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Caste-biased Expression of *fem* and *Amdsx* Genes in *Apis mellifera ligustica* (Hymenoptera: Apidae)

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Abstract

Sex determination and caste differentiation are two crucial processes for morphology building in honeybees. It is unclear whether there is an interaction between these two processes. Here, we investigated the expression of *fem* and *Amdsx* genes between female castes of honeybees. We found that the expression of *fem* and *Amdsx* is significantly higher in queens than in workers, and this expression was positively regulated by juvenile hormone (JH). Our results suggest that sex-determining genes *fem* and *Amdsx* are also involved in honeybee caste differentiation.

Introduction

Honeybees are typical social insects. A honeybee colony is usually made up of a queen, tens of thousands of workers, and hundreds of drones. The queen and workers are diploid females developed from fertilized eggs; the drones are haploid males developed from unfertilized eggs. As two female castes, the queens and workers differ significantly in morphology, physiology and behaviors (Winston, 1987). Thus, the differentiation of queen, worker, and drone contains two critical developmental processes: sex determination and caste differentiation.

Sex determination of honeybees is controlled by the cascade of *csd* > *fem* > *Amdsx* (Beye et al., 2003; Cho et al., 2007; Hasselmann et al., 2008; Gempe et al., 2009). During the early embryonic stage, *csd*, as the primary signal, transmits the sex-determining signal to the downstream genes so that the embryo develops toward female (Beye et al., 2003). *Fem* is homologous to *csd*, and its function is to maintain and stabilize the female embryo development and regulate the female-specific splicing of downstream gene *Amdsx* (Hasselmann et al., 2008; Gempe et al., 2009). *Dsx* is a conserved gene in insects located at the bottom of the sex determination cascade (Gempe & Beye, 2010). The pre-mRNA of *Amdsx* is sex-specifically spliced in males and females to produce male- and female-specific AmDSX proteins to regulate sex differentiation of honeybees (Cho et al., 2007).

Caste differentiation of female honeybees is regulated by many factors, such as nutrition (Haydak, 1970; Rembold & Dietz, 1966; Wittmann & Engels, 1987), hormones (Wirtz et al., 1972; Asencot & Lensky, 1976; Rachinsky & Beetsma, 1990), epigenetic modifications (Kucharski et al., 2008; Spannhoff et al., 2011; Wojciechowski et al., 2018), etc. The queen larvae were fed royal jelly during the whole larvae stage, while the worker larvae were just fed worker jelly for three days and then were fed bee bread till pupation. Factors such as sufficient food at the larvae stage, high titer of JH, and low level of genomic DNA methylation in the larvae can promote the queen phenotype's development. On the other hand, dicoumaric acid (Mao et al., 2015) and

plant RNAs (Zhu et al., 2017) can induce worker phenotype development. Studies confirmed that insulin/insulin-like growth factor 1 signaling (IIS) pathway (Wolschin et al., 2010; Mutti et al., 2011; Wang et al., 2013), the target of rapamycin (TOR) signaling pathway (Patel et al., 2007; Mutti et al., 2011) and epidermal growth factor receptor (EFGR) signaling pathways (Kamakura, 2011) are involved in caste differentiation. These signaling pathways transmit the initial nutritional signals to JH, which plays a dominant role in caste differentiation. Compared with worker larvae, the titer of JH in queen larvae is 26 times higher than that in worker larvae during the 4th to 5th instars (Rachinsk & Hartfelder, 1990). Meanwhile, topical application of synthetic JH to worker larvae leads to the development of queen-like traits (Rembold et al., 1974).

Although both sex determination and caste differentiation pathways are related to the development of honeybees' morphological and physiological traits, the possible link between these two pathways is poorly understood. Recent studies have shown that sex-determining genes may also play a role in caste differentiation (Brito et al., 2015; Klein et al., 2016; Roth et al., 2019). In this study, we compared the expression of *fem* and *Amdsx* between queen and worker castes and assessed the effect of JH on their expression. We found caste-biased expression of *fem* and *Amdsx*, which indicates an interaction between sex determination pathway and caste differentiation pathway.

Materials and Methods

Sample collection

The honeybees used in this study were western honeybee, *Apis mellifera ligustica* Spinola, 1806. According to standard beekeeping techniques at the Honeybee Research Institute, we raised colonies, Jiangxi Agricultural University, Nanchang, China (28°46' N, 115°49' E).

Three colonies of *A. m. ligustica* with similar populations were selected. The queen of each colony was confined on a comb to lay eggs for six hours. Then, we transferred the comb to a super box of the same colony. When the eggs hatched into larvae, some of the larvae were grafted to queen cells, and all the larvae continued to be bred in a super box. When the larvae reached 48 h, 84 h, and 120 h, the queen larvae and worker larvae were sampled respectively to analyze expression differences of *fem* and *Amdsx* between them by qRT-PCR. At 48 h, every three larvae were collected as a sample; at 84 h and 120 h, each larva is a sample. During sampling, the larvae were washed three times with double distilled water. Seven biological replicates were sampled for each time point.

The head and stomach of newly emerged queens and workers with five biological replicates were sampled. During sampling, the stomach and intestines of bees were removed. All the samples were quickly frozen in liquid nitrogen and were stored at -80 °C.

Juvenile hormone treatment

An *A. m. ligustica* colony was used in the JH treatment experiment. The queen was limited on a comb to lay eggs for six hours. When the eggs hatched, the 1-day old larvae were grafted to 24-well cell culture plates with 200 μL/hole artificial larval food (glucose 6%, fructose 6%, royal jelly 50%, yeast extract 1%, distilled water 37%). They were randomly divided into three groups. One group was topically applied with 1μL of JH III (APExBIO, purity ≥ 65.00%) solution diluted in ethanol (10 μg/μL). The second group was topically applied with 1μL of ethanol and was set as control. The third group was normal larvae without any treatment. Larvae were reared in an incubator with a temperature of 34 °C and humidity of 85%. For the next two days, the JH treatment group and ethanol treatment group continued to be treated with JH III or ethanol three times every day. Twenty-four hours after the end of the treatment, larvae of the three groups were collected to detect mRNA expression changes of *fem* and *Amdsx* through qRT-PCR. For each group, seven biological replicates were sampled. After washing three times with double distilled water, the larvae were immediately frozen in liquid nitrogen and stored at -80 °C for RNA extraction.

Quantitative real-time PCR

Total RNA was extracted from the above samples according to the instructions of the TransZol Up Plus RNA Kit (TransGen Biotech, Beijing, China). cDNA was obtained by reverse transcription following the instructions of the Primescript™ RT reagent Kit (Takara, Japan). The housekeeping gene *Amactin* of *A. mellifera* was used as an internal reference gene. Quantitative real-time PCR primers were designed based on mRNA sequences of *fem*, *Amdsx,* and *Amactin* with Primer Premier 5.0 software. The sequences of primers are listed in Table 1.

Table 1. Primer sequences used for qRT- PCR assays.

Gene	Accession number	Primer sequence (5' to 3')
fem	EU101388.1	Forward: CCAATGCGGGGTCAAGTA Reverse: TATCTGGAGGAATAAATCGTG
Amdsx	EU236954.1	Forward: CTCACACTGCGATGGTCAC Reverse: CGTCTCACTACTTCTTCCG
Amactin	NM_001185145.1	Forward: GTATTGTATTGGATTCGGGTG Reverse: TGCCATTTCCTGTTCAAAGTCA

Amplification conditions of qRT-PCR: 50 °C for 2 min, predenaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, (*fem*, 58.2 °C; *Amdsx*, 52.9 °C and *Amactin*, 52.9 °C) for 1 min. For each sample, the specificity of the PCR amplification was verified by melting curve analysis.

The data were analyzed by the comparative Ct method ($2^{-\Delta\Delta Ct}$) (Schmittgen & Livak, 2008). The expression differences of *fem* and *Amdsx* between samples were analyzed by t-test or one-way ANOVA using SPSS17.0 software (SPSS Inc., 2008).

Results

fem and *Amdsx* have expression differences between queen and worker

The expression levels of *fem* and *Amdsx* in queen larvae and worker larvae at 48 h, 84 h, and 120 hand in the head and stomach of adult queens and workers were detected by qRT-PCR analysis. The results showed that: the expression levels of *fem* in queen larvae at 84 h and 120 h were significantly higher than those in worker larvae (84 h: t=3.99, df=4.23, p=0.0146; 120 h: t=6.25, df=8.00, p=0.0002) (Fig 1), and there was no significant difference at 48 h (t=0.39, df=8.00, p=0.7053); the expression levels of *Amdsx* in queen larvae at all the three stages were significantly higher than those in worker larvae (48 h: t=-3.46, df=8.00, p=0.0085; 84 h: t=-7.56, df=8.00, p<0.0001; 120 h: t=4.04, df=5.98, p=0.0069); both *fem* and *Amdsx* expressed higher in stomach of queen than in that of worker (*fem*: t=-6.48, df=4.58, p=0.0018; *Amdsx*: t=-9.30, df=8.00, p<0.0001) (Fig 2), and there were no significant expression difference of *fem* and *Amdsx* between the head of queen and worker (*fem*: t=-1.79, df=8.00, p= 0.1107; *Amdsx*: t=-0.74, df=7.98, p= 0.4786).

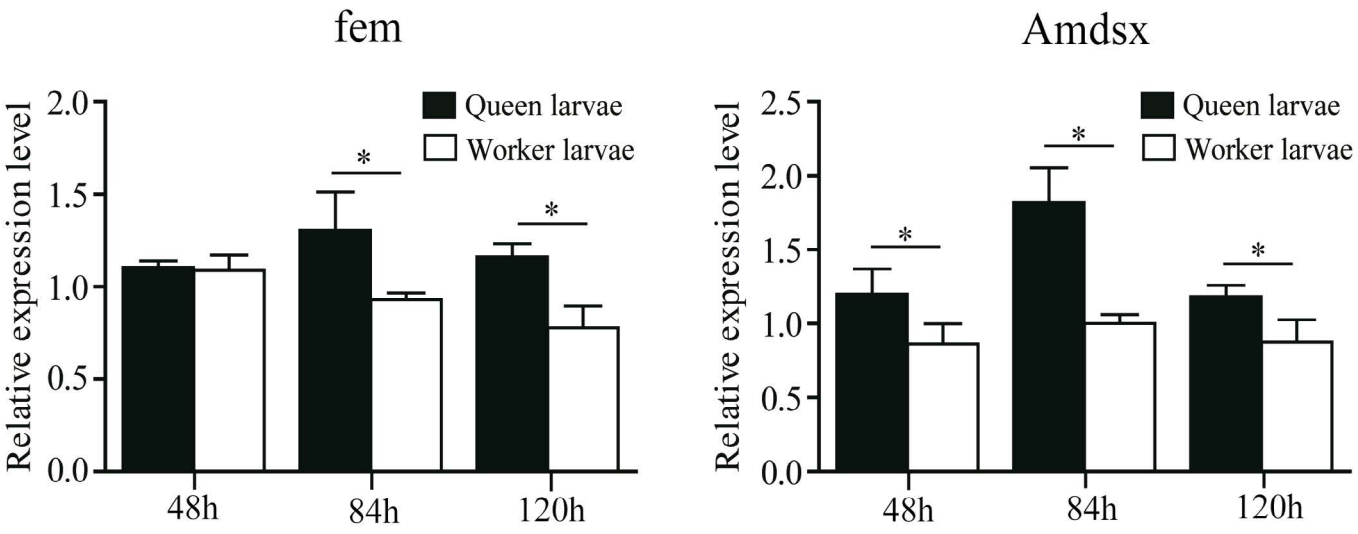

Fig 1. Expression differences of *fem* and *Amdsx* between queen larvae and worker larvae. *represent significant difference (t-test, p<0.05).

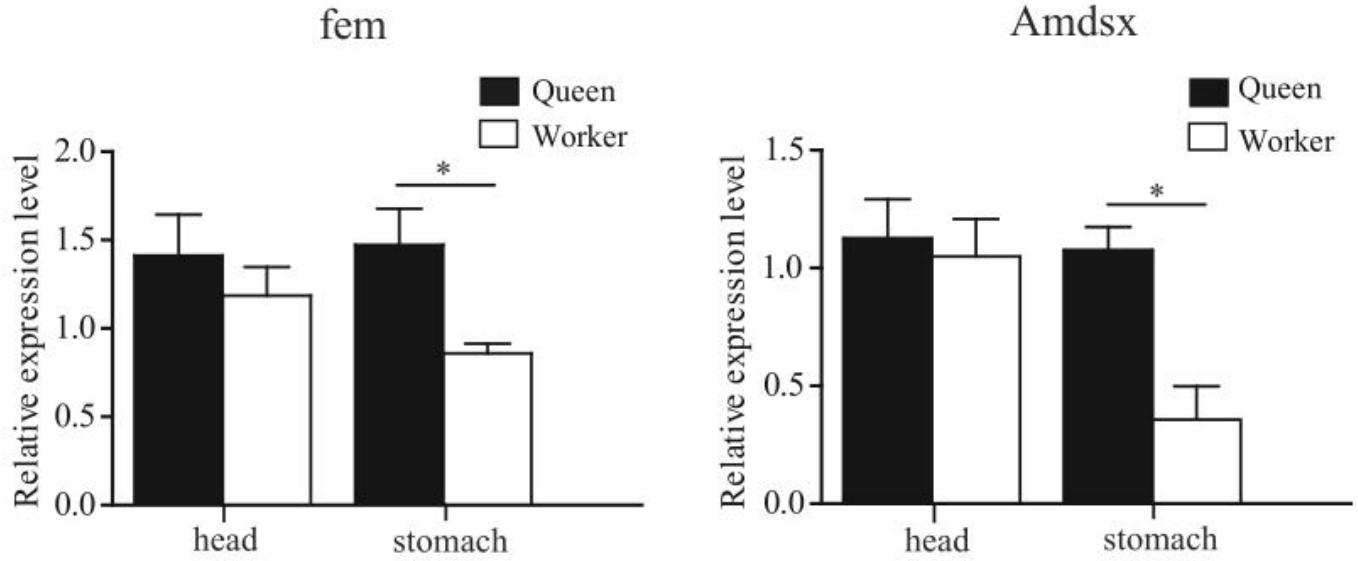

Fig 2. Expression differences of *fem* and *Amdsx* between head and stomach of queen and that of workers. *represent significant difference (t-test, p<0.05).

JH up-regulated fem and Amdsx

After treatment of larvae with JH, the expression levels of both *fem* and *Amdsx* in JH treatment group were significantly up-regulated compared with the two control groups, and there was no significant difference between the two control groups (*fem*: JH vs. normal vs. ethanol, F(2, 18)=20, p<0.0001; JH vs. ethanol, p=0.0006; JH vs. normal, p<0.0001; ethanol vs. normal, p=0.0639. Amdsx: JH vs normal vs. ethanol, F(2, 18)=20, p=0.0005; JH vs. ethanol, p=0.0003; JH vs. normal, p=0.0009; ethanol vs. normal, p=0.6773) (Fig 3).

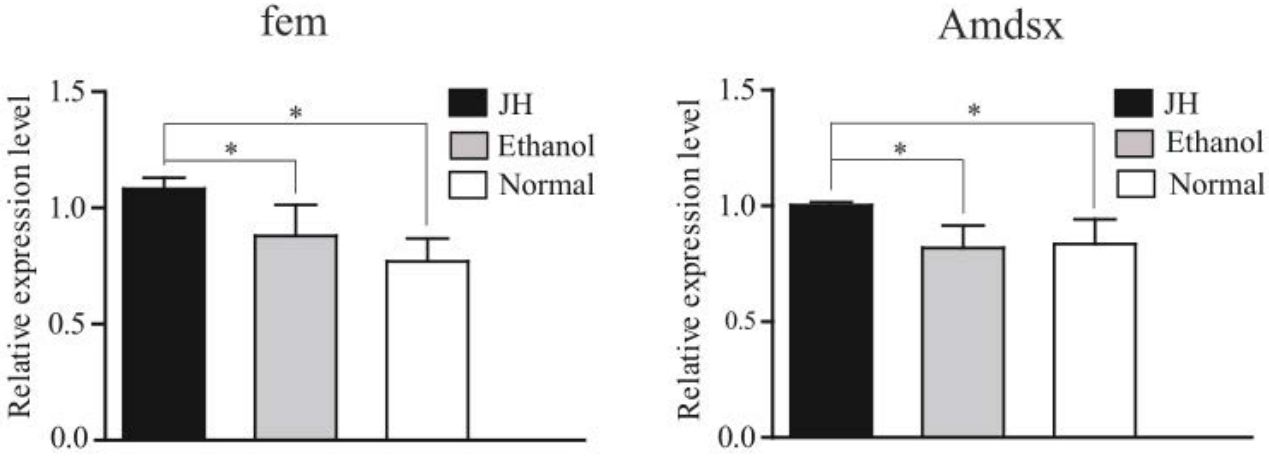

Fig 3. Expression change of *fem* and *Amdsx* after JH III treatment. *represent significant difference (ANOVA, p<0.05).

Discussion

fem and *Amdsx* are critical regulators in the sex determination cascade of honeybees. Several studies have indicated that sex-determining genes may also be involved in caste differentiation (Brito et al., 2015; Klein et al., 2016; Roth et al., 2019). A study in stingless bee *Melipona interrupta* showed that the *fem* gene expressed higher in the queen than in the worker (Brito et al., 2015). In ant *Cardiocondyla obscurior*, *dsx* exhibits both sex and form-specific expression across life stages (Klein et al., 2016). Knocking out *fem* and *Amdsx* using CRISPR/cas9, (Roth et al., 2019) found that nutrition could affect the gonad size of honeybees through the *fem* gene. In this study, we also found that *fem* and *Amdsx* had higher expression levels in the queen than in the worker. It suggests that *fem* and *Amdsx* play a critical role in sex determination and are involved in caste differentiation of female bees.

JH plays a vital role in the process of honeybee caste differentiation. The increase of JH can make female larvae develop into queens (De Oliveira Campos et al., 1975) and promote the increase in the number of ovarian tubes in workers (Dorn, 1985). We investigated the relationship between *fem*, *Amdsx,* and JH, and found that the expression levels of *fem* and *Amdsx* in the larvae were significantly increased after JH III treatment. This indicates that JH regulates the differential expression of *fem* and *Amdsx* between queen and worker. The increase of *fem* and *Amdsx* expression after JH treatment is associated with queen development.

In conclusion, our study indicates that sex-determining genes *fem* and *Amdsx* are also involved in honeybee caste differentiation, and they are positively regulated by JH. Further studies are needed to clarify how these sex-determining genes function in the caste differentiation process of honeybees.

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Authors Contribution

ZW: conceptualization, methodology
LP: resources, invetigation, writing
FC: investigation
ZW: writing

References

Asencot, M. & Lensky, Y. (1976). The effect of sugars and juvenile hormone on the differentiation of the female honeybee larvae (*Apis mellifera* L.) to queens. Life Science, 18: 693-699. doi: 10.1016/0024-3205(76)90180-6.

Beye, M., Hasselmann, M., Fondrk, M.K., Page, R.E. & Omholt, S.W. (2003). The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein. Cell, 114: 419-429. doi: 10.1016/s0092-86 74(03)00606-8.

Brito, D.V., Silva, C.G.N., Hasselmann, M., Viana, L.S., Astolfi-Filho, S. & Carvalho-Zilse, G.A. (2015). Molecular characterization of the gene *feminizer* in the stingless bee *Melipona interrupta* (Hymenoptera: Apidae) reveals association to sex and caste development. Insect Biochemistry and Molecular Biology, 66: 24-30. doi: 10.1016/j.ibmb.2015.09.008.

Cho, S., Huang, Z.Y. & Zhang, J. (2007). Sex-specific splicing of the honeybee *doublesex* gene reveals 300 million years of evolution at the bottom of the insect sex-determination pathway. Genetics, 177: 1733-1741. doi: 10.1534/genetics.107.078980.

De Oliveira Campos, L.A., Velthuiskluppell, F.M. & Velthuis, H.H.W. (1975). Juvenile hormone and caste determination in a stingless bee. Natunwissenschaften, 62: 98-99. doi:10.1007/bf00592188.

Dorn, A. (1985). Comprehensive insect physiology, biochemistry and pharmacology. International Journal of Biochemistry, 17: 1279. doi:10.1016/0020-711x(85)90021-7.

Gempe, T. & Beye, M. (2010). Function and evolution of sex determination mechanisms, genes and pathways in insects. Bioessays, 33: 52-60. doi: 10.1002/bies.201000043.

Gempe, T., Hasselmann, M., Schiøtt, M., Hause, G., Otte, M. & Beye, M. (2009). Sex determination in honeybees: Two separate mechanisms induce and maintain the female pathway. PLoS Biology, 7: e1000222. doi: 10.1371/journal.pbio.1000222.

Hasselmann, M., Gempe, T., Schiøtt, M., Nunes-Silva, C.G., Otte, M. & Beye, M. (2008). Evidence for the evolutionary nascence of a novel sex determination pathway in honeybees. Nature, 454: 519-522. doi: 10.1038/nature07052.

Haydak, M.H. (1970). Honey bee nutrition. Annual Review of Entomology. 15: 143-156. doi:10.1146/annurev.en.15.010170.001043.

Kamakura, M. (2011). Royalactin induces queen differentiation in honeybees. Nature, 473: 478-483. doi: 10.1038/nature10093.

Klein, A., Schultner, E., Lowak, H., Schrader, L., Heinze, J., Holman, L. & Oettler, J. (2016). Evolution of social insect polyphenism facilitated by the sex differentiation cascade. PLoS Genetics, 12: e1005952. doi: 10.1371/journal.pgen.1005952.

Kucharski, R., Maleszka, J., Foret, S. & Maleszka, R. (2008). Nutritional control of reproductive status in honeybees via DNA methylation. Science, 319: 1827-1830. doi: 10.1126/science.1153069.

Mao, W., Schuler, M.A. & Berenbaum, M.R. (2015). A dietary phytochemical alters caste-associated gene expression in honey bees. Science Advances, 1: e1500795. doi: 10.1126/sciadv.1500795.

Mutti, N. S., Dolezal, A.G., Wolschin, F., Mutti, J.S., Gill, K.S. & Amdam, G.V. (2011). IRS and TOR nutrient-signaling pathways act via juvenile hormone to influence honey bee caste fate. Journal of Experimental Biology, 214: 3977-3984. doi: 10.1242/jeb.061499.

Patel, A., Fondrk, M.K., Kaftanoglu, O., Emore, C., Hunt, G., Frederick, K. & Amdam, G.V. (2007). The making of a queen: TOR pathway is a key player in diphenic caste development. PLoS One, 2: e509. doi:10.1371/journal.pone.0000509.

Rachinsky, A. & Hartfelder, K. (1990). Corpora allata activity, a prime regulating element for caste-specific juvenile hormone titer in honey bee larvae (*Apis mellifera carnica*). Journal of Insect Physiology, 36: 189-194. doi: 10.1016/0022-1910(90)90121-u.

Rachinsky, A., Strambi, C., Strambi, A. & Hartfelder, K. (1990). Caste and metamorphosis: Hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. General and Comparative Endocrinology, 79: 31-38. doi:10.1016/0016-6480(90)90085-z.

Rembold, H., Czoppelt, C. & Rao, P.J. (1974). Effect of juvenile hormone treatment on caste differentiation in the honeybee, *Apis mellifera*. Journal of Insect Physiology, 20: 1193-1202. doi: 10.1016/0022-1910(74)90225-x.

Rembold, H. & Dietz, A. (1965). Biologically active substances in Royal Jelly. Vitamins & Hormones, 23: 359-382. doi: 10.1016/s0083-6729(08)60385-4.

Roth, A., Vleurinck, C., Netschitailo, O., Bauer, V., Otte, M., Kaftanoglu, O., Page, R.E. & Beye, M. (2019). A genetic switch for worker nutrition-mediated traits in honeybees. PLoS Biology, 17: e3000171. doi: 10.1371/journal.pbio.3000171.

Schmittgen, T.D. & Livak, K.J. (2008). Analyzing real-time PCR data by the comparative CT method. Nature Protocols, 3: 1101-1108. doi: 10.1038/nprot.2008.73.

Spannhoff, A., Kim, Y.K., Raynal, N.J.M., Gharibyan, V., Su, M.B., Zhou, Y.Y., Li, J., Castellano, S., Sbardella, G., Issa, J.P. & Bedford, M.T. (2011). Histone deacetylase inhibitor activity in royal jelly might facilitate caste switching in bees. Embo Reports, 12: 238-243. doi:10.1038/embor.2011.9.

SPSS. (2010). Statistical software SPSS 17.0. SPSS Inc., Chicago, IL, USA.

Wang, Y., Azevedo, S.V., Hartfelder, K. & Amdam, G.V. (2013). Insulin-like peptides (AmILP1 and AmILP2) differentially affect female caste development in the honey bee (*Apis mellifera* L.). Journal of Experimental Biology, 216: 4347-4357. doi: 10.1242/jeb.085779. doi: 10.1242/jeb.085779.

Winston, M.L. (1987). The biology of the honey bee. Cambridge, MA: Harvard University Press. doi: 10.1093/arclin/acr108.

Wirtz, P. & Beetsma, J. (1972). Induction of caste differentiation in the honeybee (*Apis mellifera*) by juvenile hormone. Entomologia Experimentalis et Applicata. 15: 517-520. doi:10.1111/j.1570-7458.1972.tb00239.x.

Wittmann, D. & Engels, W. (1987). Welche Diät ergibt Arbeiterinnenbei in vitro Aufzucht von Honigbienen? Apidologie, 18: 279-288. doi:10.1051/apido:19870307.

Wojciechowski, M., Lowe, R., Maleszka, J., Conn, D., Maleszka, R. & Hurd, P.J. (2018). Phenotypically distinct female castes in honey bees are defined by alternative chromatin states during larval development. Genome Research, 28: 1532-1542. doi:10.1101/gr.236497.118.

Wolschin, F., Mutti, N.S. & Amdam, G.V. (2010). Insulin receptor substrate influences female caste development in honeybees. Biology Letters, 7: 112-115. doi: 10.1098/rsbl.20100463.

Zhu, K., Liu, M., Fu, Z., Zhou, Z., Kong, Y., Liang, H., Lin, Z., Luo, J., Zheng, H., Wan, P., Zhang, J., Zen, K., Chen, J., Hu, F., Zhang, C.Y., Ren, J. & Chen, X. (2017). Plant microRNAs in larval food regulate honeybee caste development. PLoS Genetics, 13: e1006 946. doi: 10.1371/journal.pgen.1006946.