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

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

Department of Entomology, Pennsylvania State University, University Park, PA 16802 U.S.A.

*Corresponding author
Address: 215 Huck Life Sciences building, University Park 16802
Email: eua6@psu.edu
ORCID: 0000-0001-7935-049X

Abstract

Sexual production in social insects marks the peak of colony development, yet the mechanisms regulating it remain unclear. We investigated the role of brood in colony development and sexual production in *Bombus impatiens*. While larvae are known to reduce worker egg-laying and enhance the queen's reproductive inhibition, these effects were previously tested only in small groups. We manipulated brood size in full-size, young colonies by doubling or removing brood and monitored development. Colonies with doubled brood produced significantly more gynes, independent of colony size, while reduced-brood colonies showed a slight, non-significant increase in male production. Worker reproduction was delayed in double-brood colonies, with no change in aggression. These findings suggest brood strongly influences colony development, possibly acting as an extended phenotype of the queen or shaping worker behaviour. Understanding brood dynamics may be key to unravelling female caste determination in social insects.

Keywords: brood, sociality, caste determination, gynes, reproduction, aggression

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 number of sexuals. Annual social species like bumble bees (1) and social wasps (2) 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) (3), 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 and is regulated carefully to prevent loss of fitness. However, the factors triggering this event remain mostly unknown (4).

In hymenopteran social insects, gynes (new queens) are morphologically, physiologically, and behaviourally different from female workers (5) and their life trajectory is often determined during larval development (6, 7). The determination of female caste early in development sets the foundation for the reproductive division of labour among females, and therefore, for the social organization (7). For example, in bumble bees, the timing of gyne production is highly correlated with the onset of worker reproduction (8). Thus, understanding the triggers for producing sexuals is of a significant importance to the studies of social behaviour and the evolution of female castes.

The triggers leading to sexual production in social insects are poorly understood and research has focused on a limited number of species, mostly bumble bees, honeybees, social wasps, and some ants. Sexual production, particularly of gynes, is only weakly correlated with environmental factors (4, 9), and is more strongly associated with sociobiological factors related to the queen. For example, preventing workers from contacting the queen (10), exposing workers to gyne larvae (11), manipulating the queen’s age (12) and replacing the queen’s eggs with male eggs (13) all contributed to early gyne production in bumble bees and/or wasps. Contrary, manipulating workers age, density, or mortality have little to no effect on the onset of gyne production (12, 14, 15). Gyne production is sometimes fixed in time, as in *Polistes* wasps (*P. gallicus L*) (16), stochastic as in *Formica exsecta* (9) or seasonal as in perennial species like the honeybee, where gyne production is controlled by the queen and gynes are produced as the

population increases throughout the season (17). Gyne production is controlled by the queen also in Argentine ants, (18), and in *Aphaenogaster senilis*, where both the queen pheromone and colony size play a role in regulating sexual production (19). Overall, data on the triggers leading to sexual production vary and there is no single explanation shared across taxa.

In recent years, there was a significant growth in evidence demonstrating the role of the brood in shaping the social environment in Hymenoptera (20). Studies showed that different stages of brood regulate worker reproduction in several species, including larva and pupae *Apis mellifera* (21-23), larvae in *Novomessor cockerelly*, eggs in *Camponotus floridanus* (24, 25), larvae in *Ooceraea biroi* (26), and larvae and pupae in *Bombus impatiens* (27). 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* (23, 28, 29), and increasing the foraging activity of *Oocerae biroi* (30). Furthermore, the ratio between the brood and workers was suggested to regulate queen production in several bumble bee species in an earlier study (31). However, the data are mostly descriptive and the role of brood in regulating sexual production remained overlooked.

Here we examined directly how the amount of brood affects colony development and demography using full size colonies of bumble bees (*Bombus impatiens)*. Bumble bees are an excellent system to examine these questions since they are annual and semelparous. The life cycle of the colony starts with a single, mated queen that lays eggs following a winter diapause. Initially, the queen performs all the tasks in the colony but switches exclusively to egg laying once the first worker emerges (1). The queen monopolizes reproduction for approximately 4-5 weeks following the first emergence but loses dominance as the colony grows and transitions into the competition phase. During this phase, that highly correlates with the timing of gyne production (8), workers compete with the queen and among themselves on male production by exhibiting aggressive behaviour, oophagy and egg laying (32, 33). Gynes are produced towards the end of the season, and typically also males, though males can be produced earlier (34), and colonies differ substantially in the number and type of sexuals they produce, with some colonies specializing in producing gynes and others in producing males (35). This split sex ratio was partially explained in the diapause regime queen experienced prior to funding a colony (35).

Recent studies in this species show that (a) young larvae decrease while pupae increase worker egg laying (27); (b) the impact of the queen on worker ovary activation is stronger when combined with the brood (36), and (c) the queen pheromonal secretion is only effective when combined with brood (37). Altogether, these studies demonstrate how significant is the brood to the social organization. To test the effects of brood on worker reproduction and sexual production, 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 colonies were compared to unmanipulated colonies that served as control (c, n=6). We examined colony growth in all 16 colonies, aggressive behaviour 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 will decrease worker reproduction and aggression, and a larger amount of brood is expected to support an earlier production and a larger number of gynes. Since the brood serve as an extended phenotype of the queen, reduced amount of brood can be potentially used by workers to underestimate the quality of the queen and to lead to aggression and early worker reproduction of males.

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 that was 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 and brood (Table S1) were assigned to

three treatments. Six colonies containing queen, 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 doubled 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. 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 (208 observations of 20 min each, 65-78 per treatment, see below), sampled workers for ovary activation (a subset of workers were sampled at 5 timepoints from all colonies, n=799 workers, 250-299, see below), and measured the colony wet mass (mass was collected every other day, see 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 (3432 larvae, 904-1459 per treatment). 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 (38), and while the caste determination mechanism in *B. impatiens* is unknown, it is unlikely to be earlier than in *B. terrestris* (39).

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 emerged workers (< 24 h old) were collected daily from all worker-producing colonies and were equally redistributed across all colonies, creating a comparable growth in all colonies regardless of the amount of brood they had. A minimum of 8 and a maximum of 67 workers were collected daily in each replicate of the experiment and were equally distributed across the colonies. If the number of workers emerged did not divide by 8, extra workers were placed in colonies in the following order: nb, c and then db. While this method was effective in creating a comparable

growth across all colonies (Figure 1), it wasn't perfect since locating all newly emerged workers in colonies containing hundreds of workers before they became indistinguishable was nearly impossible. Therefore, we also examined the effect of the colony size (as measured by the number of workers on the last day) on the variables tested (see results).

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 the plastic box in which the colony resides but not its sugar reservoir. All colonies were placed in identical plastic boxes.

Aggressive behaviour: 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 analysed by an observer blind to the experimental design and hypotheses. Three different behaviours were counted and summed: (1) attack: this behaviour includes 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 (40-42). The behaviours 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 behaviours in 20 minutes.

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 (43-45). Since workers were not individually tagged, some of the workers we dissected could be from another colony (since callows were daily distributed), but since we added them to the colonies upon emergence, we assume their reproductive behaviour is similar to the rest of the workers in the host colony (42). 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 a stereomicroscope ruler. The length of the three largest oocytes was averaged (46) 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, we used the plm-v2.6.1 R package to fit a time series model and estimate the effect of treatment in colony mass while accounting for the correlation over time. For 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, depending on whether the residuals of the model were normally distributed or not (Shapiro-Wilk normality test, $p \geq 0.05$). A Poisson distribution was replaced by a negative binomial distribution in case of overdispersion which was tested using *dispersiontest* from MASS-7.3.55 package. lmm and glmm were used to compare the average oocyte size and total aggression, respectively, with maternal colony as random effect. lm was used to compare the total number of eggs, larvae, pupae, and workers across the treatments. glm was used to compare the number of new egg batches by date, glm.nb was used for aggression by date and the total number of males, and lmm was used for the total number of gynes. The linear models were fitted using lmer or lm functions from lme4-v1.1.26 R package (47) and the generalized linear models using glm or glm.nb functions from stats-v4.1.1 and MASS-7.3.55 packages included in R-v.3.6.3. All models were fitted using the treatment as a fixed effect. 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. The effect of brood on the number of sexuals was tested with and without the colony size (number of workers on the last day) as a fixed effect in the model. 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 (48, 49). 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. The R markdown file and the data

used for the statistical analysis can be found at https://github.com/pkfsantos/Brood_B_impatiens_colonies/tree/main.

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. The difference among treatments remains throughout the experiment as evidenced by an average increase in colony mass of 141.22 g in db colonies compared to control (plm; $p=5.863e-15$), and an average decrease of 152.62 g in nb colonies compared to control (plm;$p=2.2e-16$). 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.

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 (glm followed by Tukey's post hoc test $p=0.05$ for nb vs. db), 7 ($p=0.03$ for nb vs. c; $p=0.02$ for nb vs. db), 9 ($p=0.04$ for nb vs. db), and 12 ($p=0.02$ for nb vs. db). 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 ($p=0.03$ for db vs. c; Figure 2).

Aggression by and towards the queen. There were no differences in the average aggressive behaviour performed by the queen or towards the queen between the treatments (glmm followed by Tukey's post-hoc test, $p>0.22$; Figure S1). There were also no differences in the aggression performed by/towards the queen between the treatments throughout the experiments (glm.nb followed by Tukey's post-hoc test, $p>0.065$; Figure S2). One db colony was a clear outlier in the

number of the behaviours performed by the queen on day 4 (33 aggressive acts compared to 0-9 in other colonies) and towards the queen (18 compared to 0-12 in other colonies). Thus, we analysed the data also without this colony on day 4 (Figures S1 and S2) but this did not change the results.

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 $p>0.64$; Figure 3). 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 worker) so an additional two weeks brought these colonies to the competition phase where workers activate their ovaries (32, 45). The oocyte size of nb workers was approximately double the size of workers in c (lmm followed by Tukey's post-hoc test, $p=0.005$) and db ($p=0.038$) on day 7 to the manipulation. The oocyte size was approximately doubled in nb compared to db also on day 13 ($p=0.004$) and day 25 ($p=0.04$). On day 19, the differences were apparent but smaller and not significant ($p>0.05$).

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 $p>0.58$; Figure 4), meaning that the nb colonies have compensated for the initial loss of brood throughout the experiment. However, the total number of workers (including those that emerged from pupae after the last day of the experiment) was significantly different between the treatments. All colonies had at least 200 workers, but nb colonies (375±50 workers, means ± SE) were smaller than db (678±50) and control (643±46) colonies (lm followed by Tukey's post hoc test $p=0.004$ (nb vs. c); $p=0.002$ (nb vs. db); Figure 5A). db colonies produced significantly more gynes than c and nb (lmer followed by Tukey's post-hoc, $p=0.02$), with 132±51 gynes, on average, in db compared to 0.6±0.6 and 3.8±2 in nb and the control, respectively. When we controlled for the number of workers by the end of the experiment, db and c remain significantly different (lmer followed by Tukey's post-hoc, $p=0.035$; Figure 5B), but db and nb were no longer significantly different. Many more males

were produced in the nb colonies (64±27) compared to db (40±29) and c (15±13), but the variation across colonies was high and the average number of males did not significantly vary between the treatments before or after controlling for the number of workers (glm.nb followed by Tukey's post-hoc, $p>0.15$; Figure 5C).

The number of gynes 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 in *B. terrestris* is during the first or second instar, ~11 days after eggs are laid (50) and is unknown in *B. impatiens*. However, even according to a 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 23 times higher compared to c colonies.

The workers:larvae ratio by the end of the experiment was biased in favour of workers in db colonies (mean 3.4:1), nearly balanced in c colonies (mean 1.3:1) and biased in favour of larvae in nb colonies (mean 0.6:1) (Supplementary 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.

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 (ACR mode test, $p<2.2e-16$), while c and nb colonies showed a unimodal distribution, indicating the production of workers/males only (ACR mode test, $p>0.114$; Figure 6). Larval weight distribution per colony can be found in the Supplementary Figure S3.

Discussion

In this study, we demonstrate that manipulating brood quantity in a social colony significantly impacts colony development, gyne production, and worker reproduction, while showing no significant effect on aggressive behaviour or male production. Additionally, differences in gyne numbers between treatments could not be attributed to colony size. The elevated production of gynes in colonies with doubled brood suggests that brood may function as an extended phenotype of the queen, influencing not only individual reproductive decisions of workers but also colony-level strategies for investing in future queens.

In social insect species, gynes are typically larger and take longer to develop whereas males are smaller and cheaper to produce (45, 51). Investment in either depends on the availability of resources, and in social hymenopteran species, also reflects the outcomes of the conflict between the queen and the workers (52). Maintaining the social structure depends on workers gaining inclusive fitness by helping the queen rear her offspring. If the queen is no longer fecund, it is in the interest of workers to start producing males, and since the brood is the ultimate testimony to the queen's fecundity, its absence or a reduction in its amount are sufficient for the workers to break down the social structure. The effect of brood on gyne production could be a result of a direct impact of the brood or could be affected by the queen and/or the workers in response to the amount of the brood. Such indirect effects could be due to a change in the feeding regime, pheromones produced by females or predetermination of caste during egg laying.

Our data also suggest that reduction in brood may lead colonies to invest in males. While these data were insignificant, the number of males produced in nb colonies was approximately x2 compared to db and x5 compared to the control. Males can be produced by the queen (when she

stitches to lay eggs earlier in the life cycle), or by the workers when they start competing over male production but it is not clear who produced them in each of the treatments.

If the males in nb were produced by the queen, this may reflect a trade off in the queen's strategy to invest in sexuals that are cheaper to produce; A previous study (35) showed that queens following a longer winter diapause produced colonies with more males, whereas shorter diapause resulted in colonies producing more gynes, potentially resulting from the physiological state of the queen and the amount of brood she produced. Queens that switch early to male production typically produce smaller colonies, fewer gynes and more males in line with the profile of the nb colonies (32). Alternatively, the males emerging in the nb colonies could have been produced by workers that competed with the queen over male production following the absence of brood and the perception of the queen as not fecund or of lower quality. While we don't know for sure who laid the male eggs in the nb colonies, it is likely that at least some of the eggs were laid by the queen since workers emerged in nb 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 is likely too many for one individual to lay them all, thus at least some of the eggs were laid by workers in nb colonies. If males were produced by workers, it is an indication that the queen was perceived as weak. If both the queen and workers produced males simultaneously, this would represent an unusual case of peaceful competition since aggression in the nb colonies was no higher compared to the other treatments.

A few more points are worth mentioning. First, the manipulation we conducted to the amount of brood, although extreme, did not affect colony survival or health. All manipulated colonies recovered quickly, as evident by the increase in mass throughout the experiment (Figure 1) and 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 the queen's behaviour. 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, raising the question whether gynes were more likely to develop in colonies with mixed brood. In a previous study we didn't find any evidence of the impact of relatedness on brood care (27) and therefore consider it unlikely to have an impact here. We also didn't observe any differential behaviour towards the brood in our study in any of the colonies. Whether unrelated brood is more likely to develop into gynes compared to related brood is a question worth investigating. Finally, the distinct effects of colony size and brood quantity on worker reproduction and gyne production remain to be disentangled. Although these two processes are correlated (8) and often assumed to share common triggers, emerging evidence suggests they are likely regulated independently. While colony size has been shown to influence worker reproduction before (53), gyne production—despite occurring in close proximity to the onset of worker reproduction—remains unaffected by colony size. The worker-to-larvae ratio does not appear to be critical for determining the onset of gyne production although it has never been tested directly and can only be indirectly inferred from other studies (12, 13), including ours. Altogether, this may suggest that the shift toward gyne production may depend more on the queen's perception of her age (12) or the absolute amount of brood present (regardless of whether the brood is her own), rather than on the number of workers. However, further research is needed to pinpoint the exact mechanism driving this transition.

Conclusions

Overall, the results of our study show that the brood regulates not only egg laying in small groups of workers, as we found before (27), but also influences colony-level events such as the beginning of the reproductive competition and the timing of gyne production. These data suggest that the brood provides a testimony to the fecundity of the queen and leads to gyne production when it is abundant, and possibly also to the production of cheap males in its absent. Our findings in bumble bees align with data from wasps, also annual, in which partial brood removal led to increased egg laying by the queens but did not prevent worker reproduction (54). This may suggest a general mechanism in annual social species where the brood determines in an irreversible way the level of cooperation between the workers and the queens and further influences the production of sexuals. Altogether, our findings emphasize the importance of the brood in maintaining and shaping the social organization in social insects and the need to investigate its diverse roles across other taxa.

Declarations

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Author's contributions. PKFS and EA designed the experiments, PKFS collected and analyzed the data, CSM performed the behavioral observations, PKFS and EA wrote the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

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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.

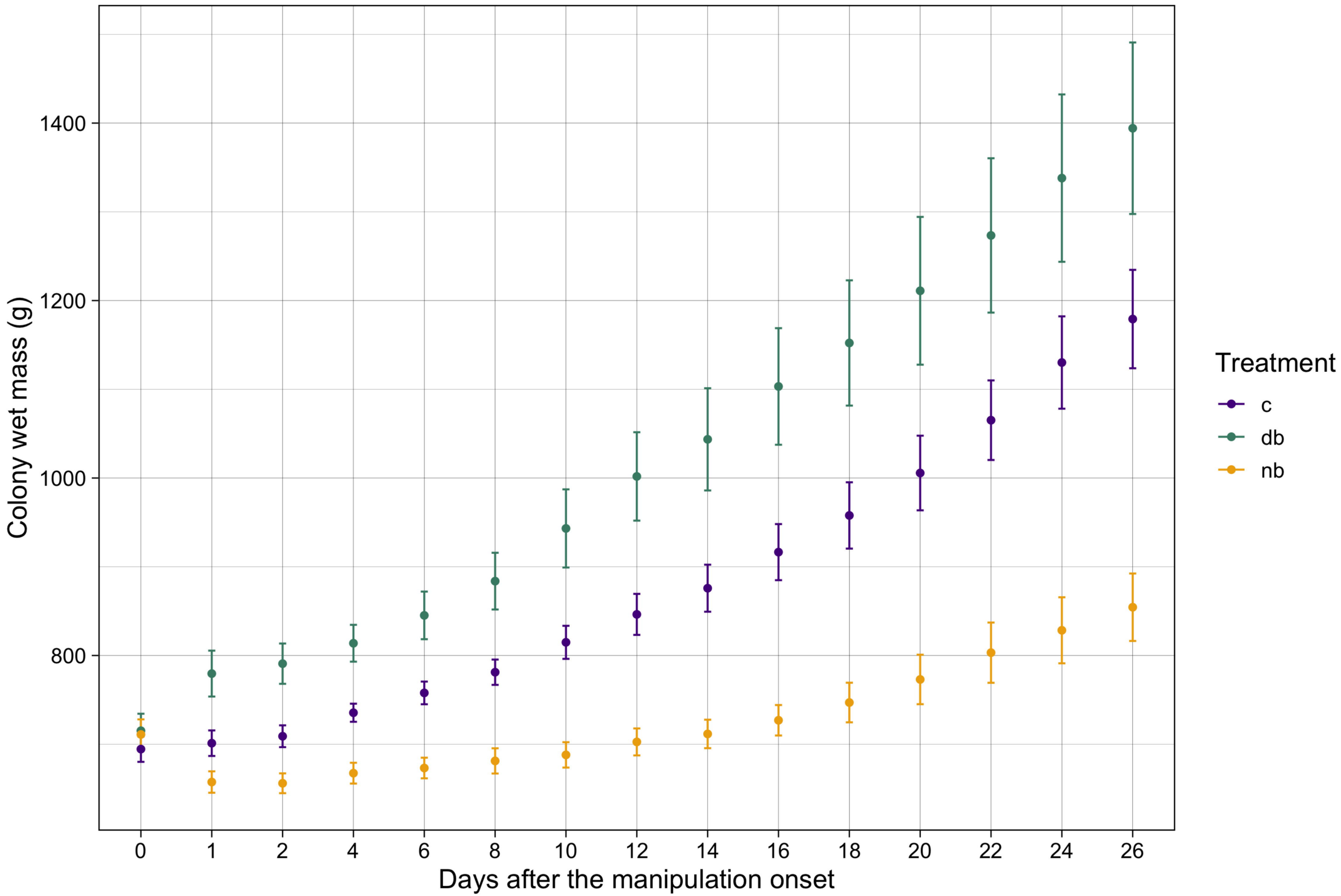
Figure 2: The average number of newly laid egg batches per day throughout the experiment in the three treatment groups: colonies containing no brood (nb), colonies containing a double amount of brood (db) and control colonies that remain unmanipulated (c). Data are presented as means ± S.E.M. Asterisks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

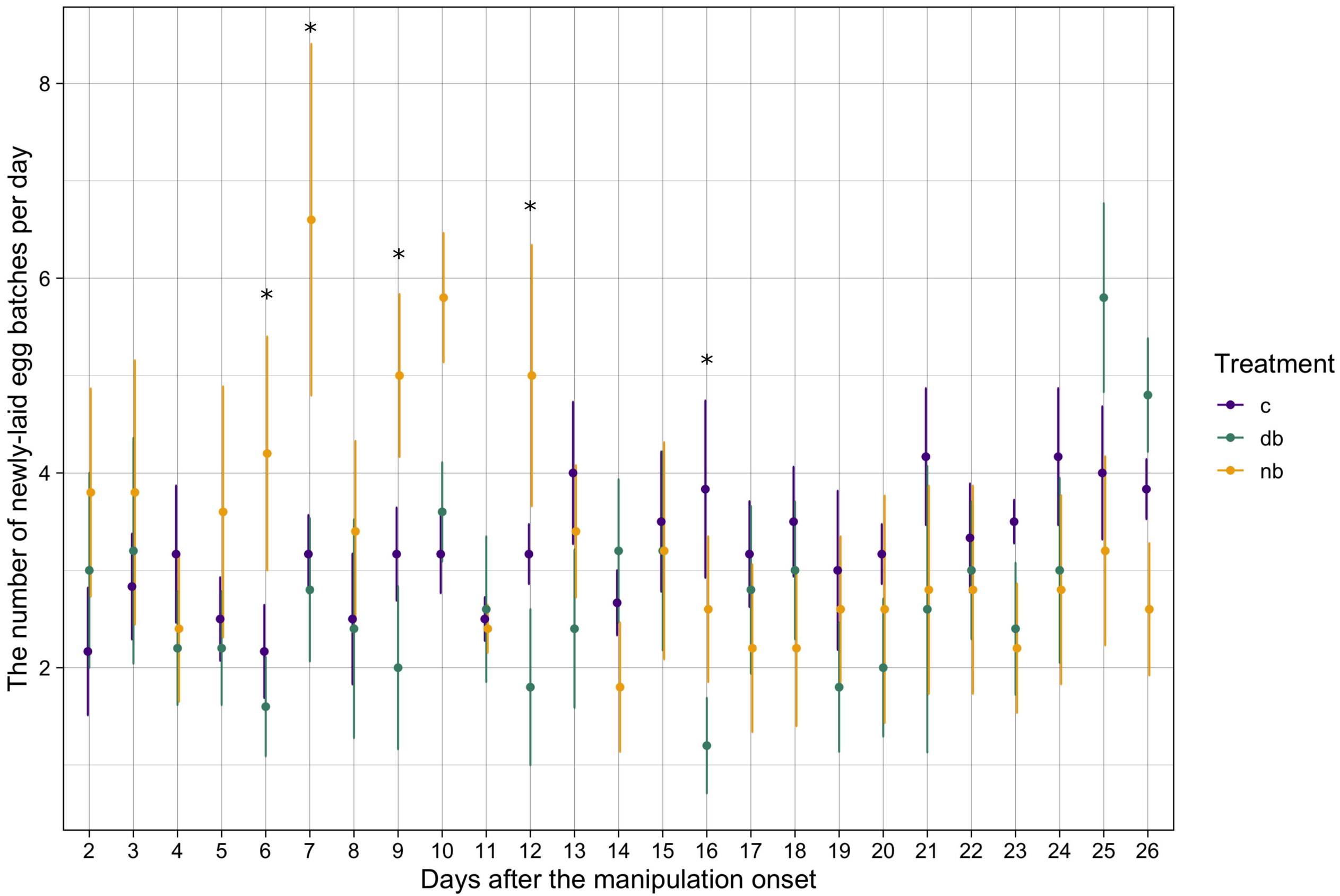
Figure 3: The average terminal oocyte size in workers in the three treatment groups: colonies containing no brood (nb), colonies containing a double amount of brood (db), and control colonies that remain unmanipulated (c). Oocyte size was measured in five timepoints throughout the duration of the experiment using 5-10 workers from each colony. The Number above columns represent the number of workers collected from all colonies. Different letters denote significant differences at $p < 0.05$.

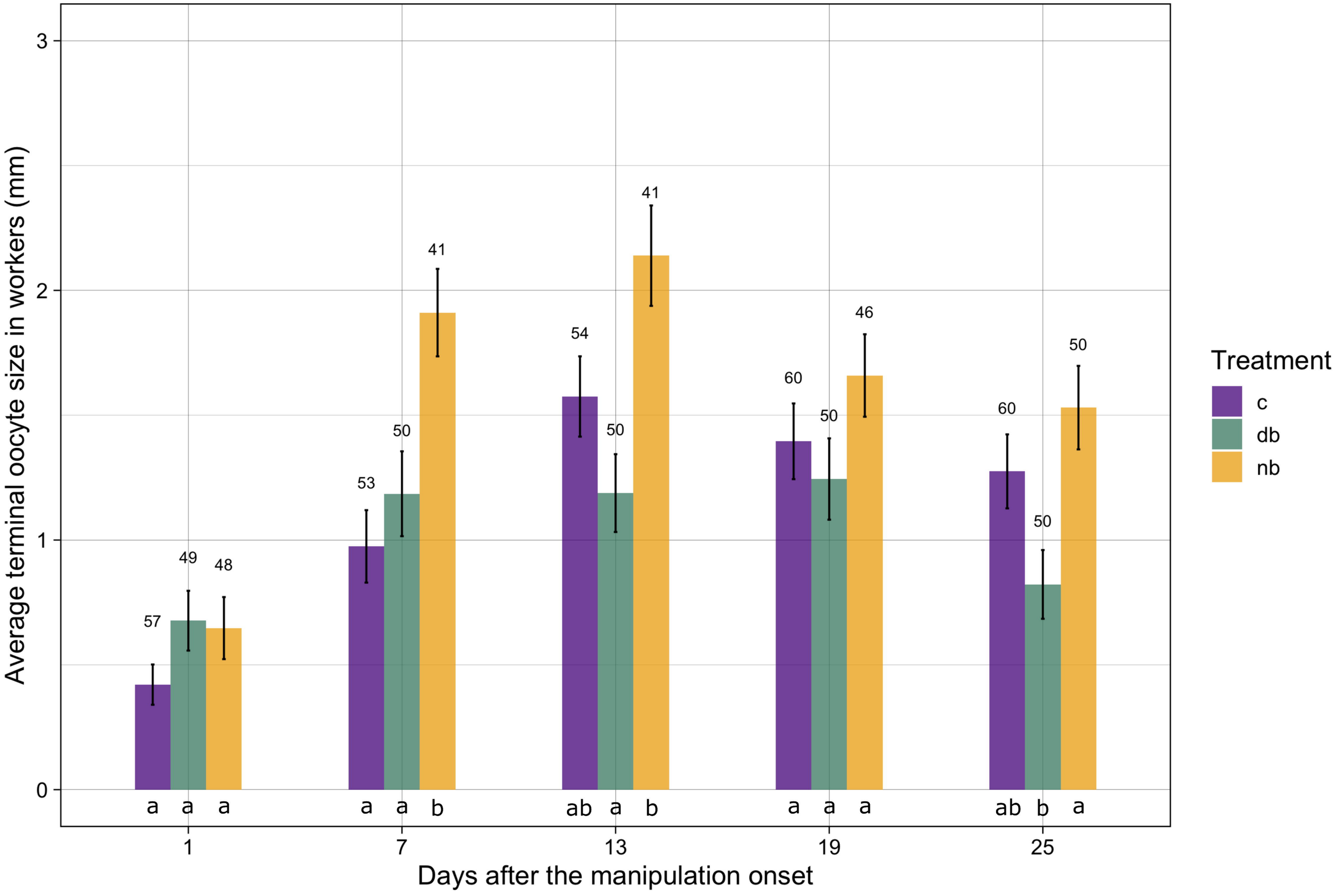
Figure 4: The effect of brood on the total number of eggs, larvae, and pupae on the last day of experiment in the three treatment groups: colonies containing no brood (nb), colonies containing a double amount of brood (db), and control colonies that remain unmanipulated (c). Different letters denote significant differences at $p < 0.05$.

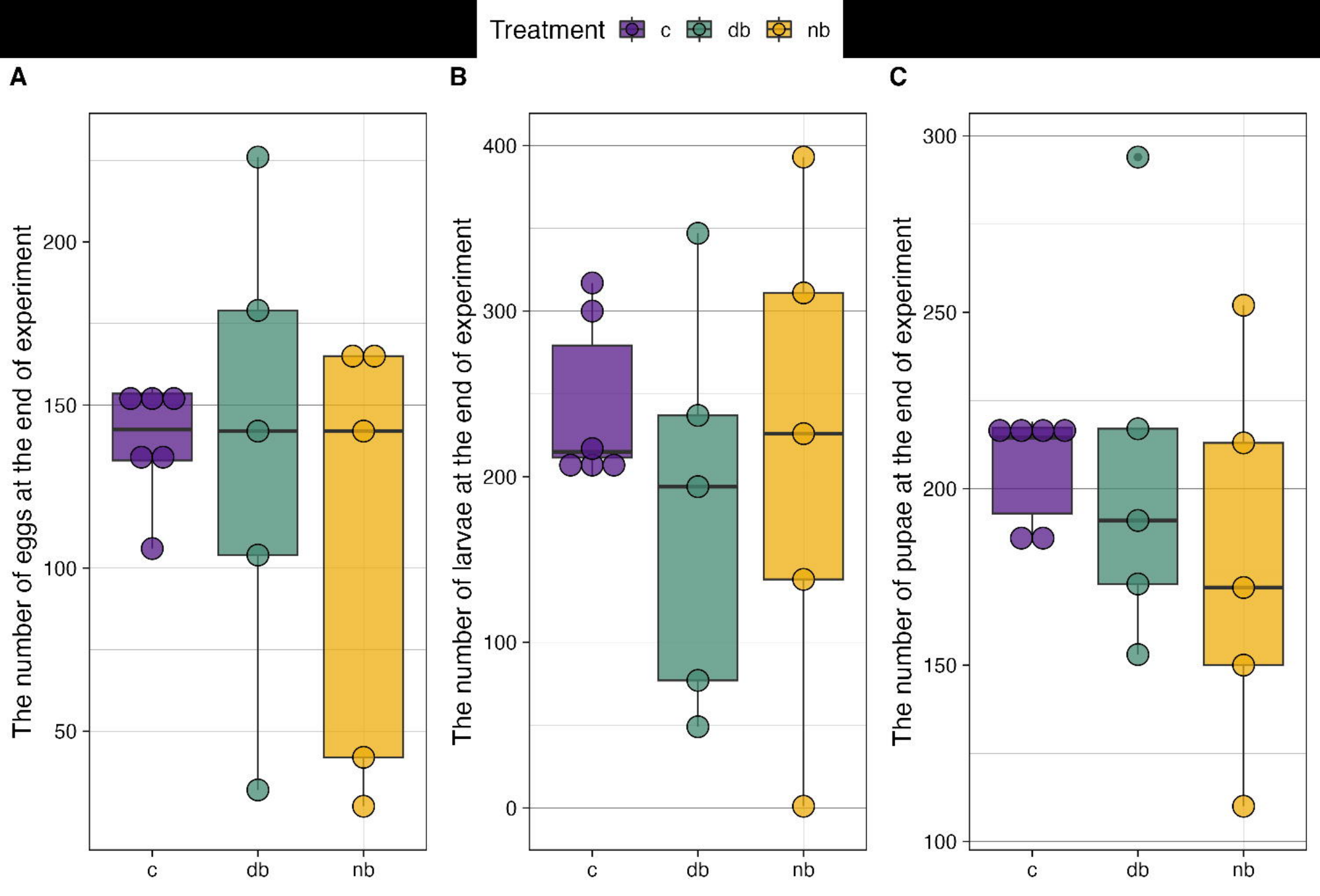
Figure 5: The effect of brood on the total number of workers, gynes and males in the three treatment groups: colonies containing no brood (nb), colonies containing a double amount of brood (db), and control colonies that remain unmanipulated (c). Different letters denote significant differences at $p < 0.05$.

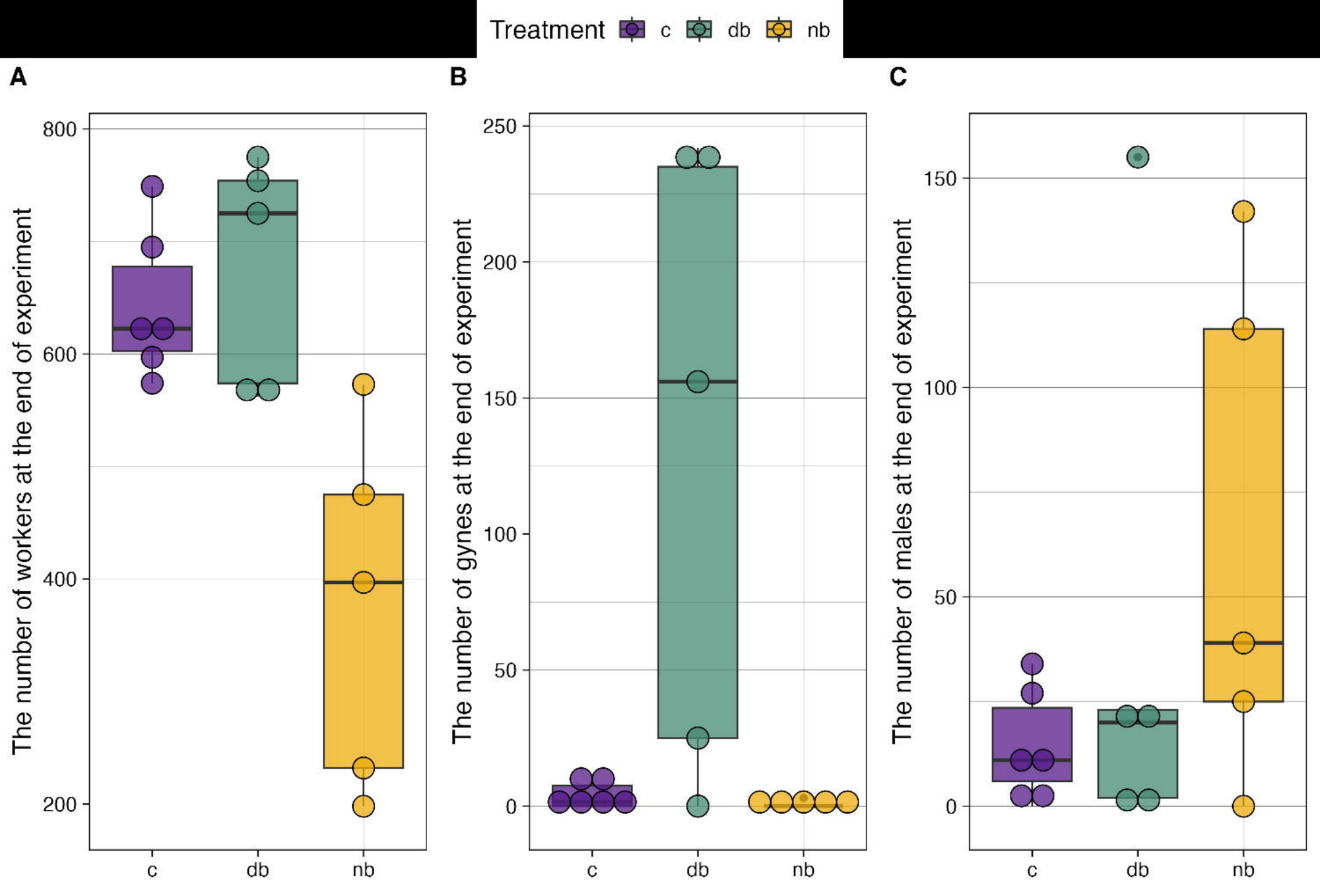
Figure 6: The effect of brood on the distribution of larval weight by the end of the experiment grouped by treatment: colonies containing no brood (nb), colonies containing a double amount of brood (db), and control colonies that remain unmanipulated (c).

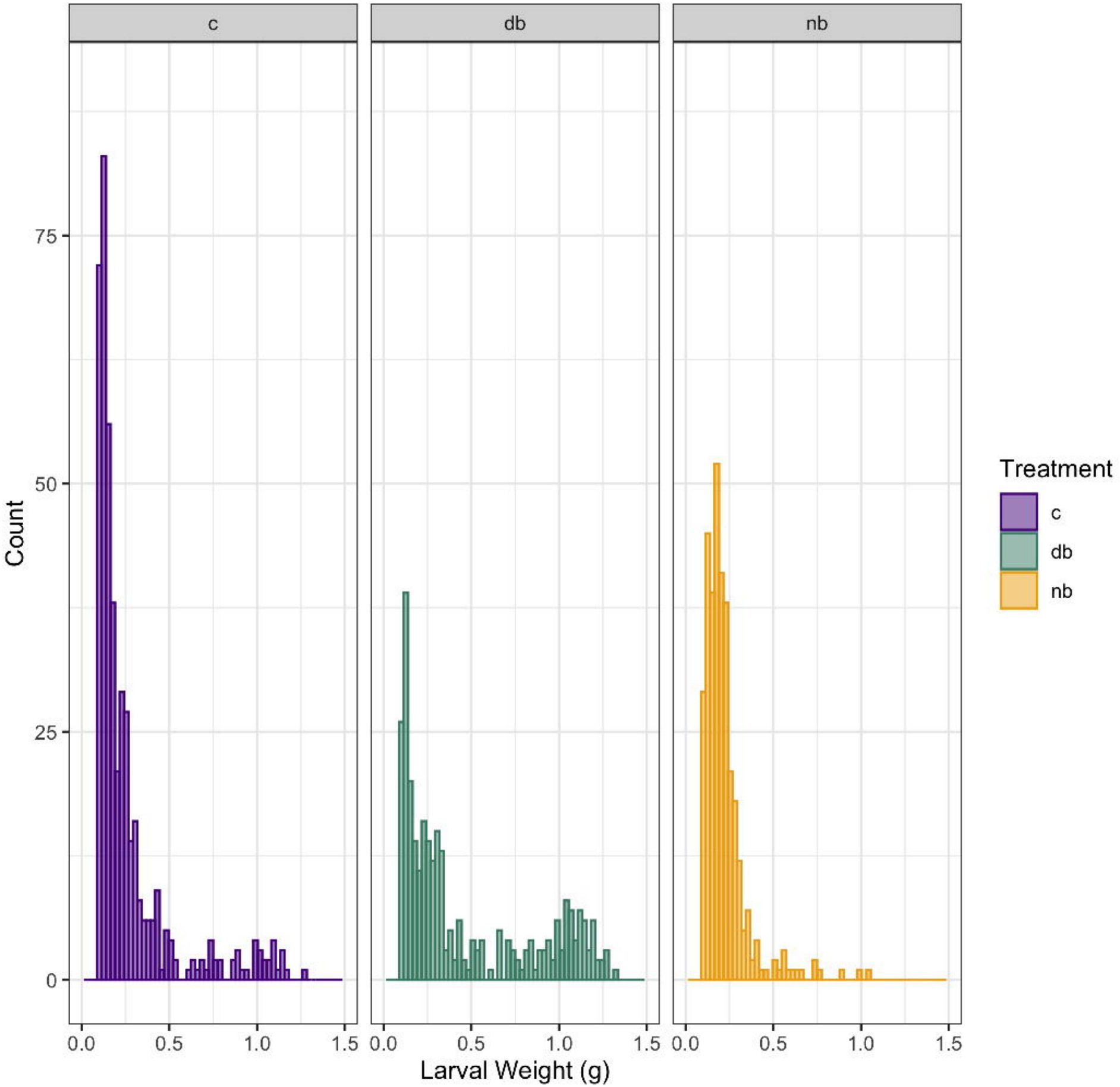

Colony wet mass (g)
1400
1200
1000
800
0
1
2
4
6
8
10
12
14
16
18
20
22
24
26
Days after the manipulation onset
Treatment
c
db
nb


The number of newly-laid egg batches per day
8
6
4
2
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
Days after the manipulation onset
Treatment
c
db
nb


Average terminal oocyte size in workers (mm)
3
2
1
0
57
49
48
53
50
41
54
50
41
60
50
46
60
50
50
a a a
a a b
ab a b
a a a
ab b a
1
7
13
19
25
Days after the manipulation onset
Treatment
c
db
nb


Treatment
c
db
nb
A
B
C
The number of eggs at the end of experiment
The number of larvae at the end of experiment
The number of pupae at the end of experiment
50
100
150
200
0
100
200
300
400
100
150
200
250
300
c
db
nb


Treatment
c
db
nb
A
B
C
The number of workers at the end of experiment
800
600
400
200
c
db
nb
The number of gynes at the end of experiment
250
200
150
100
50
0
c
db
nb
The number of males at the end of experiment
150
100
50
0
c
db
nb


c
db
nb
Count
75
50
25
0
0.0
0.5
1.0
1.5
Larval Weight (g)
Treatment
c
db
nb