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RESEARCH ARTICLE

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Effects of human sex hormones and gonadotropins on the development of worker bee larvae (*Apis mellifera* L.)

Abstract Worker bee larvae of *Apis mellifera* L. were fed artificially in an incubator under controlled temperature and relative humidity conditions. Our results showed that (35.5±0.1)°C with a relative humidity of (85±5)% was suitable for the growth of worker bee larvae. A gradually increasing content of pollen and honey, accompanied by decreasing the content of royal jelly in diet, met their fundamental needs. With environmental and nutritional needs basically met, larvae got a similar growth rate to that under natural conditions. Four kinds of human hormones, i.e., estradiol, testosterone, follicular stimulating hormone, and luteinizing hormone, were added into the daily diets of worker bee larvae at different concentrations. Their growth curves were perfectly fitted by binomial fitting complex models. Effects of a single hormone and the combinations of different hormones with a follicular stimulating hormone on the development of worker bee larvae were evaluated. Our results indicated that the development of worker bee larvae at early stage was affected greatly by exogenous hormones. Larvae of 65–89 h old were particularly sensitive to the effects of sexual and gonadotropic hormones, and interactions between different hormones were detected.

Keywords worker bee larvae, sex hormone, gonadotropins

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1 Introduction

Honeybees experience four distinct life stages: the egg, larva, pupa, and adult. Zhou et al. (2008) studied the optimal feeding condition for queen bee larvae (*Apis mellifera* L.) and demonstrated that larvae younger than 3 days could be fed artificially with the compositions of larval food carefully controlled. In our previous study (Zhou and Zhang, 2002), we found out that human estradiol benzoate (E_2) and follicular stimulating hormone (FSH) added into the diet of larvae reared by workers in the colony could increase ovariole numbers in virgin queen bees, and FSH could increase new adults' weights as well. We have already known that sex hormones and gonadotropins are closely related to the development of gonadal organs in mammals. As to the effects of gonadal hormones on the development of honeybee at different stages and the possible existence of hormone analogs in honeybee, little is known. In this study, we explored the effects of different exogenous hormones on the development of larvae under the optimum artificial condition. Human gonadal hormones, E_2, FSH, propionate testosterone (T), and luteinizing hormone (LH) were added into the daily diet of worker bee larvae, which were fed artificially in an incubator. The growth-promoting effects of different hormones were evaluated by larval weights recorded on each experimental day.

2 Materials and methods

2.1 Preparation of worker bee larvae

Worker bee larvae of *Apis mellifer* L. were obtained from the apiary in the College of Animal Science and Technology, Yangzhou University, China. An empty honeycomb was put into a healthy colony. 4–6 h after the queen bee laid eggs, we took out the comb and put it into an upper honey drawer. Eggs were hatched there and developed into worker bee larvae after 3 days. When larvae

were 65 h old, we transferred them from worker cells to aseptic culture dishes with the help of a plastic grafting tool. Artificial diets of about 0.5 cm high were spread evenly at the bottom of culture dishes. Larvae were incubated at (35.5±0.1)°C with a relative humidity of (85±5)% (incubator HSX-160B, Shanghai Fuma Laboratory Equipment Co., Ltd.). Worker bee larvae from the same battery of eggs that remained in the work cells and developed in the colony were set as a control group.

2.2 Obtain the weight of larvae and statistical analysis

The weight of each larva in the incubator was recorded daily. Each larva was rinsed quickly by drops of sterile distilled water prewarmed to 35°C to remove the adhesive diet on the body and was put on aseptic filter papers to absorb the remaining water. After being weighed, larvae were transferred to a new aseptic culture dish with fresh diet spread on it. As for the larvae that developed in the colony, when they grew older, no residue of food was left in the cells where we could not pick them out without hurting them. They were weighed only twice throughout our experiment: one at the beginning of the experiment (65 h old) and the other when they were observed to be newly capped by workers. Statistical analyses were performed using the ANOVA procedure of SAS (SAS Inst., Inc., Cary, NC). When a statistical significance was detected ($P < 0.05$), comparisons between means were carried out using Duncan's multiple-range test.

2.3 Compositions of artificial diets

Fresh royal jelly was obtained daily from the same colony, where worker bee larvae in the control group developed. Camellia pollen was collected from one healthy colony in the apiary and lyophilized. Mature honey from black locust (Yangzhou Tongren Bio-engineering Co., Ltd.) contained 20% water. Compositions of daily diets are shown in Table 1. Different ingredients were mixed thoroughly with a superfine homogenizer (F6/10, FLUKO, Germany). Diets were prewarmed to 35°C in an incubator before use.

Table 1 Compositions of diets for larvae at different stages (W/W)

larval age/h	royal jelly/%	pollen/%	honey/%	water/%
65–89	100	—	—	—
89–113	60	15	10	15
113–137	40	20	15	25
137–161	20	30	20	30

2.4 Addition of hormones into the diets

Different human hormones (Factory of Hormone Products, Cixi, Zhejiang Province, China) were added into the daily diets. Tested groups were designated as shown in Table 2. The sample number in each group is 30.

3 Results

3.1 Effects of a single hormone on the development of worker bee larvae

At two specific stages we selected, larvae in group A, and the control group had no significant difference in weight. Average larval weights were compared between different tested groups with a single hormone added in the diets and group A, as shown in Figs. 1–4.

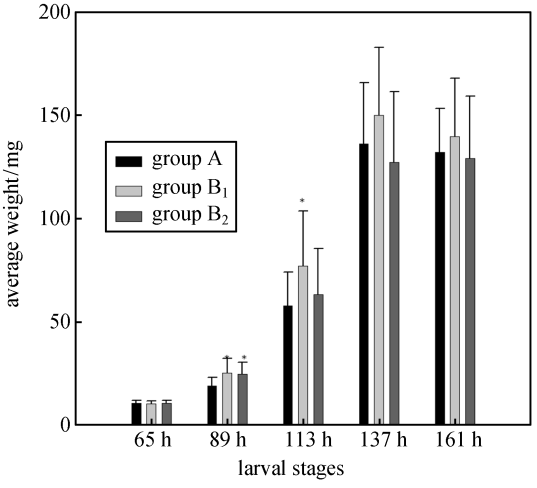

Fig. 1 Comparison of larval weights between group A and groups with E_2 in the diets
Note: Columns with * on the top had a significant difference at $P < 0.05$.

Worker bee larvae of 65 h old that we picked out from worker cells and distributed randomly in different tested groups had no significant difference in weight. 24 h later, larvae in tested groups with E_2, FSH, LH, and T added into the diets had significantly higher weights than those in group A. We concluded that larvae were extremely sensitive to the effects of hormones at this early larval stage. Exogenous hormones could improve the growth rate greatly, and this effect lasted till the larval stage of 113 h old. The difference in weight between

Table 2 Tested groups with different hormones added in the diets

group	none	E_2/(ng·g^{-1} diet)		T/(ng·g^{-1} diet)		FSH/(mIU·g^{-1} diet)		LH/(mIU·g^{-1} diet)	
	A	B_1	B_2	C_1	C_2	D_1	D_2	F_1	F_2
hormone content	—	0.06	0.30	0.125	0.625	1.2	6.0	0.18	0.90

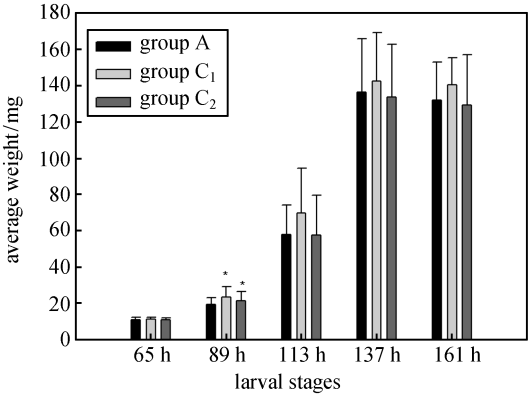

Fig. 2 Comparison of larval weights between group A and groups with T in the diets
Note: Columns with * on the top had a significant difference at $P < 0.05$.

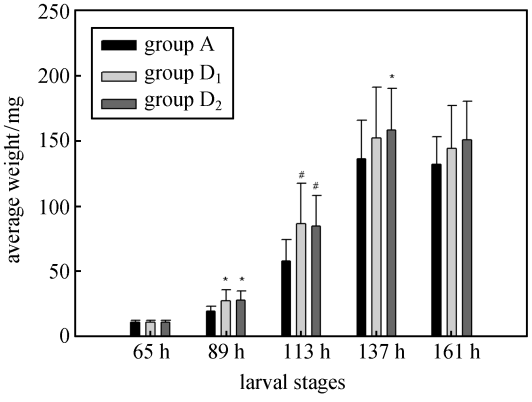

Fig. 3 Comparison of larval weights between group A and groups with FSH in the diets
Note: Columns with * on the top had a significant difference at $P < 0.05$, and columns with # on the top had a significant difference at $P < 0.01$.

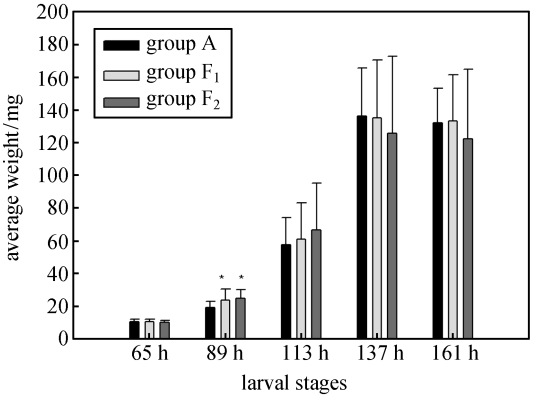

Fig. 4 Comparison of larval weights between group A and groups with LH in the diets
Note: Columns with * on the top had a significant difference at $P < 0.05$.

groups D_1 and D_2 with FSH in the diet and group A reached a significant level at $P < 0.01$, while the difference between group B_1 and group A reached a significant level at $P < 0.05$. A relatively higher concentration of E_2 in diets in group B_2 did not result in a higher growth rate proportionally.

At the larval stage of 137 h old, the positive effects of hormones on the growth of larvae could not be detected any further except larvae in group D_2, which had significantly higher weight than those in group A ($P < 0.05$). Since the larvae in group D_2 always had significantly higher weight during the whole larval stages, we suggested that FSH had a continuing effect on larval growth, and this effect could only be detected at a relatively higher concentration.

When larvae grew to be 161 h old, we detected a loss of body weight in all tested groups, which indicated the coming of the pupal stage. In the incubator, the worker bee larvae had a whole larval period of about 137–161 h (5.7–6.7 d). Collection of weight data at more specific stages within this period would enable us to determine more precisely the exact time that larvae stepped into the pupal stage.

3.2 Binomial fitting of larval growth

The developmental pattern of larvae was fitted by a nonlinear fitting method. The corresponding equations of binomial fitting complex models derived from each group were listed in Table 3. Larvae in different groups had similar developmental curves. Equations representing the larvae in group A and group F_1 were almost coincident. Larvae feeding on diets with hormones added had an average higher weight than the larvae in group A, which suggested that these exogenous hormones could promote the larval growth throughout the whole larval stage.

Table 3 Binomial equations for larval development in different groups

group	equation	R^2
A	$Y = 17.505x^2 - 45.967x + 39.535$	0.9995
B_1	$Y = 14.513x^2 - 25.461x + 20.503$	0.9989
C_1	$Y = 15.152x^2 - 31.702x + 27.088$	0.9997
D_1	$Y = 12.348x^2 - 13.296x + 9.7575$	0.9944
F_1	$Y = 15.235x^2 - 35.083x + 31.085$	0.9991

3.3 Effects of FSH combined with different hormones on the development of larvae

Three tested groups designated as group $D_2 + F_2$, group $D_2 + C_1$, and group $D_2 + B_1$ with FSH combined with different hormones added into the diets were used to evaluate the effects of FSH in the presence of different hormones on the development of worker bee larvae (Fig. 5).

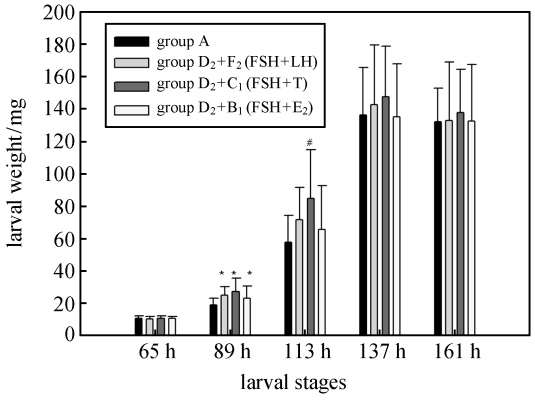

Fig. 5 Comparison of larval weights between group A and groups with FSH and other hormones added in the diets
Note: Columns with * on the top had a significant difference at $P < 0.05$, and columns with # on the top had a significant difference at $P < 0.01$.

At the beginning of our experiment, there was no significant difference in the weight of larvae distributed in different groups. Statistical analysis showed that at the larval stage of 89 h old, larvae in group A had significantly lower weight than those in any other groups with FSH added into the diets. This may be explained by the sensitivity of larvae at this stage to the effects of hormones, which was consistent with our above results.

When larvae grew to be 113 h old, significant difference in weight could be detected only between the larvae in group A and group D_2+C_1 ($P < 0.01$). Since the larval weight in group C_1 at this specific stage did not show any significant difference with the larvae in group A, we presumed this difference in weight was mainly contributed by FSH. We also noticed that FSH (group D_2) and E_2 (group B_1) could improve the larval growth individually and increase the larval weight significantly. However, the combination of FSH and E_2, as in group D_2+B_1, did not result in a significant increase in larval weight. Similar results were found in group D_2+F_2. The function of FSH in increasing the larval weight was offset by the addition of LH into the diets.

At the larval stage of 137 h old, no significant difference in larval weight could be detected between tested groups and group A. Since the positive effect of FSH on the larval growth could be detected till the near end of the larval stage (137 h old) when it was added singly, we concluded that FSH and other hormones did not appear to act in an additive manner.

We also compared the larval weights at the 89-h-old stage in two experiments. Results showed that the larvae in group D_2+C_1 had a significantly heavier weight than the larvae in group C_1, and the larvae in group D_2 heavier than those in group D_2+B_1, which confirmed a stronger function of T combined with FSH than that of T alone, reducing the effects of FSH when combined with E_2.

4 Discussion

Nutrition, temperature, and humidity are three important factors that play critical roles in the development of larvae. In our experiment, we fed worker bee larvae with a mixture of royal jelly, pollen, and honey. The optimal temperature and the relative humidity in the incubator were set according to our previous report (Zhou et al., 2008). We reduced the amount of royal jelly and increased the content of pollen and honey gradually to obtain a normal growth rate and increase the survival rate of larvae. Statistical analysis did not reveal any significant difference in the weight of larvae from control and group A at selected stages examined, from which we inferred that the developmental requirements of larvae for both environment and nutrition were basically met. Compared with the referential data for the corresponding weight of worker bees (*Apis mellifera ligustica*) developed under natural conditions (Yu and Meng, 2001), the larvae in group A had an average lighter weight, although the difference did not reach the statistically significant level. Two factors may give rise to the decreased larval weight in group A. One was the fixed temperature and humidity during the whole larval period in the incubator. As we know, workers have the capacity of modulating the temperature and humidity in the colony and making them fluctuated within a range, which may be important for the development of larvae and pupae (Jones et al., 2005). The accumulated temperature can be met by artificial control, but the subtle varying pattern is difficult to simulate exactly. Secondly, the artificial diets we prepared may meet the basic nutritional needs of larvae; however, the epimeletic behavior of worker bees in the colony could not be simulated. Worker bees check the status of larvae frequently and respond accordingly, which can never be realized when larvae are artificially fed. The detailed environmental needs of larvae still need further investigation.

When worker cells were capped by workers in the colony, it was a direct sign indicating that larvae had stepped into the pupal stage. While the larvae were developed in the incubator, we noticed that the larval weight at the stage of 161 h old was lighter than the corresponding weight at the stage of 137 h old in every tested group. It could only be explained by the reason that the larvae had stopped eating. Loss of weight could be taken as an indicator of the near ending of the larval stage under artificial conditions. When worker bee larvae were developed in the incubator, the whole period of the larval stage lasted for about 6 d, which was the characteristic of worker bee larvae under natural conditions. Compared with queen bee larvae, reaching approximately 250 mg under natural conditions with the larval stage lasting for about 5 d (Chen and Li, 2008), the developmental process of larvae in group A was the closest to that of worker bee larvae instead of queen bee larvae. Up till now, little

research was conducted about the development of honeybee larvae under artificial conditions (Zhou et al., 2003, 2008; Cao et al., 2008). Growth temperature and development rate were closely related. The artificial condition we adopted in our experiment was optimized by a series of experimental results (data not shown). In the whole experiment, we paid much attention to aseptic manipulation as far as possible, and the survival rate of larvae artificially incubated reached 97.6%, which was comparable to that under the natural condition (Xian et al., 2007a, 2007b). Binomial fitting models could be used to describe larval growth, and analogous results were obtained by Zheng et al. (2008) who fitted similar curves for pupae development in Italian honeybee. We concluded that the artificial condition we adopted successfully simulated the natural condition for the development of worker bee larvae.

On the first day of our experiment, the larval stage of 65–89 h, worker bee larvae showed extreme sensitivity to the effects of different hormones. This was consistent with the fact that the larval stage of 72–84 h was the critical period for the development of genital ridge in honeybees. Effects of gonadal hormones on the development of genital ridge could promote the growth of larvae and increase the larval weight. When larvae developed to the near end of the larval stage, larvae in the none group showed a significant difference in weight compared with larvae in group A except the larvae in group D_2 with a high concentration of FSH in the diet. In another experiment conducted (not published), we studied the growth of queen bee larvae in the incubator fed with a mixture of royal jelly, honey, and water. When FSH was added into the diets, it had the significant effects of increasing the larval weight during the whole larval stage, and the sensitivity of queen bee larvae to FSH could be detected till the beginning of the pupal stage. Similar results from both experiments supported the significant role FSH played in the development of larvae. In our previous report (Zhou and Zhang, 2002), we detected a higher concentration of FSH in queen bee larvae, which was not contributed by their taken-in food, royal jelly, or honey. We suggested that FSH or its analogs took an important part in the development of larvae, and our present work confirmed this conclusion.

In females, estradiol generally acts as a growth hormone for the tissue of the reproductive organs. If the function could be used for reference, it is interesting that we did not detect the continuing effects of E_2 on the development of larvae. Since its production is manipulated by a feedback system via the hypothalamic pituitary events in mammals, we conjectured that the concentrations we applied in this experiment may be high enough to lead to a negative feedback. The decreased larval weights in the presence of E_2 combined with FSH demonstrated this assumption on another aspect. Up till now, abundant reports were published about the effects of juvenile hormone analogs on the development of honeybee at different stages (Sullivan et al., 2000; Schulz et al., 2002; Elekonich et al., 2003; Salles and Cruz-Landim, 2004; Nocelli et al., 2007; Paes-de-Oliveira et al., 2008). Only limited research about the effects of gonadal hormones was conducted in silkworm (Keshan and Ray, 2000, 2001; Roy et al., 2007). The possible existence of these gonadal and gonadotropic hormone analogs and their potential functions in honeybee remain unknown. Long-term efforts need to be made to clarify their interactions in further research.

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