Original research article

Effects of Species and Larvae Weights on Royal Jelly Secreted by Honey Bees (*Apis mellifera ligustica* and *Apis cerana koreana*) Bred in Korea

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Abstract　Honey bees control the cast determination and colony development through the larvae's nutritional state by the Royal Jelly (RJ). However, the study of RJ dominates for RJ production of *Apis mellifera* honey bees. In any other case, RJ secretion didn't apply as a general selection characteristic to estimate the potential and to breed strong colonies. The purpose of this research was to relate the secretion of RJ against the different species and weights of grafted larvae of honey bees collected after 22 hrs. Larvae of different instars (0–2 days old) were grafted into queen cell cups and inserted into rearing colonies for the RJ collection within 22 hrs. The frames were later removed and taken to the bee breeding laboratory for measurements. It was found that the secreted RJ for feeding 1 mg *A. m. ligustica* larvae significantly differed from *A. c. koreana*: (57.84±23.32 mg) and (31.25±19.72 mg) respectively. Also, the group with the highest weight of larvae *A. m. ligustica* (16.04±5.66 mg) got significantly more RJ (147.94±30.04 mg) which was 2.59 times higher than that of larvae from the youngest group. These findings led us to understand that the weight of grafted larvae and species of honey bees influences the secretion of RJ into queen cell cups. Practically, this data can be used to estimate the RJ secretion by workers of honey bees in a short time to breed healthy strong colonies for different purposes.

Keywords　Larval weight, Species, Royal Jelly, Honey bee, *Apis mellifera ligustica*, *Apis cerana koreana*

INTRODUCTION

Royal Jelly (RJ) is secreted by the hypopharyngeal and mandibular glands of worker honey bees to feed the larvae. Scientists have researched the outstanding physiological activity of RJ and its components which contain protein, acids, vitamins, lipids, and sugar (Fratini *et al*., 2016; Maleszka, 2018; Ahmad *et al*., 2020). Due to its numerous nutritional and medicinal content (antibiotic, anti-inflammatory, antioxidant, anti-tumor, and immune-modulatory activities), coupled with the fact that RJ is fed upon by young larvae and queen bees,

some researchers have attributed the long lifespan of queen bees to RJ consumption (Maleszka, 2018). Therefore, honey bees control colony development and the cast determination of worker and queen bees through the larvae nutritional state by the nurses during the ontogenetic development (Weaver, 1957; Page *et al*., 1995; De Sousa *et al*., 2018). It was found that the start of the genetic health program of larvae takes place from the first to the third day of larvae development, after which larvae of queen bees feed on RJ, larvae of worker bees feed on mix of RJ, honey, and pollen. However, Wang *et al*.(2015) reported the weight of larvae in queen cells

above first instars was more than larvae in worker cells of *Apis mellifera ligustica* despite the same feeding condition. Regarding the fact that queen bees emerge on the 16th day while worker bees emerge on the 21st day, the mean weight of queen bees is 57.3% more than that of the worker bees (Wang *et al.*, 2015). This reveals the outstanding values of RJ to the metabolic pathways in its natural state.

To collect RJ for humans' purposes, subspecies of *A. mellifera* honey bees were selected and bred in different countries for high-level of RJ secretion (Robinson, 2011; Cao *et al.*, 2016; Gemeda *et al.*, 2020). It was found that RJ yields were significantly more with *A. m. ligustica* than *A. m. carnica* (238.46 ± 1.96 mg/cap), and (192.33 ± 1.06 mg/cap) respectively (Khan *et al.*, 2021). Moreover, the feeding of larvae depends on the subspecies, colony type (queen-less or queen-right) (Van Toor and Little-john, 1994), age of grafted larvae (Sahinler and Kaftanoglu, 1997), and harvesting interval (El-Din, 2010). Irrespective of the species of honey bees, the amount of the RJ secreted by worker bees and their number influences the honey bee colony development (Szabo and Lefkovitch, 1988) because the number of simultaneously fed larvae are restricted. Hence, RJ secretion should be studied in other honey bee species including *A. cerana*.

The purpose of our study was to relate the secretion of RJ collected after 22 hrs against the different species of honey bees (*A. m. ligustica*, and *A. c. koreana*) and different weights of grafted larvae of honey bees. Practically, this data can be used to decrease the time of the RJ collection to estimate the RJ yield in a short time. In the future, the selection of high RJ secretion colonies is the way to breed healthy strong colonies for different purposes.

MATERIALS AND METHODS

1. Study site

This study was carried out in the Bee breeding Laboratory, Department of Agricultural Biology, National Institute of Agricultural Science (NIAS), the Republic of Korea.

2. Colony selection, larvae choice

Fourteen strong queenright of *A. m. ligustica* and *A. c.* *koreana* honey bee colonies were studied from March to June 2021. These colonies were used for breeding and neither were adapted for special RJ production. In the apiary, three and four colonies were used for the selection and rearing of larvae respectively. Two queen bees were excluded for two consecutive days on the built combs without eggs for the queen to deposit eggs within 24 hours. Four days later, the larvae at the age of 0−2 days old were checked in the frames.

3. Grafting of larvae, collection of data

The study was done according to the standard methods for *A. mellifera* L. RJ research (Hu *et al.*, 2017) and the adapted method for *A. c. koreana* (Vung *et al.*, 2017). The larvae of different instars (0−2 days old) had been grafted into queen cell cups for the RJ collection and were put into the rearing colonies. Frames with grafted larvae were removed from rearing colonies after 22 hours and taken to the bee breeding laboratory for RJ collection and measurements. An electric scale balance was set to collect measurements in milligrams. The plastic queen cell cup, wax, larvae, and RJ were weighed consistently. The wax covering of the cells was studied because it keeps the environment for larval development. The data on larvae weight were grouped into six groups according to their weight which has been documented to be positively correlated with age till 28 days of age (Sahinler and Kaftanoglu, 1997; Wang *et al.*, 2015; Rinkevich *et al.*, 2016). The first number of groups 1 (1.1, 1.2), 2 (2.1, 2.2), 3 (3.1, 3.2) signifies the age of 1, 2, and 3 instars, respectively. The second number of groups 1 (1.1, 2.1, 3.1), and 2 (1.2, 2.2, 3.2) means the increased larvae weight inside the instar's development.

4. Data analysis

All data were analyzed by the Principal Component Analysis (PCA) to visualize the grouped data, by Descriptive Statistics to characterize the groups, by the Regression analysis with Pearson correlation to estimate the relations between the data, by One-way ANOVA followed by Tukey's posthoc test for comparing three and more groups, and paired samples t-test to compare two groups, where the significant levels were set at $\alpha = 0.05$. The SAS Enterprise Guide 7.1 statistical software (ver.25), MS Excel with the XLSTAT, AtteStat

application, program R were used for statistical calculations and visualization of data.

RESULTS AND DISCUSSION

1. Effect of species (*Apis mellifera ligustica* and *Apis cerana koreana*) on the royal jelly collection for 1 mg larvae

The study of the RJ secretion was carried out on two species of honey bees *A. m. ligustica* and *A. c. koreana* which were kept in the same location and used for breeding but neither were adapted for special RJ production. A paired samples t-test was performed to compare the weights of Royal Jelly (RJ) and wax covering queen cells for 1 mg larvae for both honey bees. As expected, there was a significant difference in RJ secretion between *A. m. ligustica* (m = 57.84 mg, SD = 23.32, n = 69) and *A. c. koreana* (m = 31.25 mg, SD = 19.72, n = 8); t = 3.467, df = 75, p = 0.001. Also, a significant difference was found in wax covering between *A. m. ligustica* (m = 38.30 mg, SD = 12.77, n = 69) and *A. c. koreana* (m = 22.50 mg, SD = 3.34, n = 8); t = 3.094, df = 75, p = 0.003. The amount of RJ which *A. m. ligustica* worker bees secrete to feed 1 mg larvae during 22 h, was 1.85 times more than the amount of RJ for the same 1 mg larvae of *A. c. koreana* worker bees. The species features were fixed like the more intensive feeding of *A. m. ligustica* larvae in queen cells, which queen development takes 16 days (Wang *et al*., 2015), than *A. c. koreana* larvae in queen cells provided by less feeding whose development is 15 days (Vung *et al*., 2020).

These findings confirm that the feeding and secretion of RJ by worker honey bees are influenced by the species of the honey bee. Moreover, the RJ collection trait can be recommended for the selection of honey bees for breeding strong colonies for various purposes.

2. Effect of larva weight on royal jelly, and wax covering queen cells collection of *Apis mellifera ligustica* honey bees

Data were collected after grafting larvae of different weights and rearing into the colonies of *A. m. ligustica* honey bees after 22 hours. The activity of honey bees to feed larvae depending on their weight was studied. Raw data were grouped by weight according to the body weight of European honey bee *A. mellifera* queens and workers as 1, 2, and 3 instars (Wang *et al*., 2015), each of them was grouped in relation to instar development as 1.1 and 1.2, 2.1 and 2.2, 3.1 and 3.2 by the increased larvae weight. The means and standard deviations of the groups are shown on Table 1.

The visualization of grouped data was done by Data Mining Plots which most separated the larvae 3.2 groups from other data by the principal component 1 (PC1) (66%) than by the PC2 (26%) (Fig. 1A).

The grouping of the larvae by weight from the same instar showed that the weight of RJ in cells with larvae didn't maintain by worker bees at the same amount. The weights of RJ (1.1 and 3.2) had significant differences from all other data (Table 2). The low amount of RJ food in group 1.1 could be attributed to larvae weight because older larvae in the same colony got more RJ 2.59 times. Group number 3.2 with the highest weight

Table 1. Mean and standard deviation of the larvae weights *Apis mellifera ligustica*, Royal Jelly (RJ), and wax covering per queen cell

Group	1.1		1.2		2.1		2.2		3.1		3.2	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
m (larvae), mg	1.73	0.89	2.07	0.81	5.26	1.37	6.00	1.47	10.80*	0.83	16.04*	5.66
m (RJ), mg	57.12**	17.02	102.52	15.37	68.01	12.64	105.97	17.67	89.45	7.95	147.94**	30.04
m (wax), mg	39.01	11.77	41.52	9.65	41.32	12.32	43.22	8.37	51.90	13.06	50.67	9.15
n	173		61		68		65		20		81	

Asterisk indicate general significant differences in comparing with all others data by *one-way ANOVA (F = 389.44, df = 5, P < 0.0001), Tukey post hoc test (α = 0.05); **one-way ANOVA (F = 284.04, df = 5, P < 0.0001), Tukey post hoc test (α = 0.05). The first number of groups 1 (1.1, 1.2), 2 (2.1, 2.2), 3 (3.1, 3.2) signifies the age of 1, 2, and 3 instars, respectively. The second number of groups 1 (1.1, 2.1, 3.1), and 2 (1.2, 2.2, 3.2) means the increased larvae weight inside the instar's development.
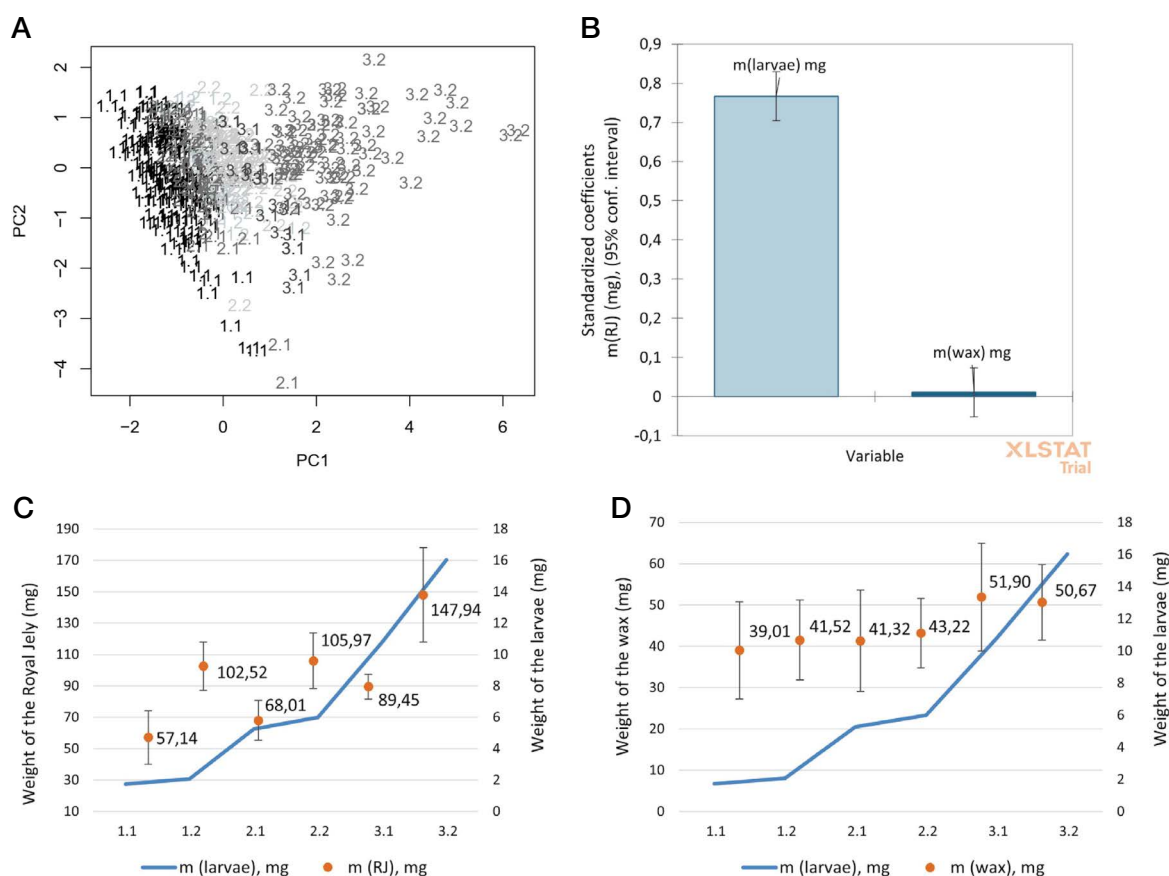
Fig. 1. Analysis of grouped data by the larvae weight of *Apis mellifera ligustica* honey bees (1.1–3.2); A - PCA analysis; B - Regression analysis of the relation of the weights of larvae, wax, and RJ. The estimation of the predictive power of two predictors showed the different scales of measurement (m (larvae) and m (wax)) in the RJ research; C, D - Effect of larva size on RJ secretion and wax covering within 22 h, respectively.

of larvae (16.04 ± 5.66 mg) had significantly higher RJ food of (147.94 ± 30.04 mg) than larvae from other groups within 22 hours by one-way ANOVA (F = 389.44, df = 5, P < 0.0001), Tukey post hoc test ($\alpha = 0.05$) (Table 1, Fig. 1C).

In a similar scenario as for RJ, wax from groups 1.1, 1.2, 2.1, and 2.2 were significantly different from groups 3.1 and 3.2 by one-way ANOVA (F = 16.09, df = 5, P < 0.0001), Tukey post hoc test ($\alpha = 0.05$) (Fig. 1D). More wax around queen cell cups (51.90 ± 13.06 mg), and (50.61 ± 9.15 mg) were collected with older larvae of group 3.1 (10.8 ± 0.83 mg), group 3.2 (16.04 ± 5.66 mg) respectively after 22 hours. Obviously, the nurse honey bees distinguished the weight of larvae, which were not only fed a suitable diet but also covered the older larvae (3.1 and 3.2) with significantly more wax cover. However, a strong positive correlation between the weight of larvae and RJ was observed (r = 0.771),

Table 2. Correlation matrix (Pearson), P < 0.05

Variables	m (larvae), mg	m (wax), mg	m (RJ), mg
m (larvae), mg	1	0.358	0.771
m (wax), mg		1	0.285

but low with the weight of wax (Table 2).

The relation of the weights of larvae, and RJ were confirmed by regression analysis. The weights of larvae and RJ had higher standardized coefficients near 1, which showed the dependence of the weight of RJ food on the weight of larvae more than the weight of the wax cover (Fig. 1B). These results discern our research from other studies on RJ, which have identified factors affecting the production and queen rearing in *Apis mellifera* honey bees including the weight of transferred larvae (Sahinler and Kaftanoglu, 1997; Jianke, 2000; Gemeda

et al., 2020).

Weights of grafted larvae had a significantly positive impact on the amount of RJ secreted by nurse bees which is most important when estimating the ability of honey bee colonies to healthy and quickly development. However, two instar larvae cannot be used for queen rearing, because newly emerged queens raised from younger larvae had higher characteristics than queens raised from older larvae (De Souza *et al.*, 2018).

CONCLUSION

The relation of the secretion of RJ against the different species of honey bees and different weights of grafted larvae of honey bees collected after 22 hrs was the purpose of our study. We refined that the weight of honey bee larvae $(16.04 \pm 5.66$ mg) got significantly more RJ $(147.94 \pm 30.04$ mg). However, RJ secretion by nurse bees differs among species (*A. cerana* and *A. mellifera*). The amount of RJ secreted into queen cells containing 1 mg *A. m. ligustica* larvae was significantly different from those of *A. c. koreana*: $(57.84 \pm 23.32$ mg) and $(31.25 \pm 19.72$ mg) respectively. Practically, our study would help breeders to select the larvae of honey bees for estimation of RJ secretion by honey bee colonies within 22 hrs and assess the potential development rate of honey bee colonies in the foraging season. In the future, the selection of high RJ secretion honey bee colonies is the way to breed healthy strong colonies for different purposes.

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CONTRIBUTION OF AUTHORS

Conceptualization, O.F. and P.N.A.; methodology, O.F. and P.N.A.; software, O.F.; validation, P.N.A., Y.-S.C.; formal analysis, D.K., E.-J.K.; investigation, resources, B.-S.P., D.K.; data curation, B.-S.P.; writing-original draft preparation, O.F., P.N.A.; writing-review and editing, P.N.A.; visualization, B.-S.P.; supervision, E.-J.K., D.K., Y.-S.C.; project administration, funding acquisition, Y.-S.C.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the correspondent author. The data are not publicly available due to privacy or ethical restrictions.

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