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Asymmetry in the ovarioles, mandibular glands and hypopharyngeal glands of honeybee (*Apis mellifera*) workers developing in queenright or queenless colonies

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Abstract – Fluctuating asymmetry, characterized by small, random deviations in the symmetry between bilaterally symmetrical traits, has been found in many species. However, it is unclear how rearing in the absence of a queen affects the asymmetry of anatomical traits in honeybee workers. To investigate this issue, we compared fluctuating and directional asymmetry between workers reared as larvae in the absence of a queen (rebel workers) and those reared in the presence of a queen (normal workers). The results showed that rebel workers had lower fluctuating asymmetry in the number of ovarioles and size of the mandibular glands than normal workers, whereas normal workers had lower fluctuating asymmetry in the size of the hypopharyngeal glands. Our results indicate that the expression of fluctuating asymmetry in an organ depends on the amount of resources allocated to its development. We did not find evidence of directional asymmetry in the studied traits in worker honeybees.

Fluctuating asymmetry / Queenlessness / Rebel workers / *Apis mellifera*

1. INTRODUCTION

Societies of the honeybee (*Apis mellifera*) are characterized by a high degree of cooperation and harmony (Wilson 1971; Seeley 1985, 2010). Typically, the queen is the only reproductive member of the colony; workers are facultatively sterile and refrain from reproducing in the presence of the queen (Wilson 1971; Bourke 1988). However, under queenless conditions, reproductive harmony collapses, and many physiological and behavioural traits of workers change (Miller and Ratnieks 2001; Martin et al.

2004; Makert et al. 2006; Malka et al. 2008). A recent study showed that under queenless conditions, also larvae perceive the lack of the queen in the colony and develop into an alternative female polyphenic form: rebel workers (Woyciechowski and Kuszewska 2012). Rebel workers develop in queenless conditions and differ from normal workers, which develop in queenright conditions, in reproductive potential, physiology and behavioural traits. Compared with normal workers, rebel workers have more ovarioles in the ovaries, larger mandibular and Dufour's glands and underdeveloped hypopharyngeal glands (Woyciechowski and Kuszewska 2012; Kuszewska and Woyciechowski 2015; Kuszewska et al. 2017, 2018). All of these traits make rebel workers more queen-like than normal workers. Several days after emergence,

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rebel workers lay their own eggs more frequently than normal workers, even if they remain in a colony with a queen (Kuszevska et al. 2018). The development of rebel workers is directly related to the absence of the queen in the colony or, more precisely, the absence of the queen's mandibular pheromones (Woyciechowski et al. 2017), during the unsealed larval stage. A queen is typically present in a colony except for a brief period during swarming, when the old queen leaves the native nest and a new queen is still in the pupal stage in a queen's cell. This short queenless period is enough to initiate the development of rebel workers from the last eggs laid by the old queen in the native nest. The conditions in which rebel workers appear can be easily established experimentally through the use of artificially split colonies that mimic the temporary lack of a queen at swarming (Woyciechowski and Kuszevska 2012; Kuszevska and Woyciechowski 2015). The evolutionary explanation for this life strategy of honeybee workers is based on the assumption of the inclusive fitness theory (Hamilton 1964) and results of the dramatic decrease in relatedness between workers following queen departure: whereas the old queen's workers are sisters, the new queen's offspring are the nephews of the old queen's workers (Woyciechowski and Kuszevska 2012).

The impact of queenlessness on adult honeybee workers has been intensively studied, and there is abundant literature concerning the various physiological and behavioural changes that occur in the absence of a queen (Miller and Ratnieks 2001; Martin et al. 2004; Malka et al. 2008). Nevertheless, little is known about the effect of queen absence during larval development on various morphological traits of adult individuals. One such morphological trait is bodily symmetry. It is predicted that under suboptimal conditions, the developmental stability of an organism may be disturbed because of an inability to compensate for the disruptions caused by exogenous environmental stressors, resulting in increased degrees of random asymmetry (van Valen 1962). Such asymmetry is called fluctuating asymmetry (FA) and is characterized by small, random deviations in the symmetry of bilaterally symmetrical traits (van

Valen 1962; Palmer and Strobeck 1986; Palmer 1994). FA is independent of body side (van Valen 1962) and can affect various body parts (Palmer 1994; Palmer and Strobeck 2003). The expression of FA depends not only on developmental stability but also the functional importance of a given trait, being greater in functionally less significant traits, as some authors have found (Palmer and Strobeck 1986; Balmford et al. 1993; Gummer and Brigham 1995; Møller and Swaddle 1997). In addition, it has been suggested that the expression of FA depends on the cost of growth of the trait, defined as the amount of structural components necessary to form a unit of length of a given character (Aparicio 2001; Aparicio and Bonal 2002). In the honeybee, FA in response to rearing temperature variation (Jones et al. 2005), hybridization (Smith et al. 1997), inbreeding (Brückner 1976; Clarke et al. 1986) and starvation (Szentgyörgyi et al. 2016, 2017) has been studied, with varying results. Another form of bilateral asymmetry—directional asymmetry—reflects a consistent bias towards greater development of one side of the body than the other (Palmer and Strobeck 1986; Palmer 1994). It is unknown how rearing in the absence of a queen affects the asymmetry of anatomical traits of workers.

The aim of our study was to describe the possible effects of queenlessness during the larval stage on the asymmetry of organs in newly emerged adult honeybee workers. Asymmetry was assessed using measurements of the number of ovarioles in both ovaries and the sizes of the left and right mandibular and hypopharyngeal glands.

2. MATERIALS AND METHODS

To study asymmetry in honeybee workers, we used data assembled from experiments carried out in the experimental apiary of the Institute of Environmental Sciences (Jagiellonian University, Krakow, southern Poland), in which two types of workers were reared: rebel workers, which develop under queenless conditions, and normal workers, which develop under queenright conditions. We collected data from 2009, 2010, 2013,

2014 and 2016, and all experiments were conducted from May to June. In total, rebel and normal workers reared in 33 colonies were studied. All colonies were maintained in Wielkopolski hives and included 20,000–40,000 workers each.

Rebel and normal workers of the same age were reared in two similarly designed ways: in colonies that were allowed to naturally swarm and in artificially split colonies that mimicked the condition of a temporary lack of a queen due to swarming (Figure 1a, b). In each colony that was allowed to naturally swarm (7 colonies), we studied two groups of workers after swarming (day 0). The first group comprised workers that emerged 12 days after swarming; as larvae, they had been fed in the presence of the mother queen before she left the native nest with the swarm. The second group comprised workers that emerged 19 days after swarming; as larvae, they had been fed in the absence of the mother queen, in the period just after she left the native nest with the swarm and before a new, young queen emerged. Experimental frames containing the newly emerged bees were moved from each colony to an incubator (36 °C). Newly emerged workers in each experimental group were killed by freezing (− 16 °C) and dissected to evaluate the effects of the larval developmental conditions on them. In the artificially split colonies (26 colonies), the queen of each colony was confined to two experimental frames to produce eggs of similar age (day 0). Three days later, the colony was divided into queenright and queenless subunits, each with one experimental frame, a similar food amount and the presence of nurses and foraging workers. The subunits were reunited when the worker cells on the experimental frames were sealed (day 13). On day 20 of the experiment, the experimental frames with newly emerged bees were moved from each colony to an incubator (36 °C). Newly emerged workers from the two experimental frames were dissected (day 21) using the same methods applied to those in the naturally swarmed colonies. For each worker, all three organs were assessed; we counted the number of ovarioles in the left and right ovaries and determined the sizes of the left and right mandibular glands and hypopharyngeal glands

under a stereomicroscope. All of the organs were stained with Giemsa reagent (approximately 10 s) before measurement. The sizes of the left and right mandibular glands were calculated as the square root of longest diameter \times shortest diameter. The hypopharyngeal glands consist of a great number of lobes, called acini, and their diameter is commonly used as an index of gland size (Nakaoka et al. 2008; Woyciechowski and Kuszewska 2012). For each of the left and right hypopharyngeal glands, the size was calculated as the average size of ten acini (with acinus size measured as the square root of the longest diameter \times shortest diameter).

All the studied workers were 1 day old; workers of the same age were used because organ size can change with age (Nakaoka et al. 2008; Wegener et al. 2009). Because the method of raising workers (naturally swarming vs. artificially split) could affect the physiology of workers, all analyses were conducted separately for the naturally swarming colonies and the artificially split colonies. If one of the dissected organs was destroyed during dissection, the measurement was not included in the analysis. The number of analysed samples from worker organs with full measurements is presented in Table I.

To compare the number of ovarioles, mandibular gland size and hypopharyngeal gland size between rebel and normal workers, we used two-way mixed-model ANOVA, with the experimental group (reared under queenright vs. queenless conditions) as the fixed effect and colony as the random effect. If the effect of an experimental treatment was found to be significant by ANOVA, a post hoc Tukey HSD test for multiple comparisons was performed, with $p=0.05$ considered significant.

To measure FA, we used index 2 (FA2) because it is independent of size (Palmer 1994). FA in the number of ovarioles and the sizes of the mandibular and hypopharyngeal glands was measured as the absolute difference between the right and left sides divided by the mean value of the right and left sides ($FA2 = |R - L| / ((R + L) / 2)$) (Palmer 1994). A higher value of this measure indicates greater asymmetry

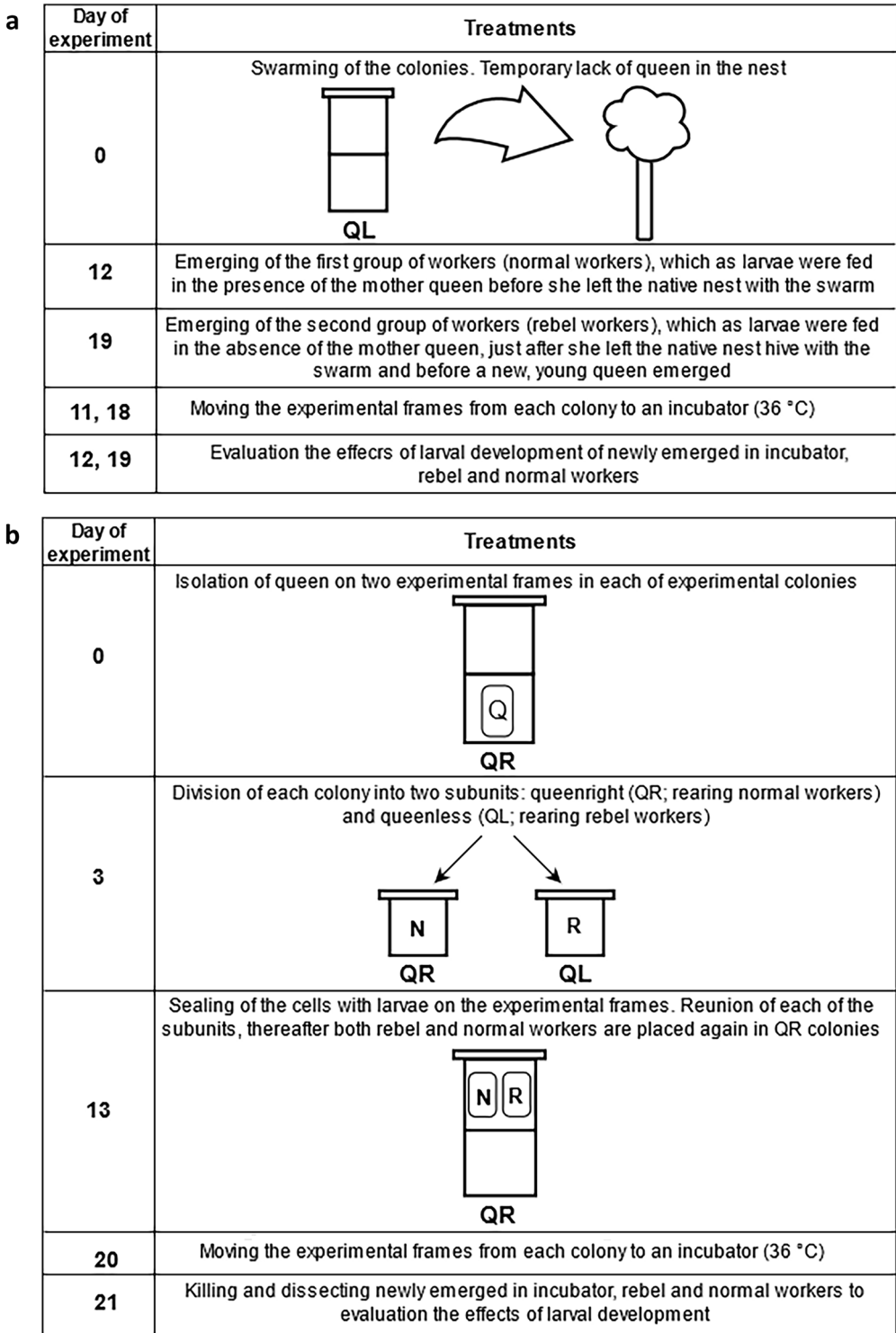


Figure 1 Timeline of the experiment, showing the manipulations on successive days. **a** Naturally swarming colonies. **b** Split colonies.

between the right and left sides of a bilateral trait for an individual. The differences in ovary, mandibular gland, and hypopharyngeal gland asymmetry between rebel and normal workers were analysed using a nonparametric Mann–Whitney *U*-test, as the data departed significantly from normality. All analyses were performed in Statistica 13.3 (StatSoft, Poland).

3. RESULTS

We found that in both the naturally swarming and artificially split colonies, workers reared under queenless conditions had more ovarioles in their ovaries (Figure 2a; swarming colonies, $df = 1$, $F = 478.49$, $p < 0.001$; split colonies, $df = 1$, $F = 613.83$, $p < 0.001$), larger mandibular glands (Figure 2b; swarming colonies, $df = 1$, $F = 166.80$, $p < 0.001$; split colonies, $df = 1$, $F = 124.51$, $p < 0.001$) and smaller hypopharyngeal glands (Figure 2c; swarming colonies, $df = 1$, $F = 139.53$, $p < 0.001$; split colonies, $df = 1$, $F = 173.54$, $p < 0.001$) than workers reared under queenright conditions. These results confirmed that the two groups of studied workers

presented different phenotypes and could be treated as different subcastes of workers.

To assess whether directional asymmetry was present in rebel and normal workers from the naturally swarming and artificially split colonies, we compared the left and right sides of each organ. No significant difference was detected in the number of ovarioles (Wilcoxon test for paired samples; $N = 1078$, $p = 0.417$), the size of the mandibular glands ($N = 1342$, $p = 0.350$) or the size of the hypopharyngeal glands ($N = 1403$, $p = 0.133$).

The degree of FA among workers from the naturally swarming and artificially split colonies was significantly lower in rebel workers than in normal workers for both the number of ovarioles (Figure 3a; Table I (part a and b)) and size of the mandibular glands (Figure 3b; Table I (part a and b)). There was no significant difference in FA in the size of the hypopharyngeal glands between rebel and normal workers in swarming colonies (Figure 3c; Table I (part a)). In contrast, in the split colonies, rebel workers had a significantly higher degree of FA in the size of the hypopharyngeal glands than normal workers (Figure 3c; Table I (part b)).

Table I Results of Mann–Whitney *U* tests for fluctuating asymmetry (FA) of organs in rebel and normal workers reared in naturally swarming colonies (a) and artificially split colonies (b); R, rebel workers; N, normal workers

Variables	Z	P	Median (quartiles)	No. of individuals
a				
No. of ovarioles FA	-4.018	$p < 0.001$	R=0.22 (0.13–0.5) N=0.4 (0.00–0.66)	R=210 N=210
Mandibular glands size FA	-2.227	$p = 0.026$	R=0.07 (0.03–0.15) N=0.09 (0.05–0.16)	R=210 N=209
Hypopharyngeal glands size FA	-0.205	$p = 0.837$	R=0.03 (0.02–0.06) N=0.03 (0.02–0.06)	R=210 N=210
b				
No. of ovarioles FA	-6.955	$p < 0.001$	R=0.22 (0.0–0.4) N=0.4 (0.00–0.66)	R=594 N=569
Mandibular glands size FA	-2.44	$p = 0.015$	R=0.06 (0.03–0.11) N=0.07 (0.03–0.13)	R=478 N=452
Hypopharyngeal glands size FA	3.772	$p < 0.001$	R=0.04 (0.2–0.07) N=0.03 (0.15–0.06)	R=509 N=483

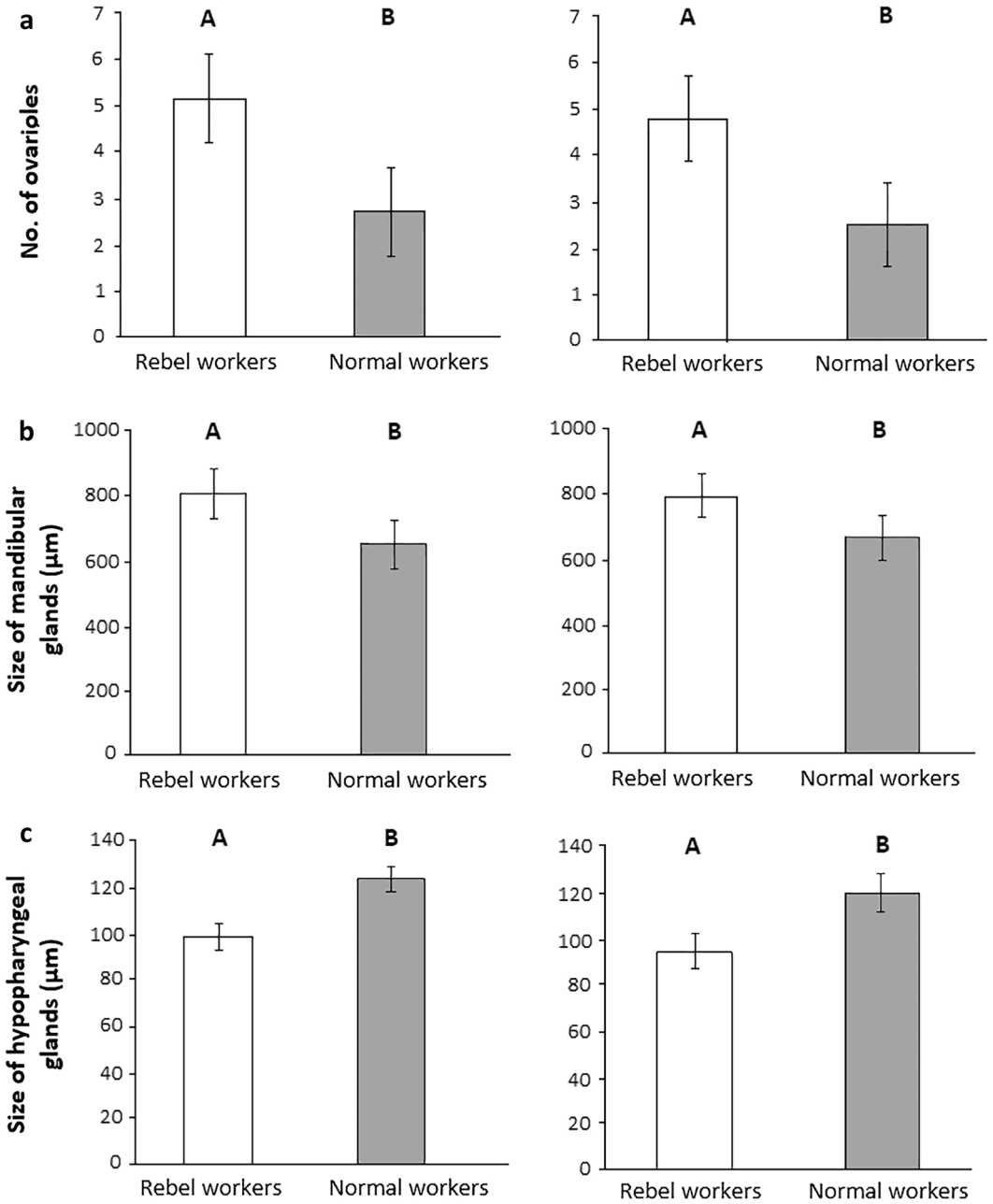


Figure 2 Anatomical parameters of 1-day-old rebel and normal workers reared in naturally swarming colonies (left column) and artificially split colonies (right column). **a** Number of ovarioles. **b** Size of mandibular glands. **c** Size of hypopharyngeal glands. Error bars show SD. Different bold letters in the graphs indicate significant differences ($P < 0.05$) between rebels and normal workers.

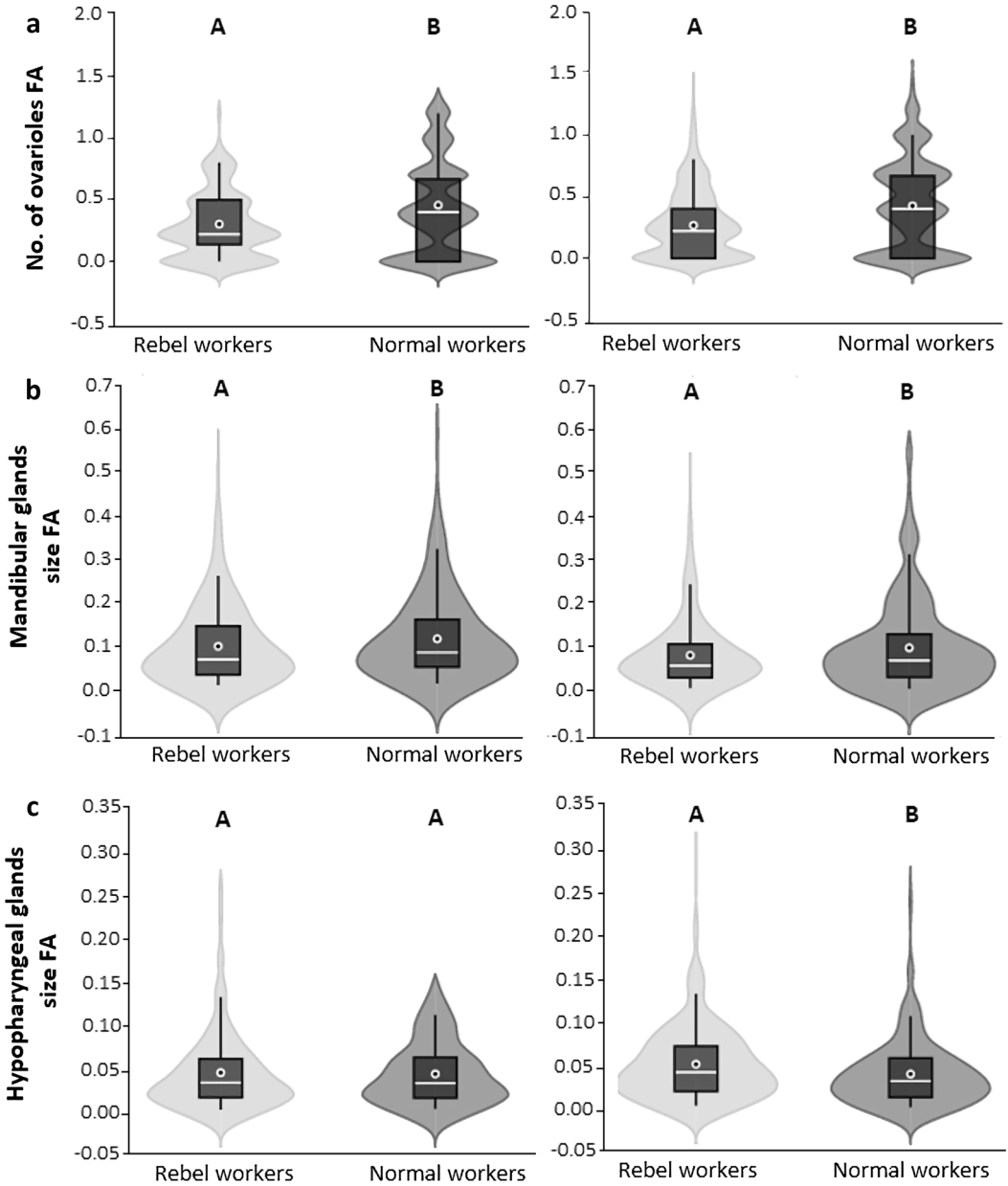


Figure 3 Fluctuating asymmetry (FA) of organs in rebel and normal workers reared in naturally swarming colonies (left column) and artificially split colonies (right column), shown as violin plots. The white line in the middle of each box plot inset within a violin plot represents the median of each distribution; the white circle inside the box shows the mean; the lower and upper edges of the box represent the first and third quartiles, respectively; and the whiskers represent the 5% and 95% confidence ranges. **a** FA in the number of ovarioles. **b** FA in the size of the mandibular glands. **c** FA in the size of the hypopharyngeal glands. Different bold letters in the graphs indicate significant differences ($P < 0.05$) between rebels and normal workers.

4. DISCUSSION

The results presented here demonstrate differences in the level of FA of organs between workers reared as larvae in the absence of a queen (rebel workers) and those reared in the presence of a queen (normal workers). Rebel workers had lower FA in the number of ovarioles and mandibular gland size than normal workers (Figure 3a, b). Both of these organs are better developed in rebel workers, which are more queen-like, suggesting that they are better prepared for reproduction than normal workers (Woyciechowski and Kuszewska 2012; Kuszewska et al. 2018). The number of ovarioles is positively associated with the potential for egg laying (Tanaka and Hartfelder 2004; Makert et al. 2006), while the mandibular glands produce queen-like pheromones (Kocher et al. 2010). Enlargement of these organs observed in rebel workers suggests high costs of their development. In turn, normal workers had lower FA in hypopharyngeal gland size than rebel workers, although the difference was significant only in artificially split colonies (Figure 3c). Hypopharyngeal glands synthesize and store brood food and are largest in nursing bees (Huang and Otis 1989). Underdeveloped hypopharyngeal glands in rebel workers suggests the low production of brood food and restricted nurse activity (Huang and Otis 1989). Larger hypopharyngeal glands (Figure 2c) suggest a higher cost of development. Therefore, our results suggest that the expression of FA depends on the input of resources to the organ, with lower FA in organs with larger input.

A less likely explanation for the differences in the FA of organs observed in the studied workers may be nutritional differences between workers feeding in queenless or queenright colonies, as some studies have shown that nutrition has an impact on FA in the honeybee (Szentgyörgyi et al. 2016; Fathy et al. 2018). Woyke (1999) showed that larvae reared under queenless conditions received more food than those reared under queenright conditions. In addition, some authors have proposed that the body mass of newly emerged workers from colonies selected for high or low pollen hoarding is correlated with

the number of ovarioles (Linksvayer et al. 2011; Wang et al. 2014). However, other studies have found no difference in body mass immediately after emergence between workers reared in a queenless colonies and those reared in queenright colonies (Woyciechowski and Kuszewska 2012; Kuszewska and Woyciechowski 2015). Woyciechowski et al. (2017) showed that workers reared with a queen or fed with a diet with the macerated queen mandibular glands added to their food under queenless conditions developed into normal workers. Only workers reared without a queen and without macerated mandibular glands in their food developed into rebel workers. These results suggest that resource allocation to particular organs is controlled by queen pheromones in larval food rather than by different feeding strategies of nurse workers.

A negative relationship between FA and trait size has been found in the secondary sexual traits of a variety of taxa (Møller 1990; Manning and Hartley 1991; Manning and Chamberlain 1993; Radesäter and Halldórsdóttir 1993), although it does not appear to be a common feature of sexual traits (Bjorksten et al. 2000). Our ovary asymmetry data correspond well with those of Ruppell et al. (2011a), who assessed the difference in the number of ovarioles between sides in honeybee workers from two strains that were bidirectionally selected for high versus low amounts of pollen stored in the colony. They found that workers from the high-pollen strain, which had a higher number of ovarioles, had lower ovary asymmetry, whereas workers from the low-pollen strain, with a lower number of ovarioles, had higher asymmetry. Similarly, Graham et al. (2011) found a strong correlation in the number of ovarioles between the left and right sides of workers in a series of crosses between Africanized and European honeybees, which had an extraordinarily high number of ovarioles.

There is a large body of evidence supporting a negative association between asymmetry and quality (reviewed by Møller and Swaddle 1997). Deviations from perfect symmetry indicate decreased quality in honeybee drones (Jaffé and Moritz 2010), male damselflies (*Lestes viridis*, de Block and Stoks 2007), male fruit

flies (*Drosophila pseudoobscura*, Markow and Ricker 1992) and male and female Japanese scorpionflies (*Panorpa japonica*, Thornhill 1991). Although there are conflicting reports on the relationship between symmetry and quality (Swaddle 1997; Pears et al. 2019; Czekońska et al. 2019), it seems very likely that the greater symmetry in the number of ovarioles and size of the mandibular glands between the two body sides in rebel workers reflects higher quality of these organs than those of normal workers.

We did not find directional asymmetry in the ovaries or mandibular and hypopharyngeal glands of rebel or normal workers. Although directional asymmetry in ovariole number in *A. mellifera* workers has been observed (Chaud-Netto and Bueno 1979), it has not been detected in other studies of this species (Thuller et al. 1998; Goudie et al. 2012) or in five other *Apis* species (Rueppell et al. 2011b). We hypothesize that the ovary from the same side is not more prevailing than that from the other side and that the same is true for the mandibular and hypopharyngeal glands.

In conclusion, in the honeybee, there were considerable differences in FA between the workers reared as larvae in the absence of a queen and those reared in the presence of a queen. Workers reared in the absence of a queen exhibited less FA in both the ovaries and mandibular glands, which were enlarged in these workers compared with workers reared in the presence of a queen. In workers reared in the presence of a queen, the hypopharyngeal glands were enlarged and characterized by lower FA. It seems that the expression of FA in a particular organ depends on the amount of resources allocated to its development. This finding deepens our understanding of the mechanisms of polyphenism in eusocial insects.

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AUTHOR CONTRIBUTIONS

WR, KK and MW conceived this research and designed the experiments. WR and KK performed the experiments. WR and HS analysed the data. WR, KK, HS and MW wrote the manuscript and participated in the revisions. All authors read and approved the final manuscript.

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DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval/declarations Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflicts of interest The authors declare no competing interests.

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