

Active rejection of non-host plants across families in *Papilio xuthus* revealed by tarsal multi-feature spike sorting

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Abstract

Herbivorous insects must identify suitable hosts for oviposition and reliably reject unsuitable ones. Whether non-host plants are detected through active deterrent cues or through the mere absence of host-recognition signals remains unclear. We investigated this question in *Papilio xuthus*, combining oviposition bioassays with multivariate spike sorting of tarsal chemosensillum tip-recording data. Females showed 51.5% oviposition on a water control after Citrus priming, establishing a high post-priming water baseline. Against this baseline, complete oviposition was suppressed to 0% on all five non-Rutaceae non-host species (spanning Apiaceae, Fabaceae, Lauraceae, and Aristolochiaceae). This consistent suppression supports active detection of non-host deterrence rather than a passive consequence of missing stimulatory input. Gaussian mixture model (GMM) clustering of six waveform features—amplitude plus five temporal and morphological parameters—separated five spike types (Types 0–4) from tarsal chemosensilla stimulated with plant methanol extracts; these types are not distinguishable by amplitude alone. Type 0 responses were robustly associated with non-host extracts (rank-biserial $r = -0.389$) and remained significant in a conservative female-ID clustered GEE analysis, whereas Type 4 responses were directionally associated with host extracts ($r = +0.454$) but did not reach significance after GEE correction ($q = 0.103$). *Orixa japonica*, a non-host Rutaceae species, partially activated Type 4 while simultaneously eliciting strong Type 0 activity, and oviposition was markedly reduced despite frequent Bending. This mixed-input pattern is consistent with an ensemble integration model in which oviposition completion reflects concurrent candidate go-associated and stop-associated peripheral inputs.

INTRODUCTION

Host plant selection determines the survival and reproductive success of phytophagous insects and is a central axis of plant–insect interactions (Bernays and Chapman 1994). In Lepidoptera, this selection is expressed as oviposition behavior by adult females, and the accept-or-reject decision begins at the periphery of the chemosensory system. Contact chemosensation—the detection of non-volatile plant compounds through direct physical contact with the leaf surface—provides the final gate that determines whether a site is accepted or rejected for egg-laying (Renwick and Chew 1994). In monophagous and oligophagous diurnal Lepidoptera, both the positive recognition of suitable hosts and the active rejection of unsuitable plants are thought to be mediated through this tarsal contact chemosensory system, yet the peripheral neural mechanisms underlying each process remain incompletely understood.

The swallowtail butterfly *Papilio xuthus* (Lepidoptera: Papilionidae) is a Rutaceae-specialist widely distributed across Japan and East Asia. Ovipositing females assess leaf chemistry by drumming the leaf surface with the ventral tarsal chemosensilla of the forelegs (Ilse 1937; Ma and Schoonhoven 1973; Nishida et al. 1987; Ohsugi et al. 1991), and each chemosensillum houses up to four gustatory receptor neurons (GRNs) and one mechanoreceptor neuron (Ma and Schoonhoven 1973; Ozaki et al. 2011). Ten non-volatile phytochemicals from *Citrus* leaves—including synephrine, stachydrine, 5-hydroxymethyltryptamine derivatives, narirutin, and *chiro*-inositol—have been identified as oviposition stimulants (Nishida et al. 1987; Ohsugi et al. 1991). None of these compounds reliably elicits oviposition in isolation; a specific combination of multiple stimulants is required (Ohsugi et al. 1991). This multi-component requirement implies that the neural code for host acceptance involves combinatorial recognition rather than detection of a single signature compound.

The neural mechanisms of this combinatorial recognition have been investigated using the tip-recording technique (Marion-Poll and van der Pers 1996). Ozaki et al. (2011) identified the gustatory receptor gene *PxutGr1* as a synephrine-specific receptor whose RNAi knockdown significantly reduced both electrophysiological responses to synephrine and oviposition behavior, establishing the molecular basis of host-plant specialization at the receptor level. Building on this, Ryuda et al. (2013) classified tarsal chemosensilla of the fifth tarsomere into three morphological types—L1, L2, and S—based on size and responsiveness to oviposition stimulants. L1-type sensilla, which are enriched in females and respond to five of the ten known stimulants, contain three GRNs (os1, os2, and os3); co-firing of these neurons above a threshold rate was proposed as the neural code for oviposition induction. Spike identification in that study relied on visual sorting into three amplitude classes (high, 1.0–1.5 mV; intermediate, 0.6–0.8 mV; low, 0.2–0.4 mV). Because the electrophysiological stimuli in that study consisted of pure oviposition stimulant solutions rather than plant extracts, only the stimulatory ("go") side of the sensory code was accessible; it remained unclear whether non-host plants are actively detected as aversive stimuli or simply perceived as an absence of host-plant signals. An additional limitation is that amplitude-based spike sorting may fail to resolve distinct spike types whose amplitude distributions overlap, particularly in recordings from intact sensilla stimulated with chemically complex plant extracts. Although a putative deterrence neuron (M2) has been described in the related species *Papilio hospiton*, where it responds preferentially to non-host plants within the family Apiaceae (Sollai et al. 2017; Sollai and Crnjar 2018), a comparable non-host-associated spike type has not been identified in *P. xuthus*, nor has active deterrence been demonstrated for non-host plants phylogenetically distant from the host family.

Here we address these questions by combining oviposition bioassays with multivariate spike sorting of tip-recording data from *P. xuthus* females. Using the oviposition rate on water in *Citrus*-primed, high-motivation females as a post-priming water baseline, we first tested whether methanol extracts of non-host plants spanning four families (Apiaceae, Fabaceae, Lauraceae, and Aristolochiaceae) suppress oviposition below the water-control level, allowing us to evaluate whether non-host rejection is an active, stimulus-driven process. We then reasoned that complex plant methanol extracts, containing numerous chemical compounds, likely activate multiple spike-generating cells simultaneously within a single sensillum; host versus non-host differences may therefore be encoded as shifts in the relative firing rates of co-active spike types rather than as categorical on-or-off differences in neural activation. To capture such a ratio code, we supplemented spike amplitude with five additional temporal and morphological waveform features—trough voltage, half-width at half-amplitude, rise slope, fall slope, and a rise-fall symmetry index—and applied PCA followed by Gaussian mixture model (GMM) clustering. This six-feature approach resolved five waveform-defined spike types (Types 0–4) that are not reliably separable by amplitude alone. Comparing type-specific firing rates across host plants, non-host plants, and *Orixa japonica*—a member of the host family Rutaceae that is nonetheless rejected for oviposition—we evaluate Type 4 as a host-associated candidate go response and Type 0 as a robust non-host-associated response. The simultaneous activation of both types by *O. japonica*, with elevated Type 0 activity and strong suppression of oviposition completion, supports an ensemble integration model in which oviposition completion reflects the concerted activity of candidate go-associated and stop-associated peripheral inputs, consistent with the stimulant-deterrent competition framework of Honda (1995). These findings extend the stimulatory neural code proposed by Ryuda et al. (2013) to a bidirectional, ensemble-based framework for host acceptance and non-host rejection.

METHODS AND MATERIALS

Insects. *Papilio xuthus* Linnaeus females were used in all experiments. A laboratory colony was established from field-caught mated females allowed to oviposit on potted *Citrus* plants; offspring were reared as the base colony. Larvae were fed a mixture of artificial diet (Insecta F-II; Nihon Nosan Kogyo) and dried *Zanthoxylum ailanthoides* leaf powder. Leaf powder was prepared by desiccating fresh leaves over silica gel followed by ball-mill grinding. Diet was prepared with ultrapure water (Merck Milli-Q IQ 7005 Q-Pod), briefly heated in a microwave, and cooled to room temperature before use. The weight ratio of artificial diet to leaf powder per Petri dish was adjusted at each larval instar: 1:1 (first), 1.3:0.7 (second), 1.5:0.5 (third), 1.7:0.3 (fourth), and 1.9:0.1 (fifth). Rearing conditions were maintained at $24 \pm 1^\circ\text{C}$, 45–60% relative humidity, and a 16 h light:8 h dark photoperiod.

Mated females aged 3–5 days post-eclosion were used for oviposition bioassays. Virgin females aged 1–5 days post-eclosion were used for electrophysiological recordings; tarsal GRN response properties in *P. xuthus* have been confirmed to be unaffected by mating status (Ozaki et al. 2011; Ryuda et al. 2013). All experiments were conducted in Takatsuki City, Osaka Prefecture, Japan.

Plant Materials and Extract Preparation. Leaf methanol extracts of nine plant species were used in both oviposition bioassays and electrophysiological recordings (Table 1). Three Rutaceae species served as host plants, and six species served as non-hosts—one from the host family and five from other families.

Plants were grown from nursery stock in the botanical garden of the JT Biohistory Research Hall (Takatsuki City, Osaka Prefecture), cultivated either in-ground or in containers. *Zanthoxylum ailanthoides* leaves were harvested from wild stands in Toyono Town, Osaka Prefecture, with landowner permission. Leaves of all species were collected between May and September during the active growing season.

Extracts were prepared by macerating fresh leaf material in methanol at 10 g fresh weight per 100 mL solvent. The crude extract was filtered through filter paper, transferred to glass vials, and stored at 4°C (short-term) or -80°C (long-term) until use. Extracts were used undiluted; at this concentration, the *Citrus* extract elicited oviposition rates exceeding those observed with fresh *Citrus* leaves.

For oviposition bioassays, plastic artificial leaves were prepared by immersing them in the methanol extract and allowing the solvent to evaporate completely at room temperature. Immediately before each trial, the leaf surface was lightly misted with water. Water-control leaves were prepared identically from untreated artificial leaves misted with ultrapure water.

Oviposition Bioassay. Only females in a motivated oviposition state were tested. Motivation was induced by providing a sucrose solution (Pocari Sweat; Otsuka Pharmaceutical) and then placing each female under approximately 6,000 lux illumination until spontaneous wing-fanning behavior equivalent to several minutes of flight was observed. Motivational state was then confirmed with a two-step pre-test: (1) contact with a water-control leaf must not elicit oviposition, and (2) contact with a *Citrus* extract leaf must elicit oviposition. Only females satisfying both criteria were admitted to the experiment.

Within 1 min of the pre-test, the test stimulus (a plant-extract leaf or a water-control leaf) was presented to the female randomly. The behavioral response was scored within 5 min. When a female was tested with more than one stimulus, a minimum inter-trial interval of 20 min was observed, after which the two-step motivational confirmation was repeated before the subsequent trial.

Pre-test *Citrus* leaves were prepared by the same protocol as the test stimuli (extract immersion, solvent evaporation, and misting). Although the precise amount of water deposited during misting could not be standardized exactly across trials, the identical procedure was applied to all test leaves, making water treatment conditions comparable across stimuli.

Behavioral responses were classified into three categories: **Oviposition** (the female curved the abdomen and made sustained ovipositor contact with the underside of the leaf, resulting in egg deposition), **Bending** (partial abdominal curving without ovipositor contact), and **No response** (drumming without abdominal movement). When multiple trials were available for the same female and treatment, the female-level exclusive category was assigned as follows: Oviposition if at least one trial showed Oviposition, Bending if no Oviposition occurred but at least one trial showed Bending, and No response if neither Oviposition nor Bending occurred. Sample sizes per treatment were: *Citrus* spp., $n = 32$; *Z. ailanthoides*, $n = 25$; *P. amurense*, $n = 10$; *O. japonica*, $n = 14$; *A. debilis*, $n = 7$; *Lespedeza* spp., $n = 6$; *C. camphora*, $n = 6$; *P. crispum*, $n = 8$; *A. keiskei*, $n = 10$; water control, $n = 33$.

Statistical Analysis of Oviposition Behavior. Oviposition behavior was analyzed with trial-level Bayesian hierarchical logistic models that retained repeated trials and repeated females. Three binary outcomes were modeled separately: (1) oviposition completion (Oviposition vs. Bending + No response), (2) oviposition-related initiation (Oviposition + Bending vs. No response), and (3) no response (No response vs. Oviposition + Bending). For each outcome, the model included plant treatment as a fixed effect, with the post-priming water control as the reference treatment, and female identity as a random intercept. Posterior effects are reported as odds ratios (ORs) relative to the water control, with posterior medians, 95% credible intervals (CrIs), and posterior probabilities for the estimated direction of the effect. Weakly informative priors were used: Normal(0, 2.5) for the intercept and plant effects on the logit scale, and a half-normal prior equivalent to Normal(0, 1.5) constrained to positive values for the female-level standard deviation. Models were fit in R using brms with the cmdstanr/Stan backend (Bürkner 2017; R Core Team 2026; Stan Development Team 2026). Each model used four chains, 4,000 iterations per chain, 1,000 warmup iterations, $adapt_delta = 0.95$, and $max_treedepth = 12$. Convergence was assessed using R-hat, effective sample size, divergent transitions, and maximum-tree-depth warnings.

Prior sensitivity was assessed by re-fitting the oviposition-completion and oviposition-or-Bending models under tighter (Normal(0, 1.5)) and wider (Normal(0, 5.0)) alternatives to the production prior for plant-level coefficients. Posterior direction probabilities were identical across all three specifications for all treatments: 1.000 for all host species and 0.984–1.000 for non-host species, confirming that the directional inferences are not sensitive to prior choice. For host plant treatments where all trials resulted in oviposition (complete separation), the likelihood function is flat for large positive coefficient values, and OR medians and CrI upper bounds scaled substantially with the prior scale (e.g., *Citrus* oviposition OR median = 83, 178, and 352; CrI upper bound = 340, 1,230, and 5,070, under tight, main, and wide priors, respectively). These extreme upper bounds reflect the data structure and should be interpreted as order-of-magnitude estimates; see Table 2 (footnote c) for further details.

Tip-recording Electrophysiology. Contact chemosensilla of the foreleg tarsi were recorded using the tip-recording technique (Marion-Poll and van der Pers 1996; Chapman 2003). Plant extract dissolved in electrolyte solution was loaded into a recording electrode (Syntech TastePROBE DTP-02), and the electrode tip was brought into contact with the distal tip of a single sensillum. Recordings were obtained exclusively from L-type sensilla.

Although L1 and L2 sensilla were not distinguished, L2 sensilla constitute a small minority of tarsal L-type sensilla in *P. xuthus* (Ryuda et al. 2013), and recordings are therefore predominantly from L1. Recordings were obtained from 28 individual virgin females. Because recordings required amputation of the foreleg and were completed within the neuron survival period of the isolated preparation, it was not possible to present all nine plant extracts to a single individual; the extracts were therefore distributed across individuals and sensilla. The same sensillum was not stimulated more than once with the same plant extract, although in some cases a sensillum was stimulated with more than one extract. The resulting dataset had a nested structure: the 1,077 recording files were distributed across 28 females (median 30.5 files per female, range 4–101). Of the 28 female recording sessions, 15 involved stimulation with more than one plant extract (median 2 extracts per session, range 1–8), accounting for 878 of 1,077 files (81.5%). Signals were amplified with a Warner Instruments DP-301 amplifier (DC-coupled mode; high-pass filter, 10 Hz; low-pass filter, 1 kHz [analog]; gain, $\times 100$) and digitized at 50 kHz using dbWave software (Marion-Poll 1996). Each recording lasted 2 s and the output was saved in text format. Experiments were conducted in a 60 Hz electrical environment (Takatsuki City, Osaka Prefecture). A total of 1,077 recording files were retained for analysis from stimulation with the nine plant extracts.

Signal Preprocessing. Raw waveform data exported from dbWave were imported into Python for analysis. A second-order Butterworth high-pass filter (cutoff, 300 Hz) was applied to remove DC drift. Recording-onset artifacts were identified and removed by automated trimming; files for which automated trimming was insufficient were manually inspected and the trim end point adjusted individually. The resulting stimulus-response duration for each file (*analysis_duration_sec*) served as the denominator for computing firing rates (Hz).

Spike Feature Extraction. Six waveform features were quantified for each spike candidate (Fig. 2):

Feature	Definition
<i>peak_v</i>	Spike peak voltage [mV]
<i>trough_v</i>	Most negative voltage of the afterhyperpolarization [mV]
<i>half_width</i>	Spike width at half-maximal amplitude [ms]
<i>rise_slope</i>	Maximum rate of voltage rise [mV/ms]
<i>fall_slope</i>	Maximum rate of voltage decay [mV/ms]
<i>symmetry</i>	Rise-fall asymmetry index (<i>rise_slope</i> / <i>fall_slope</i>)

Waveform area under the curve (*auc*) was evaluated as a seventh candidate but was excluded because of strong collinearity with *peak_v* (Pearson $r = +0.82$; $n = 58,030$ spikes). The independence of the six retained features was verified by examining PCA loadings: four principal components accounted for 99.0% of the total variance, and in PC4 (8.1% variance) *trough_v* dominated with a loading of 0.88, confirming that the afterhyperpolarization feature carries information not reducible to the amplitude index.

Because signals were recorded through a 1 kHz analog low-pass filter (Section 2.5), the absolute values of *rise_slope* and *fall_slope* reflect filtered waveform rates rather than true physiological maximum rates. The

relative ordering of these features across spike types is preserved under the identical filter conditions applied uniformly to all recordings, and therefore does not affect the validity of multi-feature classification.

Noise Filtering. Spike candidates were passed through a three-stage filtering pipeline (initial input: 58,030 spikes; final output: 57,565 spikes; retention rate: 99.2%).

Stage 1 (waveform hard thresholds). Candidates with physiologically implausible parameter values were removed using the following criteria: *half_width*, 0.2–1.7 ms; *peak_v*, 0.0–2.0 mV; *trough_v*, –1.0 to +0.05 mV; *rise_slope* ≥ 0.5 mV/ms; *symmetry* ≥ 0.1 . This stage removed 175 spikes (0.3%).

Stage 1.5 (interspike interval filter). Spike candidates occurring within the absolute refractory period (1.0 ms after the preceding spike) were discarded as duplicate detections. No spikes were removed at this stage.

Stage 2 (waveform PCA + Isolation Forest). Amplitude-normalized waveform snippets (175 sample points; normalized by *peak_v*) were projected onto five principal components (cumulative variance, 94.1%), and outliers in this PC space were identified using Isolation Forest (scikit-learn; Pedregosa et al. 2011; contamination parameter automatically estimated from the per-file noise proportion, with a minimum value of 0.005). This stage removed 290 spikes (0.5%).

Spike Classification. Multi-feature unsupervised spike sorting, in which waveform parameters beyond amplitude are used to resolve overlapping spike types, has been established as a principled approach for extracellular recording data (Quiroga et al. 2004). The 57,565 retained spikes were classified by standardizing the six features with StandardScaler, reducing dimensionality to four principal components (cumulative variance, 99.0%), and fitting a Gaussian mixture model (GMM; Souza et al. 2019; scikit-learn v1.8.0; Pedregosa et al. 2011; *covariance_type* = 'full'; *random_state* = 42; *n_init* = 10) to the resulting four-dimensional PC space.

The number of GMM components K was determined using the deposited analysis code. Models with $K = 2$ –10 were fitted, and the elbow point of the BIC and AIC curves was identified using the Kneedle algorithm (Satopaa et al. 2011) (Fig. S1). At four PCA dimensions, the BIC/AIC elbow was detected at $K = 4$; $K = 4$ and $K = 5$ were therefore examined as candidate solutions. Candidate solutions were then examined for waveform separation, assignment stability, and consistency under sensitivity analyses across PCA dimensionality, component number, and feature set (Supplementary Figs. S2–S5). This workflow selected $K = 5$ before biological interpretation of the host and non-host response profiles. Silhouette coefficient and Calinski–Harabász index both favored $K = 2$, but the maximum Silhouette value was only 0.24, failing to indicate well-separated cluster structure; because these metrics are known to favor low K when the underlying distribution is continuous and overlapping—as expected for spike waveforms recorded from intact sensilla stimulated with chemically complex extracts—they were not used as the primary selection criterion.

The robustness of $K = 5$ was further assessed through sensitivity analyses in which PCA dimensionality (3 or 4), K (3–7), and the feature set (six-feature versus a three-feature amplitude-based set) were systematically varied (Supplementary Figs. S2–S5). Varying PCA dimensionality between 3 and 4 preserved the effect directions of all primary spike types (Fig. S2). Across $K = 5$ –7, the non-host-associated Type 0 response and the host-associated Type 4 directionality were reproduced stably, whereas Type 1 and Type 3 lost stable directionality at $K \geq 6$ —Type 1 reversing from host-preferring at $K = 5$ to non-host-preferring at $K \geq 6$, and Type 3 reversing from non-host-preferring at $K = 5$ to host-preferring at $K \geq 6$ (Fig. S3). Reducing the feature set to three features

(peak_v, trough_v, and symmetry only) caused cluster assignment consistency to drop substantially (adjusted Rand index, ARI = 0.407 relative to the six-feature $K = 5$ solution) and the host-responsive cluster equivalent to Type 4 to fragment across multiple components (Fig. S4A–E), demonstrating the necessity of all six features for stable classification. A grid search across PCA dimensionality $\times K$ combinations confirmed that the bidirectional response pattern—host-associated Type 4 directionality and robust non-host-associated Type 0 activity—is consistently recovered at $K = 5$ (Fig. S5). All analyses were performed in Python 3.13.11.

Statistical Analysis of Neural Responses. Firing rate for each spike type in each recording file was computed as the spike count divided by the stimulus-response duration (*analysis_duration_sec*). The primary statistical unit was the sensillum-recording level, because each recording file corresponded to a single chemosensillum stimulated with a given plant extract (unique file identifier $\times$ plant species combination). This analysis captures stimulus-associated response profiles at the level of individual chemosensillum recordings. Because multiple sensilla were recorded from the same female and some sensilla were tested with more than one extract, these sensillum-recording-level observations are not fully independent at the biological-individual level. We therefore treated the Mann-Whitney analyses as descriptive comparisons of sensillum-recording-level response profiles and added female-ID clustered GEE as a conservative sensitivity analysis for the major neural-response contrasts. By explicitly modelling positive within-female correlation under the exchangeable working correlation assumption, GEE reduces the effective number of statistically independent observations below the raw count of recording files, producing wider confidence intervals and higher p -values than the sensillum-recording-level MWU tests; results that remain significant under GEE therefore represent a higher evidential threshold.

Main analysis (host versus non-host; Rutaceae versus non-Rutaceae). Plant extracts were categorized as host (*Citrus* spp., *Z. ailanthoides*, and *P. amurense*; three Rutaceae species) or non-host (the remaining six species), and independently as Rutaceae (*Citrus* spp., *Z. ailanthoides*, *P. amurense*, and *O. japonica*) or non-Rutaceae (the remaining five species). For each of the five spike types, firing rates were compared between groups in each categorization by two-sided Mann-Whitney U test. A non-parametric test was chosen because firing-rate distributions were right-skewed and contained numerous zero values. The ten resulting p -values (five types $\times$ two comparisons) were jointly corrected by BH-FDR.

Secondary analysis (*O. japonica* versus host Rutaceae). To investigate the neural basis of oviposition rejection by *O. japonica* despite its membership in the host family, firing rates for *O. japonica* were compared with those for the three host Rutaceae species by two-sided Mann-Whitney U test. The five resulting p -values were corrected by BH-FDR independently of the main analysis.

Female-ID clustered sensitivity analysis. To account for repeated sensillum recordings from the same female, we performed Poisson GEE on file $\times$ spike-type counts with female identity as the clustering variable, an exchangeable working correlation structure, and $\log(\textit{analysis_duration_sec})$ as an offset. Incidence rate ratios (IRRs) with 95% Wald confidence intervals were computed from model coefficients. These models were used as a conservative sensitivity analysis for the main host/non-host and Rutaceae/non-Rutaceae comparisons and for the *O. japonica* versus host Rutaceae comparison. It should be noted that within-female clustering by *female_id* does not account for the additional within-sensillum correlation arising from repeated stimulations of the same sensillum with different plant extracts (Section 2.5): observations from the same sensillum session may be more similar than the exchangeable working correlation structure assumes, which could slightly underestimate the effective number of independent data points within a female. This limitation is unlikely to

affect the qualitative direction of effects but may moderately narrow Wald confidence intervals relative to fully specified nested models.

Effect sizes were expressed as rank-biserial correlation coefficients (r), where $r > 0$ indicates higher firing in the host or Rutaceae group. Effect magnitudes were interpreted as negligible ($|r| < 0.1$), small (0.1–0.3), medium (0.3–0.5), or large (> 0.5). All computations used SciPy (Mann-Whitney U test; Virtanen et al. 2020) and statsmodels (BH-FDR correction; Seabold and Perktold 2010).

Use of AI-assisted Tools. Large language model (LLM) tools (Gemini, Claude, Claude Code, and Codex) were used to assist in the development of data-analysis code (Sections 2.6–2.10) and in the translation of this manuscript from Japanese into English. All analytical methods, results, and interpretations were verified by the authors.

RESULTS

Non-host Plant Extracts Suppress Oviposition Below the Post-priming Water Baseline. Following the two-step motivational pre-test protocol (Section 2.3), each female was confirmed to withhold oviposition on a water-control leaf and to oviposit on a *Citrus* extract leaf before the test stimulus was presented. When a water-control leaf was offered after *Citrus* priming, 17 of 33 females (51.5%) showed complete oviposition in at least one trial. Among females that did not oviposit, 12 (36.4%) showed Bending and 4 (12.1%) showed No response. Thus, 29 of 33 females (87.9%) showed either Oviposition or Bending on the water control. This water-control response was therefore treated as a post-priming water baseline under a high oviposition-motivation state.

Trial-level Bayesian hierarchical models supported strong treatment effects relative to the post-priming water baseline (Fig. 1; Table 2). All models converged well (maximum R-hat = 1.001, minimum bulk effective sample size = 2,427, no divergent transitions, and no maximum-tree-depth warnings).

All females presented with host plant extracts oviposited: oviposition rate was 100% for all three host Rutaceae species (*Citrus* spp., $n = 32$; *Z. ailanthoides*, $n = 25$; *P. amurense*, $n = 10$). The Bayesian model estimated higher oviposition odds than the water baseline for all host species, with posterior direction probabilities of 1.000 (*Citrus* spp.: OR = 177, 95% CrI = 43.2–1,256; *Z. ailanthoides*: OR = 126, 95% CrI = 30.1–912; *P. amurense*: OR = 167, 95% CrI = 25.7–2,810).

None of the five non-Rutaceae non-host species elicited complete oviposition (0% for all: *A. debilis*, $n = 7$; *Lespedeza* spp., $n = 6$; *C. camphora*, $n = 6$; *P. crispum*, $n = 8$; *A. keiskei*, $n = 10$). The Bayesian model estimated lower oviposition odds than water for all five species, with posterior probabilities for a lower-than-water effect ranging from 0.989 to 1.000 (OR medians = 0.026–0.065; Table 2). Because females were classified as ovipositing if oviposition occurred in any repeated trial for a given treatment, this 0% rate indicates that no female completed oviposition on any repeated presentation of these non-host extracts. Bending nevertheless occurred in subsets of females: *A. debilis* (3/7), *Lespedeza* spp. (2/6), *C. camphora* (2/6), *P. crispum* (2/8), and *A. keiskei* (2/10). Thus, non-host extracts did not simply abolish oviposition-related motor initiation; rather, they primarily suppressed completion of oviposition.

Orixa japonica (Rutaceae; non-host) showed an intermediate behavioral profile. Complete oviposition occurred in 2 of 14 females (14.3%), below the water-control rate of 17/33 (51.5%). The Bayesian model estimated lower

oviposition odds for *O. japonica* than for water (OR = 0.190, 95% CrI = 0.034–0.718; posterior probability lower than water = 0.994). An additional 6 of 14 females (42.9%) showed Bending, so 8 of 14 females (57.1%) showed either Oviposition or Bending. For this broader oviposition-or-Bending outcome, *O. japonica* also remained lower than water (OR = 0.372, 95% CrI = 0.137–0.945; posterior probability lower than water = 0.982), indicating that *O. japonica* suppresses completion of oviposition while retaining more oviposition-related initiation than the non-Rutaceae non-hosts.

Together, these results show that host extracts reliably elicit complete oviposition in primed females, whereas the post-priming water control elicits oviposition in about half of females and oviposition-related behavior in most. Against this elevated water baseline, non-Rutaceae non-host extracts suppressed complete oviposition to 0%, and *O. japonica* substantially reduced oviposition completion while leaving frequent Bending. Non-host plants therefore appear not merely to lack stimulatory activity but to contain inputs that suppress completion of the oviposition motor sequence.

Tip-recording Electrophysiology Resolves Five Waveform-defined Spike Types in Tarsal Chemosensilla. Of 58,030 spike candidates extracted from the 1,077 recording files retained for analysis (nine plant stimuli), 57,565 (99.2%) were retained after three-stage spike-level noise filtering (Section 2.8). Six waveform features were extracted for each spike (Fig. 2), and principal component analysis (4 components; cumulative variance, 99.0%) followed by GMM clustering (K = 5) resolved five waveform-defined spike types, designated Types 0–4 (Fig. 3). The selection of K = 5 was based on the model-evaluation workflow described in Section 2.9, including BIC/AIC elbow analysis (Fig. S1) and sensitivity analyses across PCA dimensionality, component number, and feature set (Figs. S2–S5).

The necessity of six-feature classification is illustrated by the separation of Types 2 and 3. Although Fig. 3 shows reproducible mean waveform shapes for both types, their median spike peak voltages were closely matched (Type 2: 0.78 mV; Type 3: 0.69 mV) with broadly overlapping interquartile ranges, making them indistinguishable by amplitude alone and at risk of merging into a single cluster under amplitude-based visual sorting as used in prior work (Ryuda et al. 2013). They are reliably separated only through their temporal and morphological features: median half-width (Type 2: 0.66 ms; Type 3: 0.55 ms) and afterhyperpolarization depth (Type 2: -0.237 mV; Type 3: -0.266 mV) differ between the two types.

Types 1 and 2 present a parallel challenge: their peak voltages are similarly matched, making them indistinguishable by amplitude alone. The consequence of failing to resolve them differs from the Types 2 and 3 case: rather than merging two broadly responsive classes, amplitude-based sorting would subsume the distinctively low-firing Type 1—which shows mean rates below 4 Hz for all nine plant species—into the higher-firing Type 2, rendering this potentially distinct spike type invisible to analysis (Section 4.5).

Sensitivity analyses confirmed the robustness of the K = 5 solution. Effect directions of the primary spike types (Types 0, 3, and 4) were consistent when PCA dimensionality was varied between 3 and 4 (Fig. S2). Types 0 and 4 maintained stable directionality across K = 5–7, whereas Type 1 and Type 3 both lost stable directionality at K ≥ 6—Type 1 reversing from host-preferring to non-host-preferring, and Type 3 reversing from non-host-preferring to host-preferring—indicating limited classification stability for these types (Fig. S3). Reducing the feature set to three features (peak_v, trough_v, and symmetry, omitting half_width, rise_slope, and fall_slope) caused BIC to favor K = 4, dropped cluster assignment consistency substantially (adjusted Rand index, ARI = 0.407 relative to

the six-feature $K = 5$ solution), and caused the host-associated type (equivalent to Type 4) to split across multiple clusters (Fig. S4A–E). Taken together, these analyses support the use of the six-feature $K = 5$ solution as the main descriptive classification.

Firing rates differed systematically across plant species for each spike type (Fig. 4; Fig. 5B). Mann-Whitney U tests (BH-FDR corrected; ten tests: five types $\times$ two comparisons) detected significant firing-rate differences between host and non-host stimuli for all five spike types (Sections 3.3 and 3.4; Fig. 5A).

Type 0 Spikes Respond Preferentially to Non-host Plant Extracts. Type 0 spikes fired at higher mean rates in response to non-host plants than to host plants (host: 0.54 Hz; non-host: 2.88 Hz). The host versus non-host effect was of medium magnitude (rank-biserial $r = -0.389$; $q < 0.001$), and the same direction of effect was observed in the Rutaceae versus non-Rutaceae comparison ($r = -0.338$; $q < 0.001$; Fig. 5A). The female-ID clustered GEE confirmed the non-host preference at the individual level (Host vs Non-host: IRR = 0.305, 95% CI 0.158–0.589, $q = 0.0040$; Rutaceae vs Non-Rutaceae: IRR = 0.405, 95% CI 0.227–0.725, $q = 0.012$).

Type 3 responses also showed a significant non-host preference in the host versus non-host comparison ($r = -0.207$; $q < 0.001$), but no significant difference was detected in the Rutaceae versus non-Rutaceae comparison ($r = -0.06$; n.s.), indicating that the Type 3 non-host preference does not track plant family membership.

Type 2 responses showed a modest host preference overall (host: 6.4 Hz; non-host: 5.7 Hz; $r = +0.12$; $q < 0.01$), with a consistent direction in the Rutaceae versus non-Rutaceae comparison ($r = +0.15$; $q < 0.001$). Inspection of plant-by-plant mean firing rates (Fig. 4) reveals, however, that *A. keiskei* (12.3 Hz) and *A. debilis* (11.5 Hz) elicited higher Type 2 responses than the highest-responding host plant, *Z. ailanthoides* (7.1 Hz); these elevated responses were individually significant relative to host plants combined (*A. keiskei*: $r = +0.37$, $q < 0.001$; *A. debilis*: $r = +0.34$, $q < 0.001$). For *A. keiskei*, this elevated Type 2 response was type-specific: Types 3 and 4 responded significantly below host-plant levels for the same stimulus ($r = -0.66$ and -0.56 , respectively; both $q < 0.001$). For *A. debilis*, Type 3 also responded at levels comparable to or above host plants ($r = +0.30$; $q < 0.001$), and mean Type 4 firing (9.2 Hz) was similar to *Citrus* spp. (8.1 Hz) but below *Z. ailanthoides* (17.2 Hz) and *P. amurense* (11.7 Hz).

Type 1 responses showed a slight host preference ($r = +0.14$; $q < 0.001$; Rutaceae vs. non-Rutaceae: $r = +0.19$; $q < 0.001$), but mean firing rates were low in both groups (host: 1.5 Hz; non-host: 1.3 Hz) and Type 1 directionality reversed at $K \geq 6$ in sensitivity analyses (Fig. S3), indicating that this type has limited classification stability.

Type 4 Spikes Show Host-associated Response Directionality. At the sensillum-recording level, Type 4 spikes showed the highest firing rates in response to host plant extracts. The host versus non-host effect was of medium magnitude ($r = +0.454$; $q < 0.001$), and the Rutaceae versus non-Rutaceae comparison yielded a slightly stronger effect in the same direction ($r = +0.470$; $q < 0.001$; Fig. 5A). Among host plants, *Z. ailanthoides* elicited the highest mean Type 4 response (17.2 Hz), substantially exceeding the mean response to non-host plants (5.1 Hz; Fig. 4). However, this host-associated direction did not remain significant after female-ID clustered GEE correction (Host vs Non-host: IRR = 2.16, 95% CI 1.03–4.52, $q = 0.103$; Rutaceae vs Non-Rutaceae: IRR = 2.75, 95% CI 1.09–6.94, $q = 0.103$). Thus, Type 4 is interpreted as a host-associated candidate go input rather than direct evidence for an oviposition-inducing neuron.

Orixia Japonica Simultaneously Activates Type 4 and Type 0 Responses. Neural responses to *O. japonica* were compared directly with responses to the three host Rutaceae species by Mann-Whitney U test (BH-FDR corrected; five tests: five types $\times$ one comparison; Fig. 6).

O. japonica activated Type 4 responses (mean: 8.0 Hz), but at a rate significantly below that of host Rutaceae (mean: 12.0 Hz; host Rutaceae $>$ *O. japonica*; $r = +0.228$; $q = 6.28 \times 10^{-5}$ in the sensillum-recording-level analysis; female-ID clustered GEE: $q = 0.161$, not significant). Conversely, Type 0 firing in response to *O. japonica* (mean: 1.38 Hz) significantly exceeded that in response to host Rutaceae (mean: 0.54 Hz; *O. japonica* $>$ host Rutaceae; $r = -0.297$; $q = 3.05 \times 10^{-8}$; female-ID clustered GEE: $q = 7.08 \times 10^{-8}$). Type 3 also responded more strongly to *O. japonica* (mean: 13.0 Hz) than to host Rutaceae ($r = -0.356$; $q = 6.52 \times 10^{-10}$). Firing rates of Types 1 and 2 did not differ significantly between *O. japonica* and host Rutaceae (both n.s.).

In the oviposition bioassay, *O. japonica* elicited 14.3% oviposition, below the water-control baseline of 51.5% (Bayesian trial-level model: OR = 0.190, 95% CrI = 0.034–0.718; posterior probability lower than water = 0.994; Section 3.1). Bending remained frequent (42.9%), indicating that *O. japonica* provided a mixed peripheral input: partial activation of the host-associated Type 4 response, consistent with detection of Rutaceae-like chemical features, together with elevated Type 0 activity associated with deterrent input. This mixed input was associated with strong suppression of oviposition completion rather than complete elimination of oviposition-related motor initiation.

DISCUSSION

Non-host Plants Are Rejected Through Active Deterrent Signals, Not the Absence of Oviposition Stimulants. The behavioral results support the interpretation that oviposition suppression by non-host plants is an active, stimulus-driven process. When primed females—confirmed to oviposit on *Citrus* extract—were presented with a water-control leaf, 51.5% oviposited and 87.9% showed either Oviposition or Bending. This overflow reflects a centrally maintained motivational state that persists transiently after *Citrus* contact, providing a post-priming water baseline elevated above zero. The magnitude of this overflow—51.5% oviposition on a water control—is consistent with *Citrus* contact powerfully activating go-associated chemosensory inputs, establishing a central motivational state sufficiently strong to sustain oviposition behavior transiently in the absence of ongoing peripheral stimulation; a pattern also reflected in the high mean Type 4 firing rates elicited by host Rutaceae extracts in the electrophysiological recordings (Section 3.4). All five non-Rutaceae non-host species produced 0% complete oviposition, significantly below this water baseline. This absence of oviposition is particularly informative because the female-level response was not based on a single opportunity: a female would have been counted as accepting the stimulus if oviposition had occurred in any repeated trial. A simple absence-of-stimulants hypothesis predicts that non-host plants should perform no worse than water; the finding that they suppress completion of oviposition below the water-baseline level is inconsistent with this explanation and indicates instead that non-host plants carry stimulus properties that actively deter oviposition completion. Although residual *Citrus* stimulants on the tarsi after the motivational pre-test cannot be fully excluded, this condition mirrors the natural oviposition context, in which a motivated female would similarly have had prior chemical contact with host plant tissue; the water-control response therefore reflects a biologically relevant motivated baseline rather than an experimental artifact.

The concept that oviposition acceptance or rejection is governed by the net balance of stimulatory ("go") and deterrent ("stop") sensory inputs was formulated independently by Dethier (1982) as a principle of neuroethological integration in host-plant recognition, and by Miller and Strickler (1984) as part of a general framework of host-finding and acceptance behavior in phytophagous insects. Honda (1995) synthesized these perspectives in a comprehensive review of the chemical basis of Lepidoptera oviposition, organizing the empirical evidence around a stimulant-deterrent competition model in which stimulant and deterrent inputs compete at the level of the peripheral chemosensory system. The primary experimental support Honda (1995) marshaled for this model came from *Pieris* butterflies and Brassicaceae plants—specifically, the demonstration that host-range differences between *P. rapae* and *P. napi* reflect the relative balance of glucosinolate stimulants and cardenolide deterrents (Huang and Renwick 1993), with direct tarsal tip-recording evidence showing functionally distinct stimulant-responsive and deterrent-responsive neurons contributing to these differences (Städler et al. 1995; Du et al. 1995). Extension of this framework to *P. xuthus* was presented as a plausible extrapolation rather than a direct empirical demonstration. The behavioral and chemical evidence Honda (1995) cited for *P. xuthus* specifically was entirely confined to Rutaceae: deterrent compounds chemically isolated from the non-host Rutaceae species *Orixa japonica* (Nishida et al. 1990), and competition between stimulants and deterrents within *Phellodendron amurense* (Honda and Hayashi 1995). Active deterrence of *P. xuthus* oviposition by plants outside the host family Rutaceae had not been demonstrated.

The five non-Rutaceae non-host species examined here—*A. debilis*, *Lespedeza* spp., *C. camphora*, *P. crispum*, and *A. keiskei*, spanning four plant families—consistently suppressed complete oviposition to 0%, with Bayesian posterior probabilities of 0.989–1.000 for lower oviposition odds than the water baseline. This extends the stimulant-deterrent framework to *P. xuthus* across family boundaries. A comparable cross-family limitation also characterizes prior electrophysiological work on deterrence: the putative deterrence neuron (M2) described in *Papilio hospiton* (Sollai et al. 2017; Sollai and Crnjar 2018) was characterized against non-host plants within the same family (Apiaceae) as *P. hospiton*'s host plants, leaving open whether such a response class occurs broadly for plants from phylogenetically distant families. The occurrence of Bending—incomplete oviposition in which abdominal curling begins but ovipositor-substrate contact is not made—in all five non-Rutaceae non-hosts further argues against a pure absence-of-stimulants interpretation: partial initiation of the oviposition motor sequence implies that some stimulatory input is present, with deterrent input overriding the completion of oviposition—a pattern consistent with the stimulant-deterrent competition model of Honda (1995). Type 0 spike responses provide a peripheral electrophysiological correlate of this behavioral deterrence, firing at higher rates across all five non-Rutaceae non-host species than across host plants in the sensillum-recording-level analysis ($r = -0.389$; $q < 0.001$; Section 3.3), and this non-host association remained significant in female-ID clustered GEE.

Earlier work has established several relevant precedents: tarsal contact chemosensory neurons of *Pieris brassicae* respond to a conspecific host-marking pheromone (Behan and Schoonhoven 1978); tarsal contact chemoreceptors of the congeneric *Papilio polyxenes* respond differentially to phytochemicals from host and non-host plants (Roessingh et al. 1991); and M2 deterrence neurons have been identified in the related species *Papilio hospiton* against non-host plants within a single plant family (Sollai et al. 2017; Sollai and Crnjar 2018). The identification of a non-host-associated spike type in *P. xuthus* through de novo unsupervised clustering—with a quantitative host/non-host response bias ($r = -0.389$) across non-host plants from five species belonging to four non-host families—represents a substantial extension of this earlier work.

Integrating the behavioral and electrophysiological findings, the tarsal chemosensory system of *P. xuthus* appears to operate not as a unidirectional detector that searches for a small set of host-recognition signals against a chemically silent background, but as a bidirectional evaluation system in which host-associated and non-host-associated inputs can be detected in parallel. Type 0 responses are elevated for non-Rutaceae non-host plants and for the Rutaceae non-host *O. japonica* alike, supporting Type 0 as a robust non-host-associated spike type with stop-associated behavioral correlates (where 'non-host-associated' describes the sensory firing pattern—elevated activation by non-host plant extracts—and 'stop-associated' the corresponding behavioral output, since the activating stimuli also suppress oviposition; both terms refer to the same spike type throughout this study) rather than direct proof of a single deterrence neuron. Oviposition completion appears to be linked to the relative balance of candidate go-associated and robust stop-associated inputs.

A complementary perspective at the receptor level is provided by Dong et al. (2026), who identified a specific gustatory receptor, BdorGr28b, expressed in the ovipositor sensilla of *Bactrocera dorsalis* (Diptera) that detects hesperidin as an oviposition deterrent. Hesperidin functions as an oviposition stimulant in *P. xuthus* (Ohsugi et al. 1991), illustrating that the same compound can carry opposite valences across species—the species-specific chemosensory coding that Honda (1995) and Honda et al. (2011) discussed as a central feature of host-range determination. The receptor-level deterrent coding demonstrated by Dong et al. (2026) and the spike-type-level deterrent association identified here for *P. xuthus* Type 0 are complementary lines of evidence: together they indicate that active deterrent encoding is a principle of insect peripheral chemosensation that operates across taxa and at multiple levels of the sensory hierarchy.

Orixa Japonica as a Natural Test of the Ensemble Integration Model. *Orixa japonica*—a member of the host family Rutaceae that is nonetheless rejected for oviposition—provided a natural case in which candidate go-associated and stop-associated peripheral inputs were partially dissociated (Section 3.5). *O. japonica* simultaneously activated the host-associated Type 4 response and the non-host-associated Type 0 response, and it strongly suppressed complete oviposition while leaving frequent Bending responses.

The partial activation of Type 4 by *O. japonica* (mean, 8.0 Hz) suggests that the tarsal chemosensory system detects Rutaceae-like chemical features: *O. japonica* contains compounds sufficient to activate the host-associated Type 4 response, albeit at a lower rate than host Rutaceae (mean, 12.0 Hz; $r = +0.228$; $q = 6.28 \times 10^{-5}$ in the sensillum-recording-level analysis). This Type 4 difference did not remain significant in the female-ID clustered GEE comparison of host Rutaceae versus *O. japonica* ($q = 0.161$), so Type 4 should be interpreted as a candidate go-associated response rather than definitive evidence for an oviposition-inducing channel. Concurrently, Type 0 firing in response to *O. japonica* (mean, 1.38 Hz) significantly exceeded that for host Rutaceae (mean, 0.54 Hz; $r = -0.297$; $q = 3.05 \times 10^{-8}$), and this difference remained significant after female-ID clustered GEE correction ($q = 7.08 \times 10^{-8}$). This pattern indicates mixed peripheral input. Among host Rutaceae, *Z. ailanthoides* activates Type 4 strongly (17.2 Hz) while eliciting only modest Type 0 activity (~0.6 Hz per-plant mean), yielding 100% oviposition. For *O. japonica*, Type 4 is activated at 8.0 Hz while Type 0 reaches 1.38 Hz; complete oviposition falls to 14.3%, whereas Bending remains frequent (42.9%). These data support the interpretation that *O. japonica* provides partial candidate go-associated input together with robust stop-associated input, consistent with a competitive integration rule rather than a simple threshold on either channel independently.

A further implication of the *O. japonica* result concerns the interaction between peripheral input and central motivational state. Primed females—confirmed to oviposit on *Citrus* in the motivational pre-test—showed lower complete oviposition on *O. japonica* (14.3%) than on the water-control leaf (51.5%), while Bending remained common. This indicates that elevated Type 0 activity is associated primarily with suppression of oviposition completion rather than with complete elimination of oviposition-related motor initiation. Even when partial host-associated Type 4 input is present and a motivational state favoring oviposition has been established centrally, elevated Type 0 activity accompanies suppression of the final behavioral output. Thus, the stop-associated input appears to limit oviposition completion rather than acting merely as a weak graded modulator.

Nishida et al. (1990) isolated from *O. japonica* a flavonol triglycoside—quercetin 3-O-(2'-β-D-xylopyranosylrutinoside)—that inhibited *P. xuthus* oviposition by 85% as a pure compound at concentrations exceeding estimated in-plant levels; the crude methanol extract produced complete inhibition (100%). Ono et al. (2004) subsequently characterized two hydroxybenzoic acid derivatives from the water-soluble fraction of *O. japonica* leaf extract—5-[[2-O-(β-D-apiofuranosyl)-β-D-glucopyranosyl]oxy]-2-hydroxybenzoic acid and a disyringoyl aldaric acid—that deter both oviposition and larval feeding by *P. xuthus* at the concentrations present in the tested extracts; whether these compounds reach deterrent-effective concentrations at the tarsal contact surface under natural foraging conditions remains to be determined. The strong Type 0 activation by *O. japonica* methanol extract observed here is consistent with one or more of these compound classes contributing to non-host-associated peripheral input under extract-based stimulation conditions.

The species specificity of this deterrence is highlighted by the fact that *O. japonica* serves as a host plant for the related species *Papilio macilentus* and *P. bianor* (Nishida et al. 1990). In these species, the ensemble balance of go- and stop-associated inputs for *O. japonica* presumably tilts toward acceptance, either because Type 0-equivalent spike types are less sensitive to the quercetin glycosides of *O. japonica*, because Type 4-equivalent spike types are more strongly activated by its Rutaceae compounds, or both. This inter-specific difference illustrates how a quantitative shift in the relative tuning of go- and stop-associated channels at the periphery can determine whether a plant is accepted or rejected.

Ensemble Balance of Go- and Stop-associated Inputs as a Determinant of Host Range Across Papilio Species. The ensemble integration framework offers a generalizable explanation for interspecific variation in host range within *Papilio*. Honda and Hayashi (1995) showed that differential acceptance of *Phellodendron amurense* by two sympatric swallowtail butterflies depends on the balance between co-occurring stimulant and deterrent compounds, including the deterrent flavonoid glucoside phellamurin. Within the framework of the present study, this implies that interspecific differences in host use can arise when Type 0-equivalent responses are differentially activated by the same plant compounds, when Type 4-equivalent responses differ in strength, or both. This prediction is directly testable by applying the same tip-recording approach with six-feature GMM clustering to related *Papilio* species stimulated with shared Rutaceae extracts.

The deeper principle is the species-specific valence that the same plant compound can carry across insect species. Honda (1995) provides a clear illustration: chlorogenic acid functions as an oviposition stimulant in *P. protenor* but as a deterrent in *P. memnon*. Honda et al. (2011) provide a direct empirical example of this principle: phellamurin, a prenylated flavonoid glucoside constitutive in *Phellodendron amurense*, functions as a key oviposition stimulant in *P. maackii* but acts as a potent oviposition deterrent in *P. protenor*. This reversal of valence across congeneric species implies that phellamurin activates a go-associated channel in *P. maackii* but

a stop-associated channel in *P. protenor*—a difference that may reside entirely at the peripheral receptor level. The Type 0 responses identified here represent a peripheral electrophysiological signature through which species-specific deterrent coding is likely implemented. Comparative characterization of Type 0 receptor specificity across *Papilio* species with differing host ranges would be a direct approach to understanding how host range diversity is generated and maintained at the level of peripheral taste receptor evolution.

Methodological Advantages of Six-feature Clustering. The present study differs from prior electrophysiological analyses of *P. xuthus* tarsal chemosensilla in two respects: the stimuli used and the spike-sorting strategy. Ryuda et al. (2013) stimulated sensilla with purified oviposition stimulants (synephrine, hesperidin, etc.) applied individually, conditions under which only neurons sensitive to the specific test compound are activated and the number of co-active spike types within a single recording is inherently limited. Plant methanol extracts, as used here, contain numerous phytochemicals that activate multiple spike-generating cells simultaneously within a single sensillum recording. Under these conditions, the three-amplitude-class visual classification used in prior work (Ryuda et al. 2013) rarely yielded the clear amplitude separation required to assign individual spikes to distinct response classes. The challenge is qualitatively different from that encountered in *Atrophaneura alcinous*, where host (*Aristolochia debilis*) extracts elicit multi-amplitude spikes from long-type chemosensilla while non-host (*Citrus*) extracts elicit only single-amplitude spikes (Tsuchihara et al. 2022). In that system, the qualitative difference in which spike types are recruited (some respond to host but not non-host) permits plant discrimination on the basis of amplitude alone. In *P. xuthus*, the same five waveform-defined spike types are detected under both host and non-host stimulation; what varies across plant categories is the relative firing-rate balance among the types, with Type 4 dominant under host and Type 0 dominant under non-host stimulation. This quantitative ratio code is not recoverable from amplitude-based visual sorting, which is the primary motivation for adopting multi-feature GMM clustering. A conceptually analogous population code—in which individual tastant chemicals are represented as distributed patterns across GRN types rather than through labeled-line responses—has been demonstrated in *Manduca sexta* (Reiter et al. 2015), suggesting that distributed encoding across receptor neuron populations is a broader feature of insect gustation.

Spike waveforms recorded from insect taste sensilla carry temporal and morphological information—rise slope, fall slope, half-width, afterhyperpolarization depth, and rise-fall symmetry—that prior studies of *Papilio* and related butterflies have not quantified (Ryuda et al. 2013; Sollai et al. 2017; Sollai and Crnjar 2018; Tsuchihara et al. 2022). Extracting these six features and applying automated probabilistic classification revealed that the information needed to distinguish functionally distinct spike types is present in conventional tip-recording data; it had simply not been retrieved under amplitude-only analysis. The result is a substantial increase in the information recoverable from a single recording without any modification to the recording apparatus or experimental protocol.

Types 2 and 3 illustrate this directly: their amplitude distributions overlap too broadly for reliable amplitude-based separation, yet temporal and morphological waveform features allow consistent cluster assignment (Section 3.2). The penalty for omitting these features is demonstrated in the sensitivity analysis: a three-feature solution degraded cluster assignment consistency (ARI = 0.407), caused the Type 4 cluster to fragment across multiple components, and lost the resolution needed to recover the host–non-host firing-rate balance (Fig. S4). Recovery of distinct Type 4 and Type 0 response profiles therefore requires the full six-feature representation.

The correspondence of the present spike types to neuron classes identified by Ryuda et al. (2013) requires care. Type 4 may correspond to the host-stimulant-responsive GRN class (os1) of Ryuda et al. (2013), given the shared host-preferring response direction, but a direct assignment is not possible because the stimulus conditions (pure compounds vs. whole-plant extracts) and the sensilla populations analyzed (L1-type only in Ryuda et al. vs. L-type sensilla without L1/L2 distinction in the present study) differ between studies. Type 0, which shows the non-host-associated profile, has no counterpart in the analysis of Ryuda et al. (2013). The primary reason is the choice of stimuli: purified oviposition stimulants applied individually would not strongly recruit deterrent-tuned cells, so Type 0-equivalent responses would not have been prominently active in those recordings regardless of sensillum type. Notably, Ryuda et al. (2013) also included caffeine and other bitter-tasting compounds among their stimuli, but applied amplitude-only spike classification without multi-feature waveform analysis. Two interpretations therefore remain open: Type 0-equivalent spikes may have been present in those recordings but were unresolvable by amplitude sorting, or caffeine may not activate Type 0 at all, implying that Type 0 is specifically tuned to a class of deterrent compounds distinct from canonical bitter substances. Importantly, the present classification does not demonstrate that exactly five gustatory receptor neuron classes exist in *P. xuthus* tarsi. Each waveform-defined type may include more than one cellular subtype, and the types should be interpreted as reproducible spike types with distinct stimulus-response profiles rather than as one-to-one cellular identities.

Functional Significance of the Remaining Spike Types and Future Directions. Type 3 spikes showed broadly elevated firing rates across nearly all nine plant stimuli, with mean responses exceeding 7 Hz for eight of the nine plants (Fig. 4); the sole exception was *A. keiskei* (mean, 2.1 Hz), which was anomalously low. This broad activation pattern is consistent with Type 3 representing responses to chemical constituents common across diverse plant species—widespread secondary metabolites rather than host-specific or non-host-specific compounds. Statistically, Type 3 showed a non-host preference in the overall host-versus-non-host comparison ($r = -0.207$; $q < 0.001$), but this preference did not track plant family membership (Rutaceae vs. non-Rutaceae: $r = -0.06$; n.s.), distinguishing it from the broadly non-host-associated Type 0. The anomalously low response to *A. keiskei* suggests that this plant is unusually deficient in Type 3-activating constituents. Conversely, *O. japonica* elicited the highest mean Type 3 response among all plants tested (13.0 Hz), indicating the presence of Type 3 agonists specific to *O. japonica* that are absent or scarce in the host Rutaceae. However, Type 3 directional preference showed limited stability in sensitivity analyses (Fig. S3): the non-host direction observed at $K = 5$ reversed at $K \geq 6$, paralleling the instability described for Type 1, and indicating that the apparent non-host preference of Type 3 should be interpreted with caution. The key contrast with Type 0 is selectivity: Type 0 shows approximately a fivefold difference between host (0.54 Hz) and non-host (2.88 Hz) mean firing rates, whereas Type 3 fires broadly at high rates for most plants regardless of host status (7–14 Hz). Our results suggest that Type 3 functions as a broad-spectrum response class for general plant secondary metabolites rather than as a selective deterrent detector analogous to Type 0.

Type 2 spikes showed weak host preference in both the host-versus-non-host and Rutaceae-versus-non-Rutaceae comparisons ($r = +0.12$ and $+0.15$, respectively), placing them directionally in the same category as Type 4. The effect size is, however, approximately one-quarter that of Type 4 ($r = +0.454$), and individual non-host plants such as *A. keiskei* and *A. debilis* elicited Type 2 responses that exceeded those of the highest-responding host plant (Fig. 4). Type 2 may represent a secondary go-associated response class that contributes to host recognition but is less selective and less prominently weighted than Type 4 in the

integration of oviposition signals. Type 1 spikes showed a marginal host preference ($r = +0.14$; $q < 0.001$), but mean firing rates were low in both host and non-host conditions (1.5 and 1.3 Hz, respectively), and directionality reversed at $K \geq 6$ in sensitivity analyses (Fig. S3), indicating limited classification stability; the biological significance of Type 1 therefore warrants cautious interpretation. That Type 1 is recoverable as a distinct class at all is itself a product of six-feature GMM classification: its peak voltage distribution overlaps with that of Type 2, precluding amplitude-based separation; under amplitude-only sorting, Type 1 spikes would be absorbed into the Type 2 cluster and its distinctive low-firing profile would be invisible. The uniformly low and undifferentiated firing rates of Type 1 across all nine plant stimuli are consistent with the possibility that these spikes originate from L2-type sensilla. L2 sensilla constitute a small minority of tarsal L-type sensilla in *P. xuthus* and show lower responsiveness to oviposition stimulants than L1 sensilla (Ryuda et al. 2013); because our recordings were made from L-type sensilla without distinguishing L1 from L2, the contribution of L2 sensilla to the Type 1 cluster cannot be excluded.

Two directions for future work emerge from these findings. The first is identifying the gustatory receptor gene(s) responsible for the deterrent specificity associated with Type 0. *PxutGr1*, identified by Ozaki et al. (2011) as a synephrine-specific receptor, is a plausible candidate for one host-associated input represented in part by Type 4; by analogy, Type 0 is likely served by a distinct receptor gene whose expression pattern and ligand specificity remain unknown. The quercetin triglycoside isolated from *O. japonica* by Nishida et al. (1990) and the hydroxybenzoic acid derivatives characterized by Ono et al. (2004) are logical starting points for receptor candidate screening, given that *O. japonica* reliably activates Type 0 in vivo. Recent functional characterization of deterrent GRs in Lepidoptera demonstrates that such receptors can respond to structurally diverse ligands: in *Plutella xylostella*, a bitter GR was found to be tuned to brassinolide, a plant steroid hormone not typically classified as a plant secondary metabolite, and this receptor mediated both feeding and oviposition deterrence (Yang et al. 2020), indicating that the ligand scope for Type 0-relevant receptor candidates need not be confined to canonical plant secondary metabolites. The strategy demonstrated by Dong et al. (2026)—proceeding from in vivo electrophysiology to receptor identification in *B. dorsalis*—indicates that this is an experimentally tractable problem and directly applicable to *P. xuthus*. The second direction is the characterization of how Type 4 and Type 0 inputs are integrated in higher brain centers to produce the final oviposition decision. The present study establishes a peripheral electrophysiological basis for the ensemble integration model: reproducible waveform-defined spike types, tuning biases, and behavioral associations of candidate go-associated and robust stop-associated inputs. The synaptic and circuit architecture through which these competing signals are resolved into a categorical behavioral output remains to be determined.

CONCLUSION

This study supports the view that oviposition plant selection in *Papilio xuthus* is determined not solely by the detection of host-associated ("go") signals but by the integration of these inputs with active non-host-associated ("stop") signals. In oviposition bioassays, extracts of all tested non-host plants—spanning four plant families phylogenetically unrelated to the host family Rutaceae—suppressed oviposition below the rate observed on the post-priming water control, supporting the interpretation that non-host plants are not perceived as stimulus-absent background but are detected as substrates to be positively avoided.

Spike-sorting of tip-recording data from tarsal chemosensilla, using six waveform features and Gaussian mixture model clustering, resolved Type 4 spikes—which show host-associated response directionality at the

sensillum-recording level—from Type 0 spikes, which show a robust non-host association that remains significant after female-ID clustered GEE analysis. The non-host-associated Type 0 spike type would not have been detectable under amplitude-based sorting applied to pure oviposition stimulants; its identification required stimulation with chemically complex whole-plant extracts combined with multi-feature waveform analysis. *Orixa japonica*, a Rutaceae non-host, simultaneously activated partial Type 4 responses and elevated Type 0 responses, and complete oviposition was strongly reduced despite frequent Bending. This dissociation supports the interpretation that partial host-associated input is insufficient to ensure oviposition completion when accompanied by robust non-host-associated input.

The tarsal chemosensory system of *P. xuthus* therefore appears to operate not as a device that classifies plants into host or non-host categories by detecting a fixed set of stimulatory compounds, but as a bidirectional chemical evaluation system that monitors stimulatory and non-host-associated inputs in parallel. The stop-associated Type 0 signal proved more robust under conservative female-ID clustered GEE analysis than the candidate go-associated Type 4 response—retaining statistical significance where Type 4 did not—suggesting that active deterrent detection is more reliably encoded at the peripheral level than host-stimulant detection. These findings extend the understanding of swallowtail butterfly host selection—previously framed around the neural code for oviposition induction—to an ensemble integration model in which candidate go-associated and robust stop-associated peripheral inputs act in concert to shape oviposition completion.

Declarations

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Author Contributions. Juri Nishioka: Conceptualization (behavioral experiment design), Investigation (behavioral experiments, electrophysiological recordings). Ayako Sasaki: Investigation (butterfly rearing, experimental assistance, behavioral experiments). Katsuhisa Ozaki: Conceptualization, Investigation (behavioral experiments, electrophysiological recordings), Formal analysis, Methodology, Software, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Data Availability. The raw electrophysiological recording files, processed data, and analysis code supporting this study have been deposited in Zenodo. The record is currently under restricted access for peer review and will be

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Tables

Table 1. Plant species used in this study.

Species	Family	Status
<i>Citrus</i> spp.	Rutaceae	Host
<i>Zanthoxylum ailanthoides</i> Siebold & Zucc.	Rutaceae	Host
<i>Phellodendron amurense</i> Rupr.	Rutaceae	Host
<i>Orixa japonica</i> Thunb.	Rutaceae	Non-host (same family)
<i>Aristolochia debilis</i> Siebold & Zucc.	Aristolochiaceae	Non-host
<i>Lespedeza</i> spp.	Fabaceae	Non-host
<i>Cinnamomum camphora</i> (L.) J.Presl	Lauraceae	Non-host
<i>Petroselinum crispum</i> (Mill.) Fuss	Apiaceae	Non-host
<i>Angelica keiskei</i> (Miq.) Koidz.	Apiaceae	Non-host

Table 2. Oviposition behavioral responses and Bayesian model estimates for each plant treatment relative to the post-priming water control. OR = odds ratio (posterior median); 95% CrI = credible interval; P(dir.) = posterior probability for the estimated direction. ^aOviposition (Oviposition vs. Bending + No response). ^bOviposition or Bending (Oviposition + Bending vs. No response). ^cFor host plant treatments where all trials resulted in oviposition (complete separation), the likelihood function is flat for large positive coefficient values and the CrI upper bounds are numerically influenced by the prior scale; these bounds should be read as order-of-magnitude ranges, not as precise posterior estimates. Posterior direction probabilities (P(dir.) = 1.000) are robust to prior specification under these conditions.

Plant species	N	Oviposition n (%)	Bending n (%)	No response n (%)	OR ^a (95% CrI)	P(dir.) ^a	OR ^b (95% CrI)	P(dir.) ^b
Water (control)	33	17 (51.5)	12 (36.4)	4 (12.1)	Reference	—	Reference	—
<i>Citrus</i> spp.	32	32 (100.0)	0 (0.0)	0 (0.0)	177 (43.2–1,256)	1.000	122 (20.6–1,960)	1.000
<i>Z. ailanthoides</i>	25	25 (100.0)	0 (0.0)	0 (0.0)	126 (30.1–912)	1.000	36.4 (8.88–253)	1.000
<i>P. amurense</i>	10	10 (100.0)	0 (0.0)	0 (0.0)	167 (25.7–2,810)	1.000	65.7 (9.38–1,430)	1.000
<i>O. japonica</i>	14	2 (14.3)	6 (42.9)	6 (42.9)	0.190 (0.034–0.718)	0.994	0.372 (0.137–0.945)	0.982
<i>A. debilis</i>	7	0 (0.0)	3 (42.9)	4 (57.1)	0.055 (0.0018–0.570)	0.995	0.214 (0.048–0.798)	0.990
<i>Lespedeza</i> spp.	6	0 (0.0)	2 (33.3)	4 (66.7)	0.065 (0.0017–0.722)	0.989	0.167 (0.028–0.705)	0.994
<i>C. camphora</i>	6	0 (0.0)	2 (33.3)	4 (66.7)	0.064 (0.0017–0.702)	0.992	0.168 (0.028–0.690)	0.994
<i>P. crispum</i>	8	0 (0.0)	2 (25.0)	6 (75.0)	0.042 (0.0015–0.394)	0.999	0.135 (0.027–0.518)	0.998
<i>A. keiskei</i>	10	0 (0.0)	2 (20.0)	8 (80.0)	0.026 (0.0011–0.222)	1.000	0.076 (0.017–0.254)	1.000

Figures

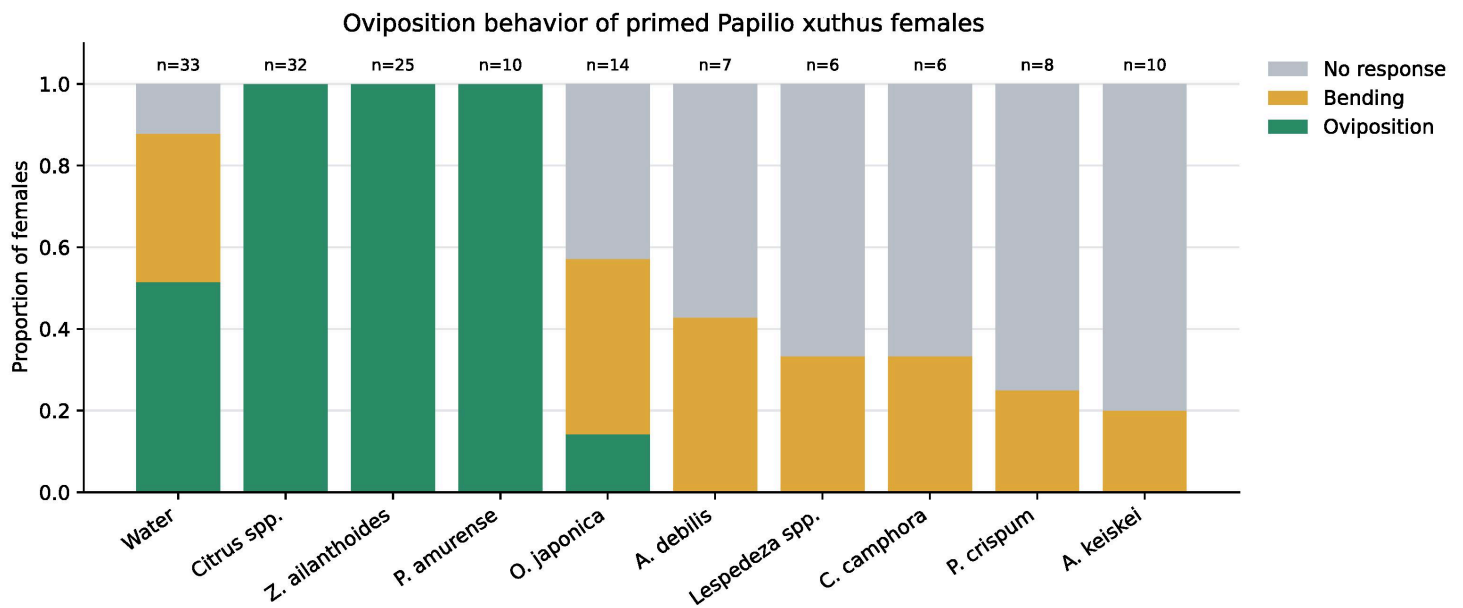


Figure 1

Oviposition behavioral responses to plant extracts. Proportions of female-level behavioral categories—Oviposition, Bending, and No response—shown for females presented with the water control, three host Rutaceae species, the Rutaceae non-host *Orixa japonica*, and five non-Rutaceae non-host species. Before each test, individual females were confirmed to withhold oviposition on a water-control leaf and to oviposit on a *Citrus* extract leaf, ensuring a motivated oviposition state. All three host plant species elicited 100% oviposition. The five non-Rutaceae non-host species elicited 0% complete oviposition, whereas *O. japonica* elicited low oviposition with frequent Bending. The figure shows observed female-level behavioral proportions only; statistical estimates are reported in the text and Table 2 from trial-level Bayesian hierarchical logistic models with female identity as a random intercept.

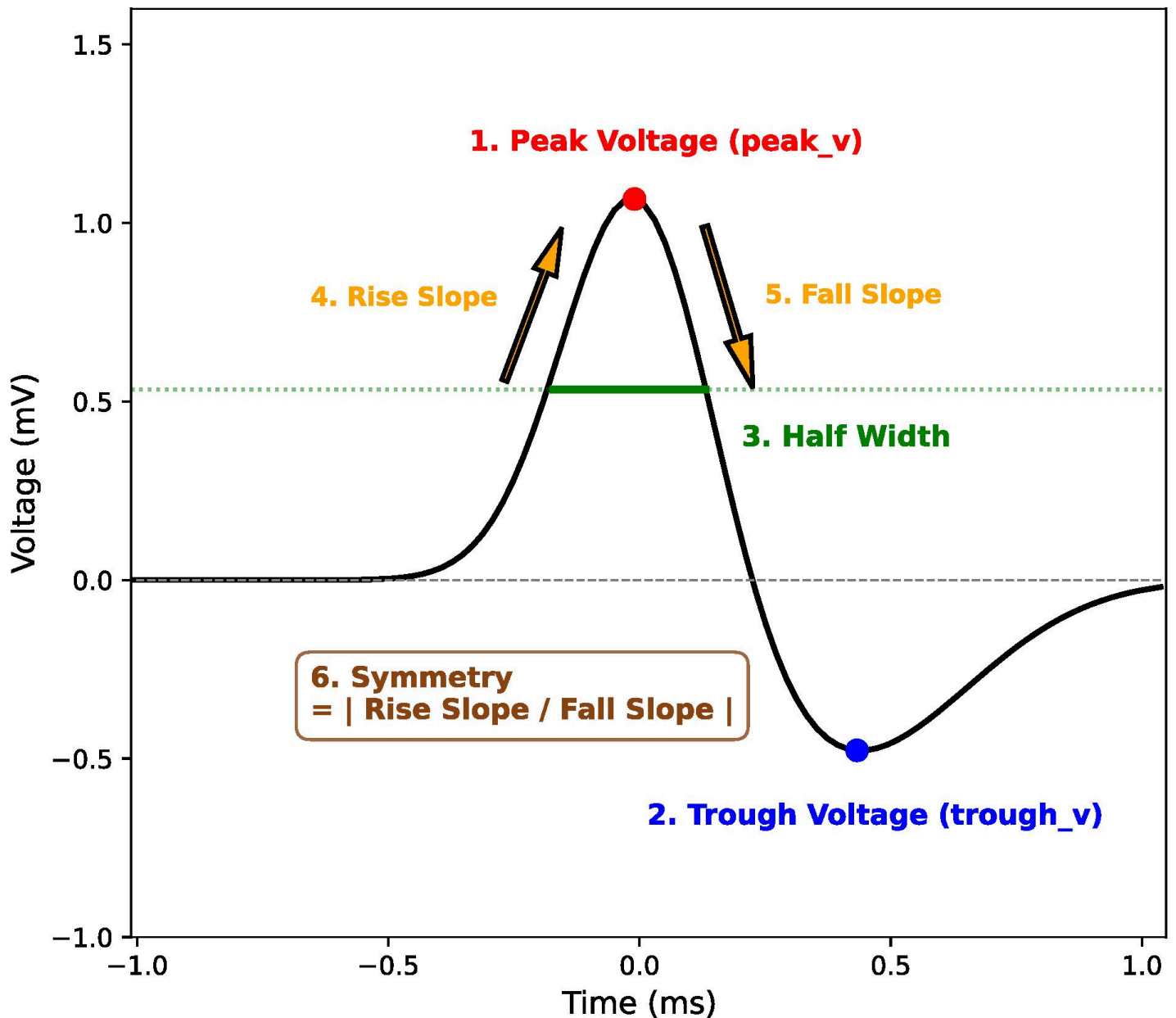


Figure 2

Six waveform features extracted from each spike. For each spike detected in tip-recording data, six waveform parameters were quantified: spike peak voltage (peak_v), afterhyperpolarization voltage (trough_v), half-width at half-maximal amplitude (half_width), maximum rate of rise (rise_slope), maximum rate of decay (fall_slope), and rise-fall asymmetry index (symmetry). Together, these features characterize spike amplitude, temporal duration, waveform slope dynamics, and morphological asymmetry. The six features were used as input for PCA dimensionality reduction and GMM clustering.

Waveform Overlay by Neural Type (K=5, up to 50 waveforms/type, total n=57,565)

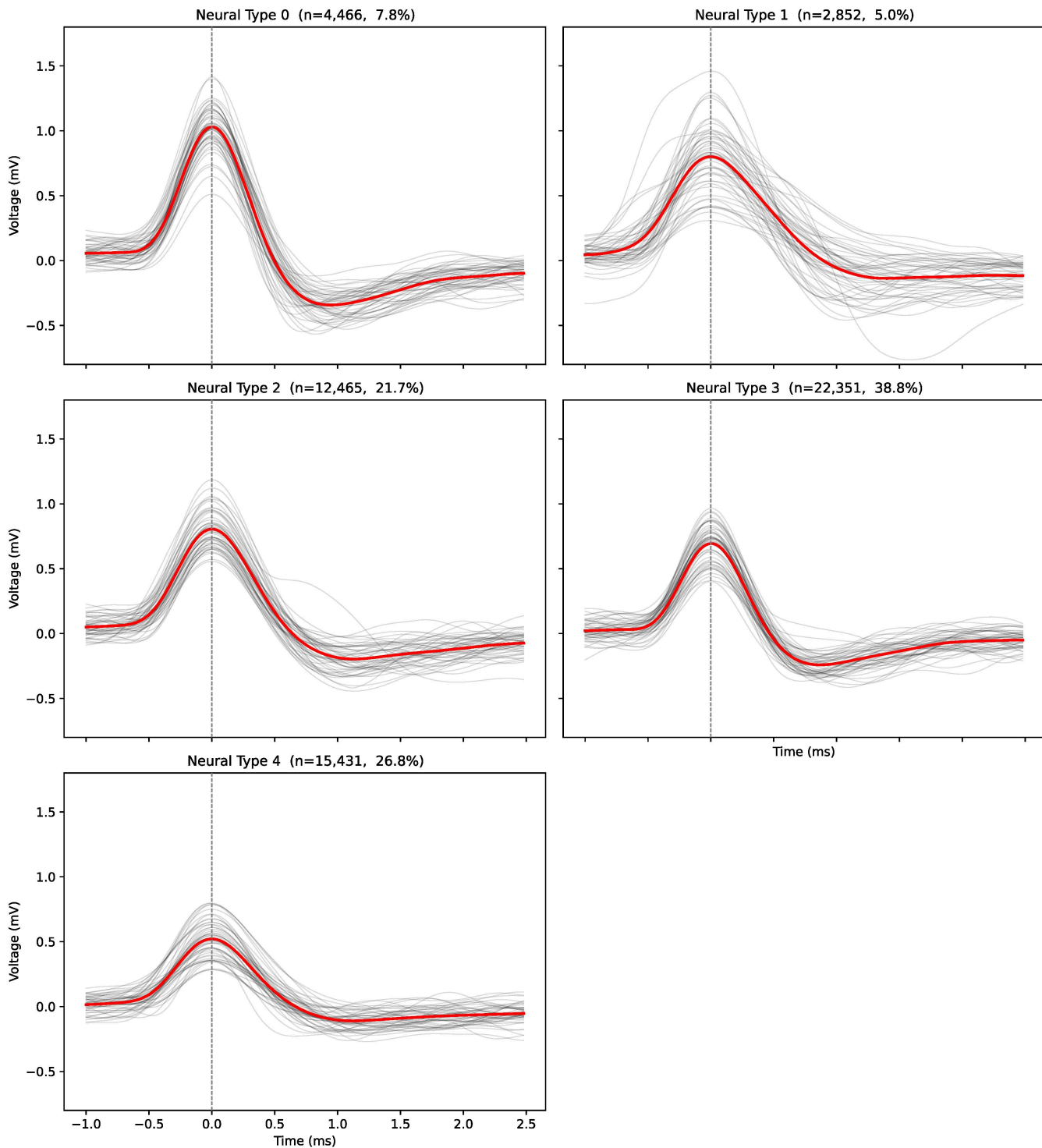


Figure 3

Five waveform-defined spike types identified by six-feature clustering. Mean spike waveforms (bold red line) with overlaid individual spike waveforms (up to 50 per type) for Types 0–4 as classified by GMM (K = 5). Classification was performed in the four-dimensional PCA space of standardized six-feature vectors. The panels show that each waveform-defined spike type has a reproducible average waveform despite variability among individual spikes. Feature-level separation of the types, including overlapping peak-voltage distributions

that require temporal and afterhyperpolarization features for resolution, is described in the text and sensitivity analyses.

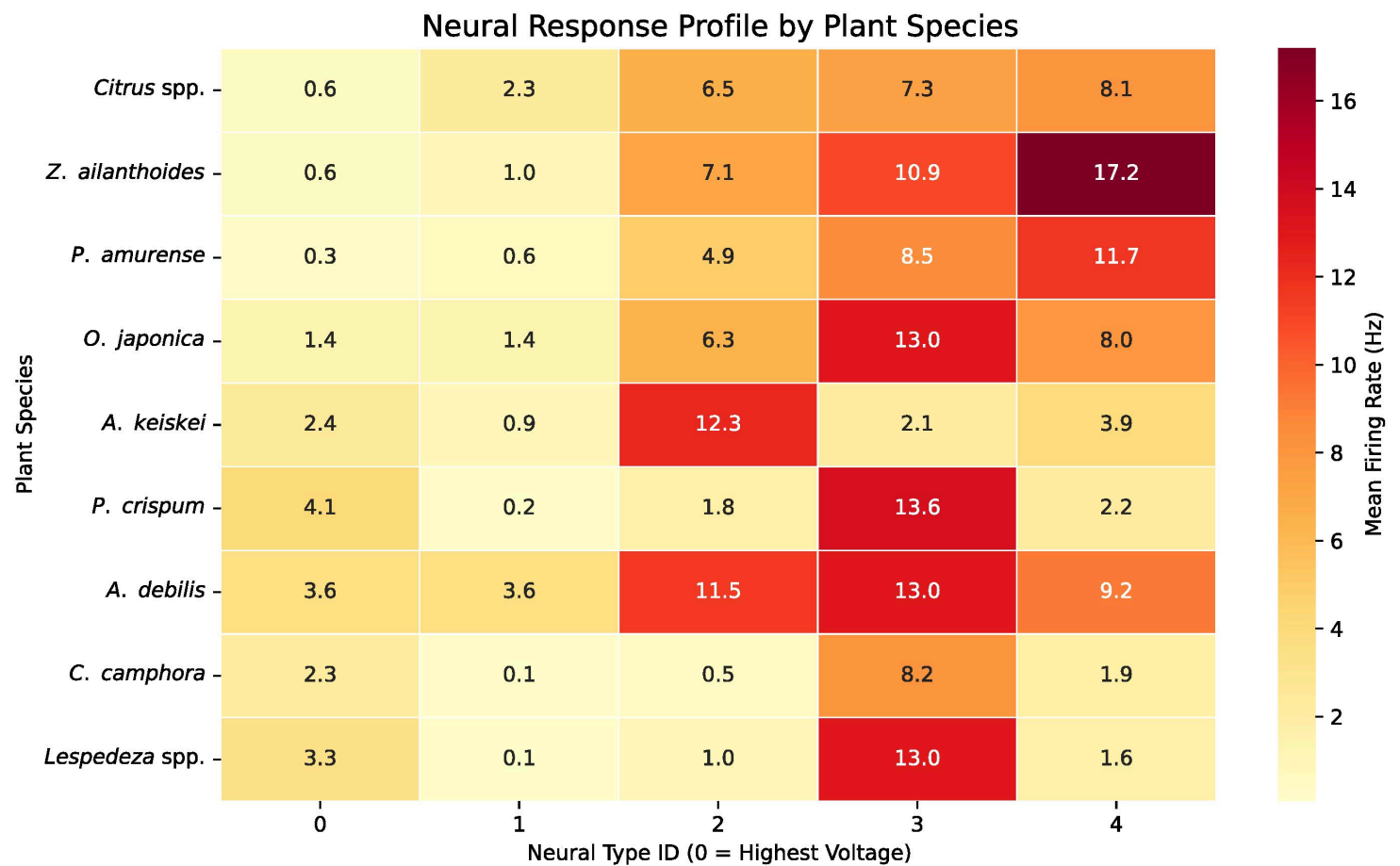


Figure 4

Heatmap of mean firing rates by spike type and plant species. Mean firing rates (Hz) of Types 0–4 in response to each of the nine plant stimuli. Type 4 showed the highest responses to host Rutaceae species, particularly *Zanthoxylum ailanthoides*, at the sensillum-recording level. Type 0 fired at higher rates in response to non-host plants than to host plants. Type 3 was broadly active across plant species. Firing rate for each spike type in each recording file was calculated as the spike count divided by the stimulus-response duration.

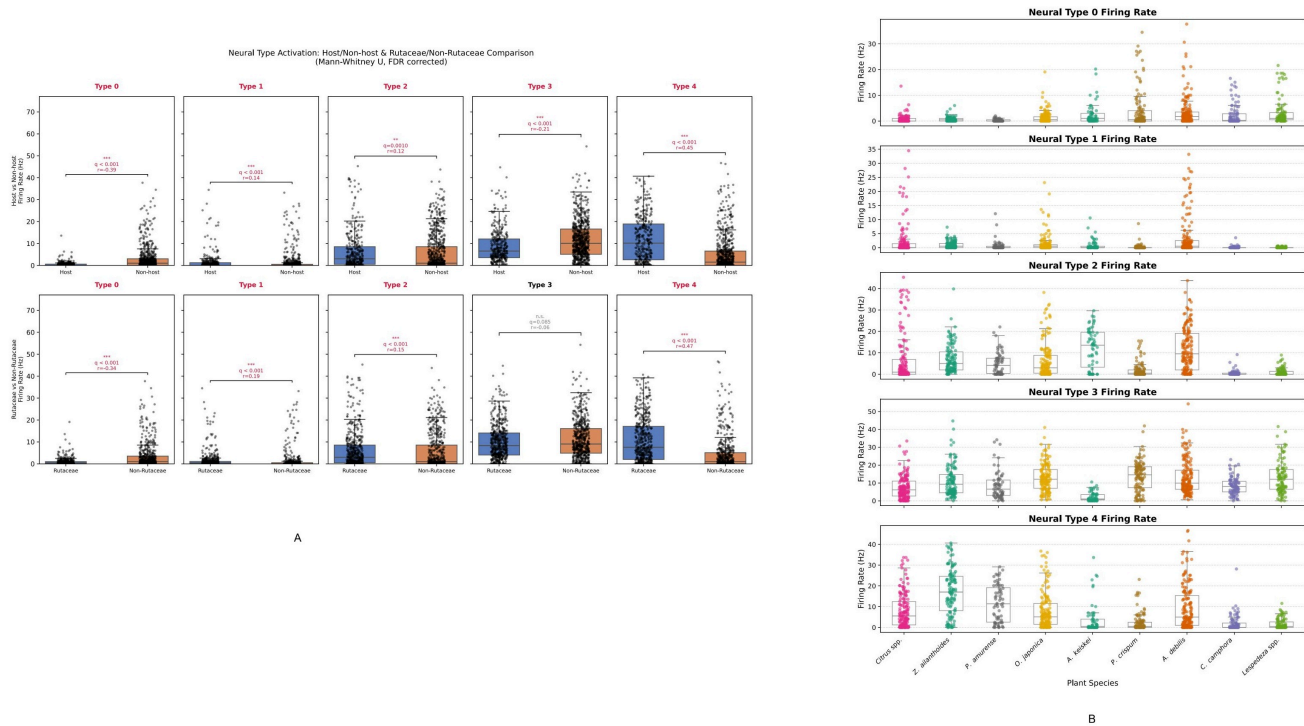


Figure 5

Spike-type firing-rate differences between host and non-host plant categories. (A) Firing-rate distributions comparing Host and Non-host plant categories (upper row) and Rutaceae and Non-Rutaceae categories (lower row) for each spike type. Annotations show the BH-FDR-corrected q value and rank-biserial correlation coefficient r ; positive r indicates higher firing in the first-named group, and negative r indicates higher firing in the second-named group. (B) Firing-rate distributions for each spike type across all nine plant stimuli (three host Rutaceae species, *O. japonica*, and five non-Rutaceae non-host species). Type 4 showed host-associated response directionality at the sensillum-recording level, whereas Type 0 showed a robust non-host-associated response. Statistical comparisons used two-sided Mann-Whitney U tests; p-values were jointly BH-FDR corrected across ten comparisons (five spike types $\times$ two category contrasts; * $q < 0.001$, $q < 0.01$, * $q < 0.05$).

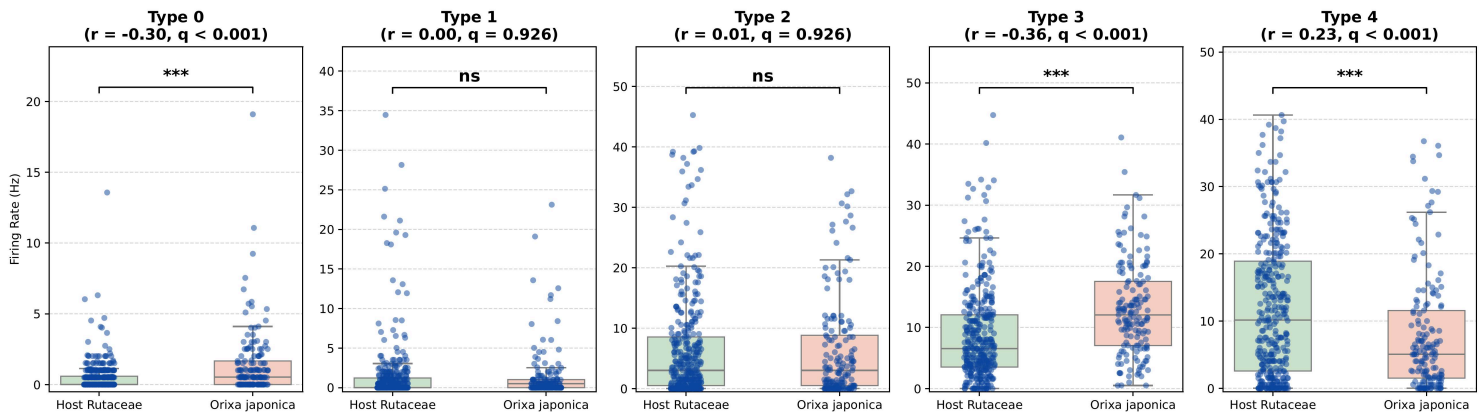


Figure 6

Neural responses to *Orixa japonica* compared with host Rutaceae. Firing rates of each spike type in response to *O. japonica* (a Rutaceae non-host) are compared with responses to the three host Rutaceae species (*Citrus* spp., *Z. ailanthoides*, and *Phellodendron amurense*). *O. japonica* activated Type 4 responses, but at a rate below that of host Rutaceae at the sensillum-recording level. In contrast, Type 0 and Type 3 responses were higher for *O. japonica* than for host Rutaceae, and the Type 0 difference remained significant in female-ID clustered GEE. These results indicate that *O. japonica*—despite sharing family membership with the host plants—provides mixed peripheral input: partial host-associated Type 4 activity together with robust non-host-associated Type 0 activity. Behaviorally, complete oviposition remained low while Bending was frequent. Statistical comparisons used two-sided Mann-Whitney U tests with BH-FDR correction across the five spike types (* **q < 0.001**, q < 0.01, * q < 0.05).

Supplementary Files

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