Behavior and feeding of two beetle pollinators of *Zamia integrifolia* (Cycadales): *Rhopalotria slossoni* (Coleoptera: Belidae) and *Pharaxanotha floridana* (Coleoptera: Erotylidae)

Avi Simon[1,2], Shayla Salzman[1,2,3], Chelsea D. Specht[1], and Robert A. Raguso[2]*

Zamia integrifolia L.f. (Cycadales) is the only cycad native to Florida (Calonje et al. 2023) and has a red list conservation status of near threatened from the International Union for Conservation of Nature (IUCN 2022). The cycad is completely reliant on pollination services from 2 native beetle species, *Rhopalotria slossoni* (Chevrolat; Coleoptera: Belidae) and *Pharaxanotha floridana* (Casey; Coleoptera: Erotylidae) (Tang 1987, Fawcett & Norstog 1993; Stevenson et al. 1998). All species from both genera form close brood-site pollination mutualisms with cycads (Tang et al. 2020) with the larval stages developing within the reproductive tissue of the pollen cone (Fawcett & Norstog 1993; Stevenson et al. 1998). *Zamia integrifolia* is a dioecious gymnosperm with pollen (male) cones and ovulate (female) cones housed on separate plants, necessitating the movement of pollinators between plants, yet ovulate cones are not thought to serve as brood sites for either pollinator (Fawcett & Norstog 1993; Stevenson et al. 1998) and only sparse nibble marks have been observed on ovulate cones in the field (Tang 1987). It has been hypothesized that *Z. integrifolia* reproduction occurs through deceptive pollination (Tang 1987; Fawcett & Norstog 1993; Stevenson et al. 1998) in a system whereby pollinators are tricked into visiting the ovulate cone as it mimics the pollen cone scent but does not provide a brood or food source (Terry et al. 2007; Salzman et al. 2020, Salzman et al. 2021).

Previous studies have found that *R. slossoni* aggregate and feed gregariously on pollen cone tissue and mostly avoid ovulate cone tissue in the field (Tang 1987; Fawcett & Norstog 1993). These weevils lay their eggs deep within the parenchyma tissue of the pollen-bearing cone scales (microsporophylls) that form the pollen cone. Developing larvae feed on the parenchyma tissue, hollowing out the microsporophyll as they develop (Fawcett & Norstog 1993). Emerged adults feed on the inner surface of the microsporophyll but never on the pollen itself (Fawcett & Norstog 1993). In previous studies, *P. floridana* also have been shown to aggregate on pollen cones but have not been observed feeding on ovulate cones (Fawcett & Norstog 1993; Stevenson et al. 1998). Larvae of the genus *Pharaxonotha* develop in the pollen cone of their cycad host where they feed on the cone axis, the microsporophyll, and the pollen (Fawcett & Norstog 1993; Chavez & Genaro 2005; Valencia-Montoya et al. 2017). Adults are reported to feed solely on pollen (Fawcett & Norstog 1993; Stevenson et al. 1998).

The lack of ovulate cone herbivory has been suggested to be due to a toxin avoidance behavior in response to the plant compound β-N-methylamino-L-alanine (BMAA). In the pollen cone, this compound is sequestered in specialized idioblast cells that pass through the gut of an insect intact such that there is no exposure to BMAA (Norstog & Fawcett 1989; Fawcett & Norstog 1993). The same compound is found dissipated in ovulate cone tissue, as idioblast cells burst during ovulate cone development (Norstog & Fawcett 1989; Fawcett & Norstog 1993). Recent research suggests that BMAA does not present as a feeding deterrent in a generalist lepidopteran herbivore (Whitaker et al. 2022), but field surveys suggest that ovulate cone tissue is largely avoided in the wild by both beetle mutualists. Here, we test whether ovulate cones might actually provide food rewards or other benefits (e.g., enhanced survivorship) to beetle pollinators in a focal laboratory study using no-choice feeding assays. Specifically, we investigate the feeding behaviors of adult *P. floridana* and *R. slossoni* beetles on ovulate cone scales and pollen cone scales (as a positive control) from *Z. integrifolia* to assess the potential for ovulate cone feeding in both species.

For both experiments, 6 pollen cones were collected from plants maintained in a greenhouse to ensure the absence of beetle larvae that could potentially emerge from inside harvested pollen cone scale tissue. Ovulate cone scales were harvested from 6 ovulate cones collected from a cultivated planting in Davie, Broward County, Florida (26.0854800 °N, 80.2514900 °W). Insects were captured as they emerged from the scales of 10 pollen cones collected at the same cultivated site in Florida. All experimental cones were reproductively mature, meaning pollen and ovules were receptive for fertilization.

To assess behavioral responses to pollen vs. ovulate cone scales, 1 insect of either species was placed in a Petri dish containing either an ovulate cone scale with ovules intact (12 *Pharaxanotha,* 9 *Rhopalotria* replicates), 2 pollen cone scales with pollen sacs intact (12 *Pharaxanotha,* 13 *Rhopalotria*), or 2 plastic 3D printed pollen cone scales (14 *Pharxanotha*, 8 *Rhopalotria*) as negative controls. Two pollen cone scales or two 3D printed scales were used to account for the much smaller size as compared to the ovulate cone scales and to provide equal surface area for insect interaction (Figs. 1 and 2). The Petri dishes were placed on white paper and backlit with a white LED light to allow the small insects to be captured in silhouette. The experiment was per-

[1]Cornell University, School of Integrative Plant Science, Section of Plant Biology and the L.H. Bailey Hortorium, Ithaca NY 14853 USA, E-mail: ams686@cornell.edu (A.S.), cdspecht@cornell.edu (C.D.S.)
[2]Cornell University, Department of Neurobiology and Behavior, Ithaca NY 14853 USA, E-mail: rar229@cornell.edu (R.A.R.)
[3]Univeristy of Georgia, Department of Entomology, Athens GA 30606 USA, E-mail: shayla.salzman@uga.edu (S.S.)
*Corresponding author; E-mail: shayla.salzman@uga.edu (S.S.)

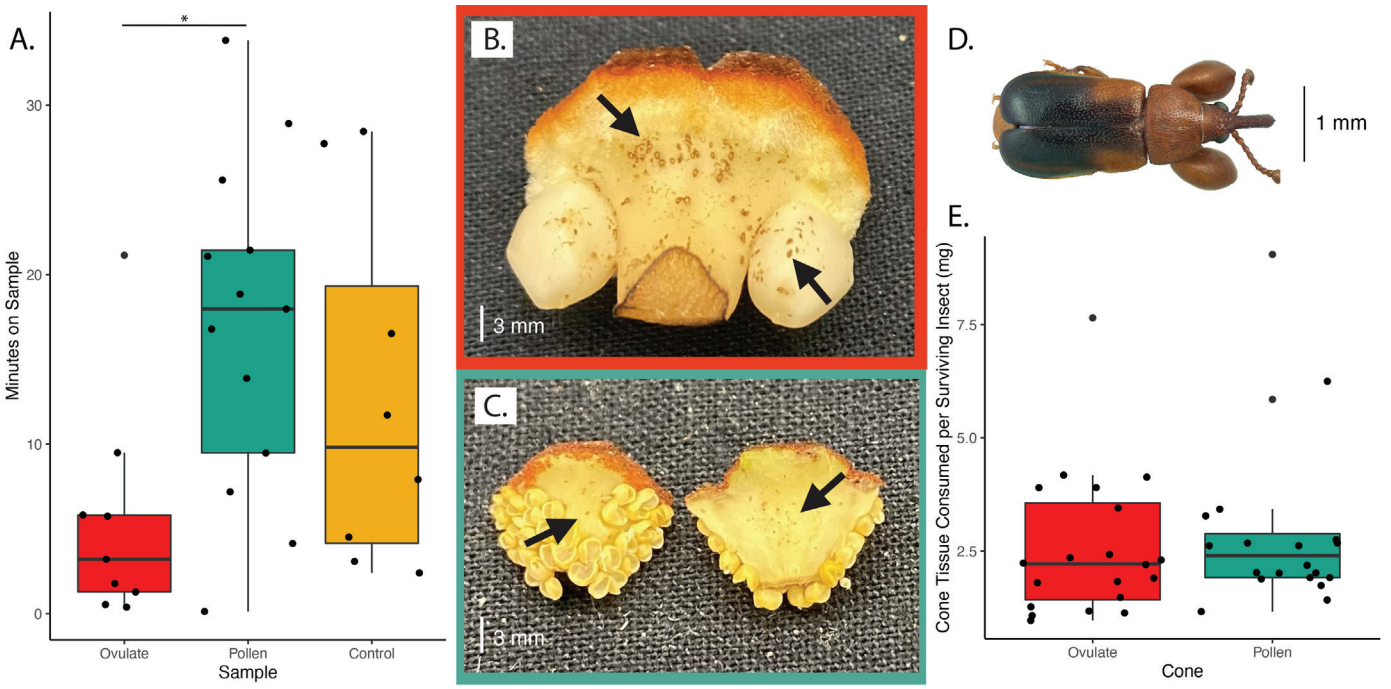

Fig. 1. A) *Rhopalotria slossoni* spend more time on pollen cone scales than ovulate cone scales ($p = 0.041$) in 30 mins no-choice behavior trials but show no statistical difference between ovulate and control. B) Ovulate cone scales show extensive feeding damage on their parenchyma tissue after 24 h. C) Pollen cone scales show feeding damage on the parenchyma tissue after 24 h. D) *Rhopalotria slossoni* E) The mass (mg) of tissue consumed per surviving weevil is equal between pollen and ovulate cone scales. In A and E, summary boxplots are shown with raw data values overlaid.

formed in a curtained enclosure within a dark room (21 °C and 45% relative humidity) so that the backlit lighting was evenly presented across all arenas. Arenas were recorded for 30 mins and videos were analyzed using EthoVision (XT-Multiple Arenas, Noldus Information Technology Inc., Leesburg, Virginia, USA). We manually outlined the area occupied by the cone scales, defining the exact location and boundary. This allowed EthoVision to precisely quantify the total time each insect spent on or off the cone scale. Each insect was counted as being on the cone

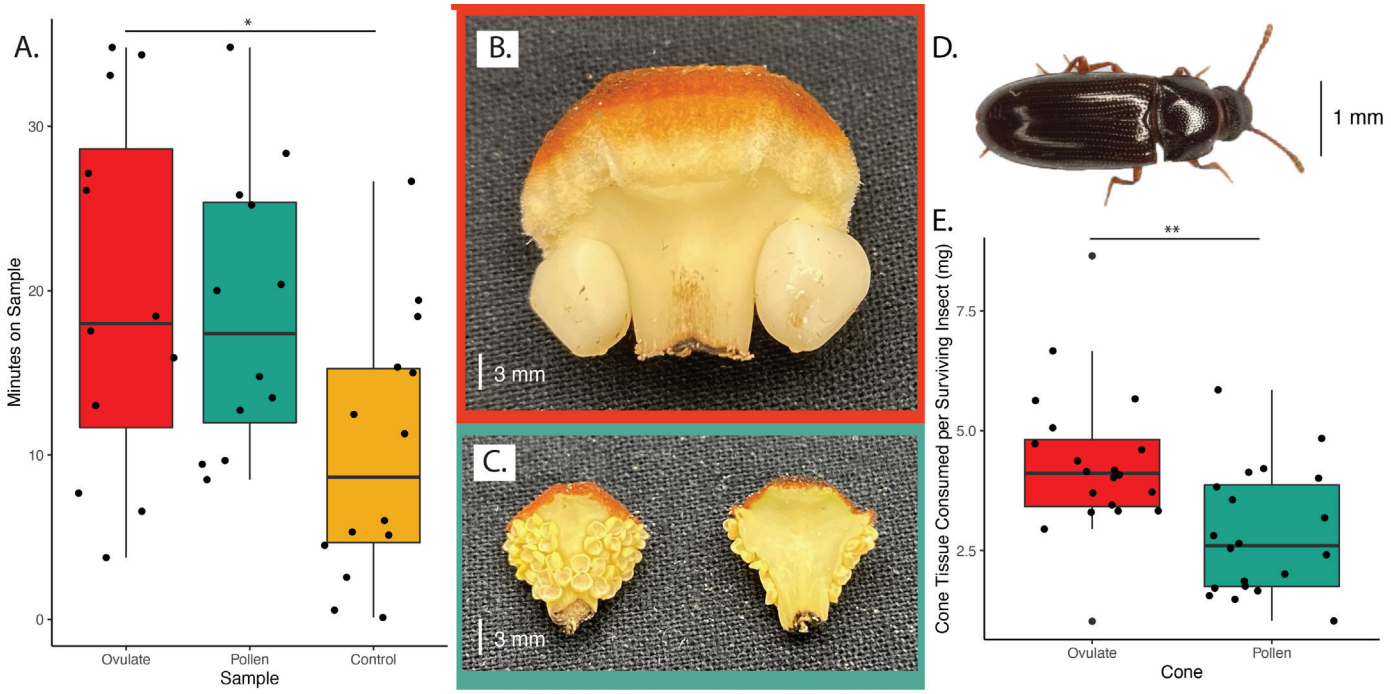

Fig. 2. A) *Pharaxanotha floridana* spend more time on the ovulate cone scale than control ($p = 0.035$) and equal amounts of time on pollen and ovulate cone scales in 30 mins no-choice behavior trials. B) No visible evidence of feeding damage on ovulate cone scale parenchyma tissue, nor on ovules after 24 h. C) No visible feeding damage on pollen cone scale parenchyma tissue after 24 h. D) *Pharaxanotha floridana* E) More mass (mg) is consumed per surviving beetle from ovulate cone scales than from pollen cone scales over 24 h ($p = 0.0033$). In A and E, summary boxplots are shown with raw data values overlaid.

when it was located within one body length (3 mm) of this boundary. The time spent on and off the cone was quantified in mins for the duration of the trial and was compared across the 3 treatments for the same species. Comparison of the differences between time spent on the 3 treatments was done using a pairwise Wilcoxon test (to account for the non-Gaussian distribution of *R. slossoni* data) or a pairwise t-test (for the *P. floridana* data, which followed a Gaussian distribution) with Bonferroni corrections for multiple comparisons.

To assess feeding on pollen vs. ovulate cones, 4 insects (mixed sex) of either species were placed in an enclosed 60 mL plastic container with either 1 pollen cone scale or 1 ovulate cone scale. Cone scales with no insects were included as a control to calculate loss of fresh mass (e.g., through desiccation) during the trial. Twenty replicates of each trial were performed. Cone scales were weighed prior to the experiment using an AL204 analytical balance (Mettler Toledo, Columbus, Ohio, USA) and containers were vented with tiny air holes before being placed into a darkened incubator (60% relative humidity, 21 °C). After 24 h, cone scales were weighed, and the number of surviving insects was recorded. The average change in fresh mass (mg) for the control cone scales was subtracted from the change in mass recorded for each test sample to standardize for desiccation during the 24 h trial period, and the resulting value was divided by the number of surviving insects to reflect individual feeding behavior more accurately. Differences between the 2 treatments of mass consumed was determined using a Mann-Whitney test and differences in the number of surviving insects was determined using an unpaired t-test.

In the present study, we observed a greater time spent on pollen cone scales in *R. slossoni* but found no significant differences in mass of tissue consumed from pollen vs. ovulate cone scales. In the 30 mins no-choice trials of insect behavior, *R. slossoni* spent significantly greater time on pollen cone scales (16.9 ± 9.80 mins [mean ± standard deviation]) as compared with ovulate cone scales (5.49 ± 6.60 mins) (p = 0.041, Fig. 1A); however, there was no difference in time spent on the control 3D printed scales (12.8 ± 10.5 mins) than either the pollen or ovulate scales (p = 1.00 and p = 0.68, respectively). In the feeding trial, *R. slossoni* survived (for 24 h) in significantly greater numbers on ovulate (3.35 ± 0.75 individuals) as opposed to pollen (2.6 ± 1.10 individuals) cone scales (p = 0.016). However, there was no significant difference in the amount of tissue consumed per surviving weevil between pollen (2.97 ± 1.93 mg) and ovulate (2.57 ± 1.60 mg) cone scales (p = 0.39, Fig. 1E). Nibble marks were visible on both pollen and ovulate parenchyma tissue (Fig. 1B and C).

In our laboratory study of *P. floridana*, we saw no difference in time spent on the pollen cone scales relative to the ovulate cone scales, yet we did observe greater feeding on the ovulate cone scales relative to the pollen cone scales. *Pharaxanotha floridana* spent significantly more time on the ovulate cone scales (19.9 ± 11.1 mins) than the control (10.2 ± 8.00 mins) (p = 0.035). There was no statistical difference in time spent on the pollen (18.6 ± 8.52 mins) vs. ovulate cone scales (p = 1.00), nor between pollen cone scales and control (p = 0.081). In the feeding trials, survivorship was similar on pollen (3.7 ± 0.57 individuals) and ovulate (3.6 ± 0.60 individuals) cone scales and significantly more ovulate cone tissue was consumed per surviving insect (4.33 ± 1.56 mg) than pollen cone tissue (2.86 ± 1.30 mg) (p = 0.0033, Fig. 2D). Nibble marks were not visible on either pollen or ovulate parenchyma tissue (Fig. 2B and C); instead, *P. floridana* were found on the reddish exterior portion of the ovulate and pollen cone scales during the feeding experiment. We thus suspect that *P. floridana* adults were consuming fine hairs on the surface of the cone scales, although there was no difference to the naked eye for damage to this surface as compared with controls. It is possible that the larger mass of tissue consumed per insect on ovulate cone scales could simply be due to the greater surface area of this tomentose exterior surface. It is noteworthy that these insects did not feed on the parenchyma tissue at all.

Our results question the assumption that *Z. integrifolia* pollinators do not, or will not, utilize the ovulate cone for feeding or sustenance. Evidence from our behavioral experiment suggests that *R. slossoni* individuals spend more time on pollen cones than they do ovulate cones, supporting previous field observations (Tang 1987; Fawcett & Norstog 1993; Stevenson et al. 1998). However, feeding trials indicated that consumption is equal between ovulate and pollen cone scales, contradicting expectations based on published observations of adult behavior in the field (Tang 1987; Norstog & Fawcett 1989; Fawcett & Norstog 1993). In this study, *R. slossoni* lived longer on the ovulate cone scales, possibly because of delayed desiccation relative to the smaller pollen cone scales, although both scales appeared to remain supple and fresh for the duration of the experiment leaving the mechanism of longevity unresolved. *Pharaxanotha floridana* showed no difference in time spent between either pollen or ovulate cone scales, although did spend more time on the ovulate cone scale than on the control. Although the literature suggests *Pharaxanotha* species do not feed on ovulate cones in the field (Tang, 1987; Norstog & Fawcett 1989; Fawcett & Norstog 1993), we did observe feeding on ovulate cone scales in the laboratory and in fact observed a greater consumption of ovulate cone scale tissue vs. pollen cone scale tissue per insect. *Pharaxanotha floridana* appeared to feed on the hairy distal surface of the ovulate cone scale, leaving the mechanism that attracts *P. floridana* into the interior of the ovulate cone for pollination an open question for future field-based studies. The micropyle drops observed by Tang (1987) in the interior of the ovulate cone are one interesting possibility for attraction (Labandeira et al. 2007; Celedón-Neghme et al. 2016) although observational data by Tang (1987) suggests this is unlikely.

The results showing greater survivorship for *R. slossoni* and greater mass consumed for *P. floridana* call into question the assumption that cycad pollination occurs by deceit alone, given that both beetle species exhibited a benefit to ovulate cone feeding in the laboratory.

The authors thank William Tang for collecting insects and cones in the wild, John Putnam for maintaining the plants in the greenhouse, and Rory Maher for making data collection possible.

Summary

Zamia integrifolia L.f. (Cycadales), a threatened cycad native to Florida, depends on 2 native beetle species for pollination: *Rhopalotria slossoni* (Chevrolat; Coleoptera: Belidae) and *Pharaxanotha floridana* (Casey; Coleoptera: Erotylidae). Both insects are brood-site pollination mutualists, known to live and feed within the pollen (male) cone. However, for pollination to occur, beetles must also visit ovulate (female) cones, which have been assumed to offer no benefits to them as food or nurseries. We tested the potential for beetle pollinator use of ovulate cones by performing no-choice behavior and feeding trials for adults of both beetle species on both ovulate cones and pollen cones of *Z. integrifolia*. *Rhopalotria slossoni* beetles showed greater survival on ovulate cone tissues despite showing no significant difference in total tissue mass consumed between cone sexes. Conversely, *P. floridana* consumed more tissue mass from ovulate cone scales yet showed no difference in survivorship on ovulate vs. pollen cone scales. Although neither beetle species is found in large numbers on ovulate cones in the field, our laboratory study suggests that both species could potentially benefit from feeding on ovulate cone tissues, questioning the standing hypothesis that *Z. integrifolia* pollination occurs by deceit.

Key Words: brood-site mutualism; cycad; Florida native plant; gymnosperm; insect feeding; insect pollination

Summario

Zamia integrifolia L. f. (Cycadales), una cicadácea nativa amenazada en la Florida, depende de 2 especies nativas de escarabajos para la polinización: *Rhopalotria slossoni* (Chevrolat; Coleoptera: Belidae) y *Pharaxanotha floridana* (Casey; Coleoptera: Erotylidae). Ambos insectos son mutualistas de la polinización del sitio de cría, y se sabe que viven y se alimentan dentro del cono de polen (macho). Sin embargo, para que ocurra la polinización, los escarabajos también deben visitar conos de ovulación (femeninos), que se supone que no les ofrecen ningún beneficio como alimento o vivero. Probamos el potencial para el uso de conos de ovulación por parte de escarabajos polinizadores, esto mediante la realización de pruebas de alimentación y comportamiento de no elección para adultos de ambas especies de escarabajos tanto en conos de ovulación como en conos de polen de *Z. integrifolia*. Los escarabajos *Rhopalotria slossoni* mostraron una mayor sobrevivencia en los tejidos del cono ovulado a pesar de no mostrar diferencias significativas en la masa total de tejido consumido entre los sexos del cono. Por el contrario, *P. floridana* consumió más masa de tejido de las escamas de los conos de ovulación, pero no mostró diferencias en la sobrevivencia en las escamas de los conos de ovulación frente a las de los conos de polen. Aunque ninguna de las especies de escarabajos se encuentra en grandes cantidades en los conos de ovulación en el campo, nuestro estudio de laboratorio sugiere que ambas especies podrían beneficiarse potencialmente al alimentarse de los tejidos de los conos de ovulación, lo que cuestiona la hipótesis actual de que la polinización de *Z. integrifolia* se produce por engaño.

Palabras Clave: mutualismo del sitio de cría; cícadas; planta nativa de Florida; gimnospermas; alimentación de insectos; polinización de insectos

References Cited

Calonje M, Stevenson DW, Osborne R. 2023. The World List of Cycads, online edition. 2013–2023 (cited 2023 Mar 07). Available from http://www.cycadlist.org (last accessed 26 June 2023).

Celedón-Neghme C, Santamaría L, González-Teuber M. 2016. The role of pollination drops in animal pollination in the Mediterranean gymnosperm *Ephedra fragilis* (Gnetales). Plant Ecology 217: 1545–1552.

Chavez, R, Genaro JA. 2005. A new species of *Pharaxonotha* (Coleoptera: Erotylidae), probable pollinator of the endangered Cuban cycad, *Microcycas calocoma* (Zamiaceae). Insecta Mundi 19: 143–150.

Fawcett PKS, Norstog KJ. 1993. *Zamia pumila* in South Florida: A preliminary report on its pollinators *R. slossoni*, a snout weevil and *P. zamiae*, a clavicorn beetle, pp. 109–119 *In* Stevenson DW, Norstog KJ [eds.], Proceedings of Cycad 90, the Second International Conference on Cycad Biology. Palm & Cycad Societies of Australia Ltd, Queensland, Australia. June 1993.

IUCN (The International Union for Conservation of Nature). 2022. The IUCN Red List of Threatened Species. Version 2022-2. https://www.iucnredlist.ord (last accessed 7 March 2023).

Labandeira CC, Kvacek J, Mostovski MB. 2007. Pollination drops, pollen, and insect pollination of Mesozoic gymnosperms. Taxon 56: 663–695.

Norstog KJ, Fawcett PKS. 1989. Insect-cycad symbiosis and its relation to the pollination of *Zamia furfuracea* (Zamiaceae) by *Rhopalotria mollis* (Curculionidae). American Journal of Botany 76: 1380–1394.

Salzman S, Crook D, Crall J, Hopkins R, Pierce N. 2020. An ancient push-pull pollination mechanism in cycads. Science Advances 6: eaay6169. DOI: 10.1126/sciadv.aay6169

Salzman S, Crook D, Calonje M, Stevenson DW, Pierce NE, Hopkins R. 2021. Cycad-weevil pollination symbiosis is characterized by rapidly evolving and highly specific plant-insect chemical communication. Frontiers in Plant Science 12: 639368. DOI: 10.3389/fpls.2021.639368

Stevenson DW, Norstog KJ, Fawcett PKS. 1998. Pollination biology of cycads, pp. 277–294 *In* Owens SJ, Rudall PJ [eds.], Reproductive Biology, Royal Botanical Gardens, Kew, London, United Kingdom.

Tang W. 1987. Insect pollination in the cycad *Zamia pumila* (Zamiaceae). American Journal of Botany 74: 90–99.

Tang W, Xu G, Marler T, Khuraijam JS, Singh R, Lindström AJ, Radha P, Rich S, Nguyen KS, Skelley P. 2020. Beetles (Coleoptera) in cones of cycads (Cycadales) of the northern hemisphere: diversity and evolution. Insecta Mundi 0781: 1–19.

Terry I, Walter GH, Moore C, Roemer R, Hull C. 2007. Odor-mediated push-pull pollination of cycads. Science 318: 70. DOI: 10.1126/science.1145147

Valencia-Montoya WA, Tuberquia D, Guzmán PA, Cardona-Duque J. 2017. Pollination of the cycad *Zamia incognita* A. Lindstr. & Idárraga by *Pharaxanotha* beetles in the Magdalena Medio Valley, Colombia: a mutualism dependent on a specific pollinator and its significance for conservation. Arthropod-Plant Interactions 11: 717–719.

Whitaker MRL, Gilliéron F, Skirgaila C, Mescher MC, De Moraes CM. 2022. Experimental evidence challenges the presumed defensive function of a “slow toxin” in cycads. Scientific Reports 12: 6013. DOI: 10.1038/s41598-022-09298-3