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

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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 differentiation 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 viable 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 differentiation in social insects.

Keyword: Trophic eggs, *Pogonomyrmex rugosus* ant, caste differentiation, 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) and are generally thought to mostly or only serve as food for offspring (Crespi 1977; Hölldobler and Wilson 1990). Trophic eggs play an important during the time of colony founding (Hölldobler and Wilson 1990). Because in most ant species queens do not forage after the mating flight, they metabolize the alary muscles and fat bodies and convert them into eggs, which serve as food to rear the first batch of larvae (Huber 1905; Keller and Passera 1989). Except for a few species such as *Crematogaster smithsi (*Heinze *et al.* 1995) and *Acanthomyrmex ferox* (Gobin and Ito 2000), the queens generally stop producing trophic eggs after the eclosion of the first workers. (see Figure 1 and Supplementary Table 1). So far the absence of trophic eggs has been reported in only one species (Supplementary Table 1),

In many ant species, workers are capable of producing haploid males but lack a spermatheca and the ability to produce diploid female offspring (Holldobler and Wilson 1990; Bourke 1988; Hammond and Keller 2004; Wenseleers and Ratnieks 2006). In some species, workers further lay trophic eggs (see supplementary Table 1). These eggs have a trophic function, in particular in genera such as *Pogonomyrmex* where there is no or only little trophallaxis (regurgitative food sharing) among workers. In such species it is has been suggested that trophic eggs may play an important role in food distribution within the colony (Gobin and Ito 2000).

Several studies suggested that the presence of trophic eggs may affect the developmental trajectory of larvae. A study in the Argentine ant *Linepithema humile* showed that the presence of queens in colonies was associated with a drastic decrease in the number of worker-laid trophic eggs as well as a decrease in the proportion of larvae developing into queens (Bartels 1988). Bartels thus proposed that trophic eggs may increase the probability of larvae to develop into queens, which are much larger than workers

(Bartels 1988). In some lineages of *Pogonomyrmex barbatus*, queens laid a higher proportion of trophic eggs upon the experimental increase of maternal juvenile hormone (Helms Cahan *et al.* 2011). Because the treatment also strongly reduced the number of workers produced but triggered a 50% increase in worker body size, trophic eggs were suggested to affect worker size. An increase in the proportion of trophic eggs has also been suggested to be associated with an increase in the proportion of larvae developing into queens (*L. humile*: Bartels 1988; *P. barbatus*: Helms Cahan *et al.* 2011). Finally, because they observed an increase in nitrogen content with increasing female caste size in the ant *Pogonomyrmex badius,* Smith and Suarez (2010) suggested that that the larger castes may consume more trophic (nutritional) eggs than the smaller caste which would feed more on foraged insects. In summary, in at least three species of ants, trophic eggs may play a role in the regulation of body size.

While conducting egg cross fostering experiments in the ant *P. rugosus* to study worker size variation, we observed a sudden increase in the frequency of females developing into queens. During these experiments, we discarded trophic eggs and only cross fostered eggs that would eventually hatch (hereafter viable eggs). This raises the possibility that the absence of trophic eggs influenced the process of caste differentiation. These observations prompted us to investigate whether trophic eggs play a role in caste differentiation in *P. rugosus*. Our experiments revealed that the presence of trophic eggs reduces the probability that female larvae develop into reproductive individuals. Metabolomic analyses also revealed profound differences between viable and trophic eggs, including in the composition of miRNAs and content of protein, triglycerides, glycogen, and sugar.

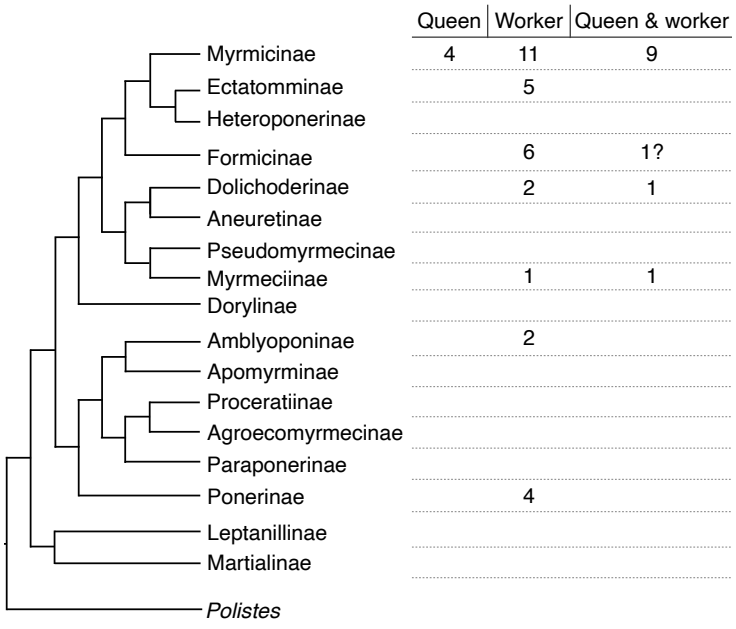


Figure 1. Trophic egg production is widespread in ants. Simplified phylogenetic tree of ant subfamilies redrawn after Romiguier *et al.* (2022). The number of species with documented trophic egg production by queens, workers or both castes is indicated for each subfamily. The question mark indicates that it is unclear whether about the evidence for the production trophic eggs by queens and workers (in *Lasius niger* trophic eggs are produced by workers and possibly queens, see supplementary Table 1). Details on the species and related references can be found in Supplementary Table 1.

Materials and methods

Some populations of *Pogonomyrmex rugosus* are characterized by a genetic caste determination system whereby development into queens or workers is determined by whether the eggs are fertilized by males of the same genetic lineage or a different genetic lineage than the queen producing the eggs (Helms Cahan *et al.* 2002; Julian *et al.* 2002; Volny and Gordon, 2002; Helms Cahan and Keller 2003). We collected queens from two populations (Bowie and Florence, Arizona, USA) known to harbor only colonies with non-genetic caste determination (Schwander et al. 2007). The queens were collected after mating flights in 2008 (Bowie) and 2023 (Bowie and Florence) to initiate colonies in the laboratory. The colonies were maintained in plastic boxes containing water tubes (glass tubes filled with water and sealed with a cotton plug) at 28°C and 60% humidity, with a 12-h/12-h light:dark cycle. They were 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 estimating the percentage of trophic eggs and from February to December 2021 for the egg content analyses.

All statistical analyses were performed with Rstudio (RStudio Team 2015).

Trophic and viable 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 (by removing the queen) 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 viable.

To determine whether queens laid variable percentages of trophic eggs over time, we isolated each of 43 *P. rugosus* queens for 8 hours every day for 2 weeks, before and after hibernation, and counted the number of trophic and viable eggs laid (see results for how to discriminate the two types of eggs). We hibernated the queens because we previously showed that hibernation is important to trigger the production of gynes in *P. rugosus* colonies in the laboratory (Schwander *et al.* 2008; Libbrecht *et al.* 2013). Hibernation conditions were as described in Libbrecht *et al*. (2013). The percentage of trophic eggs was compared using a linear mixed effect model with before vs after hibernation as the explanatory variable and colony as a random factor.

To assess whether viable and trophic eggs were laid in a random order, or whether eggs of a given type were laid in clusters, we isolated 11 queens for 10 hours, eight times over three weeks, and collected every hour the eggs laid. To determine whether viable and trophic eggs were laid in a random order, we performed a Wald–Wolfowitz runs test for each queen's egg laying sequence (package *snpar* v.1.0; this non-parametric test calculates the likelihood that a binomial data sequence is random).

Trophic egg influence on the larval caste fate

To determine whether trophic eggs influence the process of caste differentiation, we compared the development of freshly hatched (first instar) larvae placed in small recipient colonies with and without trophic eggs. From each of 22 donor colonies, we obtained approximately 30 freshly hatched larvae by isolating the queens for 16 hours (from 2pm to 6am) every day for three weeks (in October 2020), with a 24-hour break every three days. Eggs were collected every eight hours and placed during 10 days in a petri-dish with a water reservoir ensuring a high humidity until they hatched. After hatching, for each colony half of the larvae (i.e.,15 larvae) were then transferred into a recipient colony containing 20 workers, while the other half were placed in identical recipient colonies, which received in addition 45 0-4 hours-old trophic eggs (i.e., there were 3 trophic eggs per larva). There was no cross-fostering between colonies, so that larvae were always placed in recipient colonies containing workers from the same donor colony. The recipient colonies were maintained at 28°C and 60% humidity, with a 12-h/12-h light:dark cycle and fed twice a week with grass seeds, flies and 20% honey water. The caste of each newly-produced individual was recorded at the pupal stage. To compare the proportion of queen pupae produced between recipient colonies with and without trophic eggs, we used the package *lme4* (Bates *et al.* 2015) to perform a binomial generalized linear mixed effects analysis (GLMM) fit by maximum likelihood, with caste as response variable (binary categorical factor) and presence/absence of trophic eggs as an explanatory variable. Donor colony was included as a random effect. To test whether the

157 presence of trophic eggs affects survival, we performed a linear mixed effect analysis with mortality as
158 a response variable, presence/absence of trophic eggs as explanatory variable, and colonies as random
159 effects. As we found a significantly higher survival of larvae in recipient colonies with trophic eggs than
160 recipient colonies without trophic eggs (see results), we tested whether the percentage of larvae
161 developing into queens was correlated with survival by performing a linear mixed effects analysis with
162 the percentage of queen pupae as response variable, the survival as an explanatory variable and colonies
163 as a random factor.
164
165 **Volume and content of trophic and viable eggs**
166 The volumes of trophic (n=11) and viable eggs (n=14) were estimated by using the volume of an ellipse
167 ($\frac{4}{3} \times \pi \times egg\ length \times \left(\frac{egg\ width}{2}\right)^2$), with egg length and width estimated on images under 10x
168 magnification using ZEN Microscopy Software (v. 1.1.2.0).
169
170 To determine the nutritional content of viable and trophic eggs, we quantified the proteins, triglycerides,
171 glycogen, and glucose in both types of eggs. We also quantified long and small RNAs (including
172 miRNAs) as these compounds have been shown to be involved in caste differentiation in other eusocial
173 species. To obtain the two types of eggs, we isolated 12 queens for 10 hours (7am to 5pm; from March
174 to October 2021) in a dark petri-dish with three workers and a water supply. Eggs were collected every
175 hour (so all eggs were a maximum of one hour old), and trophic and viable eggs were flash-frozen
176 separately in liquid nitrogen. Twenty eggs were pooled for triglycerides-sugar-protein analyses and six
177 eggs for RNA analyses. They were kept at -80°C until the extractions were performed. After the 10 hours
178 of isolation, queens and workers were returned to their colony until the next isolation session. For each
179 of the 12 colonies, we obtained two replicates of viable and trophic egg pools (i.e., 24 replicates in total).
180 Triglycerides, glycogen and glucose were quantified as described in Tennessen *et al.* (2014), and protein
181 levels were measured using a Bradford assay (Bradford 1976). The 20 one-hour old eggs per sample
182 were homogenized with beads in 200µl of PBS buffer in a Precellys Evolution tissue homogenizer
183 coupled with a Cryolys Evolution (Bertin Technologies SAS).
184
185 For the Bradford assay, 10µl of the homogenate were put in a clear-bottom 96-well plate with 300µl of
186 Coomassie Plus Reagent (Thermo Scientific: 23200) and incubated for 10 minutes at room temperature.
187 Protein standard (Sigma: P5369) was used as standard (ranging from 0-0.5mg/ml) and protein absorbance
188 was read at 595nm on a Hidex Sense Microplate Reader.
189

190 For the triglycerides assay, 90µl of homogenate were heat treated at 70°C for 10 minutes, then 40µl were
191 mixed with 40µl of Triglyceride Reagent (Sigma: T2449) for digestion and 40µl were mixed with PBS
192 buffer for free glycerol measurement. After 30 minutes incubation at 37°C, 30µl of each sample and
193 standards were transferred to clear-bottom 96-well plate. 100µl of Free Glycerol Reagent (Sigma: F6428)
194 was added to each sample, mixed well by pipetting, and incubated five minutes at 37°C. Glycerol
195 standard solution (Sigma: G7793) was used as standard (ranging from 0-1.0mg/ml TAG) and absorbance
196 was read at 540nm on a Hidex Sense Microplate Reader. The triglycerides concentration in each sample
197 was determined by subtracting the absorbance of free glycerol in the corresponding sample.
198
199 Glucose and glycogen were quantified as in Tennessen *et al.* (2014). A 90µl aliquot was heat treated at
200 70°C for 10min and then diluted 1:2 with PBS. The standard curves for glucose (Sigma, G6918) and
201 glycogen (Sigma: G0885) were made by diluting stocks to 160µg/ml, making 1:1 serial dilution for 160,
202 80, 40, 20 and 10µg/ml. 40µl of each sample was pipetted in duplicates of a clear microplate, and 30µl
203 of each glucose or glycogen standard was pipetted in duplicates. Amyloglucosidase enzyme (Sigma,
204 A1602) was diluted 3µl into 2000µl of PBS, and 40µl diluted enzyme was pipetted to the glycogen
205 standards and to one well of the sample (for total glucose determination), 40µl PBS was pipetted to the
206 glucose standards and to the other sample well (for free glucose determination). The plate was incubated
207 at 37°C for 60 minutes. 30µl of each standard and samples (in duplicates) were transferred to a UV 96-
208 well plate and 100µl Glucose Assay Reagent (G3293) was pipetted to each well. The plate was incubated
209 at room temperature for 15 minutes and the absorbance was read at 340nm on a Hidex Sense Microplate
210 Reader. The glycogen concentration was quantified by subtracting the free glucose absorbance from the
211 total glycogen + glucose absorbance.
212
213 Concentrations of each compound (protein, triglycerides, glycogen, and glucose) were compared
214 between viable and trophic eggs using a linear mixed effects analysis (LMER; package *lme4*), with the
215 concentration as response variable and egg type as explanatory variable. Colony and extraction batch
216 were added as random effects in the model.
217
218 **Total and small RNA, and DNA**
219 RNA (>200 nt) and small RNA were isolated using the miRNeasy Mini Kit (Qiagen, cat. no. 217004)
220 and RNeasy® MinElute® Cleanup Kit (Qiagen, cat. no. 74204), respectively, following manufacturer
221 instructions. RNA (>200 nt) and small RNA concentrations were measured with a QuantiFluor® RNA
222 System (Promega). RNA (>200 nt) integrity was examined with an Agilent Fragment Analyzer (at the

223 Lausanne Genomic Technologies Facility) using a High Sensitivity Assay and small RNA were
224 examined using the small RNA kit (at the Gene Expression Core Facility at EPFL).
225 The miRNA and RNA (>200nt) concentrations were compared between viable and trophic eggs with
226 paired-t-tests (for each type of eggs we used the average of the two replicates per colony). We also
227 compared the fragment size distributions from 18 to 24 nucleotides for miRNAs (Sohel 2016) with a
228 PCA and a Mantel test.
229
230 DNA was extracted from pools of six eggs using TRIzol (Life Technologies). DNA concentration was
231 measured with a Nanodrop 3300 (ThermoFisher), and DNA integrity was examined with an Agilent
232 Fragment Analyzer (at the Lausanne Genomic Technologies Facility) using a High Sensitivity Assay.
233 DNA concentrations were compared between viable and trophic eggs using paired-t-tests (sample size is
234 5 for both types of eggs, each sample being a pool of 6 eggs).
235

236 Results

237 **Trophic and viable egg characteristics**

238 *P. rugosus* queens lay two types of eggs that are morphologically different. Viable eggs are white with a
239 bright surface and have a distinct oval shape, a homogenous content as well as a solid chorion (Figure
240 2A), while trophic eggs are rounder, have a smooth surface and a granular looking content as well as a
241 fragile chorion (Figure 2D). Trophic eggs had a significantly larger volume (94.3$\pm$4.3nL; n=11) than
242 viable eggs (n=14; 63.3$\pm$1.6nL; two-sample t-test, $t(23) = -9.54$, $p = 1.8x10^{-09}$). While viable eggs
243 showed embryonic development at 25 and 65 hours (Fig 12 B,C) there was no such development for
244 trophic eggs (Fig. 2 E,F). *P. rugosus* workers only laid viable eggs. They started to lay eggs
245 approximately three weeks after queen removal (n=12 queenless recipient colonies) and approximately
246 90% of the eggs successfully hatched. However, only approximately 5% successfully developed into
247 pupae which were all males.

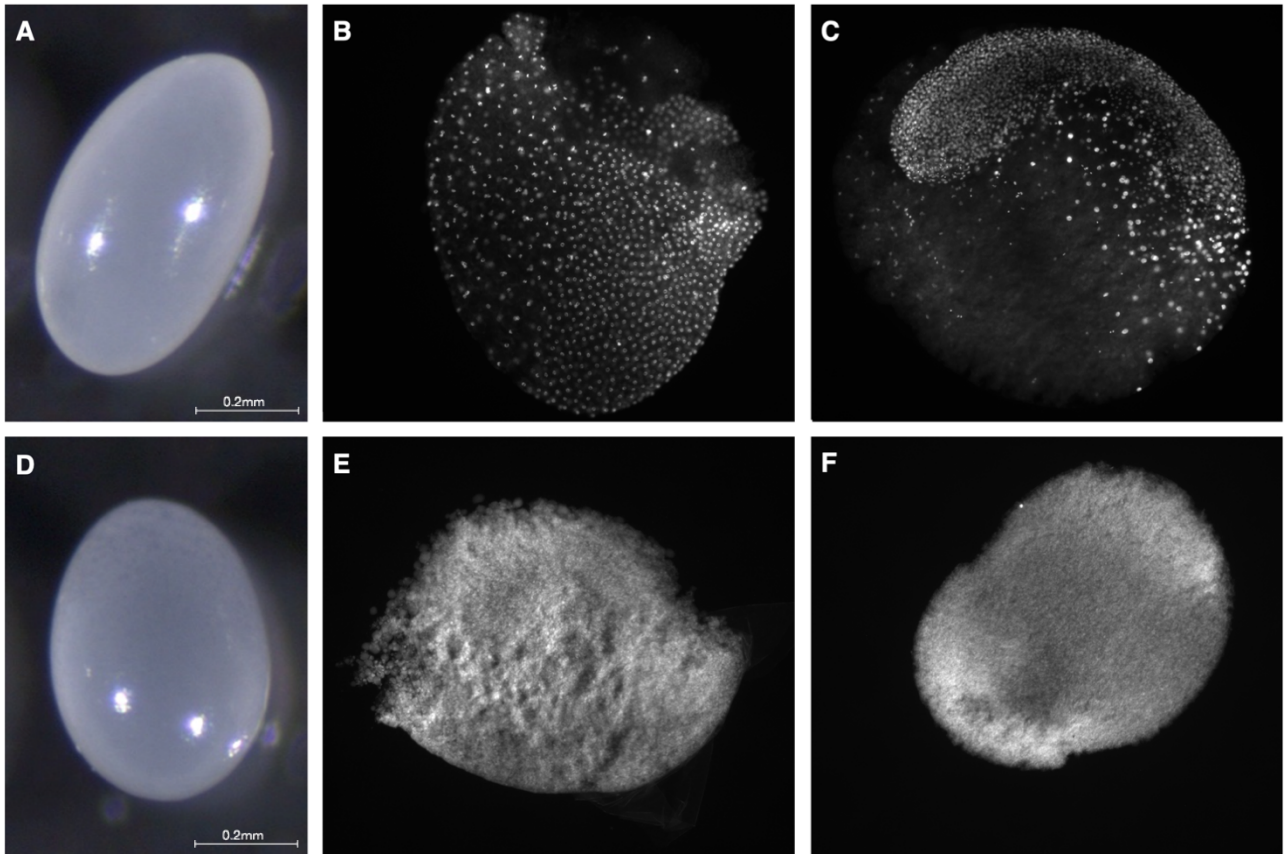
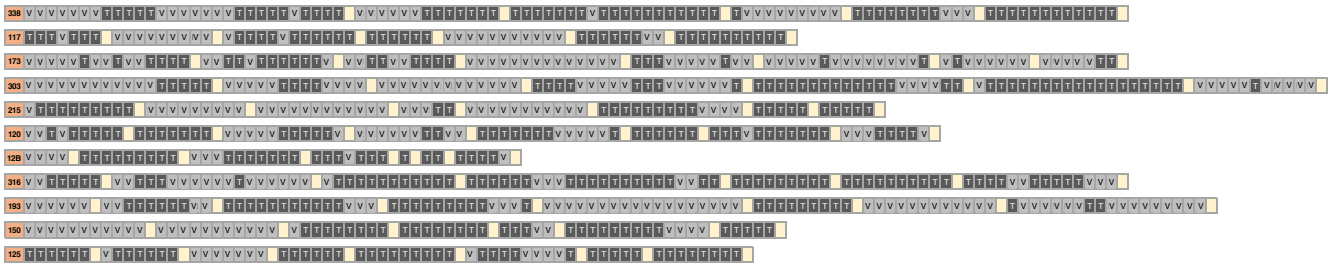


Figure 2. Morphology and of viable (**A**) and trophic (**D**) eggs laid by *P. rugosus* queens. Fluorescence images with DAPI-counterstained nuclei showing embryonic development of viable eggs at approximately 25 hours (**B**) and and 65 hours (**C**)). For trophic eggs there was no embryonic development at 25 hours (E**)** nor at 65 hours (**F**).

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



262

263 **Figure 3.** Egg-laying sequences from eleven *P. rugosus* queens. Every row shows the sequence of viable
264 (V) and trophic (T) eggs laid by a given queen (queen ID in the orange cell). Each egg laying session
265 lasted 10 hours. The yellow squares indicates the intervals (16hours- to several days) between egg-laying
266 sessions.

267

268 **Table 1.** Wald-Wolfowitz runs tests on the queen's egg sequence. Significant p-values (corrected for
269 multiple testing) indicate that queens do not lay viable 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

270

271 The concentrations of protein, triglycerides, glycogen, and glucose were significantly higher in viable
272 than trophic eggs (LMER, protein: $t = -13.11$, $p < 0.0001$; triglycerides: $t = -11.66$, $p < 0.0001$; glycogen:
273 $t = -11.98$, $p < 0.0001$; glucose: $t = -18.60$, $p < 0.0001$; Figure 4).

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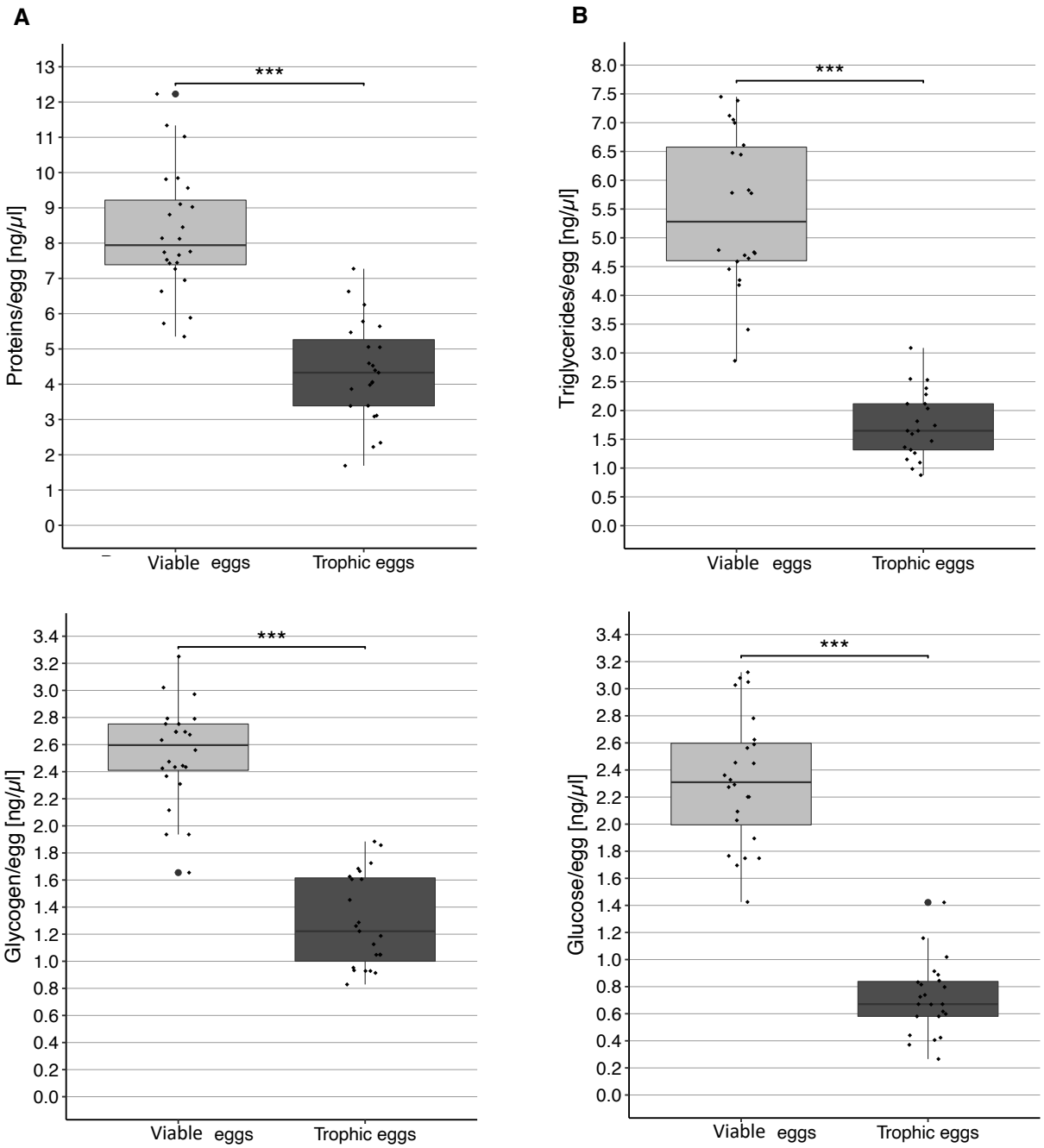

Figure 4. Concentration (± standard error) of protein (**A**), triglycerides (**B**), glycogen (**C**) and glucose (**D**) in viable and trophic eggs. Each dot represents the average of the two replicates per colony.

The amount of small RNA (<200 nt, including miRNA and tRNA; Nagano and Fraser 2011) was significantly higher in viable 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; viable eggs: 7.6±0.6ng, mean ± SE; trophic eggs: 3.6±0.3ng; paired-t-test, $t_{(23)} = 7.2$, $p = 2.7*10^{-7}$).

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

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

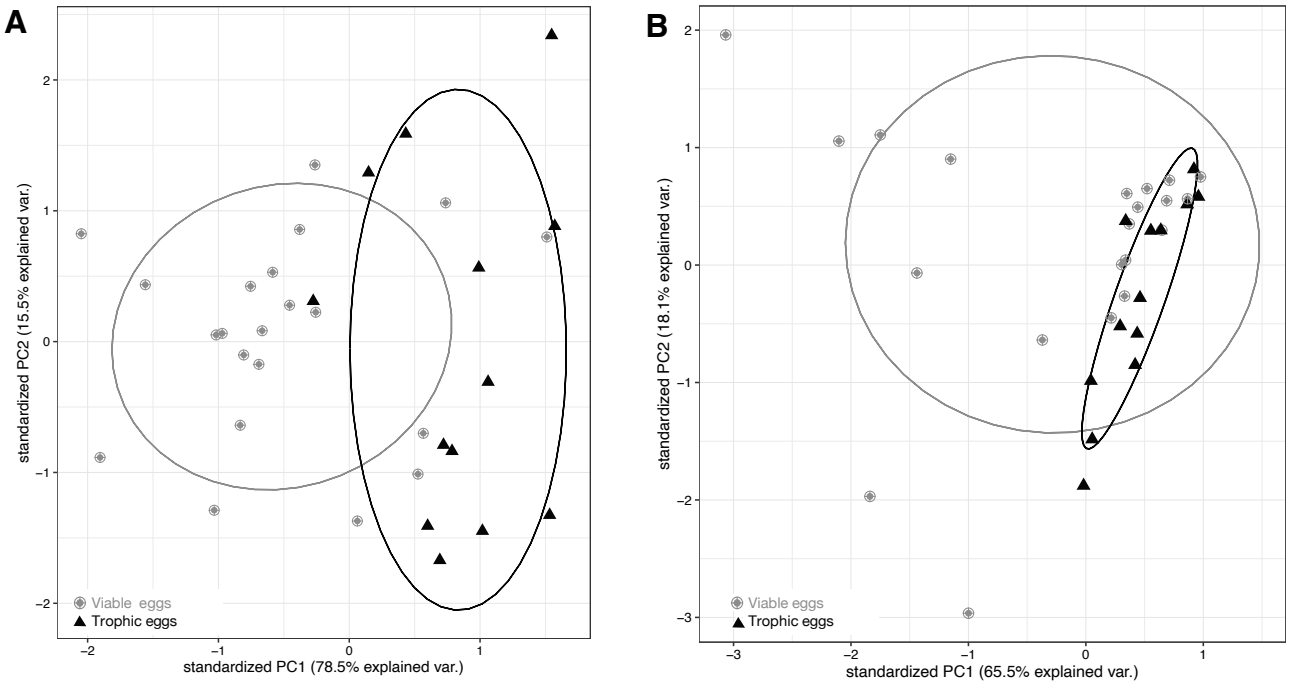

Figure 5. First two principal components (PC1 and PC2) explaining size distribution variation for (**A**) miRNA and (**B**) tRNA across egg samples, with viable eggs in grey dots and trophic eggs in black triangles. Ellipses enclose each of the egg type groups.

Trophic eggs influence caste fate of larvae

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

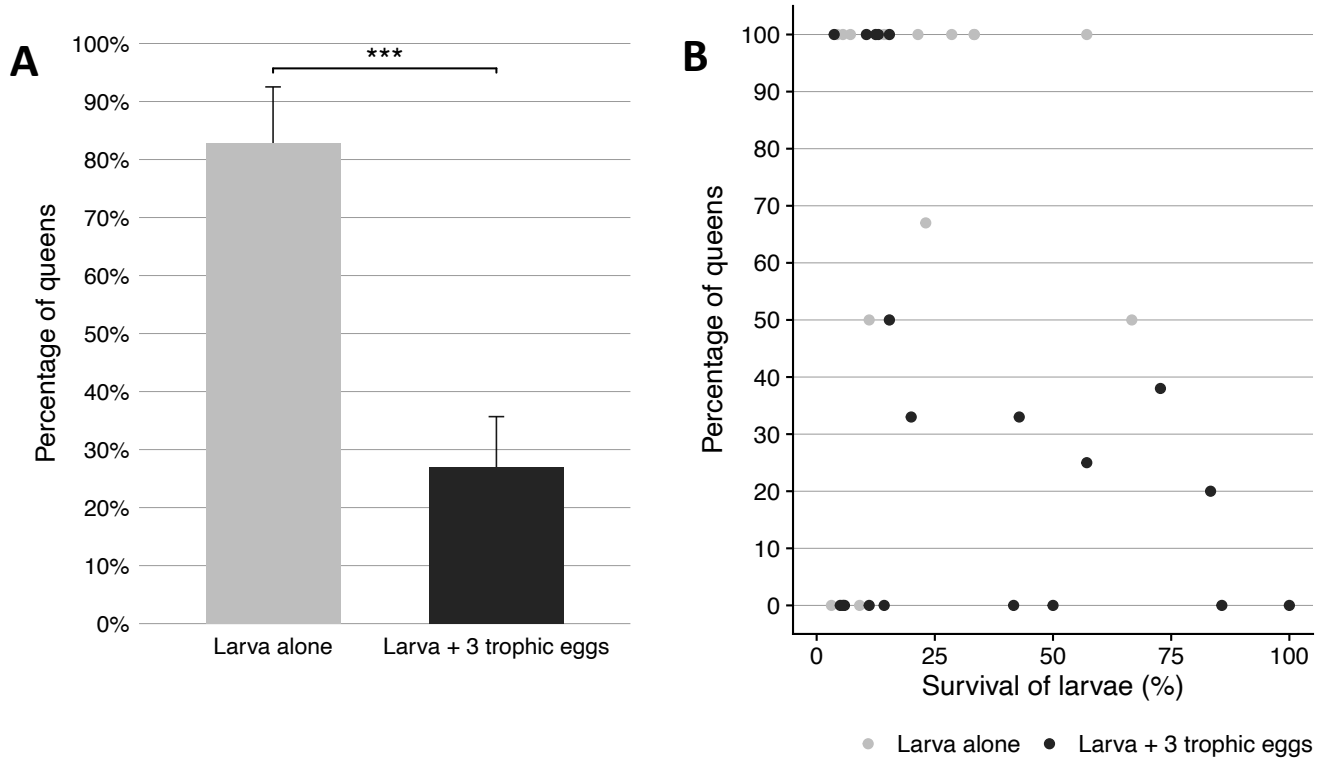

Figure 6. (**A**) Percentage (± standard error) of queens among the larvae that developed to the pupal stage in colonies without (grey) or with (black) trophic eggs. (**B**) Relationship between the percentage of larvae which developed into queens and the survival of larvae (percentage) between the larval to pupal stages.

361

362 Discussion

363 Our study reveals that *P. rugosus* queens lay a very high proportion (0.6) of trophic eggs. These eggs
364 differ in many ways from viable eggs. First, trophic eggs are larger, rounder, have a smoother surface, a
365 more granular looking content as well as a more fragile chorion than viable eggs. Similar differences
366 between trophic and viable eggs have been reported in other ant species (Wilson 1976; Wardlaw and
367 Elmes 1995; Gobin *et al.* 1998; Dietemann and Peeters 2000; Dietemann *et al.* 2002; Perry and Roitberg
368 2006; Lee *et al.* 2017). Our analyses also showed that trophic eggs are solely laid by queens; *P. rugosus*
369 workers are able to produce viable eggs which occasionally develop into males, but they do not lay
370 trophic eggs. Moreover, trophic eggs have a reduced DNA content.

371

372 Importantly, our experiments showed that the presence of trophic eggs influences the process of caste
373 differentiation. First instar female larvae fed with trophic eggs were significantly more likely to develop
374 into workers than larvae without access to trophic eggs. This was somewhat surprising because trophic
375 eggs are generally thought to be an important source of nutrients to the colony and, everything else being
376 equal, one would think that eating such eggs should increase the likelihood of females to develop into
377 queens (which are usually larger than workers). Indeed, two earlier studies suggested that an increase in
378 the proportion of trophic eggs might be associated with an increase in the proportion of larvae developing
379 into queens (*L. humile*: Bartels 1988; *P. barbatus*: Helms Cahan *et al.* 2011). However, in these two
380 studies variation in the availability of trophic eggs was associated with other differences (number of
381 queens in the colony Bartlels 1988; Administration of a JH analogue (Helms Cahan *et al.* 2011) making
382 it difficult to determine what factor had a causal effect. It would be interesting to experimentally
383 manipulate the quantity of trophic eggs in *L. humile* and *P. barbatus* to determine whether they have a
384 positive or inhibitory effect on the likelihood of larvae to develop into queens.

385

386 Our analyses revealed that trophic eggs have a lower content of protein, triglycerides, glycogen, and
387 glucose than viable eggs. A reduced protein content of trophic as compared to viable eggs has also been
388 documented in *Pheidole pallidula* (Lorber and Passera 1981). These findings are in line with the view
389 that trophic eggs do not simply have a nutritional function as it might then be expected that they should
390 at least contain as much nutrients as viable eggs. Interestingly, our analyses also revealed important
391 differences in RNA and miRNA content between the two egg types. miRNAs have already been
392 suggested to influence larval caste determination in the honeybee (Guo *et al.* 2013) with worker jelly

being enriched in miRNAs compared to royal jelly (Guo *et al.* 2013; Zhu *et al.* 2017). These studies suggest that it is not the royal jelly that stimulates larval differentiation into queen, but rather the worker jelly which stimulates the development of larvae into workers. Similarly, our study reveals that compounds found in trophic eggs, perhaps miRNAs, influence larval development towards the worker phenotype. Interestingly, it has also been recently shown that trophallactic fluid in the ant *Camponotus floridanus* contains non-digestive related proteins, microRNAs and juvenile hormone (LeBoeuf *et al.* 2016). Moreover, comparison of trophallactic fluid proteins across social insect species revealed that many are regulators of growth, development and behavioral maturation (Meurville and LeBoeuf 2021). Finally, a recent study showed that pupae of several ant species produce secretions that play an important role for early larval nutrition with young larvae 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. The presence of trophic eggs has been documented in only relatively few ant species (Table 1). However, their presence has been investigated in only few species and it is likely that trophic eggs are produced in most ant species, particularly in those with an independent mode of colony founding,

Finally, our analyses also revealed seasonal differences in the proportion of viable 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

426 an increase in the proportion of larvae developing into queens. The same logic applies to species where
427 trophic eggs are laid only by the workers in queenright colonies (Supplementary Table 1). After the
428 queen's death, workers start producing their own male offspring and lay mostly (if not only) viable eggs
429 (*Temnothorax recedens*, Dejean and Passera 1974; *Plagiolepis pygmaea*, Passera 1980; *Myrmecia*
430 *gulosa*, Dietemann *et al.* 2002), which again leads to a decrease, or cessation, in trophic egg production.
431 A decrease in trophic egg production and the development of queens were observed simultaneously in
432 freshly orphaned colonies of *Temnothorax recedens* (Dejean and Passera 1974), *Plagiolepis pygmaea*
433 (Passera 1980) and *Myrmecia gulosa* (Dietemann *et al.* 2002). These examples are consistent with the
434 view that trophic eggs may also play a role in the process of caste determination in other ant species.
435
436 Egg cannibalism has been reported in a many ant species (Wilson 1971; Sorensen et al. 1983; Bourke
437 1991; Crespi 1991; Peeters and Tsuji 1993; Aron *et al.* 1994; Heinze *et al.*1999; Schultner et al. 2013).
438 Egg cannibalism may serve many purposes, including preferential elimination of males, source of energy
439 (Sorensen et al. 1983) and intracolony conflict (Schultner et al 2014). Our study indicates a new potential
440 role of egg cannibalism as a mechanism to regulate the allocation of energy into the production of new
441 queens versus workers.
442
443 In conclusion, this study provides a new striking example of how females can influence the
444 developmental fate of their offspring. Because many ants produce trophic eggs, it is possible that this
445 mechanism of parental manipulation is widespread and plays an important role in the general process of
446 caste determination. It would be interesting to conduct manipulative experiments similar to those of this
447 study in additional species, to determine whether trophic eggs broadly play a role in the process of caste
448 determination. Of interest would also be to determine what chemical in the egg are responsible for
449 influencing the development of larvae.
450

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