



A new experimental setup for studying ants and similar-sized insects

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Abstract

Laboratory studies on insects face the dual challenge of maintaining organisms under artificial conditions, and in reduced spaces while mimicking the species' ecological requirements as much as possible. Over decades, myrmecologists have developed and continuously improved laboratory methods and artificial nests for rearing ants. However, the setups commonly used to house colony fragments of few individuals or even isolated individuals present disadvantages such as insufficient ventilation, difficult access to specific workers, and problems with water delivery. Here, we developed and tested a new setup for keeping ants or similar sized insects in small groups. The setup consisted of a Petri dish containing a piece of plaster connected underneath to a water tank by a sponge. The sponge is immersed in the water on one side and embedded in the plaster on the other side, maintaining the plaster permanently moist and thus offering a water source to the ants. We tested the setup with two ant species of different sizes, *Platythyrea punctata* and *Cardiocondyla obscurior* in feeding, starvation, and desiccation conditions. Our results showed that our new setup worked equally well for both species in all conditions in comparison to a more conventional setup with the advantage of reducing maintenance costs and ant manipulation, but also preventing death by drowning and offering water *ad libitum*. The setup was quick to build, with cheap and reusable materials for further experiments. Therefore, we are confident that it will facilitate future studies on isolated or small groups of individuals and that such a standardized setup will make future studies more comparable.

Keywords Artificial nest · Experimental design · Behavior · Long-term experiments · Plaster

Introduction

Rearing and maintaining insects in laboratory conditions are challenging as one needs to mimic species' requirements as much as possible. Experiments on ants are no exception. Although underground nests may go as deep as a few meters and workers may forage up to a hundred meters from their colonies in the field, laboratory colonies are usually kept in a more limited space depending on the species studied (Hölldobler and Wilson 1977, 1990; Nogueira et al. 2022).

Since 1753 and the first attempt by the Dutch biologist Jan Jacob Swammerdam to maintain ants in artificial nests, myrmecologists have developed and continuously improved

laboratory methods for rearing ants (Skaife 1951; Buschinger 1974; Hölldobler and Wilson 1990; Czechowski and Pisarski 1992; Cobb 2002). In general, artificial nests need to fulfill several requirements. Ideally, the nests should mimic natural conditions to limit stress and abnormal behavior. They must offer a good ventilation, an optimal humidity for both adults and brood, prevent ants from escaping, and facilitate behavioral observations while also being easy to manufacture and recycle (Buckingham 1904; Ning et al. 2019).

Experiments on ants are done at the colony level (Cook et al. 2010; Greenwald et al. 2018; Lesne et al. 2022), on colony fragments or even at the individual level. For example, several studies have investigated small groups of workers, with or without brood, or isolated individuals to test the effect of limited social interactions on behavior, development, gene expression, and lifespan (Boulay et al. 1999; Koto et al. 2015; Scharf et al. 2021). Isolation of individuals has also been used for testing treatment effects on workers' survival (Arganda et al. 2017) and disease resistance (Hughes et al. 2002; Konrad et al. 2012). In the latter cases,

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using colony fragments prevents colony damage or colony loss but also permits more replicates per condition.

For these isolation experiments, researchers have been using smaller containers such as Petri dishes, tubes closed with a cork or plastic boxes with a light-shielded nest and access to water (Bishop et al. 1980; Koto et al. 2015; Mitrus 2019). Though amenable, those small nests may have disadvantages. For example, tubes cannot be cleaned easily without disturbing the ants, and food quickly gets moldy if the tube remained tightly closed. Easy access to brood or specific workers can be another issue when using tubes. Humidity and water access are also a matter of concern. Ants usually have *ad libitum* access to water by moistened pieces of cotton, sponge, or plaster, or even drops of water directly deposited on the ground (Chang 1985; Boulay et al. 1999; Arganda et al. 2017). Nevertheless, water may dry out quickly, which necessitates daily inspection. In addition, refilling water when needed is a source of extra stress for the ants. Lastly, these devices may not be adapted for each species, which may drown in the water supply. This is problematic as it is then impossible to distinguish deaths due to specific treatment effect from “accidental” deaths.

To address these issues, we developed and tested a new setup for keeping groups of ants under controlled laboratory conditions. Our main goal was twofold: (1) to facilitate the maintenance of ants by better controlling humidity and access to water without affecting the survival of workers, and (2) to avoid accidental deaths by drowning regardless of the ant size. We used the medium-size ponerine ant *Platythyrea punctata* (Smith, 1858) and the minute myrmicine ant *Cardiocondyla obscurior* (Wheeler, 1929). The ants were maintained under three different conditions: regular

feeding, starvation, and desiccation. Those three conditions allowed us to evaluate our setup over a long period of time and to control that ants had *ad libitum* access to water. We showed that our setup worked equally well for both species. Our results are similar to those obtained with conventional devices but with much less maintenance time and no drowning accidents.

Material and methods

Nest materials and construction

Our nest design consisted of a Petri dish containing a piece of plaster (Class II alabaster plaster, Moldabaster Henry Schein 896,859) connected underneath to a water tank by a sponge. The upper part of the sponge was embedded in the plaster while the lower part was immersed in water. As a result, the plaster was permanently moist and could be used as a water source for the ants (Fig. 1).

We constructed the nests using Petri dishes (diameter: 5.5 cm) covered with mesh lids for ventilation (diameter of the ventilation window on the lids: 2 cm, Fig. 1A). We drilled a hole (diameter: 0.3 cm) at the bottom of every Petri dish at 1 cm from the wall to pass a sponge strip through (10 cm × 0.3 cm). Approximately 2 cm of the sponge strip was embedded in fresh plaster (approx. 3 mL) (Fig. 1B). The Petri dishes were then evenly spaced and arranged on a 5 × 4 array on the lid of a large plastic box (26 cm × 35 cm × 8 cm) that served as a water tank. Twenty holes (diameter: 0.5 cm) were previously drilled on the tank’s lid to allow the remaining 8 cm of the sponge strips to be immersed in water. Each

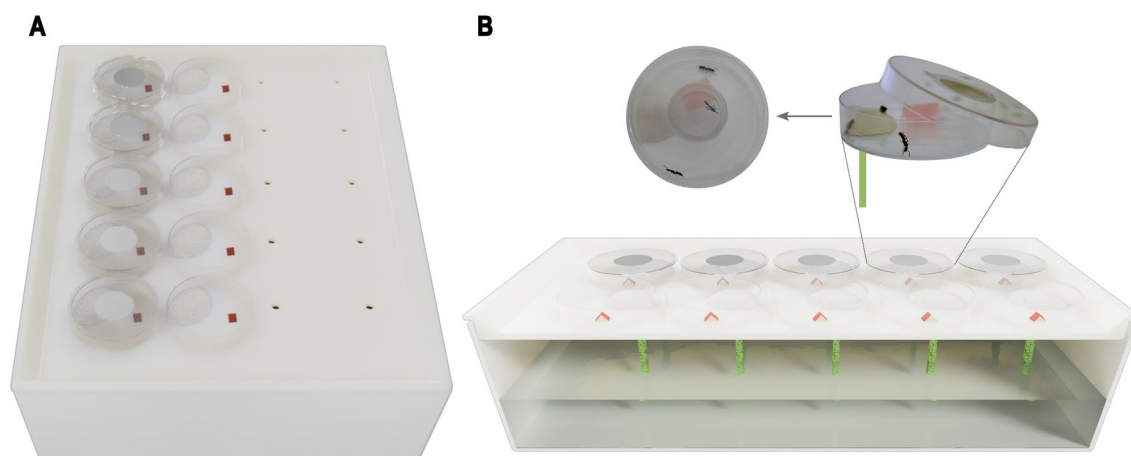


Fig. 1 Scheme of the new experimental setup, including the Petri dishes and the container box (water tank). **A** A complete view of one tank with ten prepared nests (left row with lid, right row without lid) and ten holes where the sponge connected to the plaster in each box goes through. **B** Lateral view of the complete setup cut in half with ten prepared nests (top row with lid, bottom row without lid). Close

up picture of the experimental nest (zoom) consisting of a small Petri dish that included a shelter (red plastic film) and a piece of plaster (white) with a grid lid (with three ants of *Platythyrea punctata*). The nest was connected through a sponge (green) to water contained in a tank underneath (Illustration by Henrique Galante)

tank provided water to a total of 20 Petri dishes (Fig. 1A). Humid plaster was easily recognizable as its color changed from white to light brown when the sponge strips were soaked in water (see Supplementary Fig. 1). Plasters that were not properly humidified, i.e., remained white, were replaced. This setup was hereafter referred to as “new setup” (NSU, see Supplementary Fig. 2).

Ant species

To test the reliability of our setup for different ant species, we used the ponerine ant *Platythyrea punctata* and the myrmicine ant *Cardiocondyla obscurior*. Both species differ in their worker’s size (approx. 0.8 vs. 0.2 cm for *P. punctata* and *C. obscurior*, respectively), and in their colony structure (facultative parthenogenetic vs. polygynous) (Schilder et al. 1999; Heinze 2017).

Clonal colonies of *P. punctata* were collected in 2012 at El Verde Field Station, El Yunque National Forest (Puerto Rico). Since their collection, stock colonies have been kept in plastic boxes (20 cm × 20 cm × 9 cm) with a plaster floor. Cavities dug in the plaster floor and covered by glass plates and a piece of a dark plastic film served as nest chambers. *C. obscurior* colonies were collected in 2016 in the botanical garden of the Technical University of Munich (Freising, Germany). The species was most likely introduced in that place with exotic plant pots (Schultner & Oettler, pers. com.). Colonies of *C. obscurior* were maintained in plastic boxes (20 cm × 15 cm × 5 cm) with Plexiglas connected multi-chamber frames between two glass slides and a dark plastic film as nests on the plaster floor (Heinze 2020).

Stock colonies were maintained under controlled conditions in a climate chamber (day/night cycles of 12 h 26 °C/12 h 22 °C and 75% humidity). Ants were fed with diluted honey and small cockroaches and/or fruit flies three times a week. Water was provided *ad libitum* via a plastic tube plugged by cotton. The humidity in the plastic boxes was controlled by regularly moistening the plaster during the feeding sessions.

Experiments

To evaluate nest suitability for maintaining ants under different experimental conditions, we collected in total 150 nurses (i.e., workers found in contact with or near the brood, Mersch et al. 2013; Bernadou et al. 2015) from five to six stock colonies per species. Three ants from the same stock colony were transferred and grouped into a Petri dish (diameter: 5.5 cm with a mesh lid, see setup above), in which we added a red folded plastic film that served as a shelter (i.e., ants were regularly observed resting under it, Fig. 1A and Supplementary Fig. 2). Each replicate (i.e., group of three ants) was randomly assigned to one of the following

treatments: Feeding (F), Starvation (S), or Desiccation (D). Ants were fed three times a week (on Monday, Wednesday, and Friday) with a drop of diluted honey (1:1 v/v) for the feeding treatment. In the feeding and starvation treatments, water was delivered to the ants either through humidified plaster connected to the water tank (new setup, NSU) or through a manually humidified sponge and plaster herein-after referred to as “standard setup” (SSU). In the standard setup, the sponge and the plaster had to be humidified every day (including weekends). Finally, neither water nor food was available in the desiccation treatment (D). Experimental treatments were maintained under the same controlled conditions as the stock colonies.

Ten replicates (i.e., three ants per box) were set up per condition (five conditions: F/NSU, F/SSU, S/NSU, S/SSU, and D/NSU) and per species. Mortality was checked daily until all the ants died (Starvation and Desiccation) or up to 60 days (Feeding treatment).

Statistical analysis

Survival data were analyzed using the statistical software R (version 4.1.0, R Core Team 2020 and Cox mixed-effects models (coxme, package “coxme,” Therneau 2020). For each species, we tested whether the factor “condition” (levels: F/NSU, F/SSU, S/NSU, S/SSU, and D/NSU) included as fixed effect factor influenced the survival of ant workers. The factor “replicate” nested within the factor “colony” was included in the models as a random effect factor. Statistical models were compared to null (intercept only) models using Likelihood Ratio (LR) tests to assess the significance of the predictor. Upon significance, we performed pairwise comparisons between the different conditions (glht, package “multcomp,” Hothorn et al. 2008) and *p* values were adjusted by Bonferroni correction.

Results and discussion

In this study, we developed and tested, a new setup (NSU) for keeping ants in small groups (Fig. 1). Our results showed that the new setup worked equally well for both species studied but with the advantages of considerably reducing maintenance time and preventing death by drowning.

To validate its suitability, we recorded workers’ survival of the medium-size ant *Platythyrea punctata* and the minute-size ant *Cardiocondyla obscurior* in a feeding (F) and a starvation (S) treatment. We compared our results to a setup where ants had access to water through a manually humidified sponge and plaster (SSU). We also included a desiccation (D) treatment as a control. Overall, we found a significant effect of the treatment on ants’ survival (COXME, LR-test: $\chi^2 = 240.79$, $df = 4$, $P < 0.001$ and $\chi^2 = 221.06$,

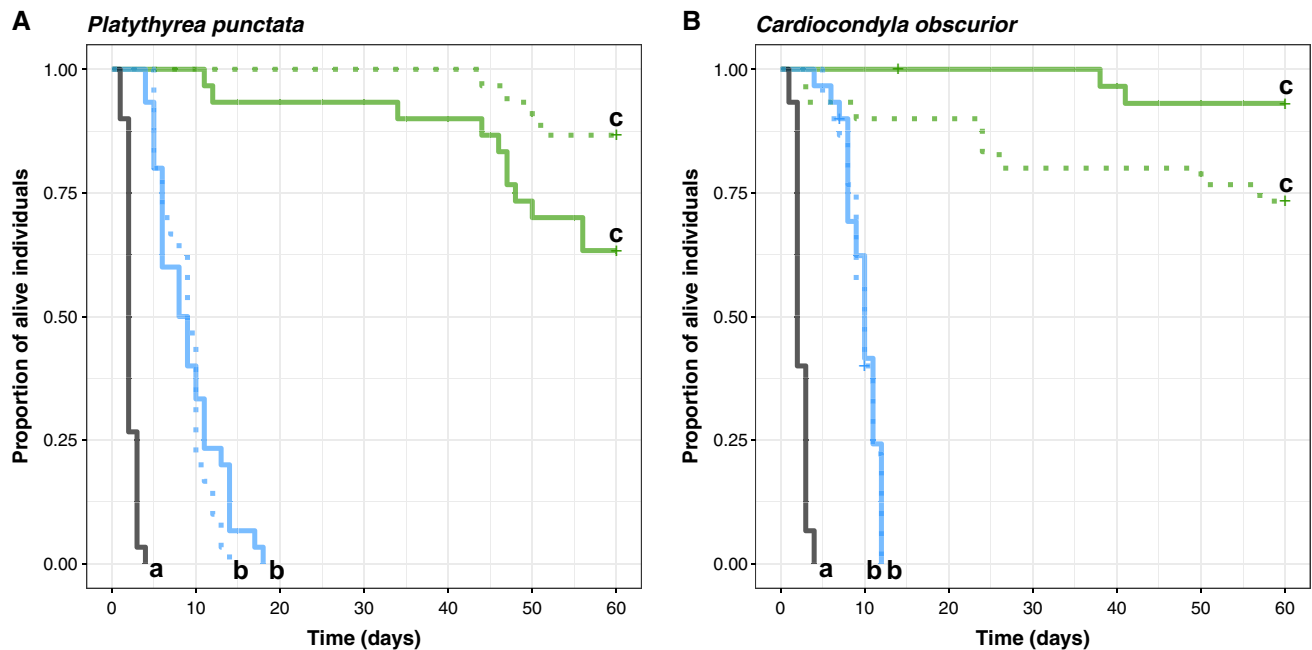


Fig. 2 Survival plots of workers of **A** the ponerine ant *Platythyrea punctata* and **B** the myrmicine ant *Cardiocondyla obscurior* ($N=30$ workers per treatment and species). Three ants from the same stock colony were grouped and randomly assigned to one of the following treatments: Feeding (green), Starvation (blue) or Desiccation (black). In the feeding and starvation treatments, water was delivered to the

ants either through humidified plaster (solid lines NSU, new setup) connected to the water tank or through a manually humidified sponge (dotted lines SSU, standard setup). No water nor food was available in the Desiccation treatment (black and solid line) (different letters indicate significant differences at $P < 0.05$ after Bonferroni corrected post hoc comparisons)

$df=4$, $P < 0.001$ for *P. punctata* and *C. obscurior*, respectively) (Fig. 2A and B). As expected, both species survived significantly longer in the feeding treatment followed by the starvation and the desiccation treatments (Bonferroni corrected post-hoc pairwise comparisons, for both species: all comparisons: $P < 0.001$ except for F/NSU versus F/SSU, and S/NSU versus S/SSU). Similar results were observed in the ant species *Camponotus fellah*, where workers survived longer when they had access to food than under starvation (Boulay et al. 1999; Koto et al. 2015). The social environment also influenced workers' survival: isolated individuals died faster than workers kept in groups (Boulay et al. 1999; Koto et al. 2015). Whether similar results hold for other species with different characteristics (e.g., colony size) needs to be tested. We believe that our setup will make future studies easier and more comparable.

Most importantly, ants' survival did not depend on the setups tested (Fig. 2A and B). The ants survived equally well in our new setup as the ants reared in the standard setup (Bonferroni corrected post-hoc pairwise comparisons, F/NSU versus F/SSU: $P = 0.428$, S/NSU versus S/SSU: $P = 1$ for *P. punctata*, and F/NSU versus F/SSU: $P = 1$, S/NSU versus S/SSU: $P = 1$ for *C. obscurior*). These results confirm that ants got *ad libitum* access to water by licking water oozing from the plaster in our new setup (LP pers. obs.). Moreover, the new setup turned out to be suitable for short

(a few days) and long-term (several months) experiments (i.e., starvation vs. feeding) with the advantage of requiring much less maintenance (i.e., no daily water refilling needed).

It is also important to note that in the standard setup we recorded one *P. punctata* (in S/) and three *C. obscurior* (two in F/ and one in S/) "accidental" deaths, i.e., ants were found dead besides or under the sponge. In addition, in the S/SSU condition, three *C. obscurior* workers died in the same Petri dish because the sponge had dried out totally between two waterings. We never had such issues within the new setup in which the plaster remained moistened and the water tank still held enough water for at least 60 days. Despite the plaster being constantly moistened, the large mesh lid openings reduced mold growing on food compared to a tightly closed setup (LP pers. obs.). Finally, a word of caution here is warranted. We observed that the sponge deteriorated after a long period (> 2 months), and in some cases plaster and sponge had to be changed to ensure proper watering (i.e., plasters became white because they dried out, see Supplementary Fig. 1).

In conclusion, our setup reduced the maintenance time considerably while also keeping the ants alive for at least 2 months and preventing accidental deaths. It was easy and quick to build, with inexpensive and readily available materials that can be reused for further experiments. Though we tested only two different species, the new setup worked

equally well with workers of more than 20 other species— isolated individuals or in groups in long-term experiments (data not shown, more than 200 days LP pers. com.). Similar-sized insects can also probably be maintained using the same setup. Finally, in case the ant behavior needs to be recorded, the mesh lids could be replaced by standard lids during the video recording. We are, therefore, confident that our setup will ease future studies on isolated or small groups of individuals and will make future studies more comparable.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00040-023-00915-6>.

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Data availability Data are available upon request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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