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

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Abstract

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

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Introduction

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

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

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

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

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

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

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

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queen that lays eggs following a winter diapause. Initially, the queen performs all the tasks in the colony but switches to mostly egg laying once the first worker emerges (Etya Amsalem et al., 2015a). The queen monopolizes reproduction for approximately 4-5 weeks following the first emergence but losses dominance as the colony grows and transitions into the competition phase. During this phase, that highly correlates with the timing of gyne production (J. Cnaani, Robinson, Bloch, Borst, & Hefetz, 2000), workers compete with the queen and among themselves on male production by exhibiting aggressive behavior, oophagy and egg laying (Duchateau & Velthuis, 1988). Gynes are produced towards the end of the season, and typically also males, though males can be produced earlier (Holland, Guidat, & Bourke, 2013), and colonies differ substantially in the number and type of sexuals they produce, with some colonies specializing in producing female sexuals (gynes) and other in producing males (Duchateau, 2004). This split sex ratio was partially explained in the diapause regime queen experienced prior to funding a colony (Duchateau, 2004).

In this study, we examined how the amount of brood affects colony development and demography using full size colonies of *Bombus impatiens*. Recent studies in this species show that (a) young larvae decrease while pupae increase worker egg laying (Starkey et al., 2019); (b) the impact of the queen on worker ovary activation is stronger when combined with the brood (M. Orlova, Starkey, & Amsalem, 2020), and (c) the queen pheromonal secretion is only effective when combined with brood (Margarita Orlova & Amsalem, 2021). Altogether, demonstrating how significant is the brood to the social organization. To test the effects of brood in full-size colonies, we manipulated the amount of brood in queenright, young colonies, prior to the stage of gyne production by transferring all the brood from one colony to another, resulting in colonies with no brood (**nb, n=5**), or with a double amount of brood (**db, n=5**) at the onset of the experiment. These

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

Material and Methods

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

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

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

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

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

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

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

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

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

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

Results

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

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highest wet mass in the double brood (**db**) colonies and the lowest in the no brood (**nb**) colonies. Control colonies exhibit intermediate values.

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

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

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

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

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

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

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

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

Discussion

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

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

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

In a previous study (Duchateau, 2004), queens produced colonies with varying amount of gynes and males following different regimes of diapause and CO_2 treatment. This effect could be mediated by the physiological state of the queen and the amount of brood she produced. Bumble bee queens are able to switch between laying diploid female eggs to haploid males and colonies are generally divided into early and late switch (Duchateau & Velthuis, 1988). The switch point is unrelated to the competition phase and the lack of correlations between these two events was established in multiple studies (Etya Amsalem, Christina M Grozinger, Mario Padilla, & Abraham Hefetz, 2015b). Queens that switches early typically produce smaller colonies, less gynes and more males in line with the profile of the **nb** colonies (Duchateau & Velthuis, 1988). Although it is unclear whether the eggs in **nb** colonies were laid by the queen (who switched to lay males) or the workers (that started the competition phase), it is likely that the majority of eggs were laid by the queen for several reasons. While workers in **nb** colonies did activate their ovaries sooner, there were no other signs for active competition (ie, worker aggression). In fact, workers in **nb** colonies exhibit low levels of aggression compared to **db** colonies. In addition, **nb** colonies produced

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

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

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

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

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

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

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

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

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Legend to figures

Figure 1: The average colony wet mass throughout the experiment in the three treatment groups: colonies containing no brood (**nb**), colonies containing a double amount of brood (**db**) and control colonies that remain unmanipulated (**c**). Timepoints 0 and 1 refers to the colony wet mass before and after brood manipulation, respectively. Data are presented as means ± S.E.M. Asterisks denote statistical difference between all treatments at $p < 0.05$.

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

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

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

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

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

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

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Fig 1

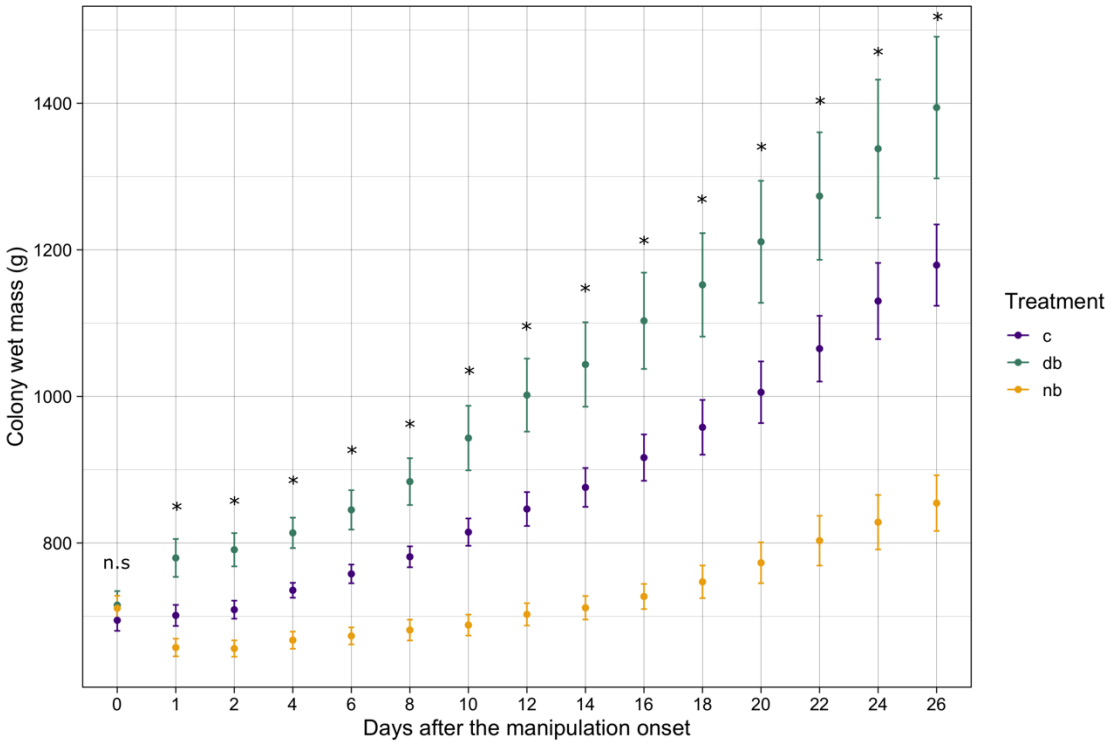

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Fig 2

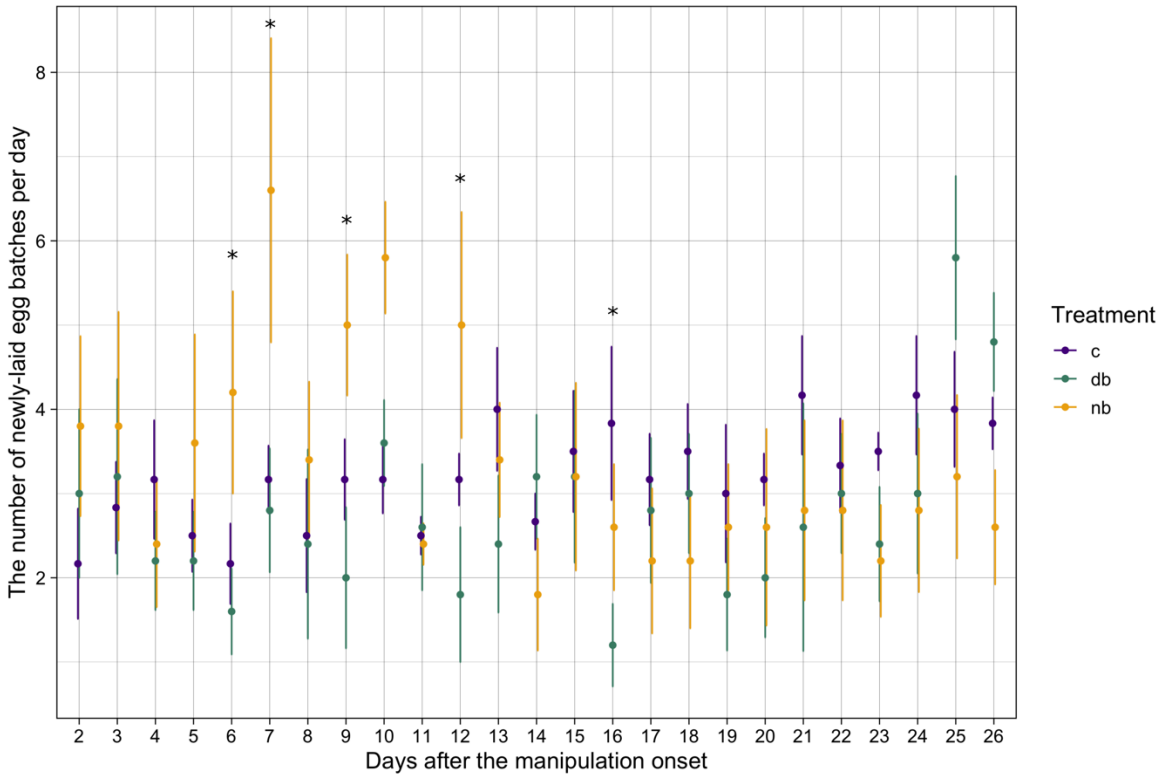

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Fig 3

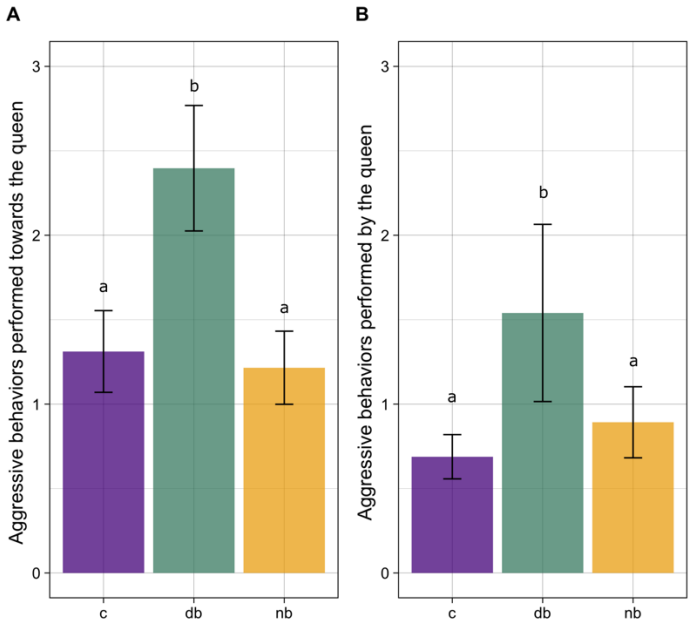

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Fig 4

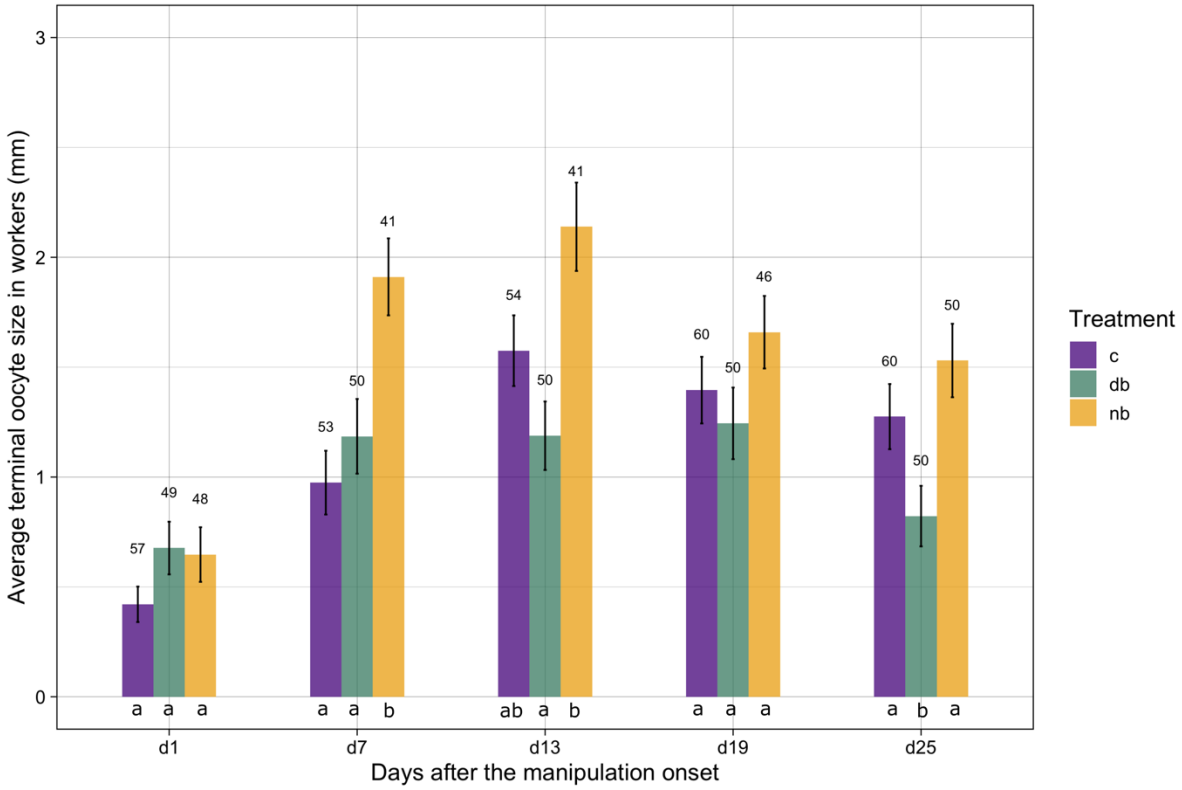

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Fig 5

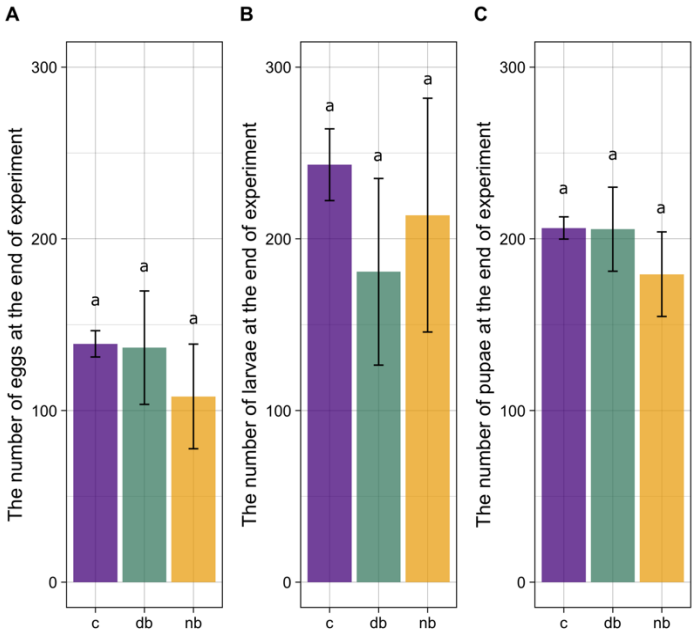

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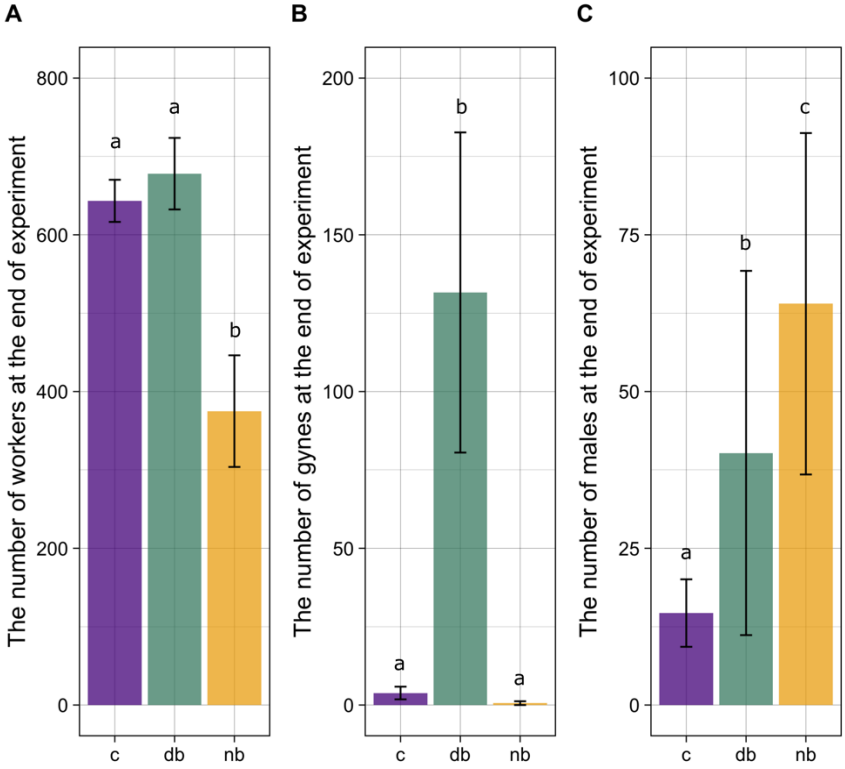
Fig 6



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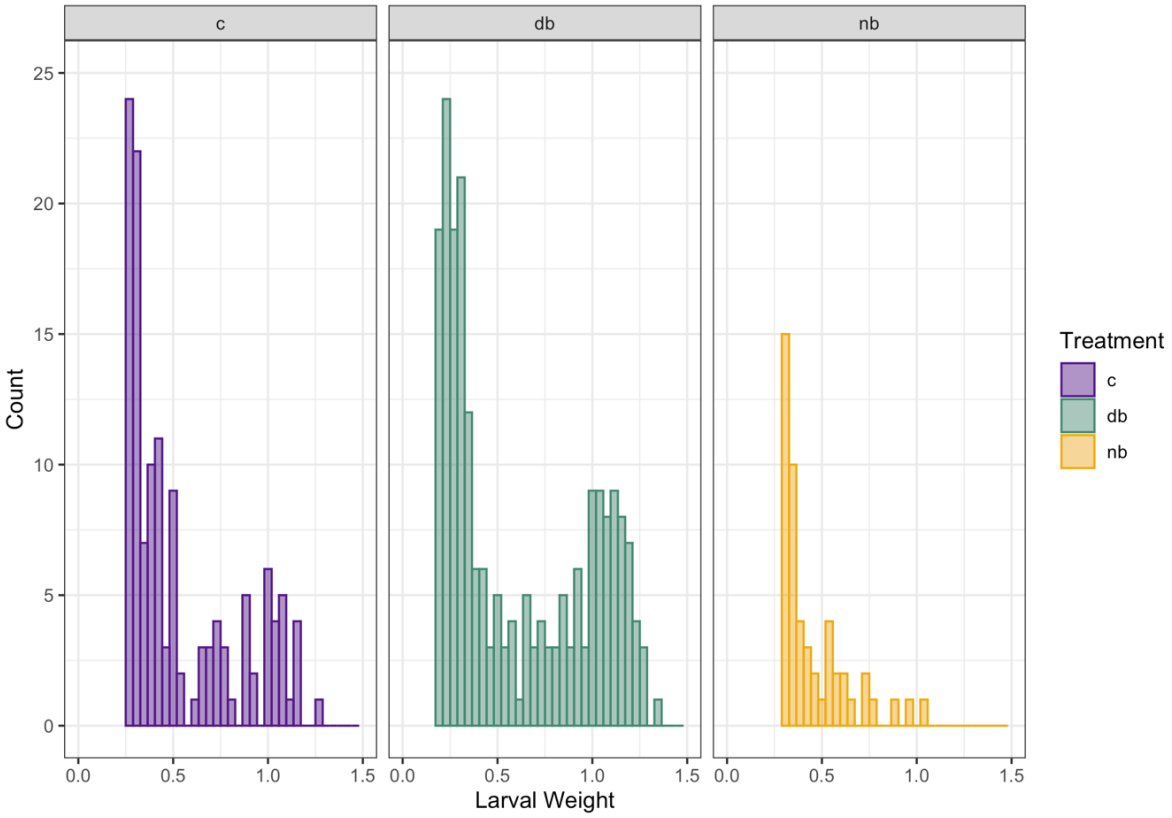
Fig 7