ABSTRACT
Target of rapamycin complex 1 (TORC1) is a master regulator of cell metabolism, and its dysregulation has been linked to an array of pathologies, including cancer and age-related diseases. Npr3, a component of GTPase-activating protein towards Rags complex 1 (GATOR1), inhibits TORC1 activity under nutrient scarcity status. The npr3 mutant exhibits some metabolic defects due to hyper TORC1 activity in Drosophila. Royal jelly (RJ) is a honeybee-secreted product and plays an essential role in caste differentiation that requires TORC1 activity. RJ is also used as a health-benefit food for its potential roles on antioxidant and anti-aging. In this study, npr3 mutant flies were used to measure the effect of RJ on metabolic modulation. Interestingly, RJ feeding significantly increased survival and decreased TORC1 activity in the npr3 mutant. RJ feeding also ameliorated the abnormal reactive oxygen species (ROS) levels and energy status in the npr3 mutant. The proteins in RJ were characterized to be the essential components in increasing npr3 mutant viability. These findings suggest that RJ modulates some metabolic defects associated with elevated TORC1 activity and that the npr3-mutant fly might be a useful tool for investigating the bioactive components of RJ in vivo.

KEY WORDS: Drosophila melanogaster, Royal jelly, Target of rapamycin complex 1, Antioxidant activity, Metabolism regulation

INTRODUCTION
Target of rapamycin complex 1 (TORC1) is a master regulator that mediates nutrient status and metabolism. It promotes synthesis and cell growth by phosphorylating downstream effectors, such as p70 S6 kinase (S6K) and elf-4E binding protein (4E-BP) (Bar-Peled and Sabatini, 2014; Kim and Guan, 2019). The inhibition of TORC1 increases the longevity of a variety of model organisms, whereas the elevated TORC1 activity has been associated with a variety of age-related diseases, including cancer, diabetes, and Parkinson’s (Johnson et al., 2013; Laplante and Sablin, 2012; Zoncu et al., 2011). Npr3 is a component of GTPase-activating protein towards Rags complex 1 (GATOR1), which inhibits TORC1 activity under nutrient starvation (Bar-Peled et al., 2013; Wei and Lilly, 2014; Wei et al., 2014). The npr3 mutant flies exhibit increased TORC1 activity and a variety of related phenotypes, such as semi-lethality, short lifespan, decreased motility, and some metabolic defects (Wei et al., 2016).

Multiple studies showed that high TORC1 activity promotes the generation of reactive oxygen species (ROS) (Chen et al., 2008; Kim et al., 2005). ROS are byproducts of aerobic metabolism during which the oxygen gets one electron and forms free radical superoxide including superoxide anion (O2−), hydrogen peroxide (H2O2), and hydroxyl radical (HO•) (Zorov et al., 2014). ROS can react and cause oxidative damage to macromolecules like protein, DNA and lipid, and thus are highly toxic for cells (Schieber and Chandel, 2014). In physiological conditions, ROS are removed by the antioxidant enzymes such as superoxide dismutases (SOD) and catalase (CAT). Thus, following either an increase in ROS generation or a decrease in antioxidant capacity, oxidative stress occurs and causes pathologies such as aging and age-related diseases (Schieber and Chandel, 2014).

Royal jelly (RJ), a nutrient-rich liquid, is secreted by the hypopharyngeal and mandibular glands of young nurse honeybees (Apis mellifera) (Ramadan and Al-Ghamdi, 2012). In Apis mellifera, RJ is an essential component for larvae differentiation to workers or queens that have the same genetic background (Colhoun and Smith, 1960; Maleszka, 2008). A few female larvae fed with RJ throughout the whole larval stage develop into large-bodied, long-lived, fertile queens, whereas most other female larvae provided RJ for the first 3 days after hatching and then switched to the beebread that contains predominantly honey and pollen develop into small-bodied, short-lived, sterile workers (Colhoun and Smith, 1960; Page and Peng, 2001). Recently, TORC1 activity is taken as an essential factor for caste differentiation in Apis mellifera. Both pharmacological treatment and genetic manipulation that decreased Apis mellifera TOR (amTOR) activity block queens’ fates (Mutti et al., 2011; Patel et al., 2007; Wheeler et al., 2014; Zhu et al., 2017). Furthermore, RJ and the RJ protein, roylaactin, promote the differentiation to queens through EGFR pathway with increased S6K activity, which is also a downstream effector of TORC1 (Kamakura, 2011). These findings suggest that TORC1 activity is required for caste differentiation of larvae into queens.

One interesting question is whether RJ supplemental food promotes TORC1 activity in other animals with similar effects to those seen in Apis mellifera. If it was true, the RJ food might have an adversarial effect on health, especially for individuals with hyper-TORC1 activity, which is closely associated with aging and aging-related diseases. RJ has been traditionally used in some countries as food supplement and been reported to have health benefits, including antioxidant, antimicrobial, anti-inflammatory, anti-tumor, and anti-aging properties (Fratini et al., 2016; Pasupuleti et al., 2017; Ramadan and Al-Ghamdi, 2012). However, RJ is not
allowed to be sold as a health-beneficial food for humans in Europe or the USA (Shorter et al., 2015).

Accordingly, the present study uses nprl3-mutant flies to investigate the effect of RJ on animals with hyper TORC1 activity. Interestingly, RJ feeding significantly alleviated the hyper TORC1 activity and increased the viability, antioxidase activity, and energy levels in nprl3 flies. Additionally, the proteins in RJ were found to be essential components for its function. These results suggest that RJ attenuates the metabolic defects associated with hyper TORC1 levels and provides a model system for quickly screening the bioactive components of RJ.

RESULTS
RJ improves the survival of nprl3-mutant flies
The nprl3-mutant flies are semi-lethal and display multiple age-related pathologies and metabolic defects, owing to elevated TORC1 activity (Wei et al., 2016). RJ exhibits a variety of beneficial properties, including metabolism regulation and antioxidant properties in multiple organisms (Ramadan and Al-Ghamdi, 2012). To determine the effect of RJ function on modulating metabolism defects with hyper TORC1 activity, the nprl3-mutant flies were fed with food containing RJ. Interestingly, RJ feeding significantly increased the viability of the nprl3 mutant (Fig. 1). Because 20% RJ had the best effect on increasing the viability of nprl3 mutants, this concentration was used in the following study.

To investigate the effect of RJ feeding on TORC1 activity, the phosphorylation level of the TORC1 downstream effectors S6K and 4E-BP were determined. Consistent with our previous report (Wei et al., 2016), the TORC1 activity significantly increased in nprl3-mutant flies (Fig. 2). Compared with slight but not significant effect on wild-type flies, RJ feeding significantly alleviated the TORC1 activity in nprl3-mutant flies (Fig. 2).

RJ reduces ROS level and enhances oxidase activity in nprl3-mutant flies
The nprl3 mutant might contain more ROS because TORC1 promotes ROS generation (Chen et al., 2008; Kim et al., 2005). To evaluate the effect of RJ feeding, we detected ROS levels in males, which are comprised primarily of somatic tissues. The Dihydroethidium (DHE) staining method is widely used to detect ROS levels in cells and tissues (Gomes et al., 2005). Interestingly, the DHE staining was stronger in nprl3-mutant flies, which can be significantly alleviated by RJ feeding (Fig. 3A,B). In animals, ROS oxidizes unsaturated fatty acids and generates malondialdehyde (MDA), which can be used as an indicator of oxidation (Tsikas, 2017). Consistent with the results of DHE staining, the nprl3-mutant flies had more MDA, which was reduced by RJ feeding (Fig. 3C). In addition, RJ feeding slightly reduced the DHE staining and MDA amount in wild-type flies (Fig. 3).

The two canonical antioxidases, SOD and CAT, play important roles in ROS elimination. The SOD and CAT activities of the nprl3-mutant flies were significantly lower than those of wild-type flies, which could be rescued by RJ feeding (Fig. 4).

The lethality of nprl3 mutants occurred at late pupal stage, which might have been caused by the defects at an earlier developmental stage (Wei et al., 2016). To confirm the effect of RJ on reducing ROS levels, we detected the MDA and antioxidase activity at the larval stage. Consistent with the effect in adult flies, RJ feeding improved the antioxidant capacity and decreased the oxidative levels in nprl3-mutant larva (Figs S1 and S2).

RJ improves energy storage and usage in nprl3-mutant flies
The nprl3-mutant flies exhibit reduced triglyceride (TG), which is the most abundant form of stored energy in the Drosophila body (Wei et al., 2016). Here we found that RJ feeding significantly increased the TG levels in the nprl3-mutant flies (Fig. 5A). In animals, TG is used to produce adenosine triphosphate (ATP), which is considered the energy currency of living cells (Bonora et al., 2012). Indeed, the nprl3-mutant flies also possessed lower levels of ATP than the wild-type flies, which could be alleviated by RJ feeding (Fig. 5B). Furthermore, the TG and ATP levels were increased in nprl3-mutant larvae, which were alleviated by RJ feeding (Fig. S3). These results suggested that RJ could alleviate the metabolic defects in nprl3-mutant flies.

RJ proteins are essential for modulating the viability of nprl3 mutants
Next, we investigated the bioactive components responsible for increasing nprl3 mutant viability. The effect of RJ components isolated using water, ethanol, or ethyl acetate were detected. The water-isolated RJ components had a similar effect to complete RJ on nprl3 mutant viability. The effect of RJ components nprl3 mutant larvae, which were alleviated by RJ feeding (Fig. S2).

Fig. 1. Effect of dietary royal jelly (RJ) on the viability of nprl3-mutant flies. The viability ratio of nprl3-mutant flies on different foods was calculated; n, number of total hatched adult flies; error bars indicate s.d. of five independent repeats; **P<0.01; ***P<0.001 compared with normal food group.
DISCUSSION

TORC1 promotes anabolic processes and stimulates oxygen consumption and mitochondrial capacity to provide energy for protein synthesis (Ramanathan and Schreiber, 2009). During mitochondrial respiration, ROS is generated as a byproduct, especially in damaged mitochondria (Zorov et al., 2014). In addition, TORC1 inhibits autophagy, the process of eliminating the damaged mitochondria. Here, we found that mutation of nprl3, a component of the TORC1 inhibitor GATOR1, results in a high ROS level in Drosophila. ROS stress causes accumulated organelle damage and accelerates aging in traditional views (Liochev, 2013). The GATOR1-mutant flies, including nprl2, nprl3 and iml1, display multiple age-related defects that might be associated with high ROS levels (Wei et al., 2016; Xi et al., 2019). Furthermore, we observed low SOD and CAT activities in nprl3 mutants, which suggests that hyper TORC1 inhibits the elimination of ROS. This result is consistent with the report that TORC1 phosphorylates and inhibits SOD1 activity in yeast and human cells (Tsang et al., 2018). The RJ food significantly alleviates the hyper TORC1 activity in nprl3-mutant flies, and this might be related to its effect on ROS clearance. Our results suggest that RJ feeding alleviates the metabolic defects of the animals with hyper TORC1 activity. The RJ exerted a similar tendency on modulating ROS and metabolism between wild-type and nprl3-mutant flies at both larval and adult stages. Furthermore, that RJ feeding increased the viability of nprl3-mutant flies might be related to its effect at the larval stage. Thus, the nprl3 mutant might be a useful tool for identifying RJ bioactive materials.

We found that some water-soluble, temperature-sensitive or -resistant RJ proteins are essential components for RJ biological function. Previously, the RJ proteins have been reported to function on modulating metabolism (Fratini et al., 2016; Guo et al., 2008; Barnuti et al., 2011; Ramadan and Al-Ghamdi, 2012). Some RJ proteins, such as the oligo form major royal jelly protein 1 (MRJP1) are temperature resistant, while the mono form MRJP1 is temperature sensitive (Moriyama et al., 2015). Further study is needed to identify the specific proteins and evaluate their function on TORC1 activity and metabolism.

In summary, the present study reveals that appropriate levels of dietary RJ can significantly improve the survival of nprl3-mutant flies, which could be a rapid and cost-effective tool for the qualitative evaluation of RJ.

Fig. 2. RJ feeding decreases TORC1 activity in the nprl3 mutant. yw and nprl3/Df flies were collected from normal food or RJ food. (A) Phosphorylated S6K (top), total S6K (middle), and Tubulin (bottom) in male flies were detected using western blot. (B) The relative intensity of phosphorylated S6K band to total S6K band. (C) Phosphorylated 4E-BP (top), total 4E-BP (middle), and Tubulin (bottom) in male flies were detected using western blot. (D) The relative intensity of phosphorylated 4E-BP band to total 4E-BP band. The ratio of p4E-BP/4E-BP in the yw was set as 1. Data are presented as mean±s.d.; values are from four independent experiments; *P<0.05; **P<0.001; NS, not significant; yw, control flies; nprl3-, nprl3/Df flies; ND, normal diet; RJ, 20% RJ diet.

essential components for rescuing the metabolic defects of nprl3-mutant flies.

In summary, the present study reveals that appropriate levels of dietary RJ can significantly improve the survival of nprl3-mutant flies, which could be a rapid and cost-effective tool for the qualitative evaluation of RJ.
Fig. 3. RJ feeding decreases the reactive oxygen species levels in the nprl3 mutant. yw and nprl31/Df flies were collected from normal food or RJ food. (A) DHE (red) and Hoechst (blue) stained fat bodies were imaged using a confocal microscope. (B) The values of DHE intensity were quantified (n=20). (C) The MDA levels were determined (n=5). Data are presented as mean±s.d. **P<0.01, ***P<0.001. yw, control flies, nprl3-, nprl31/Df flies; ND, normal diet; RJ, 20% RJ diet.
MATERIALS AND METHODS
Fly stocks and maintenance
The stocks yw, nprl31 and Df(3L) ED4515/TM6C (BDSC#9071) were described previously (Wei et al., 2016). The flies were maintained in an incubator at 25°C with a 12 h on/off light cycle and 60% relative humidity.

Fly food
The standard food contained 5% cornmeal, 1% agar, 2.4% brewer’s yeast, 3% sucrose, and 0.3% propionic acid, which was used as a normal diet (ND). The RJ was added to standard food at 5%, 10%, 20%, or 40% (weight/volume), and stirred during the food cooling step to make RJ food. For

![Graph A](image1.png)

**Fig. 4.** RJ feeding increases antioxidase activities in the nprl3 mutant. yw and nprl31/Df flies were collected from normal food or RJ food. (A) SOD activities and (B) CAT activities were determined. Data are presented as mean±s.d.; *P<0.05; **P<0.01; ***P<0.001; values are from five independent experiments; yw, control flies, nprl31; nprl31/Df flies; ND, normal diet; RJ, 20% RJ diet.

![Graph B](image2.png)

![Graph C](image3.png)

**Fig. 5.** Effect of RJ on the metabolism in the nprl3 mutant. yw and nprl31/Df flies were collected from normal food or RJ food. (A) Triglyceride levels and (B) ATP levels were determined. Data are presented as mean±s.d; values are from five independent experiments; ***P<0.001; NS, not significant; yw, control flies, nprl31; nprl31/Df flies; ND, normal diet; RJ, 20% RJ diet.
non-TM3 flies was fully viable. Thus, the viability ratio of nprl3 1/TM3 flies divided by the half number of TM3 flies.

**The viability of nprl3-mutant flies**

The nprl3/TM3 and Df(3L) ED4515/TM3 (Df) crossed and laid eggs on standard food or RJ food for 1 day. A few days later, the number of hatched adult flies on standard food or RJ food were counted. The genotypes of flies were identified using the Stubble (Sb) marker on TM3 balancer. While TM3/TM3 is lethal, the expected ratio of non-Sb (nprl3/Df) to Sb (nprl3/TM3 and Df/TM3) is 1:2, if the nprl3/Df flies were fully viable. Thus, the viability ratio of nprl3-mutant flies is the number of the non-TM3 flies divided by the half number of TM3 flies.

**MDA, oxidase, TG, and ATP levels**

The flies were crossed on standard food or RJ food. A few days later, hatched adult flies from standard food or RJ food were collected and cultured on standard food or RJ food. The 7-day-old male flies were homogenized in a buffer solution of phosphate-buffered saline (PBS) and then centrifuged for 10 min at 13,000 g. The supernatant was transferred to a new tube, the MDA (A003-1, Jiancheng Bio-Engineering, Nanjing, China), SOD oxidase (A007-1, Jiancheng Bio-Engineering, Nanjing, China), CAT oxidase (A001-3, Jiancheng Bio-Engineering, Nanjing, China), trypsin (F001-1, Jiancheng Bio-Engineering, Nanjing, China), and ATP (A095-1-1, Jiancheng Bio-Engineering, Nanjing, China) kits were used to measure the amounts of components, according to the manufacturer’s instructions. The total protein was measured using the BCA Assay Kit (CW0014, CoWin Biosciences, Beijing, China) and used for normalization. The resulting data were analyzed using one-way ANOVA.

**DHE staining**

The flies were crossed on standard food or RJ food. A few days later, hatched adult flies from standard food or RJ food were collected and cultured on standard food or RJ food. The abdomen from a 7-day-old male fly was dissected and incubated with 30 μM DHE (Beyotime, China) and Hoechst 33342 (H3570, Invitrogen, China) in PBS for 5 min and then washed three times using PBS. The fat body was dissected from the abdomen and mounted using antifade mountant (P36982, Invitrogen, USA). Images were acquired using a confocal microscope (Zeiss LSM880, Germany). The fluorescence intensities of DHE and Hoechst, used for normalization, were quantified using Image J. The resulting data were analyzed using one-way ANOVA.

**Western blot analysis**

The flies were crossed on standard food or RJ food. A few days later, hatched adult flies from standard food or RJ food were collected and cultured on standard food or RJ food for 7 days. The male flies were homogenized in 1× radioimmunoprecipitation assay (RIPA) lysis buffer (millipore, USA) containing complete protease inhibitors and phosphatase inhibitors (Roche, USA). Immunoblot analysis was performed as described previously (Wei and Lilly, 2014). The following primary antibodies were used for western blot: pS6K (1:1000; Cell Signaling Technology, 9209, USA), total S6K (1:10,000) (Hahn et al., 2010), p4E-BP (1:500; Cell
Supplementary information

Supplementary information available online at https://bio.biologists.org/lookup/doi/10.1242/bio.054999.supplemental

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Figure S1. Effect of RJ on the reactive oxygen species levels in the nprl3 mutant larvae. The 3rd star larvae of yw and nprl31/Df were collected from normal food or RJ food. The MDA levels were determined. Data are presented as mean ± SD. Values from five independent experiments. *P<0.05, **P<0.01, ***P<0.001. yw, control flies; nprl3-, nprl31/Df flies; ND, normal diet; RJ, 20% RJ diet.
Figure S2. Effect of RJ on the antioxidase activities in the nprl3 mutant larvae. The 3rd star larvae of yw and nprl3¹/Df were collected from normal food or RJ food. (A) SOD activities and (B) CAT activities were determined. Data are presented as mean ± SD. *p<0.05, **P<0.01, ***P<0.001. Values from five independent experiments. yw, control flies; nprl3⁻, nprl3¹/Df flies; ND, normal diet; RJ, 20% RJ diet.
Figure S3. Effect of RJ on the metabolism in the nprl3 mutant larvae. The 3rd star larvae of yw and nprl3\(^{1/Df}\) were collected from normal food or RJ food. (A) Triglyceride (TG) levels and (B) ATP levels were determined. Data are presented as mean ± SD. Values from five independent experiments. *p<0.05, **P<0.01, ***P<0.001. yw, control flies; nprl3\(^{1/Df}\) flies; ND, normal diet; RJ, 20% RJ diet.