

# Effect of season and behavioral activity on the hypopharyngeal glands of three honey bee *Apis mellifera* L. races under stressful climatic conditions of central Saudi Arabia

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## Abstract

Honey production gains are needed to deal with high demand in Saudi Arabia. The honey bee races are facing stressful hot-arid weather conditions that can affect different aspects of physiology and behavior. The hypopharyngeal glands (HPGs) of honey bees have prominent roles in various social behaviors through their secretions. The measurement of acini size and lipofuscin accumulation indicates the changes in HPGs in response to different factors including weather and behavioral castes. This research aimed to reveal how natural harsh environment of summer and winter can shape the HPGs in foragers and nurses of an indigenous bee race (*Apis mellifera jemenitica* Ruttner) in comparison with two exotic bee races (*Apis mellifera carnica* Pollmann and *Apis mellifera ligustica* Spinola). This study presents new information of significant differences in the HPGs of two behavioral castes (nurses and foragers) of indigenous and exotic bee races under harsh natural environmental conditions. HPGs of foragers have significantly higher lipofuscin accumulation and smaller acini size than nurse bees in all tested races during summer and winter seasons. A strong inverse correlation was found between acini size and lipofuscin accumulation in each race in both seasons. Smaller acini size and lipofuscin accumulation were detected

in the HPGs of indigenous bees (foragers and nurses) than exotic bee races during both seasons. The acini size and lipofuscin accumulation were similar between exotic bee races but higher than that of the indigenous bee race.

### Keywords

Acini, honey bee races, hypopharyngeal glands, lipofuscin

## Introduction

Beekeeping has been practiced in the Arabian Peninsula for centuries. It is an important aspect in the agriculture sector of Saudi Arabia with an additional objective to increase the income of Saudi beekeepers (Alqarni et al. 2014). Three prominent bee races are commonly domesticated in Saudi Arabia. Approximately 70–80% of bee colonies are of indigenous bees and 20–30% of total colonies include exotic European bees races (Alqarni et al. 2011).

*Apis mellifera jemenitica* Ruttner (AMJ) is an indigenous bee race that is widely used for honey production throughout the region (Alqarni 2006; Ruttner 1976). This race is well adapted to the local harsh environmental conditions (Alqarni et al. 2011; Iqbal et al. 2018a). In Saudi Arabia, the summer temperature is extremely high and often exceeds 45 °C, and the humidity is very low; in winter, the temperature can fall below 0 °C. (Ali et al. 2017; Alqarni 2006; Alqarni et al. 2011; Iqbal et al. 2018b). *Apis mellifera carnica* Pollmann (AMC) and *Apis mellifera ligustica* Spinola (AML) are exotic bee races imported annually by the beekeepers to boost agricultural production (Alqarni et al. 2014). However, exotic races face many challenges when adapting to the local hot arid environment and have higher mortality rates during the summer than indigenous races (Alattal and AlGhamdi 2015).

Hypopharyngeal glands (HPGs) in honey bees are age-dependent paired glands that are only observed in the heads of worker bees and are associated with various social behaviors via different secretions (Liu et al. 2013; Ueno et al. 2015). Each gland consists of small oval bodies (acini) that are linked to axial or terminal secretory ducts. HPGs become fully developed in young workers (6–13 days old) with large functional secreting acini (Lass and Crailsheim 1996; Rahman et al. 2014). However, these glands degenerate when the bee initiates foraging behavior outside the colony (Robinson 1992).

The activity of the HPGs is mainly dependent on the acini size, which changes with age to express age-polytheism in honey bees (Deseyn and Billen 2005; Johnson 2010; Robinson 1992). These glands exhibit change in their activities from synthesizing major royal jelly proteins in younger nurse bees to synthesis of carbohydrate metabolizing enzymes  $\alpha$ -glucosidase in forager bees (Kubo et al. 1996; Ueno et al. 2015). Royal jelly is a protein-based food fed to larvae and to produce healthy queens (Knecht and Kaatz 1990; Ohashi et al. 1999). These glands are also vulnerable to various stresses, such as starvation, heat, and *Varroa* infestation, which may result in the reduction and degeneration of the glands (Khalil 1992; Yousef et al. 2014).

Lipofuscin are undegradable lipid-protein granules enclosed in a single membrane in the HPGs, and are considered a marker of cellular aging, functional decline and mortality (Fonseca et al. 2005; Hsu and Chan 2013). Lipofuscin granules are linked to chronological age and it increases in the body as the organism ages (Munch et al. 2013b). In honey bees, lipofuscin is a good indicator for studying physiological age (Hsieh and Hsu 2011).

Beekeepers in Saudi Arabia are spending large amounts of money for importing exotic bee races because the population of indigenous bees is too scarce and honey production is too low to meet the increasing demands of the local market (Al-Ghamdi et al. 2017). Nevertheless, the exotic bee races face high mortality due to harsh climatic conditions of central Saudi Arabia. The population of indigenous bees (AMJ) is successful and more heat tolerant than the exotic bee races (AMC and AML) (Abou-Shaara et al. 2012; Alqarni et al. 2014). Therefore, the comparison of HPGs among different bee races could help us to know that which race could have less aging in summer and winter and how it can be improved to avoid early or high mortality. Moreover, this study will aid in future investigations for improvement of HPGs potentially with food in heat tolerant indigenous bee race to get high brood rearing and even royal production in natural harsh climatic conditions of Saudi Arabia. This study unveils the status of HPGs in indigenous bee race in comparison with exotic bee races. We aimed to find out the differential changes in the HPGs (acini size and lipofuscin accumulation) of nurse and forager bees of different bee races during natural summer and winter harsh environment of Saudi Arabia.

## Materials and methods

### Bee colonies and sample collection

The bee colonies of the indigenous race *A. m. jemenitica* (AMJ) and exotic races *A. m. carnica* (AMC) and *A. m. ligustica* (AML) were maintained at an agricultural farm of King Saud University (KSU), Riyadh, Saudi Arabia (24.7296° N; 46.6101° E and 558 m altitude), during 2015. The indigenous bee race was obtained from reliable local sources. Pure queens of *A. m. carnica* and *A. m. ligustica* were imported from Egypt and Jordan, respectively. These imported queens were subsequently used to raise the colonies of the exotic bee races. One strong colony of each races was selected for the experiments. The bee samples were taken from the same colony during summer and winter seasons.

The selected colonies of each race were of equal estimated colony strengths containing 5 frame bees, 3 frame brood and 2 frame of food. The plenty of flora and flowers of different plants such as *Prosopis* spp., *Eucalyptus* spp., *Ocimum basilicum* L., *Brassica rapa* L., *Eruca sativa* Mill. and some wild flowers were available in the field during the experiments. The colonies were kept healthy without any chemical treatment.

Fifty newly emerged bees of each race from cells were marked with different paint colors of Uni-Paint® (yellow and white) to determine their ages at the time of collec-

tion. Direct marking was done to keep the new emerged bees in their natural environment, no cages were used. Nurse bees were collected at 11 days, whereas foragers were collected 25 days after emergence. The separate experiments were performed during the peak summer (later June: 27–40 °C, 9.5–10% relative humidity) and winter (December: 15–20 °C, 35–38% relative humidity) seasons. A thermo-hygrometer (HANNA, HI93640N, Europe) was used to record the meteorological factors.

### **Preparation of bee samples**

The heads of ten marked nurse or forager bees were dissected with a sharp blade in the Melittology Research Lab, KSU. The HPGs were removed with fine forceps and fixed overnight in 4% formaldehyde in phosphate buffered saline (PBS, pH 7.5) at 4 °C per the protocol of Munch et al. (2013b). Then, the glands were washed three times in PBS and further incubated overnight at 40 °C with 30% glycerol in PBS. Before mounting, the glands were again incubated for 2 h at 40 °C with 50% glycerol in PBS. Slides were prepared from the samples in mounting medium (50% glycerol in PBS). The corners of the slides were locked with nail polish for long-term storage to avoid desiccation. The slides were also incubated for drying at 40 °C for one week.

### **Measurement of acini size**

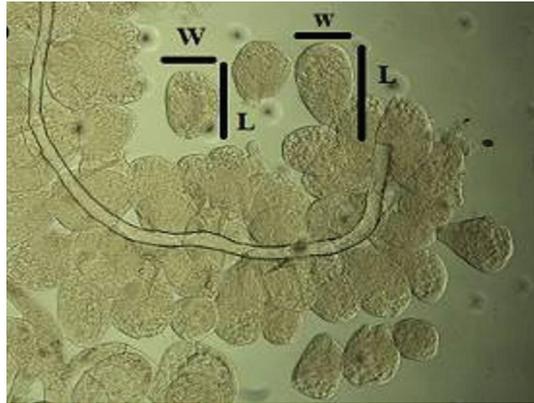
The morphological measurements and images of the acini size were recorded from mounted specimens using a 10× objective on a stereomicroscope (M165C, Leica, Germany) with a camera (DP71, Olympus). The maximum length (L) and width (W) of ten acini per slide were taken from different image locations for each individual bee (Al-Ghamdi et al. 2011a; 2011b). Two hundred acini were measured for each bee race (100 for nurses and 100 for foragers) (Figure 1). The acinal surface area (SA) was calculated using the following formula (Maurizio 1954):

$$\text{Acinal surface area} = \pi \times ((a \times b) / 2),$$

where a = maximum length, b = maximum width, and  $\pi = 3.14$ .

### **Analyses of lipofuscin accumulation**

Lipofuscin was identified by its granular appearance in the acini of HPGs for nurse and forager bees. Ten lipofuscin accumulation areas representing one individual bee were measured in one slide. Two hundred lipofuscin accumulation areas were measured for each bee race (100 for nurses and 100 for foragers). Images were taken using a 40× oil immersion objective on a Zeiss laser confocal microscope (Carl Zeiss, AG-Germany).



**Figure 1.** Microscopic measurement of acini length and width in the hypopharyngeal glands of honey bees. **L** length **W** width (Image at 20× magnification).

The longer wavelength spectrum (561 nm with emissions from 570–650 nm) was used for imaging because lipofuscin is only visible in this spectrum (Munch et al. 2013b). The laser power and detector sensitivity were kept constant for all images. The images were processed, and different regions of interest were selected to determine the lipofuscin accumulation percentage.

## Data analysis

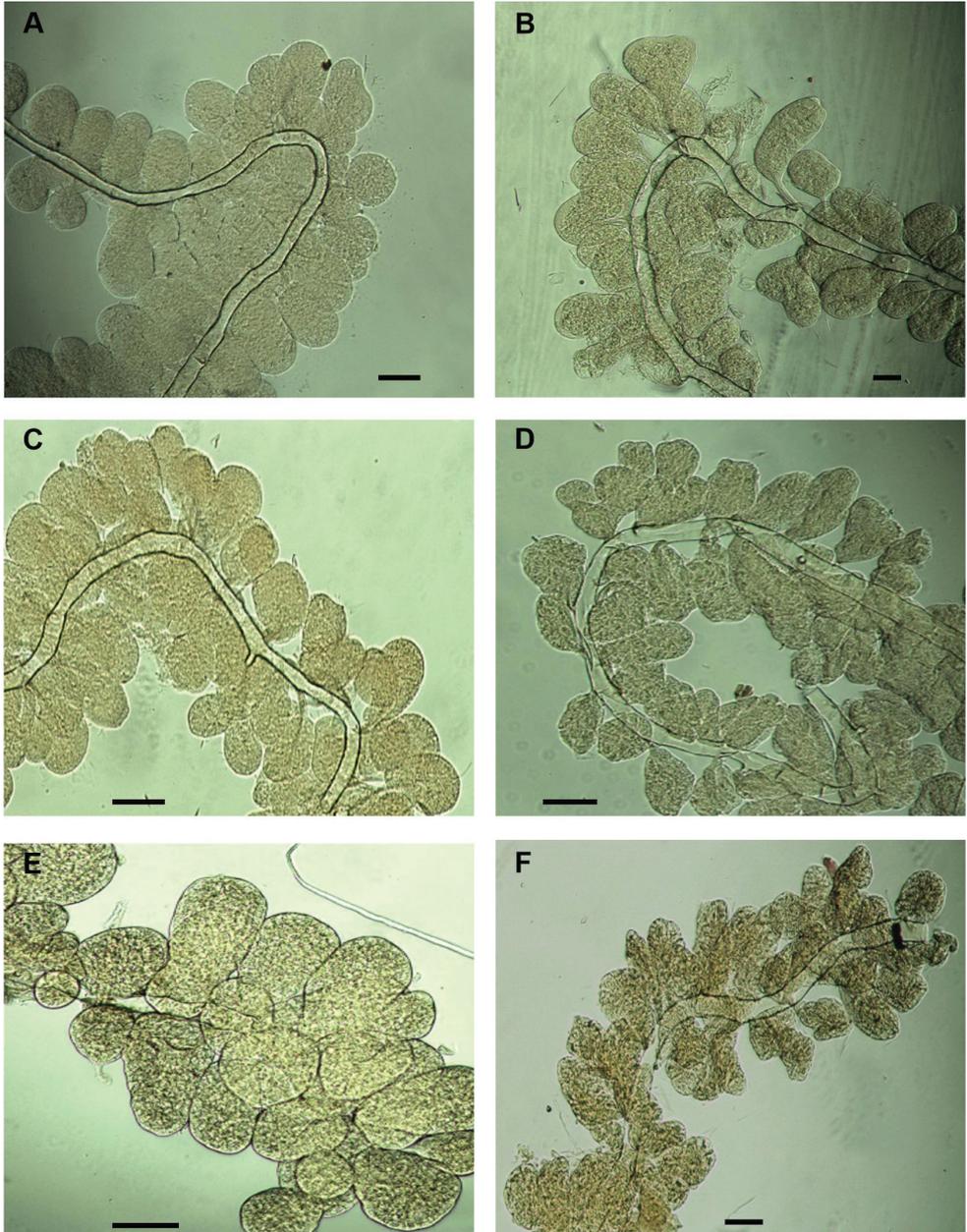
Data were examined for normality and homogeneity using the Kolmogorov-Smirnov test and Levene's test, respectively. The means were tested using an analysis of variance and subsequently separated using the LSD test at  $p \leq 0.05$ . The SPSS 22.0 program was used for the statistical analyses.

## Results

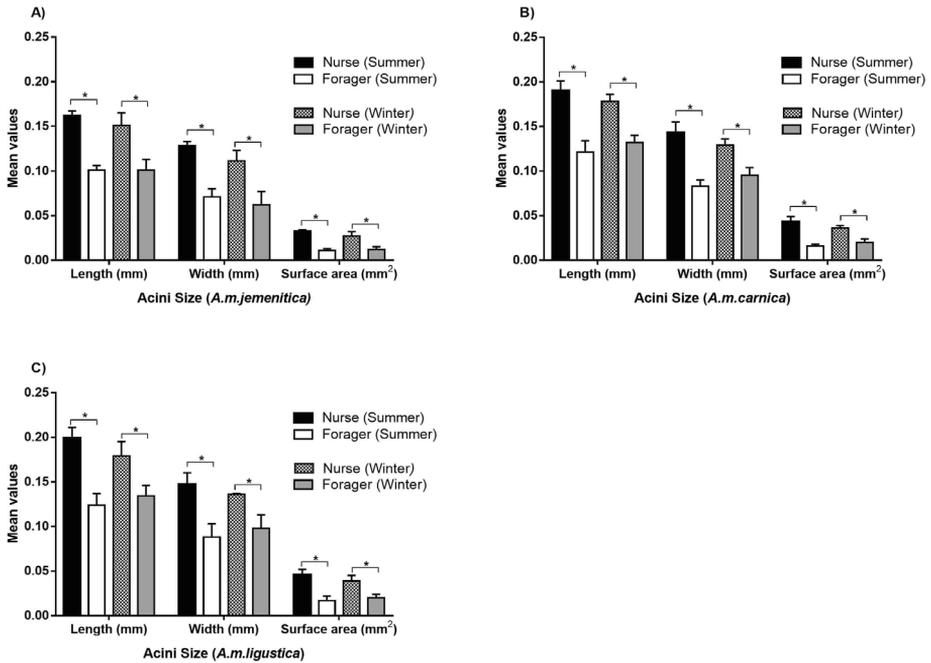
### Measurement of acini size in HPGs

Selected images of the acini of the nurses and foragers of the three bee races are shown in Figure 2. Figure 3 shows that nurse bees have larger HPG acini size measurements than forager bees of all races (indigenous = AMJ; exotic = AMC, AML) during the summer and winter seasons. Thus, irrespective of bee race and season, nurses have significantly larger acini than forager bees.

Figure 4 represents the comparison among indigenous (AMJ) and exotic bee races (AMC and AML). The acini size (L, W, and SA) was smaller in foragers and nurses of the indigenous bees than exotic bee races during the summer and winter seasons. In



**Figure 2.** Acini in the hypopharyngeal glands of the nurse and forager bees. **A** *A. m. carnica* nurse **B** *A. m. carnica* forager **C** *A. m. jemenitica* nurse **D** *A. m. jemenitica* forager **E** *A. m. ligustica* nurse **F** *A. m. ligustica* forager. Scale bars: 6.2 mm (**A**), 4.2 mm (**B**), 7.6 mm (**C**), 7.9 mm (**D**), 9.8 mm (**E**), 5.6 mm (**F**). (Images at 20× magnification).



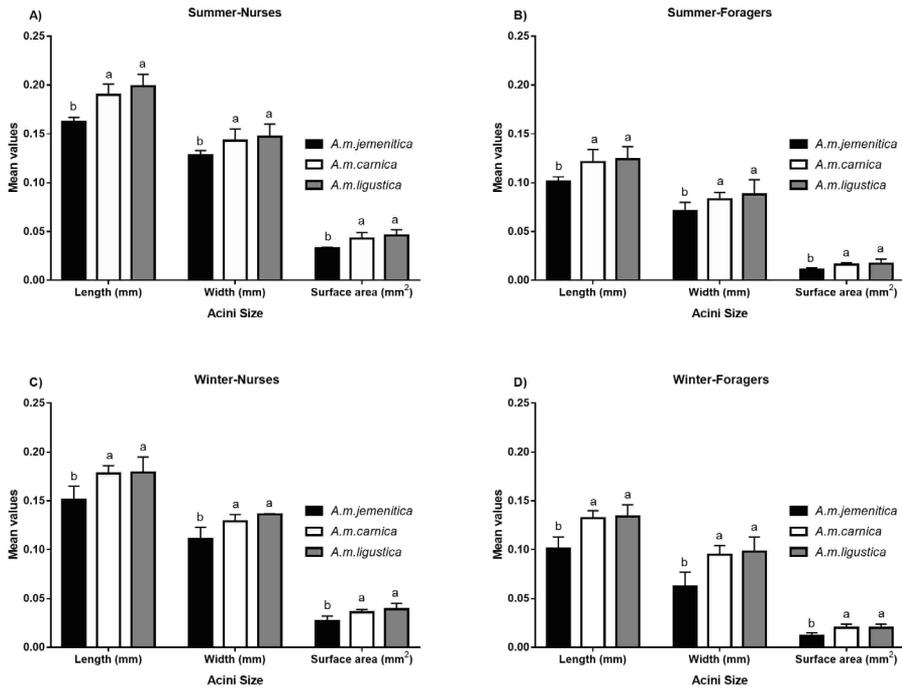
**Figure 3.** Acini sizes (length, width, and surface area) of nurse and forager bees. **A** *A. m. jemenitica* **B** *A. m. carnica* **C** *A. m. ligustica*. Nurse bees (summer & winter) had significantly larger acini than forager (summer & winter) bees in the three races. Asterisks (\*) in the graphs represent the significant differences between the groups (LSD test at  $p \leq 0.05$ ).

addition, significant differences were not observed between the two exotic races (AMC and AML) with respect to acini size.

The mean acini measurements in the AMJ nurses and foragers during the summer were as follows: L (0.162 mm and 0.101 mm, respectively), W (0.128 mm and 0.071 mm, respectively) and SA (0.033 mm<sup>2</sup> and 0.011 mm<sup>2</sup>, respectively). Similarly, the acini sizes of the AMJ nurses and foragers during the winter were as follows: L (0.151 mm and 0.101 mm, respectively), W (0.111 mm and 0.062 mm, respectively) and SA (0.027 mm<sup>2</sup> and 0.012 mm<sup>2</sup>, respectively). The sequence of bee races based on the three acini size parameters (L, W, and SA) was *A. m. ligustica* > *A. m. carnica* > *A. m. jemenitica* in both foragers and nurse bees (Table 1).

### Measurements of lipofuscin accumulation in HPGs

The microscopic lipofuscin accumulation granules in the HPGs of different bee races are shown in Figure 5. The foragers in all bee races (AMJ, AMC, and AML) presented



**Figure 4.** Inter-race comparison of acini size (length, width, and surface area) among the HPGs of *A. mellifera* **A** summer nurse **B** summer forager **C** winter nurse **D** winter forager. Graph bars headed by the same letter represent non-significant differences between the groups (LSD test at  $p \leq 0.05$ ).

a significantly higher lipofuscin accumulation percentage than nurse bees of the same race during the summer and winter seasons (Figure 6). Therefore, irrespective of bee race and season, foragers have significantly higher lipofuscin accumulation than nurse bees.

The comparison among indigenous (AMJ) and exotic bee races (AMC and AML) with respect to lipofuscin accumulation is shown in Figure 7. Nurses and foragers of AMJ presented a significantly lower lipofuscin accumulation than the nurses and foragers of exotic bee races (AMC and AML). Moreover, summer foragers of all races have higher lipofuscin accumulation than winter foragers. No significant differences were found in lipofuscin accumulation in the nurse bees of any race in either season (Figure 8).

In summer, the lipofuscin accumulation values in the nurse bees were AMJ = 12.33%, AMC = 14.13%, and AML = 15.57% and in the forager bees were AMJ = 45.43%, AMC = 56.27%, and AML = 58.23%. In winter bees, the lipofuscin accumulation values in the nurse bees were AMJ = 11.10%, AMC = 12.20% and AML = 14.80% and in the forager bees were AMJ = 40.50%, AMC = 46.00%, and AML = 46.33% (Table 1).

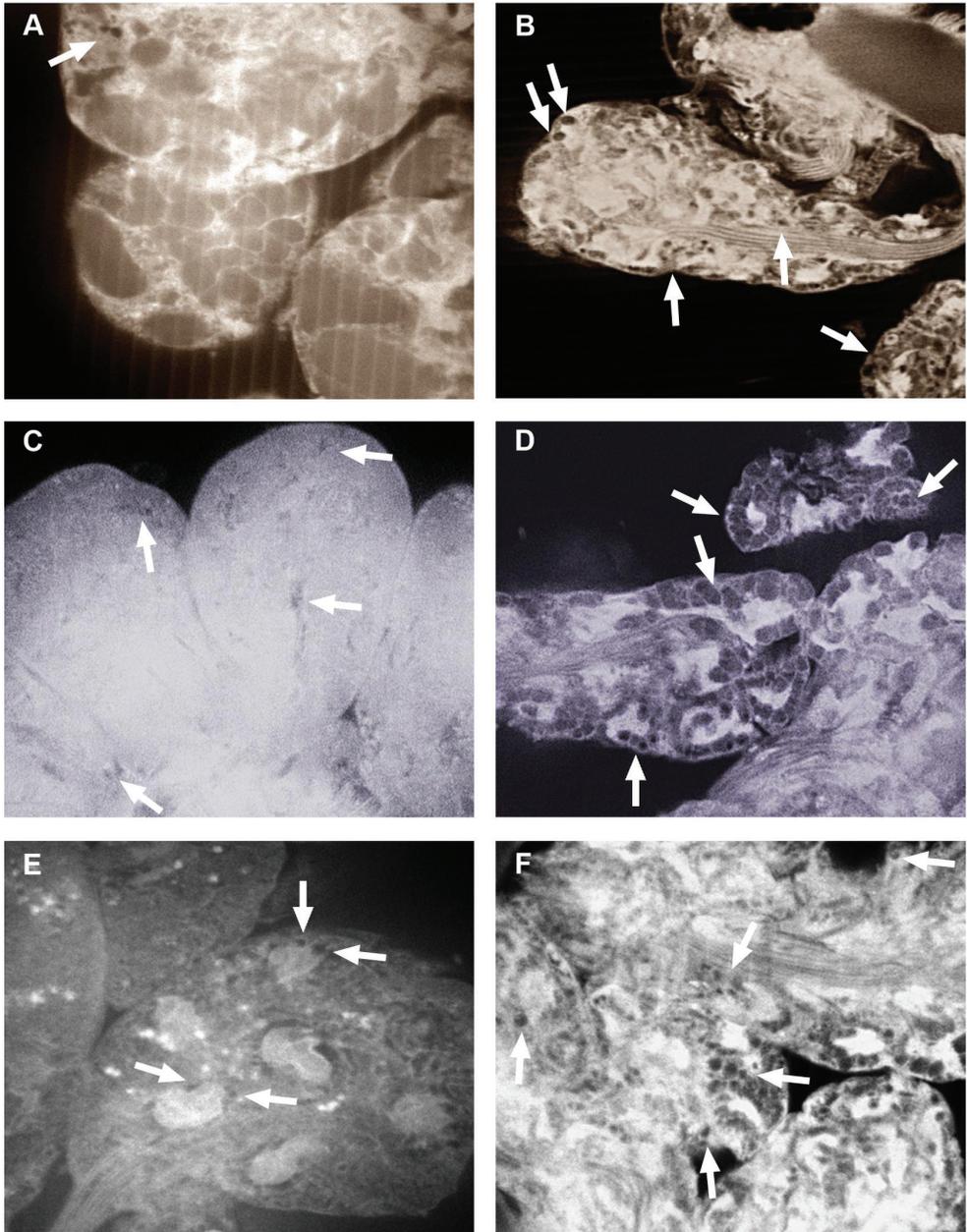
The acini size and lipofuscin accumulation were inversely correlated with each other in the nurses and foragers of all bee races. The larger acini size in the nurse bees was correlated with a lower lipofuscin accumulation, and the smaller acini sizes in the foragers was correlated with higher lipofuscin accumulation (Table 2).

**Table 1.** Measurements of acini size and lipofuscin accumulation percentages in the hypopharyngeal glands of honey bees.

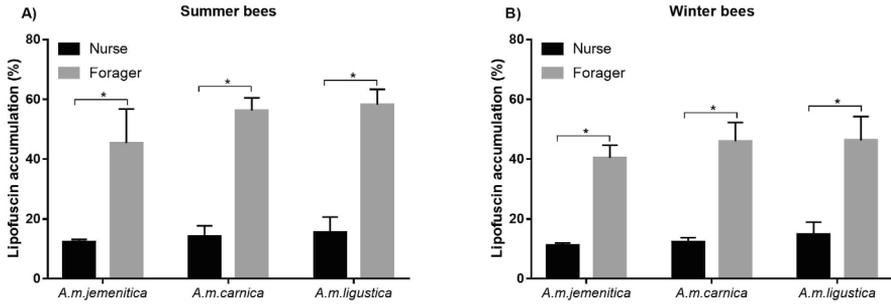
Season	Bee race	Acini size (mean ± SD)						Lipofuscin accumulation (%)							
		Length (mm)		Width (mm)		Surface area (mm <sup>2</sup> )		Nurses		Foragers		Nurses		Foragers	
		Nurses	Foragers	Nurses	Foragers	Nurses	Foragers	Nurses	Foragers	Nurses	Foragers	Nurses	Foragers	Nurses	Foragers
Summer	<i>A. m. jemenitica</i>	0.162±0.005	0.101±0.005	0.128±0.005	0.071±0.009	0.033±0.001	0.011±0.002	12.33	45.43						
	<i>A. m. carnica</i>	0.190±0.011	0.121±0.013	0.143±0.012	0.083±0.007	0.043±0.006	0.016±0.002	14.13	56.27						
	<i>A. m. ligustica</i>	0.199±0.012	0.124±0.013	0.147±0.013	0.088±0.015	0.046±0.006	0.017±0.005	15.57	58.23						
Winter	<i>A. m. jemenitica</i>	0.151±0.014	0.101±0.012	0.111±0.012	0.062±0.015	0.027±0.005	0.012±0.003	11.10	40.50						
	<i>A. m. carnica</i>	0.178±0.008	0.132±0.008	0.129±0.007	0.095±0.009	0.036±0.003	0.020±0.004	12.20	46.00						
	<i>A. m. ligustica</i>	0.179±0.016	0.134±0.012	0.136±0.001	0.098±0.015	0.039±0.006	0.020±0.004	14.80	46.33						

**Table 2.** Summary table for the inverse correlation between acini size and lipofuscin accumulation in the three bee races. Larger acini corresponded to lower lipofuscin accumulation and vice versa. L Length W width SA Surface area HPGs Hypopharyngeal glands. The arrow direction indicates the increase or decrease in size or accumulation percentage.

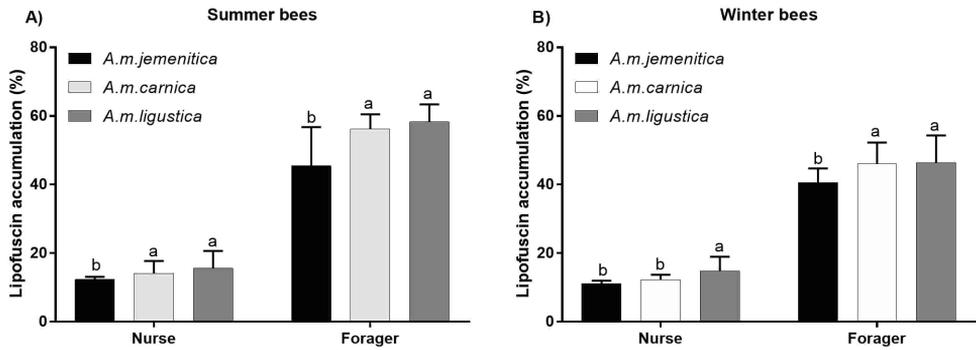
Bee race	Correlation index in the hypopharyngeal glands				Lipofuscin accumulation (%)
	Age polytheism		Acini size (L, W, SA)		
	Nurses	Foragers	↑	↓	
<i>A. m. jemenitica, A. m. carnica, A. m. ligustica</i>					↑
					↑



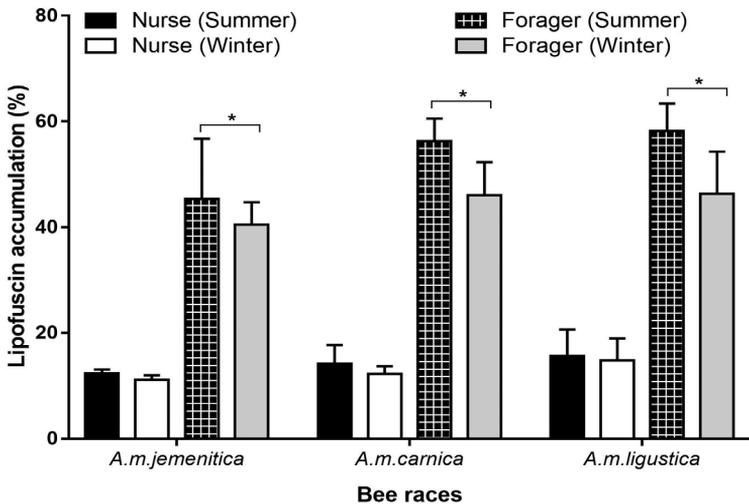
**Figure 5.** Lipofuscin accumulation (black granular structures: arrows) in the hypopharyngeal glands of nurse and forager bees **A** *A. m. carnica* nurse **B** *A. m. carnica* forager **C** *A. m. jemenitica* nurse **D** *A. m. jemenitica* forager **E** *A. m. ligustica* nurse **F** *A. m. ligustica* forager. (Images at 400× magnification).



**Figure 6.** Comparisons between nurse and forager bees in the accumulation of lipofuscin in hypopharyngeal glands. **A** summer bees **B** winter bees. Asterisks (\*) in the graph represent significant differences between the groups (LSD test at  $p \leq 0.05$ ).



**Figure 7.** Inter-race comparison of lipofuscin accumulation **A** summer bees **B** winter bees. Graph bars headed by the same letter represent non-significant differences between the groups (LSD test at  $p \leq 0.05$ ).



**Figure 8.** Seasonal variations in lipofuscin accumulation between summer and winter bees of the same race. Asterisks (\*) in the graph represent significant differences between the groups (LSD test at  $p \leq 0.05$ ).

## Discussion

### Acini size

Our results showed that acini size is linked with honey bee age-specific tasks. Nurse bees have significantly larger acini (L, W, and SA) than forager bees in all races (AMJ, AMC, and AML). These results are partially in accordance with a study by Škerl and Gregorc (2015), who reported large acini diameters in AMC nurse bees. Suwannapong et al. (2010) also found larger glands in nurses than foragers and guards in *A. cerana* Fabricius and *A. mellifera* Linnaeus. We also found that foragers have a smaller gland size, which is consistent with the findings of Ohashi et al. (2000). Therefore, all bee races exhibited identical stereotypic age-specific acini size patterns between nurses and foragers, even in the arid climate of Saudi Arabia. Moreover, age-polytheism in honey bees indicates that young nurse bees feed young bees and have larger acini, whereas older forager bees no longer feed the brood and thus present gland atrophy with smaller acini (Costa and da Cruz-Landim 2005; Deseyn and Billen 2005).

Acini size is positively correlated with HPG activity and its secretion. The larger acini in young nurses corresponds to higher HPG activity because of the production of royal jelly (RJ) for larvae. However, the reduced acini size in foragers results in decreased HPG activity (Brouwers 1982; Hrasnigg and Crailsheim 1998). The development and size of HPGs is positively linked with pollen and diet consumption (Al-Ghamdi et al. 2011a; Hrasnigg and Crailsheim 1998; Johnson 2010; Naiem et al. 1999), and nurse bees with larger glands may consume more pollen for preparing nutrients than foragers (Crailsheim 1991). In addition, the summer foragers are exposed to extreme heat stress during summer that might be a reason for less activity of HPGs than those of the nurses that remain inside the colony. The winter bees have hypertrophied HPGs due to the small number of larvae and activity reduction during winter (Škerl and Gregorc 2015). The reduced HPGs exhibit low protein synthesis rate, and secretions are presumed to be stored for the spring season until the young brood is present (Huang 1990).

Furthermore, the acini size (L, W, and SA) was smaller in indigenous bees (AMJ) than exotic bees (AMC and AML). Both nurses and forager bees (winter and summer) presented similar differences among the bee races. These findings are partially in accordance with previous studies (Al-Ghamdi 2006; Al-Ghamdi et al. 2011b), where HPG development and acini size were compared among nurse workers in one season. The present study provides a complete comparison of acini size in nurse and forager bees during the summer and winter seasons. Zeng et al. (1990) found a significant difference in acini size and number between two bee species, with *A. mellifera* presenting longer acini with more RJ secretion than *A. cerana*. In contrast, considerable differences in the size and histochemical structures of HPGs were not found between *A. mellifera* L. and *A. cerana* Fab. bee species (Suwannapong et al. 2010). However, these two studies are not comparable with ours due to taxonomic differences. The present study revealed no significant differences in acini size between the two exotic bee races AMC and AML.

Many factors, such as bee race, body size, brood pheromones, ecological factors, starvation, heat stress, *Varroa* infestation, diseases, and pesticides could play an important role in acini size in bee races (De Smet et al. 2017; Khalil 1992; Le Conte et al. 2001; Yousef et al. 2014). However, the tested colonies in this study were kept free from infestations, pesticides, and starvation. Therefore, the differences might be attributable to the other listed factors. Indigenous bees (AMJ) have significant difference in morphological characters such as smaller body size, wings and legs length than exotic bees (AMC and AML) (Alattal et al. 2014; Alqarni 1995; Alqarni et al. 2011), which could potentially explain the observed differences in acini size between the indigenous and exotic bee races. Other factors, such as geographical isolation, food preference and food consumption among different bee races, may also contribute to differences in HPG development (Alqarni 2006; Cheng 2001).

### **Lipofuscin accumulation**

In the present study, cellular senescence was observed by measuring the difference in lipofuscin accumulation in HPGs. Forager bees possessed higher lipofuscin accumulation than younger nurse bees, which is in line with the findings of Munch et al. (2013b), who reported high lipofuscin accumulation in older bees.

Lipofuscin accumulation is also known to be related to the onset of senescence and is dependent on foraging age (Heidem 2013; Munch et al. 2013a). Increased lipofuscin accumulation suggests that the organism is aging (Gray and Woulfe 2005), and such accumulations may have a significant impact on biological functions (Hsu and Chan 2013). In this study, acini size and lipofuscin accumulation showed a strong inverse correlation, with a larger acini size corresponding to lower lipofuscin accumulation in nurse and forager bees. This inverse correlation is consistent with Munch et al. (2013b). During summer, foragers are more exposed to high temperature and water stress than in winter. Thus, summer foragers might exert more energy to cope with these harsh circumstances, thereby leading to a relatively high lipofuscin accumulation compared with that of winter foragers (Figure 6).

Furthermore, indigenous bees (AMJ) showed less lipofuscin accumulation than exotic races (AMC, AML). The possible explanation may be the geographical isolation and ecological adaptations of indigenous honey bees to the local environment in the Arabian Peninsula and the different body size of these bees (Abou-Shaara et al. 2012; Alqarni et al. 2011). These bees may have evolved physiological processes to decelerate the accumulation of lipofuscin. Indigenous bees are known for their significantly lower weight loss, higher heat tolerance and greater foraging activity than AMC and AML (Alqarni 2006; Alqarni et al. 2014), and these observed features could be related to the increased adaptation of AMJ to the arid zone environmental conditions.

This study suggests the need to further investigate the association of HPGs, lipofuscin accumulation, royal jelly traits and brood quality in indigenous bees in different geographical regions of Saudi Arabia via new technologies, such as genetic characteri-

zation. It will also urge the researchers to explore the associated facts underlying the physiology of glands development with food and behavior especially in successful and heat tolerant indigenous bees of Saudi Arabia. Likewise, it will be also important to compare the acini size and lipofuscin accumulation in the other cephalic worker secretory hind brain glands. Detecting gene expression in the HPGs of indigenous bees (AMJ) compared with that of exotic bee races (AMC and AML) could be important for further understanding the mechanism of foraging and nursing behavior in the climatic conditions of Saudi Arabia.

Collectively, nurse bees presented significantly larger acini and less lipofuscin accumulation than foragers. Consequently, a strong inverse correlation was observed between acini size and lipofuscin accumulation. Inter-race comparisons showed that indigenous bees possess smaller acini and less lipofuscin accumulation in the HPGs than exotic bees during summer and winter.

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