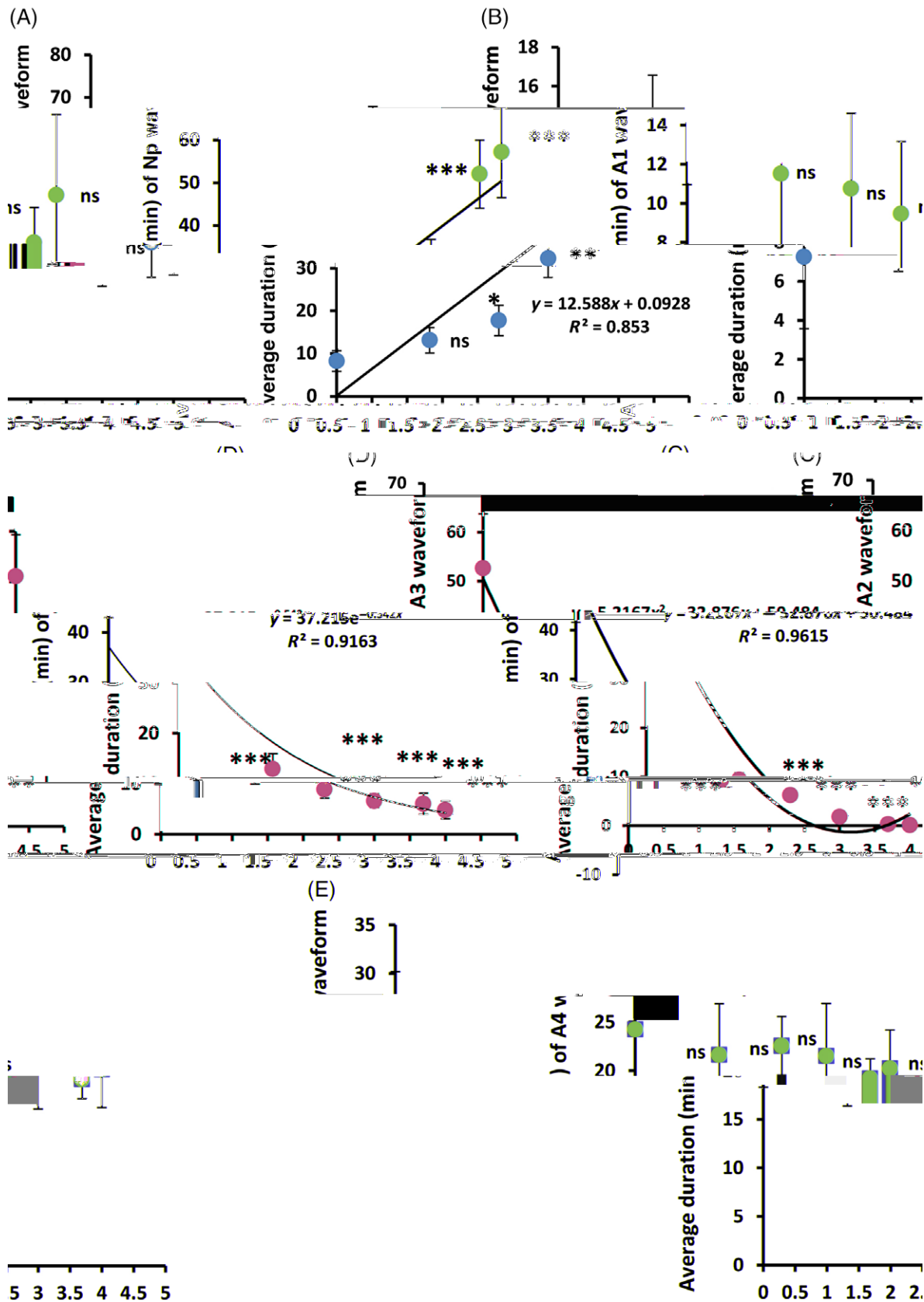
$R^2 = 0.9615$

(E)

Figure 3. Relationship between flonicamid concentrations (log) and the average duration of each EPG waveform, including the average duration of the Np waveform (A), A1 waveform (B), A2 waveform (C), A3 waveform (D) and A4 waveform (E). Each data point is presented as the mean ± SE. Asterisks *, ** and *** by a data point show a significant difference between the CK and these values ($P < 0.05$, $P < 0.01$ and $P < 0$.
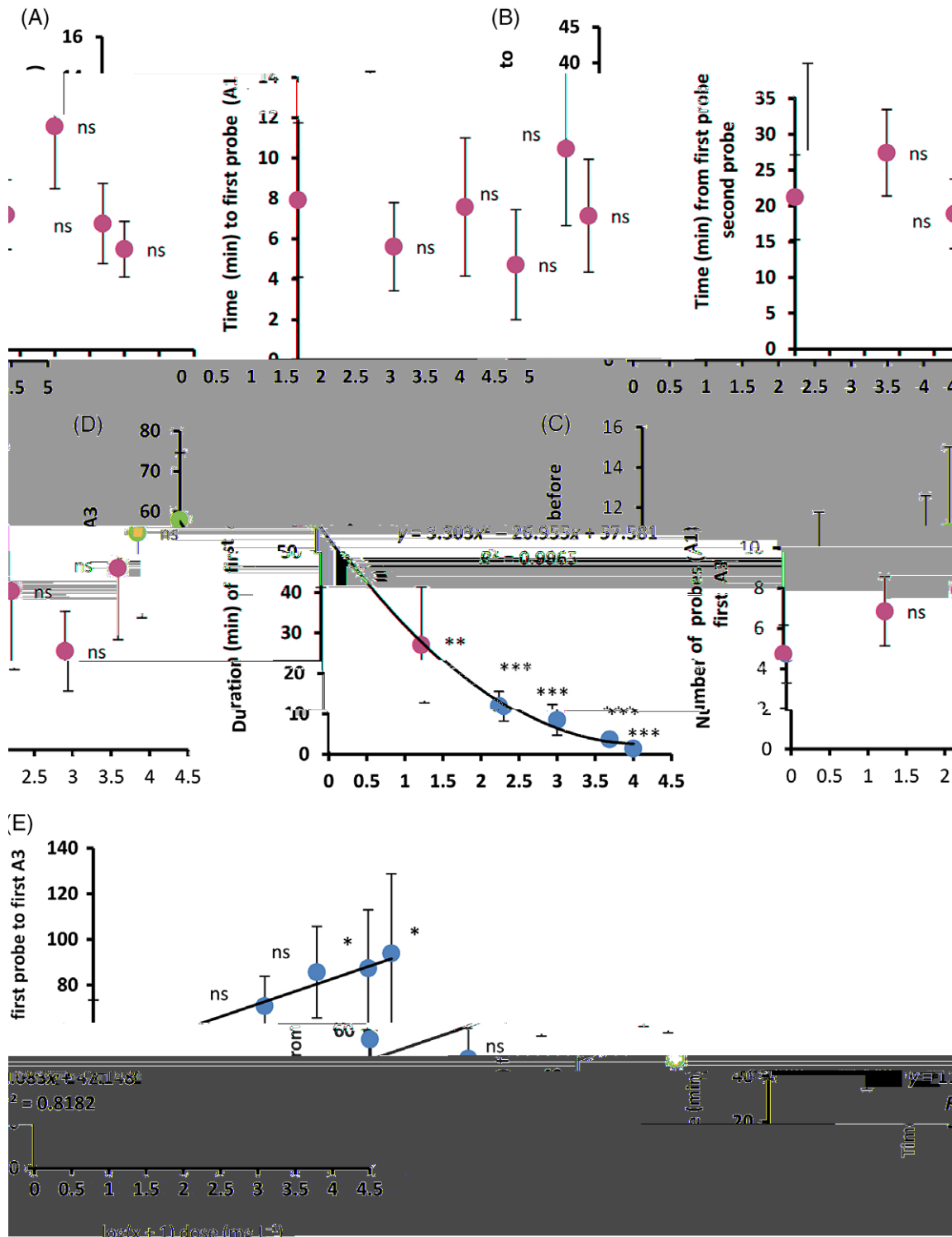
Figure 5. Relationship between flonicamid concentrations (log) and sequential variables of each EPG waveform, including the time from the start of the EPG recording to the first probe (A), the time interval from the first probe to the second probe (B), the number of probes before the first A3 (C), the duration of the first A3 (D) and the time from the first probe to the first A3 (E). Error bars indicate ± SE. Asterisks *, ** and *** by a data point show a significant difference between the CK and these values ($P < 0.05$; $P < 0.01$ and $P < 0.001$) respectively, and 'ns' denotes non-significance ($P > 0.05$).

In the present study, after leaf dipping application with floni-camid, a difference between the effects of flonicamid on active ingestion was observed, and thus effects should not be related only to the translocation of flonicamid within plant. There-fore, in addition to the leaf dipping, other methods such as injection and topical application could be used for fur-ther verification. In addition, further studies are necessary to determine any effect on the plant from flonicamid, and the speculation is that flonicamid-treated plants are distasteful to insects.
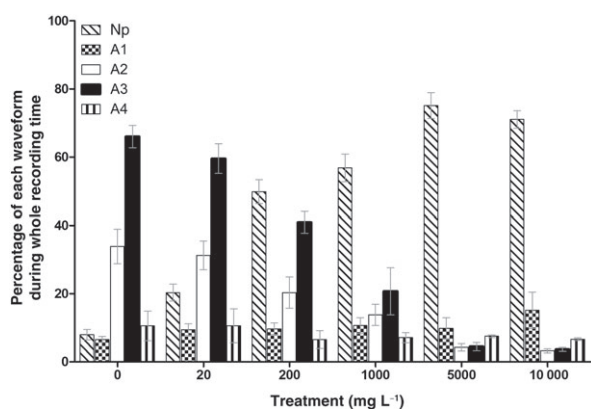
Figure 6. Percentage of each waveform phase in the whole recording time after being treated with water (CK) and different concentrations of flonicamid.

In conclusion, our EPG data showed that flonicamid significantly increased the duration of Np and decreased the active ingestion period (A2 and A3 waveforms). It may also have stimulated insects to change from phloem ingestion to mesophyll/parenchyma ingestion before dying.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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