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ORIGINAL ARTICLE

Ability to reorient is weakly correlated with central-place versus non-central-place foraging in acacia ants

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Abstract
Cognitive abilities evolve by natural selection to help an organism cope with problems encountered in the organism's typical environment. In acacia ants, coevolution with the acacia tree led workers to forage exclusively on the host plant ("in-nest" foraging), instead of the central-place foraging typical for most social insects. To test whether foraging ecology altered the orientation skills of acacia ants, we developed a novel field disorientation assay to evaluate the ability of foraging workers to quickly reorient after being disoriented (rotated) in an experimental arena. We compared 10 behaviors among disoriented and sham-treated workers of three in-nest foraging species (*Pseudomyrmex nigrocinctus*, *P. flavicornis*, and *P. spinicola*) and two central-place foraging species that regularly forage off the host tree (*P. gracilis*, *P. nigropilosus*). We predicted that experimental disorientation of workers should affect in-nest foraging species (acacia ants) more than central-place foraging species. Behavioral differences between control and disoriented ants were not consistently associated with foraging ecology, although the species least able to recover after disorientation was an acacia ant (*P. nigrocinctus*), and the species performing best after disorientation was a central-place forager (*P. gracilis*). Only one of the 10 behaviors studied consistently differed in experimentally disoriented workers compared to controls in all three species of acacia ants, whereas none of the experimentally disoriented central-place foragers differed from control workers for this specific behavior. Future studies could evaluate additional ant species living in obligate associations with plants, to further compare the cognitive abilities of in-nest versus central-place foraging organisms.

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Significance statement
Foraging ecology influences the evolution of spatial orientation abilities. Acacia ants exclusively nest and forage on acacia trees; unlike most other ants that are central-place foragers, acacia ants therefore do not face the challenge of finding the way back home after collecting food. We compared the performance of three species of acacia ants to the performance of two species of central-place foragers on a disorientation assay in the field. We found that the ability to reorient was not consistently associated with foraging ecology, although the species least able to recover after disorientation was an acacia ant (*P. nigrocinctus*), and the species performing best after disorientation was a central-place forager (*P. gracilis*). Other behaviors related with the mutualism with the acacia tree—such as pruning nearby vegetation and falling off branches to attack potential herbivores—could also select for orientation abilities because workers reorient back to the host tree.

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Central-place foraging—returning to a particular place to provision offspring—relies on spatial orientation abilities, which are key in the survival and reproduction of mobile animals (Orians and Pearson 1979; Dyer 1998; Collett et al. 2013). Social insects in the order Hymenoptera (wasps, bees, ants) are typical central-place foragers; some species show striking orientation abilities that enable them to exploit resources located hundreds of meters or kilometers away from their nest (Beekman and Ratnieks 2000; Steck et al. 2009; Zurbuchen et al. 2010; Pahl et al. 2011; Huber and Knaden 2015). Life history and ecological demands facilitating the evolution of central-place foraging therefore can influence also cognitive abilities and navigational skills of such animals (Shettleworth 2009; Smulders et al. 2010; Wystrach et al. 2013).

Orientation studies have traditionally focused on how specific ecological demands cause animals to excel at navigational tasks. For instance, bird species that store food in general outperform closely related non-storing species in spatial memory tests (Balda and Kamil 1989; Shettleworth 1990; Hilton and Krebs 1990; Clayton and Krebs 1994; Bednekoff et al. 1997; Balda and Kamil 2002; Balda and Kamil 2006). However, orientation skills of animals may also decrease in response to a relaxation of the ecological pressure selecting for navigational skills. Our study is the first to explore orientation skills in plant-associated ant species.

Ants evolved from hunting and scavenging central-place foragers (Collett et al. 2013; Ward 2014), and many extant species are predators. However, ant species that evolved obligatory mutualisms with plants are no longer predators. Plants in obligate mutualisms with ants usually provide protected nesting spaces and food (e.g., nectar and protein-rich food bodies; Hanson and Longino 2006; Rico-Gray and Oliveira 2007). Workers of such species do not leave the plant to forage because foraging co-occurs within the nesting place; hence, workers perform "in-nest" foraging. We can expect evolutionary change in cognitive abilities for spatial orientation in ants that are obligate plant-mutualists compared to typical central-place foragers, as workers are no longer orienting outside the nest.

A classical example of "in-nest" foragers are acacia ants (*Pseudomyrmex* sp.), which form obligatory mutualisms with acacia trees (Janzen 1966; Janzen 1974). Acacia ant workers exclusively feed on the nectar produced by all leaves and feed the larvae with protein-rich food bodies (called Beltian bodies) that the host tree grows on the tips of young leaves for the sole purpose of feeding the ant brood. Ants nest inside the tree's swollen and hollow spines, where workers attend the queen and brood, and store Beltian bodies. Workers only leave the acacia tree (i.e., the nest) to kill encroaching vegetation by pruning plants competing with the host tree, and to patrol neighboring acacia seedlings for possible colonization (Janzen 1966). Species of acacia ants vary in the area they keep clear from non-acacia vegetation, that is, in how far workers travel away from the host tree (Fig. 1; Amador-Vargas *in. prep.*; Janzen 1966). Acacia ants are therefore "in-nest" foragers, but workers may venture for short distances away from the nest when pruning non-acacia vegetation.

Although workers in obligate plant associations do not forage outside the nest, other ecological pressures can still have strong demands on orientation skills of workers. In obligate ant-plant mutualisms, this other ecological pressure is that workers sometimes leave the host plant to kill encroaching vegetation by pruning, and then they need to return to the host plant (Morawetz et al. 1992; Renner and Ricklefs 1998; Frederickson et al. 2005; Amador-Vargas 2012a). Hence, the behavior of pruning nearby vegetation and the distance away from the host plant that workers travel to do so may enhance or maintain their orientation abilities, counterbalancing the lower navigational demands of in-nest foraging. Acacia ants do not migrate their nest and nest founding is performed solely by the queen.

In this study, we compared the ability to reorient after an experimental disorientation of workers from in-nest and central-place foraging species of *Pseudomyrmex*. In a novel field assay, we disoriented workers that were retrieving a food item off the host tree by rotating a feeding arena. We then measured several behaviors of the presumably disoriented workers when trying to return to the host tree and compared these behaviors to control workers (not rotated, and presumably not disoriented) of the same species. If foraging inside the nest (host plant) has indeed relaxed selection on orientation skills, then we expected the experimental treatment (rotation of the arena) to have an effect of the behavior of in-nest foraging species but not of central-place foraging species. In other words, we expected our treatment to disorient in-nest foragers more than central-place foragers. We also expected the disorientation test to have a greater effect on workers of the acacia ant species that travel the shortest distance away from their host tree, *P. nigrocinctus* and *P. flavicornis* ("short-distance" travelers, ~10–30 cm), compared to workers of *P. spinicola* that travel longer distances away from the host tree ("long-distance" travelers walk about 2 m around the host tree; Fig. 1a–c).

Materials and methods

Study site and species

The study was carried out in 2012 and 2013 at Palo Verde National Park (10° 21′ N, 85° 21′ W) in Guanacaste, Costa Rica, near the facilities of the Organization for Tropical Studies. Palo Verde National Park protects a dry forest with secondary growth (1500-mm mean annual rainfall) where acacia trees *Vachellia collinsii* (formerly, *Acacia collinsii*) are

Fig. 1 Acacia ant species vary in the extent of the area that workers prune vegetation around the base of the host tree. Clearings of vegetation pruned by (**a**) *Pseudomyrmex nigrocinctus* and (**b**) *P. flavicornis* have radii about five times smaller than clearings of (**c**) *P. spinicola*

abundant. An acacia tree is usually inhabited by a single colony of ants with one queen.

We compared the behavior of three mutualistic acacia ant species with in-nest foraging (*Pseudomyrmex nigrocinctus*, *P. flavicornis*, *P. spinicola*) and two sympatric species with central-place foraging (*P. nigropilosus*, *P. gracilis*; Table 1). All three species of mutualistic ants defended their host tree against potential herbivores and kill vegetation growing around the host tree by pruning, producing a circular clearing that varies in size depending on the ant species (Fig. 1a–c). The central-place foragers *P. nigropilosus* obligatorily nests on acacia trees and exploits the plant rewards without providing protection against herbivores (Janzen 1975); workers also forage off the host plant to extract food from neighboring ant-defended acacia trees, and these workers therefore must travel back to the nesting tree to store the stolen food (Amador-Vargas 2012b). The other central-place forager, *P. gracilis*, facultatively nests on acacia trees (i.e., colonies of this species can be found nesting in hollow twigs of other tree species) where it feeds from the nectar rewards but without protecting the tree against herbivores or vegetation; workers also forage off the host tree, on other vegetation and on the ground, to prey on small arthropods to feed the larvae, relying less on the acacia Beltian bodies as protein source (pers. obs.; Clement et al. 2008).

Orientation assays

To compare the orientation abilities of the five species of acacia ants, we conducted orientation assays in the field. We placed food for the larvae on the center of a disc on the ground (Fig. 2), 20 cm away from the host tree, so when an ant found this food item, it would return to the host tree to store it. We baited ants with Beltian bodies freshly cut from the host tree as food, and we used dead mosquitoes as bait for the predatory *P. gracilis* workers. Offering different types of food to different ant species is unlikely to affect our results because our analysis compares control and treated ants of the same species. The disc (30.5 cm of diameter) had two strings attached to the

ground-facing side, which could be pulled to rotate the disc clockwise or counterclockwise (Fig. 2). When an ant worker was picking up the food item with its mandibles, we rotated the disc 45° clockwise or counterclockwise by pulling the strings. In control treatments, we rotated the disc first about 20° one direction, and then back to the original position, a total rotation summing to about 45°. This manipulation controlled for the motion of the disc without changing the visual cues at the beginning and end of the control back-and-forth rotation. This rotation was aimed at visually disorienting the ant, as it changed the position of the stripe on the disc with respect to the landscape (including the acacia tree where the ants have to return).

Because we were interested in disorienting the workers, the discs had a masking tape pasted across and painted in white color (Fig. 2). This white stripe was aligned in the direction of the host tree, pointing at one end to where ants walked onto the disc at the beginning of the experiment (Fig. 2). This line was a conspicuous visual cue (white and raised), if used by a worker for orientation, the line would lead the worker to the point where they had entered the disc on control trials, but to a different place on rotated trials. Consequently, if workers relied only on the white stripe on their way back when the disc was rotated, they would be disoriented. It is important to emphasize that we lack mechanistic studies about the orientation mechanism of acacia ants, but we would know that our manipulation had an effect in the workers' orientation if we find differences between control and rotated ants of the same species.

We sampled one to three ants per treatment and host tree (i.e., per ant colony), but we sampled from five to ten colonies per ant species, making sure they were different workers; i.e., a new worker entered the disc while we could still see the earlier workers walking with the food item on the tree (*P. nigrocinctus* N = 45 workers from six colonies; *P. flavicornis* N = 75, 10 colonies; *P. spinicola* N = 71, 10 colonies; *P. nigropilosus* N = 41, five colonies; *P. gracilis* = 27, five colonies). We consider all observations of workers to be independent regardless of colony, because

Table 1 Summary of behavioral differences between ant workers in control and rotation treatments of the disorientation tests. We sampled workers of five species of *Pseudomyrmex* ants that differed in their association with acacia trees, which in turn determines the foraging ecology (foraging exclusively on the host tree in the so-called in-nest foraging ant species or foraging also off the host tree in central-place foraging species). We expected control and treatment workers of central-place foraging species (*P. nigropilosus = P. np* and *P. gracilis = P. gr*) to differ in fewer of the measured behaviors than control vs. treatment workers of in-nest foraging species (*P. nigrocinctus = P. nc*; *P. flavicornis = P. fv*; *P. spinicola = P. sp*). Among in-nest foragers, we expected more behaviors to differ between control- and rotation-treated workers for the species that travel the least away from their host tree to kill neighboring vegetation (short distance) than control vs. treatment workers of species that travel longer distances. Shaded cells highlight results that suggest workers were disoriented, that is, were workers in control and rotation treatments differ (p < 0.05)

Species	P. nc	P. fl	P. sp	P. np	P. gr
Association with acacia tree	Obligate mutualists			Obligate parasite	Facultative parasite
Foraging ecology	In-nest			Central-place	
Distance workers travel away from host tree to kill vegetation or forage	short	short	long	long	long
Average speed	yes	no	no	no	no
Walking angle of first 3 seconds	yes	no	no	yes	yes
Walking angle entire pathway	yes	no	no	yes	no
Mean walking angle deviating from a straight line towards the acacia tree	yes	yes	no	yes	no
Returning to disc center	yes	yes	yes	no	no
Time returning to the host tree-trunk	yes	yes	yes	yes*	no
Walking off disc at a point other than the initial entrance point [a]	yes	no	yes*	no	no
Turning at the edge of the disc from the true direction of host-tree	no	no	yes	no	no
Number of spins[a]	no	no	no	no	no
Number of stops[a]	no	no	no	no	no

*$0.05 < p < 0.10$

[a] Traits that could not be tested for phylogenetic signal on the effect size of control vs. rotation treatment (see Methods)

foraging in *Pseudomyrmex* is solitary and the behavior of each worker on the experimental disc is independent from the workers in the rest of the colony. Although *Pseudomyrmex* ants rely heavily on vision for foraging and they are not known to deposit foraging trails, we wiped the disc after each trial with water and tissue to eliminate any chemical cues, and we used several discs in the experiments. In treatment and control assays, we video-recorded the worker's path back to
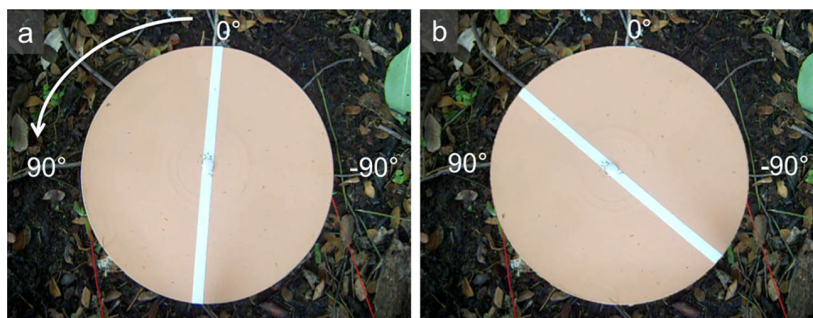
Fig. 2 Experimental arena for disorientation tests. A rotary disc was placed on the ground next to an acacia host tree inhabited by a colony of one of the five tested *Pseudomyrmex* species. **a** Workers walked onto the disc at the point nearest to the acacia tree (0° orientation, at the *12 o'clock position*) and picked up a food item placed on the center of the disc. **b** While the worker was picking up the food, the disc was rotated 45° clockwise (or counterclockwise, not shown) by pulling the red strings attached to the disc. As control treatment, the disc was rotated about 22.5° in one direction and then 22.5° on the opposite direction to return the disc back to the initial 0° orientation, as a sham treatment to control for the effect of disc movement

the acacia tree using a Nikon J1 camera capturing images at 30 frames per second.

To capture variations in behavior because of the rotation treatment, we analyzed the videos frame-by-frame to evaluate 10 behaviors that were compared between control and rotated ants: (1) We calculated the walking speed of workers using the automated function of Tracker software (Brown 2009), to assess whether ants would walk at different speeds when rotated 45° in one direction versus 20° and −20° back-and-forth in the control treatment. (2) To asses whether workers were leaving the experimental disc in a direction different than towards the host tree, we quantified in Tracker the mean angle at which workers were walking with respect to the tree. To obtain the mean angle, we obtained the angle of the ant with respect to the tree at every 10 video-frames (every 0.33 s) according to a coordinate system that positioned the host tree at 0° with respect to the entrance point on the disc (Fig. 2); we then calculated whether the circular mean of those angles deviated from the direction of the original entry point (i.e., direction towards host tree). To assess whether workers started and ended their return path with a deviation from a straight line towards the acacia tree (0°), we compared the mean angle of the worker's trajectory with respect to the acacia tree between control and treatment ants (3) at the beginning of the path (during the first 3.3 s after they picked up the food item, as it corresponds to 100 frames of video) and (4) during the entire return path on the disc. Because some discs were rotated clockwise and others counterclockwise, we standardized scores of the observed behaviors within the angular-coordinate system to have a value of 0° pointing towards the acacia tree, positive values up to 180° in the direction of the disc rotation in a particular trial, and negative values in the direction opposite to the disc rotation (Fig. 2). To score additional behaviors, we used JWatcher software (Blumstein et al. 2006) to record (5) number of times that ants returned to the disc center where they first picked up the food reward, after reaching the disc edge (Fig. 4f); (6) total time needed to return to the host tree; (7) number of times workers left the disc at a point other than the initial entrance point (Fig. S2f) (8) turned away from the host tree after reaching the disc edge (Fig. S3f), or (9) made a spin, defined as complete 360° rotation with no displacement (Fig. S4f); and (10) the number of stops (i.e., temporary absence of displacement lasting at least 1 s). We could not score behavioral observations blindly because the species identity of each tested ant and the respective treatment (disc rotation) were evident in the video recordings.

Phylogenetic corrections and statistical analyses

We first tested behavioral variables for phylogenetic signal to determine whether our analysis required a correction for phylogeny. We calculated the effect size (Cohen's D) for all variables except for the behaviors of stopping, spinning, or walking off the experimental disc at a point different than the entry point, because each tested species rarely showed these behaviors or because there were no differences between control and treatment condition for any species for these behaviors (Fig. S2, 9, 10). Second, we used the phylogenetic tree (Gómez-Acevedo et al. 2010) but pruned to include only the five *Pseudomyrmex* species included in our study, then tested whether the effect sizes for each behavioral variable had phylogenetic signal by calculating Blomberg's K (Blomberg et al. 2003). We also ran a randomization test (10,000 permutations) to assess whether the calculated variance for the independent contrasts was significantly different than expected under random trait variation, using the function "phylosig" in the phytools (Revell 2012) package in R. Walking angles cannot be treated as regular continuous variables due to their circular nature. Hence, instead of calculating a numerical effect size, we coded the effect of rotation on the mean angle as a binary variable: "1" if the mean angle of control and rotation treatments were different, and "0" if they were not different. For this binary data, we calculated the D-value or phylogenetic signal (Fritz and Purvis 2010) using the "phylo.d" function of the caper R package (Orme et al. 2013). Because none of the analyzed traits showed phylogenetic signal (Table S1), we therefore treated species as independent in all analyses.

We used a generalized linear model with Poisson distribution for the count data (counts of walking towards the acacia tree on the edge of the disc, of walking towards the center of the disc, and number of stops and spins), and a linear model for the continuous variables (walking speed and time returning to the tree, both log transformed) to test for an interaction between type of foraging (central-place vs. in-nest) and treatment (control vs. rotated). We expected the effect of rotating the disc to depend on whether workers were central-place foragers, which would result in a significant interaction term between type of foraging and treatment. We also tested whether the effect of the treatment was dependent on the distance workers typically travel away from the tree (ant species typically traveling long vs. short distances from the tree, as scored in Table 1), that is, we tested for the statistical interaction of treatment and traveling distance. Additionally, we analyzed datasets separately for each species to assess the effect of the treatment on the response variable. For circular data (the walking angle during the first 3 s, and the walking angle of the entire route), we compared angles of ants on control and rotation treatments with a Watson U^2 test using the function "watson.two.test" from the "Circular" package in R (Agostinelli and Lund 2013). We also tested whether those angles were different from zero (i.e., different from pointing towards the acacia host tree) with a modification of the Rayleigh test (also known as V-test; Zar 2010) using the function "rayleigh.test" of the "Circular" package in R, specifying an angle of zero in the term "mu"(Agostinelli and Lund 2013). We used Chi-squared tests for counts (e.g., number of

times returning to the disc center) and Mann-Whitney U tests for continuous data (e.g., speed, time to return to the tree).

Results

Of the 10 behaviors recorded, we found only one behavior (returning to disc center) that consistently altered in rotated compared to control ants of the in-nest foragers, whereas it did not change in central-place foragers (Table 1). The other nine behaviors showed differences between species (see below), but any differences were not consistently associated with foraging ecology (in-nest vs. central-place foraging; details below). Remarkably, the species showing the most differences in behavior after disorientation was an acacia ant, that is, an in-nest forager that travels short distances to prune nearby vegetation (*Pseudomyrmex nigrocinctus)*, and the species showing the least differences in behavior between control and rotated ants was a central-place forager (*P. gracilis*). However, the other in-nest forager and short-distance walker, *P. flavicornis*, showed fewer differences in behavior after the experimental rotation than expected by its ecology; rotated workers of the central-place forager *P. nigropilosus* showed similar differences in behavior than the in-nest foragers *P. spinicola* and *P. flavicornis* (Table 1).

Average speed

The effect of disc rotation on the walking speed was independent of whether ants were central-place foragers (interaction of foraging type and treatment, $F_{1,165} = 0.54$, $p = 0.46$; Fig. S1) or whether they typically walked long or short distances away from the host tree (interaction of treatment and traveling distance, $F_{1,165} = 0.68$, $p = 0.40$; Fig. S1). Analyzing by species, only an in-nest foraging species, *P. nigrocinctus*, walked more slowly on rotated disc than on control discs (Fig. S1a; Mann-Whitney $U = 257$, $p = 0.024$), whereas the other two acacia mutualists did not walk more slowly on rotated disc (*P. flavicornis*, Fig. S1b; Mann-Whitney $U = 228$, $p = 0.63$; and *P. spinicola*, Fig. S1c; Mann-Whitney $U = 170$, $p = 0.56$). The two central-place foragers did not walk more slowly on rotated discs either (*P. nigropilosus*, Fig. S1d, Mann-Whitney $U = 85$, $p = 0.47$; and *P. gracilis*, Fig. S1e, Mann-Whitney $U = 98.5$, $p = 0.42$).

Walking angle

During the first 3.3 s after the worker picked up the food item, mean walking angle of rotated workers was different from that of control workers in one of the in-nest forager species (*P. nigrocinctus*) and in both central-place foragers (*P. nigropilosus* and *P. gracilis*). Workers on rotation treatment walked at an angle about 20° greater than control workers in

P. nigrocinctus (Fig. 3a, b; $F_{1, 37} = 3.88$, $p = 0.05$) and central-place foragers of *P. gracilis* (Fig. 3i, j; Watson $U^2_{27} = 0.18$, $0.01 < p < 0.05$). The other central-place forager, *P. nigropilosus*, showed an average deviation of about 8° with respect to the control (Fig. 3g, h; Watson $U^2_{28} = 0.25$, $0.01 < p < 0.05$). Contrary to our initial prediction, rotated workers of the other two in-nest forager species showed no difference in mean angle direction to control workers (*P. flavicornis*: Fig. 3c, d; $F_{1, 39} = 0.09$, $p = 0.10$; and *P. spinicola*: Fig. 3e, f; $F_{1, 33} = 1.18$, $p = 0.28$).

When considering the entire route, workers in the rotation treatment showed deviations from control workers in one of the in-nest foraging species *P. nigrocinctus* (Fig. 3k, l; Watson $U^2 = 0.23$, $p < 0.05$), but not in the other species (*P. flavicornis*; Fig. 3m, n; Watson $U^2 = 0.09$, $p > 0.05$). As expected for central-place foragers, the initial difference between treatment and control in *P. gracilis* workers is not statistically significant when considering the entire trajectory (Fig. 3s, t; $F_{1,25} = 0.07$, $p = 0.79$). Surprisingly, rotated workers of the other central-place forager *P. nigropilosus* still differed from control workers in the walking angle when considering the entire route (Fig. 3q, r; Watson $U^2_{28} = 0.32$, $0.001 < p < 0.01$). We also found that *P. spinicola* workers, who typically travel the furthest away from their host tree, performed similarly on rotated and control discs (Fig. 3o, p; $F_{1, 33} = 1.78$, $p = 0.19$).

Did returning workers walk directly towards the acacia host tree?

As expected, the central-place forager *P. gracilis* and the long-distance traveler *P. spinicola* ants walked at an angle not deviating from the direction pointing towards the acacia tree (located at 0°), i.e., the mean walking angle was not different from zero in these two species (V test, *P. gracilis*: control $R = 0.02$, $p = 0.46$, rotated $R = 0.02$, $p = 0.44$. *P. spinicola*: control $R = 0.06$, $p = 0.63$, rotated $R = 0.16$, $p = 0.15$). In the two other in-nest foraging species, workers in both control and treatment discs had a mean walking angle different from zero: *P. nigrocinctus* (V test, control $R = 0.97$, $p < 0.0001$, rotated $R = 0.89$, $p < 0.0001$) and *P. flavicornis* (V test, control $R = 0.95$, $p < 0.0001$, rotated $R = 0.88$, $p < 0.0001$). Contrary to our prediction, workers of the central-place forager *P. nigropilosus* walked at an average angle deviating from zero, i.e., deviating from the direction pointing towards the acacia tree (V test, control $R = 0.90$, $p < 0.0001$, rotated $R = 0.89$, $p < 0.0001$).

Returning to disc center

As expected, the tendency for disoriented workers to return to the disc center depended on foraging type (interaction of treatment vs. foraging type, $Z = 3.16$, $p = 0.001$; Fig. 4) and on
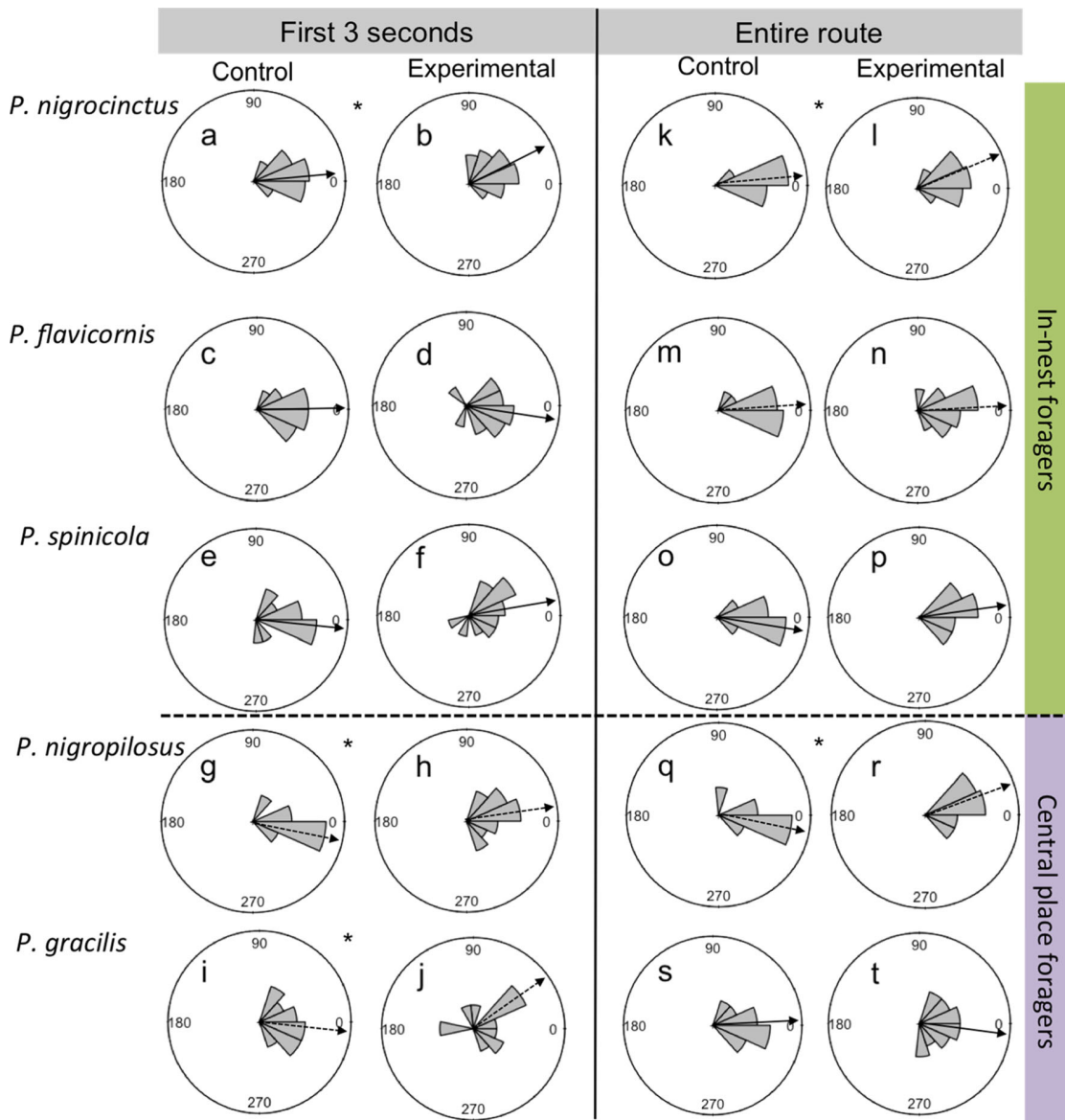
Fig. 3 Histograms of walking angles relative to the orientation towards the nest (acacia host tree at 0°) calculated in **a–j** for the first 3.3 s after a worker picked up a food item in control and rotation treatments or **k–t** calculated as the total time needed by a worker to walk off the experimental disc. *Pseudomyrmex nigrocinctus*, *P. flavicornis*, and *P. spinicola* have a mutualistic association with acacia trees and are all so-called in-nest foragers. Central-place foraging ant species include *Pseudomyrmex nigropilosus*, an acacia parasite that also extracts food from nearby acacia trees, and *Pseudomyrmex gracilis*, a species that nests on acacia trees and preys on small arthropods on the ground and nearby vegetation. The *arrow* inside each circle denotes the mean angle (*continuous arrow*, for normally distributed data) or the median angle (*dashed arrow*, for non-normally distributed data). *Asterisk* denotes significant differences ($p < 0.05$) between control and rotation trials

whether the species was a short- or long-distance traveler (interaction term of treatment vs. traveled distance, $Z = 3.16$, $p = 0.03$). Workers from all three in-nest forager species more often walked back to the center of the disc in rotation trials than in control trials (*P. flavicornis*, Fig. 4b, $X^2 = 6.93$, $d.f. = 1$, $p = 0.008$; *P. spinicola*, Fig. 4c, $X^2 = 10.20$, $d.f. = 1$, $p = 0.001$; and *P. nigrocinctus*, Fig. 4a, $X^2 = 9.38$, $d.f. = 1$, $p = 0.002$). This increase in the number of times that workers walked towards the center of the disc after reaching the disc edge was absent in both central-place foragers (*P. nigropilosus*,

Fig. 4d; $X^2 = 2.01$, $d.f. = 1$, $p = 0.15$; and *P. gracilis*, Fig. 4e, $X^2 = 0.32$, $d.f. = 1$, $p = 0.57$).

Time returning to the nest

The effect of the disc rotation on the time to return to the host tree (difference between control and rotated disc) was similar between central-place foragers and in-nest foragers (interaction of foraging type with treatment, $F_{1,66} = 2.08$, $p = 0.15$) and between short- and long-distance travelers
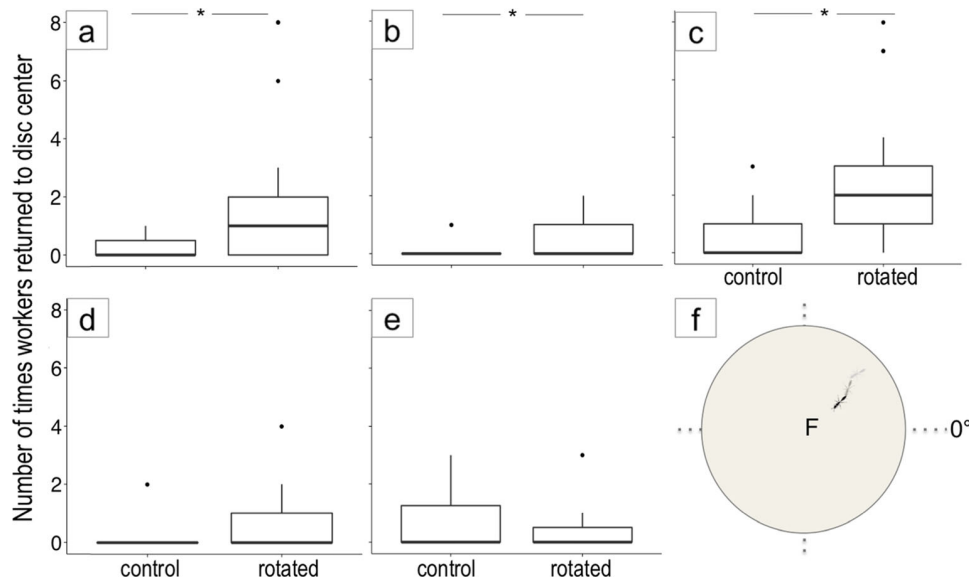
Fig. 4 Number of times that ants returned to the disc center after heading out to store the collected food, according to the treatment for workers of the in-nest foragers: **a** *P. nigrocinctus*, **b** *P. flavicornis*, and **c** *P. spinicola* and the central-place foragers **d** *P. nigropilosus* and **e** *P. gracilis*. The diagram (**f**) represents the experimental disc on which ants were tested, and the ant silhouettes depict how a worker, who had tried to return to its nest after picking up the food (located at **F**), returned to the disc center. The direction of the acacia host tree is at 0° (*three o'clock position*). Statistically significant differences between control and treatment (rotated disc) are shown (**p < 0.05*)

(interaction of treatment and traveled distance: $z = 0.16$, $p = 0.68$). When analyzed by species, workers of only one of the two central-place foragers (*P. gracilis*) returned to the host tree in about the same time in control and rotation treatments (Fig. 5e; Mann-Whitney $U = 77$, $p = 0.54$), whereas workers of *P. nigropilosus* ants tended to take longer in the rotation trials than in control trials (Fig. 5d; Mann-Whitney $U = 53.5$, $p = 0.06$). All workers of in-nest foraging species on rotation treatments took longer than control ants to return (*P. nigrocinctus:* Fig. 5a, Mann-Whitney $U = 59$, $p = 0.0002$; *P. flavicornis:* Fig. 5b, Mann-Whitney $U = 132$, $p = 0.04$; and *P. spinicola:* Fig. 5c, Mann-Whitney $U = 62.5$, $p = 0.003$).

Walking off the experimental disc

Once on the edge of the disc, ants could leave the disc by the same point from which they entered or at a different point (Fig. S2f). Contrary to our prediction, in-nest foragers and central-place foragers were equally affected by disc rotation (interaction term of treatment and foraging type: $z = 0.011$, $p = 0.99$), and the effect of the rotation was also independent of the distance workers travel away from the host tree (interaction of treatment and traveled distance: $z = 0.013$, $p = 0.99$). When analyzed by species, in two of the in-nest foragers, disc rotation caused more workers to leave the discs by a new point rather than by the entrance point when compared to control workers (*P. nigrocinctus* Fig. S2a; $X^2 = 5.06$, $d.f. = 1$, $p = 0.025$; *P. spinicola*, Fig. S2c; $X^2 = 3.22$, $d.f. = 1$,

$p = 0.072$). This was not observed in the short-distance traveler *P. flavicornis* (Fig. S2b; $X^2 = 0.89$, $d.f. = 1$, $p = 0.34$). For the central-place foragers, the probability of leaving the disc by the entrance point was not affected by the treatment of rotating the disc in *P. nigropilosus* (Fig. S2d; $X^2 = 0.71$, $d.f. = 1$, $p = 0.40$); *P. gracilis* was the only species where workers left the disc at the entrance point in all trials (Fig. S2e).

Turning away from tree on disc edge

When workers arrived at the edge of the disc, they could walk towards or away from the direction of the acacia host tree (Fig. S3f). When comparing ant species, the effect of rotating the disc on the frequency of turning away from the acacia tree did not depend on type of foraging (central-place vs. in-nest foragers; $z = 0.99$, $p = 0.32$), but the effect depended on whether ants walked short or long distances away from the tree (interaction of treatment vs. traveling distance, $z = -2.01$, $p = 0.04$). When reaching the edge of the disc, only in-nest foragers of *P. spinicola* were more likely to walk away from the acacia tree when the disc was rotated compared to control workers (Fig. S3c; Mann-Whitney $U = 379.5$, $p = 0.002$). Disc rotation had no effect on this behavior in any of the other species (*P. flavicornis*, Fig. S3b, Mann-Whitney $U = 611$, $p = 0.27$; *P. nigrocinctus*, Fig. S3a, Mann-Whitney $U = 199.5$, $p = 0.22$; *P. nigropilosus*, Fig. S3d, Mann-Whitney $U = 209$, $p = 0.82$; *P. gracilis*, Fig. S3e, Mann-Whitney $U = 58$, $p = 0.30$).
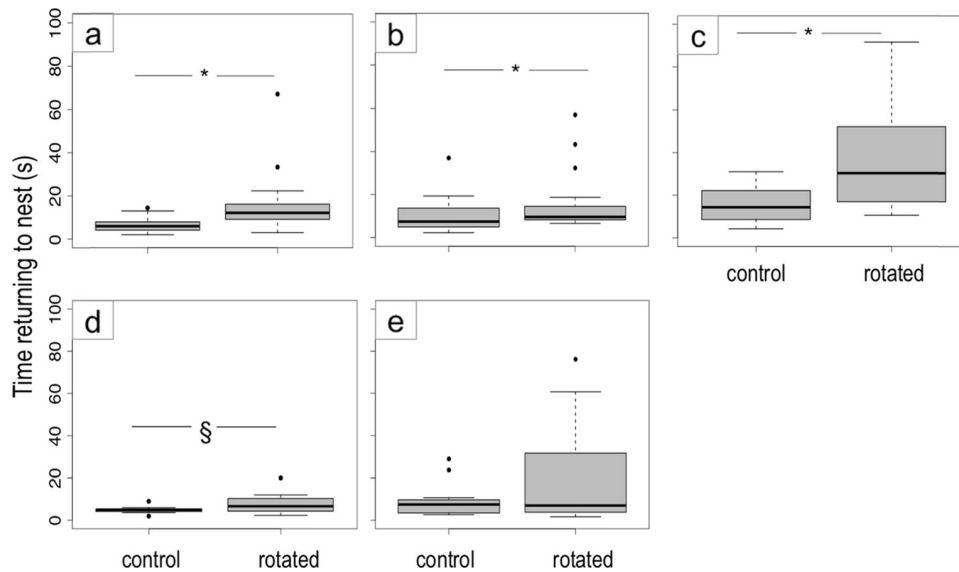
Fig. 5 Time to return to the nest after picking up the food reward in control and rotation treatments. Workers took longer to return to the nest in the rotation treatment than in the control treatment in the acacia mutualists **a** *P. nigrocinctus*, **b** *P. flavicornis*, and **c** *P. spinicola*. This difference was marginally significant in the central-place forager **d** *P. nigropilosus*. The rotation treatment had no effect on the time to return to the disc for the central-place forager *P. gracilis* (**e**). Median, interquartile range (IQR), 1.5*IQR, and outliers are represented by the *dark horizontal line*, *box*, *whisker*, and *dots* respectively. Statistically significant differences between control and rotation treatments are shown (*$p < 0.05$; §$0.05 < p < 0.10$)

Spin behavior

The effect of treatment on the number of spins depended on the type of foraging because it increased in in-nest foragers but decreased in central-place foragers (interaction of foraging type with treatment, $Z = 1.95$, $p = 0.050$). The distance workers walked away from the host tree was not associated with the number of spins in rotation versus control trials (interaction of traveling distance with treatment, $Z = 1.58$, $p = 0.11$). However, when analyzed by species, workers of any species spun similarly on rotation or control discs (*P. nigrocinctus*, Fig. S4a; Mann-Whitney $U = 233$, $p = 0.59$; *P. flavicornis*, Fig. S4b; Mann-Whitney $U = 640$, $p = 0.32$; *P. spinicola*, Fig. S4c; Mann-Whitney $U = 609.5$, $p = 0.65$; *P. nigropilosus*, Fig. S4d; Mann-Whitney $U = 169$, $p = 0.13$; *P. gracilis*, Fig. S4e; Mann-Whitney $U = 65.5$, $p = 0.82$).

Stop behavior

The difference in the number of stops performed in rotation versus control treatments was not dependent on type of foraging (interaction of foraging type and treatment, $Z = 1.05$ $p = 0.29$) or on the traveling distance (interaction of treatment and traveling distance, $Z = 0.945$, $p = 0.34$). This was also true when analyzing by species: workers of any species on rotation treatments stopped as much as control workers (*P. nigrocinctus*, Fig. S5a; Mann-Whitney $U = 206$, $p = 0.21$; *P. flavicornis*, Fig. S5b; Mann-Whitney $U = 694$,

$p = 0.94$; *P. spinicola*, Fig. S5c; Mann-Whitney $U = 628.5$, $p = 0.98$; *P. nigropilosus*, Fig. S5d; Mann-Whitney $U = 181.5$, $p = 0.52$; *P. gracilis*, Fig. S5e; Mann-Whitney $U = 57$, $p = 0.46$).

Discussion

The evolutionary transition to obligatory, mutualistic nesting on acacia trees involved marked behavioral evolution in workers, thus resulting in behavioral differentiation between mutualistic and non-mutualistic species in the genus *Pseudomyrmex* (Janzen 1966). Examples of this behavioral evolution are that acacia ant workers, compared to non-mutualistic workers, are aggressive instead of shy, prune neighboring vegetation instead of ignoring it, are nocturnal rather than strictly diurnal, and exclusively forage on the host plant rather than hunting arthropods (Janzen 1966). The cognitive abilities mediating these behaviors of acacia ant workers can be expected to be likewise affected by the association with the plant. However, we could not find consistent evidence that in-nest foraging ants (i.e., ants in obligatory mutualisms with acacia plants) would have more difficulties orienting back to the nest after an experimental manipulation than central-place foraging ants.

Acacia ants are in-nest foragers because they forage exclusively on the acacia tree where they nest, and unlike central-place foragers, they do not need to orient back to the nest after foraging. Moreover, because acacia ants walk off their host

tree to prune nearby vegetation and return to the host tree, performance in our disorientation assay may be dependent on the typical distance (long-distance vs. short distance) in-nest (acacia ant) workers typically travel away from the tree (Fig. 1). However, other behaviors might be mediated by the same mechanism in central-place and in-nest foragers, which could explain why some results are not consistent in our comparisons.

Only one of the measured behaviors consistently increased in mutualistic in-nest foragers when compared to control discs, but not in central-place foragers: returning to the disc center after heading in the wrong direction in rotation treatments (Table 1, Fig. 4). Returning to a familiar location is a known orientation strategy used by desert ants, even when it requires temporarily walking away from the nest (Wystrach et al. 2012). Similar to desert ants, acacia ants are largely visual and the optic lobes occupy a large portion of their brains (Amador-Vargas et al. 2015). However, we lack detailed mechanistic studies showing whether acacia ants are using vision to orient or how do they use it (e.g., scanning of visual patterns in the tree canopy), and more generally, how does returning to a familiar location help acacia ants to orient to their nest.

For the other nine behaviors, the effects of disorienting the ant were not consistent across foraging ecologies but there were clear differences between species in their efficiencies at performing the orientation task. Specifically, two species behaved just as predicted according to their foraging strategy and distance traveled away from the host tree: *P. nigrocinctus* and *P. gracilis*, which are, respectively, in-nest and central-place forager species. Phylogenetic evidence suggests that the obligate mutualism of *P. nigrocinctus* and acacia trees was established more recently than the mutualism of *P. spinicola* and *P. flavicornis*, even though all three species are closely related (Chomicki et al. 2015). Hence, even when the vegetation clearings of *P. nigrocinctus* superficially resemble the clearings of *P. flavicornis* (Fig. 1), it is possible that the small size of the area *P. nigrocinctus* workers clear from vegetation is in fact a consequence of the workers not exploring away from the acacia tree, whereas in *P. flavicornis* may explore further away from the host tree but stop biting the vegetation at some distance away from the tree. Anecdotally, from the five tested species, workers of *P. nigrocinctus* took the longest time to enter the experimental arena, which indirectly indicates that they are less willing to explore the vicinities of the tree than *P. flavicornis*. Hence, to understand the behavioral differences between the two short-distance travelers, it would be useful to document how accurately the size of the vegetation clearing reflects the actual distance that workers explore the vicinities of the host tree.

P. nigropilosus workers were more affected by the disorientation assay than the other central-place forager (*P. gracilis*). This species was considered as a central-place forager for this study, because workers have been observed to extract food from neighboring acacia trees (Amador-Vargas 2012b). However, this species may travel closer to the acacia than the other central-place foraging species (*P. gracilis*), because it obligatorily nest on acacia trees, only feeds on nectar and food bodies produced by acacia trees (Janzen 1975), and steals food from trees that are in closer proximity (Amador-Vargas 2012b). Hence, it is possible that *P. nigropilosus* workers experience the challenge of orienting back to the host tree but at a smaller scale than *P. gracilis* workers. More studies about the stealing behavior of *P. nigropilosus* could help to understand any differences between the three central-place foraging species tested.

The mechanisms used during navigation in acacia ants have not been studied to date, but research in other ants indicates that workers can use path integration, terrestrial or celestial cues, and systematic search to orient (Wehner 2009; Steck et al. 2011; Cheng et al. 2014; Knaden and Graham 2016). Our results show that only the central-place forager *P. gracilis* ants spun or visually scanned (i.e., performed 360° turns with no displacement, Fig. S4) several times before heading towards the acacia tree in both control and rotation treatments; this behavior was rarely observed in the other species including the sister species *P. nigropilosus*. Spinning (visual scanning) may allow *P. gracilis* ants to visually identify landscape or canopy cues to orient towards the acacia tree, a strategy used also by desert ants (Zeil et al. 2014; Wystrach et al. 2014). While our experiments were not designed to specifically address the navigational mechanisms used by acacia ants to orient, they suggest these mechanisms may differ between closely related *Pseudomyrmex* species.

Insights for comparative orientation studies The experimental design used in our study aimed to improve on typical comparative studies of animal cognition. First, comparative studies on spatial orientation traditionally compare only two species (e.g., Clayton and Krebs 1994; Day et al. 1999; Cristol et al. 2003; Odling-Smee et al. 2008; Schwarz and Cheng 2010; Bühlmann et al. 2011; Schultheiss et al. 2013), several species from different genera (e.g., Balda and Kamil 1989; Bednekoff et al. 1997; Rosati et al. 2014) or from the same genus but without phylogenetic correction (Hilton and Krebs 1990). Our study shows that comparing several closely related species could lead to different conclusions than when comparing a pair of species. For example, if we had compared only *P. nigrocinctus* (in-nest forager) with *P. gracilis* (central-place forager), our results would almost perfectly match the prediction of the acacia ant having more difficulties to reorient on the experimental assay than the central-place forager, because making mistakes may be more costly for central-place foragers. Including other closely related species complicated this tidy picture of the role of the plant mutualism in shaping foraging ecology and orientation skills. Future research could expand on our analysis to include other obligatory plant-

associates and closely related central-place foragers and thus increase the sample size of independent phylogenetic contrasts.

Comparative studies on spatial memory have also been criticized because differences between species in task performance could be due to other variables (e.g., motivation, stress) other than cognitive differences (Macphail 1982; Lefebvre 1995). This critique is valid because, typically, studies directly compare the values of each species for a particular response variable (i.e., studies may lack adequate control trials). Hence, we argue that including an experimental control (sham treatment) in the experimental design reduces the concern for these possible confounding variables, as both disoriented and sham-treated workers presumably were equally motivated and equally stressed. Performing tests in the field, where the animals passively enter the experimental apparatus, could also help reduce the stress of the animals and ensure results that better resemble what happens in nature. Also, satiation during the performance of an orientation test that uses food as reward could affect the results. We offered a reward that is not consumed by the animal, as the protein bodies that workers collected are later used to feed the larvae.

A third difficulty of comparative studies on navigational skills is standardization of behavioral assays. Even when species share similar foraging ecology, they differ in other natural history traits; species can exhibit unique behaviors that have no equivalent in other species, making it impossible to compare such behaviors across species; species are not sympatric; or live in very different environments (Bolhuis and Macphail 2001; Sherry 2006; Shettleworth 2009). Working with closely related species that vary in natural history could help overcome this problem, because closely related species typically have similar behavioral repertoires. We were able to minimize the influence of other factors in our comparison by having sympatric and closely related species, naturally nesting on the swollen spines of acacia trees and feeding mostly on nectar and food bodies produced by the tree. Species in protective plant mutualisms from other ant genera that also kill the vegetation surrounding the host tree (e.g., *Allomerus* and *Myrmelachista* species nesting on *Hirtella*, *Duroia* or *Tococa* trees; Davidson et al. 1988; Morawetz et al. 1992; Renner and Ricklefs 1998; Izzo and Vasconcelos 2002; Frederickson et al. 2005) could be used to evaluate, in another clade, the hypothesis that the obligatory mutualism shapes the orientation abilities of workers.

Conclusion

Navigation in animals has been widely studied in species that evolved remarkable orientation skills, such as long-distance foraging, migratory, or food-storing animals (Balda and Kamil 1989; Shettleworth 1990; Hilton and Krebs 1990; Clayton and Krebs 1994; Bednekoff et al. 1997; Balda and Kamil 2002;

Cristol et al. 2003; Balda and Kamil 2006; Schwarz and Cheng 2010; Bühlmann et al. 2011; Schultheiss et al. 2013). Foraging ecology is one of the forces that influence spatial memory and navigational skills in animals (Collett et al. 2013). Our results suggest that the evolutionary transition in foraging ecology of acacia ants—from central-place to in-nest foraging—necessitated by the origin of the mutualisms with a plant, could have diminished the navigational skills on acacia ant workers, although this effect was not consistent across species. Our study is the first attempt to quantify cognitive differences between sympatric, congeneric ant species that share similar ecologies, but that differ markedly in foraging ecology. Ants in obligatory mutualisms with plants present a unique opportunity to study the role of ecological factors on behavioral traits, the cognitive consequences of an obligate association with the plant, as well as the ecological pressures that shape the evolution of orientation skills.

Compliance with ethical standards

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