Study on Several Theoretical Issues of Honeybee Biology

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曾志将　等著

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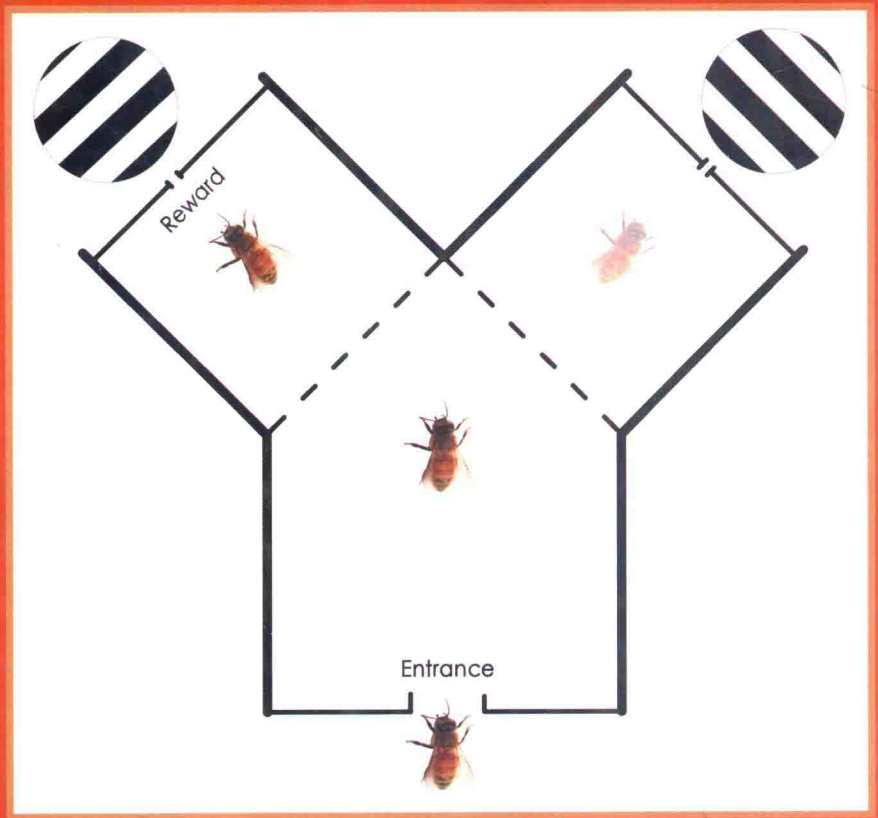

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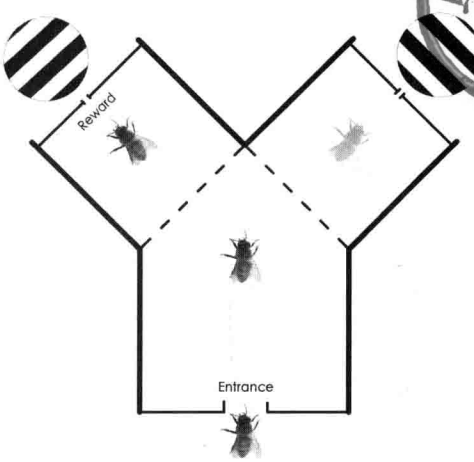

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内 容 简 介

本书是江西农业大学蜜蜂研究所曾志将教授团队在国家蜂产业技术体系、国家自然科学基金等项目连续资助下取得的研究成果。内容包括蜜蜂级型分化与基因差异表达，工蜂劳动分工，蜜蜂遗传与发育，蜜蜂性等位基因多态性分析，蜜蜂学习与记忆，工蜂监督、辨认与优惠等蜜蜂生物学主题。

本书知识新颖，理论性强，可供广大生物科技工作者及相关专业研究生参考使用，读者可藉此了解到很多蜜蜂生物学新知识。

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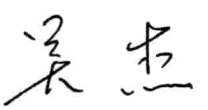
序

蜜蜂生物学是养蜂学中是一门传统学科，同时也是养蜂学中发展最迅速的领域。1961 年苏联养蜂专家塔兰诺夫出版了《蜜蜂生物学》一书，这是该领域第一部专著，该书于 1973 年被译成中文版。1987 年美国科学家 Winston 出版了专著 *The Biology of the Honeybee*; 2003 年匡邦郁和匡海鸥主编出版了我国第一部有关蜜蜂研究的专著《蜜蜂生物学》; 2006 年曾志将在该领域主编出版了我国第一本全国高等农林院校统编教材《蜜蜂生物学》。

《蜜蜂生物学理论中若干问题研究》一书是江西农业大学蜜蜂研究所曾志将教授研究团队取得的研究成果。曾志将教授是中国养蜂学会副理事长、著名蜜蜂生物学专家、国家蜂产业技术体系岗位科学家。多年来，在国家蜂产业技术体系、国家自然科学基金等项目资助下，曾志将教授带领团队成员，围绕蜜蜂生物学这一主题进行了系统研究，并在 *BMC genomics*、*PLoS One*、*Naturwissenschaften*、*Apidologie* 等世界高水平期刊发表诸多学术论文，取得了许多原创性研究成果。

我相信《蜜蜂生物学理论中若干问题研究》一书的出版发行，将让读者了解到很多蜜蜂生物学新知识，更为重要是为我国养蜂业可持续发展提供了坚实的理论基础。因此特作此序，以示祝贺。



研究员
中国养蜂学会理事长
国家蜂产业技术体系首席科学家
2015 年 1 月于北京

前　言

蜜蜂是一种资源共享、精确分工和信息交流的高度结构化社会群体。蜜蜂社会生物学特性一直受到广大生物学家关注，其原因一方面是蜜蜂通过授粉，对农业增产和生态平衡有重要作用，另一方面蜜蜂是一种典型模式昆虫，蜜蜂生物学研究结果对整个社会生物学有深远影响。

在国家蜂产业技术体系(No.nycytx-43-kxj15; No.CARS-45-kxj12)、国家自然科学基金(No.30560114; No.30760035; No.31060327; No.31260524; No.31160486)、国家公益性行业(农业)科研专项(No.nyhyzx07-041; No.200903006)、江西省赣鄱英才 555 工程领军人才培养计划、教育部博士点基金(No.20103603110003; No.20100481191)、江西省自然科学基金(No.0530031; No.2007GZN0202; No.2010GZN0044)等科研项目资助下，我们围绕蜜蜂生物学中的“蜜蜂级型分化与基因差异表达”，“工蜂劳动分工”，“蜜蜂遗传与发育”，“蜜蜂性等位基因多态性分析”，“蜜蜂学习与记忆”，“工蜂监督、辨认与优惠”六个理论问题进行了系统研究。

在七年研究过程中，有 20 多名科技人员参加了项目组研究工作，其中包括我指导培养的 1 名博士后(王子龙)、8 名博士研究生(谢宪兵、颜伟玉、刘志勇、吴小波、石元元、张丽珍、何旭江、秦秋红)、14 名硕士研究生(黄强、张含、曾云峰、石元元、何旭江、刘亭亭、管翠、秦秋红、王欢、王文祥、田柳青、曾晶、潘其忠、刘浩)。另外澳大利亚国立大学 Shao Wu Zhang 教授、美国密歇根州立大学 Zachary Y. Huang 教授、澳大利亚麦考瑞大学 Andrew B. Barron 教授、法国农业科学院 Yves Le Conte 教授、泉州师范学院孙亮先教授、上海南方基因组中心郑华军副研究员等专家参与了部分实验的设计或指导、论文撰写或修改工作。

非常值得欣慰的是，在大家的共同努力下，取得了可喜的研究成果。本书选取了江西农业大学为第一署名单位，同时为通讯作者单位的 38 篇论文，其中 25 篇论文发表在 *BMC genomics*、*PLoS One*、*Naturwissenschaften*、*Apidologie*、*Molecular Biology Reports*、*Journal of Comparative Physiology A*、*Journal of Apicultural Research*、*Insect Science*、*European Journal of Entomology*、*Journal of Apicultural Science*、*Journal of Asia-Pacific Entomology*、*Research Journal of BioTechnology* 等 SCI 刊物上，SCI 总影响因子大于 55，其中 7 篇论文 SCI 影响因子大于 3.7；另外还有 13 篇论文发表在国内的一级学报《动物学报》、《昆虫学报》、《中国农业科学》和《应用生态学报》上。

正如由于研究蜜蜂舞蹈贡献而获得 1973 年诺贝尔奖的 Karl Von Frish 教授所说：蜜蜂世界是一个“魔井”，当我们发现的新知识越多，越有更多奥秘等待我们去探索。让我们共同努力，去探索蜜蜂世界中更多的奥秘。

江西农业大学蜜蜂研究所　曾志将

2015 年 1 月于南昌

目　录

I. 蜜蜂级型分化与基因差异表达

级型分化是指在社会性昆虫群体中，相同性别个体具有不同形态结构、职能和行为的现象。蜂王和工蜂都是由受精卵发育而成的雌性蜜蜂，它们的遗传物质完全相同，但由于发育过程中得到的食物和发育空间不同，其结果是两者不仅在外观形态上差异很大，而且在生殖能力、寿命、行为等方面迥然不同。

长期以来，蜜蜂级型分化问题一直是热门领域，许多学者从不同角度研究了蜜蜂级型分化机理，并取得了很多可喜进展。

为了研究营养和空间因素对雌性蜂发育与 DNA 甲基化之间关系，以意蜂为研究对象，测定不同实验条件下 3d、5d 和 6d 幼虫头部 Dnmt3 酶活性、Dnmt3 mRNA 相对表达量、*dynactin p62* 基因甲基化水平。首次发现空间因素可以通过 DNA 甲基化来调控蜜蜂级型分化；利用甲基化 DNA 免疫共沉淀测序(MeDIP-seq)技术分析了 2d、4d 和 6d 蜂王幼虫和工蜂幼虫全基因组甲基化水平，结果发现：工蜂甲基化水平是随着幼虫日龄增加而提高，而蜂王甲基化水平是先升高然后下降，但甲基化主要发生在内含子区；应用 Illumina HiSeq^{TM} 2500 测定了 4d 蜂王幼虫和工蜂幼虫 micRNA 种类和含量，结果表明：首次发现工蜂幼虫 22 nt sRNA 显著高于蜂王幼虫。与工蜂幼虫相比，蜂王幼虫体内有 20 种 miRNA 表达下调，17 种 miRNA 表达上调。从以上结果，我们得出以下推论：幼虫食物和发育空间差异→幼虫 micRNA 差异表达→DNA 甲基化差异→级型分化。

采用荧光定量 PCR 检测不同发育时期工蜂和蜂王的 *sir2*、*hdac1* 和 *ash2* 基因表达量。结果表明：这 3 个基因在刚羽化的蜂王、产卵蜂王和产卵工蜂中的表达量都显著高于刚羽化的工蜂、哺育工蜂和采集工蜂，这表明这 3 个基因可能是类蜂王基因。

在克隆了中华蜜蜂 *Dnmt3* 基因 cDNA 序列、*dynactin p62* 基因 DNA 序列和 cDNA 序列基础上，利用荧光定量 PCR 技术检测了蜂王和工蜂 *Dnmt3* 基因和 *dynactin p62* 基因表达水平，结果表明：*Dnmt3* 基因 cDNA 序列全长 2277 bp，编码 758 个氨基酸残基，预测的蛋白分子质量为 88.24 kDa，等电点为 7.85。与西方蜜蜂 Dnmt3 氨基酸序列有 99% 同源性。*Dnmt3* 基因在工蜂和蜂王不同发育时期均有表达，其中采集蜂表达量显著高于内勤蜂，蜂王蛹表达量显著高于工蜂蛹，但产卵工蜂与产卵蜂王表达量差异不显著。因此我们推断 *Dnmt3* 可能与工蜂劳动分工及蜜蜂卵巢发育有关；*dynactin p62* 基因 DNA 序列全长为 2403 bp，cDNA 序列全长为 1491 bp，编码 496 个氨基酸残基，预测的蛋白分子质量为 56.49 kDa，等电点为 8.31。在蜂王和工蜂中，*dynactin p62* 基因表达量都是刚羽化期显著高于幼虫期，并且工蜂表达量显著高于蜂王，而该基因在雄蜂中表达量没有明显规律性。这些结果提示该基因可能与雌性蜜蜂级型分化有关。

1. Diet and cell size both affect queen-worker differenttiation through DNA methylation in honey bees (*Apis mellifera*, Apidae)

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ABSTRACT

Background. Young larvae of the honey bee(*Apis mellifera*)are totipotent; they can become either queens(reproductives)or workers(largely sterile helpers). DNA methylation has been shown to play an important role in this differentiation. In this study, we examine the contributions of diet and cell size to caste differentiation.

Methodology/Principal Findings. We measured the activity and gene expression of one key enzyme involved in methylation, Dnmt3; the rates of methylation in the gene *dynactin p62*; as well as morphological characteristics of adult bees developed either from larvae fed with worker jelly or royal jelly; and larvae raised in either queen or worker cells. We show that both diet type and cell size contributed to the queen-worker differentiation, and that the two factors affected different methylation sites inside the same gene *dynactin p62*.

Conclusions/Significance. We confirm previous findings that Dnmt3 plays a critical role in honey bee caste differentiation. Further, we show for the first time that cell size also plays a role in influencing larval development when diet is kept the same.

KEYWORDS: honey bee, caste differentiation, DNA methylation, CpG

INTRODUCTION

The honey bee(*Apis mellifera*)is a highly eusocial insect and is characterized by its sophisticated division of labor, dance communication for efficient use of resources, and its high degree of cohesion, functioning as a "superorganism" [1]. A normal honey bee colony is made up of a single reproductive queen, from a few to some thousands of haploid drones(dependent on season), and tens of thousands of non-reproductive female workers. Even though both the queen and her workers are genetically identical, the queen is the reproductive member and activates her ovaries shortly after mating, whereas workers have a highly reduced number of ovarioles and can only produce a limited number of eggs when

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activated(under queenless conditions). Besides ovariole numbers, queens and workers exhibit vast differences in morphology, behavior, physiology and longevity [2-4].

The mechanisms for differentiation between queen and worker were a hot topic in the 1950s [5]. These earlier studies established that worker larvae up to 3 days after hatching are totipotent and can still develop into virtually normal queens if they are transferred to queen cells and raised inside the colony. After 3.5 days, only intercastes can develop. This suggests that at this age, larval development is largely fixed(no queens can be produced), but also queen and worker represents the extremes of a continuum(not all become workers). Queen cells are much larger than worker cells, and are vertically oriented while worker cells are oriented horizontally. Young workers with developed hypopharyngeal and mandibular glands("nurses")detect the differences between worker and queen cells and feed the larvae different food [6]. Larvae in queen cells are provisioned with royal jelly(RJ), while those in worker cells are provided with worker jelly(WJ). The consensus now is that either different sugar concentrations or other phagastimulants in RJ enables queen larvae to eat more food, which, perhaps through stretch receptors in the gut, sends signals to the brain [7]. Higher juvenile hormone(JH)synthesis by the *corpora allata* results in reduced apoptosis and the expression of queen-specific genes, resulting in the queen phenotype [8]. Queen and worker larvae have very different activated genes [9], as well as protein profiles [10].

The most recent discovery is that DNA methylation is implicated in caste determination. Wang et al. [11] first established that honey bees have a DNA methylation system with two active orthologs of vertebrate DNA methyltransferases, Dnmt1 and Dnmt3 [11]. Dnmt3 was then shown to be involved in caste determination in honey bees [12]. Silencing this gene in newly hatched larvae results in reduced rates of methylation, which leads to a significantly higher proportion of queens. Reduced methylation therefore appears to mimic the effect of royal jelly(RJ). Kucharski et al. [12] also determined the percentage of methylation at 10 CpG sites in *dynactin p62*, a gene that responds to dietary changes in *Drosophila*. Again, injection of double-stranded Dnmt3 RNA resulted in decreased rates of methylation in *dynactin p62*, when the 10 CpG sites are considered together. This mimics the high methylation rates in worker larvae, and low methylation rates in queen larvae, when reared inside colonies.

While the effects of nutrition on caste determination have been well studied [10, 12, 13, 14], whether the size difference between worker and queen cells also contributes to caste determination is not clear. In this study we report the effects of both nutrition and cell-size on Dnmt3 activity, its gene expression, and the resulting phenotype of adult bees. We also tested the hypothesis that nutrition and cell-size affect the methylation of different CpG sites in the *dynactin p62*.

RESULTS

Individual CpG sites distributed among nearly all exons of the gene *dynactin p62* are shown in Fig. 1. Two sites(CpG9 and 13)were not detected to have any methylation and are not presented in Table 1. Two locations(CpG1, 2, CpG13, 14)had two CpG sites too close to be separated and were reported as a single site in Table 1.

Fig. 1. Schematic diagram showing primer locations, exons(top)and CpG sites within the various exons(bottom)in the gene *dynactin p62*. Polymerase chain reaction primers were designed to cover most of the CpG sites, except those in exons 6 and 9. Numbers 1–14 refer to locations of the CpG sites, and underlining highlights those units with two CpG sites tested at the same time. For CpG 9 and 13, we did not detect any methylation and they were not reported in Table 1.

Effect of diet on caste differentiation

Larvae fed with royal jelly for different durations resulted in many different physiological changes. Larvae fed RJ for a longer duration resulted in significantly lower Dnmt3 activities(Fig. 2A), significantly lower Dnmt3 mRNA expression(Fig. 2B), and

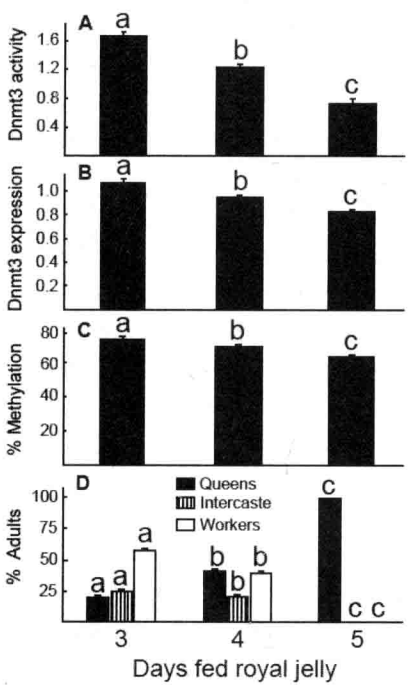

Fig. 2. Effect of feeding larvae with 3, 4, or 5 days of royal jelly. Shown are Dnmt3 enzyme activity in mmol/min(A), Dnmt gene expression(B)relative to a reference gene *calmodulin*, and percentage of methylation in the gene *dynactin p62*(C)in 6-day old larvae and percentage of adults classified as queens, intercastes, or workers(D). Different letters on top of bars indicate significant difference ($P<0.05$)among the treatments with Fisher's Protected Least Significant Difference after analysis of variance showed a significant overall effect($P<0.05$, A-C), or contingency table analysis with X^2($P<0.05$)as the test statistic(D). In D, all comparisons are among different days, not among different castes within a single day. Data for Dnmt3 activity(A)were transformed by square root transformation; Dmnt3 expression (B)and percent methylation(C)were analyzed after arcsin transformation and presented here after transformation.

Table 1. Percentage of methylation in *dynactin p62*(Mean±SE, after arcsin transformation)as affected by food type(A)and cell type(B).

Treatment	CpG-1, 2	CpG-3	CpG-4	CpG-5	CpG-6	CpG-7	CpG-8	CpG-10	CpG-11, 12	CpG-14
A. Effect of food type on queen-worker differentiation(6 day old larvae)										
Days fed RJ: 5	1.9 ± 1.8 a	1.4 ± 0.3 a	**76.6 ± 0.7 a**	**75.7 ± 4.6 a**	13.4 ± 1.5 a	12.5 ± 2.1 a	**70.2 ± 0.9 a**	80.2 ± 1.3 a	21.3 ± 5.7 a	**67.8 ± 2.3 a**
Days fed RJ: 4	2.3 ± 1.5 a	2.0 ± 0.5 a	**81.5 ± 0.8 b**	**80.1 ± 4.5 b**	14.6 ± 1.9 a	13.9 ± 1.9 a	**76.1 ± 1.3 b**	82.5 ± 1.1 a	23.2 ± 5.7 a	**72.1 ± 2.1 b**
Days fed RJ: 3	2.5 ± 1.6 a	2.6 ± 0.2 a	**86.8 ± 0.5 c**	**85.1 ± 4.4 c**	15.9 ±1.7 a	14.1 ± 2.2 a	**82.6 ± 1.1 c**	82.9 ± 0.9 a	24.7 ± 5.2 a	**78.8 ± 2.6 c**
B. Effect of cell type on queen-worker differentiation										
3 day old larvae										
Queen-cell	**2.9 ± 1.3 a**	1.1 ± 1.2 a	**70.3 ± 1.0 a**	74.6 ± 2.2 a	34.8 ± 2.1 a	**45.9 ± 1.3 a**	75.1 ± 0.9 a	**75.1 ± 2.2 a**	33.1 ± 1.9 a	63.0 ± 1.4 a
Worker-cell	**6.2 ± 1.4 b**	5.9 ± 1.4 a	**78.2 ± 1.1 b**	74.3 ± 2.0 a	35.5 ± 2.3 a	**54.8 ± 1.1 b**	77.5 ± 1.1 a	**85.9 ± 2.4 b**	34.8 ± 2.1 a	65.2 ± 1.6 a
5 day old larvae										
Queen-cell	**3.2 ± 0.6 a**	**3.1 ± 0.7 a**	**72.5 ± 1.5 a**	80.1 ± 2.2 a	24.3 ± 1.3 a	**45.8 ± 2.4 a**	**79.3 ± 2.1 a**	**76.9 ± 0.8 a**	26.7 ± 1.2 a	71.2 ± 2.2 a
Worker-cell	**8.9 ± 0.7 b**	**8.5 ± 0.9 b**	**81.0 ± 1.6 b**	82.9 ± 2.1 a	25.1 ± 1.1 a	**63.3 ± 2.0 b**	**88.9 ± 1.9 b**	**85.7 ± 0.6 b**	28.9 ± 1.1 a	72.3 ± 1.9 a

significantly lower rates of methylation for the gene *dynactin p62(*Fig. 2C)($P < 0.05$ in all tests, ANOVA). These changes were also correlated with a significantly increased proportion of queens produced, and significantly decreased proportions of both intercastes and workers in each group($P < 0.05$, Fig. 2D). Analysis of each individual methylation site indicated that at almost half of the CpG sites(4 out of 10), increased RJ feeding duration were associated with significantly lower rates of methylation(Table 1A).

Effect of cell-size on caste differentiation

When assayed at 3 and 5 days of age, larvae reared in queen cells showed significantly lower levels($P<0.05$ for all parameters, ANOVA)of Dnmt3 activity, mRNA expression, and overall rate of methylation(Fig. 3A-F, respectively). The proportion of workers was also significantly higher for larvae reared inside worker cells than those reared in queen cells(100% vs. 81%, Fig. 3G). The 19% non-workers in Fig. 3G were all intercastes and no queens were produced, despite of all the physiological differences observed. Individual analysis of each methylation site indicated that less than half of the CpG sites(4 out of 10)showed significantly reduced rates of methylation in queen-cell reared larvae compared to those reared in worker-cells in 3 day old larvae. However, in 5 day old larvae, more than half of the CpG sites(6 out of 10)showed significantly reduced rates of methylation in queen-cell reared larvae compared to those reared in worker-cells(Table 1B).

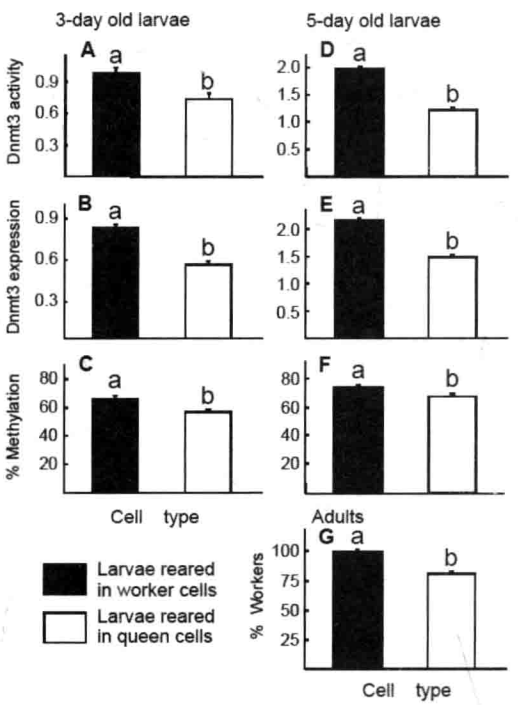

Fig. 3. Effect of cell size on honey bee larvae. Shown are Dnmt3 enzyme activity in mmol/min(A, D), Dnmt3 gene expression(B, E)relative to a reference gene *calmodulin*, percentage of methylation in the gene *dynactin p62*(C, F)in 3-day(left)and 5-day old larvae(right), and percentage of adults emerged as workers(G). The non workers here(19% for queen cells)were all intercastes. Data analysis and transformation were the same as Fig. 2.

DISCUSSION

The results of this study demonstrate that 1). Increasing the duration of royal jelly caused a graded response in decreased methyltransferase enzyme activity, decreased methyltransferase gene expression, and decreased methylation in the gene *dynactin p62*, which in turn resulted in a significant increase of queens and reduction of workers or intercastes; 2). Cell size also contributed to caste differentiation; the larger queen cells resulted in lower methyltransferase enzyme activity, lower methyltransferase gene expression, and lower percentage of methylation in the gene *dynactin p62*, resulting in a significantly lower percentage of workers produced and 3). Diet type and cell size, though both acting on caste differentiation through modulation of methyltransferase activity, its gene expression and rates of methylation of *dynactin p62*, affected different CpG methylation sites. These results suggest that cell size, a previously largely ignored factor, also contributes to caste differentiation.

Kucharski et al. [12] reported that Dnmt3 silencing caused both decreased methylation levels and increased frequency of queen phenotype. They also studied 10 CpG sites in *dynactin p62* gene and discovered decreased overall levels of methylation across these 10 sites. In our study, we designed three primers to encompass 14 CpG sites distributed more broadly across nearly all exons(Fig. 1), except exon 6(which was designated as exon 7 in both Kucharski et al. [12] and Wang et al. [11] papers; the current NCBI uses updated genomic information and annotates *dynactin p62* as having 9 exons instead of 8 exons, causing a shift in exon numbers in this study compared with the two mentioned studies)and exon 9 which we did not include. Our study manipulated the days larvae were fed with RJ and showed results consistent with Kucharski et al. [12]. Larvae fed RJ for the longest duration(3 days of RJ inside colony + 2 days of RJ in laboratory)showed the lowest Dnmt3 activity, lowest Dnmt3 gene expression, lowest overall methylation of the gene *dynactin p62* and the lowest proportion of workers or intercastes(Fig. 2). All(100%)emerged adults were queens in this treatment(Fig. 2D). Those fed RJ for 4 days(3 days of RJ inside colony + 1 day of RJ in the laboratory + 1 day of WJ in laboratory)showed intermediate levels in all the measured variables and intermediate proportions of the three castes. Those fed RJ for 3 days(3 days of RJ inside colony + 2 days of WJ in laboratory)showed the highest Dnmt3 activity, Dnmt3 gene expression, overall rates of methylation of the gene *dynactin p62* and the highest proportions of workers and intercastes. These results suggest that the response to the duration larvae are provided with RJ is graded, and is consistent with earlier results that the queen-worker difference is a continuum and not an on/off phenomenon [5].

It is well known that worker larvae up to 3 days old are still able to develop into a largely queen phenotype [5]. We therefore expected to observe no differences between the two groups of 3 day old larvae reared in different sized cells, because both received the same diet. Instead, we observed that in 3 day old larvae, all measured responses(Dnmt3 activity, expression, and percent methylation)were significantly different between the queen-cup and worker-cell reared larvae(Fig. 3 A-C). This suggested that even at 3 days old, the larvae somehow

detected differences in their rearing environment, even though larvae are still relatively small(compared to worker cell size). These differences did not become strikingly larger when larvae were 5 days old(Fig. 3D-F). Space restriction is the only explanation for the observed differences, because we removed the gravity factor(in the colony, queen cells are oriented differently from worker cells)in the laboratory larvae-rearing setting.

In addition to Dnmt3 expression and rate of methylation in the gene *dynactin p62*, we also measured enzyme activity of Dnmt3(Fig. 2A, 3A and 3D). Visual comparison of Fig. 2A vs. 2B suggests that Dnmt3 enzyme activity represents a more sensitive assay compared to Dnmt3 expression, because the differences between the 3 and 5 "Days fed royal jelly" were more striking in the Dnmt3 enzyme activity than the Dnmt3 expression levels. However the two measurements become similar in the cell type experiment(Fig. 3A, B, and D, E). These results suggest that the two factors(diet type and cell size)affect Dnmt3 activity and its gene expression differently.

Results in Table 1 suggest that diet type and cell-size affected different CpG sites. Diet type significantly affected the methylation rates of *dynactin p62* at sites 4, 5, 8, and 14, while cell-size affected sites 1/2, 3, 4, 7, 8 and 10. Only site 4 and 8 were affected by both factors and site 6 and 11/12/ were not affected by either one. This suggests that the two factors may act in two different pathways. Distinct genes might be differentially methylated when these two factors are manipulated, but this needs further experimental confirmation.

Results from Fig. 2D indicate that changing the larval diet from 3 days of RJ to 5 days of RJ decreased workers from 57% to 0%, suggesting a complete switch-over due to dietary manipulation. Fig. 3G shows that 100% of larvae when residing in worker cells became workers, while 81% became workers when residing in queen cups. The other 19% were of intercastes with no queen produced. The change of proportion of workers occurred solely due to cell size difference, because both groups were fed with the same diet(WJ harvested from 3 day old larvae). This represents a 19% reduction of workers, about one fifth of the effect of the dietary effect(that is if we ignore the fact that intercastes are not queens). These results suggest that even though cell-size can influence caste differentiation, its effect is weaker compared to the dietary effect.

DNA methylation has become a major focus in honey bee research [12, 15, 16] after its initial discovery in the system [11]. Our study measured for the first time the activities of methyltransferase Dnmt3, and provides evidence that cell types also affect DNA methylation, resulting in differences in the queen-worker development. Further studies are needed to understand how information about cell-size difference is transduced into physiological changes in the two castes.

MATERIALS AND METHODS

The Western honey bee, *Apis mellifera,* was used throughout this study. The honey bee colonies were maintained at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China(28.46°N, 115.49°E), according to standard beekeeping techniques. All experiments were conducted in a single colony to ensure genetic similarity among the larvae used.

Effect of diet on caste differentiation

Honey bee larvae were reared inside plastic queen cups inside the colony for the first three days. These larvae did not require grafting because eggs were laid directly into queen cups by confining the queen inside a special cage. These queen cups can then be detached and used for royal jelly(RJ)production [17]. After the first three days, the larvae were divided into three groups and reared in the same queen cups in an incubator(35°C and 78±7% RH). The three groups received either newly harvested RJ(harvested the same day from the same colony)for 2 days(5 day RJ), or RJ for the first day, then freshly collected worker jelly from old larvae(WJ)(4 day RJ), or WJ only for 2 days(3 day RJ). Larvae were transferred every 12 hrs to queen cups with new food(200μl per cup). On day 6, four samples each were taken for determining enzyme activity, gene expression of Dnmt3, and rate of methylation, with each sample containing 10 larval heads. The experiment was replicated in two separate trials(1060 larvae per trial), yielding 8 samples(each with 10 larval heads)per group.

Effect of cell-size on caste differentiation

Honey bee larvae were reared inside plastic queen cups or worker cells inside an incubator(35°C, 78±7% RH)from day 1(within 24 hours of larval hatching). Each cell was primed with 200μl of freshly collected WJ(harvested from 3 day old worker larvae the same day)before the larva was transferred into it. Queen cups were of the same type as the first experiment. Worker cells were on pieces of natural beeswax comb(each about 398 x 152 mm). Larvae were transferred every 8 hrs to queen cups or worker cells with new food. Larvae were sampled on day 3(whole larvae)and day 5(heads only)for the same three parameters as the first experiment, with N=8 samples for each parameter(Dnmt3 activity, gene expression, and rates of methylation)with each sample containing 10 larvae or heads. On day 6, mature larvae were transferred to 6-cell tissue culture plates(Costar, NY, USA)lined with a piece of Kimwipe and kept in an incubator(35°C and 78±7% RH)for pupation [18]. The overall emergence rate(1 day old larvae to adults)was 56.6%. We sampled 80 adult bees per group to score their morphological characteristics. We reared 1400 larvae for each trial and the experiment was replicated in two different trials.

Experimental Details

Harvest of RJ and WJ

RJ was produced according to standard practices in China [19]. Briefly, the queen was separated from the rest of the colony by a queen excluding board. Queen cups with young larvae(one day old)were introduced into the colony and allowed to be fed by workers for 2 days. Larvae were then removed and fresh RJ removed with a spatula and stored at 4°C until use. To acquire worker jelly from old larvae, we first carefully removed 3 day old larvae by using either a grafting tool or a pair of forceps, then removed the WJ using a spatula. In experiment 1, both RJ and WJ came from the same colony as the experimental larvae.