Royalactin induces queen differentiation in honeybees

Masaki Kamakura

The honeybee (Apis mellifera) forms two female castes: the queen and the worker. This dimorphism depends not on genetic differences, but on ingestion of royal jelly, although the mechanism through which royal jelly regulates caste differentiation has long remained unknown. Here I show that a 57-kDa protein in royal jelly, previously designated as royalactin, induces the differentiation of honeybee larvae into queens. Royalactin increased body size and ovary development and shortened developmental time in honeybees. Surprisingly, it also showed similar effects in the fruitfly (Drosophila melanogaster). Mechanistic studies revealed that royalactin activated p70 S6 kinase, which was responsible for the increase of body size, increased the activity of mitogen–activated protein kinase, which was involved in the decreased developmental time, and increased the titre of juvenile hormone, an essential hormone for ovary development. Knockdown of epidermal growth factor receptor (Egfr) expression in the fat body of honeybees and fruitflies resulted in a defect of all phenotypes induced by royalactin, showing that Egfr mediates these actions. These findings indicate that a specific factor in royal jelly, royalactin, drives queen development through an Egfr-mediated signalling pathway.

Caste in social insects represents one of the major transitions from one level of organization to another in evolution. The honeybee (Apis mellifera) exhibits polyphenism, that is, adult females form two interdependent castes, the queen and the worker, depending on their environment at critical periods of caste determination. This dimorphism is not a consequence of genetic difference. Queens have a larger body size and shorter developmental time than workers, have ten times the lifespan of workers, typically 1 to 2 years, and lay up to 2,000 eggs per day, whereas workers rear young larvae and gather nectar. When larvae are nourished with royal jelly, which is secreted by workers, they differentiate into queens. Royal jelly seems to contain a specific factor(s) that determines caste differentiation, but this has not previously been identified. Furthermore, the relationship between caste-specific modulation of juvenile hormone and ecdysteroid after ingestion of royal jelly and the developmental signal in caste differentiation has remained elusive. Therefore, I aimed to identify the factor(s) that induces caste differentiation in the honeybee and to investigate the mechanism through which this factor drives the caste-specific developmental pathway.

A caste differentiation–inducing factor in royal jelly

The dietary requirements for rearing queens are known, but a diet for rearing worker honeybees has not been reported. In connection with this, I found that larvae reared with royal jelly stored at 40 °C for 7 days, which did not exhibit any antifatigue effect, showed increased developmental times, decreased body weight at eclosion and decreased ovary size, compared to larvae fed a diet containing fresh royal jelly, even though they were queen–worker intermediates (Supplementary Fig. 1a–c). This result indicated that long-term storage of royal jelly at high temperature decreases the biological activity of royal jelly for queen differentiation. Therefore, royal jelly was stored at 40 °C for 7, 14, 21 and 30 days, and the effects of these royal jelly samples on caste differentiation were examined. Storage of royal jelly at 40 °C for up to 30 days caused a reduction in the growth of developing larvae, decreased weight at adult emergence, ovary size reduction and prolongation of the pre-adult development time in proportion to storage duration (Supplementary Fig. 1). Adult females reared with royal jelly stored at 40 °C for 30 days (40 °C/30 d royal jelly) developed with a full worker morphotype. These results indicate that the putative inducer of queen differentiation in royal jelly might be gradually degraded in proportion to the storage period at 40 °C, being completely degraded after 30 days. Therefore, the compositional changes in royal jelly during storage were investigated next.

First, the contents of several vitamins, 10-hydroxy-2-decenonic acid, carbohydrates and fatty acids in royal jelly samples stored at 4 °C and 40 °C for 30 days were measured. No significant differences were observed in the contents of the examined compounds, except pantothenic acid, which showed a decrease to 60% of the initial concentration during storage at 40 °C for 30 days (Supplementary Table 1). However, pantothenic acid did not induce the emergence of queens (data not shown), in agreement with a previous report. Next, compositional changes of proteins in royal jelly during storage were analysed by means of high-performance liquid chromatography (HPLC) and native polyacrylamide gel electrophoresis (PAGE). A 450-kDa protein, a 170-kDa protein and a 57-kDa protein (designated as royalactin) were degraded during storage (Supplementary Fig. 2a and Supplementary Fig. 3). Royalactin is a monomeric protein that exhibits epidermal growth factor (Egfr)-like effects on rat hepatocytes. The 170-kDa protein was completely degraded during storage at 40 °C for 21 or 30 days; because this royal jelly can still influence ovary development and growth of developing larvae, the 170-kDa protein seems to be irrelevant to caste differentiation (Supplementary Figs 1 and 2a). Royalactin was degraded proportionally to the storage period at 40 °C, being completely degraded after 30 days, whereas only 10% of the 450-kDa protein was destroyed during storage at 40 °C for 30 days, whereas only 10% of the 450-kDa protein was destroyed during storage at 40 °C for 30 days (Supplementary Fig. 2a).

Next, royalactin and the 450-kDa protein were purified (Supplementary Fig. 2b–d), and the effects of these factors on caste differentiation were examined in the same manner described above. As
Effects of royal jelly and royalactin on *Drosophila* species

Because no mutant stock of *Apis mellifera* has so far been developed, it is difficult to investigate the mechanism underlying honeybee caste differentiation at the individual level. On the other hand, fruitfly (*Drosophila melanogaster*), used as a model organism in many research fields, is available for genetic analysis in developmental biology. I considered that *Drosophila* might be suitable as a model insect for analysis of the mechanism of caste differentiation if royal jelly induced morphological and physiological changes in *Drosophila* similar to those induced in honeybee queens. Therefore, I investigated the influence of royal jelly on *Drosophila* larvae.

When *Drosophila* (Canton-S) larvae were reared with only royal jelly, they died before pupation (data not shown). However, *Drosophila* reared with medium containing 20% royal jelly, 8% yeast and 10% D-glucose had an increase in body size (body weight and body length) and fecundity, and had extended lifespan and shortened developmental time compared to flies reared with control medium or casein medium, which provide the same total energy as royal jelly medium (Fig. 2 and Supplementary Table 2). Furthermore, royal jelly medium increased cell size but not cell number (Supplementary Fig. 5). Royalactin increased body size, cell size and fecundity, extended lifespan and shortened developmental time in flies reared with 40 °C/30 d royal jelly (which did not influence morphological or physiological changes of flies), whereas 450-kDa protein or casein did not (Fig. 2, Supplementary Fig. 5 and Supplementary Table 2), in accordance with the observations that royalactin induced queen differentiation in honeybee as the major active factor in royal jelly. Thus, fresh royal jelly led genetically identical fly larvae to develop into adult individuals with phenotypes similar to queen bees, indicating that *Drosophila* could be used as a model insect for genetic analysis of caste differentiation.

**Royalactin changes *Drosophila* phenotypes via Egfr**

The insulin signalling pathway in metazoans has an important role in regulating body size, growth and metabolism. I used *Escherichia coli* and purified to homogeneity on SDS–PAGE (Fig. 1, Supplementary Fig. 2e and Supplementary Fig. 4a). Furthermore, royalactin and *E*-royalactin increased the juvenile hormone titre—which increases at the fourth larval instar to cause development into a queen—which increased in larvae given 40 °C/30 d royal jelly, and it induced larvae to develop into queens as effectively as did royal jelly at the concentration of 2.0% w/w diet (Fig. 1 and Supplementary Fig. 4a). Similar results were observed in larvae reared with recombinant royalactin (*E*-royalactin; 47 kDa), which was expressed in *Escherichia coli* and purified to homogeneity on SDS–PAGE (Fig. 1, Supplementary Fig. 2e and Supplementary Fig. 4a). Furthermore, royalactin and *E*-royalactin increased the juvenile hormone titre—which increases at the fourth larval instar to cause development into a queen—which increased in larvae given 40 °C/30 d royal jelly as potently as royal jelly, whereas the 450-kDa protein or casein had no effect (Supplementary Fig. 4b). Taken together, these results indicate that the stimulatory effect of royalactin on caste differentiation was not a nutritional effect but a morphogenetic effect, and that royalactin is the major active factor in the induction of caste differentiation by royal jelly.

**Effects of royal jelly and royalactin on *Drosophila***

Because no mutant stock of *Apis mellifera* has so far been developed, it is difficult to investigate the mechanism underlying honeybee caste differentiation at the individual level. On the other hand, fruitfly (*Drosophila melanogaster*), used as a model organism in many research fields, is available for genetic analysis in developmental biology. I considered that *Drosophila* might be suitable as a model insect for analysis of the mechanism of caste differentiation if royal jelly induced morphological and physiological changes in *Drosophila* similar to those induced in honeybee queens. Therefore, I investigated the influence of royal jelly on *Drosophila* larvae.

When *Drosophila* (Canton-S) larvae were reared with only royal jelly, they died before pupation (data not shown). However, *Drosophila* reared with medium containing 20% royal jelly, 8% yeast and 10% D-glucose had an increase in body size (body weight and body length) and fecundity, and had extended lifespan and shortened developmental time compared to flies reared with control medium or casein medium, which provide the same total energy as royal jelly medium (Fig. 2 and Supplementary Table 2). Furthermore, royal jelly medium increased cell size but not cell number (Supplementary Fig. 5). Royalactin increased body size, cell size and fecundity, extended lifespan and shortened developmental time in flies reared with 40 °C/30 d royal jelly (which did not influence morphological or physiological changes of flies), whereas 450-kDa protein or casein did not (Fig. 2, Supplementary Fig. 5 and Supplementary Table 2), in accordance with the observations that royalactin induced queen differentiation in honeybee as the major active factor in royal jelly. Thus, fresh royal jelly led genetically identical fly larvae to develop into adult individuals with phenotypes similar to queen bees, indicating that *Drosophila* could be used as a model insect for genetic analysis of caste differentiation.

**Royalactin changes *Drosophila* phenotypes via Egfr**

The insulin signalling pathway in metazoans has an important role in regulating body size, growth and metabolism. I used *Escherichia coli* and purified to homogeneity on SDS–PAGE (Fig. 1, Supplementary Fig. 2e and Supplementary Fig. 4a). Furthermore, royalactin and *E*-royalactin increased the juvenile hormone titre—which increases at the fourth larval instar to cause development into a queen—which increased in larvae given 40 °C/30 d royal jelly, and it induced larvae to develop into queens as effectively as did royal jelly at the concentration of 2.0% w/w diet (Fig. 1 and Supplementary Fig. 4a). Similar results were observed in larvae reared with recombinant royalactin (*E*-royalactin; 47 kDa), which was expressed in *Escherichia coli* and purified to homogeneity on SDS–PAGE (Fig. 1, Supplementary Fig. 2e and Supplementary Fig. 4a). Furthermore, royalactin and *E*-royalactin increased the juvenile hormone titre—which increases at the fourth larval instar to cause development into a queen—which increased in larvae given 40 °C/30 d royal jelly as potently as royal jelly, whereas the 450-kDa protein or casein had no effect (Supplementary Fig. 4b). Taken together, these results indicate that the stimulatory effect of royalactin on caste differentiation was not a nutritional effect but a morphogenetic effect, and that royalactin is the major active factor in the induction of caste differentiation by royal jelly.
rapamycin (TOR) downstream of PI3K/PDK1/Akt through signalling, but rather Egfr signalling in the fat body was implicated (Supplementary Fig. 7). These findings demonstrated that not InR Drosophila Egfr was expressed in the fat body of the wild-type flies.

To examine the stimulatory action of royalactin on Drosophila further, I investigated how Egfr signals regulate changes of body size and developmental time in response to royalactin. a Body size of female adult flies reared with casein medium (8% yeast, 11.3% D-glucose, 2.8% casein, 1.3% D-fructose, 0.4% cornstarch, 0.76% soybean oil) and royal jelly medium (8% yeast, 10% D-glucose, 20% royal jelly). b, dBody length (b, left), body weight (b, right), fecundity (c) and longevity (d) in wild-type (CS) fruit flies reared with control medium or casein medium (Supplementary Fig. 8). Royal jelly did not increase the body size of flies reared with control medium or casein medium (Supplementary Table 5 and data not shown). These results are consistent with the findings in flies reared with royal jelly.

I next investigated how Egfr signals regulate changes of body size and developmental time in response to royal jelly. Royal jelly or royalactin activated S6K—which is activated by both phosphatidylinositol-dependent kinase 1 (PDK1) downstream of PI3K and target of rapamycin (TOR) downstream of PI3K/PDK1/Akt through stimulation of Egfr23–26—and mitogen-activated protein kinase (MAPK) in the larval fat body, and the activation of these enzymes by royalactin was suppressed by Drosophila Egfr RNAi in the fat body (Supplementary Fig. 8). Royal jelly did not increase the body size of ppl>dP13KDN (Drosophila P13K dominant-negative), ppl>dPDK1RNAi, ppl>dAktRNAi, ppl>dTORDN or ppl>dS6KDNN flies, but shortened their developmental time, whereas ppl>dRafRNAi and ppl>dMKP3 (ERK-inhibitory phosphatase)27 reared with royal jelly showed increased body size but no early eclosion compared to the mutants reared with control medium or casein medium (Supplementary Fig. 6, Supplementary Table 3 and Supplementary Table 4). The increase of cell size in flies reared with royal jelly was repressed in ppl>dEgrRNAi and ppl>dS6KDNN, but not ppl>dMKP3 (Supplementary Fig. 9). Loss of S6K function in Drosophila reduces body size by decreasing cell size but not cell number28. Activity of the MAPK pathway is reported to be unaffected by nutrients29. These results indicate that royalactin activated S6K through Egfr in the fat body, acting as a morphogenetic factor to increase body size through an increase of cell size, and it also activated the MAPK pathway in the fat body to reduce the developmental time in Drosophila.

Drosophila phenotypes change in response to royalactin overexpression

To examine the stimulatory action of royalactin on Drosophila further, I investigated the effect of overexpression of royalactin using the UAS/Gal4 system30. Surprisingly, act>royalactin showed increased body size, cell size, fecundity and longevity and shortened developmental time compared with UAS-royalactin (Fig. 3b–d, Supplementary Fig. 10 and Supplementary Table 5). Moreover, overexpression of royalactin specifically in the fat body or an Egfr signal using ppl-Gal4 or Gal4 driver of rhomboid (rho), which is the essential signal-generating component of Egfr signalling during development in Drosophila31, induced the same phenotypes as act>royalactin (Fig. 3, Supplementary Fig. 10 and Supplementary Table 5). Royal jelly proteins were reported to contain royalactin, identical to major royal jelly protein (MRJP)1 and MRJP2–5 (ref. 32). I overexpressed mrjp2–5 with act-Gal4, rho-Gal4 and ppl-Gal4, and found that the body sizes of these mutants overexpressing mrjp2–5 did not change (Supplementary Table 6). Overexpression of royalactin activated MAPK and S6K in the fat body of larvae, and this activation was inhibited by Drosophila Egfr RNAi (Supplementary Fig. 11). On the other hand, when royalactin was overexpressed with P0206-Gal4 or Aug21-Gal4, it did not influence body size or developmental time of the mutants (Fig. 3b and Supplementary Table 5). Increase of body size and cell size in ppl>royalactin and rho>royalactin was suppressed by inhibition of Egfr and S6K, but not by abrogation of InR and MAPK (Fig. 3b, Supplementary Fig. 10 and data not shown). Reduction of developmental time in ppl>royalactin and rho>royalactin was suppressed by inhibition of Egfr and MAPK, but not by inhibition of S6K (Supplementary Table 5 and data not shown). These results are consistent with the findings in flies reared with royal jelly.
Royalactin changes hormone metabolism in Drosophila

To investigate the relationship between the morphological and physiological changes induced by royalactin in flies and hormone modulation, I measured changes in the biosynthesis of a biologically active ecdysteroid, 20-hydroxyecdysone (20E), and juvenile hormone in wild-type flies given royal jelly during the larval period. Moreover, changes in gene expression of yolk protein (yp) during larval development were examined because juvenile hormone induces expression in the fat body of yp, which is essential for vitellogenesis, thereby promoting egg production in Drosophila. Royal jelly and royalactin increased the 20E titre at 3 days after egg deposition (AED), and juvenile hormone titre and gene expression of yp at 4 days AED (Supplementary Fig. 12 and Supplementary Fig. 13). The increase of 20E titre in flies reared with royal jelly was suppressed in pplRNAi and pplRNAi > dMKP3, but not pplRNAi > dS6KDN (Supplementary Fig. 14a), indicating that activation of MAPK downstream of Egfr in the fat body by royalactin induced 20E synthesis to shorten the developmental time. On the other hand, the increase of juvenile hormone titre, gene expression of yp and fecundity by royal jelly was repressed in pplRNAi > dEgfrRNAi, but not pplRNAi > dS6KDN or pplRNAi > dMKP3 flies (Supplementary Fig. 14b–d and Supplementary Fig. 15). Because repression of MAPK in the fat body (pplRNAi > dMKP3) did not abrogate the increase of yp expression and fecundity, the increase of 20E by royalactin seemed not to be associated with the increase of yp expression and oviposition. Taken together, these findings indicated that Egfr signalling in the fat body is activated by royalactin via a pathway distinct from that regulating body size and developmental time, leading to induction of juvenile hormone synthesis and a consequent increase of yp expression, thereby increasing fecundity. S6K in the fat body also seemed to be associated only with the increase of body size by royal jelly.

On the other hand, increase of fecundity in flies with overexpression of royalactin was also repressed by Drosophila Egr RNAi in the fat body but not by suppression of S6K and MAPK in the fat body (Fig. 3c and data not shown). These results were consistent with those obtained in flies reared with royal jelly. Increase of longevity induced by royal jelly was also abrogated in pplRNAi > dEgfrRNAi flies, but not pplRNAi > dS6KDN or pplRNAi > dMKP3 flies, indicating that Egfr in the fat body was essential for the increase of longevity in flies reared with royal jelly (Supplementary Fig. 14e and Supplementary Fig. 16a, b). Similar results were seen in the case of overexpression of royalactin (Fig. 3d and data not shown).

Suppression of queen differentiation in honeybees with RNAi

To confirm the signalling pathway involved in caste development, I reared honeybee larvae with suppression of Apis mellifera InR (InR) and Egfr by RNAi. Knockdown of InR did not affect final adult size, developmental time or ovary size in individuals reared with royal jelly, including a double-stranded RNA for green fluorescent protein (GFP), a control of RNAi, whereas Egfr RNAi reduced adult size and ovary size, and prolonged developmental time, compared with the control (GFP) (Fig. 4 and Supplementary Fig. 17a). These inhibitory effects of Egfr RNAi on queen differentiation were also observed in individuals reared with royalactin (data not shown). Royalactin activated MAPK and S6K through Egfr in fat body of honeybee larvae as effectively as did royal jelly (Supplementary Fig. 18). These results indicate that the activation of Egfr by royalactin is also involved in caste differentiation in the honeybee. Furthermore, suppression of honeybee PI3K, PDKI, TOR and S6K with RNAi inhibited the increase to final adult size induced by royal jelly, but did not affect changes of developmental time or ovary development (Fig. 4, Supplementary Fig. 17a and Supplementary Fig. 19). Royal jelly or royalactin increased the 20E titre in 3-day-old honeybee larvae, and the juvenile hormone titre and gene expression of vitellogenin (vg), a precursor of yp, in 4-day-old honeybee larva given 40°C/30 d royal jelly, whereas the 450-kDa protein and casein did not (Supplementary Fig. 4 and Supplementary Fig. 20). Increase of the 20E titre in honeybee larvae reared with royal jelly was abolished by Egfr RNAi and PD98059, a MAPK inhibitor, but not S6K RNAi (Supplementary Fig. 20a). PD98059 prolonged developmental time in larvae reared with royal jelly (data not shown). Increase

Figure 3 | Morphological and physiological changes of Drosophila melanogaster induced by overexpression of royalactin. a. Body size of female adult flies without or with overexpression of royalactin in the fat body (UAS-Rol or ppl > Rol). b. Body length (left) and body weight (right) in flies with overexpression of royalactin and in the signal factor suppression mutants in the royalactin overexpression background. n > 40. c. d. Fecundity (c) and longevity (d) in flies with overexpression of royalactin and in Drosophila Egfr interference (dEgfr) mutants in the royalactin overexpression background (n > 50). Values are expressed as mean ± s.e.m. Values significantly different from those of UAS-royalactin are indicated by **p < 0.01.
induced queen differentiation in the honeybee. Thus, only royalactin, a monomer of MRJP1, functions as a caste determination factor. Royalactin induced prolonged longevity through Egfr in Drosophila, indicating that royalactin might have an important role in the prolongation of longevity in queens. To my knowledge, this is the first evidence that Egfr is involved in the regulation of longevity. Further research will be required to investigate the mechanism through which royalactin regulates lifespan in the fruitfly and the honeybee.

The association between royal jelly and caste formation has been known for more than 100 years, but the identity of the component(s) in royal jelly that induces queen development has been elusive. My results provide important insights into the process of caste development in the honeybee, and may also offer a valuable clue to eusociality and the evolution of social hymenopterans.

METHODS SUMMARY

Fly larvae were reared with medium containing royal jelly, D-glucose, yeast and agar at 25 °C. Honeybee larvae were reared with medium containing royal jelly, D-glucose, D-fructose and yeast extract at 34 °C with 96% humidity. Quantitative assay of juvenile hormone was carried out by high-resolution liquid chromatography-mass spectrometry (LC-MS) on a micrOTOF-Q instrument. The 20E titre of larvae was determined by the enzyme immunoassay (EIA) method. Quantitative analysis of gene expression was conducted by real-time PCR with the primers shown in Supplementary Tables 12 and 13. For honeybee RNAi experiments, the rearing diet containing enzymatically synthesized dsRNA at 150 μg ml⁻¹ was administered to second instar larvae for 2 days.

Received 2 June 2010; accepted 5 April 2011. Published online 24 April 2011; corrected 26 May 2011 (see full-text HTML version for details).


Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements I thank D. Yamamoto for provision of general fruitfly treatment methods and helpful advice; and S. Hayashi and T. Adachi-Yamada for instruction of dissection techniques in Drosophila. I thank T. Nonogaki and Y. Hasada for supply of honeybee larvae; K. Yu, M. Tatar, P. Leopold, G. Korge, Y. T. Ips, T. G. Wilson and D. Yamamoto for fly stocks. We are grateful to T. Oda for the gift of royal jelly, and to W. R. S. Steele for proofreading the article.

Author Contributions M.K. designed the research and performed the experiments. M.K. wrote the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to M.K. (kamakura@pu-toyama.ac.jp).