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Original article

The longevity of queen honey bees (*Apis mellifera*) is associated with the increase of cellular activities through the cAMP/PKA and RAS/MAPK signaling pathways

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Abstract – Queen honey bees (*Apis mellifera*) have a much longer lifespan than worker bees; however, the longevity-promoting mechanisms of queen bees are still unclear. Assaying cellular activities can explore the longevity-promoting mechanisms of queen bees because the longevity of individuals is based on the longevity of their cells. In this study, NAD^+ levels, NAD^+/NADH ratio, ATP levels, AMPK activity, lysosome activity, and ribosomal protein S6 mRNA levels were assayed to evaluate whether queen bees have higher cellular activities than worker bees. The results showed that the trophocytes and oenocytes of queen bees have higher cellular activities than that of worker bees. To explore which signaling pathway increases these cellular activities, the cAMP concentration and the mRNA levels of AC, PKA, RAS, MEK, and RSK were assayed. The results showed that the trophocytes and oenocytes of queen bees exhibit higher cAMP concentration and mRNA levels of AC, PKA, RAS, MEK, and RSK than that of worker bees. Combining these findings suggested that the cAMP/PKA and RAS/MAPK signaling pathways may increase cellular activities leading to the longevity of queen bees.

cellular activities / cAMP / RAS / longevity / honey bee

Abbreviations

NAD^+	Nicotinamide adenine dinucleotide oxidized form
NADH	Nicotinamide adenine dinucleotide reduced form
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
cAMP	Cyclic AMP
AC	Adenylyl cyclase
PKA	Protein kinase A
MAPK	Mitogen-activated protein kinase
RAS	Ras GTPase
RAF	MAPK kinase kinase
MEK	MAPK kinase
RSK	Ribosomal protein S6 kinase

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1. INTRODUCTION

Queen and worker honey bees (*Apis mellifera*) are females and share the same genome; however, the lifespan of queen bees is much longer than worker bees. Queen bees live in the hive except during mating flights and colony fission and lay 1000–2000 eggs per day throughout their lives (Bodenheimer 1937). Worker bees spend only the

first two weeks of life in the hive and perform tasks such as comb-building, cell-cleaning, and brood-nursing before transitioning to the foraging stage. In the foraging stage, worker bees collect nectar and pollen for the hive (Neukirch 1982). The difference in lifespan between queen and worker bees is mainly related to food intake. Queen bees feed on royal jelly (RJ) throughout their lives, whereas worker bees consume honey and pollen (Winston 1987).

The aging and longevity of individuals are based on the aging and longevity of their cells. In other words, studying the causes of aging and longevity of cells can clarify the aging and longevity of the individual. Trophocytes, which are large and irregularly shaped, and oenocytes, which are small and spherical, attach to form a single layer of cells around each segment of the honey bee abdomen. They are so tight that they cannot be separated. The biological functions of trophocytes and oenocytes are analogous to the white adipose tissue or liver of vertebrates (Law and Wells 1989). Trophocytes and oenocytes have been used for studying the mechanisms of aging and longevity due to the ease of isolation from the abdomen, convenient manipulation, and lack of cell division during adulthood (Hsieh and Hsu 2011a, b).

The cellular activities of trophocytes and oenocytes of worker or queen bees have been assayed, respectively, but those between worker and queen bees are unknown. The cellular activities include mitochondrial energy utilization activity, cellular energy regulation activity, cellular degradation activity, and cellular energy metabolism activity (Chuang and Hsu 2013; Hsu and Lu 2015; Hsu and Chuang 2014; Hsu and Hu 2014; Hsu et al. 2014, 2016). In this study, the cellular activities in the trophocytes and oenocytes of young and old worker bees and young and old queen bees were assayed to reveal whether queen bees have higher cellular activities than worker bees. Furthermore, which signaling pathway triggers the cellular activities in the trophocytes and oenocytes of queen bees were determined.

2. MATERIALS AND METHODS

2.1. Honey bees (Apis mellifera)

The brood combs containing pupae and a few emerged worker bees from the different colonies were transferred to an incubator (34 °C, 75% relative humidity). Seventy newly emerged worker bees were collected in a cage (15 × 10 × 12 cm), put into a 34 °C thermostat (NK system, Nippon, Japan), and daily fed honey and fresh pollen grains mixed with honey (3:1). Young (5 days old) and old (30 days old) worker bees were used for the following studies; thus, the difference between them was only age (Hsu and Chan 2013). Young (2-month-old) and old (16-month-old) queen bees were collected from different hives for the following experiments. The young and old queen bees were mated with drones and were able to lay eggs. Young and old worker bees and young and old queen bees were collected on the same dates for subsequent experiments.

2.2. NAD^+ and NADH concentration

NAD^+ and NADH levels were measured using a NAD/NADH quantification kit (K337-100; BioVision, Mountain View, CA, USA) (Chuang and Hsu 2013; Hsu and Lu 2015). Bees were anesthetized on ice. The abdomens were collected and dissected with scissors. Their digestive tracts, trachea system, and nerve system on the abdomen were carefully removed. The cuticle of the abdomen was washed with phosphate-buffered saline. Trophocytes and oenocytes were detached from the cuticle of the abdomen using a knife. Trophocytes and oenocytes from two young or old worker bees or one young or old queen bee were extracted with NADH/NAD extraction buffer. The extracted samples of queen bees were diluted to obtain a similar protein concentration as worker bees because the cells of queen bees had higher protein concentration than that of worker bees and the protein concentration interfered with the measurement

using the spectrometer. The dilution factor was used for the value calculation. The extracted samples were heated for NADH measurement. After background luminescence was recorded, the extracted samples and the extracted and heated samples were measured. The resultant luminescence was normalized to the background. Dilutions of an NADH standard solution (0, 20, 40, 60, 80, and 100 pmol l^{-1}) were processed concurrently to generate a standard curve, which was used to obtain NAD^+ and NADH values. The NAD^+ and NADH concentrations were expressed as nanomoles mg^{-1} of protein and were used to determine the NAD^+/NADH ratio. This experiment was performed with five biological replicates simultaneously using a total of ten young and ten old worker bees and five young and five old queen bees.

2.3. ATP concentration

ATP concentration was determined using an ATP determination kit (A22066; Invitrogen, Carlsbad, CA, USA) (Chuang and Hsu 2013; Hsu and Lu 2015). The manipulation of trophocytes and oenocytes was as described above. Trophocytes and oenocytes from two young or old worker bees or one young or old queen bee were homogenized in phosphate buffer containing protease inhibitors and centrifuged to obtain the resulting supernatant. The resulting supernatants of queen bees were diluted to obtain a similar protein concentration as worker bees. The dilution factor was used for the value calculation. After recording background luminescence, diluted ATP standard solution (1, 10, 100, 1000, and 5000 nM) or supernatant was added to the standard reaction solution to measure values. The ATP concentration was expressed as nanomoles per milligram of protein. This experiment was performed with five biological replicates simultaneously using a total of ten young and ten old worker bees and five young and five old queen bees.

2.4. AMPK activity

AMPK activity was evaluated using the CycLex AMPK kinase assay kit (CY-1182, CycLex, Nagano, Japan) (Hsu and Chuang 2014; Hsu and Hu 2014). The manipulation of trophocytes and oenocytes was as described above. Briefly, trophocytes and oenocytes were isolated from two young or old worker bees or one young or old queen bee, homogenized with a blue pestle and sonicator in 50 µl of cell lysis buffer containing protease inhibitors (11,697,498,001; Roche Applied Science, Indianapolis, IN, USA) and centrifuged at 5000 g for 10 min at 4 °C to obtain the supernatant. The supernatants of queen bees were diluted to obtain a similar protein concentration as worker bees. The dilution factor was used for the value calculation. The supernatant (10 µl) and kinase reaction buffer (90 µl) were applied to each well of a 96-well plate and incubated for 30 min at 30 °C. After washing, 100 µl of the anti-phospho-mouse IRS-1 S789 monoclonal antibody was added and incubated for 30 min at room temperature. After washing, 100 µl of HRP-conjugated anti-mouse IgG was added and incubated for 30 min at room temperature. After washing, 100 µl of substrate reagent was added and incubated for 15 min at room temperature. Finally, 100 µl of stop solution was added to stop the reaction. The absorption was measured at 450 nm using an ELISA plate reader (Synergy HT, BioTek, VT, USA). The AMPK activity was expressed as the absorbance at 450 nm mg^{-1} of protein. This experiment was performed with five biological replicates simultaneously using a total of ten young and ten old worker bees and five young and five old queen bees.

2.5. Lysosomal activity

The acid phosphatase activity was used to evaluate the lysosomal activity because acid phosphatase is one of the acid hydrolases in the lysosome. The acid phosphatase activities in the trophocytes and oenocytes of two young or old worker bees or one young or old queen bee were

determined using the acid phosphatase assay kit (CS0740; Sigma, Saint Louis, MO, USA) (Hsu et al. 2014, 2016). Fifty microliters of the supernatants (described in AMPK activity) (replaced with citrate buffer for the blank samples) and 50 µl of the substrate solution were mixed and incubated for 10 min at 37 °C. The reactions were stopped by adding 0.2 ml of stop solution. The absorption was measured at 405 nm using an ELISA plate reader, and the specific activity was expressed as milliunits per milliliter. One unit of acid phosphatase hydrolyzed 1 µmol of 4-nitrophenyl phosphate per minute at pH 4.8 and 37 °C. This experiment was performed with five biological replicates simultaneously using a total of ten young and ten old worker bees and five young and five old queen bees.

2.6. Quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA was extracted from the trophocytes and oenocytes of two young or old worker bees or one young or old queen bee using TRIzol® Reagent (15,596,018; Invitrogen) (Hsu et al. 2014, 2016). RNA concentration and quality were determined using a Synergy™ HT multi-mode microplate reader (7,091,000; BioTek). The complementary DNA (cDNA) synthesis was performed using an iScript™ cDNA synthesis kit (170–8891; Bio-Rad Laboratories, CA, USA). Each reaction contained 1 µg of total RNA in a 20-µl reaction volume. The qPCR was performed using a CFX connect RT-PCR detection system (Bio-Rad Laboratories) and each reaction contained 0.5 µl of 10 µM of each primer, 12.5 µl of SYBR Green (170–8882; Bio-Rad Laboratories), 1 µl of diluted cDNA, and 10.5 µl of ddH_2O in a final volume of 25 µl. The β-actin gene was used as a reference gene (Hsu et al. 2014). Primers were designed based on GenBank nucleotide sequences (Table I). The PCR program was at 95 °C for 3 min, followed by 39 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 30 s (Hsu et al. 2014). The cycle threshold (Ct) values were normalized to β-actin (ΔCt) and the gene expression was expressed as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). This experiment was performed with five biological replicates simultaneously using a

Table I Primer list for qPCR

Genes		Primer sequence (5′→3′)	Accession number
S6	Forward	AGGAAATGTGTGCTCAACCAAG	XM_026443198.1
AC	Reverse	GAACCTACAACGTGATGCCTATG	XM_026443372.1
	Forward	GTATCCGTGGAACAAGAG	
	Reverse	TGTCACCATGTAACTTCTC	
PKA	Forward	GCTCAGCTACGGAAGAGGAC	XM_393285.7
	Reverse	TCTCAGCAGCGTCAACCTTT	
RAS	Forward	ACAGCGAGAGATTTAGCAACACT	XR_003304114.1
	Reverse	GTCATGGCAAATGGCTTTAGCA	
MEK	Forward	GAACCACCAGTAATACCAGATG	XM_393416
	Reverse	ACTTTCTTGCCATTATTAGTCCAT	
RSK	Forward	ACTTGATACTGAAGGTCATATTGC	XM_026440839.1
	Reverse	TGTCATTGTTTCTTTACGATTTGC	
Actin	Forward	ATGCCAACACTGTCCTTTCTGG	NM_001185146.1
	Reverse	GACCCACCAATCCATACGGA	

S6 ribosomal protein S6, *AC* adenylyl cyclase type 10, *PKA* cAMP-dependent protein kinase A catalytic subunit, *RAS* Ras-like protein 1, *MEK* dual specificity mitogen-activated protein kinase kinase dSOR1, *RSK* ribosomal protein S6 kinase alpha-2 (p90)

total of ten young and ten old worker bees and five young and five old queen bees.

2.7. cAMP concentration

The cAMP concentration was assayed using the cAMP direct immunoassay kit (ab65355, Abcam, MA, USA) (Hsu and Chuang 2014; Hsu and Hu 2014). The manipulation of trophocytes and oenocytes is as described above. Briefly, trophocytes and oenocytes from two young or old worker bees or one young or old queen bee were treated with 110 µl of 0.1 M HCl, incubated for 20 min at room temperature, and centrifuged at 10,000 g for 10 min at 4 °C to obtain the supernatant. The supernatants of queen bees were diluted to obtain a similar protein concentration as worker bees. The dilution factor was used in the value calculation. The supernatant was used directly in the assay. After recording the background luminescence, 100 µl of the diluted cAMP standard solution (0.039, 0.078, 0.156, 0.3125, 0.625, 1.25, 2.5, 5, and 10 pmol/µl) or 100 µl of the supernatant was added to the standard reaction solution and measured spectrophotometrically at 450 nm at room temperature using an ELISA plate reader (Synergy HT, BIOTEK, VT, USA). The cAMP values, expressed as picomoles per nanogram of protein, were obtained from the luminescence measurements (standardized to background) by reference to a cAMP standard curve that was generated concurrently. This experiment was performed with five biological replicates simultaneously using a total of ten young and ten old worker bees and five young and five old queen bees.

2.8. Statistical analysis

Differences in the mean values among the four groups were determined by the Kruskal–Wallis test and followed by Mann–Whitney *U*-test for pairwise comparisons. A *P*-value of less than 0.01 was considered statistically significant (Lu et al. 2017).

3. RESULTS

3.1. NAD^+ concentration, NAD^+/NADH ratio, and ATP concentration

To determine the mitochondrial energy utilization activity and cellular energy metabolism activity in the trophocytes and oenocytes of worker and queen bees, we assayed NAD^+ levels, NAD^+/NADH ratios, and ATP levels in the trophocytes and oenocytes of young and old worker bees, and young and old queen bees. The fold changes in the mean NAD^+ levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.25 ± 0.05, 12.25 ± 2.27, and 11.51 ± 1.22, respectively. The NAD^+ levels significantly differed between young and old worker bees, between young worker bees and young and old queen bees, and between old worker bees and young and old queen bees ($n = 5$, $P < 0.01$; Figure 1A). However, there was no significant difference between young and old queen bees. The fold changes in the mean NAD^+/NADH ratios in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.25 ± 0.03, 5.49 ± 0.77, and 5.25 ± 1.11, respectively. The NAD^+/NADH ratios significantly differed between young and old worker bees, between young worker bees and young and old queen bees, and between old worker bees and young and old queen bees ($n = 5$, $P < 0.01$; Figure 1B). However, there was no significant difference between young and old queen bees. The fold changes in the mean ATP levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.82 ± 0.01, 3.25 ± 0.33, and 2.94 ± 0.33, respectively. The ATP levels significantly differed between young and old worker bees, between young worker bees and young and old queen bees, and between old worker bees and young and old queen bees ($n = 5$, $P < 0.01$; Figure 1C). However, there was no significant difference between young and old queen bees. These findings indicated that the trophocytes and oenocytes of queen bees had better mitochondrial energy utilization activity and cellular energy metabolism activity than worker bees.

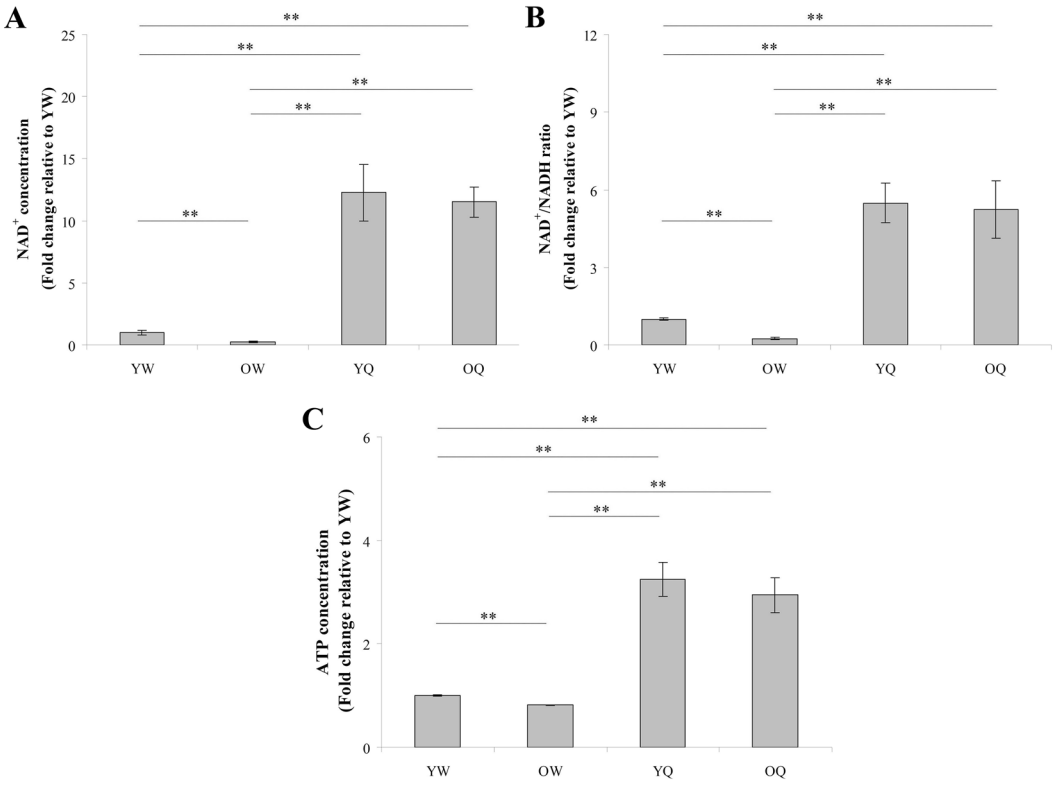

Figure 1. NAD^+ concentration (**A**), NAD^+/NADH ratio (**B**), and ATP concentration (**C**) in the trophocytes and oenocytes of young and old worker bees and young and old queen bees. The values represent the means ± standard error of the means (SEMs) ($n = 5$). Asterisks indicate statistical significance (**$P < 0.01$; Mann–Whitney U-test). YW, young worker bees; OW, old worker bees; YQ, young queen bees; OQ, old queen bees.

3.2. AMPK activity, lysosome activity, and ribosomal protein S6 mRNA level

To determine the cellular energy regulation activity, cellular degradation activity, and cellular energy metabolism activity in trophocytes and oenocytes of worker and queen bees, we assayed AMPK activities, lysosome activities, and ribosomal protein S6 mRNA levels in the trophocytes and oenocytes of young and old worker and queen bees. The fold changes in the mean AMPK activities in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.56 ± 0.08, 18.31 ± 2.67, and 16.06 ± 2.33, respectively. The AMPK activities significantly differed between young and old worker bees, between young worker bees and young and old queen bees, and between old worker bees and young and old queen bees ($n = 5$, $P < 0.01$; Figure 2A). However, there was no significant difference between young and old queen bees. The fold changes in the mean lysosome activities in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.31 ± 0.07, 2.43 ± 0.17, and 2.20 ± 0.19, respectively. Lysosome activities significantly differed between young and old worker bees, between young worker bees and young and old queen bees, and between old worker bees and young and old queen bees ($n = 5$, $P < 0.01$; Figure 2B). However, there was no significant difference between young and old queen bees. The fold changes

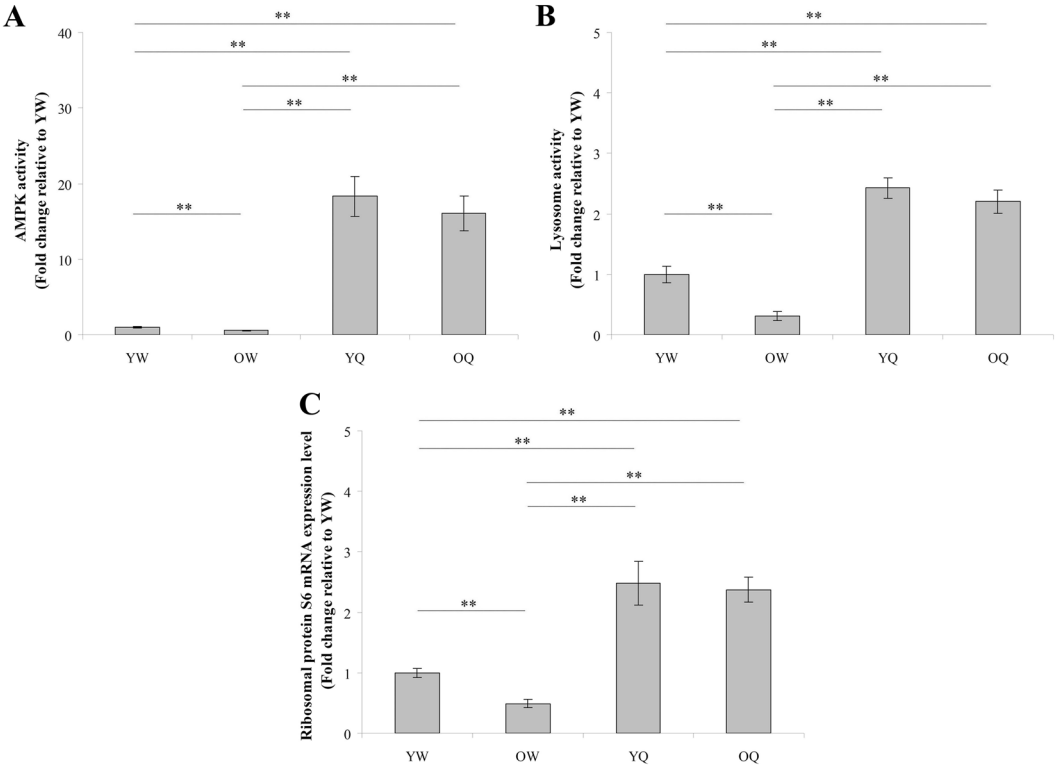

Figure 2. AMPK activity (**A**), lysosome activity (**B**), and ribosomal protein S6 mRNA level (**C**) in the trophocytes and oenocytes of young and old worker bees and young and old queen bees. The values represent the means ± SEMs ($n = 5$). Asterisks indicate statistical significance (**$P < 0.01$; Mann–Whitney U-test). YW, young worker bees; OW, old worker bees; YQ, young queen bees; OQ, old queen bees.

in the mean ribosomal protein S6 mRNA levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.49 ± 0.07, 2.48 ± 0.36, and 2.37 ± 0.21, respectively. Ribosomal protein S6 mRNA levels significantly differed between young and old worker bees, between young worker bees and young and old queen bees, and between old worker bees and young and old queen bees ($n = 5$, $P < 0.01$; Figure 2C). However, there was no significant difference between young and old queen bees. These results further revealed that the trophocytes and oenocytes of queen bees had better cellular energy regulation activity, cellular degradation activity, and cellular energy metabolism activity than worker bees.

3.3. AC mRNA level, cAMP concentration, and PKA mRNA level

To explore the signaling pathways that increased cellular activities in the trophocytes and oenocytes of queen bees, we analyzed AC mRNA levels, cAMP concentrations, and PKA mRNA levels in the trophocytes and oenocytes of worker and queen bees. The fold changes in the mean AC mRNA levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.31 ± 0.05, 7.36 ± 0.72, and 6.48 ± 0.44, respectively. The AC mRNA levels significantly differed between young and old worker bees, between young worker bees and young and old queen bees, and between

old worker bees and young and old queen bees ($n=5$, $P<0.01$; Figure 3A). However, there was no significant difference between young and old queen bees. The fold changes in the mean cAMP levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.67 ± 0.07, 3.27 ± 0.15, and 3.16 ± 0.30, respectively. The cAMP levels significantly differed between young and old worker bees, between young worker bees and young and old queen bees, and between old worker bees and young and old queen bees ($n=5$, $P<0.01$; Figure 3B). However, there was no significant difference between young and old queen bees. The fold changes in the mean PKA mRNA levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.40 ± 0.07, 2.57 ± 0.29, and 2.35 ± 0.28, respectively. The PKA mRNA levels significantly differed between young and old worker bees, between young worker bees and young and old queen bees, and between old worker bees and young and old queen bees ($n=5$, $P<0.01$; Figure 3C). However, there was no significant difference between young and old queen bees. These results indicated that the trophocytes and oenocytes of queen bees had higher signaling of the cAMP/PKA pathway than worker bees.

3.4. RAS, MEK, and RSK mRNA levels

To further investigate the signaling pathways that increased cellular activities in the trophocytes and oenocytes of queen bees, we examined the mRNA expression of RAS, MEK, and RSK

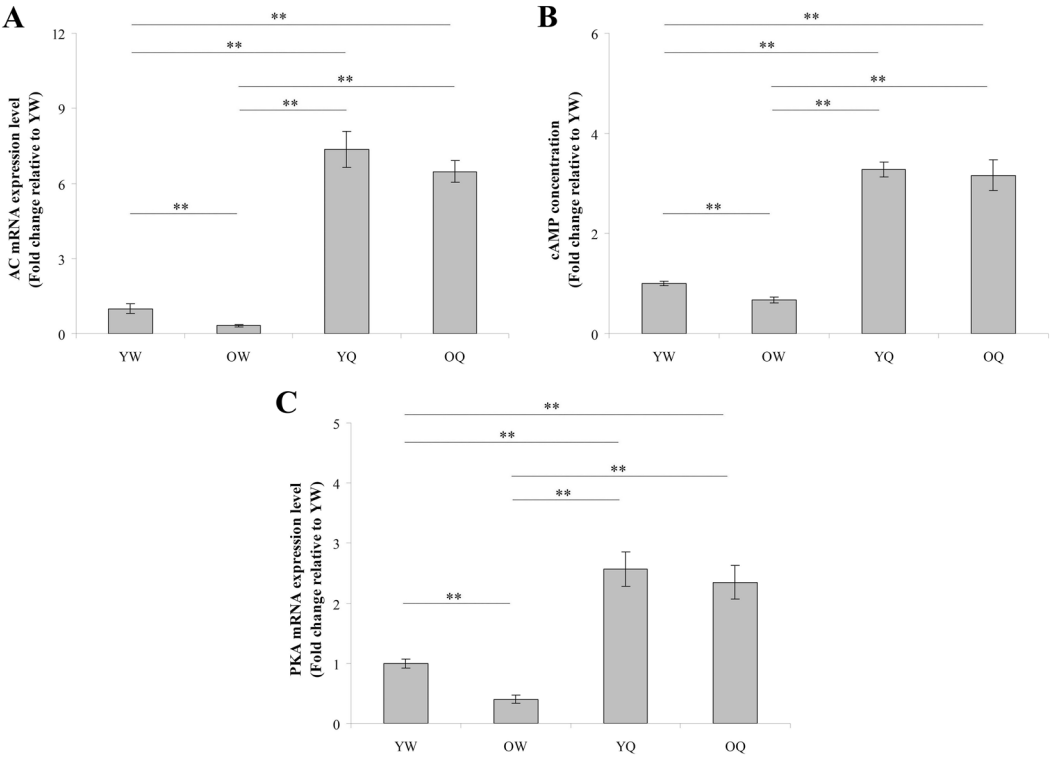

Figure 3. Adenylyl cyclase mRNA level (**A**), cAMP concentration (**B**), and PKA mRNA level (**C**) in the trophocytes and oenocytes of young and old worker bees and young and old queen bees. The values represent the means ± SEMs ($n=5$). Asterisks indicate statistical significance (**$P<0.01$; Mann–Whitney U-test). YW, young worker bees; OW, old worker bees; YQ, young queen bees; OQ, old queen bees.

in the trophocytes and oenocytes of young and old worker and queen bees. The fold changes in the mean RAS mRNA levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.62 ± 0.07, 2.87 ± 0.31, and 2.53 ± 0.22, respectively. The RAS mRNA levels significantly differed between young and old worker bees, between young worker bees and young and old queen bees, and between old worker bees and young and old queen bees ($n = 5$, $P < 0.01$; Figure 4A). However, there was no significant difference between young and old queen bees. The fold changes in the mean MEK mRNA levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.56 ± 0.05, 3.24 ± 0.20, and 3.55 ± 0.11, respectively. The MEK mRNA levels significantly differed between young and old worker bees, between young worker bees and young and old queen bees, and between old worker bees and young and old queen bees ($n = 5$, $P < 0.01$; Figure 4B). However, there was no significant difference between young and old queen bees. The fold changes in the mean MEK mRNA levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.50 ± 0.08, 5.34 ± 0.79, and 5.04 ± 0.54, respectively. The MEK mRNA levels significantly differed between young and old worker bees, between young worker bees and young and old queen bees, and between old worker bees and young and old queen bees ($n = 5$, $P < 0.01$; Figure 4C). However, there was no significant difference between young and old queen bees. These results indicated that the trophocytes and oenocytes of queen bees had higher signaling of the RAS/MAPK pathway than worker bees.

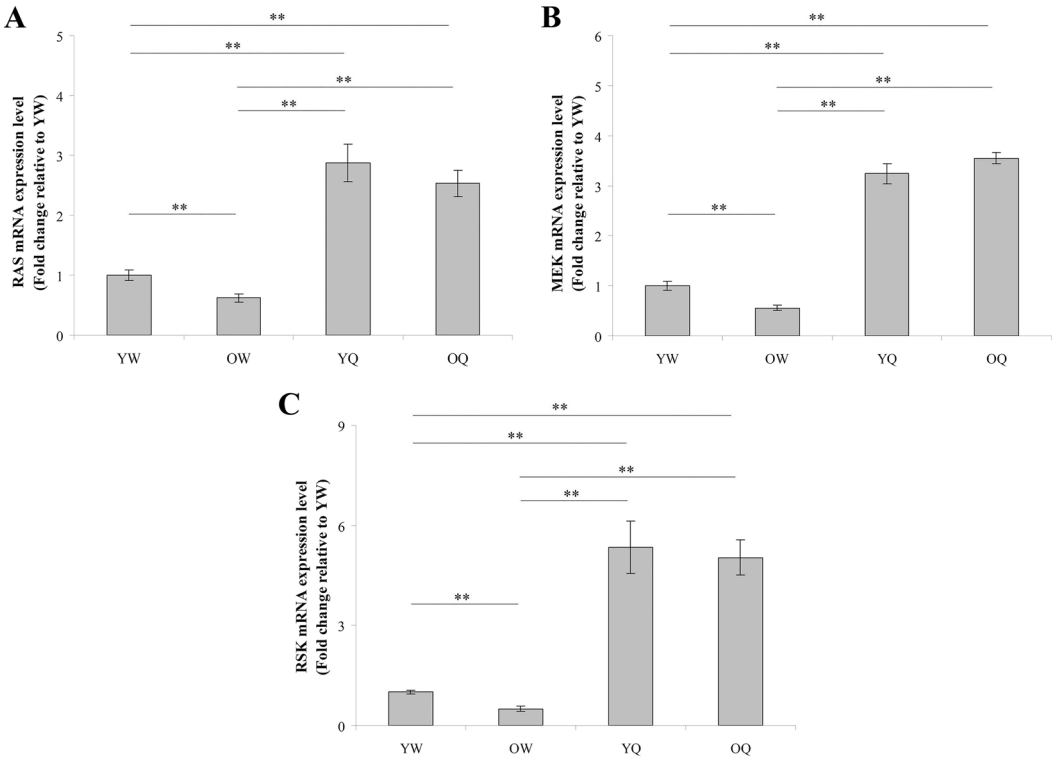

Figure 4. RAS mRNA level (**A**), MEK mRNA level (**B**), and RSK mRNA level (**C**) in the trophocytes and oenocytes of young and old worker bees and young and old queen bees. The values represent the means ± SEMs ($n = 5$). Asterisks indicate statistical significance (**$P < 0.01$; Mann–Whitney *U*-test). YW, young worker bees; OW, old worker bees; YQ, young queen bees; OQ, old queen bees.

4. DISCUSSION

Cellular activities including mitochondrial energy utilization activity, cellular energy regulation activity, cellular degradation activity, and cellular energy metabolism activity in the trophocytes and oenocytes of queen bees are higher than that of worker bees based on the results of NAD^+ levels, NAD^+/ NADH ratio, ATP levels, AMPK activities, lysosome activities, and ribosomal protein S6 mRNA levels. The molecules of cAMP/PKA and RAS/MAPK signaling pathways in the trophocytes and oenocytes of queen bees are higher than that of worker bees. These findings suggested that the cAMP/PKA and RAS/MAPK signaling pathways may increase cellular activities leading to the longevity of queen bees.

4.1. NAD^+ concentration, NAD^+/ NADH ratio, and ATP concentration

NADH can be synthesized in the tricarboxylic acid cycle through glycolysis and β-oxidation and converted to NAD^+ and H^+ by NADH dehydrogenase. H^+ increases mitochondrial membrane potential and promotes ATP synthesis by ATP synthase. Therefore, NAD^+ level, NAD^+/ NADH ratio, and ATP level have been used to evaluate mitochondrial energy utilization activity and cellular energy metabolism activity in honey bees (Chuang and Hsu 2013; Hsu and Hu 2014; Lu et al. 2017, 2018).

The NAD^+ levels and NAD^+/NADH ratios decrease with advancing age in pancreatic β cells and neurons of mice (Imai 2009), the livers, hearts, kidneys, and lungs of rats (Braidy et al. 2011), and the trophocytes and oenocytes of worker honey bees (Chuang and Hsu 2013). Additionally, caloric restriction, which extends the lifespan of organisms, is associated with high NAD^+ levels (Moroz et al. 2014). High NAD^+ levels also extend the lifespan of *Caenorhabditis elegans* (Hashimoto et al. 2010). The ATP levels decrease with advancing age in the brains of mice (Joo et al. 1999) and the hearts of rats (Guerrieri et al. 1996). Mitochondrial respiration and electron transport decrease with advancing age in the flight muscle of *Drosophila melanogaster* (Ferguson et al. 2005). The *daf-2 C. elegans* mutant *(e1370)*, which has an extended lifespan, has a higher ATP concentration than the wild-type nematode (Brys et al. 2010). Ambient temperature reduction, which can extend the lifespan of organisms, is associated with an increase in ATP concentration (Hsu and Chiu 2009). These studies indicated that young or long-lived cells have higher mitochondrial energy utilization activity and cellular energy metabolism activity than old or short-lived cells.

In this study, the trophocytes and oenocytes of young and old queen bees exhibit higher NAD^+ levels, NAD^+/NADH ratios, and ATP levels than that of young and old worker bees indicating that the trophocytes and oenocytes of queen bees have higher mitochondrial energy utilization activity and cellular energy metabolism activity than that of worker bees.

4.2. AMPK and lysosome activity and ribosomal protein S6 mRNA level

AMPK is a metabolic energy gauge and regulates cellular metabolism including the activation of glycolysis, fatty acid oxidation, mitochondrial biogenesis, and autophagy (Hardie et al. 2012; Reznick et al. 2007). Lysosomes are single-membrane organelles that contain acid hydrolase enzymes to degrade cellular macromolecules and organelles. Ribosomal protein S6 induces protein synthesis, cell growth, proliferation, and glucose homeostasis (Ruvinsky et al. 2005). Therefore, AMPK activity, lysosome activity, and ribosomal protein S6 have been used to evaluate cellular energy regulation activity, cellular degradation activity, and cellular energy metabolism activity in honey bees (Hsu and Chuang 2014; Hsu and Hu 2014; Lu et al. 2017, 2018).

AMPK activity decreases with advancing age in rat muscles (Reznick et al. 2007) and mouse left ventricular tissues (Turdi et al. 2010). Caloric restriction, which extends the lifespan of organisms, is associated with an increase in AMPK activity through AMPK-α phosphorylation (Salminen et al.

2016). The lifespan extension of *D. melanogaster* is mediated by activating AMPK (Su et al. 2019). The lysosomal activity decreased with aging in the liver of rats (Cuervo and Dice 2000) and *C. elegans* (Sun et al. 2020). Caloric restriction, which extends the lifespan of organisms, decreases lipofuscin accumulation in the lysosome of the mouse brain (Moore et al. 1995). Mitochondrial protein synthesis decreased with age in the heart of Wistar rats (Hudson et al. 1998), the skeletal muscle of humans (Rooyackers et al. 1996), and *D. melanogaster* (Yang et al. 2019). Lipoprotein lipase synthesis decreased with age in the postural skeletal muscle of Fischer 344 rats (Bey et al. 2001). These studies indicated that young or long-lived cells have higher cellular energy regulation activity, cellular degradation activity, and cellular energy metabolism activity than old or short-lived cells.

In this study, the trophocytes and oenocytes of young and old queen bees exhibit higher AMPK activities, lysosome activities, and ribosomal protein S6 mRNA levels than that of young and old worker bees indicating that the trophocytes and oenocytes of queen bees have higher cellular energy regulation activity, cellular degradation activity, and cellular energy metabolism activity than that of worker bees.

4.3. AC mRNA level, cAMP concentration, and PKA mRNA level

The cAMP is transformed from ATP by AC and as a second messenger in signaling transduction for triggering many cell processes. The cAMP can trigger calcium signaling and activate PKA which phosphorylates liver kinase B1 to activate AMPK (Park et al. 2012; Collins et al. 2000; Perisse et al. 2009).

AC mRNA expression decrease with age in mice hippocampus (Mons et al. 2004) and AC activity decline with age in rat myocardium (O'Connor et al. 1981). The cAMP levels decline with age in rat isolated aorta (Schoeffter and Stoclet 1990). PKA activity declines with age in the *Ceratitis capitata* brain (Laviada et al. 1997). These studies indicated that young cells have higher signaling of the AC/cAMP/PKA pathway than old cells.

In this study, the trophocytes and oenocytes of young and old queen bees exhibit higher AC mRNA levels, cAMP levels, and PKA mRNA levels than that of young and old worker bees indicating that the trophocytes and oenocytes of queen bees have higher signaling of AC/cAMP/PKA pathway than that of worker bees.

The studies of cAMP and PKA in this study are consistent with previous studies indicating that caffeine increase cAMP and calcium levels in the brain of honey bees (Scheiner et al. 2006), that dopamine and homovanillyl alcohol increase the cAMP level in the brain of honey bees (Beggs et al. 2007), that tyramine increase cAMP level via tyramine receptor 2 in honey bees (Rein et al. 2017), and that PKA RNAi decreased PKA activity and impaired long-term memory formation of honey bees (Fiala et al. 1999).

4.4. RAS, MEK, and RSK mRNA levels

The RAS/RAF/MEK/ERK, also called RAS/MAPK, signaling pathway is important in regulating cell survival and proliferation and in inhibiting cell apoptosis (Chung and Kondo et al. 2011). This signaling pathway phosphorylates RSK to activate ribosomal protein S6 for protein synthesis (Sawicka et al. 2016). The RAS/RAF/MEK/ERK signaling declines with age in rat hepatocytes (Hutter et al. 2000) and rat brains (Zhen et al. 1999). These studies indicated that young cells have higher signaling of the RAS/RAF/MEK/ERK pathway than old cells.

In this study, the trophocytes and oenocytes of young and old queen bees exhibit higher RAS, MEK, and RSK mRNA levels than that of young and old worker bees indicating that the trophocytes and oenocytes of queen bees have higher signaling of the RAS/MAPK pathway than that of worker bees. The study of the RAS/MAPK signaling pathway in this study is consistent with a previous study indicating that *Varroa*-parasitized honey bees with visibly deformed wing virus clinical signs activate the RAS pathway (Erban et al. 2019).

Parallel increases in AC mRNA level, cAMP concentration, PKA mRNA level, AMPK activity, RAS mRNA levels, MEK mRNA levels, RSK mRNA levels, ribosomal protein S6 mRNA levels,

and cellular functions of mitochondrial energy utilization activity, cellular energy regulation activity, cellular degradation activity, and cellular energy metabolism activity indicate that AC/cAMP/PKA and RAS/MAPK signaling pathways may be related to the increase of cellular activities. AC may increase the production of cAMP which activates AMPK activity through PKA to prompt cellular activities. This inference is supported by a previous study indicating that the activated receptor by biogenic amines stimulates AC to increase the concentration of cAMP which activates PKA in the brain to modulate the behavior of honey bees (Scheiner et al. 2006). The RAS pathway may activate RSK to activate ribosomal protein S6 for protein synthesis and cellular activities. This inference is consistent with a previous study indicating that RSK activates the ribosomal protein S6 of the brain in the mouse (Sawicka et al. 2016).

In addition, these results are consistent with our previously published transcriptional data showing that the mRNA expression of adenylyl cyclase, PKA, calcium/calmodulin-dependent protein kinase, RAS, RAF, and mitogen-activated protein kinase phosphatase 3 of queen bees are significantly higher than that of worker bees (Lu et al. 2021). Furthermore, the lifespan of queen and worker bees depends on their food intake. Queen bees eat RJ throughout their lives, and worker bees consume honey and pollen (Winston 1987). It infers that RJ might promote cellular activity through cAMP/PKA and RAS/MAPK signaling to increase NAD^+ and ATP concentration, AMPK and lysosome activities, and protein synthesis in the cells of queen bees.

Taken together, a parallel increase in cellular activities and signaling of cAMP/PKA and RAS/MAPK pathways in the trophocytes and oenocytes of queen bees suggests that the cAMP/PKA and RAS/MAPK signaling pathways may increase cellular activities leading to the longevity of queen bees.

AUTHOR CONTRIBUTION

CH designed the research; CH and YW performed research; CH, YW, and CC analyzed data; CH and CC wrote the paper. All authors read and approved the final manuscript.

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DATA AVAILABILITY

The raw data of the study are available from the corresponding author upon reasonable request.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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