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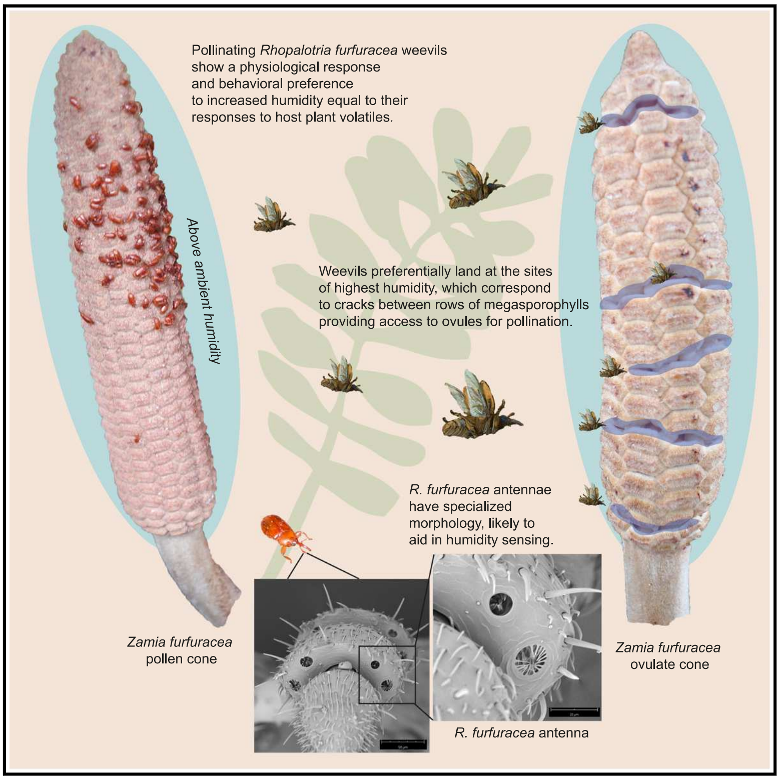
Cone humidity is a strong attractant in an obligate cycad pollination system

Graphical abstract



Pollinating *Rhopalotria furfuracea* weevils show a physiological response and behavioral preference to increased humidity equal to their responses to host plant volatiles.

Weevils preferentially land at the sites of highest humidity, which correspond to cracks between rows of megasporophylls providing access to ovules for pollination.

R. furfuracea antennae have specialized morphology, likely to aid in humidity sensing.

Authors

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In brief

Salzman et al. show that humidity acts as a strong pollination attractant. They find that plant reproductive tissues produce humidity, and morphological traits establish a strong gradient at the point of entry for pollination. Pollinators are highly attracted to higher humidity and display striking sensory morphology associated with its perception.

Highlights

- Pollinators show a strong physiological humidity response and behavioral preference
- Pollinators can discern fine differences in relative humidity
- Plant reproductive morphology concentrates humidity in a landing-strip-type fashion
- Pollinator antennae show striking morphology associated with humidity sensing

Salzman et al., 2023, Current Biology *33*, 1–11
May 8, 2023 © 2023 Elsevier Inc.
https://doi.org/10.1016/j.cub.2023.03.021

Please cite this article in press as: Salzman et al., Cone humidity is a strong attractant in an obligate cycad pollination system, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.03.021

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Article

Cone humidity is a strong attractant in an obligate cycad pollination system

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https://doi.org/10.1016/j.cub.2023.03.021

SUMMARY

Studies of pollination biology often focus on visual and olfactory aspects of attraction, with few studies addressing behavioral responses and morphological adaptation to primary metabolic attributes. As part of an in-depth study of obligate nursery pollination of cycads, we find that *Rhopalotria furfuracea* weevils show a strong physiological response and behavioral orientation to the cone humidity of the host plant *Zamia furfuracea* in an equally sensitive manner to their responses to *Z. furfuracea*-produced cone volatiles. Our results demonstrate that weevils can perceive fine-scale differences in relative humidity (RH) and that individuals exhibit a strong behavioral preference for higher RH in binary choice assays. Host plant *Z. furfuracea* produces a localized cloud of higher than ambient humidity around both pollen and ovulate cones, and *R. furfuracea* weevils preferentially land at the zone of maximum humidity on ovulate cones, i.e., the cracks between rows of megasporophylls that provide access to the ovules. Moreover, *R. furfuracea* weevils exhibit striking antennal morphological traits associated with RH perception, suggesting the importance of humidity sensing in the evolution of this insect lineage. Results from this study suggest that humidity functions in a signal-like fashion in this highly specialized pollination system and help to characterize a key pollination-mediating trait in an ancient plant lineage.

INTRODUCTION

A major focus of the study of plant-insect interactions is identifying sensory information that insects use for host plant localization. An important axis of biological and ecological diversity is represented by a subset of plant-insect interactions, i.e., pollination. The field of pollination biology has advanced through the study of the effects of visual and olfactory aspects of floral display on pollinator behavior, yet pollinators are likely to sense and utilize additional information that may be less apparent to human senses.[1–3] At its most basic, the breadth of floral trait information available to pollinators can be thought of as unavoidable cues that are beneficial only to the receiver, or signals that benefit both sender and receiver and incur a cost to the sender.[4–7] Plant cues resulting from primary metabolism (e.g., transpiration, respiration, and thermogenesis), such as humidity, CO_2, and heat, have been shown to influence pollinator behavior[8–13] but have received less attention than the more conspicuous products of secondary or "specialized" metabolism, such as volatile organic compounds (VOCs), floral pigments, infrared and ultraviolent reflectance, as well as structural features that constitute floral signals.[14–17] Although the potential for products of primary metabolism to act as signals is less clear,[18–20] it is often the interplay between different sensory channels of information that influences ecological relationships, both broadly in animal behavior[21,22] and more narrowly in plant-pollinator interactions.[23] Here, we focus on humidity, which has been suggested to serve as a cue to pollinators[8,13,24] but has recently been shown, through manipulative experiments, to function in a signal-like fashion in at least one angiosperm pollination system.[10]

Multimodal communication may especially influence evolutionary trajectories in obligate mutualisms involving nursery pollination, where pollinators spend some or all their development in close association with their host plant's reproductive tissues. Because these pollinators must re-establish the mutualism with each generation, partner-encounter signals are expected to be under strong selection[25] and are hypothesized to exist as "private channels" of chemically unique signals that would not attract a broad spectrum of visitors.[26,27] Indeed, highly specialized VOCs have been identified in nursery pollination mutualisms between figs, yuccas, and their respective pollinators[28,29] and possibly between cycads and their pollinators.[30,31] A subset of these obligate nursery pollination systems consists of deceptive, dioecious plant species that compel the pollinator to visit the ovulate plant by mistake, often through chemical mimicry of the conspecific pollen plant.[32,33] When plant sexes do not flower synchronously, the selection for a chemical private channel

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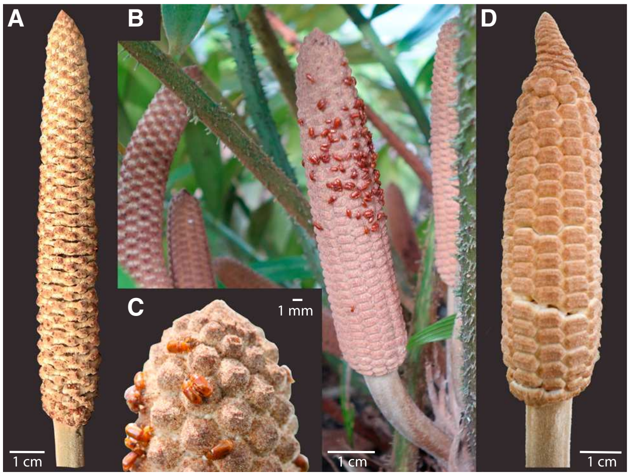

Figure 1. *Rhopalotria furfuracea* are obligate pollination mutualists of *Zamia furfuracea* and spend their entire life cycle within the reproductive tissues of the host plant

(A) Pollen cone when fully open and dehiscing pollen. Weevils are found in large numbers within cones at this stage.

(B) Pre-dehiscent pollen cone. Weevils can be found aggregating on the surface.

(C) Close up of weevils mating and laying eggs on pre-dehiscent pollen cone.

(D) Receptive ovulate cone opens with just a few cracks between rows of megasporophylls. Weevils must enter through these cracks to pollinate.

begins to break down, suggesting that partner-encounter may occur through other sensory channels.[32] Investigating the potential for humidity and other products of primary metabolism to play a role in plant-insect interactions and promote partner-encounter, rather than simply reflecting plant physiological processes (e.g., stomatal conductance), can further our understanding of the ecological and evolutionary trajectories of highly specialized mutualisms.

Due to the importance of ambient humidity to insect physiology,[34,35] the production of humidity by plants has the potential to act as a host plant localization trait. All insects have specialized hygrosensing sensilla that house neurons responding to changing environmental humidity[36] and can be exceptionally sensitive to changes in relative humidity (RH).[10,34,37–39] In a few cases, flower-visiting insects have also been shown to utilize their specific RH sensitivity to locate host plants and their associated energetic resources.[8,10,24] Floral humidity can be an outcome of either nectar evaporation[8,9] or transpiration.[10,40] Pollinators may utilize floral humidity as a profitability cue,[19] given that the evaporation of larger nectar volumes can increase RH in the floral headspace.[8] Conversely, floral morphology, such as deep-throated corolla tubes[3] or the density of floral stomata,[10] can also influence the humidity of the floral organ, but without a direct link to nectar as a reward. Floral humidity is widespread and variable in angiosperms, suggesting that it is likely to provide sensory information to pollinators[3,41] as well as to smaller arthropods that reside within flowers.[42] Nevertheless, there have been few behavioral tests of the potential for pollinators to utilize RH in flower choice[8,10,13,24]; thus, RH remains a frontier in plant-pollinator communication, with ongoing debates as to the role of RH in different pollination systems. One of the reasons for the lack of consensus on the role of humidity in pollination arises from the paucity of comparative data on this subject, especially from non-angiosperm pollination mutualisms and those whose lineages are more ancient in evolutionary history.

Cycads are an excellent model system in which to study the functional role(s) of humidity and other sensory information in pollinator behavior. Cycads are an ancient lineage of dioecious gymnosperms, producing separate plants bearing either pollen or ovulate cones. In most cases, insect pollinators live out their entire life cycle within the pollen cone in an obligate nursery pollination mutualism[43–47] that likely represents one of the earliest insect pollination mechanisms.[33] It is hypothesized that cycad pollinators are induced to leave the pollen cone, which represents their sole food source, and to visit the ovulate cone, on which they do not feed, by deceit.[46] Cycad cone VOCs appear to function as attractants and/or as repellents, pushing the pollinators out of pollen cones and attracting them to ovulate cones, depending upon dosage.[13,33,48] Cone volatile emissions change over the course of the day in association with a daily thermogenic pattern[49] that coincides with the insect's movement out of the pollen cone.[33,50–52] The metabolic burst that induces cone thermogenesis also impacts humidity, CO_2, and volatile emission.[13,53] While lower levels of VOCs are attractive to cycad pollinators, an increase in volatile emission has been shown to expel pollinators from the pollen cone in a mechanism termed "push-pull pollination."[33,48] Ovulate cones in turn produce low levels of volatiles and are not repellent to pollinators. However, the exact mechanism of pollinator attraction to ovulate cones remains largely unknown and may vary between cycad species. This attraction involves the emission of low-level volatiles[33] and could also include the products of primary metabolic processes such as humidity or CO_2. In the cycad *Zamia furfuracea*, pollinating weevils (*Rhopalotria furfuracea*) must be enticed to enter the unrewarding ovulate cones through cracks between rows of megasporophylls (Figure 1D). Given that *R. furfuracea* weevils are also found aggregating in large numbers on pre-dehiscent pollen cones (Figure 1B) when volatile production is quite low, we hypothesized that humidity may be playing a role in attraction to the ovulate cone crack as well as to the pre-dehiscent pollen cone. Here, we investigate pollinator behavioral responses to humidity in a strict brood-site mutualism between *R. furfuracea* weevils and *Z. furfuracea* cycads (Figure 1; STAR Methods), to determine (1) whether RH is used by pollinators in host plant localization, (2) if/how RH interacts with chemical signaling, and (3) how RH contributes to the successful pollination of ovulate cones.

RESULTS

Cone humidity

We set out to quantify the spatial and temporal patterns of humidity associated with cones of both sexes of *Z. furfuracea*. Both pollen cones and ovulate cones were found to produce above-ambient humidity gradients surrounding their surfaces; thus, weevils would likely experience a humidity gradient (ΔRH) as they approach different parts of the cones (Figure 2). Pre-dehiscent pollen cones measured in the lab were recorded to have the highest levels of humidity closer to the basal half of

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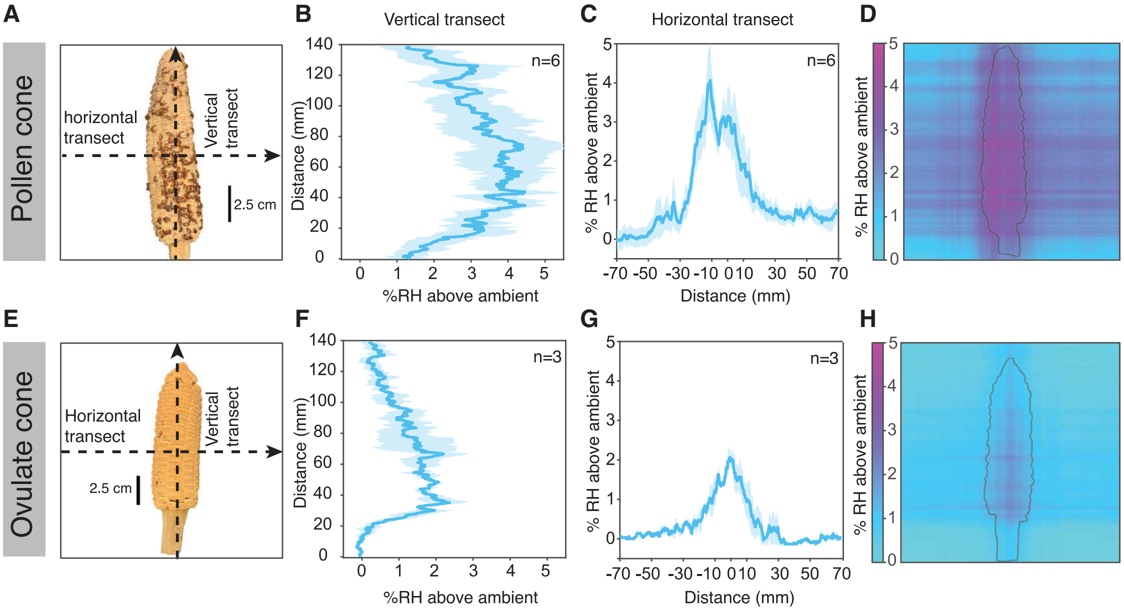

Figure 2. Cones of *Zamia furfuracea* produce a cloud of humidity higher than ambient around the surface of the cone

(A–H) Pre-dehiscent pollen cone (A–D) and receptive ovulate cone (E–H).
(A) Pollen cone with weevils showing measured transects.
(B) Vertical humidity gradient of pollen cone above ambient.
(C) Horizontal humidity gradient of pollen cone above ambient.
(D) Extrapolated humidity map of pollen cone from the horizontal and vertical transects.
(E) Ovulate cone showing measured transects.
(F) Vertical humidity gradient of ovulate cone above ambient.
(G) Horizontal humidity gradient of ovulate cone above ambient.
(H) Extrapolated humidity map of ovulate cone from the horizontal and vertical transects.
Note delta %RH is on the horizontal axis in (B) and (F) while it is on the vertical axis in (C) and (G). Standard error of the mean is shown in the shaded regions. Daily humidity patterns are found in Figure S1 and experimental setup is found in Figure S6.

the cone (Figures 2B and 2C; mean 4.4% above ambient 51.6 mm from peduncle ± 26.79 mm SD). The temperature at the highest RH location for each cone was only 0.11°C ± 0.17°C above ambient ($p = 0.15$, one-sample t test against 0; data not shown). The ovulate cones measured in the lab also were shown to have the highest humidity levels (mean 2.3% above ambient) closer to the basal half of the cone (Figures 2F and 2G; 51.69 mm from peduncle ± 17.94 mm SD) near the crack between rows of megasporophylls. The temperature of the ovulate cones 0.23°C ± 0.05°C (median = 0.20) was marginally but not significantly higher than ambient ($p = 0.25$, one-sample Wilcoxon test against 0). Figure 2 presents humidity cloud maps combining the horizontal and vertical humidity gradients for both pollen cones (Figure 2D) and ovulate cones (Figure 2H). Local humidity is much higher at the crack between megasporophylls in the ovulate cone than on the surface of the cone (see also Figure 6A; $p < 0.00001$, pairwise t test). The ambient humidity was recorded as 57.3%, while the mean surface humidity of the cones was 62.5% (mean ΔRH of 5.22%) and mean humidity at the crack was 81.7% (mean ΔRH of 24.4%). Both dehiscing pollen and receptive ovulate cones in the field produce an above-ambient humidity gradient throughout the course of the day (Figure S1). Dehiscing pollen cones produced more water vapor over the course of the day than receptive ovulate cones (pollen mean delta absolute humidity [AH] = 1.95 g/m^3, ovulate mean ΔAH = 0.79 g/m^3, $p = 2.2e{-}16$, unpaired t test) and both sexes showed a peak in water vapor production coinciding with the peak in endogenous thermogenic temperature at ~19:00 (Figure S1). Leaf tissue measured in the field showed a mean ΔRH of 0.55%. Post-receptive ovulate cones with developing seeds had a mean ΔRH of 2.89% on the surface and 0.88% at the cracks that expose the fleshy sarcotesta of the developing seeds (Figure S2).

Weevil EAG responses

We used electrophysiological methods to determine whether the antennae of *R. furfuracea* weevils can detect differences in cycad cone humidity. In physiological assays of weevil perception of humidity changes, the electroantennographic (EAG) response to high RH (wet; mean, −0.832 ± 0.997 mV SD) was significantly different from the response to ambient humidity (blank; mean, −0.156 ± 0.288 mV SD) ($p = 0.026$) yet was not significantly different than the response to the key cone volatile, 1,3-octadiene (mean, −1.18 ± 1.02 mV SD) ($p = 0.786$) (Figure 3). The antennal response to 1,3-octadiene also differed significantly from the response to the blank ($p = 0.00076$). Fourteen out of 15 weevils showed an EAG response to 1,3-octadiene in this test, whereas 11 responded to the wet stimulus and five to the blank. In control tests, weevils showed a significant antennal response to 1,3-octadiene over the carrier solvent ($p = 0.035$;

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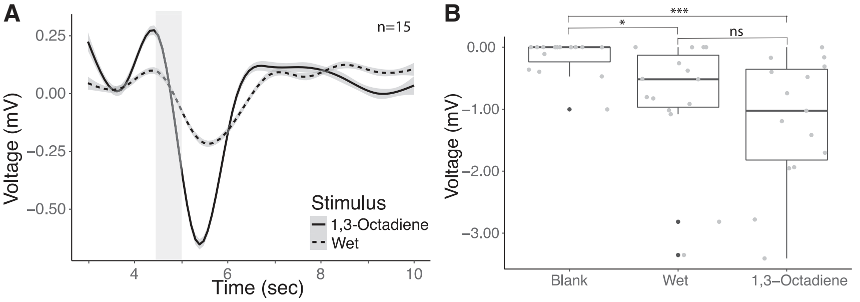

Figure 3. *Rhopalotria furfuracea* weevils show a physiological response to above-ambient humidity using electroantennographic detection

(A) Smoothed conditional mean of the antennal voltage response to the plant VOC 1,3-octadiene and to a high RH wet stimulus. Shaded box indicates the stimulus start and duration.

(B) Antennae show a significant response to the high RH wet stimulus as compared with the ambient RH blank stimulus (p = 0.026). This response is not significantly different from the antennal response to the plant VOC 1,3-octadiene (p = 0.786). Blank compared with 1,3-octadiene, p = 0.00076. *p < 0.05, ***p < 0.001, ns = not significant. Raw data are shown in gray overlaying the black box and whiskers plot. Data for corresponding controls are found in Figures S3 and S4. Experimental setup is found in Figure S7.

Figure S3). In this control, all eight weevils responded to the 1,3-octadiene stimulus (mean delta voltage, −1.07 ± 0.609 mV SD), while only two of the eight responded to the hexane stimulus (mean, −0.376 ± 0.699 V SD). Similarly, high RH did not significantly alter the resistance of the conducting gel in EAG runs with the antennae removed (conducting gel only) (p = 0.18; Figure S4). In this control trial, all responses recorded were very low delta voltage. Three changes in voltage were recorded out of eight blank tests (mean delta voltage, −0.0514 ± 0.0729 mV). Eight changes in voltage were recorded out of eight wet tests (mean delta voltage, −0.0931 ± 0.0534 mV). There was no statistically significant difference between blank and wet (p = 0.18). These controls indicate that EAG responses to both 1,3-octadiene and high RH are not methodological artifacts.

Y-tube behavior assay

We tested the behavioral responses of *R. furfuracea* weevils to humidity differences using a two-way olfactometer (y-tube). Of the 82 weevils tested for a response to VOC, 35 made a choice, with a 77% positive response to the previously identified attractive amount[33] of cone volatile, 1,3-octadiene (27/35, mean ambient humidity = 26.9%). Of the 82 weevils tested for a response to humidity alone, 27 made a choice, with an 89% positive response to higher humidity (24/27). Humidity readings in this experiment averaged 77.4% in the humid arm, 30.9% in the dry arm, and 56.7% at the base, which closely matched the 57% RH of the incubator in which weevils were housed prior to experimentation. For humidity and chemistry together, 22 of the 87 weevils tested made a choice, with 73% choosing humidity plus VOC (16/22, mean humidity readings: 78.9% in the humid arm, 27.9% in the dry arm, and 56.3% at the base). There was no statistically significant difference between responses to the three experimental conditions (mean p value based on randomly subsampling the data 1,000 times = 0.328, median p = 0.255, proportion of subsampled p < 0.05 and p = 0.333). The response to VOC + humidity was not greater than the response to either humidity alone or VOC alone (proportion of 1,000 random subsampling runs where the response to both signals was greater than to just one signal is 0.143 for humidity and 0.259 for VOC). Taken together, these results suggest that the behavioral effect of humidity is redundant but not additive with that of the volatile signal 1,3-octadiene (Figure 4).

Weevil responses to control tests of side preference, response to carrier solvent, and time of day were all trivial. There was no preference for either olfactometer arm (side) or carrier solvent. Of the 147 weevils that made a choice across all experiments, 77 (52.3%) chose the left side and 70 (47.6%) chose the right side. Similarly, exactly 50% (n = 19 out of 38) of weevils that made a choice in the dichloromethane (DCM) test trial chose the DCM solvent side as the first choice (90 weevils in total tested). The proportion of weevils that made a choice was low across all experiments, ranging from 25.3% (22/85) in the VOC + humidity test to 42.7% (35/82) in the VOC only test, and there was no effect of time of day on weevil behavior (p = 0.975, Kruskal-Wallis test).

Weevil response to quantitative differences in RH

In laboratory choice assays, *R. furfuracea* were capable of perceiving fine-scale differences in humidity and showed a strong preference for higher humidity (p < 0.0001; Figure 5). The humidity at the base of the Y maze, where weevils were introduced, averaged 73.4%. Weevils then had the choice between a high humidity arm (mean 81.9%) or a lower humidity arm (mean 67.0%). Of the 90 total weevils tested, 25 (28%) made a choice, with 100% of those choosing the higher humidity arm (8.5% higher RH on average than the base).

Weevil approach to ovulate cones

In field assays, wild *R. furfuracea* weevils preferentially landed at the crack between rows of megasporophylls where the highest humidity is recorded (Figure 6). Receptive ovulate cones in the field had between two and three horizontal cracks, usually one close to the base and one near the apex of the cone. Eighty-five total fluorescent marks of pollinators that landed on ovulate cones were observed. Fluorescent marks were equally visible on the surface and at the cracks (Figures 6 and S5). Most marks were located on the cracks or the contiguous megasporophylls. The fluorescent mark that was farthest from a crack was 22.2 mm away from it. A one-sample t test (t = 0.613, df = 84, p = 0.271) and 95% confidence intervals (−0.912, 1.74) indicated that a mean of zero for the distribution of distances from the crack cannot be excluded and that mean is not significantly greater than zero.

Antennal sensory morphology

Scanning electron micrography (SEM) of *R. furfuracea* antennae was used to characterize the sensory sensilla, whose morphology is tightly linked with function in insects.[54] The antennal flagella of *R. furfuracea* are composed of a scape, pedicel, and nine flagellomeres, with the majority of sensilla distributed

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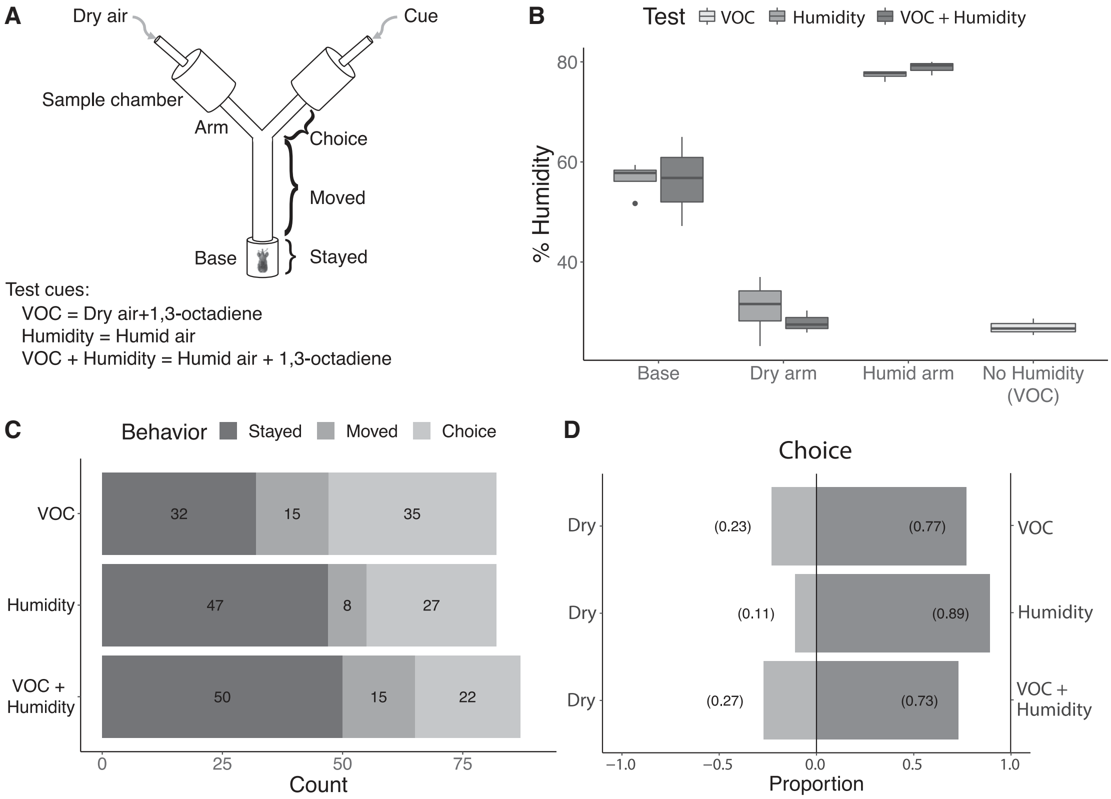

Figure 4. *Rhopalotria furfuracea* weevils use humidity as a redundant signal that is neither additive nor synergistic with VOC

(A) Experimental setup of two-choice olfactometer showing location of humidity measurements, location of behavior scores, and the three test stimuli.

(B) Humidity measurements for all tests. Note that “no humidity” corresponds to the entire olfactometer in the VOC test.

(C) Weevil behavior across experimental conditions. Counts of weevils in each location are noted.

(D) There is no significant difference between weevils’ first choice responses to the three different test conditions (mean p for 1,000 runs = 0.328, median p = 0.255, proportion of $p < 0.05$ and p = 0.333).

Proportions are given in parentheses and sample size noted in (C).

across flagellomeres 7–9 (Figures 7A and 7B). Flagellomeres 7 and 8 show the presence of six sensory pockets at the distal end (Figures 7C and 7D). These sensory pockets are filled with peg-like, blunt-ended, smooth sensilla that are morphologically consistent with hygrosensing and can be divided into two categories based on macro-morphology, mostly with smooth edges (SEs) or heavily lobed (Sarlacc) (Figure 7D). The inner and outer edges of the distal ends of flagellomeres 7 and 8 have one Sarlacc (S) sensory pocket between two SE sensory pockets for a total of six sensory pockets. The S pocket has an opening diameter of ∼10 μm, whereas the SE pocket has a diameter of ∼5 μm. It is unclear how many individual sensory pegs reside within each of these pockets. The other three types of sensilla are found on the surface of the flagellomeres and are morphologically consistent with chemosensory function, particularly with odor perception. The Trichodea type I sensilla (T1) are ∼45 μM long, sharp barb-shaped with grooves on the surface and a socket at the base. They either point laterally at approximately 45° angles on flagellomeres 7–9 or lie parallel to the surface of the antennae, often extending beyond the distal end of the flagellomere. In this latter orientation, the T1 sensilla are distributed throughout the antennae, including on the scape and pedicel. The trichodea type II sensilla (T2) also are sharply barbed with grooves and a socket at the base, but are smaller, at ∼20 μM long. T2 sensilla lie along the surface of the antennae and are found on flagellomeres 5–9, increasing in density toward the distal end of the antennae. The basiconic sensilla type (B) is much smaller (∼5 μM long) with a relatively blunt end and lacking a socket at the base. These are sparse and are found on flagellomeres 7–9 near the SE and S sensory pockets. Antennae are held at an angle toward the ventral surface of the insect, directing the three most densely sensilla-covered flagellomeres and the sensory pockets toward any substrate the insect may be crawling on (Figure 7; Video S1 at https://doi.org/10.7910/DVN/XX6JVB).

DISCUSSION

Cone humidity benefits both partners

Pollinators have access to a wealth of plant-produced sensory information, yet not all information is useful for both the plant and the pollinator. Sensory information often is categorized as either a cue or a signal, based primarily on outcomes for both the sender and the receiver. A cue represents information conveyed inadvertently between partners that primarily benefits the receiver (e.g., via eavesdropping), whereas a signal benefits both the sender and the receiver and is hypothesized to evolve

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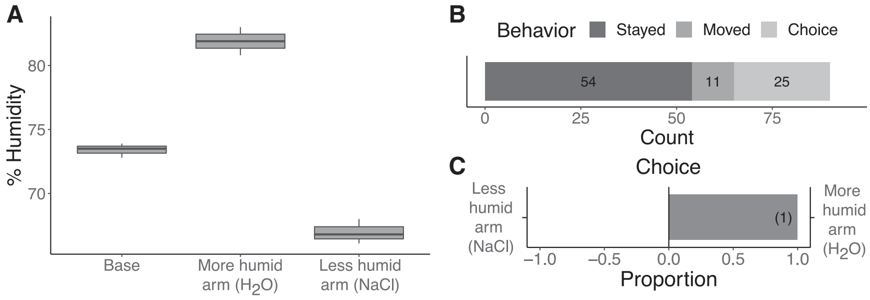

Figure 5. *Rhopalotria furfuracea* can perceive quantitative differences in relative humidity (RH)

(A) Humidity measurements of the three olfactometer positions.

(B and C) (B) Weevil behavior shows that 60% (n = 54) of weevils stayed in the base and 28% (n = 25) made a choice, and (C) all (100%) of weevils that made a choice chose the more humid arm (8.5% higher RH than the base). Proportion in parentheses.

from cues.[21,55–57] Differing definitions of "signal" can range from very basic (simply benefiting both partners at cost to the sender)[4–7] to stringent, with more conservative definitions requiring evidence that signals have evolved for the purpose of communication.[55,58–60] In this study, we find that *R. furfuracea* weevils respond positively to high cone humidity, likely to aid in localization of both the pollen and ovulate cones of host species *Z. furfuracea* to the benefit of both partners, with humidity acting as a signal *sensu lato*. Determining whether cone humidity has evolved explicitly for the purpose of communication with pollinators would require a phylogenetically rigorous study of RH gradients and pollinator responses, at least across the genus *Zamia* if not the entire Cycadales, which is far beyond the scope of the present study.

Nevertheless, our findings provide a clearer understanding of the multiple sensory channels involved in this highly specialized mutualism, while adding to the growing body of literature demonstrating the effect of humidity as a sensory stimulus in plant-pollinator interactions.[8,10,13,24] As obligate mutualists depending on their host plants for shelter and food, *R. furfuracea* weevils are exposed to the full suite of metabolic signals and cues and may utilize all of them synchronously or asynchronously throughout their life cycle when making decisions regarding their interactions with *Z. furfuracea*. To produce viable seeds, dioecious *Z. furfuracea* plants must induce their insect partners to depart the pollen cone at the right time and simultaneously encourage the insects to visit and thereby pollinate a neighboring conspecific ovulate cone. Our data show that *R. furfuracea* weevils show a strong physiological response to higher humidity (Figure 3) and that humidity alone can function as an attractant at close-range, regardless of plant VOC (Figure 4D). Together with plant measurements and field experiments, these findings suggest that humidity is in part responsible for mediating weevil behavior that enhances partner encounter and plant reproductive success. The reciprocal benefits to both the sender and the receiver within a symbiotic relationship indicate that humidity in *Zamia* cones functions in a signal-like fashion in coordination with other plant-produced signals in this obligate nursery pollination mutualism.

R. furfuracea weevils may use humidity to locate ideal host plants in a manner that is neither additive nor synergistic with host volatiles (Figure 4), suggesting that humidity and VOC serve a similar function of partner encounter in this mutualism, at least at close range orientation. Cone humidity may aid in attraction and localization to ideal mating and egg laying sites on pre-dehiscent and pre-thermogenic pollen cones where volatile production is quite low. Humidity measurements show that pre-dehiscent pollen cones have a high dissipation of humidity, averaging ~4% ΔRH around the entire cone (Figure 2) at the time that weevils aggregate on the outer surface (Figures 1B and 1C). Female weevils drum the surface of the pre-dehiscent cone with their antennae searching for suitable egg laying sites until they come to the softer, and likely more humid, tissue[61] at the junction where two microsporophylls meet (Video S1). Here, they use their rostrum to burrow a hole (Figure 1C) in which they lay a single egg.[45] Local differences in humidity may also aid in attraction and localization to ideal microhabitats in the interior of pollen cones. Pollen cones remain tightly closed, with no separation between microsporophylls until the cone reaches reproductive maturity. At the time of pollen dehiscence, cones elongate and all microsporophylls separate from one another, creating open spaces that result in an overall morphology similar to what mechanical engineers recognize as an ideal radiator (Figure 1A).[46,62] This complete opening of the pollen cone allows for rapid dissipation of heat along with plant volatiles, humidity, and CO_2 and provides weevils access to the softer parenchyma tissue that serves as their sole food source.[45]

The humidity of ovulate cones also appears to be functionally redundant with VOC in mediating pollen transfer, at least at close ranges. As opposed to the full opening of pollen cones (Figure 1A), *Z. furfuracea* ovulate cones remain tightly closed, with only a few horizontal cracks forming between discrete rows of megasporophylls (Figure 1D). In ovulate cones measured in the lab, the high humidity cloud was localized around the basal half of the cone where cracks were open between megasporophylls. The RH of ovulate cones was recorded at an average ΔRH of 2% at the measurement arm located 5–10 mm above the surface of the cone (Figure 2), with humidity at the cracks measuring about 20% higher than the surrounding cone surface (Figure 6A). It is noteworthy that pollinating weevils must enter through this crack to pollinate the plant, and that *R. furfuracea* can discriminate fine-scale differences in humidity (±8% RH), choosing the more humid environment 100% of the time in our bioassays (Figure 5C). Thus, the humidity gradient at the crack is analogous to a floral landing strip,[63] with weevils preferentially landing directly on this crack (Figure 6), as the humidity gradient guides them to the point of entry. It is also possible that VOC or CO_2 gradients or visual contrast may additionally influence pollinator attraction to the landing site, as the cracks between megasporophylls expose the white inner surface of the ovulate cone (Figure 1D). However, the data presented here are sufficient to indicate that humidity alone is effective at close range for attracting weevils. Although not explicitly tested, the data suggest that humidity may function as a redundant backup for VOC (as

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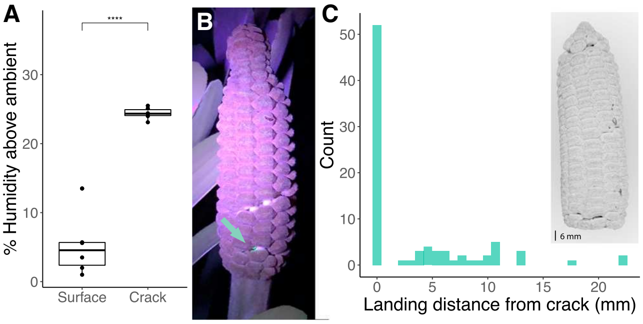

Figure 6. Fluorescent dye-based tracking confirms that *R. furfuracea* preferentially land at the highest humidity location of receptive ovulate cones

(A) Humidity measurements of the surface and crack of six ovulate cones shows an ~20% higher humidity at the crack ($p < 0.00001$).

(B) Ovulate cone photographed under UV light showing a mark of green-fluorescent dye left by a pollinator on a crack.

(C) Frequency distribution of the distance of fluorescent marks to cone cracks ($n = 85$) shows that most marks are located directly on the cracks of ovulate cones. One cone scale is about 6-mm tall.

Control measurements of other plant tissues are shown in Figure S2 and additional fluorescent marks are shown in Figure S5.

suggested by the efficacy backup hypothesis[21,64,65]) when the local environment presents high chemical variation or saturation of volatiles, as is likely where these cones are found, deep within the dense canopy of cycad leaves. By way of comparison with other pollination systems, similar research on a brood-site-deceptive plant, the "dead horse arum" (*Helicodiceros muscivorus*), showed that while carrion-like volatile sulfides were sufficient to attract blowfly pollinators, the flies were more likely to enter the floral chamber (and thus effect pollination) when the floral appendix leading to that chamber was heated.[66] Additional experimentation would be required to tease apart the interplay of all sensory information and to determine the relative contribution at differing spatial scales. Combined, these data indicate that weevil attraction to low-level volatile production[33] plus high humidity is functionally linked with specific aspects of cone morphology and life history, creating an effective suite of traits mediating weevil attraction to cycad ovulate cones and successful pollination.

Evolutionary implications

The volatile chemical signal has likely evolved under selective pressure in the *Zamia* lineage as a means of maintaining pollinator specificity.[67] However, any trait associated with primary metabolism, such as CO$_2$ or humidity, is less likely to be under pollinator-mediated selective pressure, given the general importance (and great antiquity) of these products in basic plant physiology and homeostasis. Thus, the morphological features associated with primary metabolite signaling and sensing, such as cone opening and insect sensilla, may be the targets of selection as signal modifiers rather than the metabolites themselves.

From the sender viewpoint, the morphology of the *Z. furfuracea* ovulate cone allows for the generation of a steep humidity gradient that attracts weevils to the exact location required for cone entry and effective pollination. The highest humidity on the ovulate cone is recorded at the crack between megasporophylls, exploiting the weevils' preference for humidity and rendering a by-product of primary metabolism into a strong localization trait. Ovulate cone developmental morphology varies across the Cycadales, and it is possible that evolutionary changes in cone morphology are linked to harnessing humidity as a localization trait. *Zamia* and *Dioon* are the only cycad genera with tightly closed ovulate cones that open with just a few (or one in *Dioon*) horizontal cracks, and these genera are both pollinated in part by *Rhopalotria* weevils and their sister lineages. Ovulate cones in the cycad genera *Encephalartos*, *Stangeria*, *Ceratozamia*, and *Macrozamia*, with some exceptions, open with small spaces between every megasporophyll and thus are not likely to produce as steep a humidity gradient as is found in *Zamia* cones. Indeed, *Cycadothrips chadwicki* (Thysanoptera), the pollinators of the Australian cycad species, *Macrozamia lucida* and *M. macleayi*, prefer dry conditions and avoid humidity.[13] In this case, thrips avoid higher humidity in a weaker but redundant fashion to their avoidance of higher cone temperature, increased volatile emission, and external light.[13]

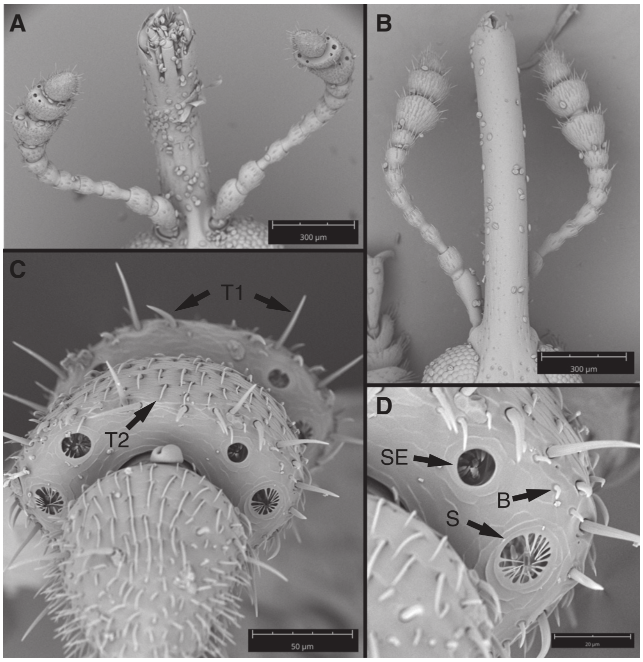

Figure 7. Scanning electron micrographs of *R. furfuracea* antennae show likely humidity sensing organs

(A) Ventral view of antennae at 175× magnification.

(B) Dorsal view of antennae at 185× magnification. Note in (A) and (B) that antennae angle ventrally.

(C) Flagellomeres 1–3 at 1,300× magnification.

(D) Antennal sensory pockets at flagellomere 2 at 3,700× magnification. T1, trichoid type 1 sensilla; T2, trichoid type 2 sensilla; B, basiconic type sensilla; SE, smooth edged sensory pocket; S, Sarlacc sensory pocket. Note the presence of pollen grains across the rostrum and antennae.

From the receiver viewpoint, *R. furfuracea* weevils appear to have specialized antennal morphology dedicated to the perception of humidity and temperature as well as chemical stimuli. The distal ends of the 7th and 8th antennal flagellomeres of *R. furfuracea* contain deep pockets full of sensilla that are likely hygrosensing organs (SE and S; Figure 7D). Although we are

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unable to visualize the fine morphology of these sensilla, they appear similar to coeloconic or basiconic sensilla in that they are smooth-sided, peg-like sensilla with rounded tops.[68] Across insects, similar aporous peg-in-pit sensilla types are generally innervated with three sensory neurons responding to changing humidity and temperature.[39,69–71] The unusual morphology seen here, consisting of deep pockets filled with many aporous peg-like sensilla, has only been found in fruit flies,[72] spittle bugs,[68] and—to a lesser degree—in dermestid beetles,[73] all of which live in high-humidity environments. It is possible that this morphology increases the sensitivity of the hygrosensing organ at a local scale and/or protects the sensilla from very wet environments. Such sensory fields or pockets have been suggested as a diagnostic character for the Oxycorini tribe of weevils that includes the genus *Rhopalotria*[74] and appear to coincide with the evolution of cycad feeding. Some closely related non-cycad feeding species within Oxycorini have weak sensory fields comprising short, rounded basiconic sensilla surrounded by overlapping long barbed golden sensilla (*Hydnorobius hydnorae*) and others have small clusters of golden sensilla (*Oxycraspedus cribricollis*).[74] However, with the shift to cycad feeding in subtribe Allocorynina, the antennal sensory fields show an increased density of rounded basiconic sensilla, and in some cases the antennal surface has invaginated into pockets, as seen across the genus *Rhopalotria*.[74,75] These pockets further show high levels of morphological diversity across the *Rhopalotria* genus.[75] Therefore, *R. furfuracea* humidity preference and sensory morphology may be considered an exaptation[76] coopted from a preexisting innate humidity preference and currently experiencing selective pressure related to a cycadivorous life history. Although it is not conclusive that these deep pockets of aporous peg-like sensilla are humidity- and temperature-responsive organs, they provide the best morphological matches in comparison with well-established case studies.[39,68–71] Notwithstanding, the sensory morphology seen here is striking and it is reasonable to expect the evolution and diversification of traits associated with either volatility or humidity and temperature sensing organs in this lineage, given its close ecological ties with thermogenic host plants such as cycads.

The current study of weevil behavioral responses to a primary plant metabolite (humidity) coupled with the previously published description of weevil attraction to secondary plant metabolites (VOC)[33] demonstrates how a suite of plant traits may function in tandem. Weevil behavioral response and cycad cone signal production coordinate across these two sensory channels to mediate reproductive success for both parties. Weevil preference for a higher humidity environment coupled with their aversive response to higher VOC emissions[33] coincides with plant diel signaling. Dehiscing pollen and receptive ovulate cones produce humidity gradients throughout the course of the day, with a peak in water vapor production coinciding with peak thermogenesis at around 19:00 (Figure S1).[49] Work done in other thermogenic cycad cones has shown that an increase in respiration causes a subsequent increase in temperature, followed by an increase in VOC production.[53] Weevils are attracted to higher humidity but are repelled by higher VOC production and remain inside the dehiscing pollen cone during the thermogenic event when humidity is the highest (Figure S1), leaving the cone en masse 30-min to 1-h post peak temperature/humidity, when VOC emission has reached its highest point.[33] It is worth noting that the behavioral responses shown in this work relate to a preference for humidity decoupled from temperature, as the behavioral experimental design held temperature constant with differences only in RH. It would, however, be valuable to study the diel rhythm of all sensory stimuli (thermogenesis, VOC, CO_2, and humidity) and their corresponding weevil behavioral response to further tease apart the multimodal mediation of this mutualism. Our findings suggest that to understand the ecology and evolutionary trajectory of highly specialized mutualisms, researchers should consider the complex repertoire of signals and cues that could mediate interactions under varying conditions in space and time.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.03.021.

ACKNOWLEDGMENTS

We thank the staff of Montgomery Botanical Center for their dedication to cycad research, specifically Joanna Tucker-Lima for collecting and shipping weevils. We thank Jennifer Svitko for guidance in SEM, Neil Rosser for assistance in the field, and Rory Maher for making data collection possible. We thank Mike Tevelonis and Botanics Wholesale for the generous donation of plants for the *in situ* experiments, as well as Day Glo and Peter Kashian from Super Materials for contributing the fluorescent dyes. Funding was provided by a National Science Foundation Postdoctoral Research Fellowship in Biology to S.S. (1906333) and Cornell Sigma Xi to A.D.

AUTHOR CONTRIBUTIONS

S.S., C.D.S., and R.A.R. conceived of the project; S.S. did all behavior work and conducted data analyses; A.D. collected RH cloud data; W.K. performed EAG experiments; W.A.V.-M. and M.C. collected insect distribution data; and W.A.V.-M. analyzed these data. All authors edited and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research. One or more of the authors of this paper self-identifies as a member of the LGBTQIA+ community. One or more of the authors of this paper self-identifies as living with a disability. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

Received: January 20, 2023
Revised: March 6, 2023
Accepted: March 8, 2023
Published: April 3, 2023

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
1,3-ocatdiene	ChemSampCo	CAS# 1002-33-1
n-hexane	Supelco	N/A
Dichloromethane	Honeywell	Cat# 650463
Fluorescent dyes	DayGlo Color Corp	Eco-14 OrangeEco-21 MagentaEco-18 Signal Green
NaCl	Sigma Aldrich	Cat# 22,351-4
Deposited data		
Olfactometer responses, EAG responses, humidity readings, and Video S1 of weevil behavior	This study	https://doi.org/10.7910/DVN/XX6JVB
Experimental models: Organisms/strains		
Cycad weevil *Rhopalotria furfuracea*	Montgomery Botanical Center housed on *Zamia furfuracea* cones	N/A
Zamia furfuracea pollen and ovulate cones	Montgomery Botanical Center	N/A
Software and algorithms		
R 3.6	The R Project for Statistical Computing	https://cran.r-project.org/mirrors.html

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Shayla Salzman (sms728@cornell.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data have been deposited at the Harvard Dataverse and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Our work was conducted during the summer (July and August) of 2021 and 2022. *Rhopalotria furfuracea* weevils were collected in the field at Montgomery Botanical Center in Miami Florida, USA and shipped live along with *Zamia furfuracea* ovulate and pollen cones at multiple stages of development to the lab environment at Cornell University. Cones and weevils were held in a Precision low-temperature illuminated incubator (Thermo Fisher Scientific, Waltham MA USA) set to lighting and temperature conditions that mimic their Florida environment (RH 57%; photophase: 7:00-21:00 hrs at 29 °C; scotophase: 21:00-7:00 hrs at 26 °C). Prior studies show that excised cycad cones continue to function physiologically for upwards of 14 days.[49,53,77] All bioassays were performed within 5 days of cone excision.

METHOD DETAILS

Study system and collections

Zamia furfuracea is a dioecious gymnosperm that is native to the coastal regions of Veracruz, Mexico[78] and is pollinated by the weevil *Rhopalotria furfuracea*[50] (as *R. mollis*). The plants and their pollinators were introduced together in large numbers to the southeastern United States, specifically southern Florida, where they have naturalized and now form communities in horticultural plantings. Both

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pollen and ovulate cones of *Z. furfuracea* are rather small, dull-colored, cylindrical projections that emerge near to the ground (Figure 1). *Zamia furfuracea* pollen cones are composed of rows of tightly compressed cone scales (microsporophylls) that bear pollen sacs (microsporangia) on their abaxial surface. Pollen-releasing cones must open along seams between the microsporophylls, thereby exposing the interior of the cone and the pollen in a process called "dehiscence" (Figure 1A). Ovulate cones are composed of rows of tightly compressed cone scales bearing ovules (megasporophylls), and open along a few large cracks between rows of megasporophylls when the ovules are receptive (Figure 1D).

Rhopalotria furfuracea weevils complete their entire lifecycle within the parenchyma tissue of the pollen cone of *Z. furfuracea* during the time that the plant is reproductive.[45] While *R. furfuracea* weevils can be found in large numbers within a dehiscing pollen cone (Figure 1A), they also aggregate on the outside of the cone prior to dehiscence, where they can be found mating and laying eggs on the surface (Figures 1B and 1C; Video S1). Because cones are only present on *Z. furfuracea* for approximately two months during their reproductive phase, the weevils go into diapause as late-stage larvae during the ten months of the year that *Z. furfuracea* plants do not present a viable food source (i.e. pollen cones).[45] *Rhopalotria furfuracea* are attracted to low concentrations (10 μg) of the VOC 1,3-octadiene but are repelled by high concentrations (650 μg) and ovulate cones are likely visited by mistake.[33] *Rhopalotria furfuracea* do not aggregate on ovulate cones nor feed on their parenchyma tissue.[45] Large populations of plants and pollinators thrive as part of the extensive *ex situ* cycad conservation collection at Montgomery Botanical Center (MBC) in Coral Gables, Miami-Dade Co., Florida, USA.

For all studies, weevils were collected from MBC and housed on *Z. furfuracea* pollen cones. Weevil development lasts for 7-9 days with the larval stage entirely enclosed within the microsporophyll of the pollen cone, such that most microsporophylls are inhabited.[45] This precludes the ability to assay only freshly emerged adult insects for behavioral analysis. Weevils were allowed to freely feed on the cones until bioassay in order to minimize stress prior to experimentation. This may reduce behavioral attraction in the artificial environment of the olfactometer, making a significant response all the more noteworthy. Weevils were assessed in mix-set groupings of five individuals for each olfactometer run following the method established in the cycad pollination literature,[79,80] which best approximates the natural environment and behavioral landscape of these highly aggregative insects.

Humidity of pollen and ovulate cones

The humidity of ovulate and pollen cones was measured using a custom built automated movable hygrometer (Figure S6).[8,10] Humidity gradients were measured at various distances from the cone surface, rather than at a point source, to provide a more detailed picture of the spatial distribution of humidity that weevils would encounter when approaching a cone. Pollen cones (n=6) were tested at the pre-dehiscent stage (prior to the cone opening or thermogenic events) when previously reported to be attractive to weevils.[50] Ovulate cones (n=3) were tested at the receptive stage, indicated by large horizontal cracks between rows of megasporophylls and not during peak thermogenesis. Cones were held vertically on a metal stand using a binder clip (base) and a rubber band (tip) (Figure S6B). Two hygrosensors (Omega 314A) were used to measure relative humidity (RH) gradients, one to measure cone humidity and another to measure ambient humidity, with a probe placed 10–20 cm from the cone. Cone RH was measured along horizontal and vertical transects of ∼13.5 cm each with the probe held at 0.5cm from the cone surface. The probe was attached to a kdScientific (model 100) syringe pump dispenser which moved the probe at approx. 0.2 mm/sec with resolution set at 1 datapoint/sec across the transect. During our measurements, ambient humidity was 56.57 % ± 1.76 and temperature was 23.25ºC ± 0.11 (mean ± std. dev.). Humidity maps of pollen and ovulate cones were produced by generating a running average of 3 datapoints from the average delta RH values of both transects of pollen and ovulate cones. The running averages were downsampled 5 times such that each millimeter around the cone received one representative RH value. Values for horizontal and vertical transects were summed and the average was plotted using the heatmap function in MATLAB 2019.

In addition, the relative humidity of a total of six ovulate cones at both the cone surface and the crack opening between rows of megasporophylls was measured using a HMP42 humidity and temperature probe attached to a HIMI41 indicator (Vaisala, Helsinki Finland) inside the incubator where the cones were housed (Ambient Temperature: 29 ºC). Cones were measured during off-peak thermogenesis. Relative humidity of leaf surface and both the surface and crack of post-receptive ovulate cones were measured in the field (GSP-6 hygrometer, Elitech, San Jose, CA). The ovulate cone crack at this point is exposing the fleshy sarcotesta of the seed.

Finally, the humidity cue of fourteen dehiscing pollen cones and seven receptive ovulate cones was measured (GSP-6 hygrometer, Elitech, San Jose, CA) over a 24-hour period in the field to determine the daily variation. Probes were placed onto the cones and ambient measurements were taken from probes hanging in leaves nearby at similar heights. Absolute humidity was determined from these values as relative humidity depends on ambient temperature which changes throughout the day.

Weevil Electroantennographic response

Rhopalotria furfuracea weevils were tested for a physiological response to humidity as compared to the VOC stimulus 1,3-octadiene using electroantennography (EAG). The whole antennal EAG represents the sum of all action potentials elicited by a stimulus and propagated along the antennal nerve to the antennal lobe, visualized as the deflection (- mV) from the resting electrophysiological baseline of an antenna.[81,82] Headspace samples were prepared in 5 $^{3/4}$'' Pasteur Capillary Pipettes. The four pipette conditions were plant VOC: 1,3-octadiene, solvent control: Hexane, high relative humidity (RH): Wet, or ambient RH: Blank. The 1,3-octadiene sample was prepared by spotting 1μl of 100 ng/μl 1,3-octadiene (CAS# 1002-33-1, ChemSampCo Dallas TX USA) diluted in SupraSolv chromatography grade n-hexane (Supelco, Bellefonte, PA USA) onto a 0.5 cm x 3cm piece of filter paper (Whatman plc, Maldstone UK). The hexane solvent was allowed to dry (i.e. until the spot was no longer visible after 8-12 seconds) and the paper was inserted into a clean 5 $^{3/4}$'' Pasteur Capillary Pipette. The hexane sample was prepared in the same way using 1 µl of hexane. The Blank sample consisted of a piece of un-altered filter paper and the Wet sample received a piece of filter paper saturated with deionized water. All pipettes were immediately sealed on both ends with parafilm and stored at -20C° until ∼3-5 minutes before use in an EAG run when they were allowed to equilibrate to room temperature at 22C°.

EAG was conducted as described in Raguso et al.[83] using a Syntech Stimulus Controller CS-05 and Syntech IDAC-232 (Ockenfels Syntech GmbH, Kirchzarten DE), with the major exception that the humidifier/bubbler was removed from the system. Stimulus pulses were delivered through the alternate line in a 0.5-second pulse. The software records the 4.5 seconds prior to the stimulus (front baseline) and then recovery after the impulse for a total of 10 seconds. Under a dissecting microscope, the entire head of the specimen was excised with a scalpel blade (Figure S7). No incisions or modifications were made to the antenna including the apical antennal segments or its cuticle. A salt-free electrode gel (Spectra 360, Davie FL, USA), was used to affix the occiput of the excised head to the negative electrode and the apical antennal segment to the positive electrode to serve both as an adhesive and conducting element to complete the antennal circuit. Antennae were given ∼3 min to equilibrate in the circuit prior to the EAG run.

The EAG trials consisted of two tests, 1) response to plant VOC (1,3-octadiene) verses carrier solvent (Hexane) and 2) high relative humidity (Wet) verses ambient humidity (Blank) and plant VOC (1,3-octadiene). Eight weevils, four male and four female, were tested for response to 1,3-octadiene as compared with the carrier solvent hexane. Fifteen weevils (five males and ten females) were tested for comparison between Wet, Blank, and 1.3-octadiene. In order to control for the potential for a false-positive response to humidity the conducting gel alone (no antenna) was tested for mV deflection in resistance in response to high RH stimulus as compared to a blank. In this case, blank and humidity stimuli were tested on a gel bridge connecting the positive and negative electrodes eight times each. Differences in mV response to stimuli were tested for significance using a pairwise Wilcox test and Bonferroni corrected for multiple comparisons.

Y-tube behavior assay

A series of two-way olfactometer experiments were used to assay weevil responses to VOC and humidity, independently and in combination. The olfactometer was built from a clear glass y-tube that served as the observation chamber (3.8 cm in diameter, base length 16.5 cm, arm lengths 8.9 cm; see Figure 3A). Sample chambers consisted of glass cups nestled within the arms of the y-tube and connecting to air flow hoses. The sample chambers were covered on their open end with a filter constructed from white synthetic tulle so that weevils could not leave the observation chamber, but air was able to flow through. Rubber septa charged with the chemical stimuli were placed in the sample chamber during experiments. The distance from the joint of the y-tube to the synthetic tulle filter was 4.5 cm. Weevils were introduced into the observation chamber via a glass tube base that fit securely into the bottom of the y-tube. The base was open at the top and covered with synthetic tulle on the bottom to allow air flow to exit the system after blowing over the weevils.

All glassware was washed with scent free soap and baked in a Precision oven (Thermo Fisher Scientific, Waltham MA USA) at 80 °C for at least 3 hours prior to use and between every 6 replicates. Tulle filters located at the sample chambers and base were changed every 6 replicates. This y-tube olfactometer was set up at an angle of 20 degrees and lit from above with an evenly spaced array of LED lights illuminating the arena to 0.59 micromole mM photons /M^2/ sec measured with an LI-250 light meter (LI-COR Biosciences, Lincoln NE, USA). House air was regulated to 300mL/min using two ports from the 4-choice olfactometer air delivery system w/vacuum pump (Model# OLFM-4C-ADS+VAC, Analytical Research Systems, Gainesville, FL USA). Any potential scent was removed from the air by passing it through a glass vial filled with 12x40 mesh size granular activated charcoal (EnviroSupply and Service, Irvine CA USA) prior to introducing it to the olfactometer.

A test of weevil response to the chemical carrier solvent (control) was conducted with charcoal purified air and 10 µl of HPLC grade Dichloromethane (DCM) (Cat # 650463, Honeywell, Muskegon MI USA) pipetted onto a clean 8mm rubber septum (Cat # C4013-30, Thermo Fisher Scientific, Waltham MA USA). New septa with 10 µl DCM were used for each replicate.

Weevils were introduced into the olfactometer in cohorts of five weevils (mixed sex) as established in the literature[79,80] and were given 5.5 minutes within the observation chamber to make decisions. Trials were run between 9:45 and 17:30. Due to the low number of individuals and short lifespan of these insects, each set of five weevils was tested for responses to each of the three experimental conditions introduced in random order. Each replicate group of weevils was noted and accounted for in statistical analysis described below. In some cases, weevils escaped or died and were replaced with new weevils. The sample side was changed every three replicates and the glass y-tube was cleaned after every six replicates. Videos were recorded using a Panasonic CCTV video camera (Model # WV-BP334, Kadoma OSA Japan) and first choice was scored blind as the moment weevils made a definitive move beyond 1 cm into a given arm of the y-tube.

Three experimental conditions were presented: 1) VOC in one arm with both arms dry, 2) dry arm versus humid arm, and 3) dry arm versus humid arm plus VOC (Figure 3A). For humid arms, air was pushed through distilled water after passing through the activated charcoal filter. For VOC experiments, 10 µg of 97% 1,3-octadiene (CAS# 1002-33-1, ChemSampCo Dallas TX USA) was diluted in 10 µl of DCM (Cat # 650463, Honeywell, Muskegon MI USA) and pipetted onto 8 mm clean rubber septa (Cat # C4013-30, Thermo Fisher Scientific, Waltham MA USA). New septa were used for each replicate. Humidity was recorded from the test arms and base using an HMP42 humidity and temperature probe attached to an HIMI41 indicator (Vaisala, Helsinki Finland). The sample side was changed every three replicates and eighteen replicates were performed for each experimental condition.

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Weevil response to quantifiable differences in humidity

The ability of weevils to discern between fine-scale differences in humidity was tested using the olfactometer described above. In this case weevils had the choice between a finer scale difference in humidity generated by pushing charcoal filtered air through either distilled water in one arm (mean 81.9% RH) or a saltwater solution in the other arm (28.52 M solution of NaCl (Cat # 22,351-4, Sigma Aldrich, Milwaukee WI USA), mean 67.0% RH). Delta RH% was 14.9% between the two arms with a difference of ± 8% from the base (mean 73.4%RH). Eighteen replicates with five weevils each were performed, and weevil choices were recorded as described above.

Weevil approach to ovulate cones

Field experiments were used to determine the location of highest attraction on ovulate cones. Weevil pollinators in the field were marked with biodegradable, non-toxic, UV fluorescent dyes: Eco-14 Orange, Eco-21 Magenta, and Eco-18 Signal Green (DayGlo Color Corp, Cleveland OH USA) following Valencia-Montoya et al.[84] The experiment was conducted at MBC from July 30–August 3, 2021. A different color dye was applied each day for five consecutive days at 17:00 hours on pollen cones that were releasing pollen. Receptive ovulate cones were examined every day using a UV-lamp at 23:00 hours to look for new fluorescent marks left by the weevils. The distance between the landing mark and the closest crack between megasporophylls was measured using an electronic digital caliper (Adoric 0-6'', AdoricLife Corp, Orlando FL, USA). Fluorescent marks were only included with different colors or new marks from the color applied the same day to ensure independence. Because absolute distances to the cracks were measured, the data are zero-truncated with the cracks as reference points. Given a normal distribution (as calculated with a random sampling of data points), a one-sided T-test was conducted to determine if the mean distance between florescent landing mark and closest crack was significantly greater than 0 mm, and confidence intervals were calculated by bootstrapping the transformed dataset 5,000 times using the package *boot*,[85,86] in R. v. 3.6.[87]

Morphological basis of humidity perception

Scanning electron micrographs (SEM) of weevil antennae were used to search for potential humidity perceptual (hygrosensory) organs. *Rhopalotria furfuracea* weevils collected from *Z. furfuracea* plants at MBC were air dried, mounted onto 12.7 mm SEM aluminum mount stubs (Cat# 75174, Electron Microscopy Sciences, Hatfield PA USA) and sputter coated with gold for 80 seconds under 0.05 mBar pressure using a 108 Auto Sputter Coater (Ted Pella, Redding CA USA). Prepared weevils were imaged using a Phenom XL scanning electron microscope using a 10 kV beam voltage with background scatter (Thermo Fisher Scientific, Waltham MA USA).

QUANTIFICATION AND STATISTICAL ANALYSIS

Weevil choice in behavioral experiments was tested for statistical significance using a proportion test (*prop.test* R. v. 3.6).[87] For the fine scale humidity experiment (Figure 5) a proportion of positive responses was tested against a null hypothesis of 0.5 (no preference). For the comparative tests of humidity, VOC, and humidity + VOC, individual cohorts of insects were attributed to only one condition to account for replication by equally dividing the cohorts randomly amongst the three conditions (each condition receiving 33% of the raw data). The proportion of positive responses for the subset of data in each experimental condition was then calculated and used to determine the p-value for that condition via a proportion test. This was repeated 1000 times with random assignments of cohorts to experimental conditions and the mean p-value and proportion of significant runs ($p\text{-values} < 0.05$) was calculated for each condition. A null hypothesis was set to equal proportions between humidity, VOC, or VOC + humidity conditions (Figure 4). In order to determine if humidity and VOC have additive effects on weevil choice (Figure 4) the proportion of positive responses to VOC + humidity was subtracted from the proportion of positive responses to VOC for each of the 1000 runs and the resulting proportion of negative values was calculated.