Insectes Sociaux (2019) 66:601–609
https://doi.org/10.1007/s00040-019-00724-w

Insectes Sociaux

RESEARCH ARTICLE

A new type of egg produced by foundress queens of *Atta texana* (Attini, Formicidae)

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Received: 4 June 2019 / Revised: 25 August 2019 / Accepted: 6 September 2019 / Published online: 20 September 2019

Abstract
In the fungus-growing ant genus *Atta*, foundress queens nourish their brood and incipient fungus gardens with nutrients derived from trophic eggs. We discovered a third kind of egg laid by *Atta* foundresses in addition to reproductive and trophic eggs. We use fluorescent microscopy to show that this third type of eggs represents reproductive eggs that are unviable and fail to develop. Unviable reproductive eggs are somewhat larger (≈490 µm × 317 µm) than regular reproductive eggs (≈425 µm × 240 µm), but smaller than trophic eggs (≈640 µm × 530 µm). Trophic eggs liquify by some endogenous process within 24 h after oviposition to release nutrient contents. Unviable reproductive eggs do not liquify, but unviable reproductive eggs can be digested by the fungus, whereas reproductive eggs are not digested by the fungus to complete their development. We also report the first observation for *A. texana* of nanitic males (presumably diploid males) that were killed by the foundress queen shortly after the males' eclosion.

Keywords Attine ant · Fungus-growing ant · Nest founding · Oviposition · Trophic egg

Introduction

Fungus-growing ants (attines) are unique among ants because they depend on a mutualistic fungus that they grow as food (Mehidabadi and Schultz 2009; Augustin et al. 2011; Mueller et al. 2017, 2018). The fungus is vertically transmitted between ant generations, as a female reproductive takes an inoculum of the fungus from her natal nest, stores a pellet of fungus for the duration of the mating flight in a pocket in her mouth, then uses this pellet as a starter culture for her first fungus garden in her incipient nest (Huber 1905, 1907; Autuori 1942; Bazire-Bénazet 1957; Weber 1972; Mueller et al. 2001; Mueller 2002; Augustin et al. 2011). Most attine foundresses forage occasionally at this early stage for plant substrate to sustain the growth of the incipient garden (Wheeler 1907; Weber 1958; Mueller et al. 1998; Fernández-Marín et al. 2005), but *Atta* foundresses have claustral nest founding where they seal themselves into an incipient nest, precluding foraging. An *Atta* foundress, therefore, has to sustain the growth of the incipient garden entirely on the resources carried by the foundress (von Ihering 1898; Huber 1905, 1907; Autuori 1942; Weber 1972; Seal 2009; Augustin et al. 2011). Only after the queen has raised her first cohort of workers does a colony forage for leaf material to sustain fungal growth. To nourish the incipient garden and the first brood, a foundress queen lays two types of eggs: reproductive eggs that develop into workers and trophic eggs that the queen processes to feed to her brood, or that the queen ingests herself to digest and convert into nourishment for the fungus in the form of the queen's feces (Huber 1905, 1907; Augustin et al. 2011).

Trophic eggs are non-embryonated eggs incapable of development, and they serve as a supplementary nutrient for queens and larvae (Huber 1905, 1907; Autuori 1942; Wilson 1971; Weber 1972; Glancey et al. 1973; Diehl-Fleig and de Araújo 1996; Augustin et al. 2011; Lee et al. 2017; Peeters 2017). In ants, the size of trophic eggs varies markedly between different species, and trophic eggs can either be larger or smaller than reproductive eggs within a species (Glancey et al. 1973; Augustin et al. 2011; Lee et al. 2017). In some ant species, workers lay trophic eggs to help feed

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00040-019-00724-w) contains supplementary material, which is available to authorized users.

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the larvae (Gobin et al. 1998; Lee et al. 2017; Peeters 2017); whereas in other species, trophic eggs are produced only by foundress queens during the early nest-founding stage to nourish the first cohort of workers (Weber 1972; Glancey et al. 1973). In incipient colonies of *Atta*, for example, larvae are fed only trophic eggs laid by the newly mated foundresses (Huber 1905, 1907; Autuori 1942; Bazire-Bénazet 1957; Weber 1972), presumably because consumption of fungus garden by the brood would attenuate garden growth. Trophic eggs represent the most important food resources for both foundresses and larvae at the nest-founding stage of *Atta sexdens* (Bazire-Bénazet 1957; Augustin et al. 2011), and foundresses of *At. colombica* can rear brood to eclosion even in the absence of fungal garden (Fernández-Marín and Wcislo 2005), indicating that trophic eggs are a sufficient food for complete development of *Atta* larvae (i.e., fungal food is not necessary to complete larval development).

During experiments to characterize embryological development in *Atta texana* (Fang et al. in preparation; Fang 2019), we discovered a third kind of egg laid by foundress queens in addition to reproductive and trophic eggs. We (a) identify this third type of egg with fluorescent microscopy as unviable reproductive eggs; (b) document experimentally that these unviable reproductive eggs are eventually digested by the fungus, whereas reproductive eggs are not digested and thus complete embryonic development; (c) show that trophic eggs liquify by some endogenous process within 24 h after oviposition; and (d) report one incipient nest that produced nanitic males (possibly diploid males), which were killed by this foundress shortly after eclosion.

Methods

Ant colonies

Dealate foundresses of *Atta texana* were collected on 5. May 2018 at Brackenridge Field Laboratory ($N = 25$ females) and on 21. May 2018 at Commons Ford Metropolitan Park ($N = 30$ females), both in Austin, Texas, USA. Foundresses were collected as they were excavating nests during the day following mating flights at dawn (Marti et al. 2015). Each foundress was kept in a round plastic dish (Pioneer Plastics, Inc.; 6 cm diameter, 4 cm height), as described in detail by Marti et al. (2015). Each dish had a bottom layer of 2.5 cm moistened plaster, leaving enough room above the plaster to approximate the natural chamber dimensions dug by *At. texana* foundresses. The plaster was saturated with distilled water to maintain 100% humidity in the chamber. Prior to the experiment, the layer of plaster in each dish had been exposed to UV light for 10 min to reduce contamination. All lab colonies were maintained in a temperature-controlled room (25 ± 1 °C) during the experiments. Foundresses were not given any substrate for gardening, and foundresses, therefore, used their stored body resources for oviposition, brood rearing, and gardening, as is typical for natural claustral colony founding of *At. texana* and other *Atta* species (Della Lucia et al. 1995; Augustin et al. 2011; Fujihara et al. 2012; Seal 2009; Marti et al. 2015).

Colony maintenance and egg collection

Atta foundresses begin oviposition and expel a pellet of fungal hyphae during the first 2 days of nest founding and shortly after that lay the first eggs (Autuori 1942; Weber 1972; Augustin et al. 2011; Marti et al. 2015). Because of the high mortality typical for incipient colonies (typically more than 50%; Hölldobler and Wilson 1990, 2010; Peeters and Ito 2001; Brown and Bonhoeffer 2003; Marti et al., 2015; Camargo et al. 2016), we waited with egg collection until 1 week after the first brood of workers emerged. The first workers eclosed about 45 days after the day of the mating flight (at 25 ± 1 °C). Of the 55 foundresses collected, 20 foundresses succeeded at cultivating a healthy fungus garden by 28. June 2018; however, only six of these colonies (all collected on 5. May) produced workers. Experimental eggs were collected from these 6 colonies every 3 days from 28. June to 16. August. Because eggs can be difficult to locate in gardens, we separated each queen for egg collection from her garden and placed her for 24 h in a separate round plastic dish (5.5 cm diameter; 3.7 cm height) together with three of her minima workers and a small fragment from her garden ($\sim 5 \times 5 \times 2$ mm^3). Each of these dishes had a bottom layer of 1% agarose and was maintained at 25 ± 1 °C. The agarose maintained 100% humidity in a dish, but allowed also easy visual identification of any eggs on the smooth, translucent agarose substratum. Workers moved any newly laid eggs to the small fungus fragment, from which the eggs could then be collected with the help of a moistened fine brush. Because the main focus of our study was to characterize reproductive and trophic eggs, we did not collect behavioral observations on queens and workers systematically, but recorded observations sporadically while monitoring experimental dishes during the experiments.

Egg-deliquescence experiments

We observed reproductive eggs (R-egg), unviable reproductive eggs (UR-egg), and tropic eggs (T-egg) during egg collection (see details below; Figs. 1 and 2). Only the trophic T-eggs have been known so far as a source of nutrients for brood, the queen, and the garden in *Atta* (Huber 1905, 1907; Autuori 1942; Bazire-Bénazet 1957; Weber 1972; Augustin et al. 2011), but both UR-eggs and T-eggs could be potential resources of nutrients for developing brood, the queen, or the garden. Preliminary observations indicated that T-eggs

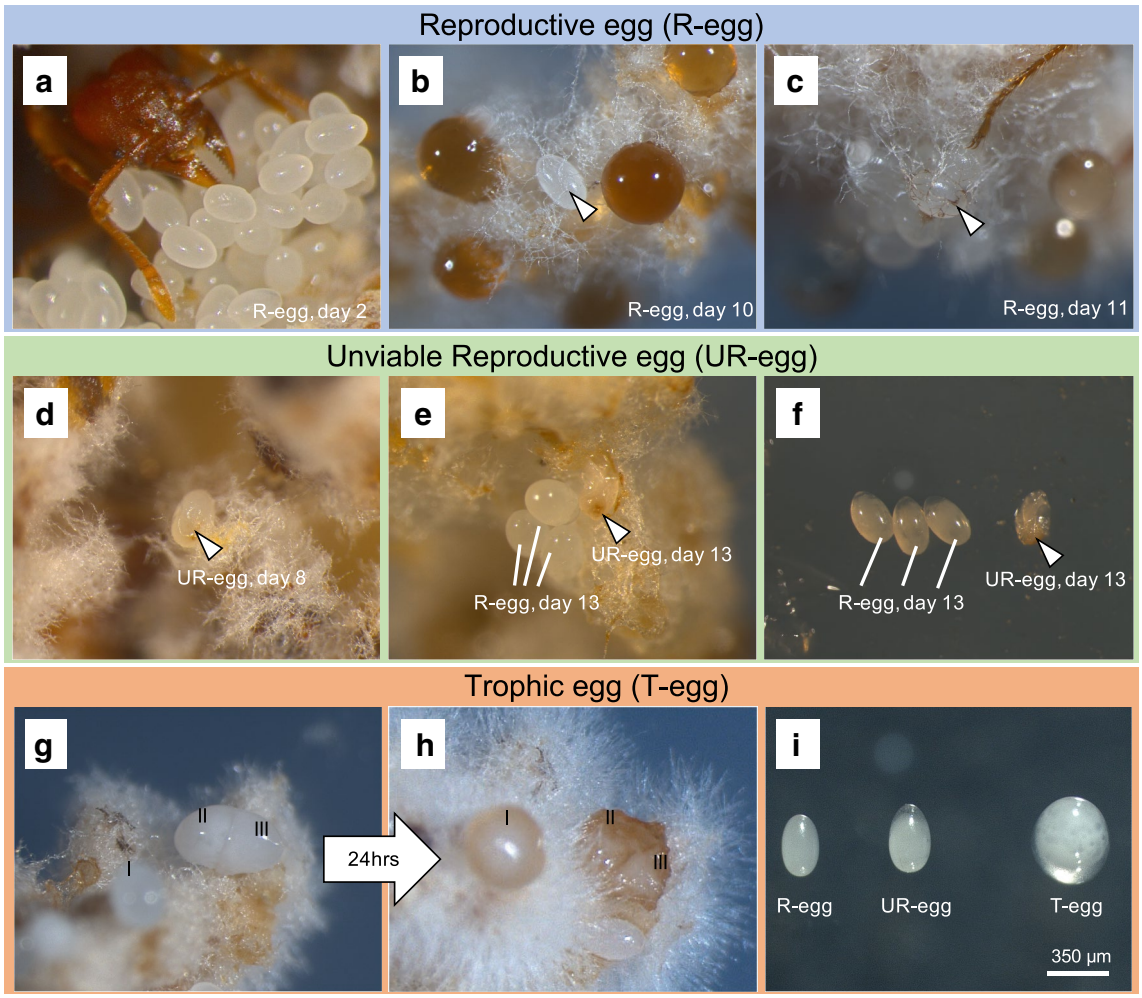

Fig. 1 Comparative morphology of three types of eggs laid by *Atta texana* foundresses: reproductive eggs (R-eggs), unviable reproductive eggs (UR-eggs), and trophic eggs (T-eggs). **a** A cluster of R-eggs tended by an *At. texana* worker. **b** A 10-day-old R-egg (see arrow) with healthy mycelium growing on the egg surface. **c** An 11-day-old R-egg with dead or stressed mycelium on the egg surface (see arrow). **d** An UR-egg starting to be digested by the mycelium, causing a depression on the surface (see arrow). **e**, **f** An UR-egg that had been digested by mycelium by day 13 (see arrow). **g**, **h** Three T-eggs (labeled I, II, and III) all laid on the same day, then all three eggs deliquesced within 24 h. **i** Morphological aspects of a R-egg, UR-egg, and T-egg, showing that T-eggs are larger than the other two types of eggs, and that UR-eggs are slightly larger than R-eggs (see Table 1 for exact size measurements)

deliquesced within 24 h after oviposition. Specifically, T-eggs liquified when the eggshell disintegrated, typically within a day; while, R-eggs and UR-eggs did not deliquesce during that time. To quantify the deliquescence process more rigorously, and to characterize differences among the three types of eggs, we set up three simple experiments to test factors that may cause the T-eggs to liquify. First, the fungus may secrete compounds, such as enzymes, that digest and liquify the T-eggs; this hypothesis predicts that contact with fungal garden is necessary, and that the T-eggs will not liquify (or liquify at a slower rate) when isolated from contact with fungal garden. Second, T-eggs may liquify by some unknown endogenous process, such as self-digestion of the thin eggshell; this hypothesis predicts that contact with fungal garden or with other eggs is not necessary for liquefaction. Third, R-eggs or UR-eggs may secrete compounds that cause liquefaction of T-eggs; this hypothesis predicts that T-eggs will liquify when in contact with other eggs, but not when isolated without any egg contact in garden and when resting on an inert surface.

To test the predictions of these hypotheses, we set up simple experiments to observe whether liquefaction of T-eggs occurred under different conditions. To test whether contact with fungus was sufficient, we conducted an experiment where newly collected T-eggs were placed either onto a small piece of garden (~ $2 \times 2 \times 2$ mm^3) or alternatively were placed isolated on an inert surface. For these experiments, eggs on garden or eggs in isolation were conducted in the absence of workers by placing them onto a central island (see Fig. 3) in a sealed dish with a moat of distilled water

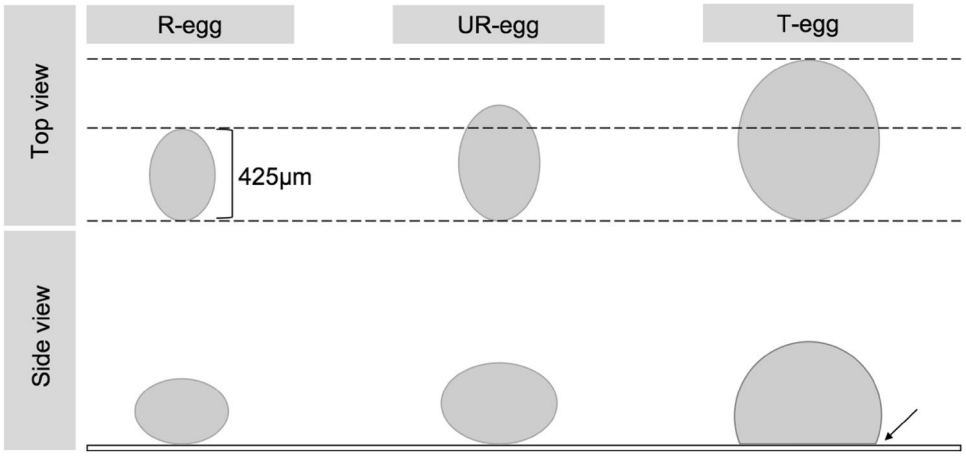

Fig. 2 Size comparison of a reproductive egg (R-egg), an unviable reproductive egg (UR-egg), and a trophic egg (T-egg) laid by an *Atta texana* foundress. The top view shows the difference in length between a reproductive egg (average of 425 μm), unviable reproductive egg (490 μm), and trophic egg (640 μm). The side view shows the morphological deformation at the bottom (see arrow) in a trophic egg (T-egg) when resting on a solid surface, whereas R-eggs and UR-eggs have a firm eggshell and do not deform when resting on a surface

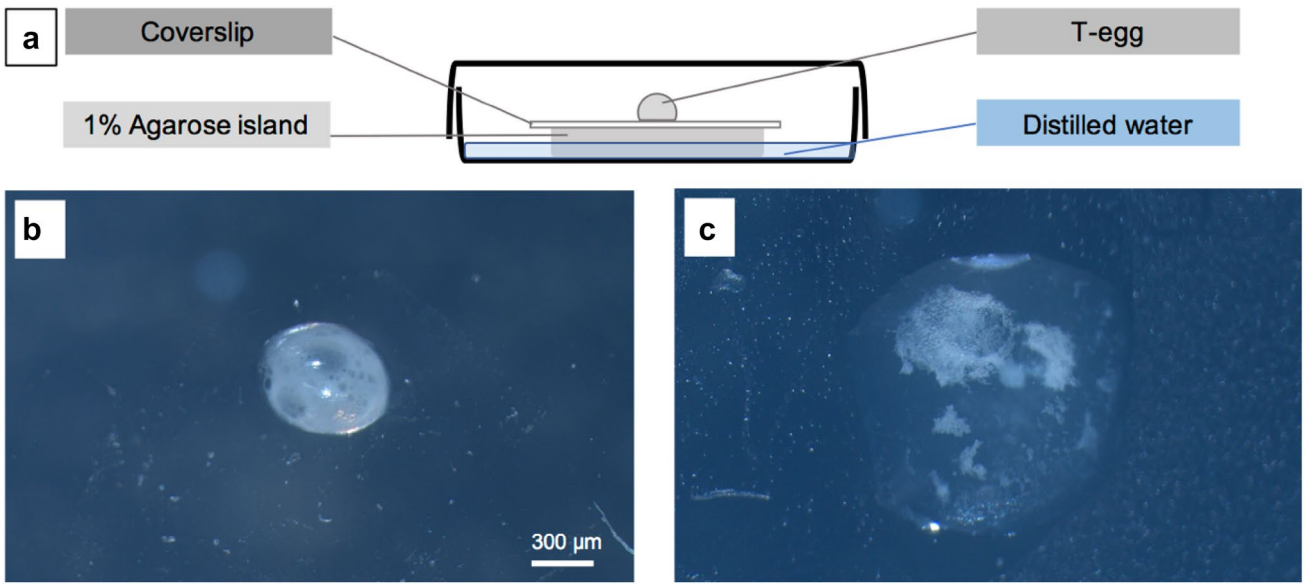

Fig. 3 **a** Experimental setup testing whether trophic eggs (T-eggs) liquify by themselves as a result of an endogenous processes. **b** At the beginning of the experiment, one newly laid T-egg was placed on an island as shown in (**a**) and resting on a cover slip, then maintained at 25 ± 1 °C. To maintain the humidity, distilled water was added into the Petri dish, and then the dish was sealed with parafilm. **c** A T-egg that had liquified within 24 h. Experiments with trophic eggs in isolation were conducted in the absence of workers by placing them onto a central island in a sealed dish, with a moat of distilled water surrounding the island to maintain humidity

surrounding the island to maintain humidity. This test chamber was a 6 cm diameter Petri dish with a central island of 1% agarose and a microscopy coverslip (Fig. 3) onto which we transferred, with the help of a moistened fine brush, 10 newly laid T-eggs (4–6 h after oviposition). We then sealed the dish with Parafilm to maintain 100% humidity, and we observed the liquification process for the next 24 h. In a parallel experiment testing whether contact with fungal garden is necessary, we repeated this experiment with 20 T-eggs placed isolated, without contacting other eggs, onto a garden fragment (Fig. 4). To test whether contact between eggs was necessary, we added T-eggs and R-eggs to garden-fragments in test chambers such that T-eggs were in contact with R-eggs or UR-eggs (Fig. 5bII, III). We repeated this

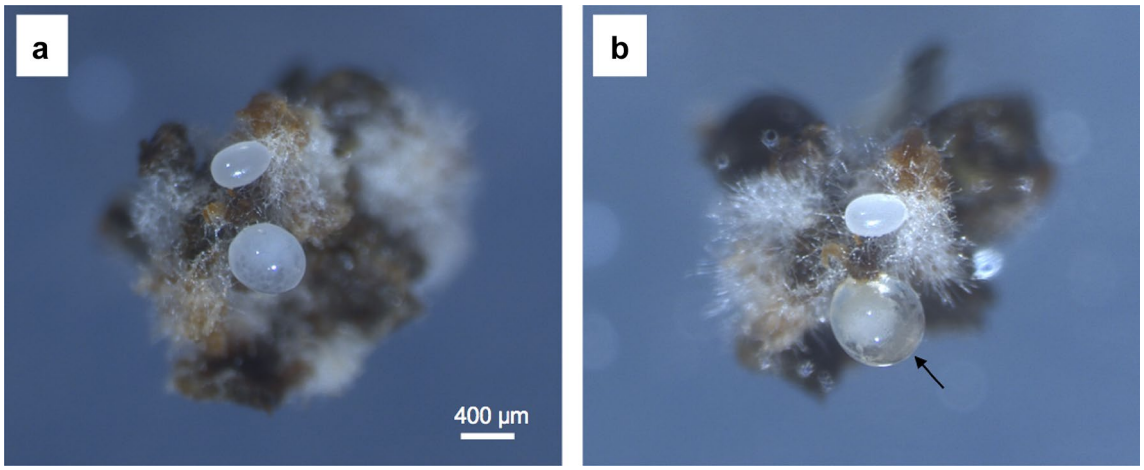

Fig. 4 **a** Experimental setup testing a reproductive egg (R-egg) and a trophic egg (T-egg) placed on a small fragment of fungus garden. **b** The T-egg (see arrow) liquified within 24 h. Experiments with one reproductive egg and one trophic egg on garden were conducted in the absence of workers by placing them onto a layer of 1% agarose in a Petri dish

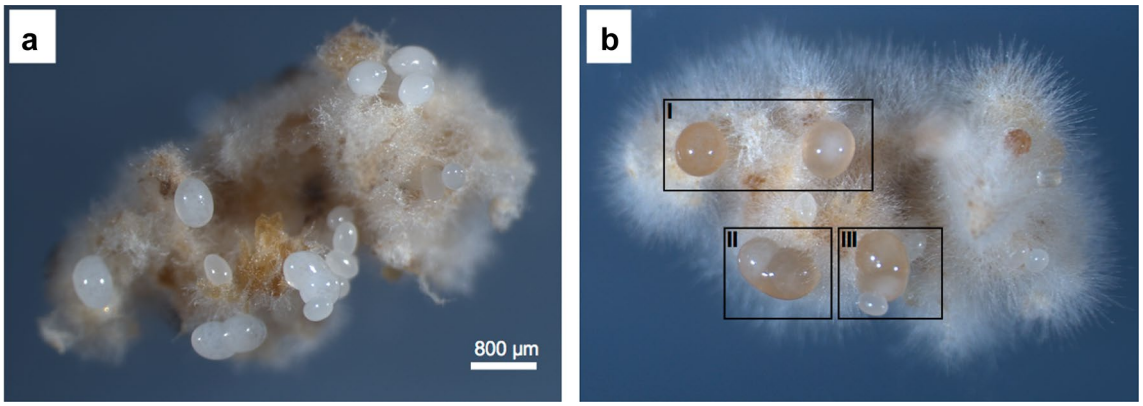

Fig. 5 **a** Experimental setup testing several reproductive eggs (R-eggs) and several trophic eggs (T-eggs) placed on a small fragment of fungus garden. T-eggs were placed on the garden either in isolation (without touching any other eggs, but resting on the fungus garden) or placed such that T-eggs touched other eggs. **b** Touching other eggs was not necessary for T-eggs to liquify, and T-eggs in isolation and those touching other eggs both liquified within 24 h. The experiments with the mixture of eggs on garden were conducted in the absence of workers by placing the eggs onto a garden fragment resting on a layer of 1% agarose in a Petri dish

experiment with 20 T-eggs placed in contact with other eggs onto a garden fragment (Fig. 5). We scored whether T-eggs had liquified after 24 h, and we photographed representative results to illustrate the deliquescence process (Figs. 3, 4 and 5).

Microscopy

To collect eggs for fluorescent microscopy, we moved a foundress into a small round container (Pioneer Plastics, Inc.; 6 cm diameter, 4 cm height) with a bottom of 1% agarose to provide humidity, a small garden fragment (~3 mm diameter), and 5 minima workers to move eggs laid by the queen into the small garden. We allowed the queen to lay eggs for 24 h, then returned the queen back to her colony, but permitted the 5 minima workers to tend to the newly laid egg in the agarose chamber (detailed methods in Fang et al. in preparation; Fang 2019). After 5 days, we used standard methods of embryo fixation (Fang et al. in preparation) to fix 5-day-old reproductive eggs and unviable reproductive eggs, to compare embryo development between these two types of eggs. Protocols for embryo fixation and DAPI staining are described in Fang (2019). We generated fluorescent microscopy images with a Zeiss Axiovert Fluorescent Light Microscope at the Microscopy and Imaging Facility at the University of Texas at Austin (http://sites.cns.utexas.edu/cbrs/microscopy). We measured the length and width of reproductive eggs and unviable reproductive eggs using a Nikon Eclipse Ni Compound Light Microscope. Due to the fragility of tropic eggs that easily burst when transferred

onto a microslide, we imaged T-eggs on the agarose substratum onto which they had been placed by the queen, using a Leica MZ16 Stereomicroscope fitted with a DFC420 digital camera, then calibrated each egg's length and width with ImageJ (https://imagej.nih.gov/ij/). Sample sizes for measurements of the three types of eggs are listed in Table 1.

Statistical analysis

For comparing the egg length differences, we performed statistical analyses in RStudio, version 1.1.463 (R Core Team 2017). We include R scripts used for analyses (Online Resource 1) and the raw data of egg length measurement (measured in microns) as.csv file in Online Resource 2 (Table S1).

Results and discussion

Comparison of the three types of eggs produced by *Atta texana* queens

Trophic eggs (T-eggs) are larger than the other two eggs, and unviable reproductive eggs (UR-eggs) are slightly larger than reproductive eggs (R-eggs) (Figs. 1i and 2). The average length and width (mean ± SD) of R-eggs, UR-eggs, and T-eggs are listed in Table 1. The average length of the three types of eggs was significantly different from each other (Tukey's post hoc test, $P < 0.01$, Fig. 2), suggesting that workers may be able to recognize the three types of eggs by their sizes. The length of reproductive eggs of *At. texana* (≈ 425 µm length) is similar to those of other leaf-cutting ants studied so far. For example, we deduced from published figures that the length of reproductive eggs is ≈ 420 µm long in *At. cephalotes* and ≈ 440 µm in *At. sexdens* (Dijkstra et al. 2005); ≈ 470 µm in *Acromyrmex rugosus rugosus* (Verza et al. 2017); and ≈ 460–620 µm in *Ac. echinatior* and ≈ 460–625 µm in *Ac. octospinosus* (for presumably pooled samples of reproductive and trophic eggs; Dijkstra et al. 2005).

Table 1 Average length and width of the three types of eggs (in µm, ± SD) laid by *Atta texana* foundresses: reproductive eggs (R-eggs), unviable reproductive eggs (UR-eggs), and trophic eggs (T-eggs)

Type	Length	Width	*N*
Reproductive egg	426.09 ± 16.98	239.75 ± 11.67	44
Unviable reproductive egg	491.04 ± 35.53	317.96 ± 17.33	49
Trophic egg	640.54 ± 80.01	530.00 ± 70.82	28

T-eggs do not have a rigid eggshell and easily deform when resting on a surface (Figs. 2 and 3a), which may have contributed to the greater variance of T-egg measurements compared to the variance of R-eggs and UR-eggs measurements

T-eggs deliquesced (i.e., liquified) within 24 h after being laid by the queen (Fig. 1g, h). Previous studies had reported the morphology of T-eggs from *At. sexdens* (Augustin et al. 2011), but the process of egg-deliquescence is fully described here for the first time (Fig. 1g, h, 3, 4 and 5). Because of the similar size between R-eggs and UR-eggs, it appears that UR-eggs have been overlooked in previous studies of oviposition behavior in *Atta*, but fluorescent microscopy documents that UR-eggs are clearly distinct from R-eggs (Fig. 6). R-eggs produced by a fertilized *At. texana* foundresses undergo embryogenesis and develop for about 15 days at the egg stage (at 25 ± 1 °C; Fang 2019) before hatching, but UR-eggs do not develop and do not hatch. For example, when comparing R-eggs with UR-eggs that are both 5 days old (Fig. 6), DAPI staining revealed that germ band elongation is in progress by day 5 in R-eggs, showing a high density of nuclei where the future head lobe is located (see arrow in Fig. 6a). In contrast, 5-day-old UR-eggs show a single nucleus (see arrow in Fig. 6b), indicating that no embryo developed in UR-eggs. UR-eggs, therefore, fail to develop properly, and it is likely that UR-eggs are eventually consumed by the ants or digested by the fungus (Fig. 1d–f). Because UR-eggs comprised about 10% of all the eggs that we collected for our experiments, it is possible that UR-eggs are a maladaptive artifact produced by *At. texana* under the unusual laboratory conditions. Alternatively, UR-eggs may represent a second kind of trophic egg that does not liquify within 24 h (see further observations below), whereas T-eggs are a readily liquifying trophic egg.

Fate of the three types of eggs in incipient gardens

In our observations of incipient colonies, minima workers gathered the R-eggs from the queen, placed them on the incipient garden, and tended to the eggs with continuous grooming and guarding (Fig. 1a). Some R-eggs appeared to be neglected occasionally by the workers. Although these neglected R-eggs can be covered by live mycelium, the eggs maintained their integrity without being damaged or digested by the fungus (Fig. 1b, c). In contrast, putative UR-eggs were digested by mycelium by the eighth day after oviposition (Fig. 1d). The nutrients of UR-egg were presumably absorbed by the mutualistic fungus (Fig. 1e, f), suggesting recycling of nutrients from defunct eggs by the fungus, or perhaps that UR-eggs may represent a second type of trophic egg. T-egg invariably deform morphologically (Fig. 1g) when placed on a hard surface (see arrow in Fig. 2), indicating that trophic eggs do not have a rigid eggshell maintaining the shape of the egg, therefore readily liquify and can, thus, be readily imbibed by the ants or digested by the fungus. Eggshells of T-egg are clearly more fragile than the eggshells of the rigidly chlorinated R-eggs and UR-egg. Similar egg properties were also found

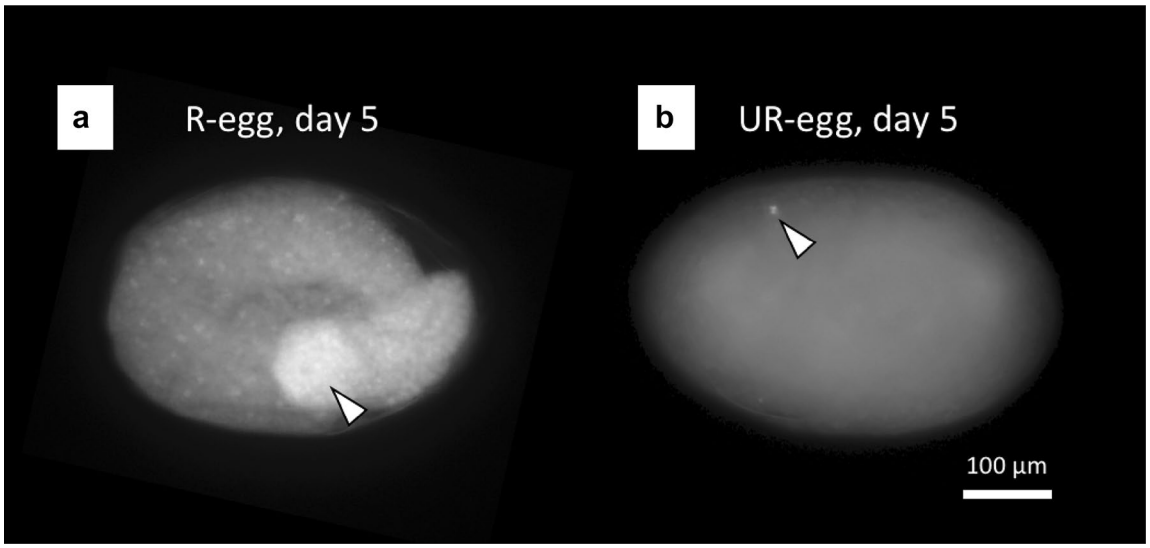

Fig. 6 Comparison of a reproductive egg (R-egg) and an unviable reproductive egg (UR-egg) five days after oviposition by an *Atta texana* foundress. **a** The developing ant embryo growing by germ band extension in the R-egg. The arrow indicates the region with a high density of nuclei that will develop into the head lobe. **b** Only a single nucleus and no developing embryo are visible in an UR-egg of comparable age (5 days)

by Gobin et al. (1998) in the ant *Gnamptogenys menadensis*, where the chorion of T-eggs is thinner than in R-eggs. Additional studies are needed to fully characterize eggshell properties of *At. texana*.

Egg-deliquescence experiments

Trophic eggs (T-eggs) deliquesce by themselves within 24 h (Fig. 3b, c, $N=10$, for example when placed on a solid surface in a humid experimental chamber (Fig. 3a). This indicates that T-eggs are sufficiently sturdy to withstand oviposition and the initial transport by the ants, but that T-eggs become quickly fragile by some endogenous process, and eventually liquify (Fig. 3b, c) to facilitate ingestion by the ants or digestion by the fungus. T-eggs placed experimentally on a fungus garden fragment liquified within 24 h, regardless of whether these T-eggs were placed in isolation (Fig. 4, $N=20$) or placed in contact with other eggs onto a garden fragment (Fig. 5, $N=20$). Contact between eggs and contact with fungus, are, therefore not necessary for deliquescence of T-eggs in garden. These experiments establish the first complete timeline of trophic egg-deliquescence in *Atta*, an important adaptation for nourishing both brood and incipient fungus garden.

Foundress behavior

Our study was not designed to collect systematic observations of foundress behavior in *At. texana*, but we sporadically observed two interesting behaviors of foundresses towards T-eggs. First, an *At. texana* foundress uses her mandibles as a scoop to gently scoop up a trophic egg from her abdomen during oviposition, such that a T-egg comes to rest on the closed mandibles functioning as a scoop; whereas, a foundress picks up the smaller R-eggs and UR-eggs by holding them with her mandibles functioning as forceps. This behavioral difference indicated that the foundress recognizes the fragility of the T-eggs, and the scooping behavior appears to minimize the chance of bursting the egg during transport. Interestingly, workers carried T-eggs using the mandibles as forceps, possibly because the mandibles of minima workers may be too small to function as an effective scoop. As a second behavioral observation, we observed one foundress that produced only nanitic males, but no workers, during the colony-founding stage (Fig. 7). This *At. texana* foundress had been collected after the mating flight on 5. May 2018, and we found two nanitic male adults and one nanitic male pupa on 19. July 2018 in this queen's garden. The nanitic males may have been diploid males that were homozygous at the sex-determining locus, most likely because of inbreeding (Ross and Fletcher 1985; Duchateau and Marien 1995; Gerloff et al. 2003), but other explanations cannot be ruled out without further study, such as developmental mutations in a diploid female, or unusual development of diploid eggs under the accelerated embryogenesis and larval ontogeny typical for brood of foundress queens (see Fang 2019; Fang et al. in preparation). Diploid males were found in the field in some mature colonies of *Atta sexdens* from Panamá, and these diploid males are somewhat larger than normal haploid males of *At. sexdens* (Armitage et al. 2010). If the nanitic males of *At. texana* can be shown

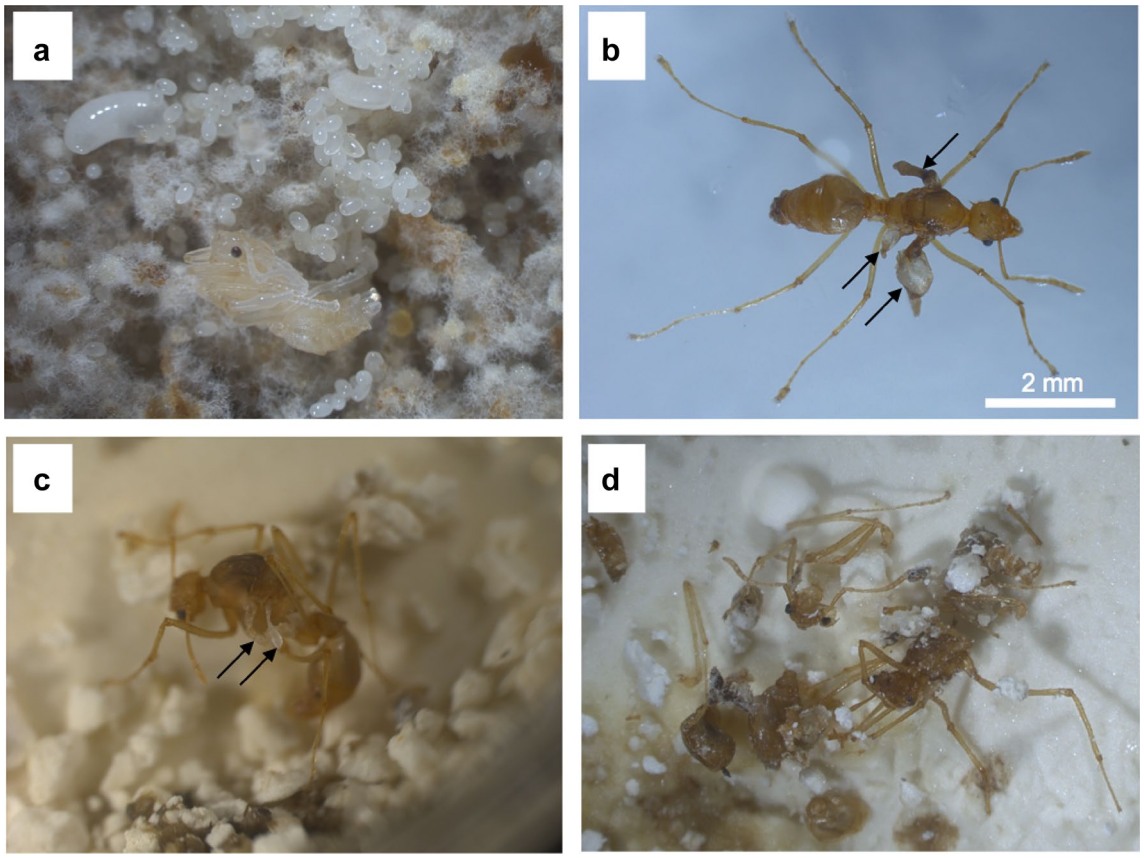

Fig. 7 Nanitic males produced by an *Atta texana* foundress. Only one foundress produced such nanitic males, of a total of six foundresses that produced any offspring; the other five foundresses raised a typical brood of only workers. **a** A nanitic male pupa and larvae and eggs resting on the incipient fungus garden. **b** A nanitic male with three stubby wings that failed to expand (see arrows). **c** A nanitic male with two stubby wings (see arrows) that was later attacked and killed by the *At. texana* foundress. **d** Two nanitic males that had been killed and dismembered by the *At. texana* foundress within 48 h after the males' eclosion

to be diploid, their ontogeny would, therefore, have to be different from those of the larger diploid males of *At. sexdens*. In our incipient nest of *At. texana*, the foundress groomed the male pupa before that male's eclosion (Fig. 7a), but the same queen killed and then dismembered the two nanitic males within 48 h after the males' eclosion (Fig. 7d). Because this foundress queen killed the nanitic males quickly, it is possible that such males may have been overlooked in previous studies of incipient *Atta* nests.

Acknowledgements We thank Zach Phillips, Emma Dietrich, Yao-Lun Yang for ant collection; and Ryan Bailey for colony maintenance. The study was funded by a Texas Ecolab award to CCF, a National Science Foundation award DEB-1354666 to UGM, and the W.M. Wheeler Lost Pines Endowment from the University of Texas at Austin.

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