



Differential Feeding Responses of Several Bee Species to Sugar Sources Containing Iridomyrmecin, an Argentine Ant Trail Pheromone Component

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Abstract Pollinators such as bees need reliable access to nectar resources. However, competition for these carbohydrate rewards can be high among floral visitors. Moreover, invasive insects may further restrict pollinator access to flowers. For example, invasive Argentine ants (*Linepithema humile*) are known to harass and displace pollinators from flowers and reduce nectar reward acquisition. The foraging behavior of bees is strongly influenced by numerous chemical cues and signals at the flower. Thus, to avoid aggressive floral visitors, bees may use chemical cues when deciding whether or not they forage on the flower. Here, we demonstrate that Argentine ants deposit a pygidial gland chemical, iridomyrmecin, on a sugar source while feeding. Based on two-choice laboratory assays conducted with three commercially available bee species, the presence of iridomyrmecin in the sugar resource influenced the foraging decisions of *Megachile rotundata* and *Osmia lignaria*, but not those of *Apis mellifera*. Detailed insights into the chemical signals left by ants at floral resources and their effects on foraging decisions by pollinators can provide vital information for understanding the magnitude and mechanisms of impacts invasive ant species might have on pollinators.

Keywords Foraging · chemical communication · competition · *Megachile rotundata* · *Osmia lignaria* · *Apis mellifera*

Introduction

Pollinators provide ecological and economic benefits via pollination in agricultural and natural ecosystems (Ashman et al. 2004; Potts et al. 2010). However, we are currently experiencing a global insect biodiversity decline (Sanchez-Bayo and Wyckhuys 2019), and insect pollinators are no exception (Goulson et al. 2015; Marshman et al. 2019). Threats to pollinator populations include habitat loss, disease, insecticide exposure and biological invasions (Vanbergen et al. 2013). In particular, the impacts of invasive insects on local pollinator populations can be extensive; they disrupt plant-pollinator mutualisms (Geslin et al. 2017; Sahli et al. 2016), spread disease (Loope et al. 2019), outcompete native pollinators and even prey upon them (Wilson and Holway 2010; Wilson et al. 2009). The Argentine ant, *Linepithema humile*, is a globally distributed invasive species that is known to directly interfere with insect pollinators (Hanna et al. 2015; Lach 2008a, b; Miner 2018; Sidhu and Wilson Rankin 2016) and hummingbird pollinators (Rankin et al. 2018). Therefore, it is critical that we understand the magnitude and mechanisms of invasive Argentine ant impacts on ecologically important pollinator taxa, particularly bees.

Some of interspecific interactions between Argentine ants and pollinators can be mediated via chemical

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stimuli. Floral visitation by bees is known to be affected by diverse chemical cues and signals. The chemicals involved in this process may be produced by the flower itself or be deposited by previous floral visitors (Ballantyne and Willmer 2012). These chemicals may serve as attractants to recruit nestmates to a resource as seen in ants (Choe et al. 2012) and stingless bees (Nieh 2004). Alternatively, chemical cues may be associated with previous visitation, such that bee foragers that avoid such cues may decrease nectar competition (Eltz 2006; Gawleta et al. 2005; Saleh et al. 2007; Stout and Goulson 2001; Witjes and Eltz 2009). Since Argentine ants actively visit flowers to collect nectar (Buys 1987; Markin 1970; Visser et al. 1996), it is possible that the foraging Argentine ants may leave some chemical cues behind on the flower, potentially affecting the foraging behavior of subsequent floral visitors.

Several possibilities exist in terms of the types of chemical cues that may be left on flowers by Argentine ants. Similar to some ant species that mark their territories or foraging area with chemical compounds (Lenoir et al. 2009; Offenberg et al. 2004), Argentine ants may chemically mark visited flowers. Previous studies documented Argentine ants depositing chemical trails (dolichodial and iridomyrmecin) while recruiting their nestmates to food resources (Choe et al. 2012). Once Argentine ants numerically dominate a food source due to their extremely efficient recruitment systems relative to other recruiting species (Flanagan et al. 2013; Holway 1999), they will also aggressively defend these resources against interspecific competitors (Leonetti et al. 2019). During such aggressive interactions, Argentine ants may produce large quantities of exocrine gland chemicals (dolichodial and iridomyrmecin) for alarm as well as defensive purposes (Welzel et al. 2018). These types of chemicals may serve as a reliable cue indicating the current or recent presence of aggressive species or even their identities (Kats and Dill 1998). To avoid these aggressive floral visitors, foraging pollinators may use chemical cues or indirect chemical traces when deciding whether or not to visit a specific flower (Cembrowski et al. 2014).

Here, we investigated whether Argentine ants leave any pheromone (chemical signature) traces on a sugar source while feeding. Furthermore, we identified and quantified the compound deposited. Unlike previous research in which a corolla was extracted in an organic solvent to examine the chemical residues left by visiting insects (Eltz 2006), the current research focused on the

sugar source itself by implementing an “artificial nectary” that can be extracted for chemical analyses. Once the chemical compound left by the foraging ants was identified, we subsequently conducted choice tests with three bee species that are commercially available for pollination to determine if the presence of the compound (iridomyrmecin, see results) in the carbohydrate resource would differentially influence foraging decisions across pollinator species.

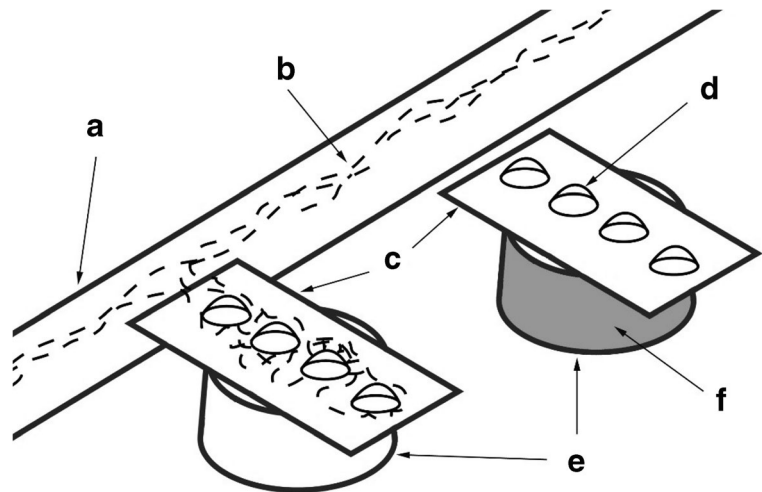
Methods

Collection of Chemicals To collect chemical compounds that are left by foraging Argentine ants at sugar sources, polyacrylamide (PAA) hydrogels were used as the “artificial nectary” in which sugar water was provided to foraging ants. Because the PAA hydrogel is not soluble in organic solvents (e.g. hexane) (IPCS 1985), it was possible to extract the compounds left on the surface of the hydrogel by foraging ants for subsequent chemical analyses.

The experiment was conducted using a field population of Argentine ants at the biological control grove on the University of California, Riverside campus (33.972442, -117.317799). PAA hydrogel beads (Rainbow Water Beads, Greenville, SC) were prepared by soaking in 25% (w/v) sucrose solution overnight. The final beads (≈ 1.2 cm in diameter) were bisected using a clean razor blade. Four halves were placed flat-side down along a glass microscope slide (25 mm \times 75 mm \times 1 mm, Fisher Scientific) with 3–5 mm in between each half bead (Fig. 1). These slides were then placed on top of a cylindrical plastic stage (6 cm diameter, 2.5 cm height). In the treatment group, the stages with slides were placed close to active foraging ant trails (on top of irrigation lines), such that foraging Argentine ants could access the hydrogels on the slides (Fig. 1, left). In the control group, the stages were also set near to the ant trails, but with a 10 mm gap such that ants were prevented from reaching the slide (Fig. 1, right) and the outer surface of the plastic stage was treated with a fluoropolymer resin (PTFE-30, Insect-a-Slip Insect Barrier, BioQuip Products Inc., Rancho Dominguez, CA) to prevent ants from accessing the hydrogels via the stage.

Three hours after the initiation of ant foraging, all hydrogels were carefully collected in glass tubes (borosilicate glass, 16 mm \times 100 mm, Fisherbrand) using clean forceps. For the treatment hydrogels, a brief

Fig. 1 Experimental field set-up for collecting Argentine ant pheromone at a sugar source. The set-up on the left is for the treatment (i.e., ants were allowed to forage on the sucrose solution absorbed on polyacrylamide (PAA) hydrogel beads), and the set-up on the right is for the control (i.e., ants were prevented from accessing the sucrose solution absorbed on PAA hydrogel beads). **a** Irrigation line, **b** ant trail, **c** glass slide, **d** PAA hydrogel, **e** plastic stage, **f** a fluoropolymer resin coating



exposure to carbon dioxide (~ 1 min) was used to aid in the removal of ants from the slides and hydrogels. Based on a separate preliminary study, this brief anaesthetization with carbon dioxide did not influence the production or release of volatile pheromones by Argentine ants (D.H. Choe, unpublished data). The tubes were immediately sealed with laboratory film (Parafilm®, Bemis Company Inc., Oshkosh, WI) and transported to the laboratory in a refrigerated container. Treatment and control collections were replicated five times each.

Chemical Analyses All hydrogels were extracted on the same day as field collection. We added 1 mL hexane (Fisher Scientific) to each test tube containing hydrogels, gently shook each test tube and then extracted the solvent. Each hexane extract was then run through a glass pipette column containing 10 mg of sodium sulfate anhydrous (Fisher Scientific) to remove moisture. The final extracts were collected in 2 mL glass vials (Agilent Technologies) and further concentrated to 80 μ L in conical inserts (250 μ L in capacity, Agilent Technologies).

The hexane extracts were analyzed using gas chromatography equipped with a flame ionization detector (GC-FID) and gas chromatography coupled with mass spectrometry (GC-MS). For GC-FID, an Agilent 7890 gas chromatograph equipped with a DB-5 column (30 m \times 0.25 mm inner diameter, Agilent Technologies) and a flame ionization detector was used. One microliter of sample was injected with an automatic liquid sampler. Samples were injected in split mode, with a temperature program of 50 $^{\circ}$ C for 1 min and

then 10 $^{\circ}$ C min^{-1} to 300 $^{\circ}$ C with a 20-min hold. Helium was used as the carrier gas. For GC-MS, electron impact mass spectra (70 eV) were taken with an Agilent 5975C mass selective detector interfaced to an Agilent 7890A gas chromatograph fitted with a DB-5 column (Agilent Technologies). The injection mode and temperature program were identical with the GC-FID analyses. Helium was used as the carrier gas. Chemical identification was based on comparisons (mass spectra and retention times) with the synthetic standard.

Once the compound was identified as iridomyrmecin (see results), we conducted the following processes for its quantification. We prepared a series of iridomyrmecin standards of known concentrations (0.000195–0.1 mg/mL) in acetone, and analyzed 1 μ L of each sample in an Agilent 7890 gas chromatograph equipped with a DB-5 column and an FID as described above. Iridomyrmecin shows two peaks in the GC trace due to thermal isomerization in the GC injector; the peaks from iridomyrmecin were integrated and a calibration curve was established. The quantity of iridomyrmecin per hydrogel sample (i.e., four halves of hydrogel beads) was estimated based on this calibration curve.

Bioassays To determine if the presence of iridomyrmecin in the sugar resource would affect pollinator foraging and sucrose consumption, we conducted a series of choice tests with a sucrose solution containing synthetic iridomyrmecin at 0.01 mg/mL (“low”) and 0.1 mg/mL (“high”) concentrations. The concentrations tested were based on the findings from the pheromone

collection and analyses (see results). We selected three commercially important pollinator species for these bioassays: alfalfa leafcutter bees (*Megachile rotundata* Fab.), blue orchard bees (*Osmia lignaria* Say) and honey bees (*Apis mellifera* L.). Bees were obtained from Watts Solitary Bees (Bothell, WA), Mountain West Mason Bees (Riverton, UT), and apiaries located on the University of California, Riverside campus (Riverside, CA), respectively.

For each bee species, we collected 60 newly emerged (and thus, ant-naïve) individuals and randomly assigned half to each of two choice treatments: control vs. 0.01 mg/mL of iridomyrmecin (low) or control vs. 0.1 mg/mL of iridomyrmecin (high). Each bee was placed in an individual arena, which consisted of an inverted, clear 59-mL plastic soufflé cup lined with filter paper (Ahlstrom 610, 1.5- μ m pore size, Thomas Scientific, Swedesboro, NJ). Two different feeders (0.6 mL microcentrifuge tubes with 2-mm hole near tip) were installed through opposite walls of each arena such that each bee could access two feeder options from inside. One feeder contained 50 μ L of 20% (m/m) sucrose solution (control solution, dyed yellow with food coloring) and the other contained 50 μ L of 20% sucrose solution with iridomyrmecin (pheromone solution, dyed red with food coloring). After 24 h, all filter papers were collected and photographed, noting any spots of colored fecal matter, which would indicate consumption. Based on the color of the fecal deposits, we scored each bee as consuming just the control solution (yellow), both control and the pheromone solutions (both yellow and red spots), or just the pheromone solution (red). One *M. rotundata* died during the 24-h period and was excluded from analysis. Pilot choice tests demonstrated no color preference by the bees when given the choice of yellow and red sucrose solutions.

For each species and concentration of iridomyrmecin, we then used chi-square goodness-of-fit tests in package 'stats' (R Core Team 2020) to compare these counts to our null expectations, which represent no preference among feeding on the control solution, pheromone solution or both solutions. We used a Bonferroni correction for multiple comparisons, such that the alpha level was 0.00278. We calculated the adjusted Pearson residuals to determine if specific categories differed from the null expectation. All statistics were conducted in R v. 3.6.2 (R Core Team 2020) and all means are reported \pm standard error.

Results

Pheromone Collection and Analyses GC-FID and GC-MS analyses indicated that iridomyrmecin was consistently present on the surface of hydrogels visited by foraging Argentine ants. Iridomyrmecin was not found in any extract from control hydrogels. Based on quantification using external standards, the quantity of iridomyrmecin per sample (i.e., four halves of hydrogel beads) was estimated as $0.43 \pm 0.16 \mu\text{g}$ ($n = 5$). Assuming iridomyrmecin was extracted from the thin layer of sugar water (e.g., 10 μ L) that was available on the surface of the hydrogels at the time of extraction, using 0.01 and 0.1 mg/mL concentrations (equivalent to 0.1 and 1 μg in 10 μ L, respectively) of iridomyrmecin for the subsequent bioassays was justified.

Bioassays *Megachile rotundata* and *Osmia lignaria* both exhibited a strong preference for the control solution and an avoidance of feeding only on the pheromone solutions (Fig. 2: *M. rotundata* low concentration pheromone: $X^2_2 = 21$, $p < 0.0001$; *M. rotundata* high pheromone concentration: $X^2_2 = 14$, $p = 0.00091$; *O. lignaria* low concentration pheromone: $X^2_2 = 24.8$, $p < 0.0001$; *O. lignaria* high pheromone concentration: $X^2_2 = 22.2$, $p < 0.0001$). In stark contrast, honey bees exhibited no preference for either solution, significantly feeding on both feeders more than expected (Fig. 2: *A. mellifera* low concentration pheromone: $X^2_2 = 34.6$, $p < 0.0001$; *A. mellifera* high pheromone concentration: $X^2_2 = 15$, $p = 0.00055$). There was no effect of pheromone concentration on bee feeding behavior for any of the three species tested ($p > 0.05$).

Discussion

A higher rate of floral visitation by insects often increases seedset (Chen et al. 2018; Waters et al. 2020), although visitation is not a perfect proxy for plant reproductive success (Morales and Traveset 2009). However, resource competition often occurs when multiple species seek out the same floral resources, like nectar (Balfour et al. 2015). Such competition has important implications for foragers, because the presence of other floral visitors may influence the ultimate foraging success of pollinators. Here we examined whether a nectar competitor, the Argentine ant, deposits any chemical

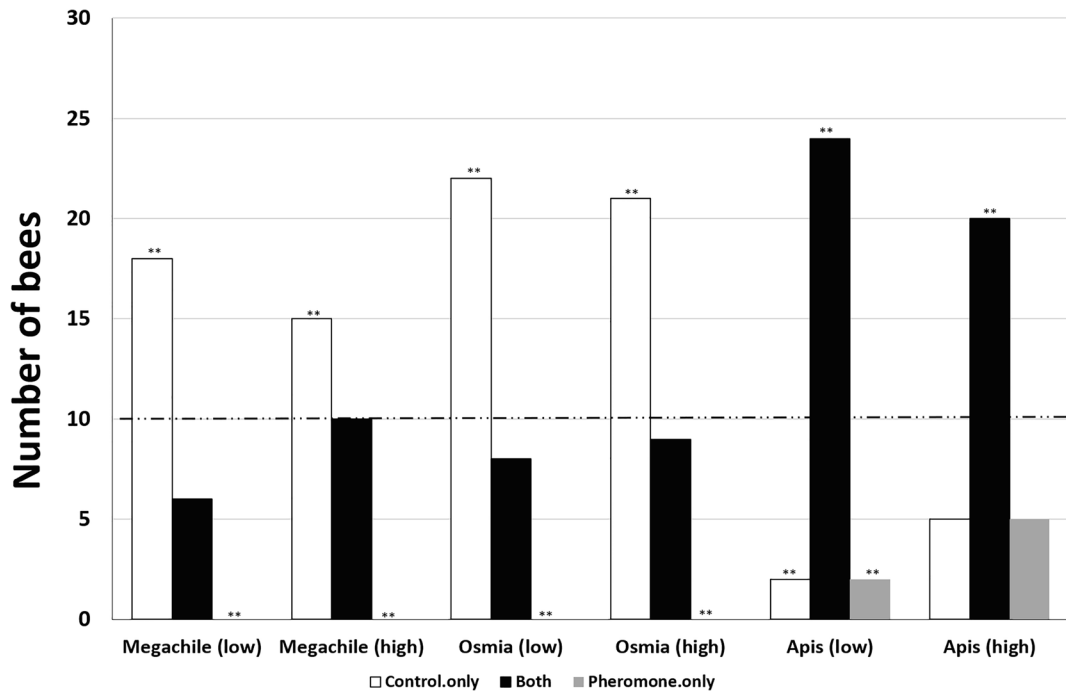


Fig. 2 Number of bees ($n = 30$ individuals per bee species) that fed either on control sucrose solution only, on both control and pheromone sucrose solutions, or on pheromone-impregnated sucrose solution only. The dashed line indicates the expected number of bees in that category assuming no feeding preference (one-third

of individuals). “Low” indicates the pheromone, iridomyrmecin, at a concentration of 0.01 mg/mL, and “high” indicates the pheromone at a concentration of 0.1 mg/mL. $\alpha = 0.00279$ with Bonferroni correction. ** $p < 0.001$

cues at the carbohydrate source during foraging, and whether those cues influence subsequent resource consumption by three different bee species.

We detected the ant-derived chemical, iridomyrmecin, on the sugar source after only 3 h of Argentine ant feeding. Iridomyrmecin is one of the iridoids produced and stored in the pygidial gland (located in the abdomen) of Argentine ants (Cavill and Houghton 1974; Cavill et al. 1976). While we did not explicitly observe the process by which iridomyrmecin was transferred to the surface of the hydrogels, it is likely that some amount of iridomyrmecin will be present on other parts of the ant’s body, such as the mouthparts, antennae, and tarsi (D.H. Choe, unpublished data). These body parts are likely to come into contact with the PAA hydrogel, subsequently transferring trace amounts of iridomyrmecin. Additionally, the foraging ants may have deposited iridomyrmecin while walking on the surface of the PAA hydrogel to find a spot to feed. Iridomyrmecin has been detected from the chemical trails laid by Argentine ants for foraging and for nest relocation (Choe et al. 2012). Lastly, it is possible that

the ants deposited iridomyrmecin around the PAA hydrogel, but the compound was volatilized and adhered to the surface of the hydrogel. Elucidating the exact mechanisms of pheromone deposition at the sugar source requires future research. Furthermore, the presence of dolichodial (another minor constituent of Argentine ant trail pheromone) (Choe et al. 2012) in the sugar source and its impact on pollinators should be examined.

The presence of iridomyrmecin in the sucrose solution had strong effects on artificial nectar consumption by two solitary bee species in the family Megachilidae: *M. rotundata* and *O. lignaria*. While solitary bees have been documented to change their foraging behavior based on the identity of the bee species occupying a flower (e.g., Yokoi and Fujisaki 2009; Yokoi and Fujisaki 2011), this is the first report, to our knowledge, of solitary bees responding specifically to ant chemicals at a food resource. The pheromone was presented in the sugar water at biologically relevant concentrations (0.1 and 1 mg/mL). Along with our quantification study of iridomyrmecin in the sugar source, our bioassay

results suggest that the chemical cues left by foraging Argentine ants significantly alter the foraging behavior and resource collection of megachilid bees. Repellent or irritant properties of iridomyrmecin for other ant species have been previously reported when the compound is either presented on the prey item or used to topically treat the ants (Stökl et al. 2012; Welzel et al. 2018).

It is important to point out that naïve *A. mellifera* neither avoided nor preferred the sucrose solution containing iridomyrmecin. Rather, we observed honey bees foraging from both control and treated artificial nectars. This lack of avoidance behavior is similar to that observed by the Asian honey bee (*Apis ceranae*) in response to chemical compounds produced by red imported fire ants, *Solenopsis invicta* (Wu et al. 2016). However, this lack of avoidance is in stark contrast to the responses of two megachilid bee species (this study) and other flower visiting insects (Wu et al. 2016).

Here, life history may partly explain the difference found between the solitary bee species and the honey bee in their response to sucrose solution with iridomyrmecin. Both *M. rotundata* and *O. lignaria* are solitary, and as such do not maintain food stores akin to the honey and pollen stores of *A. mellifera*. Thus, the innate avoidance to iridomyrmecin by both *M. rotundata* and *O. lignaria* may be indicative of the risk averse nature of solitary species. A female solitary bee is likely to have decreased fitness if she struggles to find resources (Zurbuchen 2010), is harassed (Rossi et al. 2010) or is preyed upon at a food source. This is in contrast to the European honey bee, whose eusocial life history may suggest that an individual forager is expendable and can be lost with no significant impact on the inclusive fitness of the colony as a whole. Alternatively, there may be a phylogenetic pattern: both species responding to iridomyrmecin are in the family Megachilidae, while honey bees are in the family Apidae. Future research into which different bee taxa exhibit an aversion to iridomyrmecin would be the first step toward identifying the underlying mechanism of the differential responses observed across species of bees.

In the field, previous experience by a bee forager may play into its subsequent foraging decisions (Leadbeater and Chittka 2009; Miner 2018). Learning of chemical cues is particularly common in pollinators, who may use olfaction to locate flowers (Wright and Schiestl 2009) and to avoid competitors (Sidhu and Wilson Rankin

2016) or predators (Wang et al. 2016). Ballantyne and Willmer (2012) demonstrated that naïve bumble bees in a laboratory were not inherently repelled by scent marks of the ant, *Lasius niger*. However, the bumble bees could readily associate ant scent marks with a lack of reward while foraging on sugar rewards (Ballantyne and Willmer 2012). Here, by including only naïve bees in the assay, we could exclude the possibility that experience with ants or the learning of ant cues influenced consumption of sucrose solution. These newly emerged bees had no opportunity to gain any exposure to Argentine ants or any chemical cues associated with the ants before participating in assays. Given that naïve *M. rotundata* and *O. lignaria* both avoided iridomyrmecin, it is likely that these bees have an innate avoidance to the ant pheromone. Additional research is needed to explicitly test this hypothesis and to determine if such an aversion is widespread.

Summary

Our data suggest that flowers or other sugar sources that are visited by Argentine ants are likely to have iridomyrmecin deposited on them. Laboratory bioassays indicated that the presence of iridomyrmecin in an artificial nectar source at biologically relevant concentrations significantly affects the sucrose-solution feeding behavior of the “naïve” bees of the solitary species, *Megachile rotundata* and *Osmia lignaria*, but not of the eusocial *Apis mellifera*.

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Author Contributions DHC designed chemical experiments; QYL, EP, and DHC conducted chemical experiments; EWR designed the bioassays; EWR, JMC and NHP conducted the bioassays; EWR conducted statistical analyses; and DHC, JMC and EWR wrote the manuscript.

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Compliance with Ethical Standards**Conflict of Interest** Not applicable.**Ethics Approval** Not applicable.**Consent to Participate** Not applicable.**Consent for Publication** Not applicable.**References**

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