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Field evaluation of honeybee colonies (*Apis mellifera L.*) for selecting breeding lines

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ABSTRACT

Honeybees (*Apis mellifera L.*) are pollinators with immeasurable benefits, contributing to the human diet and economic sustainability through the production of hive products. Beekeepers are faced with the challenges of selecting desirable colonies for breeding. It is necessary to evaluate individual honeybee colonies to select breeding lines with high productivity. We bred honeybees in controlled mating stations and selected colonies with similar conditions and placed at the experimental apiary and used for this study. We studied the hygienic behavior of colonies using the pin-killed brood assay and evaluated the production of some hive products (royal jelly (RJ), propolis, and wax) without any adaptation of colonies. The percentage of dead brood removal varied significantly while larval acceptance rate was marginally significant between colonies. The weight of propolis, RJ and wax did not show any significant differences between colonies. RJ production differed between hygienic and non-hygienic bee colonies, with higher values recorded in non-hygienic bee colonies compared to hygienic bee colonies (1.61 ± 0.22 g and 0.78 ± 0.07 g, respectively). Non-hygienic colonies showed better performance in selection for comb-building and the production of RJ. The hygienic condition of colonies did not significantly influence the production of propolis and wax. Thus, it is necessary for beekeepers to evaluate individual colonies for selecting breding lines.

Introduction

Honeybees (*Apis mellifera L.*) are known to be active promoters in the development of the biodiversity of many ecosystems. They provide valuable services in crop pollination (Calderone, 2012) and equally play an essential role as ecological factors by maintaining environmental health (Clement, 2009; Nanetti et al., 2021). The benefits of pollinators are immeasurable, contributing to the human diet (Klein et al., 2007; Goulson et al., 2015) and economic sustainability (Gallai et al., 2009; Rucker et al., 2012). The most populous and commonly known pollinators (honeybees) equally produce natural products derived from pollination services. The honeybee is known to produce honey, royal jelly (RJ), bee wax, propolis, and pollen that are used by man in pharmaceutical industries, cosmetics, food, and income generation (Lowore et al., 2018; Jagdale et al., 2021). Also, the tremendous benefit of *A. mellifera* has promoted bee farming as a conservation positive activity (Russell, 2008).

Irrespective of the numerous importance of honeybees, a number of factors are contributing to the decline in their abundance, distribution (Potts et al., 2010; Tehel et al., 2016), and colony losses (Breeze et al., 2014). For instance, in recent years, increases in the mortality rate of *A. mellifera* colonies in many beekeeping operations around the world (Goulson et al., 2015; Wilfert et al., 2016) may extend to their wild populations (De la Rúa et al., 2009; Thompson et al., 2014) and consequently a decrease in the population of some plant species that could be important to ecosystems (Potts et al., 2016) are attributed to climate change, habitat destruction and degradation, pests, agro-chemicals, and nutrition (DeGrandi-Hoffman et al., 2021). Also, colony collapse and honeybee pests have been attributed to poor colony management which results to varroa infestation (Kütükoğlu et al., 2012).

The challenges of beekeeping have called on the attention of beekeepers, researchers, and other stakeholders to advance knowledge to improve on their status and output of hive products. This can be done by improving beekeeping technology through honey bee breeding to

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mitigate beekeeping challenges and increase the chances of good pollination services. To achieve this, it is important to practice controlled breeding to maintain desirable traits (Plate et al., 2019). The difficulties in selecting and maintaining honeybee colonies with desirable characteristics have pushed some beekeepers to relent their efforts in performing controlled breeding while it could be achieved through multiple testing.

Honeybee colonies may show different behavioral abilities in performing tasks including queen rearing, RJ secretion, comb-building, hygiene, and honey, wax, and propolis production. Performing some of these behavioral patterns is thought to be governed by both instinct and cognition (Gallo and Chittka, 2018). For instance: the ability of bees to learn from past experiences permits them to improve motor skills (Mirwan et al., 2015; Abramson et al., 2016); forecast the outputs of their own actions (Webb, 2004). Studies have documented the link between different aspects of some characteristics of honeybee colonies as a result of bees expressing a particular behavior. For example: the hygienic behavior of honeybee colonies has been used as an indicator to measure the rate of dead brood removal, which is a positive test in controlling brood diseases and varroa (Peng et al., 1987; Boecking and Drescher, 1992; Spivak and Gilliam, 1998; Harris, 2007); Colonies with good ability in wax production and comb-building can store more honey and pollen when conditions are favorable (nectar flow, good brood rearing, presence of a queen, temperatures higher than 15 °C), ease communication network in the colony (Bogdanov, 2016).

Hive products are substantially important to both bees and man. The propolis produced by worker bees play multiple roles: seal holes and gaps in the hive (Hegazi, 1998); protect the colony from microbes, spores producing organisms, and a wide range of pathogens (Simone-Finstrom and Spivak, 2010; Evans and Schwarz, 2011; Wagh, 2013); provide the colony with social immunity by mummifying heavy-dead invaders (Evans and Spivak, 2010; Wagh, 2013). The RJ secreted by worker bees is a nutritive substance used in raising quality queen bees (Pyrzanowska et al., 2014), and by man, for cosmetics and medicine (Kunugi and Ali, 2019; Ahmad et al., 2020). Hive products has become an increasing source of income to beekeepers (Bogdanov, 2011; Clarke and McDonald, 2017; Al-Kahtani et al., 2020). However, their quantity and quality are still being affected by several biotic and abiotic factors (Andrich et al., 1987; Helaly, 2018; Gemeda et al., 2020; Xun et al., 2020). For instance, queen cell acceptance rate is a prerequisite for RJ secretion while colony management may increase the probability of producing more of other hive products. Regarding the value of hive products to both bees and man, it is important to select and breed colonies with high-productive potentials. Therefore, it is the priority of beekeepers to identify and select colonies with multiple desirable characteristics for breeding. This is to maximize the time and money spent in maintaining bred lines with just a single valuable output. Beekeepers intend to breed colonies with the maximum cost-benefit return. However, the complexity in the ecology of honeybees which is highly attributed to their natural environment is a critical problem in many beekeeping operations, in selecting and maintaining breeding lines.

Few studies have evaluated the relationship between some characteristics of honeybee colonies. For example, colonies with higher levels of hygienic behavior could produce more RJ (Khan and Ghramh, 2022) and reduce mite infestation (Rinderer, 1986; Rosenkranz et al., 1997). Beekeepers (especially in developing countries) still find it difficult to ascertain the fact that single multipurpose colonies can be selected. To our knowledge, there is need to evaluate some characteristics of honeybee colonies placed under similar conditions, in selecting breding lines. In this study, we bred *A. mellifera* in isolated mating stations (controlled mating) and conducted field analysis to evaluate some properties of the selected honeybee colonies. We repeatedly examined the hygienic behavior of colonies, the queen cell acceptance rate, the comb-building ability, RJ production, beeswax production, and propolis production to determine the workers' ability to express multiple traits. We also identified hive products that are simultaneously produced in honeybee colonies. This is a baseline study for honeybee breeders and beekeepers to maximize their time by selecting profitable colonies.

Material and methods

This study was conducted in an apiary (35.591° N, 126.278° E) at the honeybee breeding laboratory at the Department of Agricultural Biology, National Institute of Agricultural Science (NIAS), Wanju, Republic of Korea.

Selection of experimental colonies

Honeybee (*Apis mellifera L.*) colonies were bred from April-June 2022 and kept in standard Langstroth hives in the experimental apiary for colony development. Five queenright colonies with similar conditions (number of combs, approximately 70% brood area and 70% worker bee population, fertile queens, good performance, temperature, and relative humidity) were selected and used in this study from July to September 2022. Digital thermosensors (ONSET, HOBO ext temp/RH logger, UX100-023A) were inserted to monitor hive temperature and relative humidity (RH) over 3 days, to ensure that there was consistency among selected colonies. These colonies were sorted and labeled as A, B, C, D, and E. Selected colonies were fed equal amounts of sugar syrup (0.25L) and one piece of pollen patty made of soybean powder, sugar powder, pollen substitute and water at a ratio 1:1:1:1/2.

Evaluating hygiene behavior

The freeze-killed and pin-killed broods are the two assays commonly used to test the hygienic behavior in honeybee colonies (Spivak and Reuters, 1998). The use of which depends on individual objectives and resource availability. We performed a pin-killed brood assay to evaluate the hygienic condition of each colony (Palacio et al., 2000). Sealed brood combs were selected and a section of the brood area (5 cm by 6 cm) containing 100 cells on each side was marked (Fig. 1a) and pin-killed by perforating brood cells (Fig. 1b) (Khan and Ghramh, 2021). Five sections were marked on each side of the brood comb, making a total of 10 sections per brood. Initially, in each marked brood area, the number of uncapped cells was recorded. In each colony, one frame with pin-killed broods was inserted at the center of brood nest. After 24 h, frames were removed and the percentage of uncapped cells and dead brood removal was recorded (Fig. 1c). We considered only uncapped cells with dead brood removal as a positive score for a hygienic behavior. Hygienic colonies were considered with a positive score of at least 50 % dead brood removal within 24 h. This study was repeated three times with seven-day interval between trials. The percentage of dead brood removal was calculated (**Appendix A. S2**).

Queen cell larval acceptance

Two empty built combs of honey bees (*Apis mellifera L.)* were marked and inserted into the selected queenright colony for the queen to deposit eggs. After 24 h, the presence of eggs was checked and recorded as day 1. Within a period of 3 days when eggs are expected to hatch into larvae, the combs were removed to transfer the freshly hatched larvae (≥20 h-old) into artificial queen cell cups (Doolittle, 1915). Queen cell cups were attached to rearing frames and placed in the rearing colonies 2 h before grafting for worker bees to clean the cells. The larvae were transferred into each cup containing one drop (5 ml syringe) of diluted RJ in water at a ratio of 1:1 (v/v) using the Chinese grafting tool. The queen bee in each colony was excluded using a queen excluder. Grafted larvae on rearing frames were placed into each colony and fed equal amounts of sugar syrup (powdered sugar dissolved in water at a ratio of 1:1, v/v). Sixty queen cell cups containing grafted larvae were inserted into each colony and reared for 24 h. This process was replicated three times with ten-day interval between trials. Standard procedures for

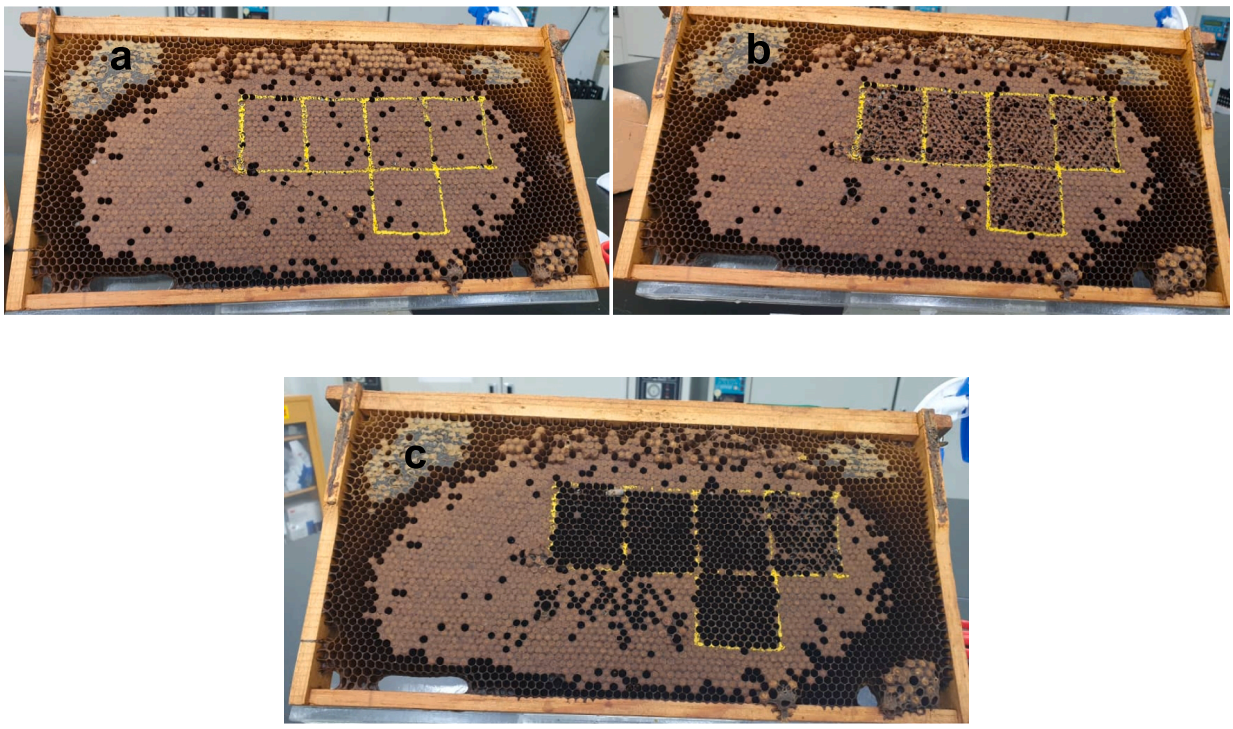

Fig. 1. Testing the hygienic behavior of honeybee colonies; marked brood comb (a), Pin-killed brood area (b), and dead brood removal by worker bees (c).

larval acceptance and RJ production were adopted (Li et al., 2003) with some modifications in the duration of larval rearing (24 h instead of 48–72 h). It is anticipated that colonies that have the ability to produce reasonable amounts of RJ within 24 h, will perform better in 48 h. After 24 h, rearing frames were removed and taken to the laboratory for RJ and wax collection.

Wax and RJ production

The weight of queen cells containing wax, larvae and RJ was recorded (W_1). The wax on the plastic queen cells was removed and the weight of the queen cell containing larva and RJ was recorded (W_2). The larvae in the cells were removed and the weight of queen cell with RJ was recorded (W_3). The RJ was scrapped out using the RJ scrapper and the weight of the empty queen cell cups was recorded (W_4). An electric scale balance (HS220S, HANSUNG Instrument Co., Ltd.) was used for the measurements (**Appendix A. S3**). The weight of wax and RJ was gotten from $W_1 - W_2$ and $W_3 - W_4$ respectively.

Comb building and propolis collection

One empty foundation comb was inserted into each colony just before the feeder, for the workers to perform their duty of comb-building. A small amount of sugar syrup (powdered sugar dissolved in water at a ratio of 1:1, v/v) was applied at the top of each comb as a comb-building stimulant. The combs were removed and checked after three weeks to record the percentage of comb area built. One plastic net for collecting propolis was placed in each colony, at the top of the frames below the cover for three weeks. After three weeks, the propolis nets were removed and placed in plastic bags and frozen at – 4 °C for four days (96 h). The propolis was scrubbed out of the net by flexing above a clean paper sheet in the laboratory. An electric scale balance (HS220S, HANSUNG Instrument Co., Ltd.) was used to weigh propolis from each net (each colony). Both experiments were repeated three times.

Data analysis

Data were characterized using Descriptive Statistics. One-way analysis of variance (ANOVA) was used to compare the means of more than two groups, followed by the Tukey post-hoc test. The non-parametric Kruskal-Wallis test followed by multiple pairwise comparison of variance using Dunn's procedure was used to compare the means of propolis, RJ per cell and RJ per colony. Two-tailed Student's *t*-test was used to compare the means of two groups. Pearson's correlation was used to evaluate the relationship between RJ and the amount of wax on queen cell cups. The results are presented as mean ± standard error of the mean. The XLSTAT statistical software version 2007.8.04 was used to conduct the analysis with levels of significance set at 5%.

Results and discussion

The hygienic condition of colonies and larval acceptance rate

The hygienic condition of colonies and larval acceptance rates were evaluated (Table 1).

The percentage of dead brood removal varied significantly (F_4 =

Table 1
Variation in the hygienic condition of colonies and larval acceptance rate.

Colony	Queen cell Acceptance			Hygienic condition
	Number of larvae grafted	Number of larvae accepted	Larval acceptance rate (%) (mean ± SEM)	Percentage of dead brood removal (Mean ± SEM)
A	180	101	56.11 ± 10.64a	31.67 ± 7.26b
B	180	53	29.44 ± 5.64a	65.00 ± 5.77a
C	180	70	38.89 ± 9.88a	38.33 ± 4.41b
D	180	116	64.45 ± 2.22a	33.33 ± 4.41b
E	180	64	35.56 ± 8.89a	52.33 ± 5.36ab

*Means within columns followed by different letters are significantly different at $P < 0.01$, $\alpha = 0.05$.

6.571, $P = 0.007$) between colonies while larval acceptance rate was marginally significant among groups ($F_4 = 3.330$, $P = 0.056$). Though the rate of larval acceptance was insignificant among groups, colonies D and A showed the highest levels of acceptance (64.45 ± 2.22 % and 56.11 ± 10.64 %, respectively) (Table 1). Colonies B and E had the highest percentage of dead brood removal (hygienic response) (65.00 ± 5.77 % and 52.33 ± 5.36 %, respectively) (Table 1) within 24 h. Criteria for a hygienic score vary with study and time of evaluation. Our values within 24 h are consistent with those of other studies. For instance, the percentage of dead brood removal for hygienic bee colonies at 48 h was more than 95 % (Medina-Flores et al, 2014), 20–80 % (Araneda et al., 2008) and 71.75 % (Vásquez et al., 2016).

Larval acceptance rate was high in less-hygienic bee colonies compared to hygienic bee colonies (Table 1). Colonies B and E with the highest level of hygiene, recorded the lowest rate of larval acceptance (29.44 ± 5.64 % and 35.56 ± 8.89 %, respectively). In other studies, queen cell acceptance rate was higher in hygienic colonies compared to non-hygienic colonies (64.33 ± 2.91 % and 29.67 ± 1.20 %, respectively) (Khan and Ghramh, 2022). These results are inconsistent with our findings. Honey bee colonies with fully hygienic behavior are not common. For example, only 1 out of 30 and 31 colonies in a Canadian and England studies recorded more than 95 % dead brood removal within 24 h (Harpur et al., 2014) and 2 days (Péres-Sato et al., 2009) respectively. Although hygienic colonies showed low larvae acceptance rate, selective breeding of hygienic honeybee stocks is important in fighting against brood diseases (Spivak and Reuter, 2001; Wilson-Rich et al., 2009).

Production of RJ, wax and propolis in honeybee colonies

The weight of propolis and RJ per cell did not differ significantly between colonies ($K = 9.325$, df = 4, $P = 0.053$ and $K = 5.067$, df = 4, $P = 0.281$ respectively). The weight of RJ per colony differed significantly among colonies ($K = 10.567$, df = 4, $P = 0.032$). The weight of wax per cell and wax per colony did not show any significantly among colonies ($F_4 = 0.452$, $P = 0.769$ and $F_4 = 2.032$, $P = 0.166$ respectively). Though most variables did not differ significantly among colonies, some colonies have the potentials of producing hive products more than others. For instance, high propolis producing colonies showed the potentials of producing more RJ per cell compared to other colonies (Table 2). However, the ability of these colonies to build wax on queen cells did not vary significantly. Colonies A and C have the tendency of producing more propolis and RJ compared to B, D, and E (Table 2). The amount of wax built on queen cell cups could be influenced by the age of grafted larvae (Frunze et al., 2022). In this study, we grafted first instar larvae and our results are consistent with those of a similar study which also found no significant differences in wax built on queen cell cups and RJ collection when larvae of the same age were used (Frunze et al., 2022). The ability of colonies producing simultaneously propolis and RJ could be attributed to their foraging capacity and pollen collection. For example, higher RJ producing bees have higher levels of foraging capacity, brood pheromone recognition and pollen collection compared to low RJ producing bees (Han et al., 2017). Foraging honey bees were found to collect raw materials from living plants to make propolis after mixing with wax (Hegazi, 1998; Bankova et al., 2000).

Table 2
Variation in the weights of propolis, RJ per cell, RJ per colony, wax per cell and wax per colony.

Colony	Propolis (g) (Mean ± SEM)	RJ per cell (mg) (Mean ± SEM)	RJ per colony (g) (Mean ± SEM)	wax per cell (mg) (Mean ± SEM)	wax per colony(g) (Mean ± SEM)
A	5.37 ± 1.34a	57.87 ± 7.42a	2.03 ± 0.62b	40.03 ± 2.09a	1.36 ± 0.31a
B	1.50 ± 0.29a	44.97 ± 2.37a	0.78 ± 0.12a	47.64 ± 14.97a	0.89 ± 0.36a
C	2.17 ± 0.44a	62.90 ± 14.91a	1.30 ± 0.12ab	35.29 ± 6.31a	0.76 ± 0.13a
D	1.17 ± 0.17a	39.40 ± 5.1a	1.51 ± 0.14b	49.30 ± 6.71a	1.89 ± 0.19a
E	1.57 ± 0.57a	39.27 ± 6.1a	0.78 ± 0.09a	46.59 ± 8.51a	1.08 ± 0.47a

*Means within columns followed by different letters are significantly different at $P < 0.05$, $\alpha = 0.05$.

Influence of the hygienic condition of colonies on hive products

The percentage of dead brood removal was used to quantify the hygienic behavior of honeybee colonies because every colony has a maximum level of dead brood removal ability. Bee hive products collected from these colonies were evaluated (Fig. 2).

The RJ collected significantly differed between hygienic and non-hygienic honey bee colonies (t = 2.16, P = 0.009). The RJ production was higher in non-hygienic bee colonies compared to hygienic bee colonies (1.61 ± 0.22 g and 0.78 ± 0.07 g respectively) (Fig. 2). The amounts of propolis and wax production did not differ significantly between hygienic and non-hygienic honeybee colonies. However, the mean weight of propolis was higher in non-hygienic honeybee colonies compared to hygienic honeybee colonies (2.9 ± 0.76 g and 1.53 ± 0.29 g, respectively) and that of wax was higher in non-hygienic honeybee colonies compared to hygienic honeybee colonies (1.34 ± 0.20 g and 0.99 ± 0.27 g respectively) (Fig. 2). The amount of wax secreted to seal queen cells was not influenced by the amount of RJ secretion (r = 0.069, P = 0.807) (Fig. 3). Frunze et al. (2022) reported that the amount of wax and RJ in queen cell cups could be affected by age of grafted larvae. In other studies, RJ production was higher in hygienic honeybee colonies compared to non-hygienic honeybee (Khan and Ghramh, 2022). Although these results are not in accordance with our study, it is thought that the hygienic behavior in honeybee colonies is a heritable trait and can be controlled by multiple genes (Bigio et al., 2014; Oxley et al., 2010; Lapidge et al., 2002). However, it is necessary to conduct selective breeding in many generations as this could improve on the genetic output of most populations (Bigio et al., 2014). The reliability on a single colony in performing multiple tasks would depend on the objectives of individual beekeeper. Nevertheless, a baseline guide for selecting and breeding colonies is still needed.

The ability of honeybee colonies to build comb was evaluated among hygienic and non-hygienic colonies. The percentage of comb area built differed significantly between hygienic and non-hygienic colonies (t = 7.633, P < 0.0001). Hygienic colonies showed less ability in comb-building compare to non-hygienic colonies (7.50 ± 3.096 % and 53.33

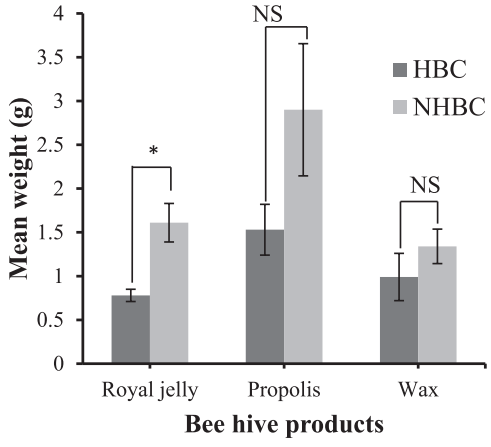

Fig. 2. Mean weight of bee hive products between hygienic (HBC) and non-hygienic (NHBC) bee colonies (Means ± SEM). NS, not significant; *, significant (Student's *t*-test, $p < 0.05$).

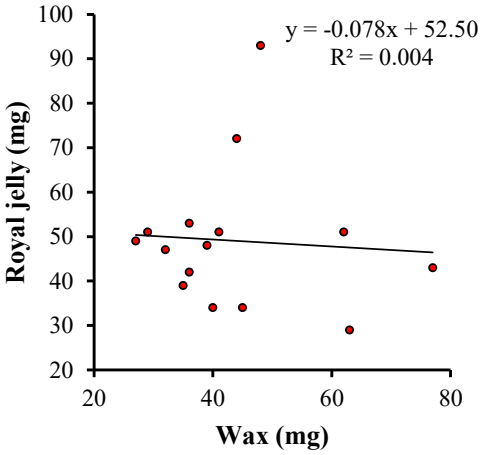

Fig. 3. Correlation between weight of royal jelly per cell and wax cover per cell.

± 4.41 % respectively). Though we recorded a great difference in the percentage of comb area built between hygienic and nnon-hygienic colonies, many factors are associated to comb-building in honeybee colonies which may not rely on their hygienic condition. For instance, comb-building is thought to be instinct as well as cognitive (Gallo and Chittka, 2018). The dependence on environmental factors (temperature, season and nectar flow) for comb-building by honeybees could tilt their attention towards performing other tasks in the hive during off seasons to maximize the energy demand for comb-building. It was reported that variation in the hygienic behavior of colonies at different periods could be attributed to workers performing other tasks (Scannapieco et al., 2016) and changes in season (Boutin et al., 2015).

Conclusions

The results of the present study indicate that the production of RJ and the percentage of dead brood removal vary among honeybee colonies. Queen cell acceptance was significantly higher in non-hygienic honeybee colonies compared to hygienic honeybee colonies. Non-hygienic colonies showed better performance in selection for comb-building and the production of RJ. The hygienic condition of colonies did not significantly influence the production of propolis and wax. However, their values were found to be higher in non-hygienic honeybee colonies than hygienic honeybee colonies. Based on the objectives of individual beekeeper, it is necessary for beekeepers to evaluate the productivity of each colony when selecting breeding lines.

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Authors' contributions

P.N.A, B-S.P and Y-S.C; designed the experiments, P.N.A; conducted the experiments and analyses, D-G.O, D-W.K,Y-Y.J. and K-M.K; validated the resources, P.N.A; wrote the first draft, Y.-S.C and B-S.P; funding acquisition and project administrators, all authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aspen.2023.102101.

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