

1           **Sexual production is regulated by the brood in a social bee (*Bombus impatiens*)**

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3                           Priscila K. F. Santos, Cameron S. Murray, Etya Amsalem\*

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5   Department of Entomology, Center for Chemical Ecology, Center for Pollinator Research, Huck  
6   Institutes of the Life Sciences, Pennsylvania State University, University Park, PA 16802 U.S.A.

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8   \*Corresponding author

9   Address: 215 Huck Life Sciences building, University Park 16802; +1 814 8637675

10   Email: eua6@psu.edu

11   ORCID: 0000-0001-7935-049X

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18

19 **Abstract**

20 The production of sexuals in social insects often marks the climax of colony development and  
21 the ultimate purpose for building the worker force. However, the mechanisms regulating this  
22 process are largely unknown. Here we examined the role of the brood in regulating colony  
23 development and sexual production using the bumble bee *Bombus impatiens*. Previous studies in  
24 this species demonstrate that the presence of larvae reduces worker egg laying and enhances the  
25 inhibitory effect of the queen. However, these studies were conducted using small groups, and  
26 the effect of the brood on colony-level events, such as the onset of worker reproduction and  
27 sexual production, remained unexplored. To examine these effects, we doubled or removed the  
28 brood in full-size, young colonies at the onset of the experiment and monitored colony  
29 development thereafter. We show that double-brood colonies produced significantly more gynes,  
30 whereas colonies with a reduced amount of brood produced significantly more males.  
31 Additionally, worker reproduction started sooner in colonies with a reduced amount of brood and  
32 was delayed in double-brood colonies, while aggression overall was the highest in double-brood  
33 colonies. Overall, our findings indicate that the brood has significant impacts on colony  
34 development and sociobiology. The mechanisms determining female caste development in social  
35 species are still mostly unknown and the brood may be key to understanding how demographical  
36 changes in colony development shape social behavior. The variation in the amount of brood may  
37 also explain the various reproductive strategies across bumble bee colonies and other social  
38 species.

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41

## 42 **Introduction**

43 Social insects can produce giant colonies, however most of the individuals are sterile helpers that  
44 are produced to support the production of a limited amount of sexuals. Annual social species like  
45 bumble bees (Etya Amsalem, Christina M. Grozinger, Mario Padilla, & Abraham Hefetz, 2015a)  
46 and social wasps (Jandt, Tibbetts, & Toth, 2013) slowly build up the worker force to support sexual  
47 production in a single event via a strategy called “bang-bang” theory (an abrupt shift from  
48 producing workers to sexuals) (Macevicz & Oster, 1976), whereas perennial species like honey  
49 bees and many species of ants alternate between cycles of worker and sexual production. Timing  
50 the production of sexuals is critical for synchronizing sexual emergence with mating and floral  
51 availability. However, the factors triggering this event remain mostly unknown (Hovestadt, Degen,  
52 & Mitesser, 2018).

53  
54 Gynes (new queens) are morphologically, physiologically, and behaviorally different than workers  
55 (Erin D. Treanore, Derstine, & Amsalem, 2020) and their life trajectory is often determined during  
56 larval development (Trible & Kronauer, 2017; Wilson, 1979). The determination of female caste  
57 early in development sets the foundation for the reproductive division of labor among females, and  
58 therefore, for the social organization (Wilson, 1979). Thus, understanding the triggers for  
59 producing sexuals is of a significant importance to the study of social insect sociobiology and the  
60 evolution of female castes.

61  
62 Most studies on the triggers leading to sexual production were conducted in bumble bees and social  
63 wasps and point to weak correlations between sociobiological / environmental factors and sexual  
64 production (Hovestadt et al., 2018). Several studies in bumble bees examined factors related to the

65 queen and/or the workers (see below), and further studies emphasize the importance of the brood,  
66 but to the best of our knowledge, no study experimentally tested the role of the brood in regulating  
67 sexual production. Preventing workers from contacting the queen (Lopez-Vaamonde et al., 2007),  
68 or transferring old queens to young colonies (C. Alaux, Jaisson, & Hefetz, 2005), or allowing  
69 workers a contact with gyne larvae (Cédric Alaux, Jaisson, & Hefetz, 2006) in *Bombus terrestris*  
70 resulted in earlier gyne production. Gynes were also produced earlier when the number of workers  
71 was doubled and the queen eggs were replaced with male eggs (Bloch, 1999). Contrary, decreasing  
72 workers' age or keeping the number of workers constant and low (and supposedly below the  
73 minimum threshold needed for the production of gynes) (C. Alaux et al., 2005), or manipulating  
74 worker density and egg laying rate by the queen (Shykoff & Muller, 1995) did not influence the  
75 onset of gyne production. In another bumble bee species (*Bombus lucorum*), an increase in worker  
76 mortality rate in either young or old colonies did not affect gyne production, but stressed colonies  
77 invested less in males compared to controlled colonies (Muller & Schmid-Hempel, 1992). In  
78 *Polistes* wasps (*P. gallicus* L), gyne production occurs after a fixed time interval, irrespective of  
79 the production of workers (Deleurance, 1950), and in *Vespula vulgaris*, it has been shown that  
80 when old queens are transferred into a young colony, they immediately initiate queen production  
81 (Potter, 1964), pointing again to the physiological age of the queen as a potential trigger. In  
82 perennial species, the switch to rearing sexuals is presumably determined by seasonal changes in  
83 population size. In the honeybee, males and gynes are produced as the population increases in late  
84 spring and early summer (Seeley, 2010) and their production is controlled by the queen, whereas  
85 in ants, the data is species-dependent. In *Formica exsecta*, gyne production varies stochastically  
86 and could not be explained using geographic and demographic variables (Liautard, Brown, Helms,  
87 & Keller, 2003). In the Argentine ants, gyne production is controlled by the presence of the mated

88 queen (Vargo & Passera, 1993) and in *Aphaenogaster senilis*, both queen pheromone and colony  
89 size play a role in regulating sexual production (Boulay et al., 2007). Overall, data on the triggers  
90 leading to sexual production vary and are sometimes controversial and limited to a small number  
91 of species.

92

93 In recent years, there was a significant growth in evidence demonstrating the role of the brood in  
94 shaping the social environment in Hymenoptera (Schultner, Oettler, & Helantera, 2017). Studies  
95 showed that different stages of brood regulate worker reproduction in several species, including  
96 larva and pupae in *Apis mellifera* (Jay, 1972; Maisonnasse, Lenoir, Beslay, Crauser, & Le Conte,  
97 2010; Mohammedi, Paris, Crauser, & Le Conte, 1998), larvae in *Novomessor cockerelly*, eggs in  
98 *Componotus floridanus* (Ebie, Holldobler, & Liebig, 2015; Endler et al., 2004), larvae in *Oocerae*  
99 *biroi* (Ravary, Jahyny, & Jaisson, 2006), and larvae and pupae in *Bombus impatiens* (Starkey,  
100 Brown, & Amsalem, 2019). The brood can also regulate workers' task allocation like accelerating  
101 the transition from nursing to foraging tasks, increasing the number of foraging trips and the size  
102 of pollen loads in *Apis mellifera* (Maisonnasse et al., 2010; T. Pankiw, 2004; Tanya Pankiw, Page  
103 Jr, & Kim Fondrk, 1998), and increasing the foraging activity of *Oocerae biroi* (Ulrich, Burns,  
104 Libbrecht, & Kronauer, 2016). Furthermore, the ratio between the brood and workers was  
105 suggested to regulate queen production in several bumble bee species in an earlier study (Free,  
106 1955). However, the data are mostly descriptive and the role of brood in regulating sexual  
107 production remained overlooked.

108

109 Bumble bees are an excellent system to examine whether the brood triggers sexual production  
110 since they are annual and semelparous. The life cycle of the colony starts with a single, mated

111 queen that lays eggs following a winter diapause. Initially, the queen performs all the tasks in the  
112 colony but switches to mostly egg laying once the first worker emerges (Etya Amsalem et al.,  
113 2015a). The queen monopolizes reproduction for approximately 4-5 weeks following the first  
114 emergence but loses dominance as the colony grows and transitions into the competition phase.  
115 During this phase, that highly correlates with the timing of gyne production (J. Cnaani, Robinson,  
116 Bloch, Borst, & Hefetz, 2000), workers compete with the queen and among themselves on male  
117 production by exhibiting aggressive behavior, oophagy and egg laying (Duchateau & Velthuis,  
118 1988). Gynes are produced towards the end of the season, and typically also males, though males  
119 can be produced earlier (Holland, Guidat, & Bourke, 2013), and colonies differ substantially in the  
120 number and type of sexuals they produce, with some colonies specializing in producing female  
121 sexuals (gynes) and other in producing males (Duchateau, 2004). This split sex ratio was partially  
122 explained in the diapause regime queen experienced prior to funding a colony (Duchateau, 2004).  
123  
124 In this study, we examined how the amount of brood affects colony development and demography  
125 using full size colonies of *Bombus impatiens*. Recent studies in this species show that (a) young  
126 larvae decrease while pupae increase worker egg laying (Starkey et al., 2019); (b) the impact of  
127 the queen on worker ovary activation is stronger when combined with the brood (M. Orlova,  
128 Starkey, & Amsalem, 2020), and (c) the queen pheromonal secretion is only effective when  
129 combined with brood (Margarita Orlova & Amsalem, 2021). Altogether, demonstrating how  
130 significant is the brood to the social organization. To test the effects of brood in full-size colonies,  
131 we manipulated the amount of brood in queenright, young colonies, prior to the stage of gyne  
132 production by transferring all the brood from one colony to another, resulting in colonies with no  
133 brood (**nb, n=5**), or with a double amount of brood (**db, n=5**) at the onset of the experiment. These

134 colonies were compared to unmanipulated colonies that served as control (**c, n=6**). We examined  
135 colony growth, aggressive behavior towards and by the queen, worker ovary activation, and the  
136 production of egg batches, brood, workers and sexuals for 26 days. We continued monitoring all  
137 the adults that emerged in the colonies from day 27 until the emergence of the last bee. We  
138 hypothesized that an increased amount of brood at the onset of the experiment will decrease worker  
139 reproduction, as was found previously in small groups of workers (M. Orlova et al., 2020; Starkey  
140 et al., 2019). A larger amount of brood is expected to support an earlier production and a larger  
141 number of gynes, whereas aggression could be either higher or lower. If aggression follows the  
142 levels of worker reproduction, it should be lower after doubling the brood amount, but could also  
143 be higher if it follows the increased colony size and the presence of gynes.

144

## 145 **Material and Methods**

146 *Bumble bee rearing.* 16 *Bombus impatiens* colonies at the approximate age of 3-4 weeks from the  
147 emergence of the first worker were obtained from Koppert Biological Systems (Howell, MI, USA).  
148 All colonies contained a queen, workers ( $72 \pm 5$ , mean  $\pm$  SE, the number of workers per colony is  
149 provided in Table S1), and brood of different developmental stages. The colonies were kept in the  
150 laboratory under constant darkness, 60% relative humidity, and temperature of 28-30° C. They  
151 were provided with unlimited 60% sugar solution and fresh pollen collected by honeybees and  
152 purchased from Koppert. Colonies were handled under red light.

153

154 *Experimental design.* Sixteen colonies of approximately the same wet mass (see Methods and  
155 Figure 1) and approximately the same number of workers (Table S1), and therefore, approximately  
156 the same amount of brood, were assigned to three treatments. Six colonies containing queen,

157 workers and brood remained unmanipulated and served as controls (**c**). The remaining 10 colonies  
158 were randomly divided to two, and all the brood from five colonies was removed and transferred  
159 to the other five, resulting in colonies with no brood (**nb**) and in colonies with a double amount of  
160 brood (**db**) in the first day of the experiment. Since we were not able to count the brood precisely,  
161 the ‘double’ refers to brood from two colonies, and not to a double amount of brood. All colonies  
162 remained with their own original queen and workers. The experiment was conducted in two  
163 consecutive replicates, each containing eight colonies. Each replicate included all three treatments.  
164 Colonies were provided unlimited food and kept in the conditions above. During the first 26 days,  
165 we controlled for colony growth (see below), observed aggression towards and by the queen  
166 (below), sampled workers for ovary activation (below), and measured the colony wet mass  
167 (below). The number of new egg batches was counted daily. On day 26, we removed all the bees  
168 and the brood excluding the pupae. The numbers of eggs and larvae were counted, and the larva  
169 body mass was measured. The pupae were also counted but were kept in the colonies until they all  
170 emerged. All individuals that emerged from the pupae were counted for the total number of adults  
171 (workers, gynes, males) produced in each colony. Larva body size distribution in each colony is  
172 presented using all larvae with a body mass larger than 0.1 g. This cutoff was chosen since  
173 differences in body mass between castes (queen/workers) are measurable only in the third instar,  
174 corresponding to approximately 0.1 g (Jonathan Cnaani, Borst, Huang, Robinson, & Hefetz, 1997),  
175 and while the caste determination mechanism in *B. impatiens* is unknown, it is unlikely to be earlier  
176 than in *B. terrestris*.

177

178 *Control for colony growth:* To control for differences in the growth of colonies throughout the  
179 experiment (i.e., colonies with no brood could not produce new workers right away), newly-



180 emerged workers (< 24 h old) were collected daily from all worker-producing colonies and were  
181 equally redistributed across all colonies, creating a normal growth in all colonies regardless of the  
182 amount of brood.

183

184 *Colony mass:* Colonies were weighted twice at the beginning of the experiment before and after  
185 the social manipulation and then every other day throughout the experiment using an electronic  
186 scale. This measurement was done by placing the entire colony on a scale and is not intrusive nor  
187 stressful. The mass reported includes the bees, brood, cells and also the plastic box in which the  
188 colony resides but not its sugar reservoir.

189

190 *Aggressive behavior:* colonies were video-recorded for aggression towards and by the queen every  
191 other day. Video recording (20 minutes per colony) was performed between 9 a.m. to 1 p.m.  
192 Approximately 70 hours of videos were analyzed by an observer blind to the experimental design  
193 and hypotheses. Three different behaviors were counted and summed: (1) attack: this behavior  
194 included overt aggression in the form of pulling, climbing, dragging or an attempt to sting; (2)  
195 darting: a sudden movement of a bee towards another bee without a body contact, and (3)  
196 humming: a series of short wing vibrations towards another bee that are conducted while the bee  
197 is in movement (E. Amsalem & Grozinger, 2017; Etya Amsalem & Hefetz, 2010; Duchateau,  
198 1989). The behaviors performed by the queen towards workers (“queen aggression”) and by  
199 workers towards the queen (“worker aggression”) were summed separately and are presented as  
200 the average of the sum behaviors in 20 minutes.

201

202 *Worker ovarian activation:* 10-15% of the workers in each colony were collected at five timepoints  
203 (on days 1, 7, 13, 19, and 26), and a subset of them (5-10 workers / colony / time point) were  
204 dissected for ovary activation. The number of workers removed was negligible in relation to the  
205 number of workers in the colonies and did not change the colony size in a meaningful way. Similar  
206 samples were taken in previous studies and are representative of the overall reproductive state of  
207 the colony. For example: (Padilla, Amsalem, Altman, Hefetz, & Grozinger, 2016; E. D. Treanore,  
208 Kiner, Kerner, & Amsalem, 2020). To measure the terminal oocyte size, worker abdomen was  
209 dissected under a stereomicroscope. The two ovaries were transferred to a drop of water and the  
210 three largest oocytes were measured using the stereomicroscope ruler. The length of the three  
211 largest oocytes was averaged and is presented in mm.

212

213 *Statistical analyses.* Statistical analyses were performed using R Studio-v1.2.5033. To examine  
214 the effect of treatment on colony wet mass, new egg batches, worker oocyte size, aggression, the  
215 total number of eggs, larvae, pupae, workers, males, and gynes, we used either a linear model (lm),  
216 or a generalized linear model (glm) with a Poisson distribution, depends on whether the residuals  
217 of the model were normally distributed or not (Shapiro-Wilk normality test,  $p \geq 0.05$ ). lm was used  
218 to compare the average oocyte size and the total number of eggs, larvae, pupae, and workers across  
219 the treatments. glm was used to compare colony wet mass, new egg batches, aggression, total  
220 gynes and males. The linear models were fit using lm function from lme4-v1.1.26 R package  
221 (Bates, Mächler, Bolker, & Walker, 2015) and the generalized linear models using glm function  
222 from stats-v4.1.1 package included in R-v.3.6.3. All models were fitted using the treatment as a  
223 fixed effect. When data were collected throughout the experiment (i.e., colony wet mass, new egg  
224 batches, oocyte size, and aggression), we also included the timepoint and the interaction between

225 the timepoint and treatment as fixed effects. Since the experiment was conducted in two  
226 consecutive replications, we also included the term “repeat” as a random effect when it improved  
227 the model fit. The best model (with or without the repeat variable) was determined using ANOVA.  
228 Post-hoc pairwise comparisons across the treatments were performed using estimated marginal  
229 means using emmeans-v1.5.4 R package with Tukey test as adjustment method for multiple  
230 comparisons. To test the effect of treatment on the bimodality of larval body mass distribution, we  
231 use the ACR method implemented in the multimode-v1.5 R package (Ameijeiras-Alonso,  
232 Crujeiras, & Rodriguez-Casal, 2021; Ameijeiras-Alonso, Crujeiras, & Rodríguez-Casal, 2019).  
233 Figures were created using ggplot2-v3.3.5 and ggpubr-v0.4.0. Statistical significance was accepted  
234 at  $\alpha=0.05$ . Data are presented as means  $\pm$  S.E.M.

235

## 236 **Results**

237 *Colony wet mass.* The wet mass of the colonies at the beginning of the experiment, prior to the  
238 manipulation (“day zero”, Figure 1), was similar across all three treatments and was modified  
239 (“day 1”) according to the desired manipulation with the **db** colonies weighing more compared to  
240 the **nb** colonies and the control colonies being an intermediate group. Colony wet mass increased  
241 throughout the experiment in all three treatments, reflecting the increase in worker populations  
242 and brood and indicating a normal and healthy development of all colonies. It should be noted that  
243 the **nb** colonies, although had no brood at the onset of the experiment, started to produce new  
244 brood right after the manipulation and continue to do so until the experiment was terminated on  
245 day 26. Post-hoc comparison showed that the colony wet mass was significantly different among  
246 the three treatments in all days following the manipulation (Tukey’s post hoc test  $p<0.05$ ) with the

247 highest wet mass in the double brood (**db**) colonies and the lowest in the no brood (**nb**) colonies.

248 Control colonies exhibit intermediate values.

249

250 *Production of new egg batches.* The number of newly-laid egg batches increased significantly in  
251 the **nb** colonies, approximately one week after the onset of the experiment as compared to at least  
252 one of the other two treatments (Figure 2). These differences were maintained for about two weeks  
253 and were significantly higher in **nb** compared to the other treatments on days 6, 7, 9, and 12 (glmm  
254 followed by Tukey's post hoc test  $p \leq 0.05$ ). **db** colonies produced less egg batches than the other  
255 treatments throughout the experiment, but a significant difference compared to the control was  
256 observed only on day 16 ( $p < 0.05$ ; Figure 2).

257

258 *Aggression by and towards the queen.* On average, workers in **db** colonies presented significantly  
259 more aggressive behaviors towards the queen (glmm followed by Tukey's post-hoc test,  $p < 0.05$ )  
260 and the queen presented significantly more aggressive behavior towards workers (glm followed  
261 by Tukey's post-hoc test,  $p < 0.05$ ; Figure 3) compared to workers in **nb** and **c** colonies. However,  
262 one **db** colony was a clear outlier in the number of behaviors performed by the queen on day four  
263 (33 aggressive acts compared to 0-9 in other colonies) and towards the queen (18 compared to 0-  
264 12 in other colonies). Reanalyzing the data without this colony resulted in similar outcomes for  
265 worker aggression towards the queen (higher in **db** compared to **nb** and **c** colonies) but not for the  
266 amount of aggression presented by the queen (insignificant differences between all treatments).  
267 Throughout the experiment, there were significant differences between all treatments in the  
268 aggression performed by the workers on days 8 and 12 (glmm followed by Tukey's post hoc test

269  $p < 0.05$ ) and no differences in the aggression performed by the queen (glm followed by Tukey's  
270 post hoc test  $p < 0.05$ ) (Supplementary Figure S1).

271

272 *Workers' ovary activation.* On the first day of the experiment, all workers had inactivated ovaries  
273 and there were no significant differences in the average terminal oocyte of workers, as we would  
274 expect from young, queenright colonies (lmm followed by Tukey's post hoc test  $p > 0.05$ ; Figure  
275 4). The control colonies exhibit normal development throughout the experiment, as evidence by  
276 an increase in the average terminal oocyte of workers about two weeks after the experiment onset.  
277 The manipulation was conducted on colonies that are estimated to be approximately 3-4 weeks old  
278 (counted from the emergence of the first workers) so an additional two weeks brought these  
279 colonies to the competition phase where workers activate their ovaries (J. Cnaani, Schmid-Hempel,  
280 & Schmidt, 2002; Duchateau & Velthuis, 1988). A significant increase in the average terminal  
281 oocyte size of workers was observed in the **nb** colonies compared to the other two treatments on  
282 day 7 to the manipulation, and between **nb** and **db** in days 13 and 25 (lmm followed by Tukey's  
283 post hoc test  $p < 0.05$ ). On day 19, the differences were apparent but smaller and non-significant.

284

285 *Production of brood and adults.* The total number of brood (eggs, larvae, and pupae) did not vary  
286 across treatments on the last day of the experiment (day 26) (lm: Eggs or Larvae or Pupae ~  
287 Treatment; Tukey's post hoc test  $p < 0.05$ ; Figure 5), meaning that the **nb** colonies have  
288 compensated for the initial loss of brood throughout the experiment. However, the total number of  
289 adults (including those that emerged from pupae after the last day of the experiment) was  
290 significantly different among treatments. **nb** colonies produced fewer workers (lm followed by  
291 Tukey's post hoc test  $p < 0.05$ ) and more males than **db** and **c** (glm followed by Tukey's post hoc

292 test  $p < 0.05$ ), while **db** colonies produced more gynes compared to the other treatments (lmm  
293 followed by Tukey's post hoc test  $p < 0.05$ ; Figure 6).

294

295 The workers:larvae ratio by the end of the experiment was biased in favor of workers in **db**  
296 colonies (mean 3.4:1), nearly balanced in **c** colonies (mean 1.3:1) and biased in favor of larvae in  
297 **nb** colonies (mean 0.6:1) (Table S2). Although these ratios were not assessed at the beginning of  
298 the experiment, they can be inferred from the data, with the highest W:L ratio in **nb** colonies (~70  
299 workers, no brood) and a much lower ratio in the **db** colonies compared to the control, as they  
300 maintained a similar number of workers but had brood from two colonies. Overall, the ratios by  
301 the beginning of the experiment were flipped towards the end of the experiment. All **db** colonies,  
302 except one, produced gynes, and the total number of gynes produced in **db** colonies was higher  
303 than the other colonies. On the other hand, only one **nb** colony and half of the control colonies  
304 produced gynes during the experiment (Supplementary Table S1).

305

306 *Distribution of larva body mass.* The differences between the treatments in gyne production are  
307 reflected also in the body mass distribution of larvae that were collected and weighted on the last  
308 day of the experiment (day 26). The larvae in **db** colonies showed a bimodal distribution of larva  
309 mass, corresponding to larvae that will develop into workers/males and gynes, while **c** and **nb**  
310 colonies show a unimodal distribution, indicating the production of workers/males ( $p < 0.05$ ; Figure  
311 7). The data per colony is also provided in Supplementary Table S2.

312

313 **Discussion**

314 In this study we show that manipulating the amount of brood in a colony has significant impacts  
315 on colony development, sexual production and workers' behavior and reproduction. Colonies with  
316 double amount of brood produced more gynes, while colonies with reduced brood produced more  
317 males. Furthermore, increased amount of brood led to an increase in worker aggression towards  
318 the queen whereas decreased amount of brood led to workers activating their ovaries sooner.  
319 Overall, these findings shed light on the impacts of brood on worker reproduction, colony  
320 development and sexual production in *B. impatiens*, and suggest the role of brood in shaping the  
321 social structure in social insects is larger than previously assumed.

322

323 In natural colonies, different amount of brood may be determined by environmental conditions  
324 (availability of resources) or the intrinsic quality of the queen. Either of these may shape the  
325 strategy of the colony to invest on either gynes that are larger and take longer to develop or in  
326 males that are smaller and cheaper to produce (J. Cnaani et al., 2002). In our study, the 'decision'  
327 of the colony to invest in sexuals could not be explained by brood differentiating to gynes prior to  
328 the experimental manipulation since all the gyne-producing colonies, excluding two, produced  
329 gynes 25-43 days following the manipulation (Table S1). Thus, at the time of the brood  
330 manipulation, the diploid brood was not produced yet or was too young to commit to being a  
331 worker or a gyne. The critical period for female differentiation is known in *B. terrestris* (first or  
332 second instar, ~11 days after eggs are laid, out of 32 days of development, on average) (J. Cnaani,  
333 Robinson, & Hefetz, 2000), but not in *B. impatiens*. However, in *B. impatiens* it is likely to be at  
334 the fourth instar (15-17 days after egg are laid, out of 36 days of development on average) as  
335 hypothesized in *Bombus* species that are phylogenetically closer to *B. impatiens* (Barie &  
336 Amsalem, 2022; Cameron, Hines, & Williams, 2007; J. Cnaani et al., 2002). Even according to a

337 conservative estimate of an early critical period as in *B. terrestris*, the first gyne emerging in most  
338 colonies was unlikely to be committed to be a gyne at the onset of the manipulation. In two  
339 colonies, the first gyne emerged 19 days after the onset of the manipulation and could potentially  
340 be committed to be gynes at the manipulation onset, but both these colonies produced >200 gynes  
341 in total over an extended period, so the majority of gynes produced in these colonies differentiate  
342 after the manipulation onset. Moreover, although the number of colonies producing gynes in **db**  
343 and control was close (4/5 **db** and 3/6 **c**), the total number of gynes produced in the **db** colonies  
344 was 30 times higher compared to **c** colonies.

345  
346 In a previous study (Duchateau, 2004), queens produced colonies with varying amount of gynes  
347 and males following different regimes of diapause and CO<sub>2</sub> treatment. This effect could be  
348 mediated by the physiological state of the queen and the amount of brood she produced. Bumble  
349 bee queens are able to switch between laying diploid female eggs to haploid males and colonies  
350 are generally divided into early and late switch (Duchateau & Velthuis, 1988). The switch point is  
351 unrelated to the competition phase and the lack of correlations between these two events was  
352 established in multiple studies (Etya Amsalem, Christina M Grozinger, Mario Padilla, & Abraham  
353 Hefetz, 2015b). Queens that switches early typically produce smaller colonies, less gynes and more  
354 males in line with the profile of the **nb** colonies (Duchateau & Velthuis, 1988). Although it is  
355 unclear whether the eggs in **nb** colonies were laid by the queen (who switched to lay males) or the  
356 workers (that started the competition phase), it is likely that the majority of eggs were laid by the  
357 queen for several reasons. While workers in **nb** colonies did activate their ovaries sooner, there  
358 were no other signs for active competition (ie, worker aggression). In fact, workers in **nb** colonies  
359 exhibit low levels of aggression compared to **db** colonies. In addition, **nb** colonies produced



360 workers that emerged more than 30 days after the onset of the experiment, meaning that at least  
361 some eggs in these colonies were diploid and were laid by the queen. On the other hand, the number  
362 of egg batches produced per day in these colonies may be too high for one individual to lay them  
363 all, which can support the hypothesis that at least some of the eggs were laid by workers in **nb**  
364 colonies. It is not unreasonable to assume that colonies specializing in male production have more  
365 worker-destined males compared to colonies that specialize in gyne production. However, this  
366 question is yet to be examined. If it is indeed the queen that laid the eggs in the **nb** colonies, the  
367 higher investment in males could indicate a switch in the queen's strategy to invest in sexuals that  
368 are cheaper to produce. These strategies make sense given that **nb** colonies were also smaller, and  
369 thus, they did not only lack brood at the onset of the experiment (and therefore lacked the future  
370 worker force needed to support gyne production), but also contained smaller worker population  
371 throughout the experiment that could support gyne production at a later point. None of the colonies  
372 in the study were small by any mean (on average **nb** had slightly less than 400 workers whereas  
373 the controls and the **db** colonies had slightly more than 600 workers), but differences in the  
374 population size accumulated throughout the experiment despite our daily effort to redistribute  
375 newly-emerged workers, likely due to the inability to locate all newly-emerged workers in colonies  
376 with hundreds of workers before they became indistinguishable. Colony size and the reduced  
377 amount of brood likely correlate also in non-manipulated colonies, and both could influence the  
378 queen to invest on either queens or males.

379

380 Two more points are worth of mentioning. First, the manipulation we conducted in the amount of  
381 brood, although extreme, did not affect colony survival or health. All manipulated colonies  
382 recovered quickly, as evident by the normal mass gain throughout the experiment (Figure 1) and

383 by no significant differences in the amount of brood by the end of the experiment (Figure 5). This  
384 quick recovery was partially achieved by a temporary increase in egg laying in **nb** colonies (Figure  
385 2), indicative of the plasticity of colonies. The second point is the potential impact of relatedness  
386 on the results. All colonies contained non-related workers (as newly emerged workers were  
387 distributed daily across all the colonies, including the controls), but only the **db** colonies contained  
388 partially unrelated brood. In a previous study we didn't find any evidence to the impact of  
389 relatedness on brood care (Starkey et al., 2019) and therefore find it unlikely to have an impact  
390 here as well. We also didn't observe any differential behavior towards the brood in our study in  
391 any of the colonies. That being said, whether unrelated brood is more likely to develop into gynes  
392 compared to related brood, is a question worth investigating.

393

394 Previous studies in bumble bees pointed to many factors that do not trigger gyne production, but  
395 several studies did provide positive results. The first by Alaux et al 2005 showed that transferring  
396 an old queen into a young colony resulted in an earlier competition and gyne production (C. Alaux  
397 et al., 2005). They concluded that age-dependent change in the queen triggers gyne production.  
398 However, it is interesting to note that the treatment groups in this experiment (C17/Q10 and  
399 C10/Q17 corresponding to colony of a certain age that was matched with a queen of a certain age)  
400 also differed in the worker number and in the ratio of larvae to workers. Young colonies with old  
401 queens (Q17/C10) that initiated gyne production earlier, were also smaller (~14 workers vs ~35)  
402 and with higher larva/worker ratio, so the differences in the timing of gyne production could simply  
403 be the results of differences in brood/worker ratio. Another study by (Bloch, 1999), showed that  
404 replacing the queen eggs with male eggs or doubling the number of workers also advanced gyne  
405 production in *B. terrestris*. These findings contradict the findings in both (C. Alaux et al., 2005)

406 and the current paper since higher worker/larvae ratio led to early production of gynes (though see  
407 results about the flipped patterns of W:L ratio at the time of the manipulation and at d26). One  
408 point worth noting in this study is that the author replaced all the eggs laid by the queen with either  
409 male or female eggs of a donor but 50% of the male eggs and 20% of the female eggs did not  
410 survive to the last day of the experiment. Gyne production was earlier in the groups with doubled  
411 workers and male eggs, thus, again, in the groups where the ratio between workers and brood was  
412 higher. Gyne production in the two studies occurred in either smaller colonies (C. Alaux et al.,  
413 2005) or larger colonies (Bloch, 1999) which could explain why the colony size is controversial  
414 across different bumble bee studies. We believe that a closer look into other studies will likely  
415 reveal that the brood amount was a confounding factor in many of them, and that these studies  
416 together with the current one, provide a holistic explanation to the factors controlling sexual  
417 production in bumble bee colonies.

418

419 Despite the increase in workers' ovary activation in **nb** colonies, they did not exhibit or received  
420 more aggressive towards/by the queen compared to the control colonies. In fact, the aggression  
421 levels were much higher in the **db** colonies where lower activation of ovaries was observed. This  
422 may indicate that aggression and ovary activation are not necessarily interlinked, despite previous  
423 correlations in small groups of workers (Almond, Huggins, Crowther, Parker, & Bourke, 2019;  
424 Etya Amsalem & Hefetz, 2010; Van-Honk, Roeseler, Velthuis, & Hogeveen, 1981; Van Doorn &  
425 Heringa, 1986). It is possible that aggression is triggered by the density of workers (**db** colonies  
426 were larger), close to the production of gynes (**db** colonies produced gynes) or simply precedes  
427 ovarian activation and disappear once hierarchies are determined (Etya Amsalem & Hefetz, 2010).  
428 The lack of aggression in **nb** workers is in line with Bourke and Ratnieks (2001) study about the

429 conflict over male parentage (A. F. G. Bourke & Ratnieks, 1999). They proposed that workers  
430 from male-specialist colonies (early-switch) may have a delay identifying the male brood until late  
431 stages of larval development which result in a delay of the competition point (A. F. Bourke &  
432 Ratnieks, 2001; Duchateau & Velthuis, 1988).

433

434 Overall, the results of our study show that the brood regulates not only egg laying in small groups  
435 of workers, as we found before (Starkey et al., 2019), but also influences colony level events such  
436 as the beginning of the competition and the timing of gyne production. These data support and  
437 extend our previous findings showing that the effect of the queen on worker reproduction and  
438 aggression was stronger in the presence of brood (M. Orlova et al., 2020). The current study shows  
439 that workers activate their ovaries in colonies without brood, even in the presence of the queen in  
440 relatively young colonies (Figure 4), emphasizing the limited impact of the queen and the ability  
441 of workers to gather information from multiple sources to meet their reproductive interests. We  
442 further found that doubling the amount of brood induces an earlier transition to gyne production  
443 whereas the removal of the brood induces worker reproduction and an increase in the production  
444 of males. These findings emphasize the importance of the brood in maintaining and shaping the  
445 social organization in social insects and the need to investigate its diverse role across other taxa.

446

447

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605 Legend to figures

606 **Figure 1:** The average colony wet mass throughout the experiment in the three treatment groups:  
607 colonies containing no brood (**nb**), colonies containing a double amount of brood (**db**) and  
608 control colonies that remain unmanipulated (**c**). Timepoints 0 and 1 refers to the colony wet mass  
609 before and after brood manipulation, respectively. Data are presented as means  $\pm$  S.E.M.

610 Asterisks denote statistical difference between all treatments at  $p < 0.05$ .

611 **Figure 2:** The average number of newly-laid egg batches per day throughout the experiment in the  
612 three treatment groups: colonies containing no brood (**nb**), colonies containing a double amount  
613 of brood (**db**) and control colonies that remain unmanipulated (**c**). Data are presented as means  $\pm$   
614 SEM. Asterisks denote statistical difference at  $p < 0.05$ .

615 **Figure 3:** The average sum of aggressive behavior performed towards the queen (A) or by the  
616 queen (B) in the three different treatments groups: colonies containing no brood (**nb**), colonies  
617 containing a double amount of brood (**db**) and control colonies that remain unmanipulated (**c**).  
618 Data are presented as means  $\pm$  S.E.M. Different letters denote significant differences at  $p < 0.05$ .

619 **Figure 4:** The average oocyte size in workers in the three treatment groups: colonies containing  
620 no brood (**nb**), colonies containing a double amount of brood (**db**) and control colonies that remain  
621 unmanipulated (**c**). Oocyte size was measured in five timepoints throughout the duration of the  
622 experiment using 5-10 workers from each colony. The Number above columns represent the  
623 number of workers. Different letters denote significant differences at  $p < 0.05$ .

624 **Figure 5:** The effect of brood on the total number of eggs, larvae, and pupae on the last day of  
625 experiment averaged by treatment: **nb**, colonies containing no brood; **db**, colonies containing a  
626 double amount of brood and control colonies that remain unmanipulated, **c**. Different letters denote  
627 significant differences at  $p < 0.05$ .

628 **Figure 6:** The effect of brood on the total number of workers, gynes and males averaged by  
629 treatment: **nb**, colonies containing no brood; **db**, colonies containing a double amount of brood  
630 and control colonies that remain unmanipulated, **c**. Different letters denote significant differences  
631 at  $p < 0.05$ .

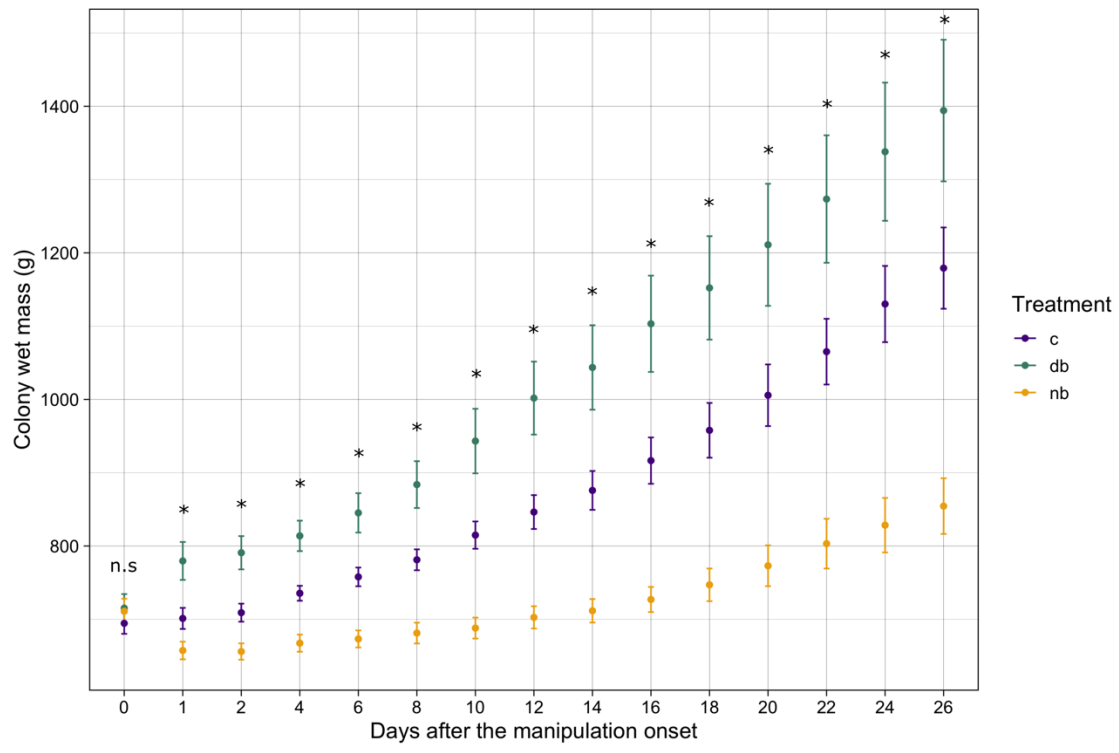
632 **Figure 7:** The effect of brood on the distribution of larval weight by the end of the experiment  
633 grouped by treatment: **nb**, colonies containing no brood; **db**, colonies containing a double amount  
634 of brood and control colonies that remain unmanipulated, **c**.

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Fig 1

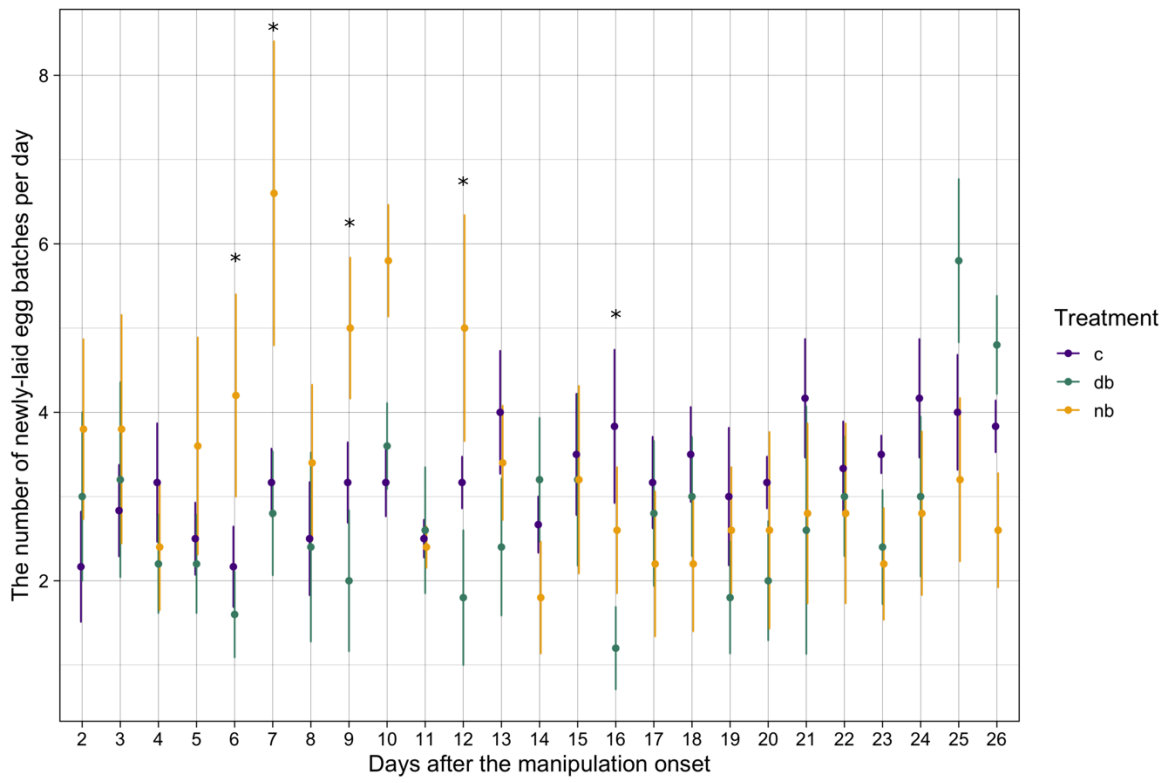


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Fig 2

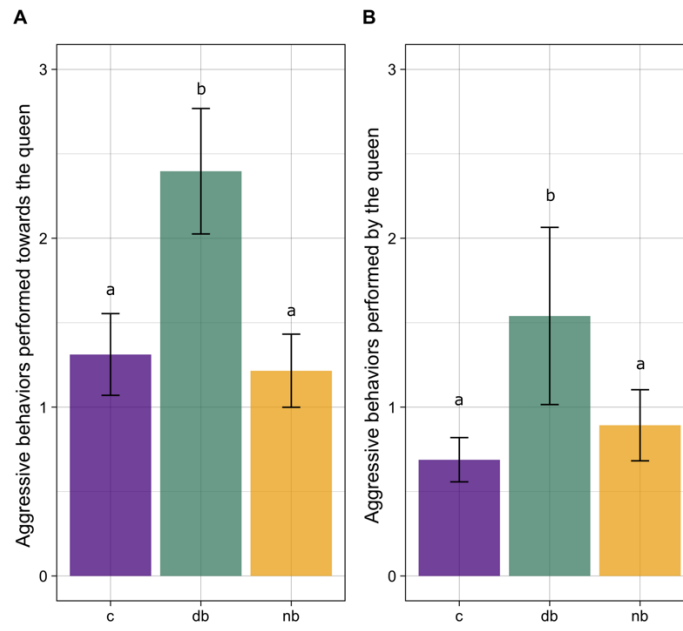


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Fig 3

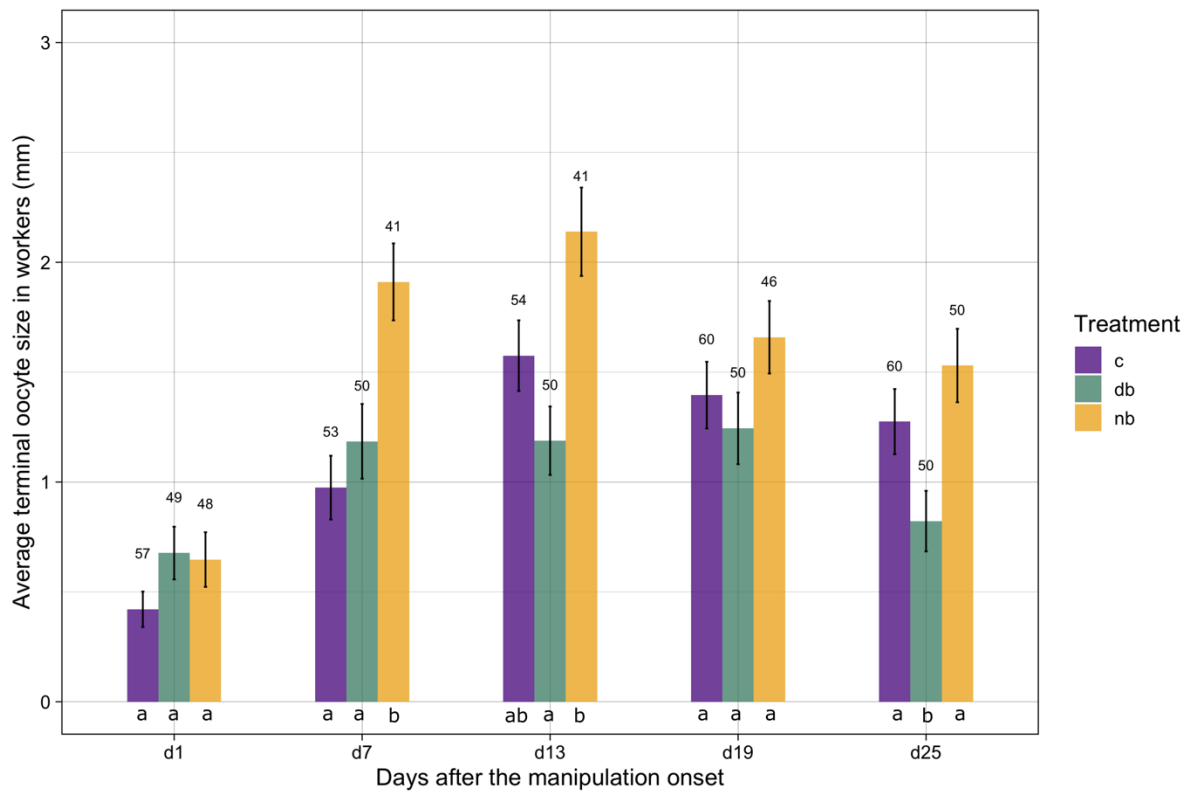


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Fig 4



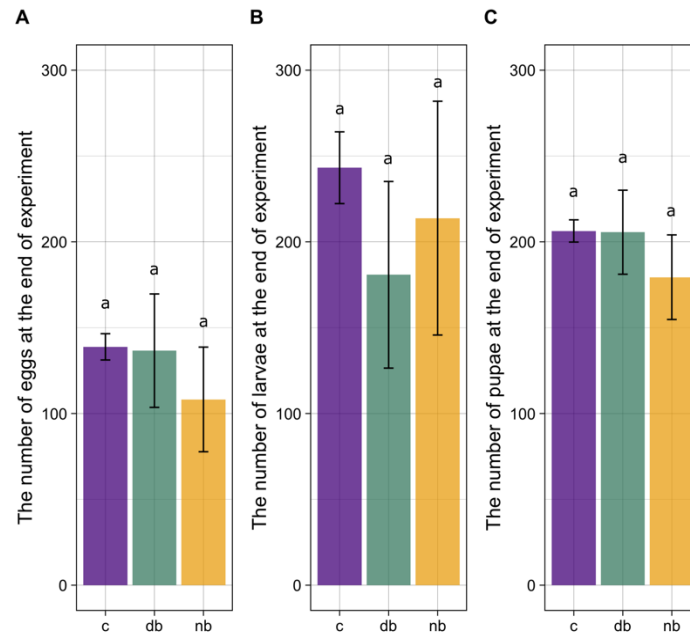
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Fig 5

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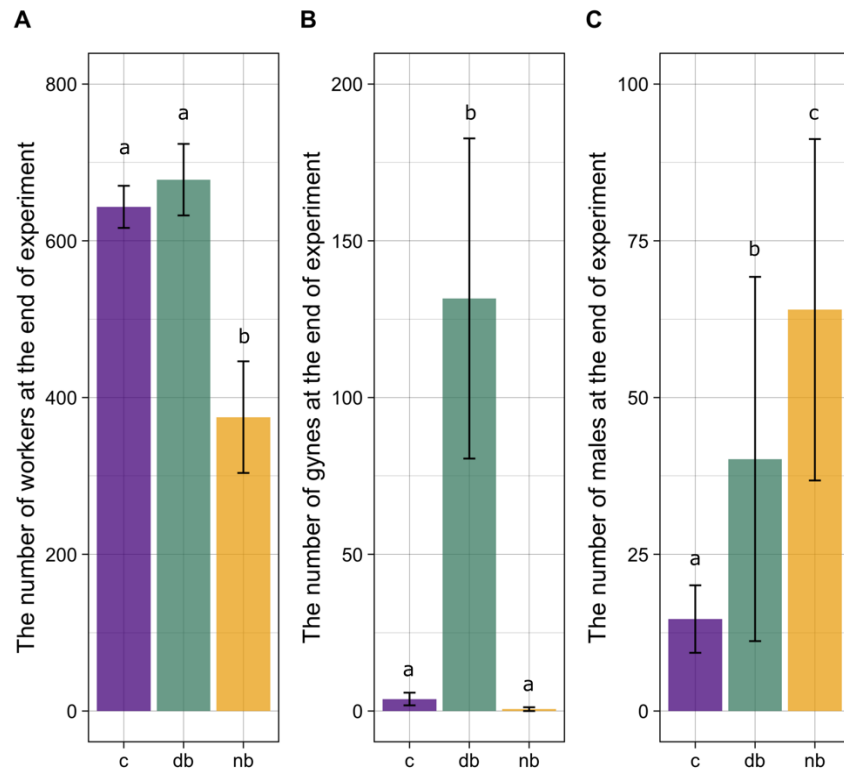
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Fig 6

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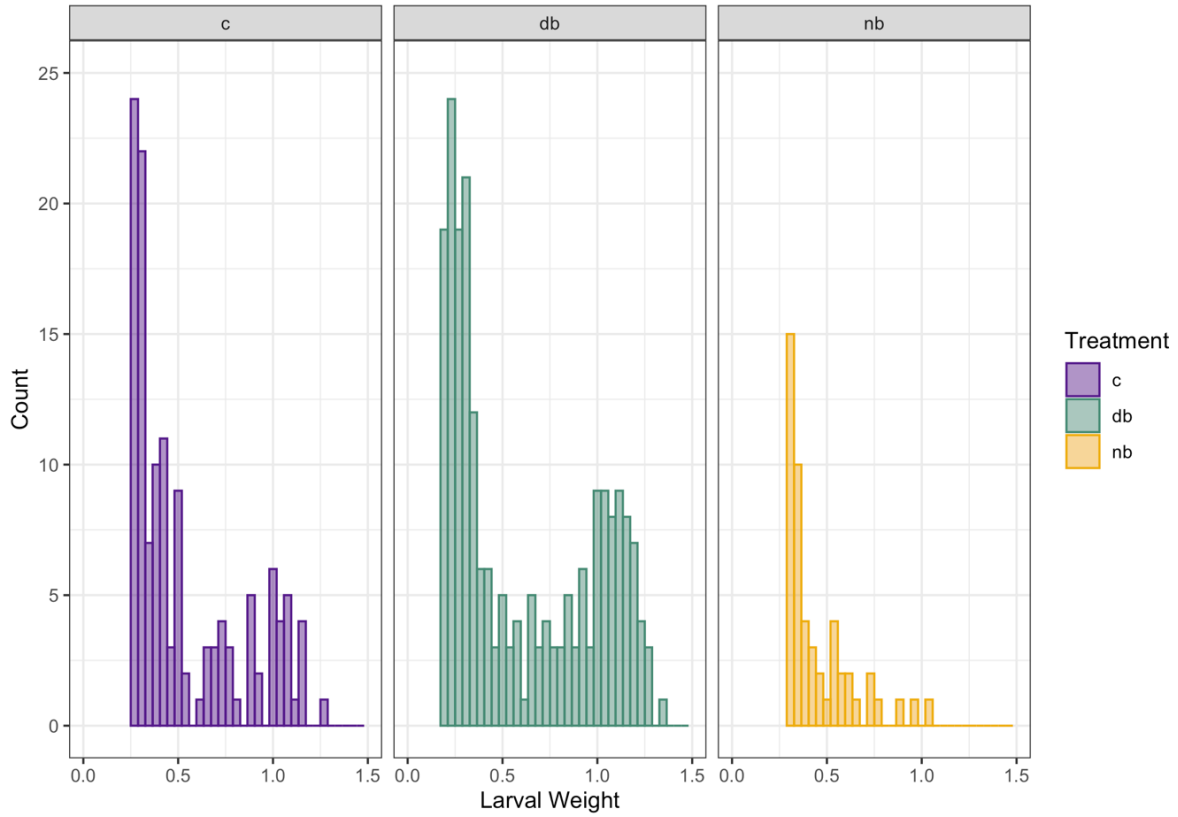
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Fig 7

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