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SHORT COMMUNICATION

Trophic specialization of a newly described spider ant symbiont, *Myrmecicultor chihuahuensis* (Araneae: Myrmecicultoridae)

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Abstract. The spider *Myrmecicultor chihuahuensis* Ramírez, Grismado & Ubick (Myrmecicultoridae) was described in 2019 and hypothesized to be a myrmecophile, living inside the nests of *Novomessor* (Myrmicinae) and perhaps also *Pogonomyrmex* (Myrmicinae) ants. To test the hypothesis that *M. chihuahuensis* are chemical mimics of their host ants, we carried out behavioral bioassays to observe interactions between the spiders and the host ants. We compared the cuticular hydrocarbon (CHC) profiles of the spiders and the ants. We discovered that this new species of spider is a myrmecophage, displaying hunting strategies similar to other myrmecophagous spiders, particularly those in the genus *Zodarion* Walckenaer, 1826 (Zodariidae). The CHC profile of *M. chihuahuensis* did not match the profile of the ants with which it associates, *Novomessor albisetosus* (Mayr), but ants and spider shared several compounds, potentially involved in species recognition.

Keywords: Myrmecophage, *Zodarion*, *Novomessor*, *Pogonomyrmex*, ant guest

The spider, *Myrmecicultor chihuahuensis* Ramírez, Grismado, & Ubick (Myrmecicultoridae) was described in 2019 (Ramírez et al. 2019) (Fig. 1) and was hypothesized to be an ant inquiline. This small spider has only been found in association with ant nests, specifically nests of *Novomessor albisetosus* (Mayr, 1886) (Myrmicinae), *N. cockerelli* (André, 1893), and *Pogonomyrmex rugosus* Emery, 1895 (Myrmicinae). Ramírez et al. (2019) and Azevedo et al. (2021) carried out multi-locus phylogenetic analyses suggesting that *M. chihuahuensis* does not belong to any known spider family. Therefore, Ramírez et al. (2019) placed this species in its own family, the Myrmecicultoridae. *Myrmecicultor chihuahuensis* shares some morphological similarities with spiders in the families Zodariidae or Prodidomidae (Ramírez et al. 2019) but, based on the molecular data, is distinct from those families.

David Lightfoot observed these mysterious spiders moving on the mound of a *P. rugosus* nest in the Chihuahuan Desert in Mexico (fig. 11F in Ramírez et al. 2019). This was the only known instance that live spiders of this species have ever been observed on the surface of ant colonies. Lightfoot (pers. comm.) indicated that the spiders appeared to approach and contact a worker ant using their front legs. This type of contact has been reported in other species of myrmecophilous spiders living inside or in close proximity to ant colonies (Donisthorpe 1927; Allan & Elgar 2001; Erthal & Tonhasca 2001; reviewed in Cushing 1997) and may be a mechanism for myrmecophiles to acquire the cuticular hydrocarbon (CHC) profile of host ants (reviewed in Akino 2008, Nash & Boomsma 2008; von Beeren et al. 2011, 2012a; Parker 2016).

Lightfoot's observations, the fact that these spiders have only been collected from pitfall traps placed 0.5 – 1 m from the nest entrances of ants, and the collection of specimens from soil sifted from excavated ant nests (see Ramírez et al. 2019) supported the hypothesis that these spiders are myrmecophiles that live inside nest chambers. It was further hypothesized in the 2019 paper that these small spiders (adults slightly < 3 mm in length) are likely feeding on other small arthropods living inside the colonies as has been demonstrated for other species of spider myrmecophiles (Porter 1985; Cushing 1995).

To live alongside ants, the spiders either need to evolve behavioral avoidance strategies, mechanical defense mechanisms, or chemical or morphological adaptations that allow them to avoid being detected by the hosts (Lenoir et al. 1999; Cushing 1997, 2012; Geiselhardt et al. 2007; Akino 2008; Ceccarelli 2013; Parmentier et al. 2017; Fischer et al. 2020; Lorenzi & d'Ettorre 2020). Based on field observations, we hypothesized that *M. chihuahuensis* do live closely associated with or inside the host colonies. We further hypothesized that these spiders may show some degree of behavioral interaction with the host ants that could suggest some form of chemical mimicry. To test our hypotheses, we: (1) carried out behavioral bioassays with living spiders and host ants to determine the level of behavioral interaction and (2) compared the CHC profiles of spiders and ants collected from the same nests in 2019 and 2020 to determine if there was evidence for chemical mimicry.

In October 2019, we traveled to the Dalquest Desert Research Station (DDRS) located in the Chihuahuan Desert in Texas, United States to excavate ant nests and look for spiders inside. During an excavation of a *N. albisetosus* nest carried out 23–24 October 2019, one juvenile *M. chihuahuensis* and one adult female were collected from an excavation pit at the DDRS (29.55420° N, 103.78640° W; DMNS ZA.41776 in https://scan-bugs.org/portal/index.php). The one female and juvenile spider along with samples of the respective host ants from the same colony were flash frozen and sent to AB to compare the CHC profile of the spiders and the ants. From 28 September to 5 October 2020, we revisited DDRS and used dry pitfall trap sampling to capture live spiders that could be used for behavioral bioassays and could also be subsequently sent to AB for CHC analysis along with additional samples of host ants.

We set six dry pitfall traps 0.5 – 1.0 m from primary nest openings of *N. albisetosus*. Each trap consisted of two plastic cups set flush to the surface of the ground with a smaller plastic cup set inside these with the bottom cut out; a flat rock was suspended over these cups to
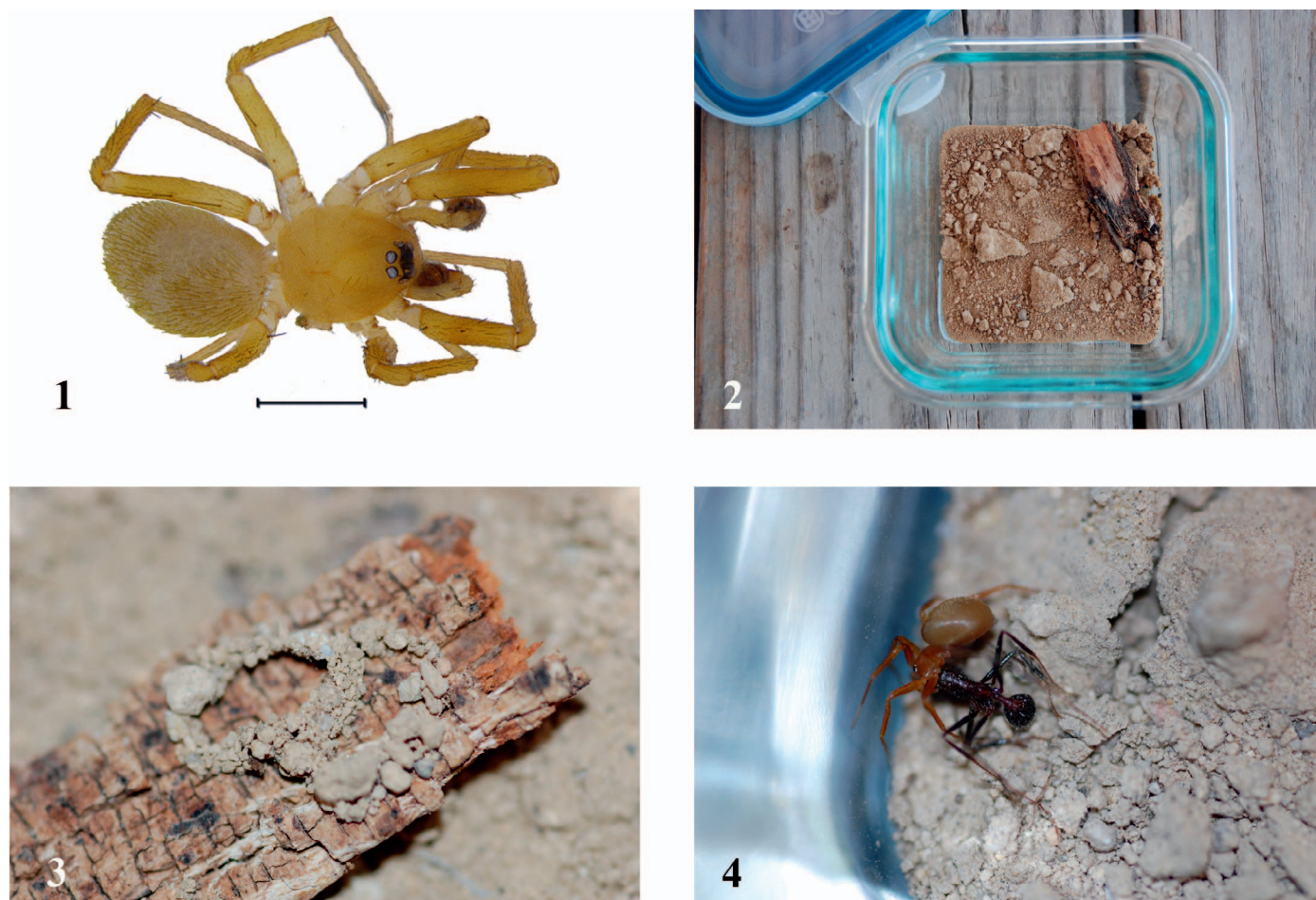
Figure 1–4.—The myrmecophagous spider *Myrmecicultor chihuahuensis* (Myrmecicultoridae). (1) *Myrmecicultor chihuahuensis* male (DMNS ZA.41782). Scale bar = 1 mm. (2) 10 × 10 cm diameter Pyrex dish used for behavioral observations. The small piece of bark provided a hiding place for the spiders. (3) Circular corrals made from soil granules by *Myrmecicultor chihuahuensis* females. (4) *M. chihuahuensis* feeding on *Novomessor albisetosus* with fangs inserted behind ant's head.

keep the interior of the trap from overheating in the desert habitat. Dry pitfall traps (one per nest) were placed at the beginning of the fieldwork and monitored each morning thereafter to check for spiders.

Two live female spiders (*M. chihuahuensis*) were collected from a single pitfall trap set next to one *N. albisetosus* nest. No other live spiders were collected in the vicinity of the remaining nests. Seven *N. albisetosus* host ants from the same nest were also collected to use in the behavioral bioassay. The spiders were introduced into a small glass Pyrex dish 10 cm^2 x 4 cm deep. A thin layer of soil was placed in the bottom of this container and a small piece of bark was placed against one wall to provide a hiding place for the spiders (Fig. 2). At 18:30 in the evening, we began observations of any interactions between spiders and ants using a red light to reduce disturbance from light. Since spiders had previously only been recorded on the surface of ant nests at night (Lightfoot, pers. obs.), it was assumed that this species was nocturnal.

For the analysis and comparison of CHCs of spiders and their putative host ants, freeze-killed specimens of potential host ants (10 *N. albisetosus* from both the 2019 and 2020 trips) and spiders (4 *M. chihuahuensis* including the two females from 2020 as well as the female and juvenile from 2019) were used. The live females and the *N. albisetosus* ants collected in 2020 were flash frozen using dry ice after the behavioral bioassay was completed; the other two spiders

collected in 2019 from excavation pits were also flash frozen with dry ice and sent to AB for analysis along with host ants.

The flash frozen ants and spiders were submersed in 150 μl hexane with an internal standard for 20 min. For chemical profiling, we used a GCMS-QP2020 gas chromatography/mass-spectrometry system (Shimadzu, Kyōto, Japan) equipped with a ZB-5MS fused silica capillary column (30 m x 0.25 mm ID, df = 0.25 μm) from Phenomenex (Torrance, CA, USA). Samples (1 μl for ants and 1 μl for spiders) were injected using an AOC-20i autosample, into split/splitless-injector in splitless-mode at a temperature of 310°C. Helium was used as the carrier-gas with a constant flow rate of 2.13 ml/min. The start temperature was set to 40°C with a 1-minute hold; after that the temperature was increased 30°C/min to 250°C and further increased 50°C/min to a final temperature of 320°C and held for 5 minutes. Electron impact ionization spectra were recorded at 70 eV ion source voltage, with a scan rate of 0.2 scans/sec from m/z 40 to 650. The ion source of the mass spectrometer and the transfer line were kept at 230°C and 320°C, respectively. CHCs were identified using diagnostic ions and retention indices calculated based on a standard series of n-alkanes (Carlson et al. 1998) and compared to CHC available for *N. cockerelli* (Smith et al. 2009). The double bond positions in alkenes were determined by iodine-catalyzed methyl-thiolation of double bonds using DMDS (dimethyl-disulfide, Sigma-Aldrich, St. Louis, MO) and subsequent mass spectrometry according

to Carlson et al. (1989). For statistical comparison of the CHC profiles, we used a PERMANOVA analysis on a Bray-Curtis similarity matrix using species as fixed effect in R 3.6.1 (R Core Team 2019) based on a previously published code (Brückner & Heethoff 2017).

After introducing the two female spiders to the Pyrex enclosure (Fig. 2), they built arena-shaped retreats out of sand particles beneath the wood fragment (Fig. 3) (apparent the morning after introduction into the enclosure). When the ants were introduced to the enclosure, both spiders were beneath the wood fragment. Soon after introducing the ants, the spiders emerged from under the wood, presumably in response to the ants' movements in the container. We watched and filmed several successful hunting sequences under red light so as not to disrupt the spiders' behaviors. The behavior of the spiders towards the ants was consistent: a spider repeatedly approached an ant, quickly moving away when the ant turned toward the spider (see supplemental videos 0212-03 and 0215-09, online at https://doi.org/10.1636/JoA-S-21-072.s1 and https://doi.org/10.1636/JoA-S-21-072.s2 respectively). In no instance did either spider approach an ant frontally. After pursuing an ant around the enclosure, the spider rushed at the ant and bit a rear leg, then retreated (see supplemental video 0215-09). The effects of the spider's venom were apparent within a few seconds (See supplemental video 0215-11, https://doi.org/10.1636/JoA-S-21-072.s3). The ant's movement slowed, the body bent, and after a few minutes, paralysis from the venom was complete. The spider then re-approached, inserted its fangs behind the ant's head, lifted, and carried the ant toward the wood fragment (Fig. 4).

In one hunting sequence, one of the spiders bit a single ant repeatedly before moving away. We also observed instances when a live (unbitten) ant approached the encumbered spider. In each of these instances, when an active ant approached the spider encumbered with a paralyzed ant, the spider immediately repositioned, turned, or flipped itself so that the approaching ant would encounter the paralyzed nest mate, rather than the spider. The spider easily manipulated the paralyzed ant even though it was larger than the spider and utilized the dead (or dying) ant as a shield. The spider's attack of the host ants could be described as an ambush. In no instance did we observe any evidence of spiders luring the ants or of spiders using their front legs to "pseudo-antennate" the hosts as was suggested by David Lightfoot's observations in the field.

Comparing the CHC profiles of the spider and the putative host ants (*N. albisetosus*), we found 61 compounds from cuticle extractions (Table 1; Fig. 5), seven of which remain to be elucidated. Sixty of the compounds were detected for the spider, while we found 42 CHC-related compounds for *N. albisetosus* (Table 1). A majority of compounds was shared by both species including most n-alkanes (C27–C32), the respective mono-methyl alkanes of similar length (see Table 1 for details and Fig. 5). The eight CHCs which appeared to be unique to the spider species included mainly monomethyl and dimethyl alkanes with a C26 backbone (Table 1). Although the chemical profiles (Fig. 5) and overall composition of CHCs between ants and spiders was different (PERMANOVA: N_{permu} = 9999; *pseudo*F = 4.34; P = 0.0011), both species shared several compounds in similar ratios (17/61 CHC; ~25%).

It is clear from these observations that *M. chihuahuensis* is a myrmecophage. The hunting behavior of this spider is similar to that reported for other species of myrmecophages (reviewed in Cushing 2012). These spiders avoid frontal attacks, instead approaching ants from the rear. Their hunting strategy is very similar to that of species of *Zodarion* Walckenaer, 1826 (Pekár 2004). After paralyzing an ant, the spider carries and uses it as a shield, presenting the dead ant to any approaching nest mate. This behavior is very similar to the hunting strategy noted in various species of *Zodarion* (Zodariidae) (Pekár & Král 2002; Couvreur 1990a, b). Such a rear attack and even the use of dead ants as "shields" has also been noted in several other genera of myrmecophagous spiders in the family Thomisidae such as

Table 1.—Cuticular hydrocarbons of *Novomessor albisetosus* and *Myrmecicultor chihuahuensis*. The retention indices (RI), identified CHCs and their mean (±SD) abundances. Double bonds of alkenes (*) were determined by DMDS derivatization. n.d. denotes non detected compounds.

| RI	compound	*N. albisetosus*	*M. chihuahuensis*
2500	C25	1.88±1.12	0.73±0.62
2536	11-; 13-Me-C25	0.07±0.09	0.29±0.17
2575	3-Me-C25	0.09±0.09	0.63±0.67
2600	C26	0.53±0.15	1.76±1.88
2606	8-Me-C26	n.d.	0.92±0.88
2628	10-,14-Me-C26	0.11±0.12	0.24±0.28
2664	2- or 4-Me-C26	n.d.	0.24±0.24
2670	Me-C26	n.d.	0.21±0.22
2688	4,12-Dime-C26	n.d.	0.17±0.19
2700	C27	4.61±2.4	4.88±1.78
2731	9-,11-,13-Me-C27	1.79±0.82	3.14±2.72
2736	Me-C27	0.06±0.09	0.33±0.23
2742	7-MeC27	0.54±0.38	0.9±0.51
2751	5-MeC27	0.88±0.55	0.67±0.53
2774	3-MeC27	0.93±0.63	4.43±5.5
2800	C28	2.13±1.14	2.28±1.71
2805	3,7-;3,11-Dime-C28	n.d.	1.67±1.47
2829	10-,14-Me-C28	0.75±0.84	4.7±5.27
2858	unknown	n.d.	0.39±0.33
2863	2- or 4-Me-C28	n.d.	0.5±0.43
2871	meC28	n.d.	0.67±0.34
2875	C29-9-ene*	1.79±0.39	2.01±3.26
2888	4,12-Dime-C28	n.d.	1.69±1.17
2900	C29	10.46±7.21	3.75±2.19
2930	9-,11-,13-,15-Me-C29	2.97±0.98	2.59±1.26
2939	7-Me-C29	0.89±0.57	0.72±0.7
2952	5-Me-C29	1.46±1.18	0.61±0.3
2965	2-Me-C29	n.d.	0.91±0.52
2973	3-Me-C29	1.81±0.62	1.49±0.88
2986	5-,x-Dime-C29	n.d.	0.51±0.48
3000	C30	1.28±0.28	1.32±1.06
3024	10-,14-Me-C30	1.63±0.45	1.37±0.27
3055	unknown	n.d.	0.55±0.36
3064	2- or 4-Me-C30	n.d.	0.36±0.42
3080	C31-9-ene*	15.13±1.55	0.49±0.19
3100	C31	1.2±0.65	5.6±4.27
3130	9-;11-;13-;15-Me-C31	8.27±1.53	5.88±1.83
3142	7-Me-C31	1.08±1.05	0.96±1.06
3153	5-Me-C31	4.09±0.62	5.24±1.97
3162	unknown	2.26±0.67	n.d.
3179	unknown	0.93±0.27	1.13±0.37
3200	C32	1.09±0.12	2.63±0.85
3230	10-,14-Me-C32	3.91±0.67	3.82±1.51
3255	unknown	0.76±0.1	2.25±3.12
3268	2- or 4-Me-C32	n.d.	0.85±1.1
3277	Me-C32	0.19±0.14	0.71±0.52
3290	C33-ene	0.93±0.09	0.4±0.35
3310	unknown	n.d.	0.81±0.91
3344	9-;11-;13-;15-Me-C33	9.7±1.57	7.5±1.36
3353	7-Me-C33	1.4±1.58	1.1±1.3
3371	Dime-C33	2.96±0.98	2.91±0.82
3388	5-,x-Dime-C33	n.d.	0.28±0.24
3400	C34	0.02±0.07	0.27±0.32
3437	Me-C34	0.39±0.19	0.65±0.12
3472	Dime-C34	1.65±1.03	1.24±0.32
3495	unknown	1.34±1.43	0.55±0.95
3500	C35	n.d.	1.11±0.67
3624	10-,14-Me-C36	3.27±0.63	2.65±1.1
3658	Dime-C36	2.8±0.4	3.28±0.45
3740	9-;11-;13-Me-C37	n.d.	0.04±0.08
3750	7-Me-C37	n.d.	0.02±0.03
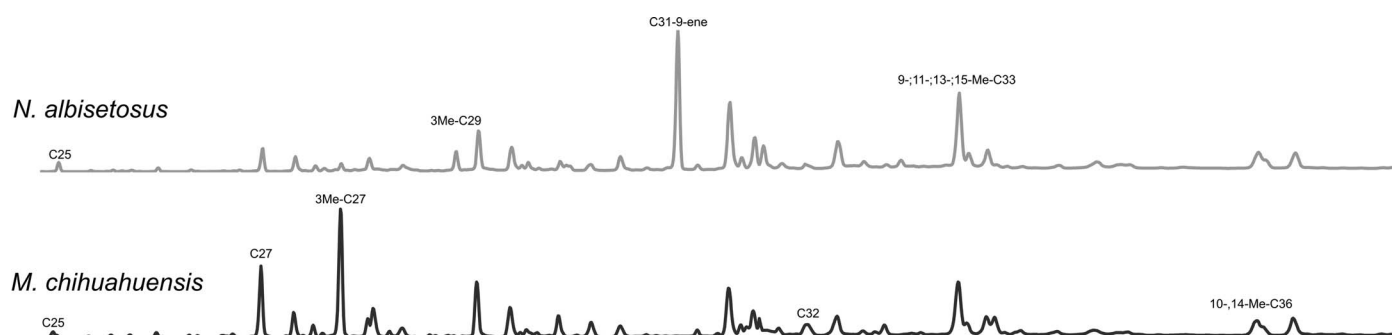
Figure 5.—Cuticular hydrocarbon (CHC) profiles of the spider *Myrmecicultor chihuahuensis* and *Novomessor albisetosus* ants. Example gas chromatographic traces of CHCs extracted from the spider and ant species. Major CHCs are labeled; complete list by retention index can be found in Table 1.

Aphantochilus rogersi O.P.-Cambridge, 1871 (Piza 1937; Oliveira & Sazima 1984; Castanho & Oliveira 1997) and *Bucranium* spp. (Thomisidae) (Piza 1937; Bristowe 1941; Oliveira & Sazima 1984; Castanho & Oliveira 1997; reviewed in Cushing 2012). The similarity in trophic hunting behavior between these spiders in the new family Myrmecicultoridae and those in the family Zodariidae is particularly interesting given the observation in Ramírez et al. (2019) that the new species shares certain morphological characters as those in the family Zodariidae such as the short posterior spinnerets, the strongly procurved anterior eye row, and similarities in the leg setae.

The two female spiders killed nine ants in the course of two nights. All ants added to the Pyrex container were rapidly killed by the spiders, although they were observed feeding only on one or two of these ants. This level of carnage, or "over-kill," has also been documented for species of *Zodarion* (Pekár 2005; Pekár & Toft 2015). This over-kill is hypothesized to be a strategy related to the specialized hunting of dangerous prey (Líznarová & Pekár 2013). However, it may be an artifact of the small size of the enclosure and the inability of the spiders or ants to escape one another.

There are many strategies ant symbionts (myrmecophiles and myrmecophages) use to achieve association with their hosts, ranging from behavioral adaptations and morphological defense to chemical mimicry and weaponry (e.g., Akino 2008; Kronauer & Pierce 2011; von Beeren et al. 2012b; Parker 2016; Parmentier 2019). Here, we gathered preliminary data to test whether the ant associated spider, *M. chihuahuensis*, uses chemical mimicry of its host's CHCs to live in close proximity with the ant. We found that the spider CHCs did not match that of *N. albisetosus*, but did show some similarity to the chemical profile of the ants. As with our study, Pekár & Jiroš (2011) found some similarity in CHC profiles between the myrmecophagous *Zodarion alacre* (Simon, 1870) spider that feeds on *Iberoformica subrufa* (Roger, 1859) (Formicinae). These authors hypothesized that the ant-associated CHC compounds found in *Z. alacre* might be produced *de novo* by the myrmecophagous spider. The mechanism by which *M. chihuahuensis* acquire their CHCs (or manufacture these chemical signals) remains unknown. It is also unclear whether or not the chemical signature of *M. chihuahuensis* is similar enough to allow the spider to become fully integrated into the colony or just allow the spider to hunt these dangerous prey without risk of attack. The eight CHCs that appear to be unique to the spider profile are likely to contain enough information content for the ants to recognize *M. chihuahuensis* as a foreign organism and elicit a defensive response, although this also remains to be tested. *Novomessor* ants are not particularly aggressive towards non-colony members (Whitford et al. 1980) hence we hypothesize that this non-aggressiveness may enable the spider to live in close nest proximity even while displaying a mismatching CHC profile. Like zodariids, this spider may build its corral-shaped retreats under debris in the vicinity of the nests

(Cushing & Santangelo 2002). Our observations do not support the hypothesis that these spiders are inquilines.

Many aspects of the natural history of this ant myrmecophage remain to be discovered including how they locate their host ant colonies (assuming they are stenophagous and prey only on particular species of ants). It has been suggested for other species of myrmecophagous spiders that these specialized stenophagous hunters may eavesdrop on ant pheromones as a strategy for locating their prey (Allan et al. 1996; Clark et al. 2000; Cárdenas et al. 2012; Fischer 2019; Adams et al. 2020). It is also unknown what juvenile spiders feed on. Clearly much remains to be discovered about the biology and chemical ecology of this interesting new species of myrmecophage.

ACKNOWLEDGMENTS

SUPPLEMENTAL MATERIALS

Video clips taken with iPhone under red light of *Myrmecicultor chihuahuensis* females hunting *Novomessor albisetosus* ants.

Supplemental Video 1 (0212-03) Hunting sequence – spider biting rear legs of ant, online at https://doi.org/10.1636/JoA-S-21-072.s1

Supplemental Video 2 (0215-09) Hunting sequence – spider ambushing ant from rear and biting multiple times, online at https://doi.org/10.1636/JoA-S-21-072.s2

Supplemental Video 3 (0215-11) Effect of spider venom on bitten ant, online at https://doi.org/10.1636/JoA-S-21-072.s3

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