Trophic eggs affect caste determination in the ant *Pogonomyrmex rugosus*

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Author contributions: EG, TS and LK designed the study. EG performed the experiment and analysed the data. EG and LK wrote the manuscript with input from TS.

Abstract

Understanding how a single genome creates distinct phenotypes remains a fundamental challenge for biologists. Social insects provide a striking example of polyphenism, with queen and worker castes exhibiting morphological, behavioural, and reproductive differences. Here we show that trophic eggs, which do not contain an embryo and are primarily regarded as a source of food, play a role in the process of caste determination in the harvester ant *Pogonomyrmex rugosus*. When first instar larvae were given access to trophic eggs, they mostly developed into workers. By contrast, larvae without access to trophic eggs developed into queens. We found that trophic eggs differ in many ways from reproductive eggs, including texture, morphology and their contents of protein, triglycerides, glycogen, sugar and small RNAs. Moreover, comparison of miRNA fragment size distributions suggests differences in the composition of miRNAs between the two egg types. This is the first demonstration of trophic eggs playing a role in caste determination in social insects.

Keyword: Trophic eggs, *Pogonomyrmex rugosus* ant, caste determination, miRNA, triglycerides, glycogen, proteins

Introduction

Many species of insects, spiders, amphibians, marine invertebrates and sharks produce trophic eggs, a special type of eggs that do not contain an embryo (Levin and Bridges 1995; Blake and Arnofsky 1999; Collin 2004; Kudo and Nakahira 2004; Perry and Roitberg 2006; Strathmann and Strathmann 2006; Gibson *et al.* 2012; López-Ortega and Williams 2018). It is generally assumed that these non-developing eggs are either a by-product of failed reproduction or that they serve as nutrition for offspring (Perry and Roitberg 2006). However, the suggestion that trophic eggs solely provide a nutritional function is based on surprisingly little evidence. We here report a direct function of trophic eggs in the determination of alternative phenotypes in ants.

Trophic eggs have been reported in many ant species (Figure 1 and Supplementary Table 1), where they are also thought to mostly or only serve as food for the offspring (Crespi 1977). Although they are believed to be generally laid by workers, they can also be laid by the queens (see Figure 1 and Supplementary Table 1). While conducting egg cross fostering experiments in the ant *Pogonomyrmex rugosus* to study worker size variation, we observed a sudden increase in the frequency of females developing into queens. During these experiments, we only cross fostered reproductive eggs, raising the possibility that the absence of trophic eggs influenced the process of caste determination. Similarly, changes in the ratio of trophic and reproductive eggs has been proposed as a possible explanation for changes in worker size in another *Pogonomyrmex* species following treatments with a JH analogue (Helms Cahan *et al.* 2011). Because these treatments resulted in an increased proportion of trophic eggs, the authors proposed that the presence of trophic eggs might have altered the nutritional environment of developing larvae, thereby leading to increased worker size. These observations prompted us to investigate whether trophic eggs play a role in caste determination in *P. rugosus*. Our experiments show that the presence of trophic eggs reduces the probability that female larvae develop into reproductive individuals. Metabolomic analyses also revealed profound differences between reproductive and trophic eggs, including in the composition of miRNAs and content of protein, triglycerides, glycogen, and sugar.

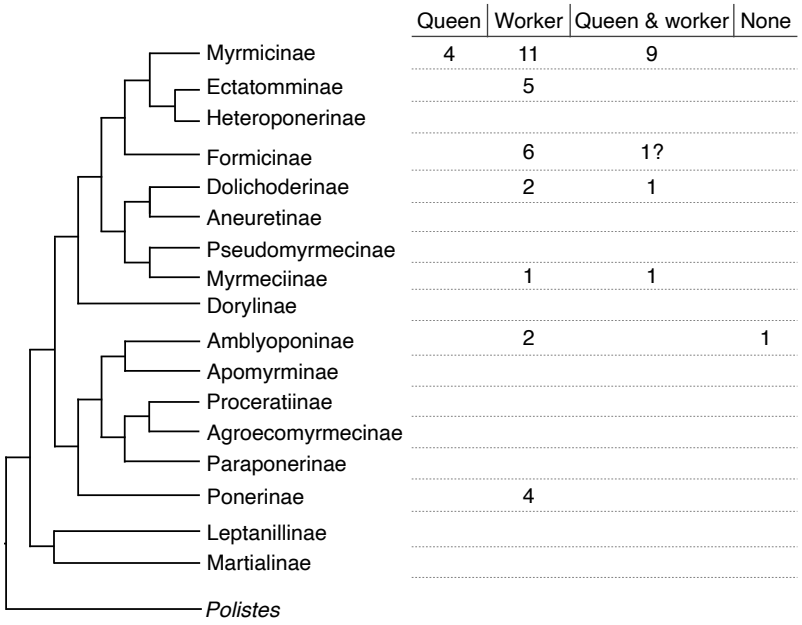


Figure 1. Trophic egg production is widespread in ants. Simplified phylogenetic tree of ant families redrawn after Romiguier *et al.* (2022). The number of species with documented trophic egg production by queens, workers or both castes, as well as absence of trophic eggs, is indicated for each family. Details on the species and related references can be found in Supplementary Table 1.

Materials and methods

Pogonomyrmex rugosus colonies were initiated by queens collected after mating flights in 2008 (Bowie, Arizona, USA) or 2013 (Bowie and Florence, Arizona, USA). The colonies were maintained at 28°C and 60% humidity, with a 12-h/12-h light:dark cycle and fed once a week with grass seeds, flies and 20% honey water. Eggs were collected in October 2020 for the experiment investigating the effect of trophic eggs on larval caste fate, in November 2021 for the percentage of trophic eggs and from February to December 2021 for the egg content analysis.

Trophic and reproductive egg production

To verify that workers do not lay trophic eggs, as previously shown for other *Pogonomyrmex* species (Supplementary Table 1), we created 12 queenless colonies and waited approximately three weeks until workers started laying eggs. From each of these colonies, we isolated two groups of five workers for 12 hours every two days for two weeks in November 2020 to obtain eggs. Collected eggs were then placed for 10 days in a petri dish containing a water reservoir to study their development and distinguish whether they were trophic or reproductive.

To determine whether queens lay variable percentages of trophic eggs over time, we isolated 43 *P. rugosus* queens for 8 hours every day for 2 weeks, before and after hibernation, and counted the number of trophic and reproductive eggs laid (see results for how to discriminate the two types of eggs). The percentage of trophic eggs was compared using a linear mixed effect model within Rstudio (RStudio Team 2015), with before vs after hibernation as the explanatory variable and colony as a random factor.

65 To inform on the mechanisms of oogenesis, we assessed whether reproductive and trophic eggs were laid in a
66 random order, or whether eggs of a given type were laid in clusters. To this end, we isolated 11 queens for 10
67 hours, eight times over three weeks, and collected every hour the eggs laid. To determine whether reproductive
68 and trophic eggs were laid in a random order, we used Rstudio (package *snpar* v.1.0; RStudio Team 2015) to
69 perform a Wald–Wolfowitz runs test for each queen's egg laying sequence (this non-parametric test calculates the
70 likelihood that a binomial data sequence is random).

71 Trophic egg influence on the larval caste fate

72 To determine whether trophic eggs influence the process of caste determination, we compared the development
73 of freshly hatched (first instar) larvae placed in small recipient colonies with and without trophic eggs. From each
74 of 22 donor colonies, we obtained approximately 30 freshly hatched larvae by isolating the queens for 16 hours
75 (from 2pm to 6am) every day for three weeks (in October 2020), with a 24-hour break every three days. Eggs were
76 collected every eight hours and placed during 10 days in a petri-dish with a water reservoir ensuring a high humidity
77 until they hatched. After hatching, half of the larvae were then transferred into a recipient colony containing 20
78 workers, while the other half of the larvae were placed in identical recipient colonies, which received in addition
79 three 0-4 hours-old trophic eggs. There was no cross-fostering between colonies, so that larvae were always
80 placed in recipient colonies containing workers from the same donor colony. The recipient colonies were
81 maintained at 28°C and 60% humidity, with a 12-h/12-h light:dark cycle and fed twice a week with grass seeds,
82 flies and 20% honey water. The caste of each individual was recorded at the pupal stage. To compare the
83 proportion of queen pupae produced between recipient colonies with and without trophic eggs, we used Rstudio
84 (RStudio Team 2015) and the package *lme4* (Bates *et al.* 2015) to perform a binomial generalized linear mixed
85 effects analysis (GLMM) fit by maximum likelihood, with caste as response variable (binary categorical factor) and
86 presence/absence of trophic eggs as an explanatory variable. Donor colony was included as a random effect. To
87 test whether the presence of trophic eggs affects survival, we performed a linear mixed effect analysis with
88 mortality as a response variable, presence/absence of trophic eggs as explanatory variable, and colonies as
89 random effects. As we found a significantly higher survival of larvae in recipient colonies with trophic eggs than
90 recipient colonies without trophic eggs (see results), we tested whether the percentage of larvae developing into
91 queens was correlated with survival by performing a linear mixed effects analysis with the percentage of queen
92 pupae as response variable, the survival as an explanatory variable and colonies as a random factor.

93 Volume and content of trophic and reproductive eggs

94 The volumes of trophic (n=11) and reproductive eggs (n=14) were estimated by using the volume of an ellipse
95 ($\frac{4}{3} \times \pi \times egg\ lenght \times \left(\frac{egg\ width}{2}\right)^2$), with egg length and width estimated on images under 10x magnification using
96 ZEN Microscopy Software (v. 1.1.2.0).

97 To determine the nutritional content of reproductive and trophic eggs, we quantified the proteins, triglycerides,
98 glycogen, and glucose in both types of eggs. We also quantified long and small RNAs (including miRNAs) as these

99 compounds have been shown to be involved in caste determination in other eusocial species (Suzzoni *et al.* 1979;
100 Schwander *et al.* 2008a; Helms Cahan *et al.* 2011; Cameron *et al.* 2013; Guo *et al.* 2013; Libbrecht *et al.* 2013;
101 Søvik *et al.* 2015; Collins *et al.* 2017). To obtain the two types of eggs, we isolated 12 queens for 10 hours (7am
102 to 5pm; from March to October 2021) in a dark petri-dish with three workers and a water supply. Eggs were
103 collected every hour (so all eggs were a maximum of one hour old), and trophic and reproductive eggs were flash-
104 frozen separately in liquid nitrogen. Twenty eggs were pooled for triglycerides-sugar-protein analyses and six eggs
105 for RNA analyses. They were kept at -80°C until the extractions were performed. After the 10 hours of isolation,
106 queens and workers were returned to their colony until the next isolation session. For each of the 12 colonies, we
107 obtained two replicates of reproductive and trophic egg pools (i.e., 24 replicates in total).

108 Triglycerides, glycogen and glucose were quantified as described in Tennessen *et al.* (2014), and protein levels
109 were measured using a Bradford assay (Bradford 1976). The 20 one-hour old eggs per sample were homogenized
110 with beads in 200µl of PBS buffer in a Precellys Evolution tissue homogenizer coupled with a Cryolys Evolution
111 (Bertin Technologies SAS).

112 For the Bradford assay, 10*µ*l of the homogenate were put in a clear-bottom 96-well plate with 300*µ*l of Coomassie
113 Plus Reagent (Thermo Scientific: 23200) and incubated for 10 minutes at room temperature. Protein standard
114 (Sigma: P5369) was used as standard (ranging from 0-0.5mg/ml) and protein absorbance was read at 595nm on
115 a Hidex Sense Microplate Reader.

116 For the triglycerides assay, 90*µ*l of homogenate were heat treated at 70°C for 10 minutes, then 40*µ*l were mixed
117 with 40*µ*l of Triglyceride Reagent (Sigma: T2449) for digestion and 40*µ*l were mixed with PBS buffer for free
118 glycerol measurement. After 30 minutes incubation at 37°C, 30*µ*l of each sample and standards were transferred
119 to clear-bottom 96-well plate. 100*µ*l of Free Glycerol Reagent (Sigma: F6428) was added to each sample, mixed
120 well by pipetting, and incubated five minutes at 37°C. Glycerol standard solution (Sigma: G7793) was used as
121 standard (ranging from 0-1.0mg/ml TAG) and absorbance was read at 540nm on a Hidex Sense Microplate
122 Reader. The triglycerides concentration in each sample was determined by subtracting the absorbance of free
123 glycerol in the corresponding sample.

124 Glucose and glycogen were quantified as in Tennessen *et al.* (2014). A 90*µ*l aliquot was heat treated at 70°C for
125 10min and then diluted 1:2 with PBS. The standard curves for glucose (Sigma, G6918) and glycogen (Sigma:
126 G0885) were made by diluting stocks to 160*µ*g/ml, making 1:1 serial dilution for 160, 80, 40, 20 and 10*µ*g/ml. 40*µ*l
127 of each sample was pipetted in duplicates of a clear microplate, and 30*µ*l of each glucose or glycogen standard
128 was pipetted in duplicates. Amyloglucosidase enzyme (Sigma, A1602) was diluted 3*µ*l into 2000*µ*l of PBS, and
129 40*µ*l diluted enzyme was pipetted to the glycogen standards and to one well of the sample (for total glucose
130 determination), 40*µ*l PBS was pipetted to the glucose standards and to the other sample well (for free glucose
131 determination). The plate was incubated at 37°C for 60 minutes. 30*µ*l of each standard and samples (in duplicates)
132 were transferred to a UV 96-well plate and 100*µ*l Glucose Assay Reagent (G3293) was pipetted to each well. The
133 plate was incubated at room temperature for 15 minutes and the absorbance was read at 340nm on a Hidex Sense

134 Microplate Reader. The glycogen concentration was quantified by subtracting the free glucose absorbance from
135 the total glycogen + glucose absorbance.

136 Concentrations of each compound (protein, triglycerides, glycogen, and glucose) were compared between
137 reproductive and trophic eggs with Rstudio (RStudio Team 2015) and the package *lme4* (Bates *et al.* 2015) using
138 a linear mixed effects analysis (LMER), with the concentration as response variable and egg type as explanatory
139 variable. Colony and extraction batch were added as random effects in the model.

140 Total and small RNA, and DNA

141 RNA (>200 nt) and small RNA were isolated using the miRNeasy Mini Kit (Qiagen, cat. no. 217004) and RNeasy®
142 MinElute® Cleanup Kit (Qiagen, cat. no. 74204), respectively, following manufacturer instructions. RNA (>200 nt)
143 and small RNA concentrations were measured with a QuantiFluor® RNA System (Promega). RNA (>200 nt)
144 integrity was examined with an Agilent Fragment Analyzer (at the Lausanne Genomic Technologies Facility)
145 using a High Sensitivity Assay and small RNA were examined using the small RNA kit (at the Gene Expression
146 Core Facility at EPFL).

147 The miRNA and RNA (>200nt) concentrations were compared between reproductive and trophic eggs with paired-
148 t-tests in Rstudio (RStudio Team 2015; for each type of eggs we used the average of the two replicates per colony).
149 We also compared the fragment size distributions from 18 to 24 nucleotides for miRNAs (Sohel 2016) with a Mantel
150 test.

151 DNA was extracted from pools of six eggs using TRIzol (Life Technologies). DNA concentration was measured
152 with a Nanodrop 3300 (ThermoFisher), and DNA integrity was examined with an Agilent Fragment Analyzer (at
153 the Lausanne Genomic Technologies Facility) using a High Sensitivity Assay. DNA concentrations were compared
154 between reproductive and trophic eggs using paired-t-tests (sample size is 5 for both types of eggs, each sample
155 being a pool of 6 eggs).

156 Results

157 Trophic and reproductive egg characteristics

158 *P. rugosus* queens lay two types of eggs that are morphologically different. Reproductive eggs are white with a
159 bright surface and have a distinct oval shape, a homogenous content as well as a solid chorion (Figure 2A), while
160 trophic eggs are rounder, have a smooth surface and a granular looking content as well as a fragile chorion (Figure
161 2D). Trophic eggs had a significantly larger volume (94.3$\pm$4.3nL; n=11) than reproductive eggs (n=14; 63.3$\pm$1.6nL;
162 two-sample t-test, $t(23) = -9.54$, $p = 1.8\times10^{-09}$). *P. rugosus* workers only laid reproductive eggs. They started to lay
163 eggs approximately three weeks after queen removal (n=12 queenless recipient colonies) and approximately 90%
164 of the eggs successfully hatched. However, only approximately 5% successfully developed into pupae which were
165 all males.

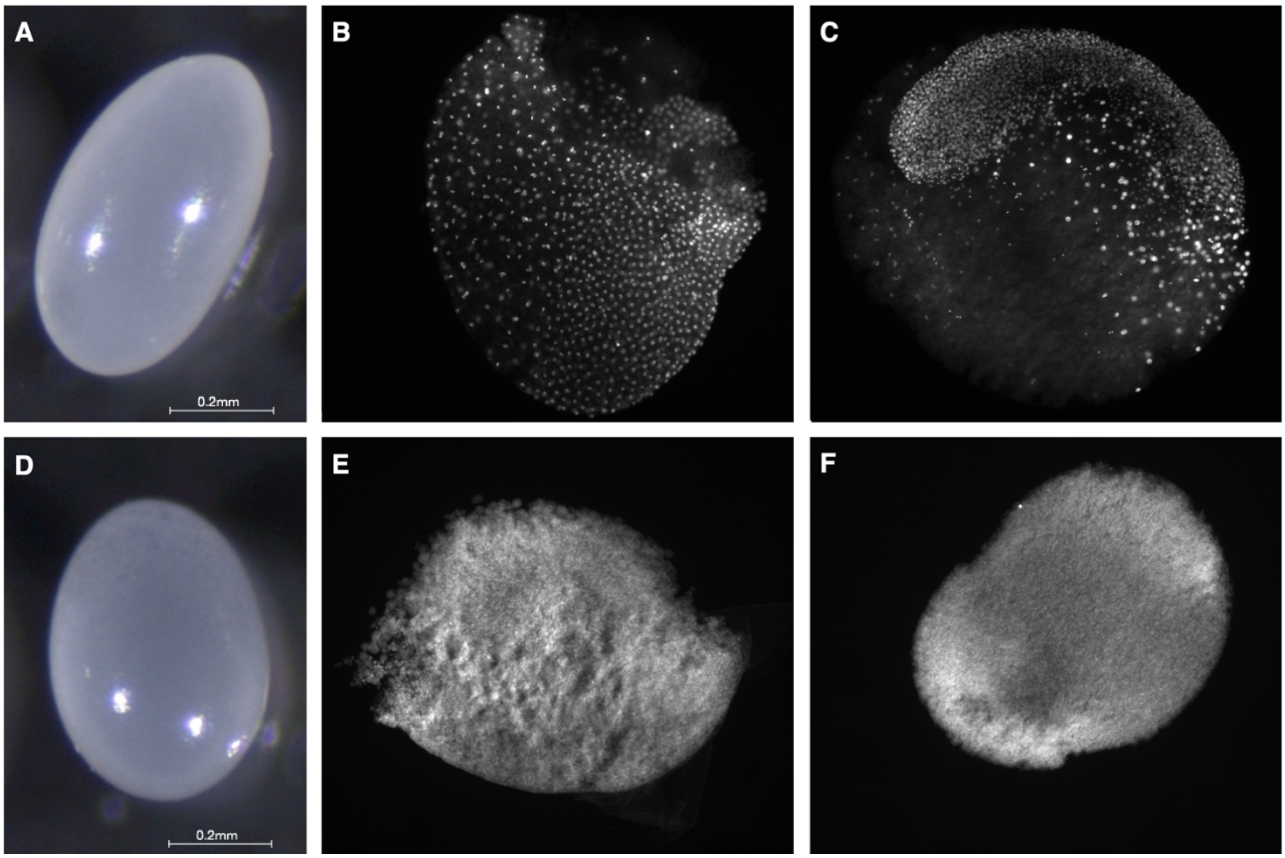


Figure 2. Morphology and development of eggs laid by *P. rugosus* queens. Reproductive egg general morphology (**A**), with embryonic development at approximately 25 hours (**B**) and 65 hours (**C**). In trophic eggs (**D**), there is no embryonic development at 25 hours (**E**) nor at 65 hours (**F**). Panels B, C, E, F represent fluorescence images with DAPI-counterstained nuclei.

The percentage of eggs that were trophic was higher before hibernation (61.6±1.4% mean± SE; n=43 colonies) than after (50.3±2.0%; LMER, t(86)=5.04, $p=9x10^{-6}$). This higher percentage was due to a reduced number of reproductive eggs, the number of trophic eggs laid remained stable. The production of the two types of eggs was not random (Wald-Wolfowitz runs tests, p-values for the 11 queens in Table 1). Instead, each of the 11 queens tended to lay relatively long sequences of either reproductive (6.1±0.7; mean number per sequence ±SE) or trophic eggs (6.0±0.5; Figure 3).

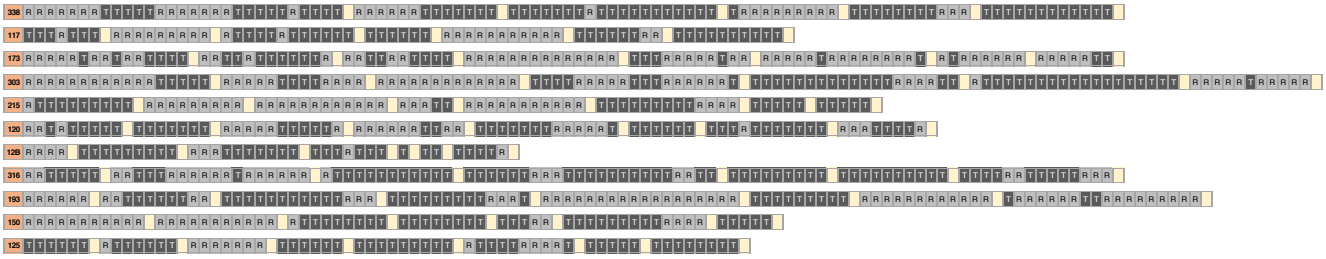


Figure 3. Egg laying sequences from eleven *P. rugosus* queens. Every row shows the sequence of reproductive R and trophic T eggs laid by a given queen (queen ID in the orange cell). Each egg laying session lasted 10 hours. Each yellow square separates two egg laying sessions and represents an interval of minimum 16 hours to several days.

181

182 **Table 1.** Wald-Wolfowitz runs tests on the queen's egg sequence. Significant p-values (corrected for multiple testing)
183 indicate that queens do not lay reproductive and trophic eggs in a random sequence.

Queen ID	P-value for egg sequence	P-value of random sequence	Number of eggs per sequence
338	$4.1*10^{-11}$	0.419	94
117	$4.3*10^{-07}$	0.567	63
173	$1.7*10^{-03}$	0.755	92
303	$4.8*10^{-13}$	0.765	110
215	$9.8*10^{-11}$	0.292	70
120	$1.4*10^{-05}$	0.518	75
12B	$1.9*10^{-03}$	0.298	38
316	$3.4*10^{-09}$	0.737	93
193	$4.3*10^{-12}$	0.655	101
150	$1.4*10^{-10}$	0.630	62
125	$1.5*10^{-05}$	0.404	58

184

185 The concentrations of protein, triglycerides, glycogen, and glucose were significantly higher in reproductive than
186 trophic eggs (LMER, protein: $t = -13.11$, $p < 0.0001$; triglycerides: $t = -11.66$, $p < 0.0001$; glycogen: $t = -11.98$,
187 $p < 0.0001$; glucose: $t = -18.60$, $p < 0.0001$; Figure 4).

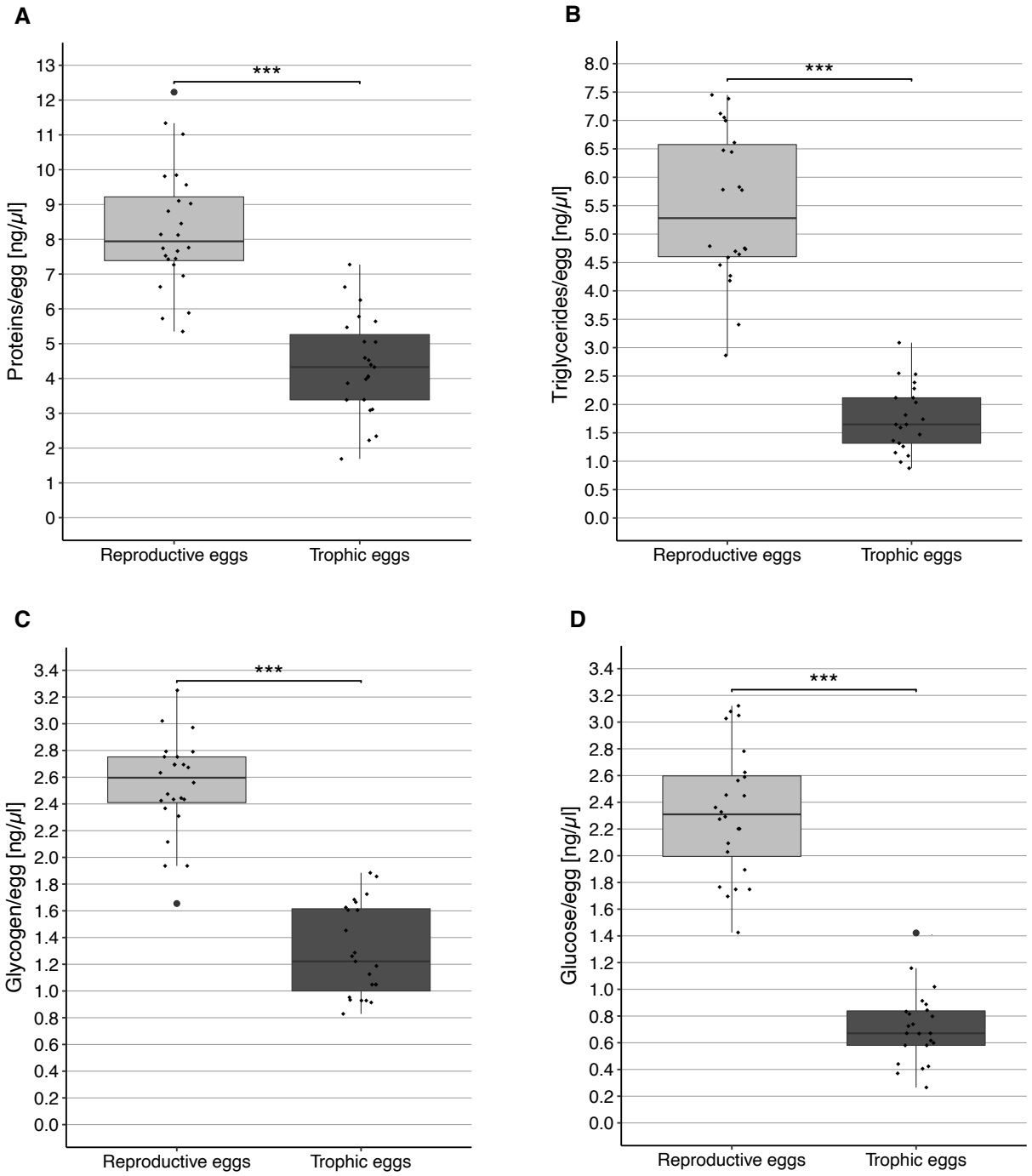
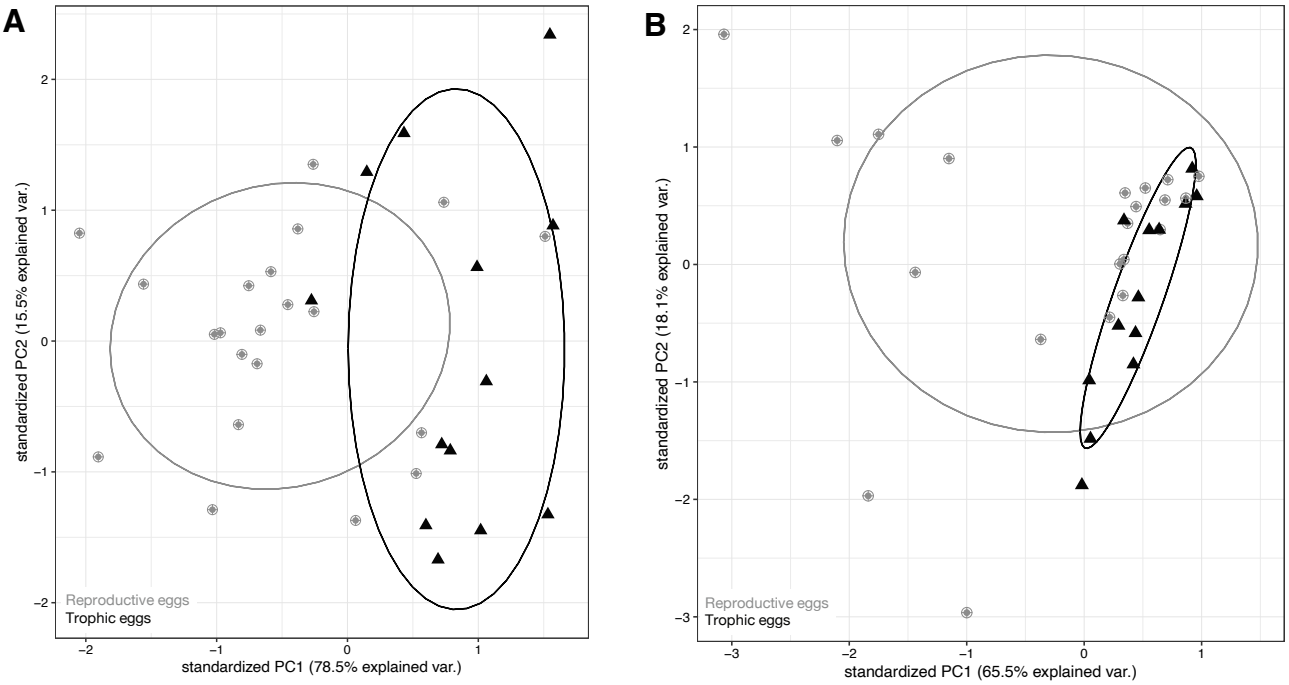


Figure 4. Concentration of protein (**A**), triglycerides (**B**), glycogen (**C**) and glucose (**D**) in reproductive and trophic eggs. Each dot represents the average of the two replicates per colony.

The amount of small RNA (<200 nt, including miRNA and tRNA; Nagano and Fraser 2011) was significantly higher in reproductive eggs (44.3±1.4ng, mean ± SE) than in trophic eggs (22.3±1.1ng; paired-t-test, $t_{(23)} = 15.9$, $p = 6.5*10^{-14}$). The same was true for longer RNAs (>200 nt; reproductive eggs: 7.6±0.6ng, mean ± SE; trophic eggs: 3.6±0.3ng; paired-t-test, $t_{(23)} = 7.2$, $p = 2.7*10^{-7}$).

196 The DNA quantification showed that the amount of DNA was about twice higher in reproductive (15.9±1.9ng/μl)
197 than trophic eggs (8.8±1.9ng/μl; t-test, $t_{(4.7)} = 2.7$, $p = 0.045$).

198 There was a significant difference in the miRNA fragment size distribution between reproductive and trophic eggs
199 (Mantel test, $r_M = 0.26$, $p<.0001$), as shown on the PCA (Figure 5A). There was no difference in the tRNA fragment
200 size distribution between the two types of eggs (Mantel test, $r_M = 0.01$, $p=0.30$, Figure 5B).



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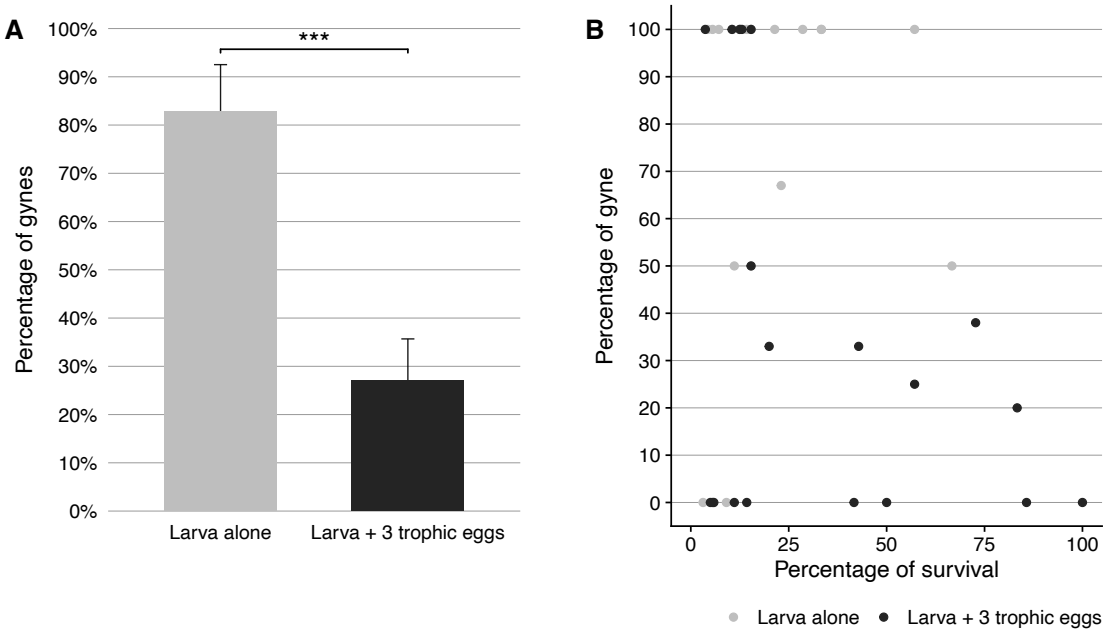
202 **Figure 5.** First two principal components (PC1 and PC2) explaining size distribution variation for (**A**) miRNA and (**B**)
203 tRNA across egg samples, with reproductive eggs in grey and trophic eggs in black. There is a separation of the samples
204 by egg types for miRNAs, but not for tRNA. Ellipses enclose each of the egg type groups.

205 Trophic eggs influence caste fate of larvae

206 The percentage of larvae that developed into queens was significantly lower in recipient colonies that received
207 trophic eggs (27±9% mean±SE; n=22) than in recipient colonies without trophic eggs (83±10%; n=22; binomial
208 GLMM, z = 4.25, p = $2x10^{-5}$; Figure 6A and Supplementary Table 2). The survival of larvae until the pupal stage
209 was also significantly lower in the colonies without trophic eggs (16.9±3.8%; n=22; LMER, z = 2.66, p = 0.008)
210 than in colonies with trophic eggs (30.2±6.7%; mean±SE; n=22) , but the 1.8 fold survival decrease cannot fully
211 account for the 3 fold difference in queen percentage between the two treatments. Furthermore, there was no
212 significant correlation between larval mortality and the percentage of larvae developing into queens (n=44 recipient
213 colonies; LMER, z = 0.97, p = 0.34; Figure 6B). These analyses allow us to exclude differential survival between
214 castes as an explanation for the higher percentage of queens developing in the recipient colonies without trophic
215 eggs.



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217 **Figure 6.** (**A**) Percentage of larvae which developed into queens in recipient colonies without (grey) or with (black)
218 trophic eggs. (**B**) Scatterplot of the relationship between the percentage of larvae which developed into queens and the
219 percentage of survival from larvae to pupae.

220

221 Discussion

222 Our study reveals that *P. rugosus* queens lay a very high proportion (0.6) of trophic eggs. These eggs differ in
223 many ways from reproductive eggs. First, trophic eggs are larger, rounder, have a smoother surface, a more
224 granular looking content as well as a more fragile chorion than reproductive eggs. Similar differences between
225 trophic and reproductive eggs have been reported in other ant species (Wilson 1976; Wardlaw and Elmes 1995;
226 Gobin *et al.* 1998; Dietemann and Peeters 2000; Dietemann *et al.* 2002; Perry and Roitberg 2006; Lee *et al.* 2017).

227 Our analyses also showed that trophic eggs are solely laid by queens; *P. rugosus* workers are able to produce
228 reproductive eggs which occasionally develop into males, but they do not lay trophic eggs. Moreover, trophic eggs
229 have a reduced DNA content.

230 Importantly, our experiments showed that the presence of trophic eggs influences the process of caste
231 determination. First instar female larvae fed with trophic eggs were significantly more likely to develop into workers
232 than larvae without access to trophic eggs. This was somewhat surprising because trophic eggs are generally
233 thought to be an important source of nutrients to the colony and, everything else being equal, one would think that
234 eating such eggs should increase the likelihood of females to develop into queens (which are usually larger than
235 workers). Indeed, a study in the Argentine ant *Linepithema humile* showed that the presence of queens in colonies
236 was associated with a drastic decrease in the number of trophic eggs (laid by workers) fed to the larvae as well as
237 a decrease in the proportion of larvae developing into queens (Bartels 1988). Bartels thus proposed that the
238 deprivation of trophic eggs may have an inhibitory effect on the probability of larvae to develop into queens (Bartels
239 1988), but no experiment was performed to show a causal effect of trophic eggs. In some lineages of *P. barbatus*,
240 the experimental increase of maternal juvenile hormone resulted in a 50% increase in worker body size, as well
241 as a sharp reduction in total number of progeny reared and a higher proportion of trophic eggs laid by queens
242 (Helms Cahan *et al.* 2011). This was interpreted as an effect of trophic egg availability or brood/worker ratio on
243 the nutritional environment. Importantly, in these two studies the consumption of trophic eggs was suggested to
244 either increase the size of the individuals produced or the likelihood to develop into queens (which are larger than
245 workers). By contrast, our study reveals that the consumption of trophic eggs reduces the likelihood of developing
246 into queens.

247 Our analyses revealed that trophic eggs have a lower content of protein, triglycerides, glycogen, and glucose than
248 reproductive eggs. A reduced protein content of trophic as compared to reproductive eggs has also been
249 documented in *Pheidole pallidula* (Lorber and Passera 1981). These findings are in line with the view that trophic
250 eggs do not simply have a nutritive function as it might then be expected that they should at least contain as much
251 nutrients as reproductive eggs. Interestingly, our analyses also revealed important differences in RNA and miRNA
252 content between the two egg types. miRNAs have already been suggested to influence larval caste determination
253 in the honeybee (Guo *et al.* 2013) with worker jelly being enriched in miRNAs compared to royal jelly (Guo *et al.*
254 2013; Zhu *et al.* 2017). These studies suggest that it is not the royal jelly that stimulates larval differentiation into
255 queen, but rather the worker jelly which stimulates the development of larva into worker. Similarly, our study
256 reveals that compounds found in trophic eggs, perhaps miRNAs, influence larval development towards the worker
257 phenotype. Interestingly, it has also been recently shown that trophallactic fluid in the ant *Camponotus floridanus*
258 contains non-digestion related proteins, microRNAs and juvenile hormone (LeBoeuf *et al.* 2016). Moreover,
259 comparison of trophallactic fluid proteins across social insect species revealed that many are regulators of growth,
260 development and behavioral maturation (Meurville and LeBoeuf 2021). Finally, a recent study showed that pupae
261 of several ant species produce secretions that play an important role for early larval nutrition with young larvae
262 exhibiting stunted growth and decreased survival without access to the fluid (Snir et al. 2022). This raises the

possibility that chemicals delivered in trophic eggs, trophallactic fluids and pupae secretions play previously unsuspected roles in communication and caste development. Given that some ants do not perform trophallaxis, it would be interesting to determine whether there are differences in the content of trophic eggs of species performing trophallaxis and species which do not.

Maternal effects on the process of caste determination have been demonstrated in several social insect species, including *P. rugosus*, either by queen behaviour or content of the eggs being produced (De Menten *et al.* 2005; Linksvayer 2006; Schwander *et al.* 2008b; Libbrecht *et al.* 2013; Wei *et al.* 2019). This is, to our knowledge the first experimental demonstrations that provisioning of trophic eggs influences caste fate. Since only queens produce trophic eggs in *P. rugosus,* trophic egg provisioning could be the main mechanism underlying the previously documented maternal effects on the process of caste determination. In species where workers produce trophic eggs (Supplementary Table 1), the same mechanism could allow workers to influence colony level caste ratios.

Finally, our analyses also revealed seasonal differences in the proportion of reproductive and trophic eggs, with a higher ratio of trophic eggs before hibernation than after. In *Pogonomyrmex* the production of new queens occurs after hibernation (Smith and Tschinkel 2006) or when the queen dies or is removed from the colony (pers. obs). Thus, new queens are typically produced when there are fewer trophic eggs. Our results predict that under natural conditions, a decrease in the proportion of trophic eggs should lead to an increase in the larvae developing into queens. The same logic applies to species where trophic eggs are laid only by the workers in queenright colonies (Supplementary Table 1). After the queen's death, workers start producing their own male offspring and lay mostly (if not only) reproductive eggs (*Temnothorax recedens*, Dejean and Passera 1974; *Plagiolepis pygmaea*, Passera 1980; *Myrmecia gulosa*, Dietemann *et al.* 2002), which again leads to a decrease, or cessation, in trophic egg production. A decrease in trophic egg production and the development of queens were observed simultaneously in freshly orphaned colonies of *Temnothorax recedens* (Dejean and Passera 1974), *Plagiolepis pygmaea* (Passera 1980) and *Myrmecia gulosa* (Dietemann *et al.* 2002). These examples are consistent with the view that trophic eggs may also play a role in the process of caste determination in other ant species.

In conclusion, this study provides a new striking example of how females can influence the developmental fate of their offspring. Because many ants produce trophic eggs, it is possible that this mechanism of parental manipulation is widespread and play an important role in the general process of caste determination.

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