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Oxidative Stress and Anti-Oxidant Enzyme Activities in the Trophocytes and Fat Cells of Queen Honeybees (*Apis mellifera*)

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Abstract

Trophocytes and fat cells of queen honeybees have been used for delayed cellular senescence studies, but their oxidative stress and anti-oxidant enzyme activities with advancing age are unknown. In this study, we assayed reactive oxygen species (ROS) and anti-oxidant enzymes in the trophocytes and fat cells of young and old queens. Young queens had lower ROS levels, lower superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities, and higher thioredoxin reductase (TR) activity compared to old queens. These results show that oxidative stress and anti-oxidant enzyme activities in trophocytes and fat cells increase with advancing age in queens and suggest that an increase in oxidative stress and a consequent increase in stress defense mechanisms are associated with the longevity of queen honeybees.

Introduction

Aging is a complicated process that leads to decreasing cellular proliferative potential and increasing cellular deterioration, causing a progressive decline in biological function and an increased incidence of age-associated diseases.[1] Studies on the biology of aging not only contribute to the understanding of aging mechanisms but also provide insight into age-associated diseases and possible treatment strategies.

The oxidative stress hypothesis indicates that aging results from the accumulation of oxidative damage and that life span is determined by the rate at which oxidative damage occurs.[2] Cellular oxidative stress results when the generation of reactive oxygen species (ROS) exceeds the capacity of cellular anti-oxidant defenses to remove these toxic species. ROS include a diverse variety of chemical species, including superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2). ROS are mainly produced through reactions of the mitochondrial electron transport chain, the oxidation of polyunsaturated fatty acids, and nitric oxide generation.[3] In addition, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase (NO) and xanthine oxidase (XO) also produce superoxide and H_2O_2. ROS can damage lipids, proteins, carbohydrates, and nucleic acids. The subsequent dysfunction of organelles as well as the damage to cellular integrity and functionality that result from these molecular insults lead to cellular senescence.[4] An intricate anti-oxidant defense system that includes catalase (CAT), glutathione peroxidase (GPx)/glutathione reductase (GR) system, superoxide dismutase (SOD), and the thioredoxin peroxidase (TPx)/thioredoxin reductase (TR) system has evolved to neutralize the burden of ROS production. CAT, which is abundant in peroxisomes but less prevalent in mitochondria and the endoplasmic reticulum, converts H_2O_2 to water and O_2. GPx, which is present in the cytoplasm and mitochondrial matrix, removes H_2O_2 by coupling its reduction to water with the oxidation of glutathione to glutathione disulfide. Subsequently, GR reduces glutathione disulfide to glutathione. GPx can also reduce other peroxides, such as fatty acid hydroperoxides. SODs are metal-containing enzymes that catalyze the removal of superoxide to generate H_2O_2. Cu,Zn-SOD is present in the cytoplasm and nucleus, whereas Mn-SOD is primarily located in mitochondria.[5] TPx, which is present in the cytoplasm, mitochondrial matrix, and nucleus, removes H_2O_2 by coupling its reduction to water with the oxidation of reduced thioredoxin to oxidized thioredoxin. Subsequently, TR reduces oxidized thioredoxin to reduced thioredoxin.[6,7]

Despite the presumptive link between oxidative stress and aging, recent studies have shown that increased oxidative stress promotes the longevity and metabolic health of organisms[8,9] and that mitochondrial oxidative stress is not causal with respect to aging.[10–13] These phenomena highlight the

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concept of mitohormesis, in which increased mitochondrial metabolism and ROS formation induce an adaptive response that increases stress resistance and extends the life span.[8,9]

Trophocytes and fat cells of honeybees (*Apis mellifera*) attach to one another to form a single layer of cells around each segment of abdomen. The ease with which these cells can be isolated from the abdomen and manipulated as well as the fact that no cell division occurs during adulthood make them attractive targets for cellular senescence studies in honeybees.[14–18] Aging-related molecule assays in the trophocytes and fat cells of young or old workers and young or old queens showed that the trophocytes and fat cells of queens had longevity-promoting mechanisms that prolonged life span and could serve as target cells for the study of delayed cellular senescence.[14,15] If trophocytes and fat cells of queens are to be used for longevity-promoting mechanisms studies, it is important to investigate the changes in oxidative stress and anti-oxidant enzyme activities in young and old queens. Such data could provide clues for understanding delayed cellular senescence mechanisms in future aging studies. In this study, the levels of ROS and the activities of anti-oxidant enzymes were evaluated in the trophocytes and fat cells of young and old queens to clarify the relationship of oxidative stress and aging in queen honeybees.

Materials and Methods

Queen honeybees

The queens were purchased from a single commercial breeder (Hsinchu, Taiwan). As described in a previous study,[15] young (2-month-old) and old (16-month-old) queens were mated with drones and were able to lay eggs. They were collected from different hives on the same dates for the following studies. Young queens have dense and light yellowish-brown fuzz and light yellowish-brown epidermis. However, old queens have sparse and dark brown fuzz and dark blackish-brown epidermis.

ROS assays in trophocytes and fat cells

Individual queens of each group were dissected, and their abdominal trophocytes and fat cells were detached from the cuticle in honeybee saline (156.4 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 22.2 mM glucose, pH 7.3).[19] Trophocytes and fat cells were used for ROS assays.

Dihydroethidine (HET) was used to evaluate ROS, including superoxide, hydroxyl radicals, and H_2O_2.[20–22] The trophocytes and fat cells from one young or old queen were incubated with 5 μM HET (D7008; Sigma, Saint Louis, MO) for 10 min at 37°C and then stained with 0.2 μM Hoechst 33342 for 5 min at 37°C (H3570; Invitrogen, Carlsbad, CA). ROS (red fluorescence) was detected by observing cells at room temperature under a confocal laser scanning microscope (Leica TCS SP2; Leica, Wetzlar, Germany) at an excitation wavelength of 520 nm and an emission wavelength of 610 nm; nuclei (blue fluorescence) were detected at excitation and emission wavelengths of 350 and 461 nm, respectively.[22,23]

2′,7′-Dichlorodihydrofluorescein-diacetate (H_2DCF-DA) was used to evaluate ROS, including H_2O_2, hydroxyl radicals, and peroxyl radicals.[22,23] The trophocytes and fat cells from one young or old queen were incubated with 5 μM H_2DCF-DA (D6886; Sigma) for 20 min at 37°C and then stained with 0.2 μM Hoechst 33342 (Invitrogen) for 5 min at 37°C. Confocal laser scanning microscopy and image analyses were performed as described above using excitation and emission wavelengths of 498 nm and 522 nm, respectively, to detect ROS (green fluorescence).[22–24] The nuclei were detected as described for HET.

The average intensities and areas of red fluorescence (ROS) and green fluorescence (ROS) in the cells were determined using QWin image processing and analysis software (version 2.5, Leica, Wetzlar, Germany). The ratio of average intensity/area of red and green fluorescence in the cells represented ROS levels, respectively. Both the HET and H_2DCF-DA experiments were biologically replicated four times and used a total of four young and four old queens.

Supernatant preparations of trophocytes and fat cells

Trophocytes and fat cells were isolated from one young or old queen, homogenized in 500 μL of phosphate-buffered saline containing protease inhibitors (11697498001; Roche Applied Science, Indianapolis, IN), and centrifuged at $5{,}000 \times g$ for 10 min at 4°C. The resulting supernatant was collected and assayed immediately. The protein concentration was determined using a protein assay reagent (500-0006; Bio-Rad Laboratories, Hercules, CA) by monitoring the wavelength of 595 nm at room temperature.

ROS assays in supernatants

A total of 10 mM H_2DCF-DA (2 μL) was added to 200 μL of fresh supernatant (described above), and the fluorescence was monitored at room temperature for 120 min at 5-min intervals at excitation and emission wavelengths of 485 nm and 530 nm, respectively.[20] The ROS levels are expressed as DCF min^{-1} mg^{-1} of protein. This experiment was biologically replicated six times in queens and used a total of six young and six old queens.

NO activity assay

NO activity was measured as previously described.[25] Briefly, a final 200 μL of reactive solution containing 5 μL of fresh supernatant (described in Supernatant preparations of trophocytes and fat cells, above), 5 μL of NADPH (1 mM), and 2 $\mu$of lucigenin (20 μM) initiated the reaction followed by immediate measurement of chemiluminescence in a ultraviolet-visible (UV/VIS) spectrophotometer (Spectramax M2, Molecular Devices, NY) for 12 min. Appropriate blanks were established. The resultant chemiluminescence was normalized to the blank. The specific activity was expressed as micromole min^{-1} mg^{-1} of protein. This experiment was biologically replicated six times and used a total of six young and six old queens.

XO activity assay

XO activity was measured using the XO Assay Kit (ab102522; Abcam, Cambridge, MA). Briefly, trophocytes and fat cells were isolated from one young or old queen, homogenized in 60 μL of assay buffer, and centrifuged at $16{,}000 \times g$ for 10 min at 4°C. The protein concentration was determined (described in Supernatant preparations of trophocytes and fat cells, above). According to the manufacturer's instructions, 50 μL of supernatant was incubated at 37°C in the reaction

mixture for 30 min and the measurement of fluorescence was performed in a microplate reader at 570 nm. XO supplied in the kit was used as the standard, and XO activity was determined by comparing the fluorescence of supernatants with that of standards. The specific activity was expressed as unit mg^{-1} of protein. This experiment was biologically replicated six times and used a total of six young and six old queens.

CAT activity assay

CAT assays were performed as previously described[26] with slight modifications. Briefly, 890 μL of 50 mM potassium phosphate buffer (pH 7.0), 100 μL of 30% H_2O_2, and 10 μL of fresh supernatant (described in Supernatant preparations of trophocytes and fat cells, above) were mixed at 25°C. A blank was prepared with 900 μL of 50 mM potassium phosphate buffer and 100 μL of 30% H_2O_2. The rate of absorbance change (ΔA/min) at 240 nm, indicating the decomposition of H_2O_2, was recorded, and the activities were calculated using the molar extinction coefficient of H_2O_2 at 240 nm (43.59 L/mol-cm). One unit of CAT corresponds to the amount of enzyme that degrades 1 μmol H_2O_2 per minute at 25°C. The specific activity was expressed as micromole min^{-1} mg^{-1} of protein. This experiment was biologically replicated eight times and used a total of eight young and eight old queens.

GPx activity assay

GPx activity was measured as previously described.[27] Briefly, 100 μL of fresh supernatant (described in Supernatant preparations of trophocytes and fat cells, above) was incubated with 875 μL of coupling reagent (50 mM Tris-HCl, pH 7.6, 2 mM EDTA, 1 mM NaN_3, 0.1 mM reduced glutathione, 0.2 mM NADPH, and 1 U/mL GPx) for 2 min at 25°C, and the reaction was initiated by adding 25 μL of 1 mM H_2O_2. Then, the ΔA/min at 340 nm was recorded; the ΔA/min of a blank, in which the fresh supernatant was replaced with 100 μL of Tris-HCl buffer, was also recorded. The net ΔA/min of the samples after subtracting the blank rate was used to calculate the GPx activity using the molar extinction coefficient of NADPH at 340 nm (6,220 L/mol-cm). The specific activity was expressed as nanomole min^{-1} mg^{-1} of protein. This experiment was biologically replicated six times and used a total of six young and six old queens.

SOD activity assay

SOD activity was measured using the SOD Assay Kit-WST (S311; Dojindo, MD). Briefly, 200 μL of the supernatant (described in Supernatant preparations of trophocytes and fat cells, above) or 200 μL of the SOD standard solution was applied to each well of a 96-well plate, and 200 μL of the WST working solution was added. After mixing, 20 μL of enzyme working solution was added, and each sample was incubated at 37°C for 20 min. The total SOD activity was obtained by measuring the absorbance at 450 nm using a microplate reader (DU-70; Beckman, NJ). Mn-SOD activity was measured by adding 4 μL of 0.33 M sodium cyanide to the mixture, and the Cu,Zn-SOD activity was calculated through subtracting the Mn-SOD activity from the total SOD activity.[28] The specific activity was expressed as unit mg^{-1} of protein. This experiment was biologically replicated six times and used a total of six young and six old queens.

TR activity assay

TR activity was measured using the Thioredoxin Reductase Assay Kit (CS0170; Sigma, Saint Louis, MO) according to the manufacturer's instructions. This assay is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) with NADPH to 5-thio-2-nitrobenzoic acid (TNB), which produces a strong yellow color that is measured at 412 nm. Briefly, 14 μL of the supernatant (described in Supernatant preparations of trophocytes and fat cells, above) or 10 μL of the supernatant (described in Supernatant preparations of trophocytes and fat cells, above) and 4 μL of inhibitor solution were mixed with 180 μL of working buffer and 6 μL of DTNB in 96-well plate. The absorption was measured at 412 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader for 1 min. TR activity was calculated through subtracting the rate of DTNB reduction in the presence of sample plus an inhibitor of TR from the rate of DTNB reduction in the presence of sample. The specific activity was expressed as unit μg^{-1} protein. This experiment was biologically replicated six times and used a total of six young and six old queens.

Statistical analysis

Differences in mean values between the two age groups were examined using two-sample *t*-tests. A p value of less than 0.05 was considered statistically significant.

Results

ROS levels increase with age

ROS, determined through measuring the levels of superoxide, hydroxyl radicals, and H_2O_2,[20–22] showed a change with the queen's age. The trophocytes and fat cells of young queens exhibited lower ROS levels than old queens (Fig. 1A). The average intensity/cellular area of ROS in old queens was significantly higher compared to young queens ($n=4$, $p<0.05$; Fig. 1B), demonstrating that ROS levels increase with age in queens.

In addition, ROS, determined through measuring the levels of H_2O_2, hydroxyl radicals, and peroxyl radicals,[22,23] also showed a change with the queen's age. The results showed that ROS levels in the trophocytes and fat cells of queens increased with age (Fig. 1C). The average intensity/cellular area of ROS in old queens was significantly higher compared to young queens ($n=4$, $p<0.05$; Fig. 1D), indicating that overall oxidative stress increases with age in queens.

The ROS levels in trophocytes and fat cells were confirmed through assaying ROS levels in cellular supernatants again. The mean values of ROS were 164.27 ± 5.80 and 219.67 ± 9.61 DCF min^{-1} mg^{-1} protein in young and old queens, respectively ($n=6$, $p<0.05$; Fig. 1E), demonstrating that ROS increase with age in queens.

The activities of NO and XO

To evaluate ROS production, we assayed the activities of NO and XO. The NO activity was 2.33 ± 0.44 μmol min^{-1} mg^{-1} protein in young queens and 4.56 ± 0.44 μmol min^{-1} mg^{-1} protein in old queens ($n=6$, $p<0.01$; Fig. 2A), indicating that NO activity increases with age in queens. This result demonstrated that ROS production increases with age in queens. The XO activity was 4.68 ± 0.44 unit mg^{-1} protein in young queens and 5.28 ± 0.20 unit mg^{-1} protein in old queens ($n=6$, $p>0.05$; Fig. 2B), indicating that XO activity is not significantly different between young and old queens.

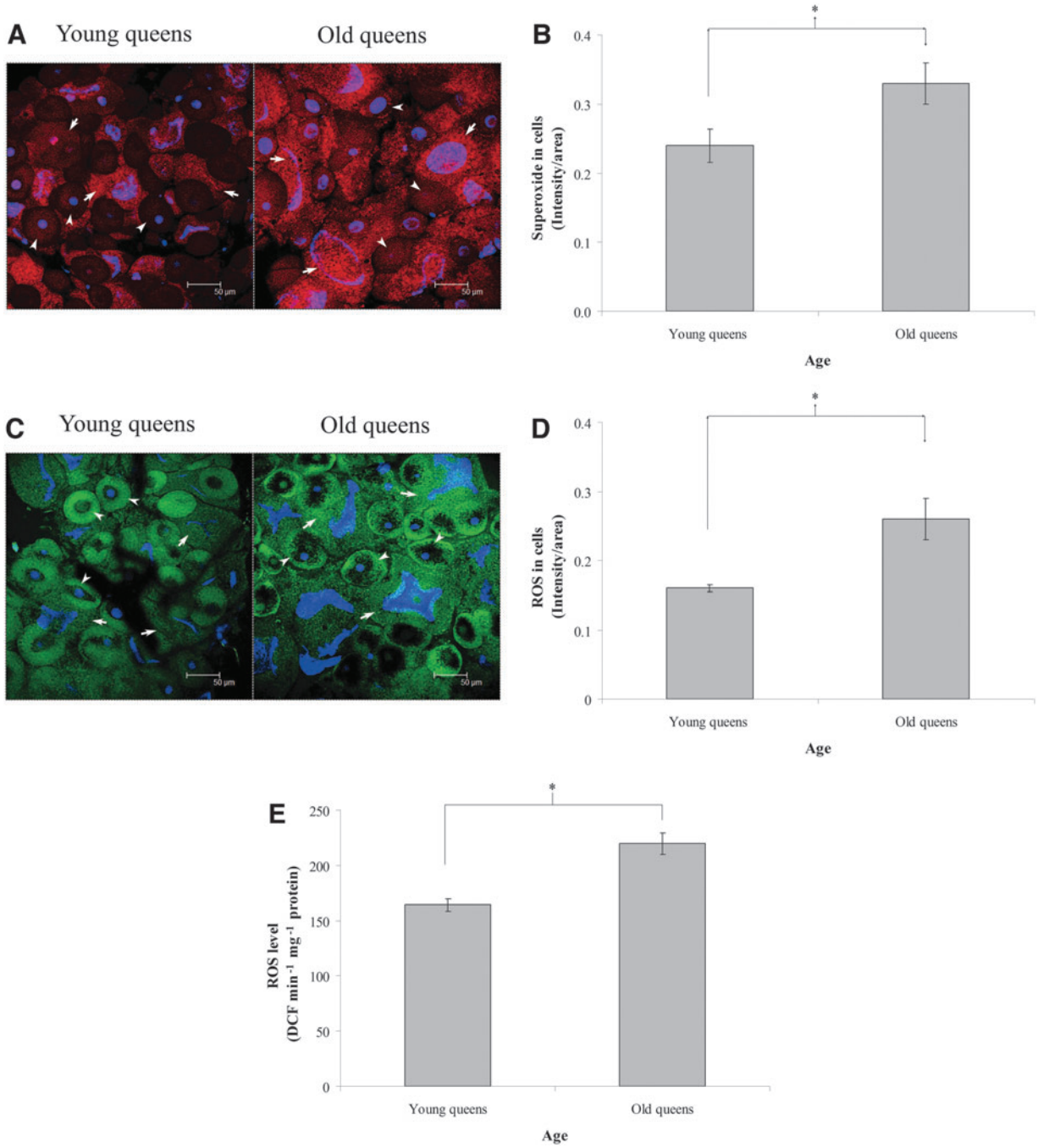

FIG. 1. Reactive oxygen species (ROS) levels in the trophocytes and fat cells of young and old queens. (**A**) Red fluorescence indicates the presence of ROS in young and old queens. Purple fluorescence indicates nuclei. Arrows point to trophocytes. Arrowheads point to fat cells. Scale bar, 50 μm. (**B**) Quantification of ROS in young and old queens. Bars represent mean ± standard error of the mean (SEM) ($n = 4$). (**C**) Green fluorescence indicates the presence of ROS in young and old queens. Blue fluorescence indicates nuclei. Arrows point to trophocytes. Arrowheads point to fat cells. Scale bar, 50 μm. (**D**) Quantification of ROS in young and old queens. Bars represent mean ± SEM ($n = 4$). (**E**) The ROS levels in the supernatants of the trophocytes and fat cells of young and old queens. Bars represent mean ± SEM ($n = 6$). Asterisks indicate statistical significance as determined by two-sample t test (*) $p < 0.05$).

The activities of CAT, GPx, Mn-SOD, Cu,Zn-SOD, and TR

The CAT activity was 91.31 ± 10.98 μmol min^{-1} mg^{-1} protein in young queens and 277.18 ± 12.99 μmol min^{-1} mg^{-1} protein in old queens ($n = 8$, $p < 0.01$; Fig. 3A), and the GPx activity was 169.95 ± 19.57 nmol min^{-1} mg^{-1} protein in young queens and 223.22 ± 13.98 nmol min^{-1} mg^{-1} protein in old queens ($n = 6$, $p < 0.05$; Fig. 3B). The Mn-SOD activity was 5.19 ± 1.57 units/mg protein in young queens and 38.04 ± 2.74 units/mg protein in old queens ($n = 6$, $p < 0.01$; Fig. 3C), and the Cu,Zn-SOD activity was 138.26 ± 3.60 units/mg

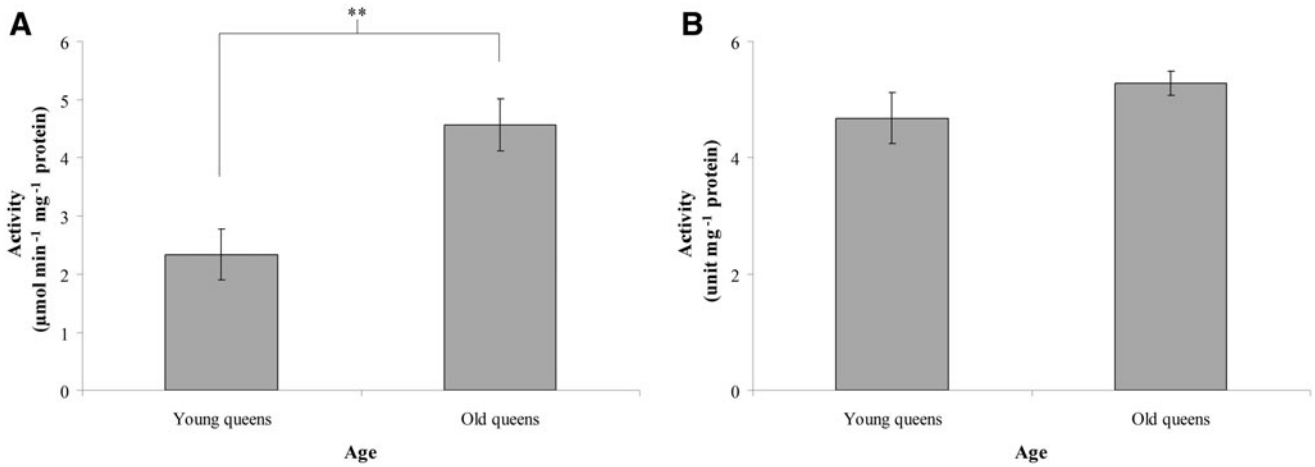

FIG. 2. The activities of nicotinamide adenine dinucleotide phosphate hydrogen (NADHP) oxidase (NO) (**A**) and xanthine oxidase (XO) (**B**) in the trophocytes and fat cells of young and old queens. Bars represent mean ± standard error of the mean (SEM) ($n = 6$). Asterisks indicate statistical significance as determined by two-sample t test (**) $p < 0.01$).

protein in young queens and 216.77 ± 14.99 units/mg protein in old queens ($n = 6$, $p < 0.05$; Fig. 3D). The TR activity in trophocytes and fat cells was 25.39 ± 1.65 μmol min^{-1} mg^{-1} protein in young queens and 7.97 ± 3.10 μmol min^{-1} mg^{-1} protein in old queens ($n = 6$, $p < 0.05$; Fig. 3E). These results showed that the activities of CAT, GPx, Mn-SOD, and Cu,Zn-SOD increased and TR decrease with age in the trophocytes and fat cells of queens.

Discussion

In this study, ROS levels and anti-oxidant enzyme activities in the trophocytes and fat cells of queens were assayed and showed that young queens have lower ROS levels, CAT, GPx, and SODs activities, and higher TR activity than old queens, showing that oxidative stress increases with age in the trophocytes and fat cells of queen honeybees. The increase of oxidative stress might be associated with the cellular longevity-promoting mechanisms of queens.

ROS levels increase with age

ROS are generated as byproducts of energy metabolism in cells.[5] ROS levels are higher in the trophocytes and fat cells of old queens compared to young queens. The higher levels of ROS in old queens may be due to higher energy metabolism, which increases the production of superoxide and other ROS.[6] This high-energy metabolism in old queens may be closely related to the longevity of queens. This inference is consistent with previous studies showing that: (1) Increased mitochondrial metabolism induced by glaucarubinone extends the life span of *Caenorhabditis elegans*,[29] (2) increased mitochondrial energy metabolism protects against cardiac failure in mice,[30] and (3) increased mitochondrial energy metabolism extends the life span of an annual fish (*Nothobranchius rachovii*) under conditions of ambient temperature reduction, which has been shown to effectively extend the life span of organisms.[31]

In addition, an increase in ROS levels concomitant with an increase in energy metabolism in the trophocytes and fat cells of old queens is consistent with previous studies indicating that the trophocytes and fat cells of queens had longevity-promoting mechanisms.[14,15]

NO and XO produced ROS, such as superoxide and H_2O_2.[32,33] NO activity increased with the age of queens, indicating that ROS production also increased with the age of queens. This result is in accord with the results of ROS levels in this study and previous studies showing that NO increased with aging in rat liver, aorta, and myocardium.[34–36] Nevertheless, ROS production by mitochondria can significantly surpass the amount of ROS produced by NO.[32,37] Therefore, the higher levels of ROS in old queens may be due to higher energy metabolism, which increases the production of superoxide and other ROS.[6]

Although XO activity increased with aging in human plasma, rat plasma, rat aorta, rat gastrocnemius muscle, mice cerebral cortex, mice liver, mice plasma, mice spleen, and mice thymus,[38–40] XO activity did not change with aging in mice kidney, mice lung, long-lived mice liver, long-lived mice kidney, and long-lived mice plasma.[39,40] In this study, XO activity is not significantly different between young and old queens. This phenomenon is consistent with a previous study, showing that XO activity is not significantly different between young and long-lived mice.[40] The explanation is most likely that XO does not participate in the impairment of trophocytes and fat cells with aging in queens. This inference is in agreement with a previous study showing that XO does not contribute to impaired peripheral conduit artery endothelium-dependent dilatation with aging in human.[41]

Activities of CAT, GPx, Mn-SOD, Cu,Zn-SOD, and TR

The ROS levels and the activities of CAT, GPx, Mn-SOD, and Cu,Zn-SOD in the trophocytes and fat cells of queens increased with age, indicating that CAT, GPx, Mn-SOD, and Cu,Zn-SOD were responsible for scavenging ROS. The biological function of SOD is to remove superoxide to generate H_2O_2, which is then scavenged by CAT, the TPx/TR system, and the GPx/GR system. The trophocytes and fat cells of old queens might have a higher energy metabolism, which increases the production of superoxide. The higher level of superoxide induces an increase in the activity of SODs, producing more H_2O_2, which is then scavenged by CAT, the TPx/TR system, and the GPx/GR system. TPx removes H_2O_2 by coupling its reduction to water with the oxidation of

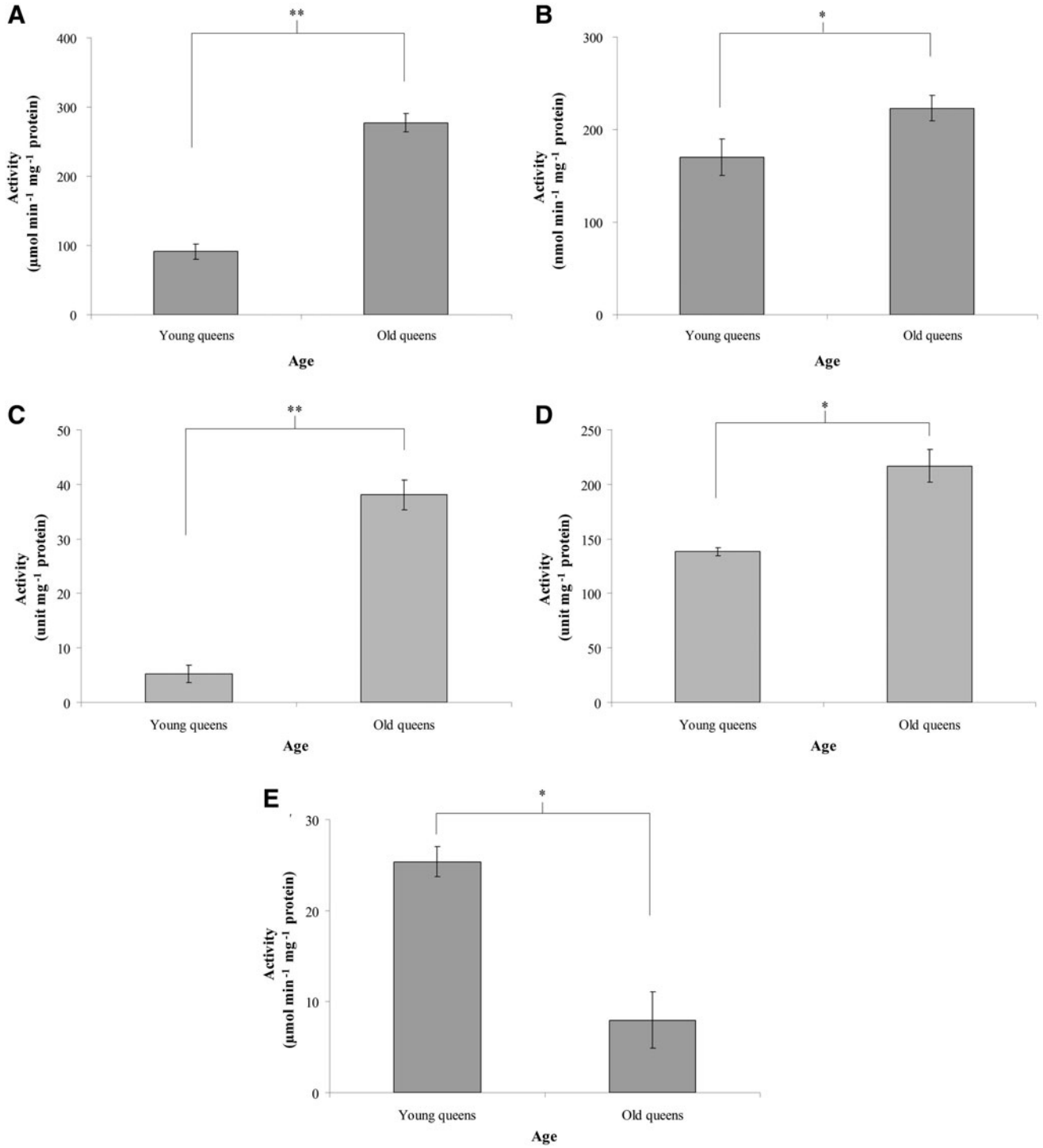

FIG. 3. The activities of catalase (CAT) (**A**), glutathione peroxidase (GPx) (**B**), Mn-superoxide dismutase (SOD) (**C**), Cu,Zn-SOD (**D**), and thioredoxin reductase (TR) (**E**) in the trophocytes and fat cells of young and old queens. ***Bars*** represent mean ± SEM ($n = 8$ in (**A**), $n = 6$ in (**B**), $n = 6$ in (**C**), $n = 6$ in (**D**), $n = 6$ in (**E**)). Asterisks indicate statistical significance as determined by two-sample *t*-test (*) $p < 0.05$; (**) $p < 0.01$).

reduced thioredoxin to oxidized thioredoxin. Subsequently, TR reduces oxidized thioredoxin to reduced thioredoxin using NADPH.[6,7] Therefore, TR activity can represent the activity of the TPx/TR system. Likewise, GPx removes H_2O_2 by coupling its reduction to water with the oxidation of reduced glutathione to oxidized glutathione. Subsequently, GR reduces oxidized glutathione to reduced glutathione using NADPH.[6] Therefore, GPx activity can represent the activity of GPx/GR system. The anti-oxidant enzyme activity results of the queens obtained in this study are consistent with those of a previous study, which showed that mated queens exhibited higher CAT activity in the ventriculi and spermathecae and higher SOD activity in muscles and spermathecae compared to virgin queens.[42]

CAT, the TPx/TR system, and the GPx/GR system are used to scavenge H_2O_2. CAT activity is higher than TR activity and TR activity is higher than GPx activity in young queens. Likewise, CAT activity is higher than TR activity and TR activity is higher than GPx activity in old queens. This phenomenon suggests that the CAT might be the principal scavenger for H_2O_2 in queens, with the TPx/TR system and the GPx/GR system playing a secondary role and a third role. The fact that TR activity is higher than GPx activity in queens is consistent with a previous study, showing that TR is higher H_2O_2 detoxification than GPx in rat brain mitochondria.[43] Thioredoxin and glutathione are the substrates of TPx and GPx and are reduced by TR and GR using NADPH,[6] which can be supplied through higher cellular energy metabolism. Therefore, the TPx/TR system and the GPx/GR system more actively degrade H_2O_2 under conditions of higher cellular energy metabolism because of the greater availability of NADPH. This explanation is consistent with the results of previous studies showing that NADPH levels are closely related to anti-oxidant capacity[6] and that the injection of reduced glutathione into the antennal lobes of workers prior to treatment with an oxidative stress inducer blocks oxidative stress-mediated inhibitory effects.[44] Nevertheless, TR activity is higher in young queens as opposed to CAT and GPx activities, which are lower in young queens, indicating that TR as well as CAT and GPx are regulated in a reverse manner in queens. This phenomenon is consistent with a previous study, showing that TR and GPx are regulated in a contrasting manner in the cancer systems tested.[45]

The activities of anti-oxidant enzymes have been reported to decrease with age in vertebrates, including in the rat brain,[46,47] rat liver,[48] mouse brain,[49] and fish muscle.[50] However, the results of these previous studies are not consistent with our observations in queens, in which the activities of CAT, GPx, Mn-SOD, and Cu,Zn-SOD increased with age. The high activities of anti-oxidant enzymes in queens may be involved in longevity-promoting mechanisms, which is a speculation supported by previous reports that anti-oxidant enzyme activities are elevated in rodents under a calorie-restricted diet.[48,51,52] Calorie restriction has been shown to successfully extend the life span of organisms, ranging from ciliates to mammals.[53]

Taking these findings together, we hypothesize that an increase in metabolism concomitant with an increase in oxidative stress and a consequent increase in stress defense mechanisms are associated with the longevity of queens. This postulate is consistent with previous studies, showing that increased ROS extends the life span of *Caenorhabditis* elegans,[54–58] *Saccharomyces* cerevisiae,[59,60] *Schizosaccharomyces pombe*,[61] and naked mole-rat.[62] This postulate is also supported by previous studies, showing that decreasing ROS levels by anti-oxidants and over-expression of ROS scavenge enzymes prevent health-promoting effects of physical exercise in humans and do not increase the life span of mice.[63,64]

Acknowledgments

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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