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Original article

The cholesterol-hydroxyecdysone-vitellogenin pathway is involved in the longevity of trophocytes and oenocytes of queen honey bees (*Apis mellifera*)

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Abstract – Trophocytes and oenocytes in the abdomen of honey bees do not divide after eclosion; however, trophocytes and oenocytes of queen bees have a longer lifespan and maintain better cellular function than those of worker bees. To explore this phenomenon, we assayed the molecules involved in the cholesterol-hydroxyecdysone-vitellogenin (Vg) pathway in the trophocytes and oenocytes of young and old worker and queen bees. The results showed that Vg and cholesterol levels in hemolymph and cholesterol levels, 20-hydroxyecdysone (20E) levels, and the messenger RNA levels of *cytochrome P450 314A1 20-hydroxylase* (*Cyp314A1*), *ecdysone receptor isoform A* (*EcR-A*), *ecdysone receptor isoform B1* (*EcR-B1*), *ultraspiracle* (*USP*), *ecdysone-induced protein 74* (*E74*), *ecdysone-induced protein 75* (*E75*), *broad-complex* (*BR-C*), *Vg*, and *Vg receptor* (*VgR*) in trophocytes and oenocytes were increased in queen bees compared with worker bees. These findings indicated that queen bees have higher expression of molecules in the cholesterol-hydroxyecdysone-Vg pathway than worker bees.

cholesterol / 20-hydroxyecdysone / vitellogenin / longevity / honey bee

1. INTRODUCTION

Queen and worker honey bees are females, however, queen bees can live up to 3 years or 30 times longer than worker bees, which have an average lifespan of 35 days (Remolina & Hughes, 2008). Thus, queen bees have a much longer lifespan than worker bees, although they share the same genome. Therefore, honey bee (*Apis mellifera*) is a model animal for studies of aging and longevity (Keller & Jemielity, 2006). In addition, they live in large colonies and are easily manipulated, and their genome has been sequenced and is similar to vertebrate genomes. Genome sequence of honey bees is 47.5% similar to that of human beings (The Honeybee Genome Sequencing Consortium, 2006; Wang et al., 2006; Elsik et al., 2014).

The longevity of individuals depends on the longevity of their cells. In addition, trophocytes and oenocytes of honey bees are immersed in hemolymph and can be used to test anti-aging drugs when drugs are microinjected into the hemolymph. Therefore, trophocytes and oenocytes are chosen for cellular senescence and longevity studies (Hsieh and Hsu, 2011a). Furthermore, trophocytes and oenocytes are used in cellular senescence and longevity studies due to their

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analogous biological functions as white adipose and liver cells, ease of isolation from the abdomen, and convenient manipulation (Chan et al., 2011; Nilsen et al., 2011; Seehuus et al., 2013). Most importantly, these cells do not divide after eclosion, a necessary requirement for studying aging and longevity (Hsieh and Hsu 2011a, b). Trophocytes and oenocytes of honey bees are located in the abdomen and tightly attach to each other with gap junctions to construct a single layer of cells around each abdominal segment (Kuterbach and Walcott, 1986). Trophocytes are large and irregularly shaped, and oenocytes are small and spherical (Hsieh and Hsu 2011a, b; Paes-de-Oliveira and Cruz-Landim 2003).

Recent studies have shown that cellular functions including mitochondrial energy utilization, energy regulation activity, cellular degradation activity, and cellular metabolism in the trophocytes and oenocytes of worker bees decrease with age (Chuang and Hsu 2013; Hsu and Chuang 2014; Hsu et al. 2014; Lu et al. 2017). However, these processes are maintained with age in queen bees (Hsu and Hu 2014; Hsu and Lu 2015; Hsu et al. 2016). These findings indicate that the trophocytes and oenocytes of worker bees exhibit aging phenotypes and that those of queen bees show longevity phenotypes, as these cells do not proliferate after eclosion in either worker or queen bees (Hsieh and Hsu 2011a, b). Thus, the longevity of these cells is associated with that of queen bees. Exploring the longevity mechanism of trophocytes and oenocytes can promote the understanding of longevity mechanism of queen bees.

Vitellogenin (Vg), a precursor of yolk protein, is synthesized in the abdominal fat body (Sappington and Raikhel 1995) and is highly expressed in queen and long-lived winter worker bees (Seehuus et al. 2006; Corona et al. 2007; Aurori et al. 2014). Knockdown of Vg decreases the lifespan of worker bees (Nelson et al. 2007). These findings suggest that Vg may be related to the longevity of queen bees in addition to the deposition of yolk in developing oocytes. The transcription of Vg is regulated by the transcription factors including ecdysone-induced protein 74 (E74), ecdysone-induced protein 75 (E75), and broad-complex (BR-C) (Sun et al. 2002; Yang et al. 2014) and by the ecdysone receptor (EcR)/ultraspiracle (USP)/20-hydroxyecdysone (20E) nuclear receptor complex (Paul et al. 2005). The nuclear EcR/USP complex functions as the 20E receptor (Hill et al. 2013; Hansen et al. 2014; Mello et al. 2014). 20E, an active ecdysteroid, is synthesized from ecdysone by cytochrome P450 314A1 20-hydroxylase (Cyp314A1). Ecdysone is synthesized from cholesterol in the ecdysteroid biosynthesis pathway (Petryk et al. 2003; Yamazaki et al. 2011). Thus, the cholesterol-hydroxyecdysone-Vg pathway seems to be potentially involved in the longevity of queen bees. However, it remains unclear whether the cholesterol-hydroxyecdysone-Vg pathway is active in the trophocytes and oenocytes of worker and queen bees and whether this pathway is related to the longevity of trophocytes and oenocytes of queen bees.

2. MATERIALS AND METHODS

2.1. Honey bees

Young (5-day-old) and old (30-day-old) worker bees and young (2-month-old) and old (16-month-old) queen bees were collected on the same dates for the same experiments. The breeding and collection of bees were described in our previous studies (Hsu and Chan 2013; Hsieh and Hsu 2013).

2.2. Hemolymph collection

Hemolymph was collected from two young or two old worker bees and from one young or one old queen bee between the second and third abdominal segments by capillary tubes (1-000-0500; Drummond Scientific Company, PA, USA). Hemolymph from two worker bees was combined for each sample.

2.3. Vg levels in hemolymph

Four microliters of hemolymph (described in Section 2.2) were mixed with 10.5 μl of the luciferase culture cell lysis reagent, 0.5 μl of protease inhibitor (11697498001; Roche Applied Science, IN, USA), and 5 μl of SDS sample buffer. The mixture was centrifuged and resolved on a 10%

SDS gel. The gel was stained using Coomassie blue. Vg is 180 kDa protein (Wheeler and Kawooya, 1990). Five replicates were performed: five samples containing hemolymph from two young and old worker bees and five samples containing the hemolymph of individual young and old queen bees.

2.4. Cholesterol levels in hemolymph

Cholesterol levels were quantified using a cholesterol quantitation kit (MAK043; Sigma, MO, USA). Briefly, 1 µl of hemolymph (described in Section 2.2) was mixed with 200 µl chloroform/isopropanol/IGEPAL CA-630 (7:11:0.1) and centrifuged at 13,000*g*. The organic phase was transferred to a new tube and dried at 50 °C. Then, the pellet was dissolved in 200 µl cholesterol assay buffer to obtain the supernatant. After background luminescence was recorded, 50 µl of a diluted standard cholesterol solution (0, 20, 40, 60, 80, and 100 ng μl^{-1}) or 50 µl supernatant were mixed with 44 µl cholesterol assay buffer, 2 µl cholesterol probe, 2 µl cholesterol enzyme mix, and 2 µl cholesterol esterase and incubated for 60 min at 37 °C in the dark. The cholesterol levels were measured at 570 nm using a spectrophotometer (Synergy HT; BioTek, VT, USA) and are expressed as nanograms per microliter. A calibration curve using cholesterol standards was used to quantify cholesterol levels. Five replicates were performed: five samples containing hemolymph from two young and old worker bees and five samples containing the hemolymph of individual young and old queen bees.

2.5. Cholesterol levels in trophocytes and oenocytes

The assay of cholesterol levels in trophocytes and oenocytes was modified from Section 2.4. Briefly, 10 mg of trophocytes and oenocytes from two young or two old worker bees and one young or one old queen bee were detached from the cuticle of the abdomen using a knife, homogenized with 200 µl chloroform/isopropanol/IGEPAL CA-630 (7:11:0.1), and centrifuged at 13,000*g*. The remaining method is the same as Section 2.4. Five replicates were performed: five samples containing supernatant from two young and old worker bees and five samples containing the supernatant of individual young and old queen bees.

2.6. 20E levels in trophocytes and oenocytes

20E levels were measured using a 20E enzyme immunoassay kit (A05120; Bertin Pharma, MLB, France). Briefly, 10 mg of trophocytes and oenocytes from two young or two old worker bees and one young or one old queen bee was detached from the cuticle of the abdomen using a knife, homogenized with 400 µl of methanol, and centrifuged at 5000*g*. Supernatants were transferred into a new tube, dried, and re-dissolved in 200 µl of EIA buffer. The 96-well plate was pre-washed with wash buffer, after which 50 µl of a diluted 20E standard solution (0, 39.1, 78.1, 156.3, 312.5, 625, 1250, 2500, and 5000 pg ml^{-1}), and 50 µl each of samples was loaded onto the plate in duplicate. The plate is incubated at 4 °C overnight, then emptied and washed with 300 µl wash buffer. Finally, the plate was developed with 200 µl of Ellman's reagent and incubated in the dark at room temperature on a shaker. 20E levels were measured at 410 nm using a spectrophotometer (Synergy HT; BioTek) and are expressed as picograms per milliliter. A calibration curve using 20E standards was used to quantify 20E levels. Five replicates were performed: five samples containing supernatant from two young and old worker bees and five samples containing the supernatant of individual young and old queen bees.

2.7. Quantitative real-time polymerase chain reaction analysis

Trophocytes and oenocytes were isolated from two young or two old worker bees and from one young or one old queen bee. Total RNA was extracted from these cells using TRIzol® Reagent (15596018; Invitrogen, CA, USA). RNA concentration and quality were determined using a Synergy™ HT multi-mode microplate reader (7091000; BioTek). 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. Quantitative real-time polymerase chain reaction (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. Primer sequences were shown in supplemental Table S1. The *actin* gene was used as a reference gene (Lourenco et al., 2008). Five replicates were performed: five samples containing total RNA from two young and old worker bees and five samples containing the total RNA of individual young and 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 the Mann-Whitney *U* test for pairwise comparisons. The *P* value less than 0.01 was considered statistically significant.

3. RESULTS

3.1. Vg and cholesterol levels in hemolymph

To discover molecules associated with the cellular function of trophocytes and oenocytes, we assayed the proteins in hemolymph. The results showed that Vg concentration in the hemolymph of queen bees was significantly higher than worker bees (Fig. 1a). The fold changes in the mean Vg concentration in hemolymph in old worker bees, young queen bees, and old queen bees relative to young worker bees were 1.36 ± 0.38, 21.71 ± 5.90, and 24.08 ± 5.44, respectively. Vg levels significantly differed between young worker bees and young and old queen bees and between old worker bees and young and old queen bees (n = 5, P = 0.008; Fig. 1b).

We propose here a hypothetical cholesterol-hydroxyecdysone-Vg pathway as underlying differential longevity in honey bees. In this pathway, dietary cholesterol would be the driver and Vg production by fat body trophocytes and oenocytes would be the final outcome. To confirm Vg levels, we assayed cholesterol levels in hemolymph because cholesterol is related to Vg synthesis in the cholesterol-hydroxyecdysone-Vg pathway. The mean cholesterol levels in the hemolymph of young and old worker bees and young and old queen bees were 299.37 ± 29.70, 181.95 ± 41.61, 692.38 ± 111.16, and 867.05 ± 131.14 ng μl^{-1}, respectively. Cholesterol levels significantly differed between young worker bees and young and old queen bees and between old worker bees and young and old queen bees (n = 5, P = 0.008; Fig. 1c). These findings demonstrated that queen bees had higher Vg and cholesterol levels in hemolymph than worker bees.

3.2. Cholesterol levels, 20E levels, and Cyp314A1 messenger RNA expression

Cholesterol in hemolymph can enter into trophocytes and oenocytes (Clark and Block, 1959). The mean cholesterol levels in the trophocytes and oenocytes of young and old worker bees and young and old queen bees were 39.33 ± 3.07, 16.83 ± 1.68, 97.98 ± 14.06, and 82.73 ± 13.17 ng μl^{-1}, respectively. Cholesterol 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 = 0.008; Fig. 2a).

Cholesterol can be used to synthesize 20E in the cholesterol-hydroxyecdysone pathway (Petryk et al. 2003; Yamazaki et al. 2011). We assayed 20E levels in the trophocytes and oenocytes of young and old worker bees and young and old queen bees. The mean 20E levels in young and old worker bees and young and old queen bees were 55.41 ± 11.80, 46.84 ± 11.16, 608.15 ± 27.78, and 809.21 ± 90.95 ng μl^{-1}, respectively. 20E levels significantly differed between young worker bees and young and old queen bees and between old worker bees and young and old queen bees (n = 5, P = 0.008; Fig. 2b). Queen bees have an average 14 times 20E levels than worker bees.

To further confirm ecdysteroid biosynthesis in the trophocytes and oenocytes of young and old worker bees and young and old queen bees,

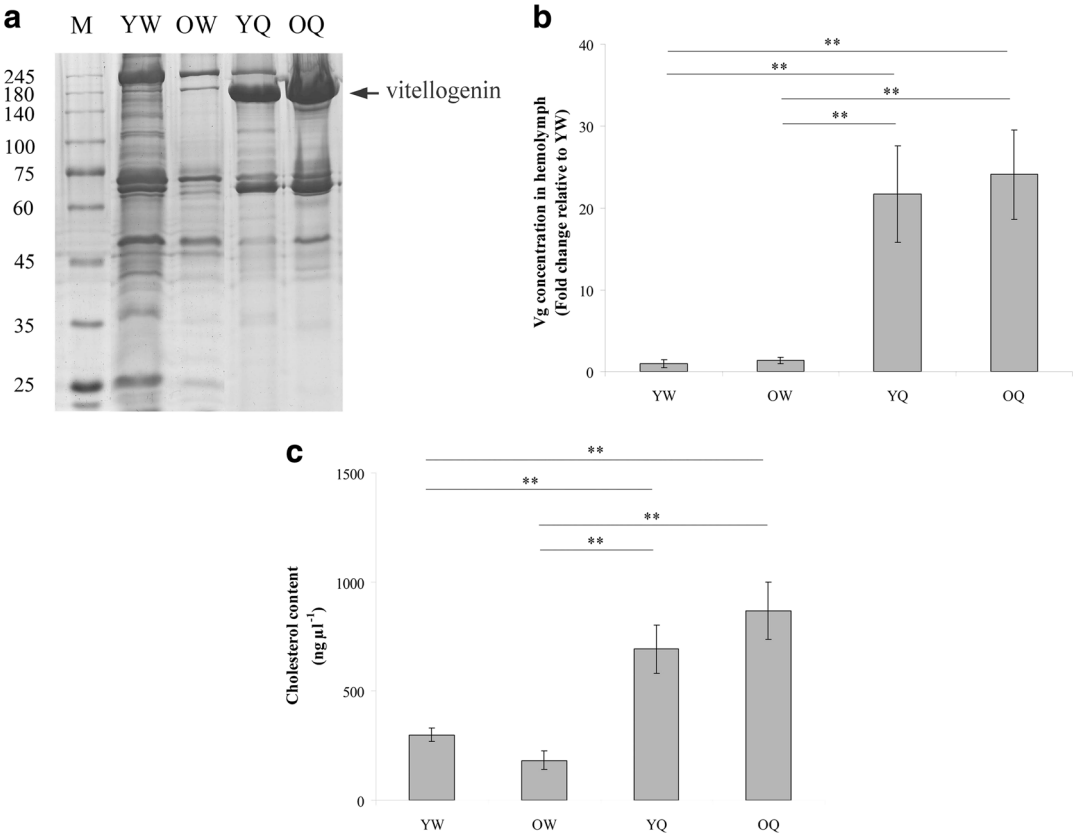

Figure 1. Vg expression and cholesterol levels in the hemolymph of young and old worker bees and young and old queen bees. **a** Vg was analyzed using SDS-PAGE. **b** The results are normalized to the expression in young worker bees; they are presented as fold changes and represent the mean ± standard error of the mean (SEM) ($n = 5$). **c** Cholesterol levels. The bars indicate the mean ± SEM ($n = 5$). M, markers; YW, young worker bees; OW, old worker bees; YQ, young queen bees; OQ, old queen bees. The asterisks indicate significant differences (**$P < 0.01$; Mann-Whitney U test).

we assessed their Cyp314A1 messenger RNA (mRNA) expression levels. The fold changes in the mean Cyp314A1 mRNA expression levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 1.09 ± 0.21, 13.97 ± 2.39, and 14.06 ± 4.22, respectively. Cyp314A1 mRNA expression significantly differed between young worker bees and young and old queen bees and between old worker bees and young and old queen bees ($n = 5$, $P = 0.008$; Fig. 2c). These findings indicated that the trophocytes and oenocytes of queen bees had higher cholesterol levels, 20E levels, and ecdysteroid biosynthesis than worker bees.

3.3. EcR-A, EcR-B1, and USP mRNA expression

To determine 20E receptor expression, we measured the mRNA expression of *EcR-A* , *EcR-B1* , and *USP* in the trophocytes and oenocytes of young and old worker bees and young and old queen bees. The fold changes in the mean EcR-A mRNA expression levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 1.14 ± 0.11, 2.07 ± 0.10, and 2.21 ± 0.25, respectively. EcR-A mRNA expression significantly differed between young worker bees and young and old queen bees and between old worker bees and young and old

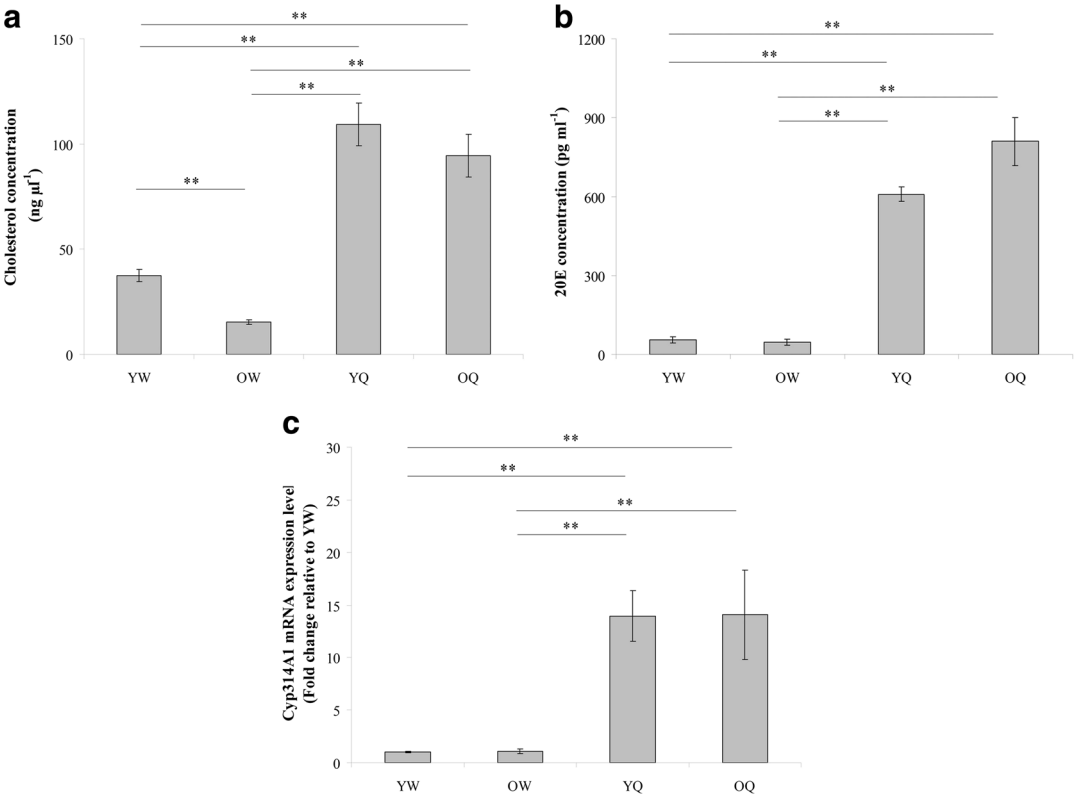

Figure 2. Cholesterol (**a**) and 20E (**b**) levels and *Cyp314A1* mRNA expression (**c**) in the trophocytes and oenocytes of young and old worker bees and young and old queen bees. In **a** and **b**, the bars indicate the mean ± SEM ($n = 5$). In **c**, the results are normalized to the expression in young worker bees; they are presented as fold changes and represent the mean ± SEM ($n = 5$). YW, young worker bees; OW, old worker bees; YQ, young queen bees; OQ, old queen bees. The asterisks indicate significant differences (**$P < 0.01$; Mann-Whitney U test).

queen bees ($n = 5$, $P = 0.008$; Fig. 3a). The fold changes in the mean EcR-B1 mRNA expression levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.75 ± 0.14, 1.28 ± 0.12, and 1.17 ± 0.08, respectively. EcR-B1 mRNA expression significantly differed between young worker bees and young and old queen bees and between old worker bees and young and old queen bees ($n = 5$, $P = 0.008$; Fig. 3b). The fold changes in the mean USP mRNA expression levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.57 ± 0.10, 1.62 ± 0.14, and 1.81 ± 0.18, respectively. USP mRNA expression 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 ($n = 5$, $P = 0.008$; Fig. 3c). These findings indicated that the trophocytes and oenocytes of queen bees had higher expression of EcR genes than worker bees.

3.4. E74, E75, and BR-C mRNA expression

To determine the expression of transcription factors of Vg, we measured the mRNA expression of E74, E75, and BR-C in the trophocytes and oenocytes of young and old worker bees and young and old queen bees. The fold changes in the mean E74 mRNA expression levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.62 ± 0.10, 2.36 ± 0.47, and 2.15 ± 0.28,

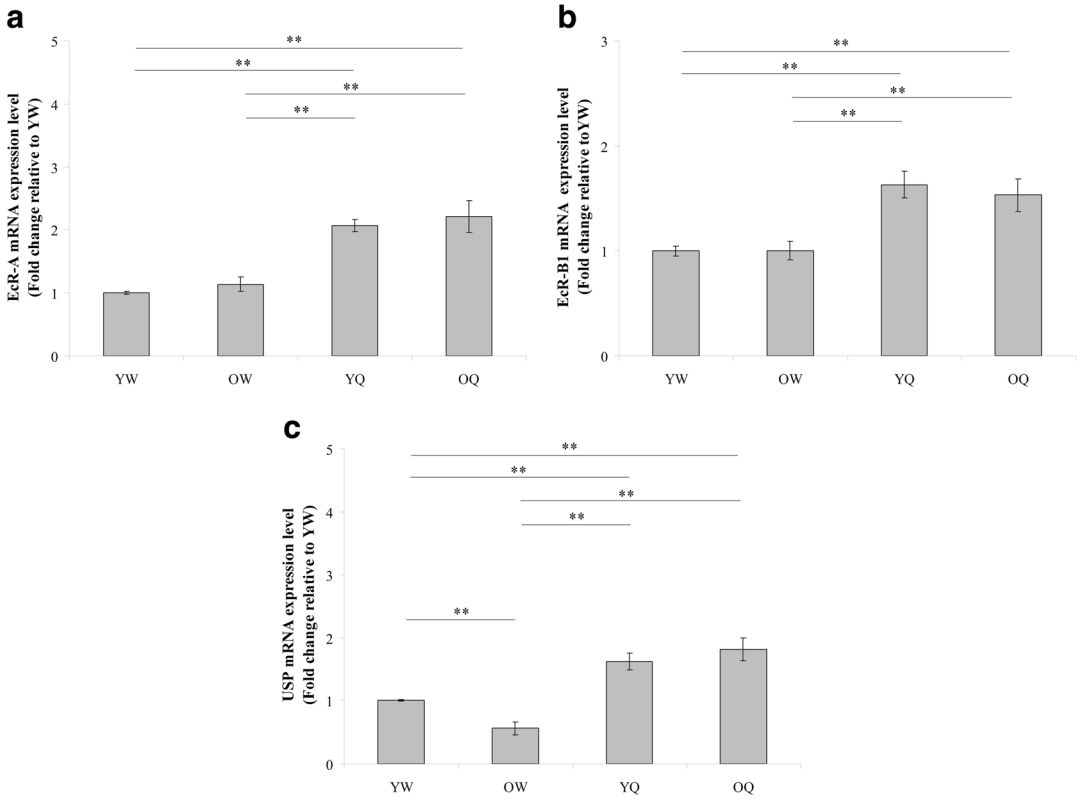

Figure 3. The mRNA expression of *EcR-A* (**a**), *EcR-B1* (**b**), and *USP* (**c**) in the trophocytes and oenocytes of young and old worker bees and young and old queen bees. The results are normalized to the expression in young worker bees; they are presented as fold changes and represent the mean ± SEM ($n = 5$). YW, young worker bees; OW, old worker bees; YQ, young queen bees; OQ, old queen bees. The asterisks indicate significant differences (**$P < 0.01$; Mann-Whitney U test).

respectively. E74 mRNA expression 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 ($n = 5$, $P = 0.008$; Fig. 4a). The fold changes in the mean E75 mRNA expression levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 1.20 ± 0.35, 2.39 ± 0.36, and 3.17 ± 0.22, respectively. E75 mRNA expression significantly differed between young worker bees and young and old queen bees and between old worker bees and young and old queen bees ($n = 5$, $P = 0.008$; Fig. 4b). The fold changes in the mean BR-C mRNA expression levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 1.21 ± 0.18, 5.91 ± 0.74, and 5.35 ± 1.62, respectively. BR-C mRNA expression significantly differed between young worker bees and young and old queen bees and between old worker bees and young and old queen bees ($n = 5$, $P = 0.008$; Fig. 4c). These findings indicated that the trophocytes and oenocytes of queen bees had higher expression of transcription factors of Vg than worker bees.

3.5. Vg and VgR mRNA expression

To understand *Vg* and *VgR* expression, we measured Vg and VgR mRNA expression in the trophocytes and oenocytes of young and old worker bees and young and old queen bees. The fold changes in the mean Vg mRNA expression

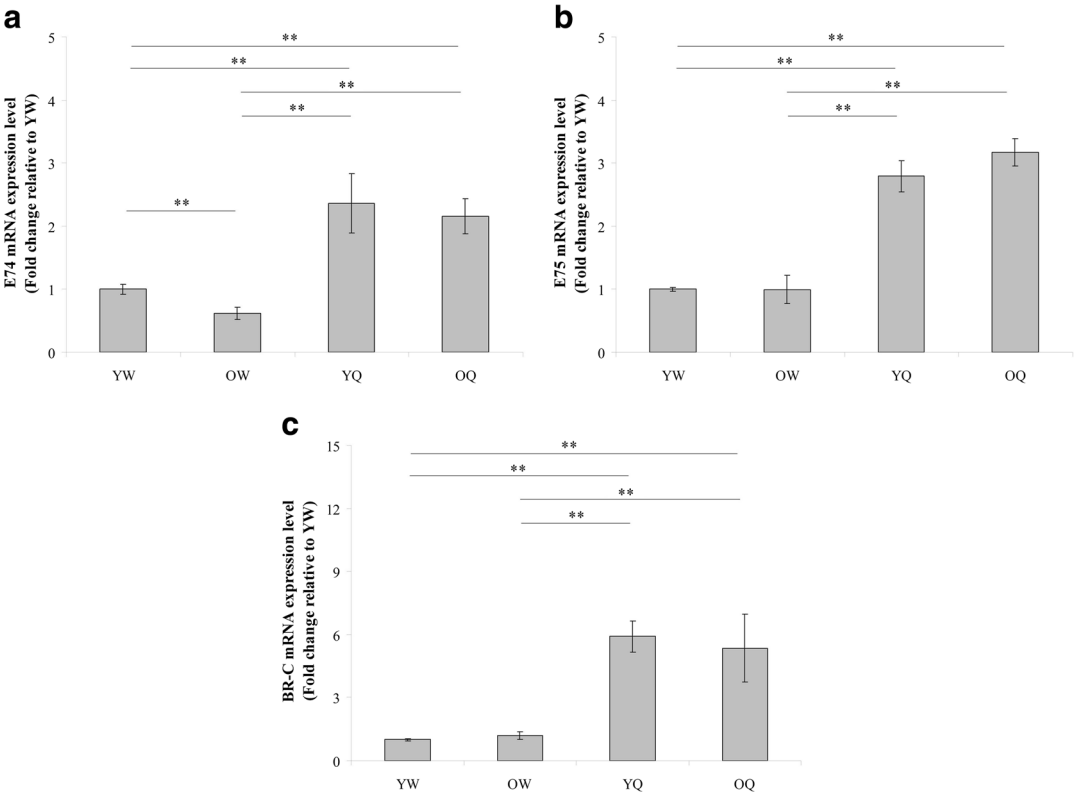

Figure 4. The mRNA expression of *E74* (**a**), *E75* (**b**) and *BR-C* (**c**) in the trophocytes and oenocytes of young and old worker bees and young and old queen bees. The results are normalized to the expression in young worker bees; they are presented as fold changes and represent the mean ± SEM ($n = 5$). YW, young worker bees; OW, old worker bees; YQ, young queen bees; OQ, old queen bees. The asterisks indicate significant differences (**$P < 0.01$; Mann-Whitney U test).

levels in old worker bees, young queen bees, and old queen bees were 0.92 ± 0.25, 280.81 ± 22.95, and 233.37 ± 62.31, respectively. Vg mRNA expression significantly differed between young worker bees and young and old queen bees and between old worker bees and young and old queen bees ($n = 5$, $P = 0.008$; Fig. 5a). Queen bees have an average 250 times Vg mRNA expression than worker bees. The fold changes in the mean VgR mRNA expression levels in old worker bees, young queen bees, and old queen bees relative to young worker bees were 0.99 ± 0.22, 11.09 ± 2.51, and 18.26 ± 3.80, respectively. VgR mRNA expression significantly differed between young worker bees and young and old queen bees and between old worker bees and young and old queen bees ($n = 5$, $P = 0.008$; Fig. 5b). These findings indicated that the trophocytes and oenocytes of queen bees had higher Vg and VgR expression than worker bees.

4. DISCUSSION

We have explored the longevity mechanism of queen bees by assessing mitochondrial energy utilization, energy regulation activity, cellular degradation activity, and cellular metabolism in the trophocytes and oenocytes of worker and queen bees and have demonstrated that these cells exhibit aging phenotypes in worker bees but maintain longevity phenotypes in queen bees (Chuang and Hsu 2013; Hsu and Chuang 2014; Hsu et al. 2014; Lu et al. 2017; Hsu and Hu 2014; Hsu and Lu 2015; Hsu et al. 2016). To explore this mechanism further,

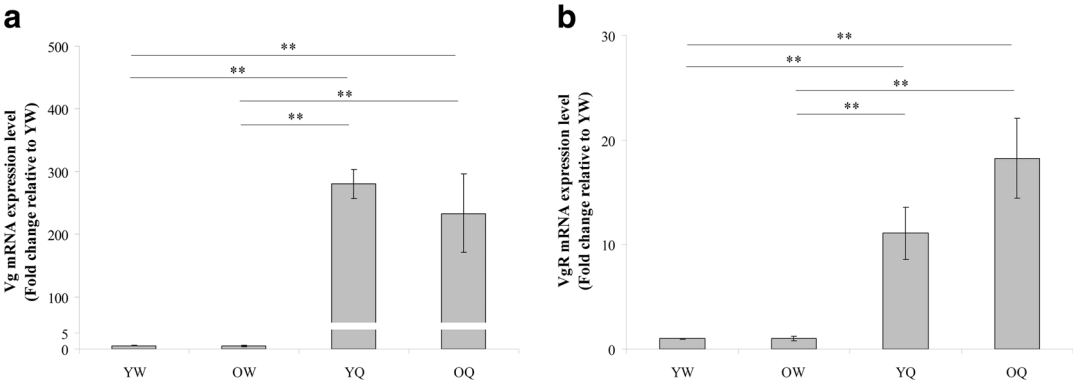

Figure 5. The mRNA expression of *Vg* (**a**) and *VgR* (**b**) in the trophocytes and oenocytes of young and old worker bees and young and old queen bees. The results are normalized to the expression in young worker bees; they are presented as fold changes and represent the mean ± SEM ($n = 5$). YW, young worker bees; OW, old worker bees; YQ, young queen bees; OQ, old queen bees. The asterisks indicate significant differences (**$P < 0.01$; Mann-Whitney U test).

in this study, the molecules involved in the cholesterol-hydroxyecdysone-Vg pathway were assessed. The findings indicated that the trophocytes and oenocytes of queen bees have increased ecdysteroid and Vg biosynthesis compared with worker bees and parallel increases in the activation of the cholesterol-hydroxyecdysone-Vg pathway and the cellular function of queen bees, suggesting that the cholesterol-hydroxyecdysone-Vg pathway is involved in the longevity of trophocytes and oenocytes of queen bees, which may be associated with the longevity of queen bees.

4.1. Vg and cholesterol levels in hemolymph

Trophocytes and oenocytes are immersed in hemolymph as honey bees have an open circulatory system. The cellular function of trophocytes and oenocytes is regulated by molecules in hemolymph. The hemolymph of queen bees has higher Vg levels than worker bees, indicating that Vg is not only associated with oogenesis but also contributed to cellular longevity. This inference is supported by previous studies showing that queen bees and long-lived winter worker bees have higher Vg expression (Seehuus et al. 2006; Aurori et al., 2014), knockdown of Vg decreases the lifespan of worker bees (Nelson et al. 2007), Vg is necessary for proper immune function (Amdam et al., 2004, 2005), Vg acts as an antioxidant in female honey bees (Seehuus et al. 2006; Corona et al. 2007), and Vg increases synthesis under *Nosema ceranae* infection (Lee and Nieh 2017). Vg may induce the capacity to respond to stress to increase the lifespan of queen bees.

Vg synthesis is regulated by 20E, which is derived from cholesterol (Hansen et al. 2014; Yamazaki et al. 2011). Honey bees cannot synthesize cholesterol, which is derived from pollen in worker bees and from royal jelly in queen bees (Clark and Block, 1959). The hemolymph of queen bees has higher cholesterol levels than worker bees, indicating that queen bees have high cholesterol, which is used to synthesize 20E.

4.2. Cholesterol levels, 20E levels, and Cyp314A1 mRNA expression

The cholesterol of trophocytes and oenocytes is taken up from hemolymph. The cholesterol levels of trophocytes and oenocytes are proportional to those of hemolymph. The cholesterol of trophocytes and oenocytes can be converted to ecdysone, which can be further converted to 20E via Cyp314A1 20-hydroxylase in fruit flies (Petryk et al. 2003; Huang et al. 2008) and honey bees (Feldlaufer et al. 1986; Yamazaki et al. 2011). In this study, cholesterol, 20E, and Cyp314A1 mRNA expression levels in the trophocytes and oenocytes of queen bees were

higher than those measured in worker bees, suggesting that ecdysteroid biosynthesis was increased in queen bees. This inference is consistent with a previous study showing that ecdysteroid hemolymph titers of queen bees are higher than those of laying worker bees and foragers (Robinson et al., 1991).

Cyp314A1 is expressed in the brains, ovaries, and fat bodies of worker honey bees (Yamazaki et al. 2011). The ovaries of egg-laying worker and queen honey bees express higher Cyp314A1 mRNA than those of nurse bees and foragers (Yamazaki et al. 2011). The egg-laying worker and queen honey bees also synthesize higher ecdysteroid than nurse bees and foragers (Robinson et al. 1991). In addition, queen bumble bees also express higher Cyp314A1 mRNA than worker bumble bees (Geva et al. 2005). These studies demonstrated that long-lived female bees highly express Cyp314A1 mRNA. This phenomenon indicated that 20E is associated not only with oogenesis but also with longevity. 20E effect on oogenesis and longevity may be mediated by Vg via the hydroxyecdysone-Vg pathway.

4.3. EcR-A, EcR-B1, and USP mRNA expression

The EcR-A/EcR-B1/USP complex functions as 20E cytoplasmic receptor in fruit flies (Yao et al. 1992; Talbot et al. 1993; Hill et al. 2013), mosquitos (Wang et al. 1998; Wang et al. 2002; Hansen et al. 2014), and honey bees (Takeuchi et al. 2007; Barchuk et al. 2008; Mello et al. 2014). *EcR-A* and *EcR-B1* are EcR subtypes in fruit flies (Talbot et al. 1993) and honey bees (Takeuchi et al. 2007). EcR-A is expressed in the brains and ovaries of honey bees (Takeuchi et al. 2007). This study demonstrated that *EcR-A*, *EcR-B1*, and *USP* were also expressed in the trophocytes and oenocytes of female honey bees. Higher expression of EcR-A, EcR-B1, and USP mRNA in the trophocytes and oenocytes of queen bees indicated that ecdysone-triggered regulatory hierarchy was highly activated in queen bees than worker bees. These findings are consistent with the results of cholesterol, 20E, and Cyp314A1 in this study.

4.4. E74, E75, and BR-C mRNA expression

E74, E75, and BR-C are the transcription factors of Vg in fruit flies (Burtis et al. 1990; Segraves and Hogness 1990; Tzolovsky et al. 1999), mosquitos (Pierceall et al. 1999; Sun et al. 2002; Zhu et al. 2007), tobacco hornworms (Zhou et al. 1998), wax moths (Jindra et al. 1994), honey bees (Paul et al. 2005, 2006), and silkworm (Yang et al. 2014). In this study, *E74*, *E75*, and *BR-C* were expressed at higher levels in the trophocytes and oenocytes of queen bees than worker bees, demonstrating that *E74*, *E75*, and *BR-C* were expressed in the trophocytes and oenocytes of female honey bees. Higher expression of E74, E75, and BR-C mRNA in the trophocytes and oenocytes of queen bees confirmed that ecdysone-triggered regulatory hierarchies were activated in queen bees. These findings are consistent with the results of cholesterol, 20E, Cyp314A1, EcR-A, EcR-B1, and USP in this study.

4.5. Vg and VgR mRNA expression

Vg is expressed in the fat bodies of mosquitos (Deitsch et al. 1995), honey bees (Wyatt and Davey 1996), and silkworm (Yang et al. 2014). *Vg* and *VgR* were expressed at higher levels in the trophocytes and oenocytes of queen bees than worker bees, indicating that queen bees synthesized greater amounts of Vg and VgR. *Vg* mRNA expression in trophocytes and oenocytes is consistent with Vg expression in hemolymph. The high *Vg* and *VgR* mRNA levels in trophocytes and oenocytes and high Vg levels in hemolymph suggest that the trophocytes and oenocytes of queen bees might perform the autocrine function through Vg binding to VgR to promote their longevity. *VgR* has been found in the fat bodies, ovaries, head, hypopharyngeal gland, and midgut of honey bees (this article, Guidugli-Lazzarini et al. 2008). Vg may increase the cellular lifespan of *VgR*-expressing cells, leading to the longevity of queen bees.

Vg expression is promoted by the EcR/USP/20E and E74/E75/BR-C complexes in fruit flies (Hansen et al. 2014), mosquitos (Pierceall et al. 1999; Sun et al. 2002), and silkworm (Yang et al.

2014). In this study, we demonstrated that Vg expression is correlated with the EcR/USP/20E and E74/E75/BR-C complex in the trophocytes and oenocytes of queen bees and that the cholesterol-hydroxyecdysone-Vg pathway is active in the trophocytes and oenocytes of queen bees, and this pathway is related to the longevity of trophocytes and oenocytes of queen bees.

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AUTHOR CONTRIBUTIONS

C.Y.L. and C.Y.H. designed the research; C.Y.L. performed the research; C.Y.L., P.J.H, and C.Y.H. analyzed the data; C.Y.H. wrote the paper.

La voie cholestérol-hydroxyecdysone-vitellogénine est impliquée dans la longévité des trophocytes et des oenocytes de reines *(Apis mellifera)*

Cholestérol / 20-hydroxyecdysone / vitellogénine / longévité / abeille.

Der Cholesterol-Hydroxyecdyson-Vitellogenin-Stoffwechselweg beeinflusst die Lebensdauer von Trophozyten und Oenozyten der Bienenkönigin *(Apis mellifera)*

Cholesterol / 20-Hydroxyecdyson / Vitellogenin / Lebensdauer / Honigbiene.

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