

RESEARCH ARTICLE

Trail pheromone modulates subjective reward evaluation in Argentine ants

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ABSTRACT

The Argentine ant, *Linepithema humile*, is native to South America but has become one of the most invasive species in the world. These ants heavily rely on trail pheromones for foraging, and previous studies have focused on such signals to develop a strategy for chemical control. Here, we studied the effects of pre-exposure to the trail pheromone on sugar acceptance and olfactory learning in Argentine ants. We used the synthetic trail pheromone component (Z)-9-hexadecenal, which triggers the same attraction and trail-following behavior as the natural trail pheromone. We found that pre-exposure to (Z)-9-hexadecenal increases the acceptance of sucrose solutions of different concentrations, thus changing the ants' subjective evaluation of a food reward. However, although ants learned to associate an odor with a sucrose reward, pheromone pre-exposure affected neither the learning nor the mid-term memory of the odor-reward association. Taking into account the importance of the Argentine ant as a pest and invasive organism, our results highlight the importance of pheromonal cues in resource evaluation, a fact that could be useful in control strategies implemented for this species.

KEY WORDS: Social insects, *Linepithema humile*, Trail pheromone, Sucrose acceptance, Appetitive olfactory learning

INTRODUCTION

The Argentine ant, *Linepithema humile*, is a well-known invasive pest species. It is native to South America and one of the most successful and widely distributed invaders, having expanded towards Mediterranean and subtropical areas all around the world (Suarez et al., 2001). One of the reasons for this expansive success is a genetic bottleneck (Tsutsui et al., 2000), which has led to reduced intraspecific aggression, allowing the species to redirect resources to colony growth (Holway et al., 1998) and thus reach high population densities. These introduced populations are able to displace native ants (Suarez et al., 1998), other arthropods (Cole et al., 1992) and

vertebrates (Alvarez-Blanco et al., 2017; Suarez and Case, 2002), disrupt mutualisms (Bond and Slingsby, 1984) and facilitate honeydew-producing hemipteran pests such as mealybugs (Holway et al., 2002). Another potential factor facilitating the ants' invasive success is their mass recruitment strategy towards food sources through the use of trail pheromones (Carpintero and Reyes-López, 2008).

Pheromones are ubiquitous chemical signals used in animal communication. They are released to the environment by exogenous glands, and elicit behavioral or physiological responses in individuals of the same species (Karlson and Lüscher, 1959). Pheromones typically elicit stereotyped, innate responses that are species specific (Karlson and Lüscher, 1959; Wyatt, 2014). Among social insects, ants make extensive use of pheromones in a broad spectrum of behavioral contexts [including nest defense and alarm, foraging, social recognition and social interactions, and sexual communication (Vander Meer et al., 1998)]. A conspicuous pheromone-dependent behavior in various ant species is the pheromonal marking of trails leading to and from a profitable food source. Information about the quality of these sources is encoded in the variable amount of trail pheromone released, by increasing or reducing the scent-marking intensity per ant (Jackson and Châline, 2007) or the percentage of marking ants (Mailleux et al., 2000).

The trail pheromone of Argentine ants is produced by the ventral gland of workers (Cavill et al., 1980; Wilson and Pavan, 1959) and has (Z)-9-hexadecenal as a main aggregation compound (Cavill et al., 1979). Early studies showed that (Z)-9-hexadecenal alone was able to elicit trail following (Cavill et al., 1979; Van Vorhis Key and Baker, 1982) and generate attraction towards treated areas (Choe et al., 2014). In the field, this trail following can be disrupted by a high concentration of synthetic (Z)-9-hexadecenal, which can thus be used as an efficient and natural control method (Nishisue et al., 2010). Dispensers of this component positioned appropriately resulted in a reduction of Argentine ants' foraging activity (Tanaka et al., 2009; Westermann et al., 2016). However, only combining these dispensers with insecticidal sugary bait had a significant effect of population reduction after a year (Sunamura et al., 2011).

In the framework of animal communication, the study of pheromones remains limited to their capacity to trigger stereotyped responses at the time of pheromone detection. However, recent findings indicate that the biological effects of pheromones are more complex than previously thought. Pre-exposure to pheromones modulates behaviors that are not explicitly related to the specific message that they convey, such as responsiveness to reward and noxious stimuli (Baracchi et al., 2017, 2020; Rossi et al., 2018). They can also replace reinforcement stimuli in learning experiments (Carew et al., 2018; Coureaud et al., 2006), or modulate experience-dependent behaviors such as

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associative learning (Baracchi et al., 2020; Urlacher et al., 2010; Vergoz et al., 2007), even when the pheromone is no longer present.

Associative learning is an essential component of the foraging and recruitment behavior of social insects. Ants learn landmark configurations characterizing sites of interest (food sources and the nest) and also the routes connecting them (Collett and Collett, 2002). In addition, they can learn visual and olfactory cues at the food source (Dupuy et al., 2006; Josens et al., 2009; Yilmaz et al., 2017). Robust olfactory memories arise via individual experience or via odor perception in the context of trophallaxis (mouth-to-mouth contacts). These memories play a fundamental role in subsequent individual foraging decisions and, therefore, in the foraging organization of the colony (Provecho and Josens, 2009).

Even though the Argentine ant is a species of global relevance, its learning abilities have not been well explored. In one study, it was found that ants learned visual cues, but when conflictive information was presented, chemical trails were preferred to visual and spatial cues (Aron et al., 1993). In another work, Argentine ants were shown to learn and discriminate cuticular hydrocarbons appetitively in harnessing conditions (van Wilgenburg et al., 2012). Thus, investigating the cognitive abilities of Argentine ants could provide further cues to understand the ecological and evolutionary success of this species in the colonization of new habitats.

Here, we studied the effect of (Z)-9-hexadecenal on the evaluation of appetitive resources (sucrose) and on the learning of odors associated with such resources. In our experiments, we analyzed the effect of the trail pheromone component when it was no longer present and in a context different from the one in which the pheromone was presented. We thus aimed at assessing the response modulation of the individual's appetitive motivation rather than the reflexive response of trail-following behavior. Specifically, we pre-exposed ants to different doses of (Z)-9-hexadecenal and examined whether this pre-exposure affected their ingestion of sucrose solution. After identifying an adequate dose of synthetic pheromone to modify ingestion, we tested whether its pre-exposure also changed the subjective evaluation of sucrose rewards of varying concentration. Finally, we investigated whether ants can learn to associate an odor spot within a circular arena with sucrose solution, and whether pheromone pre-exposure modulates learning.

MATERIALS AND METHODS

Experiments were performed between May 2017 and January 2020 using three queenright *Linepithema humile* (Mayr 1868) colonies that had been collected between March 2017 and August 2019 from the native range in Argentina on the campus of the University of Buenos Aires (34°32'48.3"S, 58°26'21.0"W). Ants were kept under controlled conditions (26±1°C, 56±6% humidity, natural light:dark cycle) for at least 2 months before experiments. Colonies were reared in artificial nests that consisted of large plastic boxes (30×50×30 cm) with Fluon-painted walls to prevent escapes. The floor was covered with plaster (Paris type), on which a stack of acrylic plates (12×8 cm) separated from each other by ~2 mm served as a refuge. Colonies were fed daily with honey water (outside the experimental periods, except for Experiment 2, for which ants were deprived of food a few days before the experiment to increase their motivation to forage) and once a week with cockroaches (*Blattella germanica*). Water was provided *ad libitum*.

Experiment 1: sucrose solution acceptance

Pheromone dose effects

We first established the effects of different volumes of pheromone (0.2, 0.4, 0.8, 1.6 and 3.2 µl) on the feeding behavior of ants. Ants

were collected each day from the nest and separated into groups of three, maintained in acrylic pots (2.6 cm diameter, 3 cm height). As *L. humile* workers are monomorphic, all ants were of similar size. After 2 h of rest in darkness, each pot containing the three ants was placed within a larger plastic pot containing a filter paper (1×5 cm) soaked with either 0.2, 0.4, 0.8, 1.6 or 3.2 µl (Z)-9-hexadecenal (Carbosynth, Compton, UK). This larger pot (216 ml) was covered with a lid, and ants were confined therein for 15 min. Therefore, ants were exposed to the pheromone as a vapor but had no direct contact with it. The whole procedure was performed under air extraction. Control ants were subjected to the same conditions but no pheromone was presented within the larger pot.

After the 15-min pre-exposure, each pot with the three ants was removed from the larger one. Ants were then offered a 3-µl drop of 5% w/w sucrose solution at the center of their container. They were video recorded over 3 min using a SONY Handycam HDR-XR260 camera, and videos were analyzed using BORIS software (Friard and Gamba, 2016). Ants' feeding responses were coded as a binary response (feeding, 1; not feeding, 0). Ants that did not touch the drop of sucrose solution were excluded from analyses (~14% of the total ants).

Sucrose acceptance

We next studied whether and how pheromone pre-exposure affects the subjective evaluation of sucrose solutions. To this end, we compared the acceptance of sucrose solutions of different concentrations between pheromone-pre-exposed and non-pre-exposed ants, as sucrose concentration is a crucial parameter for foragers as an estimator of food quality (Scheiner et al., 2004).

The pheromone pre-exposure was performed as described above, but in groups of four ants, and lasted 20 min. Half of the four-ant groups were pre-exposed to 1.6 µl (Z)-9-hexadecenal and the other half was subjected to the same confining conditions but in the absence of pheromone (control groups). The whole procedure was performed under air extraction. After pre-exposure, each ant of one group was individually offered only one of four different sucrose concentrations: 1, 5, 10 or 20% w/w. Thus, in no case were two ants of the same group tested for the same concentration; they were never in the same sucrose concentration group, and each ant was only used once. The ants' acceptance responses were evaluated individually based on a protocol established by Sola et al. (2013). Briefly, an ant was gently placed on a bridge (2×50 mm²) that ended in a feeding arena containing a 3-µl drop of sucrose solution. The individual responses of each of the four ants were observed while the following group of four ants was pre-exposed to the synthetic pheromone.

We compared the feeding behavior of pheromone-pre-exposed and non-pre-exposed ants for the four different sucrose concentrations. Individual feeding responses to the sucrose concentrations were recorded as a binary response (feeding or not). We excluded ants that did not touch the drop or fell from the bridge, which constituted ~15% of the total ants assayed. Ants were filmed from a lateral view for 3 min while they were drinking, using a camera-fitted stereomicroscope (Leica MZ8 at 25× magnification with a Leica ICA camera). Feeding time (s) was obtained from the videos and was defined as the time during which an ant's mandibles were in contact with the solution until the ant had finished ingesting and left the feeding arena. As the video recording lasted 3 min, ants that fed longer were only considered for the binary response, but not for the feeding time (~5% of the total ants). We also excluded ants for which the feeding time could not be recorded (~2% of the total ants, i.e. owing to recording errors or poor visibility of the ants' mouthparts).

Experiment 2: appetitive olfactory conditioning of free-walking ants

Ants were trained to choose an odorant that was associated with sucrose solution within an experimental arena. Before conditioning, we made sure that the odorant used as a conditioning stimulus did not elicit spontaneous responses in ants.

Acquisition

The protocol was based on Piqueret et al. (2019). Linalool was used as the conditioned stimulus (CS) and was paired with 20% (w/w) sucrose solution as an unconditioned stimulus (US). Before conditioning, foragers were individually marked with water-based paint while ingesting a sucrose solution in the foraging area of their colony. They were then pre-exposed to (Z)-9-hexadecenal for 20 min (Ph+O+ group) or not pre-exposed for the same amount of time (Ph-O+ group), as in Experiment 1. After pre-exposure, ants were placed in another context where the pheromone was no longer present. A marked ant was placed in the center of the experimental arena (a circular plastic pot; 5.5 cm diameter, 4.5 cm height), with a clean filter paper covering the bottom and lateral walls painted with Fluon to prevent escapes. A semi-circular white wall was placed around the setup to prevent external visual stimulation. The arena had two holes in the wall facing each other. Eppendorf tubes were inserted into the holes with their openings pointing towards the center of the arena. A semi-circular zone (3 cm²) was drawn on the floor around each tube. One tube contained a piece of filter paper (1 cm²) soaked with 1 µl of 3% (v/v) linalool (Sigma-Aldrich, 97% pure) diluted in mineral oil. The opposite tube facing the odor tube contained a piece of filter paper soaked with 1 µl solvent (mineral oil). On each tube, a mesh prevented the ant from entering it. A small plastic disc (6 mm diameter) was placed in front of each tube, where 1 µl of 20% (w/w) sugar solution (US) or water was offered. The reward was placed in front of the odor, with the water placed in front of the solvent.

Every time an ant was placed in the experimental arena, it was allowed to familiarize for 1 min with the new environment within a central circular ring (3 cm diameter, 4.5 cm height) with Fluon-painted walls. Then, the ant was released and the time needed to find the sucrose solution (latency) was recorded during each conditioning trial. The ant was allowed to drink the drop of sugar solution and was then transported back to its colony, where it could perform trophallaxis to ensure high and stable motivation along trials. The inter-trial interval depended on the individual's motivation and whether or not it performed trophallaxis (~8 min). During this interval, the filter paper at the bottom of the arena and the plastic discs were replaced with clean ones, and the Eppendorf tubes were cleaned with ethanol (96%) to remove possible chemical cues left by the ant. The orientation of the arena was also randomly changed between trials to prevent the use of visual or spatial cues. Ants were subjected to three consecutive conditioning trials. Trials were video recorded using a SONY Handycam HDR-XR260 camera, and videos were analyzed with BORIS software (Friard and Gamba, 2016). From the videos, the latency to find sucrose was measured as the time (s) from the moment at which the ring was removed until the ant found the sucrose solution.

Memory test

Memory tests were performed in the absence of reward. This evaluation took place ~9 min after the third conditioning trial, depending on the ant's motivation and whether they spent time performing trophallaxis in the nest. For the tests, we used the same experimental arena but empty plastic discs were placed in front of

the two tubes containing either linalool or mineral oil. As for conditioning, a semi-circular zone was drawn around each tube, which allowed recording of the time spent by the ant near the odor (CS) or the solvent over 2 min (duration), as well as the number of times the ant entered each zone (occurrence). The orientation of the arena was also randomly changed between the last conditioning trial and the memory test to prevent the use of visual or spatial cues. Each ant underwent only one memory test.

Memory tests were video recorded using a SONY Handycam HDR-XR260 camera, and videos were analyzed with BORIS software (Friard and Gamba, 2016). From the videos, we quantified the occurrence as the number of times the ant entered each zone (linalool or mineral oil) and the duration as the time (s) the ant spent in each zone.

Control group for the associative learning process

The protocol of appetitive olfactory conditioning of free-walking ants used in Experiment 2 had never previously been used in *L. humile*. To make sure that the behavior observed for the two other groups of ants (Ph+O+ and Ph-O+) was the sole result of associative learning, we submitted an additional group (Ph-O-) to the same training procedure without any odorant (US-only group). This group was not exposed to the pheromone and faced tubes with no odor during three visits to the arena. The reward was placed in front of one of the empty tubes and water in front of the other one. The rest of the protocol was exactly the same.

The evaluation for this control group, at the fourth visit, was exactly the same as the memory tests for the odor groups (Ph+O+, Ph-O+); all cases were performed in the absence of reward. To this end, we used the same arena as during training, but empty plastic discs were placed in front of the two tubes containing either linalool or mineral oil. As for training, a semi-circular zone was drawn around each tube, which allowed recording of the time spent by the ant near the odor or the solvent over 2 min (duration), as well as the number of times the ant entered each zone (occurrence). The orientation of the arena was also randomly changed between the last training trial and the evaluation test to limit the use of visual or spatial cues. Each ant underwent only one evaluation test.

Evaluation tests were video recorded using a SONY Handycam HDR-XR260 camera, and videos were analyzed with BORIS software (Friard and Gamba, 2016). From the videos, we quantified the occurrence as the number of times the ant entered each zone (linalool or mineral oil) and the duration as the time (s) the ant spent in each zone.

Data analysis

Ants' feeding responses to the sucrose drop after pre-exposure to the different doses of pheromone (Experiment 1) were examined using a binomial generalized linear mixed model (GLMM; binomial error structure with logit-link function, glmer function, lme4 package; Bates et al., 2015). The response variable was 'feeding' (i.e. 1/0) and 'dose' (i.e. 0, 0.2, 0.4, 0.8, 1.6 and 3.2 µl) was the predictor. The container of the three-ant group was added as random factor to account for the fact that the experiment was performed in groups, although ants' responses were assessed individually. *Post hoc* comparisons between treatments and control were performed with the emmeans package (<https://github.com/rvlenth/emmeans>), and the false discovery rate correction was applied.

The ants' sucrose solution acceptance after pre-exposure to the pheromone or not (Experiment 1) was examined using a binomial (logit-link function) additive GLMM (glmer function, lme4 package; Bates et al., 2015) with 'concentration' (i.e. 1, 5, 10 and

20%) and ‘treatment’ (i.e. pheromone or nothing) as predictors, and ‘colony’ as a random factor. Feeding time was log transformed and analyzed using a linear mixed model (LMM; lme function, nlme package; <https://svn.r-project.org/R-packages/trunk/nlme/>) with ‘concentration’ (i.e. 1, 5, 10 and 20%) as predictor and ‘colony’ as a random factor.

The model used to analyze the time spent by the ants (latency) to find the drop of sucrose solution during acquisition (Experiment 2) was an LMM with ‘trial’ (i.e. Trial 1, Trial 2, Trial 3) as the predictor and individual as a random factor to account for the data dependency (lme function, nlme package). Individuals were nested into the colonies of origin to account for the fact that ants belonging to a given colony were more likely to behave similarly than those tested in different colonies. The response variable was log transformed to meet the criteria of the LMM.

For the memory tests (Experiment 2), two response variables were analyzed separately: the time spent close to each tube (duration) over 2 min and the visit frequency (occurrence) of these zones. The GLMM retained to analyze the visit frequency contained ‘zone’ (i.e. linalool or solvent) as predictor and individuals nested in colonies as random factors, given the dependence of the data. A Poisson family and a log link were chosen, given the data distribution (glmer function, lme4 package; Bates et al., 2015). The LMM retained to analyze the time spent in each zone contained ‘zone’ (i.e. linalool or solvent) as predictor and individuals nested in colonies as random factors (lme function, nlme package). The response variable was log transformed to meet the criteria of the LMM.

The visitation occurrence of the two zones surrounding the linalool and mineral oil for the Ph–O– group (Experiment 2) was analyzed with a Poisson (log-link function) GLMM (glmer function, lme4 package; Bates et al., 2015) with ‘zone’ as predictor and individuals nested in their colonies as random factors. The time spent by the ants in the two zones was square-root transformed before analysis with a similar LMM (lme function, nlme package).

Tukey *post hoc* tests were performed for comparisons after LMMs with the emmeans package. In all analyses, we retained the significant model with the highest explanatory power (i.e. the lowest Akaike’s information criterion value). All statistical analyses were two-tailed tests performed with R 3.6.1 (<https://www.r-project.org/>) and the significance threshold was set at 0.05.

RESULTS

Experiment 1: sucrose solution acceptance

Pheromone dose effects

The dose of pheromone used during pre-exposure (in a pot of 216 ml) had a significant effect on ants’ feeding behavior (GLMM; dose: $\chi^2=15.95$, d.f.=5, $P=0.007$), with more ants feeding on the sucrose solution ($88.10\pm 5.06\%$, mean \pm s.d.) after pre-exposure to 1.6 μ l pheromone compared with the control ($51.02\pm 7.22\%$) (GLMM *post hoc* test; 0 versus 1.6, $P=0.002$; others, n.s.) (Fig. 1).

Sucrose acceptance

We next determined whether pheromone pre-exposure affected the subjective evaluation of sucrose solutions differing in their concentration. The percentage of feeding ants increased with sucrose concentration, both for the pheromone-pre-exposed and the control groups (GLMM; concentration: $\chi^2=85.69$, d.f.=3, $P<0.001$; Fig. 2A). Pheromone pre-exposure effectively increased the acceptance of the sucrose solutions, i.e. in the number of feeding ants at a given concentration (GLMM; treatment: $\chi^2=7.96$, d.f.=1,

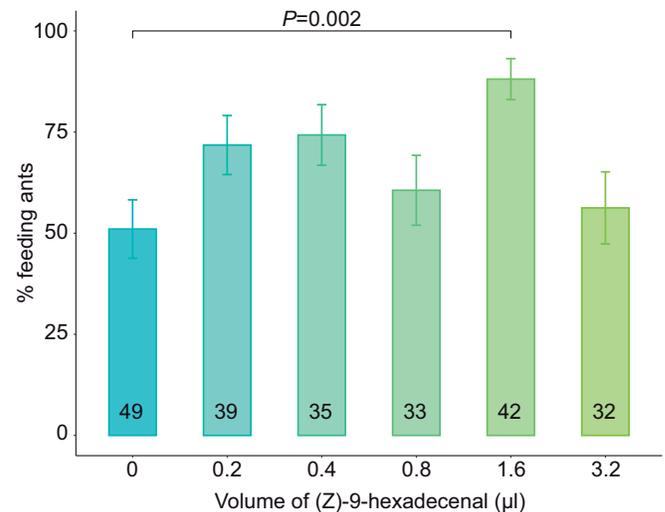


Fig. 1. Volume-dependent feeding responses after pheromone pre-exposure. Percentage of ants feeding on a 5% w/w sucrose drop (3 μ l) after pre-exposure to different volumes of (Z)-9-hexadecenal (0, 0.2, 0.4, 0.8, 1.6, and 3.2 μ l) in a 216 ml closed pot. The dose of 1.6 μ l (Z)-9-hexadecenal induced an increase in the percentage of ants feeding compared with control ants. Bars represent means \pm s.e.m.; numbers inside bars indicate sample sizes. Generalized linear mixed model (GLMM) *post hoc* test with estimated means and false discovery rate correction.

$P=0.005$; Fig. 2A). Despite the fact that the interaction between treatment and concentration was not significant and therefore removed from the statistical model, the difference between pre-exposed and control ants was particularly visible at the lowest sucrose concentration (control $21.52\pm 4.65\%$ versus pheromone $39.47\pm 5.64\%$), and almost inexistent at the highest sucrose concentration (control $79.75\pm 4.55\%$ versus pheromone $78.21\pm 4.70\%$; Fig. 2A). The time spent feeding also increased with sucrose concentration (LMM; concentration: $F=70.13$, d.f.=3, $P<0.001$) but was not influenced by pheromone pre-exposure (Fig. 2B). Thus, pheromone pre-exposure affected the subjective evaluation of appetitive resources. This effect may have rendered food sources of low-level quality more attractive. This change in evaluation was translated into the decision to feed but not to stay longer on a food source.

Experiment 2: appetitive olfactory conditioning of free-walking ants

Acquisition

Ants trained to find the odor spot associated with sucrose solution within the experimental arena reduced the latency to find the food during the three consecutive learning trials (LMM; trial: $F=5.37$, d.f.=2, $P=0.006$; Fig. 3A). This reduction was particularly visible when comparing the latency of the first and the third trial (LMM *post hoc* test, $P=0.005$; others, n.s.; Fig. 3A). The interaction between treatment and trial was not significant and therefore removed from the model. Pheromone pre-exposure did not affect this variable so that both groups showed similar performances.

Memory test

Ants tested for memory retention ~ 9 min after the last conditioning trial remembered the rewarded odor and preferred the zone of the arena containing it to an equivalent zone presenting mineral oil (solvent), despite the absence of reinforcement. Pre-exposed and control ants visited the odor zone more times (Ph–O+; 6.30 ± 4.64 ;

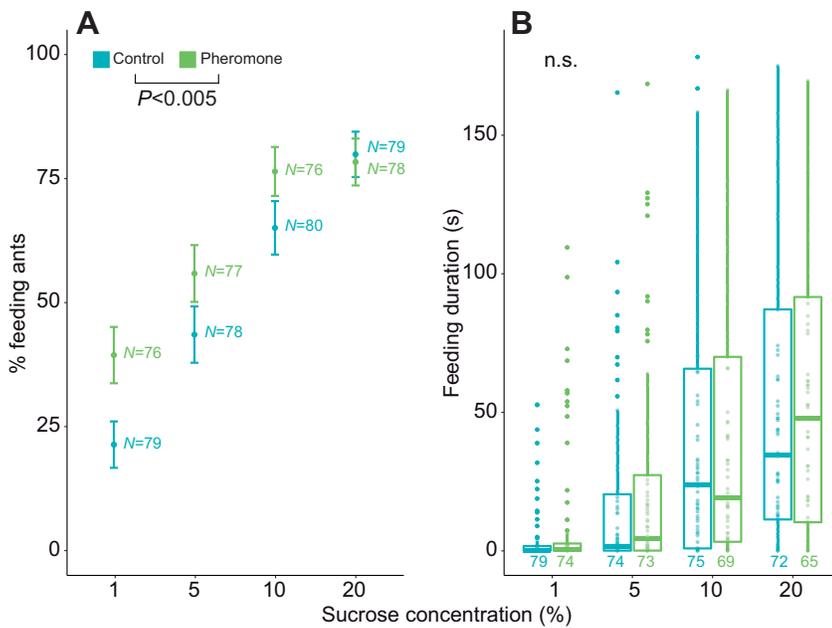


Fig. 2. Effect of pheromone pre-exposure on feeding responses to four sucrose concentrations. (A) Sucrose-solution acceptance was recorded as the percentage of ants that fed on a given drop of sucrose at four different concentrations (1, 5, 10 and 20%). Pheromone pre-exposure increased the number of ants feeding on sucrose solutions. Dots represent means \pm s.e.m.; *N* indicates sample size. GLMM. (B) Time spent feeding at drops of different sucrose concentrations. There was no effect of pheromone pre-exposure. Box plots represent median, quartiles, and 10th and 90th percentiles (lower and upper whiskers); dots represent individual ants. Numbers under box plots indicate sample sizes; differences in sample size with respect to A are due to the exclusion of ants that fed longer than 3 min and ants for which feeding time could not be recorded (see Materials and Methods for details). Linear mixed model (LMM). n.s., not significant.

Ph+O+: 5.81 ± 3.06 , means \pm s.d.) than the solvent one (Ph-O+: 3.77 ± 3.05 ; Ph+O+: 3.13 ± 2.31) (GLMM; zone: $\chi^2=44.73$, d.f.=1, $P<0.001$; Fig. 3B). The interaction between treatment and zone was not significant and therefore removed from the model. Pheromone pre-exposure had no effect on memory retention, consistently with the absence of effect on learning (Fig. 3A).

A similar pattern of results was obtained when analyzing the time spent by the ants in the two zones (LMM; zone: $F=30.32$, d.f.=1, $P<0.001$; Fig. 3C). Both groups of ants spent more time in the odor zone (Ph-O+: 46.60 ± 29.06 s; Ph+O+: 49.07 ± 28.44 s, means \pm s.d.) than in the solvent one (Ph-O+: 26.33 ± 28.75 s; Ph+O+: 19.56 ± 21.71 s), and there was no effect of pheromone pre-exposure

(Fig. 3C). The interaction between treatment and zone was not significant and therefore removed from the model. Thus, Argentine ants established a memory of the odor-sucrose association that could be retrieved at least a few minutes after learning.

Control group for the associative learning process

The US-only group that was not pre-exposed to the pheromone component and that experienced three consecutive rewarded visits to the arena with no odor (Ph-O-) did not visit one zone of the arena significantly more than the other (linalool: 5.30 ± 1.56 ; mineral oil: 6.47 ± 3.04 , means \pm s.d.) (GLMM; zone: $\chi^2=3.46$, d.f.=1, $P=0.063$; Fig. 4A). The same results were obtained when

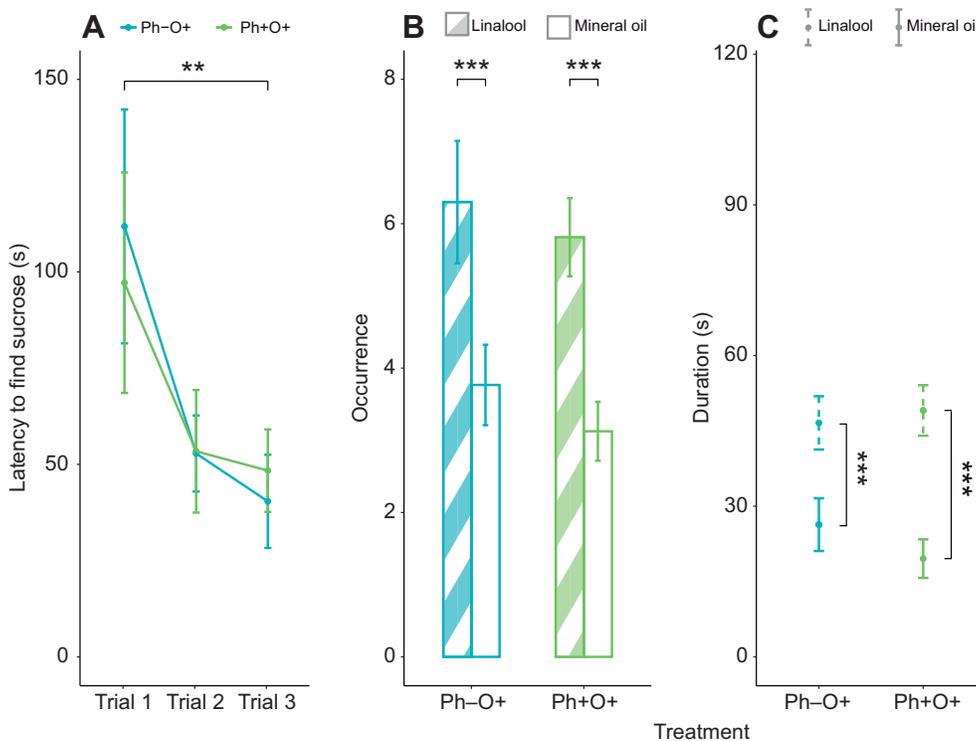


Fig. 3. Appetitive olfactory conditioning of free-walking ants. Ants were trained to associate linalool with a reward after pre-exposure to the pheromone (Ph+O+) or not (Ph-O+). (A) Latency (time until finding the sucrose) for the three conditioning trials. The latency to find the reward reduced with the number of trials but there was no effect of pheromone pre-exposure. (B) Occurrence (number of times ants entered each zone) in the memory test for both groups. Ants visited the zone containing the odor to which they had been trained more often than they visited the zone containing the solvent (mineral oil), thus showing significant retention. Pheromone pre-exposure did not affect retention. (C) Duration (time spent in each zone) during the memory test. Both groups showed a similar pattern. Dots and bars represent means \pm s.e.m. Ph-O+, *N*=30; Ph+O+, *N*=32. GLMM (B) and LMM (A and C). ** $P<0.01$, *** $P<0.001$.

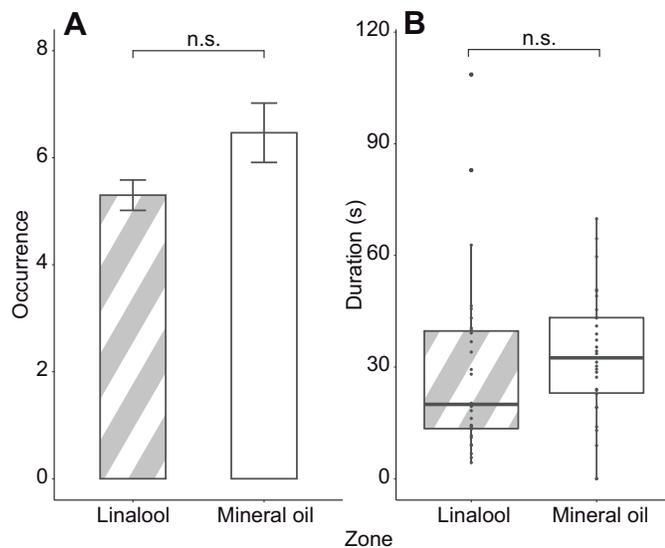


Fig. 4. Evaluation test for the group of ants trained without odorant (Ph-O-, N=30). This group of ants followed the same training procedure as the Ph-O+ and Ph-O+ groups of ants but without any odorant (US-only group). During the evaluation test, an ant was placed in the center of an arena with two Eppendorf tubes, one containing linalool (used as a conditioned stimulus during Experiment 2) and one containing mineral oil (solvent), with no reward. (A) There was no significant difference in the occurrence of visits between the linalool and mineral oil zones. Bars represent means \pm s.e.m. GLMM. (B) There was no significant difference in the time spent by the ants in the two zones. Box plots represent median, quartiles, and 10th and 90th percentiles (lower and upper whiskers); dots represent individual ants. LMM. n.s., not significant.

comparing the time spent by the ants in the two zones (linalool: 28.83 ± 23.63 s; mineral oil: 32.95 ± 17.30 s) (LMM; zone: $F=0.65$, d.f.=1, $P=0.427$; Fig. 4B). Thus, the test results of the group conditioned with odor and sucrose were not affected by spontaneous odor biases during the memory test.

Taken together, these results indicate that Argentine ants were able to learn and retained the odor-reward association after a short/mid-term delay, but were unaffected in their performance by pheromone pre-exposure.

DISCUSSION

Pre-exposure to the trail pheromone component modulates subjective reward evaluation

In this study, we analyzed if and how a pheromonal signal affected the subjective evaluation of reward (Experiment 1) and the cognitive capacities (Experiment 2) of Argentine ants, *L. humile*. We pre-exposed ants to (Z)-9-hexadecenal, a component of the trail pheromone that triggers the same trail-following response as the entire trail pheromone in this species, and determined whether this treatment affected these behavioral traits when the pheromone was no longer present. In doing this, we investigated, for the first time, olfactory learning and memory in a foraging context in *L. humile*.

In Experiment 1, (Z)-9-hexadecenal significantly modulated the acceptance of sucrose solutions of varying concentration by ant foragers. These results should be differentiated from previous reports indicating that the presence of trail pheromone, or of a trail pheromone component, increases the number of feeding ants at a food source. For instance, Greenberg and Klotz (2000) showed that adding (Z)-9-hexadecenal to a sucrose solution in the foraging area of Argentine ants' colonies increased both the number of ants attracted to the solution and its consumption at the population level.

Such an effect is expected for a trail pheromone leading to or marking an appetitive resource. In our work, the pheromone component did not mark the path to the food or the food itself. The pheromone was pre-exposed before foraging so that the effects observed reflected a prior tuning of the insects' motivation to search for and subsequently respond to food. Moreover, pre-exposure occurred in a context (a closed pot) unrelated to foraging, and ants were only exposed to the pheromone vapor (i.e. they had no direct contact with it on a substrate as in natural conditions). In nature, during foraging or recruitment, ants have access to other signals provided by recruiters, which deposit the trail pheromone, and by other foragers on the trail itself (Czaczkes et al., 2015). In our study, these sources of information were deliberately absent. This shows that the prior stimulation with the trail pheromone prepared the ants for foraging, enhancing their subjective evaluation of reward. Another difference between our study and that of Greenberg and Klotz (2000) is that we analyzed ants' consumption behavior at the individual level, not at the population one. This level of analysis allows us to infer about the mechanisms underlying such behaviors.

In honey bees, a similar increase in sucrose responsiveness was found after pre-exposure to geraniol, a pheromone associated with appetitive events (Baracchi et al., 2017, 2020). Accordingly, in the same work, pre-exposure to 2-heptanone, a pheromone that signals aversive events, decreased sucrose responsiveness. Pheromones thus modulate the subjective value of reward according to their valence. In our experiment, (Z)-9-hexadecenal increased the ants' acceptance of sucrose solutions, but the largest effect was observed for the lowest-concentration sucrose solution (1%), which generated an increase of nearly two times the percentage of ants feeding compared with ants without pheromone pre-exposure (from ~20% to 40%). However, no differences were found for the highest sucrose concentration (20%). The modulation of appetitive motivation would thus be particularly visible for food sources perceived as poor under normal conditions and would become 'valuable' after pheromone pre-exposure. In honey bees, modulation of responsiveness by pheromone components has typically been found in the case of low intensities of reinforcing stimuli (sucrose solution, electric shock) (Baracchi et al., 2017; Rossi et al., 2018). This can be understood by considering that responses to lower intensities can experience upward modulation, whereas in the case of higher intensities, such modulation would not be possible due to a ceiling effect. In honey bees, the observed change in appetitive responsiveness as a consequence of pheromone exposure is due to a modulation of aminergic circuits controlling appetitive motivation. This effect therefore accounts for the changes observed in subjective reward evaluation (Baracchi et al., 2020).

Trail-laying behavior is strongly modulated by the quality (Jackson and Châline, 2007; Verhaeghe, 1982) and quantity (Mailleux et al., 2000) of food sources. Therefore, trail pheromones provide both directional information leading to the food source and information on the food quality assessed by the recruiting ant. The latter informational component would make ants more prone to accepting poor-quality solutions.

The significant effect of pheromone pre-exposure on feeding acceptance was not verified when considering the feeding time. Ants spent more time feeding at higher concentrations of sucrose, but pheromone pre-exposure did not modify the time spent feeding. Thus, although the pheromone enhanced the acceptance of low-quality food sources, the resource quality could still eventually be assessed to avoid spending too much time and energy exploiting these resources to optimize foraging.

In a study conducted in parallel to our work (Oberhauser et al., 2020), the presence of pheromone trails leading to a sucrose solution did not influence food acceptance or feeding behavior of *Lasius niger* ants, a result that, in principle, contradicts our findings. These ants had to run once on a 20-cm channel covered with filter paper, onto which different amounts of trail pheromone were applied. At the end of the channel, a low-quality sucrose solution was offered to determine whether the immediate experience of running on the trail pheromone enhanced acceptance of that food source. Ants were analyzed during a single run in all experiments, in which starvation and food properties were varied. Important differences between this work and ours are the duration and method chosen for pheromone exposure. Whereas in our work, Argentine ants were pre-exposed for 20 min in an enclosure before they departed to the food source, in Oberhauser et al. (2020), *L. niger* ants were not pre-exposed to the pheromone, but exposed during a single short run in the channel. This means that their experience with the pheromone was significantly shorter than that of the Argentine ants in our work. As the *L. niger* ants were allowed to run only once to the food source, evaluation of the cumulative effects of the trail pheromone on their foraging behavior was not possible. Had the exposure to the pheromone been longer and continuous, it could have perhaps affected the foraging decisions of the *L. niger* ants in a way similar to that observed for our Argentine ants.

Alternatively, the difference in the impact of trail pheromone on foraging decisions observed between these two species could reflect differences in social information use and relevance. Whereas *L. niger* seems to prioritize individually acquired information over social information (Grüter et al., 2011), Argentine ants follow the opposite trend (Aron et al., 1993; von Thienen et al., 2016). The relative weight of trail pheromone use in *L. niger* and *L. humile* can be discussed in the light of their respective ecologies: *L. niger* forms smaller colonies than *L. humile* (Aron et al., 1993; Beckers et al., 1989; Stradling, 1970) and is a sedentary species usually foraging at long-lasting food sources (Völkl et al., 1999), whereas *L. humile* forms large societies (Beckers et al., 1989) and frequently emigrates during the course of a single season (Markin, 1970). In changing environments with unpredictable resources, pheromones would allow rapid displacement in response to any local disturbance for opportunistic species such as *L. humile*, rather than relying on the development of individual memories leading to spatial fidelity (Aron et al., 1993).

Pre-exposure to the trail pheromone component does not affect appetitive learning or memory retention

In Experiment 2, and irrespective of the treatment received, ants learned the odor-reward association and retrieved the olfactory memory to find the food during a retention test. We can assure that this was the result of an associative learning process as the Ph-O-control ants that underwent the same training procedure without any odor did not show any preference between linalool and mineral oil during the evaluation (Fig. 4).

To our knowledge, this is the first evidence of olfactory associative learning and memory in *L. humile* studied in a foraging context and in free-walking conditions. Previous studies showed that this species can orient towards learned visual cues (Aron et al., 1993) and form associations between cuticular hydrocarbons and sucrose in harnessing conditions (van Wilgenburg et al., 2012).

The fact that *L. humile* ants retrieved the olfactory memory ~9 min after the last conditioning trial indicates that olfactory

memories can drive their foraging choices, at least during the interval used in our experiments. In current models of honey bee memory (Menzel, 1999), this interval corresponds to the interface between short-term (from seconds to a few minutes) and mid-term (from a few minutes to ~24 h) memories, defined on the basis of the different molecular cascades activated by learning trials during these temporal windows. Our study thus raises the question of the durability of olfactory memories formed in the brain of Argentine ants. Future studies should address the issue of long-term memory formation and its temporal characterization in these ants.

No changes were detected in learning and memory retention with respect to pheromone pre-exposure. Both pheromone-pre-exposed and non-pre-exposed ants reduced to the same extent the latency to find the rewarded odor in the arena during the three conditioning trials, and exhibited a similar preference for that odor in the retention test. This result was unexpected, given the fact that the trail pheromone component changed the subjective evaluation (i.e. the salience) of sucrose solution, which was used as the US during conditioning. Experiments in honey bees have shown that a high responsiveness to sucrose solutions of variable concentration correlates with better olfactory and tactile learning (Scheiner et al., 2001a,b). Similarly, in ants, foragers with higher responsiveness to sucrose concentrations than nurses learn better appetitive associations between odor and sucrose (Perez et al., 2013). Moreover, in honey bees, pre-exposure to pheromones modulates not only reward responsiveness but also appetitive olfactory learning and memory formation (Baracchi et al., 2020). There are, however, at least two plausible explanations for the absence of effect of the trail pheromone component on the cognitive performances studied. First, the sucrose concentration used for conditioning was 20%, which yielded the same level of acceptance in pre-exposed and control ants (ceiling effect; see above and Fig. 2A). It is thus possible that the modulatory effect of the trail pheromone component was lost owing to the intrinsic high-motivational value of the sucrose solution used as reward. The slightly bigger difference in the time spent by the pheromone-pre-exposed ants in the two zones of the memory test compared with non-pheromone-pre-exposed ants could be the manifestation of such modulation. Using a lower sucrose concentration during olfactory conditioning could have revealed a modulatory effect of the pheromone on learning; however, the possibility of conditioning the ants with such a low-quality reward would have been significantly reduced. Second, the size of the arena was small (5 cm diameter), thus diminishing the potential penalty of wandering around in search of the reward. In a large arena, with an increased surface area, rendering the localization of the odor spot more difficult, differences between pheromone-pre-exposed and control ants could have been visible. Alternatively, keeping the same arena but rendering the problem more complex by adding a second odor during the memory test (instead of mineral oil), or even conditioning pheromone-pre-exposed and control ants to discriminate two odorants with different outcomes, could reveal a modulatory effect of the pheromone component on cognition.

The neural underpinnings of pheromone-based modulation of reward evaluation

The neural mechanisms underlying the observed modulation of sucrose acceptance by the trail pheromone component remain unknown in Argentine ants. Nonetheless, in honey bees, a recent work has shown that biogenic amines are involved in the modulation of appetitive responsiveness and learning (Baracchi

et al., 2020). This effect is consistent with their well-known role as neuromodulators of several aspects of animal behavior, including appetitive responsiveness (Scheiner et al., 2002). In honey bees, octopamine mediates appetitive responses (Scheiner et al., 2006) and the reinforcing properties of sucrose rewards (Hammer, 1993). Recent work also indicates the involvement of tyramine in similar functions (Scheiner et al., 2017). In the fruit fly *Drosophila melanogaster*, sucrose reward is represented by a specific subset of dopaminergic neurons in the brain (Burke et al., 2012; Liu et al., 2012). In all these cases, providing that octopaminergic, tyraminergetic and dopaminergic neurons play a similar role in Argentine ants, pheromone pre-exposure could activate these signaling circuits, resulting in a direct enhancement of appetitive motivation. Alternatively, the modulation by the trail pheromone component may be inhibitory rather than excitatory. In this case, serotonergic signaling could be the target of pheromonal action, given its depressing role in feeding behaviors. In *Camponotus mus* ants, for instance, serotonin diminishes feeding activity (Falibene et al., 2012) without modifying the sucrose acceptance threshold. Thus, if serotonin depresses feeding responses, the trail pheromone component could disinhibit such depression, rendering animals more responsive to food.

In *Pheidole dentata* ants, lowering serotonin levels experimentally by oral administration of the serotonin-synthesis inhibitor α -methyltryptophan resulted in reduced trail-following behaviors (Muscedere et al., 2012). Treated ants were less likely to initiate trail following, and oriented along pheromone trails for significantly shorter distances than untreated, similar-age workers. Exposure to the trail pheromone could modulate this behavioral component rather than the feeding itself. As a result, pheromone-exposed ants would be both more responsive to food and more accurate in trail following.

Social and individual information use and food-source profitability

By studying the effect of social information on individual foraging behavior, we set individual foraging decisions within a social context, thus raising the question of how individuals value these two informational sources. In leaf-cutting ants, appetitive motivation is modulated by both the social information received during recruitment and by their own evaluation of the resource (Roces, 1990, 1993). In carpenter ants, social information seems to dominate in this evaluation process, as ants receiving social instructions via trophallaxis followed such instructions even when the food presented to them was toxic (Josens et al., 2016). Argentine ants rely heavily on their trail pheromone, even more than other mass-recruiting ants (Aron et al., 1993). Aron and colleagues (1993) found that more than 90% of the foragers laid a trail during their first trip to and from the nest, and a great majority of them chose to follow the trail pheromone over visual cues learned in a Y maze, even when presented with very weak trail concentrations. Whether Argentine ants would drink more of poor or toxic solutions following social instructions provided by a recruiter remains to be studied. It has been suggested that following social instructions, instead of evaluating the food quality and weighing up the gains and losses of energy in engaging in foraging behavior, is less costly and much faster for individuals (Rendell et al., 2010). This social bias can help groups to monopolize food sources in a competitive environment (Detrain and Deneubourg, 2008). This mechanism could therefore be a key point to explain the invasive success of mass-recruiting ants such as Argentine ants (Carpintero and Reyes-López, 2008).

Perspectives for Argentine ant control

The synthetic trail pheromone component (*Z*)-9-hexadecenal as a disruptor of the trail-following behavior has received attention as a novel control agent for this invasive species (Nishisue et al., 2010; Suckling et al., 2008, 2011; Tanaka et al., 2009). This synthetic component has several advantages for control strategies: it is commercially available, species specific and non-toxic. However, the use of pheromone dispensers alone to disrupt the ants' trails is not effective enough to decrease ants' populations (Nishisue et al., 2010). Sunamura et al. (2011) showed that combining synthetic trail pheromone dispensers with insecticidal bait effectively reduced Argentine ants' populations. This study demonstrated the validity of combining pheromone and bait treatments as a novel strategy to manage Argentine invasive ants in a small area, but could also be applicable to larger-scale eradication programs. Here, we show an additional advantage of using (*Z*)-9-hexadecenal combined with toxic baits, namely the fact that the pheromone component not only disrupts the trails of the ants but also predisposes them to a better evaluation of the bait, thereby increasing its potential acceptance.

Given the scarcity of studies on the learning abilities of *L. humile* and the species' high environmental and economic impact, more studies are required to characterize the cognitive processes involved in its foraging activities and invasive success. Integrating the study of cognition with control strategies in the case of target species is still a relatively unexplored field of research, which could provide important advantages to manipulate the decision-making processes of the Argentine ant. This approach could be used in specific scenarios, allowing us, for example, to transform the rejection of toxic bait into a better acceptance of the harmful food, both at the individual and colony level (Josens et al., 2016).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.R., M.G., P.d.E., R.J.; Methodology: N.R., R.J.; Validation: N.R.; Formal analysis: N.R.; Investigation: N.R., M.P.; Resources: P.d.E., R.J.; Data curation: N.R., M.P., M.A.M.; Writing - original draft: N.R., M.G., R.J.; Writing - review & editing: N.R., M.G., P.d.E., R.J.; Visualization: M.G., P.d.E., R.J.; Supervision: R.J.; Project administration: P.d.E., R.J.; Funding acquisition: P.d.E., R.J.

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Data availability

Data are available from the Figshare Digital Repository at doi:10.6084/m9.figshare.12445067.

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