

# Royalactin induces queen differentiation in honeybees

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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<sup>1</sup>. 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<sup>2,3</sup>. This dimorphism is not a consequence of genetic difference<sup>4,5</sup>. 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 2,000 eggs per day, whereas workers rear young larvae and gather nectar<sup>6,7</sup>. When larvae are nourished with royal jelly, which is secreted by workers<sup>2,3</sup>, 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

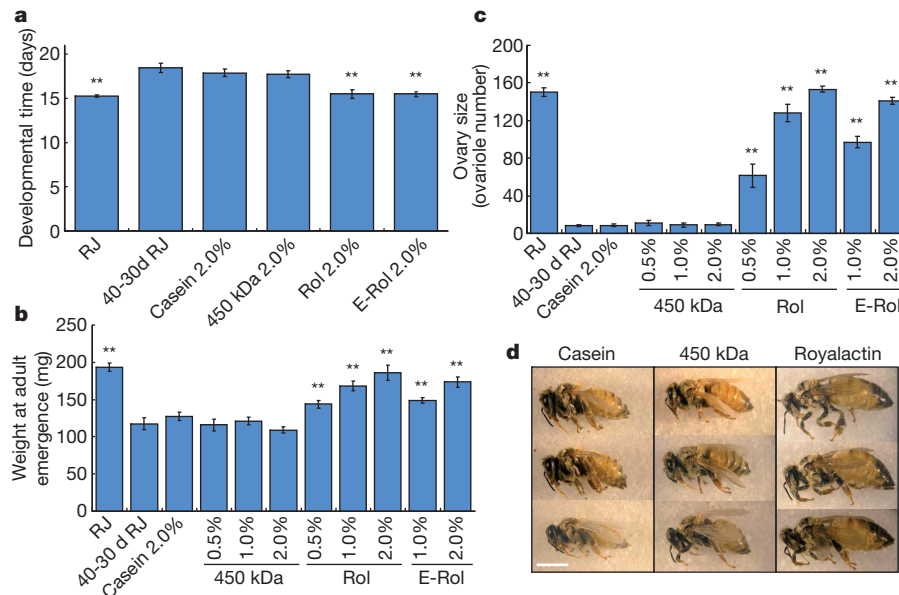
The dietary requirements for rearing queens are known<sup>8</sup>, but a diet for rearing worker honeybees has not been reported. In connection with this, I found that larvae reared with royal jelly stored at 40 °C for 7 days, which did not exhibit any antifatigue effect<sup>9</sup>, showed increased 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-decenoic 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<sup>10</sup>. 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<sup>11</sup>) were degraded during storage (Supplementary Fig. 2a and Supplementary Fig. 3). Royalactin is a monomeric protein that exhibits epidermal growth factor (Egf)-like effects on rat hepatocytes<sup>11,12</sup>. The 170-kDa protein was completely degraded during storage at 40 °C for 14 days, being undetectable in royal jelly stored 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 period of storage, and was completely lost 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

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**Figure 1 | Effects of casein, 450-kDa protein, royalactin and recombinant royalactin on caste characters in the honeybee.** a–c, Developmental time (a), weight at adult emergence (b) and ovary size (c) in individuals ( $n = 10$ –28) reared with royal jelly (RJ), royal jelly stored at 40 °C for 30 days (40-30d RJ) or 40-30d RJ containing casein, 450-kDa protein, royalactin (Rol) or E-royalactin

shown in Fig. 1, the 450-kDa protein (0.5% to 2.0% w/w diet) and casein (2.0% w/w diet), which was used as a control for evaluating nutritional effect, did not change the final adult size, developmental time, or ovary size in individuals reared with 40 °C/30 d royal jelly. In contrast, royalactin shortened developmental time and increased both weight at adult emergence and ovary size in proportion to the concentration added to a diet containing 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<sup>13,14</sup>—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 morphogenic 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

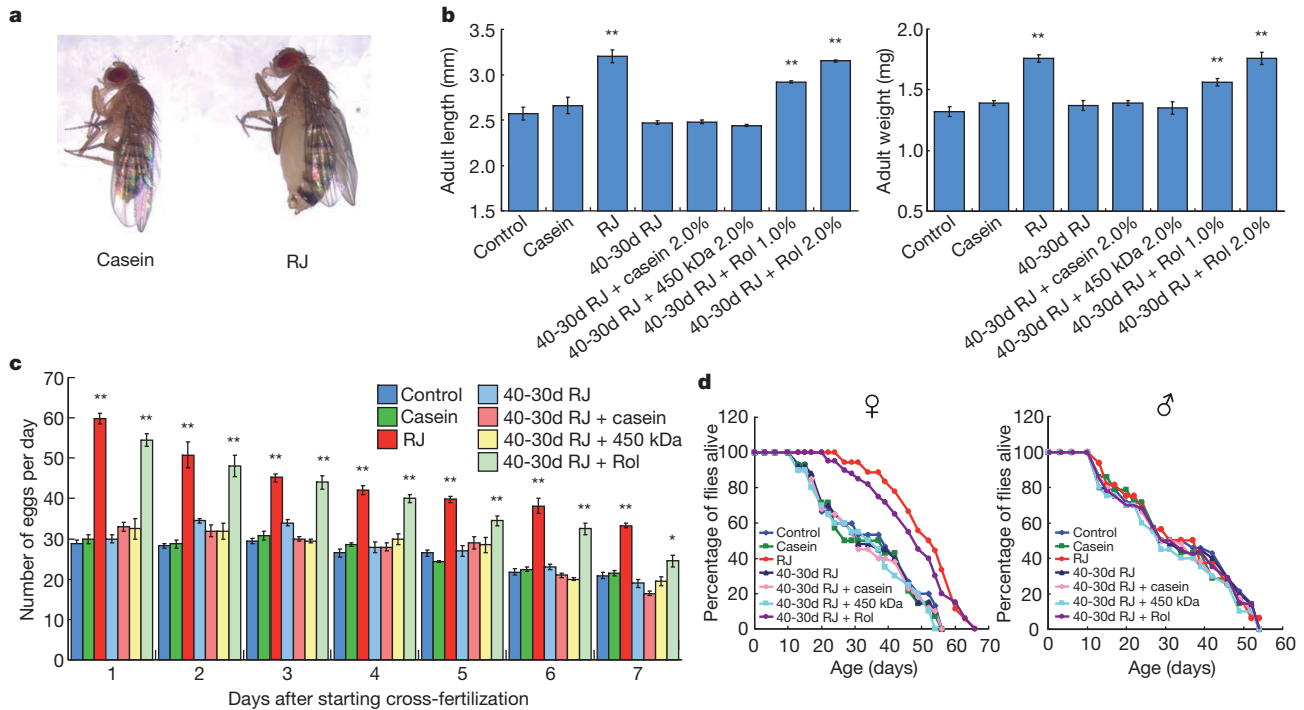
(E-Rol) were measured. d, Final adult size after eclosion is shown. Values are expressed as mean  $\pm$  s.e.m. Values significantly different from those of larvae reared with 40-30d RJ are indicated by \*\* $P < 0.01$ . Royalactin accounted for approximately 2.0% of RJ. Scale bar, 5 mm.

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<sup>15,16</sup>. First, I examined the effects of royal jelly on body size of *insulin receptor* (*InR*) mutants (*InR<sup>E19</sup>/InR<sup>E19</sup>* and *InR<sup>p5545</sup>/InR<sup>E19</sup>*)<sup>15</sup> and mutant showing elevated levels of phosphatidylinositol-3 kinase (PI3K) activity in prothoracic gland and corpora allata with *P0206-Gal4* (*P0206>dP13K*)<sup>16</sup>, all of which show reduced body size and weight. The *InR* mutants and *P0206>dP13K* reared with royal jelly medium had larger body size and shorter developmental time than individuals reared with control medium or casein medium (Supplementary Fig. 6 and Supplementary Table 3).

I previously found that royalactin functions similarly to Egf in rat hepatocytes<sup>11,12</sup>. Therefore, I investigated the effects of royal jelly on body size of *Epidermal growth factor receptor* (*Egfr*) mutants (*Egfr<sup>tsla</sup>/Egfr<sup>J24</sup>*)<sup>17</sup>. Royal jelly did not influence body size or developmental time in the *Egfr* mutants (Supplementary Fig. 6 and Supplementary Table 3). Next, to determine the tissue specificity of royal jelly action in flies, I examined the influence of royal jelly on body size and developmental time in mutants in which expression of *Egfr* was silenced in the prothoracic gland, corpora allata or fat body, which are involved in body size regulation of *Drosophila*<sup>16,18–21</sup>. I used *Aug21-Gal4* (ref. 19) or *pumless* (*pp1*)-*Gal4* (refs 16, 22) as a line with specific *Gal4* expression in corpora allata or fat body, respectively. Royal jelly increased body size and shortened developmental time in *P0206>dEgfrRNAi*



**Figure 2 | Morphological and physiological changes of *Drosophila melanogaster* induced by royal jelly and 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–d**, Body length (**b**, left), body weight (**b**, right), fecundity (**c**) and longevity (**d**) in wild-type (CS) fruit flies

reared with control medium (8% yeast, 10% D-glucose), casein medium, royal jelly (RJ) medium or medium containing royal jelly stored at 40 °C for 30 days (40-30d RJ) or 40-30d RJ with casein, 450-kDa protein or royalactin (Rol) ( $n > 40$ ). Values are expressed as mean  $\pm$  s.e.m. Values significantly different from those of flies reared with control medium are indicated by \* $P < 0.05$  or \*\* $P < 0.01$ .

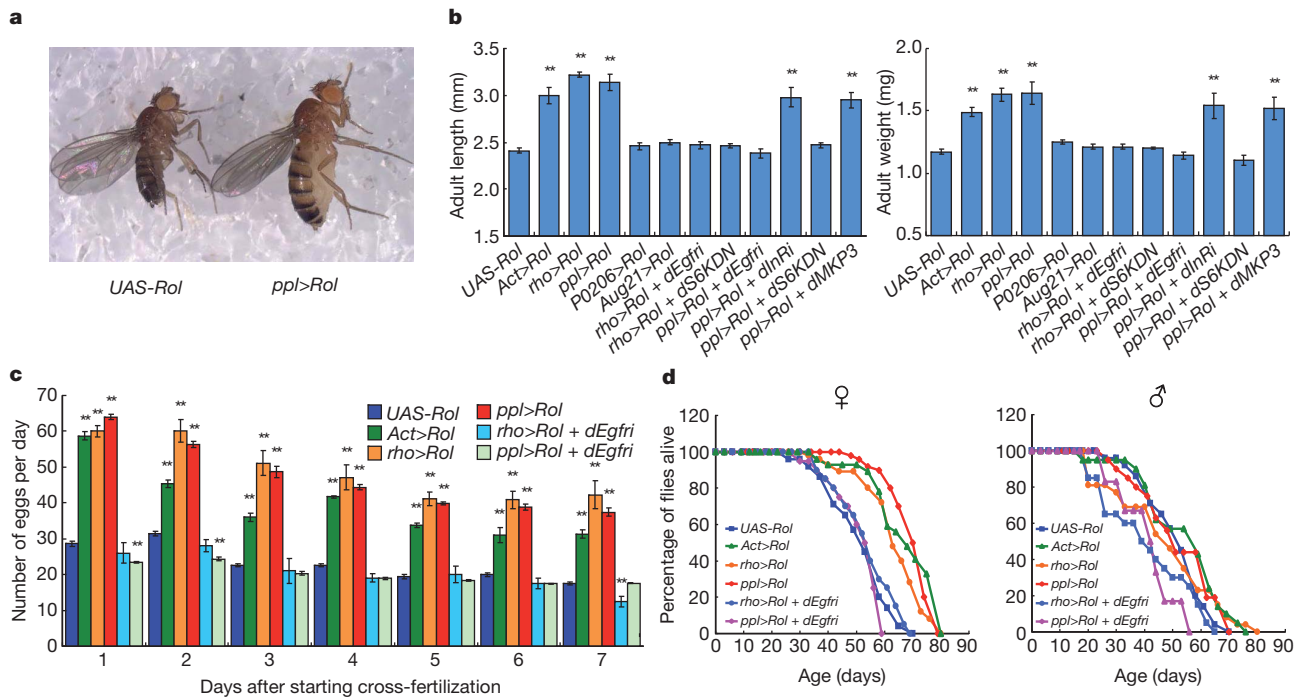
flies (an RNA interference (RNAi) line for *Drosophila* Egfr and *Aug21>dEgfrRNAi* flies, whereas it did not affect body size or developmental time in *ppl>dEgfrRNAi* flies (Supplementary Fig. 6 and Supplementary Table 3). Similar results were observed in *ppl>dEgfrRNAi* flies reared with royalactin (data not shown). I confirmed that *Drosophila* Egfr was expressed in the fat body of the wild-type flies (Supplementary Fig. 7). These findings demonstrated that not InR signalling, but rather Egfr signalling in the fat body was implicated in the increase of body size and reduction of developmental time by royal jelly or royalactin.

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 Egfr<sup>23–26</sup>—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>dPI3KDN* (*Drosophila* PI3K dominant-negative), *ppl>dPDK1RNAi*, *ppl>dAktRNAi*, *ppl>dTORDN* or *ppl>dS6KDN* flies, but shortened their developmental time, whereas *ppl>dRafRNAi* and *ppl>dMKP3* (ERK-inhibitory phosphatase)<sup>27</sup> 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>dEgfrRNAi* and *ppl>dS6KDN*, but not *ppl>dMKP3* (Supplementary Fig. 9). Loss of S6K function in *Drosophila* reduces body size by decreasing cell size but not cell number<sup>28</sup>. Activity of the MAPK pathway is reported to be unaffected by nutrients<sup>29</sup>. These results indicate that royalactin activated S6K through Egfr in the fat body, acting as a morphogenic 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 examined the effect of overexpression of royalactin using the UAS/Gal4 system<sup>30</sup>. 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 *Drosophila*<sup>31</sup>, 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 (MRJP1 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 repressed 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.



**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 (**b**, left) and body weight (**b**, 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  $\pm$  s.e.m. Values significantly different from those of *UAS-royalactin* are indicated by  $**P < 0.01$ .

### Royalactin changes hormone metabolism in *Drosophila*

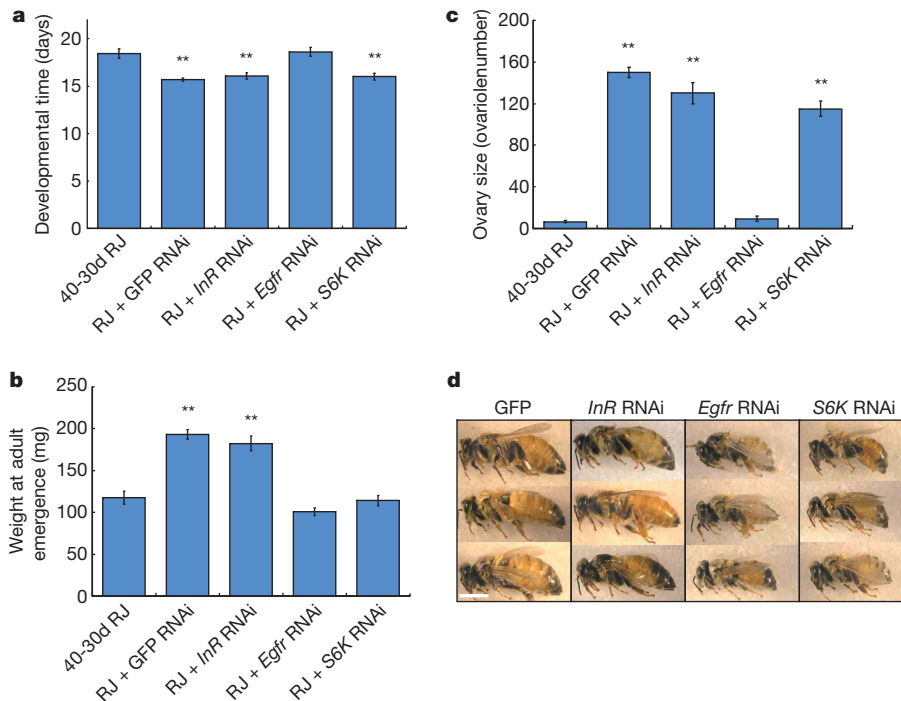
To investigate the relationship between the morphological and physiological changes induced by royalactin in flies and hormone modulation, I measured changes in the biosynthesis of a biologically active ecdysteroid, 20-hydroxyecdysone (20E), and juvenile hormone in wild-type flies given royal jelly during 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*<sup>33</sup>. 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 *ppl>dEgfrRNAi* and *ppl>dMKP3*, but not *ppl>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 *ppl>dEgfrRNAi*, but not in *ppl>dS6KDN* or *ppl>dMKP3* flies (Supplementary Fig. 14b–d and Supplementary Fig. 15). Because repression of MAPK in the fat body (*ppl>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 Egfr* 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 *ppl>dEgfrRNAi* flies, but not *ppl>dS6KDN* or *ppl>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*, *PDK1*, *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 larvae 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 4 | Suppression of queen differentiation in honeybee with RNAi.** **a–c**, Developmental time (**a**), weight at adult emergence (**b**) and ovary size (**c**) in individuals ( $n = 10–16$ ) reared with royal jelly stored at 40 °C for 30 days (40-30d RJ) and royal jelly (RJ) containing dsRNA of GFP and signal factors. Values

of juvenile hormone titre and *vg* expression in honeybee larvae reared with royal jelly was inhibited by *Egfr* RNAi but not *S6K* RNAi and PD98059 (Supplementary Fig. 20b, c). Thus, I found that activation of *S6K* by royalactin through *Egfr* was involved in the increase of body size in queens, whereas MAPK activity downstream of *Egfr* in response to royalactin was responsible for the increase of 20E synthesis, thereby shortening developmental time in the honeybee. Topical application of juvenile hormone to worker larvae results in the emergence of queen-like individuals with ovary development, but which display body sizes consistent with workers<sup>34,35</sup>. Therefore, an increase in juvenile hormone titre downstream of *Egfr* signalling activated by royalactin may have a function in ovary development in queens. These mechanisms are consistent with those of the morphological and physiological changes induced by royalactin in flies.

Here I provide the first evidence, to my knowledge, that royalactin acts on *Egfr* in the honeybee to induce queen differentiation. Furthermore, I found that administration and overexpression of royalactin in *Drosophila* caused morphological and physiological changes resembling the phenotypes of queen bee, through a similar mechanism to that of caste differentiation in the honeybee. These results provide new evidence that *Egfr* signalling has an important role in growth regulation.

The 450-kDa protein consists of apalbumin (420 kDa) and an oligomer of apisimin (5.5 kDa)<sup>36</sup>. Apalbumin is an oligomer of the gene product of *mrjp1* (ref. 36). On the other hand, royalactin is derived from *mrjp1* (ref. 37), but is present as a monomeric glycoprotein with a molecular mass of 57 kDa in royal jelly, and is structurally distinct from apalbumin (antibodies to royalactin do not recognize apalbumin)<sup>11</sup>. Apalbumin binds strongly to apisimin to form a stable complex (450-kDa protein); apalbumin was not separated from apisimin in the absence of detergent<sup>36</sup>. These results indicate that royalactin is not derived from apalbumin in royal jelly. The 40 °C/30 d royal jelly, which contained 90% of the initial concentration of 450-kDa protein, did not induce queen development, and the 450-kDa protein did not increase the rate of emergence of queens when it was added to a diet containing 40 °C/30 d royal jelly. However, both royalactin and E-royalactin induced queen differentiation in the honeybee. Thus, only royalactin,

are expressed as mean  $\pm$  s.e.m. Values significantly different from those of larvae reared with 40-30d RJ are indicated by \*\* $P < 0.01$ . **d**, The final adult size after eclosion is shown in the photograph. Scale bar, 5 mm.

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  $\mu\text{g ml}^{-1}$  was administered to second instar larvae for 2 days.

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