

# Oxidative stress decreases in the trophocytes and fat cells of worker honeybees during aging

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**Abstract** Trophocytes and fat cells of honeybees have been used for cellular senescence studies, but their oxidative stress and antioxidant enzyme activities with aging in workers is unknown. Here, we assayed reactive oxygen species and the activities of antioxidant enzymes in the trophocytes and fat cells of young and old workers. Young workers had higher reactive oxygen species levels, higher superoxide dismutase and thioredoxin reductase activities as well as lower catalase and glutathione peroxidase activities compared to old workers. Adding these results up, we propose that oxidative stress decreases with aging in the trophocytes and fat cells of workers.

**Keywords** Radical oxygen species · Antioxidant enzyme · Aging · Trophocyte · Fat cell · Honeybee

## Abbreviations

ROS Reactive oxygen species  
SOD Superoxide dismutase

CAT	Catalase
GPx	Glutathione peroxidase
GR	Glutathione reductase
TPx	Thioredoxin peroxidase
TR	Thioredoxin reductase
NO	NADPH oxidase
XO	Xanthine oxidase
ATP	Adenosine triphosphate
SA-β-Gal	Senescence-associated β-galactosidase
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
HET	Dihydroethidine
H <sub>2</sub> DCF-DA	2',7'-dichlorodihydrofluorescein-diacetate
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide oxidized form
NADH	Nicotinamide adenine dinucleotide reduced form
AMPK	AMP-activated protein kinase
Sir2	Silent information regulator 2
PPAR-α	Peroxisome proliferator-activated receptor-α

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## Introduction

Oxidative stress hypothesis posits that aging results from the accumulation of oxidative damages (Harman 1956). Cellular oxidative stress results when the production of reactive oxygen species (ROS) surpasses

the capacity of cellular antioxidant defenses to remove them. ROS include principally superoxide ( $O_2^{\bullet-}$ ), hydroxyl radicals ( $OH^{\bullet}$ ), and hydrogen peroxide ( $H_2O_2$ ). They are produced by reactions of the mitochondrial electron transport chain, the oxidation of polyunsaturated fatty acids, and nitric oxide production (Miquel 1992). In addition, NADPH oxidase (NO) and xanthine oxidase (XO) also produce superoxide and hydrogen peroxide. ROS can damage lipids, proteins, carbohydrates, and nucleic acids which lead to cellular senescence (Terman and Brunk 2006). An intricate antioxidant defense system that includes catalase (CAT), glutathione peroxidase (GPx)/glutathione reductase (GR) system, superoxide dismutase (SOD), and thioredoxin peroxidase (TPx)/thioredoxin reductase (TR) system has evolved to neutralize the burden of ROS generation.

Trophocytes are large and irregularly shaped, and fat cells are small and spherical. They attach to each other to construct a single layer of cells around each abdominal segment. No cell division during adulthood, the ease of isolation from the abdomen, and convenient manipulation make them suitable targets for the studies of cellular senescence (Hsieh and Hsu 2011a, b, 2013; Hsu and Chan 2013; Chan et al. 2011; Chuang and Hsu 2013; Hsu and Chuang 2013). A recent study showed that trophocytes and fat cells of young queens had lower ROS levels, lower SOD, CAT, and GPx activities as well as higher TR activity compared to old queens. These results show that oxidative stress and antioxidant enzyme activities in trophocytes and fat cells increase with advancing age in queens (Hsieh and Hsu 2013). Queens and workers shared the same genome. However, queens have a much longer lifespan than workers. Therefore, the study of oxidative stress and antioxidant enzyme activities in the trophocytes and fat cells of young and old worker honeybees (*Apis mellifera*) not only realizes the oxidative stress of workers but also understands the difference between workers and queens as compared to queens. In addition, if trophocytes and fat cells of workers are to be used for future cellular senescence studies, it is important that changes in oxidative stress and antioxidant enzyme activities in young and old workers be explored. Such data could be used as reference values for evaluating cellular rejuvenation or senescence in future aging studies, especially anti-aging-drug-screening studies. In this study, the levels of ROS and the activities of

antioxidant enzymes were evaluated in the trophocytes and fat cells of young and old workers reared in a field hive to clarify the relationship between oxidative stress and aging in worker honeybees.

## Materials and methods

### Worker honeybees

The breeding of worker honeybees and the selection of worker age were carried out as described previously (Hsieh and Hsu 2011a; Chuang and Hsu 2013; Hsu and Chuang 2013). Young (1-day-old) and old (50-day-old) workers were collected from the same hive on the same dates for the following studies.

### ROS assays in trophocytes and fat cells

ROS assays in trophocytes and fat cells were carried out as described previously (Hsieh and Hsu 2013). Dihydroethidine (HET) was used to evaluate superoxide, hydroxyl radicals, and hydrogen peroxide (Benov et al. 1988; Bindokas et al. 1996; Münzel et al. 2002). 2',7'-Dichlorodihydrofluorescein-diacetate ( $H_2DCF-DA$ ) was used to evaluate hydrogen peroxide, hydroxyl radicals, and peroxy radicals (Münzel et al. 2002; Tarpey et al. 2004). Both the HET and  $H_2DCF-DA$  experiments were biologically replicated eight times and used a total of eight young and eight old workers.

### Supernatant preparations of trophocytes and fat cells

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

### ROS assays in supernatants

ROS assays in supernatants were carried out as described previously (Hsieh and Hsu 2013). The

ROS levels are expressed as DCF  $\text{min}^{-1} \text{mg}^{-1}$  of protein. This experiment was biologically replicated five times and used a total of fifteen young and fifteen old workers.

#### NO activity assay

NO activity assays were measured as previously described (Cheng et al. 2013; Hsieh and Hsu 2013). The specific activity was expressed as micromole  $\text{min}^{-1} \text{mg}^{-1}$  of protein. This experiment was biologically replicated ten times and used a total of thirty young and thirty old workers.

#### XO activity assay

XO activity assays were carried out as described previously (Hsieh and Hsu 2013). The specific activity was expressed as unit  $\text{mg}^{-1}$  of protein. This experiment was biologically replicated ten times and used a total of thirty young and thirty old workers.

#### CAT activity assay

CAT activity assays were carried out as described previously (Hsieh and Hsu 2013). The specific activity was expressed as micromole  $\text{min}^{-1} \text{mg}^{-1}$  of protein. This experiment was biologically replicated five times and used a total of fifteen young and fifteen old workers.

#### GPx activity assay

GPx activity assays were carried out as described previously (Hsieh and Hsu 2013). The specific activity was expressed as nanomole  $\text{min}^{-1} \text{mg}^{-1}$  of protein. This experiment was biologically replicated four times and used a total of twelve young and twelve old workers.

#### SOD activity assay

SOD activity assays were carried out as described previously (Hsieh and Hsu 2013). The specific activity was expressed as unit  $\text{mg}^{-1}$  of protein. This experiment was biologically replicated five times and used a total of fifteen young and fifteen old workers.

#### TR activity assay

TR activity assays were carried out as described previously (Hsieh and Hsu 2013). The specific activity was expressed as unit  $\mu\text{g}^{-1}$  protein. This experiment was biologically replicated five times and used a total of fifteen young and fifteen old workers.

#### 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.

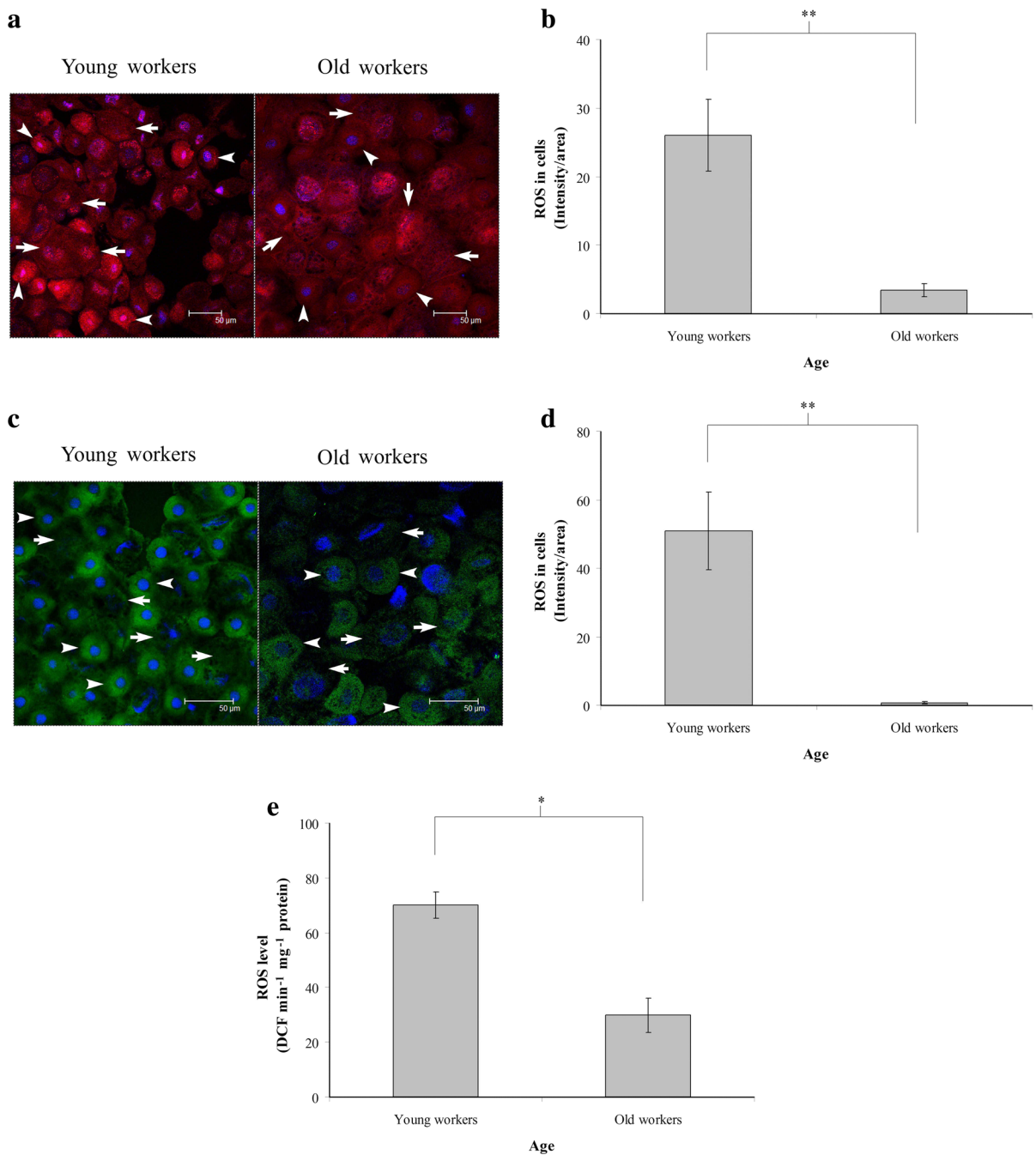
## Results

### ROS levels decrease with aging

To examine the oxidative stress in the trophocytes and fat cells of worker honeybees, we assayed ROS levels in these cells and ROS levels in cellular supernatants. ROS levels detected by HET were decreased in the trophocytes and fat cells of workers with aging (Fig. 1a). The statistical analysis showed that the fluorescence intensity/cellular area of red fluorescence was significantly higher in young workers than old workers ( $n = 8$ ,  $P < 0.01$ ; Fig. 1b). In addition, ROS levels detected by  $\text{H}_2\text{DCF-DA}$  were decreased in the trophocytes and fat cells of workers with aging (Fig. 1c). The fluorescence intensity/cellular area was significantly higher in young workers than old workers ( $n = 8$ ,  $P < 0.01$ ; Fig. 1d). Furthermore, the ROS levels in trophocytes and fat cells were confirmed through assaying ROS levels in cellular supernatants. The mean values of ROS were  $70.08 \pm 4.87$  and  $29.83 \pm 6.35$  DCF  $\text{min}^{-1} \text{mg}^{-1}$  of protein in young and old workers, respectively ( $n = 15$ ,  $P < 0.05$ ; Fig. 1e), revealing that ROS decrease with aging in workers.

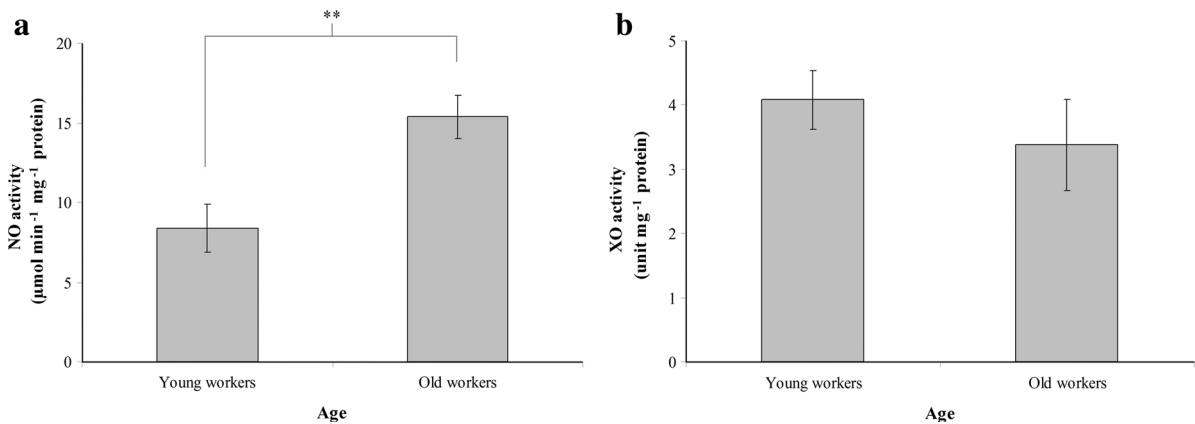
### The activities of NO and XO

To evaluate ROS production, we assayed the activities of NO and XO. The NO activity was  $8.40 \pm 1.49$   $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein in young worker and  $15.38 \pm 1.35$   $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein in old workers ( $n = 30$ ,  $P < 0.01$ ; Fig. 2a), indicating that NO



**Fig. 1** ROS levels in the trophocytes and fat cells of young and old workers. **a** Red fluorescence indicates the presence of ROS in young and old workers. Purple fluorescence indicates nuclei. Arrows point to trophocytes. Arrowheads point to fat cells. Scale bar 50  $\mu\text{m}$ . **b** Quantification of ROS in young and old workers. Bars represent mean  $\pm$  standard error of the means (SEMs) ( $n = 8$ ). **c** Green fluorescence indicates the presence of ROS in young and old workers. Blue fluorescence indicates nuclei.

Arrows point to trophocytes. Arrowheads point to fat cells. Scale bar 50  $\mu\text{m}$ . **d** Quantification of ROS in young and old workers. Bars represent mean  $\pm$  SEMs ( $n = 8$ ). **e** The ROS levels in the supernatants of the trophocytes and fat cells of young and old workers. Bars represent mean  $\pm$  SEMs ( $n = 15$ ). Asterisk indicates a statistically significant difference ( $*P < 0.05$ ,  $**P < 0.01$ ; two-sample  $t$  test). (Color figure online)



**Fig. 2** The activities of NO (a) and XO (b) in the trophocytes and fat cells of young and old workers. Bars represent mean  $\pm$  SEMs ( $n = 30$ ). Asterisks indicate statistical significance as determined by two-sample  $t$  test (\*\* $P < 0.01$ )

activity increases with aging in workers. This result demonstrated that ROS production increases with aging in workers. The XO activity was  $4.08 \pm 0.46$  unit  $\text{mg}^{-1}$  protein in young workers and  $3.38 \pm 0.71$  unit  $\text{mg}^{-1}$  protein in old workers ( $n = 30$ ,  $P > 0.05$ ; Fig. 2b), indicating that XO activity is not significantly different between young and old workers.

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

To investigate the ROS scavenging capacity in the trophocytes and fat cells of worker honeybees, we assayed CAT, GPx, SOD, and TR activities in these cells. The CAT activity was  $96.39 \pm 12.91$   $\mu\text{mol min}^{-1} \text{mg}^{-1}$  of protein in young workers and  $485.40 \pm 51.72$   $\mu\text{mol min}^{-1} \text{mg}^{-1}$  of protein in old workers ( $n = 15$ ,  $P < 0.01$ ; Fig. 3a), whereas GPx activity was  $242.38 \pm 16.81$   $\text{nmol min}^{-1} \text{mg}^{-1}$  of protein in young workers and  $415.46 \pm 34.07$   $\text{nmol min}^{-1} \text{mg}^{-1}$  of protein in old workers ( $n = 12$ ,  $P < 0.05$ ; Fig. 3b). Thus, both CAT and GPx activity increased with aging in the trophocytes and fat cells of workers. In contrast, SODs and TR activities were significantly decreased with aging in the trophocytes and fat cells of workers. The Mn-SOD activity was  $162.28 \pm 5.92$  unit  $\text{mg}^{-1}$  of protein in young workers and  $115.24 \pm 10.07$  unit  $\text{mg}^{-1}$  of protein in old workers ( $n = 15$ ,  $P < 0.05$ ; Fig. 3c), whereas the Cu, Zn-SOD activity was  $652.33 \pm 53.86$  unit  $\text{mg}^{-1}$  of protein in young workers and  $310.29 \pm 22.68$  unit  $\text{mg}^{-1}$  of protein in old workers ( $n = 15$ ,

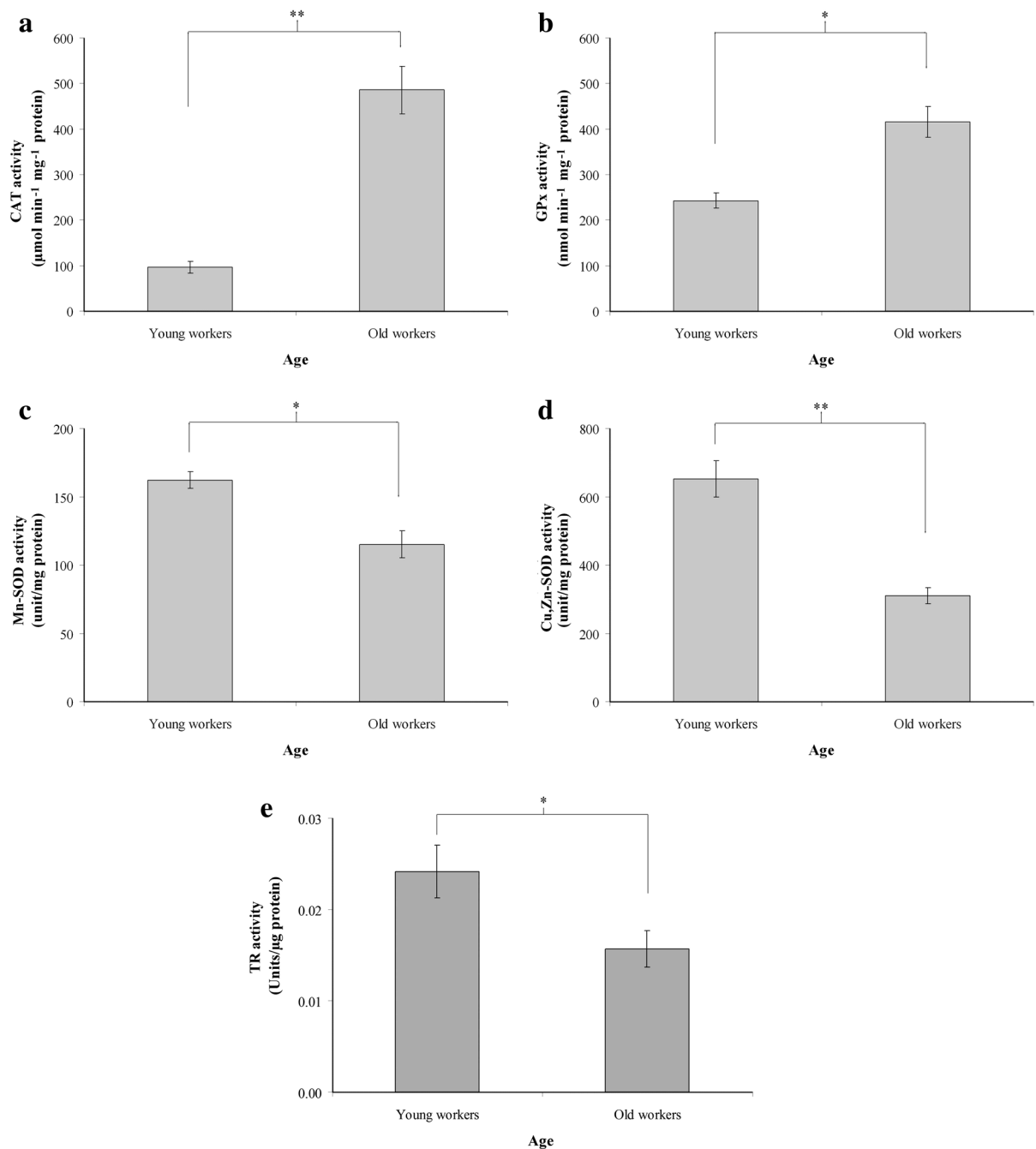
$P < 0.01$ ; Fig. 3d). The TR activity in trophocytes and fat cells was  $0.024 \pm 0.003$  unit  $\mu\text{g}^{-1}$  of protein in young workers and  $0.016 \pm 0.002$  unit  $\mu\text{g}^{-1}$  of protein in old workers ( $n = 15$ ,  $P < 0.05$ ; Fig. 3e).

## Discussion

In this study, ROS levels and antioxidant enzyme activities in the trophocytes and fat cells of workers were assayed and showed that young workers have higher ROS levels, higher SODs and TR activities, and lower CAT, GPx, and NO activities than old workers. According to the results of ROS levels and SOD activities, oxidative stress decreases with aging in workers. However, based on the results of NO, CAT, and GPx activity, oxidative stress increases with aging in workers. ROS generation by mitochondria can significantly exceed the amount of ROS produced by NO (Dikalov 2011; Ago et al. 2010). Therefore, adding these results up, we propose that oxidative stress decreases with aging in workers.

### ROS levels decrease with aging

ROS are generated as by-products of energy metabolism in cells (Halliwell and Gutteridge 1999). In this study, the trophocytes and fat cells of young workers contained higher ROS levels than old workers. The high levels of ROS in young workers may be due to higher energy metabolism (Kowaltowski et al. 2009).



**Fig. 3** The activities of CAT (a), GPx (b), Mn-SOD (c), Cu,Zn-SOD (d), and TR (e) in the trophocytes and fat cells of young and old workers. Bars represent mean  $\pm$  SEMs [ $n = 15$  in (a),  $n = 12$  in (b),  $n = 15$  in (c),  $n = 15$  in (d),  $n = 15$  in (e)]. Asterisk indicates a statistically significant difference ( $*P < 0.05$ ,  $**P < 0.01$ ; two-sample  $t$  test). CAT converts hydrogen peroxide to water and oxygen. GPx removes hydrogen peroxide 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 hydrogen peroxide (Halliwell and Gutteridge 1999). TPx removes hydrogen peroxide by coupling its reduction to water with the oxidation of reduced thioredoxin to oxidized thioredoxin. Subsequently, TR reduces oxidized thioredoxin to reduced thioredoxin (Kowaltowski et al. 2009; Arnér and Holmgren 2000)

This inference is supported by our recent studies, which showed that young workers had higher mitochondrial membrane potential, ATP concentrations, nicotinamide adenine dinucleotide oxidized form (NAD<sup>+</sup>) concentrations, NAD<sup>+</sup>/nicotinamide adenine dinucleotide reduced form (NADH) ratio, AMPK activity, Sir2 activity,  $\beta$ -oxidation, and PPAR- $\alpha$  expression than old workers (Hsu and Chan 2013; Chuang and Hsu 2013; Hsu and Chuang 2013). Our explanation is also supported by previous studies showing that increased energy metabolism promotes the increased formation of ROS (Harman 1956; Sohal and Weindruch 1996; Furukawa et al. 2004; Barja de Quiroga 1992; McDevitt and Speakman 1994; Hannon 1960; Yan and Sohal 2000). Therefore, high levels of ROS in the trophocytes and fat cells of young workers most likely reflect higher cellular energy metabolism.

Conversely, the low levels of ROS in old workers may be the result of low energy metabolism, which may be a consequence of the disruption of organellar and/or macromolecular function from oxidative damage. This interpretation is consistent with our recent studies showing that old workers have low energy metabolism (Hsu and Chan 2013; Chuang and Hsu 2013; Hsu and Chuang 2013). This explanation is also in agreement with previous studies indicating that oxidative damage and mitochondrial dysfunction increased with aging (Hsieh and Hsu 2011a; Yan and Sohal 2000; Farooqui 2007, 2008; Trifunovic and Larsson 2008). Therefore, low levels of ROS in the trophocytes and fat cells of old workers most likely reflect lower cellular energy metabolism.

NO and XO are able to produce superoxide and hydrogen peroxide (Dikalov 2011; Berry and Hare 2004). NO activity increased with aging in workers, indicating that ROS production by NO increased with aging in workers. This result is consistent with previous studies (Hsieh and Hsu 2013; Laurent et al. 2012; Li et al. 2010; Wang et al. 2010). This finding indicated that oxidative stress increases with aging in workers. However, ROS generation by NO is lower than that by mitochondria (Dikalov 2011; Ago et al. 2010). Therefore, ROS levels principally derive from energy metabolism, not from NO.

XO activity is not significantly different between young and old workers. This phenomenon is in agreement with previous studies (Hsieh and Hsu 2013; Vida et al. 2011a, b). The reason is most likely that XO doesn't participate in the impairment of

trophocytes and fat cells with aging in workers. This inference is consistent with previous studies (Hsieh and Hsu 2013; Eskurza et al. 2006).

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

CAT and GPx activities increased with aging, whereas Mn-SOD and Cu, Zn-SOD activities decreased with aging in the trophocytes and fat cells of workers. This phenomenon is consistent with previous reports (Corona et al. 2005; Inal et al. 2001; Saraymen et al. 2003; King et al. 1997; Tatone et al. 2006; Li et al. 2010; Rippe et al. 2010; Castro Mdel et al. 2012). Parallel decreases in SOD activity and ROS with aging remarkably indicate that SOD is used to scavenge superoxide which derives from energy metabolism in workers. According to this scenario, young workers should produce high hydrogen peroxide due to high ROS and SOD. However, young workers have low CAT and GPx activities. The most likely reason is that hydrogen peroxide has important roles as a signaling molecule in the regulation of biological processes in young workers (Veal et al. 2007; Giorgio et al. 2007), resulting in a decrease of CAT and GPx activities. In contrast, old workers have high CAT and GPx activities. The possible reason is that hydrogen peroxide plays lesser roles in the regulation of biological processes with the accumulation of oxidative damage, leading to an increase of CAT and GPx activities. More work is needed to clarify this point.

Comparison between workers and queens

Comparing workers and queens, young workers, young queens, and old queens have high ROS levels, which propose having high cellular energy metabolism (Hsu and Chan 2013; Chuang and Hsu 2013; Hsieh and Hsu 2013; Hsu and Chuang 2013). High cellular energy metabolism may be necessary for growth in young workers (Hsu and Chuang 2013), whereas high cellular energy metabolism may be due to the longevity-promoting mechanisms in queens (Hsieh and Hsu 2011b, 2013). Parallel decreases in ROS and SOD activity with aging in workers and parallel increases in ROS and SOD activity with aging in queens obviously indicate that SOD is used to scavenge superoxide in honeybees and ROS principally derives from cellular energy metabolism

(Corona et al. 2005; Rippe et al. 2010; Hsu and Chan 2013; Chuang and Hsu 2013; Hsieh and Hsu 2013; Hsu and Chuang 2013).

Similarity in CAT, GPx, TR, NO, and XO activity between workers and queens implies that (1) hydrogen peroxide might play biological functions in young individuals resulting in a decrease of CAT and GPx activities; (2) hydrogen peroxide might play lesser roles in the regulation of biological processes with the accumulation of oxidative damage, leading to an increase of CAT and GPx activities. (3) oxidative stress is low in young individuals and high in old individuals in the light of low NO, CAT, and GPx activities in young individuals. However, mitochondria produce significantly high ROS levels than NO (Dikalov 2011; Ago et al. 2010). Taking these phenomena together, we propose that oxidative stress decreases with aging in workers and oxidative stress increases with aging in queens (Hsieh and Hsu 2013).

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