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ORIGINAL RESEARCH ARTICLE

Effects of 10-Hydroxy-2-decenoic acid on the development of honey bee (*Apis mellifera*) larvae

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Summary

Honey bee queens and workers, both of which are female, develop from the same type of genome, but they have substantial morphological and physiological differences. Epigenetic modifications have been suggested to be involved in caste differentiation that is induced by royal jelly. 10-hydroxy-2(E)-decenoic acid (10-HDA), which can inhibit histone deacetylation, is a main component of royal jelly. In this study newly hatched larvae were artificially fed with diets containing 1.10%, 1.70%, 2.30%, 2.90% 10-HDA, referred to as control (C), treatment 1 (T1), treatment 2 (T2) and treatment 3 (T3), respectively, to study its effects on larval development. Compared to the control group, the weight of emerged adults was significantly reduced in the 10-HDA-treated groups. The expression level of the histone deacetylase 3 (HDAC3) gene was significantly up-regulated in the 10-HDA-treated groups than in the control group. The expression level of DNA methyltranferase 3 (DNMT3) gene was initially down-regulated, but subsequently up-regulated with increasing 10-HDA concentration. The results suggested 10-HDA not only reduces the weight of the newly emerged workers but also regulates activation of HDAC3 and DNMT3 that control epigenetic changes.

Efectos del ácido 10-hidroxi-2-decenoico en el desarrollo de larvas de la abeja de la miel (*Apis mellifera*)

Resumen

Las abejas de la miel reinas y obreras, ambas femeninas, se desarrollan a partir del mismo tipo de genoma, pero tienen diferencias morfológicas y fisiológicas sustanciales. Se ha sugerido que las modificaciones epigenéticas participan en la diferenciación de castas inducida por la jalea real. El ácido 10-hidroxi-2(E)-decenoico (10-AHD), que puede inhibir la desacetilación de las histonas, es un componente principal de la jalea real. En este estudio se alimentaron artificialmente larvas recién nacidas con dietas que contenían 1,10 %, 1,70 %, 2,30 % y 2,90 % de 10-AHD, referidas como control (C) , tratamiento 1 (T1), tratamiento 2 (T2) y tratamiento 3 (T3), respectivamente , para estudiar sus efectos sobre el desarrollo larvario. En comparación con el grupo de control, el peso de los adultos nacidos se redujo significativamente en los grupos tratados con 10-AHD. El nivel de expresión del gen de la histona desacetilasa 3 (HDAC3) fue significativamente mayor en los grupos tratados con 10-AHD que en el grupo control. El nivel de expresión del gen de la metiltranferasa3 de ADN (DNMT3) disminuyó inicialmente, pero posteriormente aumentó conforme se incrementó la concentración de 10-AHD. Los resultados sugirieron que 10-AHD no sólo reduce el peso de las obreras recién emergidas, sino que también regula la activación del HDAC3 y DNMT3 que controlan los cambios epigenéticos.

Keywords: honey bee, 10-HDA, histone acetylation, DNA methylation

Introduction

The honey bee *Apis mellifera* provides an excellent model for identifying the epigenetic mechanisms of developmental plasticity (Maleszka, 2008). Worker and queen honey bees develop from an identical genome, but there are remarkable morphological, behavioural and physiological differences (Winston, 1987). It is well known that the developmental plasticity of honey bees results from differential intake of royal jelly at an early larval stage (Page and Peng, 2001). For the first three days of life, all larvae are fed with royal jelly. Worker larvae are then switched to a diet of pollen and nectar, whereas queen larvae remain on a royal jelly diet throughout their larval development. Royal jelly is

a complex mixture of proteins, essential amino acids, sugars, fatty acids and mineral salts, and is produced by the hypopharyngeal and mandibular glands of nurse workers (Drapeau *et al.*, 2006).

It has been reported that silencing DNA methyltransferase 3 (DNMT3) expression in newly hatched larvae makes worker-destined larvae develop into queens with well-developed ovaries, as well as royal jelly does (Kucharski *et al.*, 2008). Recent reports have demonstrated that (E)-10-Hydroxy-2-decenoic acid (10-HDA), a main component of royal jelly, has histone deacetylase inhibitor (HDACi) activity, suggesting that histone acetylation may be involved in the developmental plasticity of honey bee larvae (Spannhoff *et al.*, 2011). Histone acetylation is one of the most intensively studied epigenetic modification triggers, and provides a mechanism for coupling external information with gene transcription through regulated acetylation and deacetylation (Shahbazian and Grunstein, 2007; Haberland *et al.*, 2009). The steady -state level of histone acetylation is controlled by the antagonistic actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Eberharter and Becker, 2002). HDACi inhibits the activity of HDACs, which will induce increasing levels of histone acetylation (Dokmanovic and Marks, 2005; Dokmanovic *et al.*, 2007; Xu *et al.*, 2007).

In order to study the effects of this deacetylase inhibitor on honey bee development, we fed freshly emerged young larvae with 10-HDA.

Material and methods

Honey bees and 10-HDA

The honey bee (*Apis mellifera*) colonies were maintained at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China. 10-HDA was ordered from Yuancheng Gongchuang Technology CO. Ltd.; Wuhan, China. The purity of the chemical was over 98%.

The *in vitro* rearing of honey bee larvae

Larval food

Fresh pure royal jelly was obtained according to standard protocol (Zeng *et al.*, 2009) and frozen at -20°C. In order to determine the quantity of 10-HDA added, 10-HDA concentration from the fresh pure royal jelly was determined by high-performance liquid chromatography (HPLC) (Ferioli *et al.*, 2007; Zhou *et al.*, 2007). 10-HDA concentration in the fresh pure royal jelly was 1.65% (Fig. 1). Honey bee larvae were reared with basic larval food (BLD) containing 66.7% royal jelly, 6% fructose, 6% glucose, 1% yeast extract and 20.3% ddH_2O (Kucharski *et al.*, 2008). 10-HDA accounted for 1.10% of the BLD.

Newly hatched larvae were fed with BLD that differed in the quantity of 10-HDA. 10-HDA contents in BLD range from 1.10%, 1.70%, 2.30%, to 2.90%, referred to as control (C), treatment 1 (T1), treatment 2 (T2) and treatment 3 (T3), respectively. The total weight of ddH_2O and the added quantity of 10-HDA accounted for 20.3% of larval food. Larval

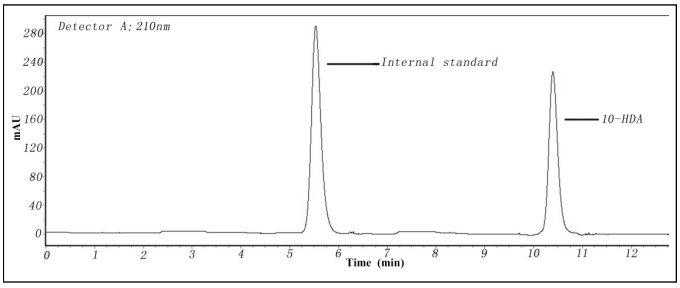

Fig. 1. HPLC chromatogram of 10-HDA and internal standard. Internal standard: methyl-p-hydroxy benzoate. Separation conditions: column Luna 5μm C_{18} (2), 250 × 4.6 mm id, 5μm particle size; mobile phase: (0.03 mol/l HCL)/ methanol/ ddH_2O = 10:55:35 (v/v); flow rate: 1.0ml/min; injection volume: 4μl; detection performed at 210nm.

food for the whole experimental period was prepared upon initiation of study and stored at -80°C. The food was warmed at 35°C for 15 mins before usage.

Larvae rearing

Newly hatched larvae were removed from the comb and transferred to 24-well cell culture plates with 200μl of larval food per well in the incubator. The temperature was maintained at 35°C and humidity was 90%. Larvae were fed every 12 hours with 200μl fresh food per well. When larvae began to defecate, they were removed from the cell culture plates, dried on Kimwipe™ tissues, and transferred to the pupation plates. The pupation plates were placed in an incubator at 35°C and 75% relative humidity, until adult bees emerged (Kucharski *et al.*, 2008).

cDNA synthesis and real-time quantitative PCR (qRT-PCR)

3rd instar larvae were removed from the 24-well cell culture plates and placed gently on pre-cleaned Kimwipes to remove excess food. The larvae were preserved in liquid nitrogen until RNA was extracted (E.Z.N.A.™ Total RNA Kit Ⅱ;OMEGA). RNA concentration and quality were spectrophotometrically measured using NanoPhotometer™ P 300 (IMPLEN). RNA was extracted from pools of five 3rd instar larvae. cDNA was synthesized by mixing 400ng RNA with 3μl (0.5μg/μl) oligo-dT 18 primer (Invitrogen) in 24μl DEPC-treated water and incubated for 10 min at 70°C. Then 10μl 5×RT M-MLV buffer (Takara), 10μl dNTPs (2.5mM each), 1.5μl M-MLV Revertase (50U/μl, Takara), 1.5 μl Ribonuclease inhibitor (50U/μl, TransGen) were added to the RNA extracts and incubated at 42°C for 1h and afterwards for 15 min at 70°C. Lastly, the synthesized cDNA was diluted by 1:10 with DEPC-treated water for subsequent qRT-PCR reactions.

1μl of diluted cDNA was mixed with 5μl of SYBR® *Premix Ex Taq™* II, 0.4μl of specific gene primer (10mM) (Table 1) and 3.2μl of DEPC-treated water for qRT-PCR assay. The qRT-PCR assay was begun with an initial phase of 95°C for 30s, then the temperature cycle

Table 1. LGene-specific primers used in real-time quantitative PCR.

Gene name	Forward primer	Reverse primer	Reference Sequence or References
HDAC 1	TCCTGGCACTGGTGACCTCCG	GCAACCTAACCGATCCCCTGTCA	XM_394976.4
HDAC 3	TTGGAGCAGAAAGTGGGAGA	CTATTGCTGTCGGTTGGAAAA	XM_395811.4
HDAC 4	CATAGCACGATGTTCGGTTGT	CACGTTGTCACGCTTCAGATT	XM_391882.4
HDAC 6	AGAAAGGAATGGGTGATGC	CTAAAGAAGATAGCCAATGTGTC	XM_001121726.2
DNMT 3	AGCCTGGGCTAAAATTGCCGGG	ACGGGAGCAATAATCCTTGGAGGC	NM_001190421.1
GAPDH 1	GCTGGTTTCATCGATGGTTT	ACGATTTCGACCACCGTAAC	Huang *et al.* 2012
GAPDH 56	GGATCAGGAAATTGGGGTTC	CGGAAGCTTATGTCCTGGAA	Huang *et al.* 2012

followed: 95°C for 10s; 60°C for 1 min over 40 cycles. Finally, melting curves were recorded by increasing the temperature from 50°C to 90°C.

Statistical analysis

The Bio-Rad iQ5 2.1 Standard Edition Optical System Software was used to calculate the C_t values and the qpcR package (Spiess and Ritz, 2010; Hornik, 2011) was used to calculate the PCR amplification efficiency of each gene. The relative gene expression level was calculated according to Huang *et al.* (2012). The variance of larvae weight was analysed using ANOVA from the StatView package (v 5.01, SAS Institute, Gary, NC, USA).

Results

The effect of 10-HDA on the weight at adult emergence, development time and survival rate of adult honey bee

1st instar larvae were fed larval food with various concentrations of 10-HDA (1.10, 1.70, 2.30 and 2.90%). Compared with the control group, the weight of freshly emerged bees in the treated groups was significantly decreased ($P < 0.05$). Also within the treated groups the weight of freshly emerged bees decreased with increasing dosage of 10-HDA (Fig. 2). We also obtained data of development time and survival rate (from larvae to adult emergence). There were no significant differences between the control and three treatment groups (Table 2).

Table 2. Effect of 10-HDA on survival rate and developmental time of honey bee. Note: Values are means ± SD. The same letter in the same low indicates no significant difference (P > 0.05).

	Survival rate (%)	Developmental time (days)
Control （C）	82.61±1.26a	21.00±0.28a
Treatment 1（T1）	83.91±2.07a	21.04±0.32a
Treatment 2（T2）	82.05±1.69a	21.00±0.34a
Treatment 3（T3）	83.33±0.96a	21.07±0.28a

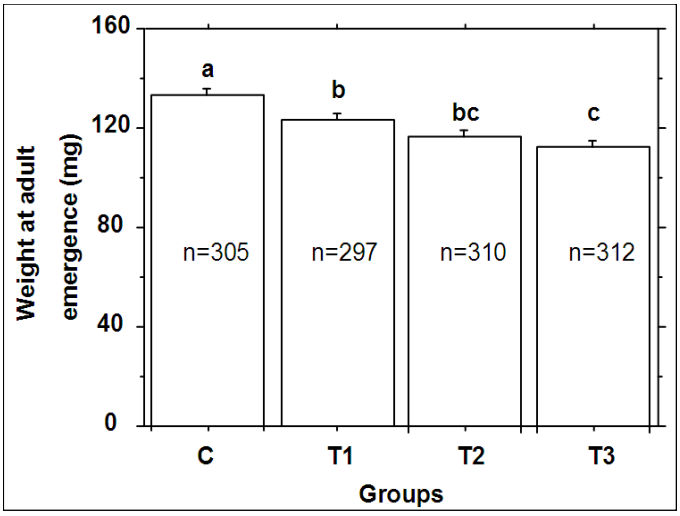

Fig. 2. Effects of 10-HDA on weight of newly emerged honey bees. Values are means ± S.E. Different letters on top of bars indicate significant difference (P < 0.05). n represents the number of newly emerged honey bees in each group. C, T1, T2, T3 represent diets containing 1.10%, 1.70%, 2.30%, 2.90% of 10-HDA respectively.

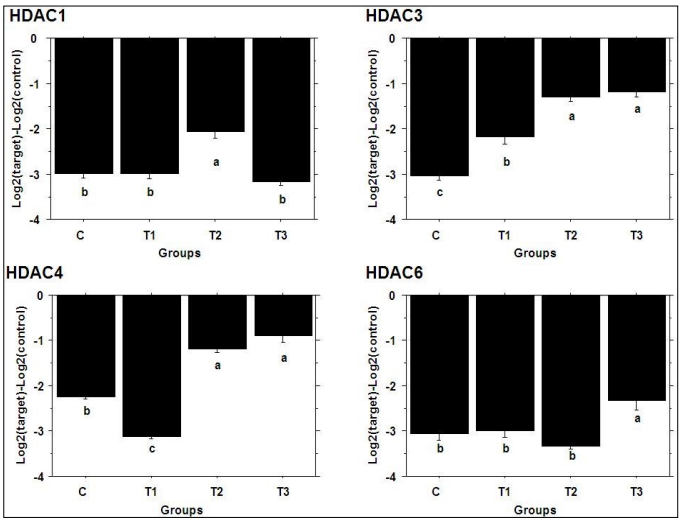

Fig. 3. Effects of 10-HDA on HDACs gene expression levels (log transformed) normalized for two reference genes (GPDH-1 and GPDH-56). Values are means ± S.E. Different letters on bottom of bars indicate significant difference (P < 0.05). In the y axis, target means the expression levels of HDACs gene and control stands for the expression levels of reference genes. C, T1, T2, T3 represent diets containing 1.10%, 1.70%, 2.30%, 2.90% of 10-HDA, respectively.

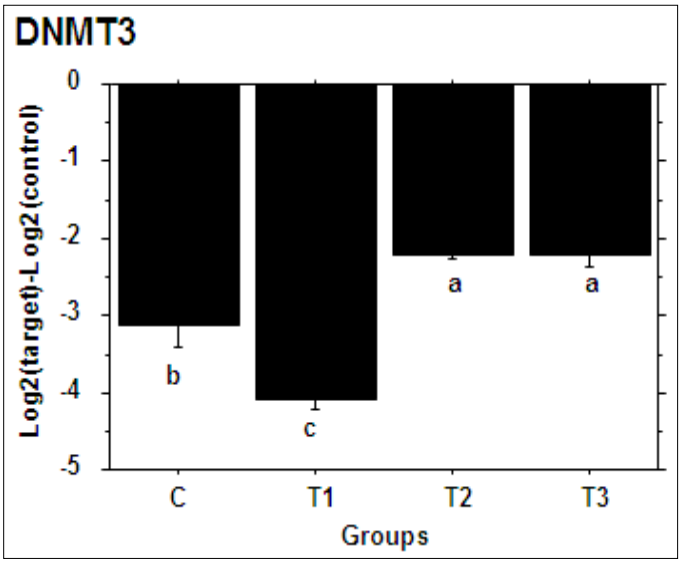

Fig. 4. Effects of 10-HDA on DNMT3 gene expression level (log transformed) normalized for two reference genes (GPDH-1 and GPDH-56). Values are means ± S.E. Different letters on bottom of bars indicate significant difference (P < 0.05). In the y axis, target means the expression level of DNMT3 gene, control stands for the expression levels of reference genes. C, T1, T2, T3 represent diets containing 1.10%, 1.70%, 2.30%, 2.90% of 10-HDA, respectively.

Expression levels of HDACs genes and DNMT3 gene

HDAC3 expression was significantly up-regulated in treated groups ($P < 0.05$) (Fig. 3). Compared to the control group, HDAC4 was initially significantly down-regulated, however significant up-regulation in treated groups was subsequently observed ($P < 0.05$). The genes of HDAC1 and HDAC6 were up-regulated significantly in T2 and T3 groups, respectively. The expression level of DNMT3 gene was similar with HDAC4, initial significant down-regulation followed by significant up-regulation in treated groups compared to the control (Fig. 4).

Discussion

In this study, we provided experimental evidence for an association between 10-HDA and growth of honey bee larvae. Furthermore, the expression levels of HDACs and DNMT3 were significantly regulated by the dosage of 10-HDA fed to developing larvae. The results suggest 10-HDA not only reduces the weight of newly emerged workers, but also regulates the activation of HDAC3 and DNMT3 that control epigenetic changes.

The differences in emergence weight of 10-HDA-treated bees compared to controls were significant. Emergence weight reduced with increasing dosage of 10-HDA fed to the larvae. 10-HDA inhibits the activity of HDACs, which might increase histone acetylation. Acetylation of ε–amino groups of lysine residues within histone tails relaxes chromatin structure, and increases the accessibility of transcription factors to their target genes (Shahbazian and Grunstein, 2007). Therefore, increasing levels of histone acetylation are generally associated with gene expression. The cyclin-dependent kinase (CDK) inhibitor p21, which plays an important role in the arrest of cell growth, is one of the most consistently induced genes by HDACi (Archer *et al.*, 1998; Richon *et al.*, 2000; Gui *et al.*, 2004). In the development stages of honey bee larvae, 10-HDA might increase the expression of p21 gene and arrest cell growth. Consequently, the weight of newly emerged honey bees is reduced.

In the honey bee (*Apis mellifera*) genome, four HDAC genes have been identified so far (Spannhoff *et al.*, 2011). These HDACs are classified into two classes based on their homology to yeast proteins (Bolden *et al.*, 2006; Ma and Schultz, 2008). Class I includes HDAC1 and HDAC3 and have homology to yeast RPD3. HDAC4 and HDAC6 belong to class II and share domains with similarity to yeast HDA1. HDAC1 is the most thoroughly studied HDAC at a functional level. It has been reported that HDAC1 in yeast can deacetylate all four core histones and serve as a direct transcriptional repressor (Robyr *et al.*, 2002; Kurdistani and Grunstein, 2003). In *Drosophila* and colon cancer cell lines, silencing of HDAC3 leads to cell growth inhibition (Foglietti *et al.*, 2006; Wilson *et al.*, 2006). HDAC4 interacts specifically with and represses the myocyte enhancer transcription factor (MEF2) and Runt related transcription factor 2 (RUNX2) which play essential roles in the control of chondrocyte hypertrophy and bone formation (Vega *et al.*, 2004; Cohen Jr, 2006). HDAC6 is the main gene involved in tubulin deacetylase and has important functions in modulating the misfolded protein response and cytoskeletal dynamics (Zhang *et al.*, 2008; Haberland *et al.*, 2009). All four HDACs contain zinc in their catalytic site and HDACi (such as 10-HDA) could chelate the zinc atom in the active site of HDACs. In this study, we found that 10-HDA also has an impact on transcription level of HDACs, which is consistent with a previous report (Dokmanovic *et al.*, 2007). Compared with HDAC1 and HDAC6 genes, 10-HDA had a stronger effect on HDAC3 and HDAC4 genes. This result suggests that although 10-HDA can alter the expression of four HDAC genes, the minimum concentration required to elicit an effect on each HDAC gene varies is different. Moreover there was a negative relationship between emergent weight of bees and the expression levels of HDAC3. It has been reported that silencing of HDAC3 stimulates p21 promoter activity and expression level (Wilson *et al.*, 2006). The reduced weight of emerged honey bees might be due to the inhibition of HDAC3 activity by 10-HDA.

It is well known that the developmental processes of honey bees are epigenetically influenced. Accumulating evidence suggests that the interaction between histone acetylation and DNA methylation are involved in developmental differentiation (Dobosy and Selker, 2001; Miller *et al.*, 2008). In this study, we found that 10-HDA has a significant influence on the expression level of DNMT3. What is more, the expression level of DNMT3 correlated with HDAC4, which means the activity of HDAC4 may have a vital effect on transcription of DNMT3. There is an

existing notion that royal jelly does not harbour the activity to inhibit DNA methylation (Spannhoff *et al.*, 2011). Therefore, DNA methylation may not be directly affected by royal jelly. DNA methyltransferases may even take cues from 10-HDA in the developmental processes of honey bee larvae, adjusting the expression level. Interestingly, when the concentration of 10-HDA is increased up to 1.70% in larval food, equivalent with the 10-HDA content in royal jelly, the expression level of DNMT3 in worker larvae was significantly decreased, in addition to the expression level of DNMT3 in queen-destined larvae (Kucharski *et al.*, 2008). This suggests that acetylation influences DNA methylation and the development of honey bee larvae.

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