# Reproductive traits and mandibular gland pheromone of anarchistic honey bee workers *Apis mellifera* occurring in China

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Abstract – In honey bee colonies, workers, in particular of "anarchistic" lineages, can activate their ovaries and lay eggs, even in the presence of the queen. We identified three queentight colonies showing typical signs of worker reproduction. To characterize this new lineage, we extracted the andibular gland and analyzed it using gas chro atography. The total a ounts of the five ain co ponents of the andibular gland, na ely ethyl p-hydroxyben-zoate (HOB), 9-oxo-2(E)-decenoic acid (9-ODA), (S)-9-hydroxy-(E)-2-decenoic acid (9-HDA), 10-HDA, and 10-hydroxyde-canoic acid (10-HDAA) were significantly higher in the andibular gland profiles of workers with activated ovaries (AWs,  $8.88\pm1.71 \mu$ g) co pared to workers with inactivated ovaries (IAWs,  $4.00\pm2.09 \mu$ g). Further ore, the che ical profiles of IAWs were do inated by the "worker substances" 10-HDA ( $34.64\pm8.19 \%$ ) and its precursor 10-HDAA ( $22.88\pm4.95 \%$ ), while the che ical profiles of two precursor substances 10-HDAA ( $40.04\pm7.55 \%$ ). The ratios of two precursor substances 10-HDAA/9-HDA of IAWs were ore worker like (>1.0) whereas AWs were ore queen like ( $\leq$ 1.0). These results suggest that the andibular phero ones of anarchistic workers rese ble a ore queen-like reproductive active profile and that these workers ay represent a reversion to a ore basal reproductive phenotype.

# *Apis mellifera* / anarchistic colony / ovary activation / egg production / worker policing / worker mandibular gland pheromone

### 1. INTRODUCTION

Like any eusocial insects, the honey bee A m L. is characterized by extre e reproductive division of labor between the queen and workers (Michener 1974). Under nor al conditions, the queen is usually the sole fe ale reproductive active individual in a colony, while workers refrain fro reproducing despite having functional ovaries and in general, can lay only haploid ale eggs (arthenotoky) (Winston 1987; Hepburn and Radloff 1998; Hoover et al. 2003; Slessor et al. 2005). In wild-type colonies of European subspecies, approxi ately 0.01 % of workers lay 7 % of all ale eggs, of which only one egg in 1000 is reared and the other are reoved by other workers; the so-called worker



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policing (Ratnieks 1993; Visscher 1996). However, effective worker policing is not present in a rare strain of *An* "anarchistic" colonies, in which a large nu ber of worker-laid eggs are because 9-ODA that is produced in regular colonies by a functional queen has been shown to inhibit ovary activation and suppress the production of a queen-like phero one signal in other workers (Crewe 1988; Moritz et al. 2000, 2004, 2008; Diete ann et al. 2007). Thus, the ratios of 9-ODA/(9-ODA + 9-HDA + 10-HDA + 10-HDAA) and 10-HDAA/9-HDA have been used as strong indicators of individual fecundity (Plettner et al. 1993; Moritz et al. 2004; Hoover et al. 2005a; Schäfer et al. 2006).

Anarchists, as a special case of worker reproduction, provide an experi ental resource for investigating the proxi ate echanis s underlying the worker reproduction in wild-type colonies. However, anarchy is a co plex syndro e, which is influenced by phero ones, worker policing, queen and worker genetic co ponent, and other factors. The ajority of research of the anarchistic traits is based on the selectively bred line aintained by the University of Sydney, which reliably show higher levels of anarchistic phenotype (Oldroyd et al. 1999; Oldroyd and Osborne 1999).

In 2012, we first saw drone eggs and larvae appeared above the queen excluder in several queenright An L. colonies, in Shanxi province, China. In order to confir their anarchistic traits, we investigated the following: (1) the percentage of worker with stage 4–5 activated ovaries (Hess 1942; Pirk et al. 2010) (2) and if worker-laid eggs are able to escape worker policing. In order to further understand the role of the worker andibular gland secretions in the acquisition of reproductive status and in the proxi ate

echanis s underlying the anarchistic syndro e, (3) we investigated the co position of andibular gland profiles of workers with inactivated ovaries (IAWs) and workers with activated ovaries (AWs) sa pled in three colonies with typical anarchistic traits and deter ined if AWs produce queen-like substances in their andibular gland secretions.

# 2. MATERIALS AND METHODS

Eggs and larvae were found above the queen excluder in ten queen right *An* L. colonies, in the apiary of Shanxi province, China, of which three were transported to Hangzhou and the University's apiary. All of the were suspected of being "anarchistic" colonies. Further ore, three colonies not showing any features of anarchistic activities were used as discri inator colonies.

#### 2.1. Experiment 1. Ovary activation analysis

Honey bee abdo ens were dissected according to Dade (1977) and assigned as being inactivated (ovaries thread-like and lacking defined ova, stages 1–3) or fully activated (clearly defined ova, stage 4, or at least one egg present in ovarioles, stage 5) (Hess 1942; Pirk et al. 2010). Sixty workers per anarchistic colony (=3) were obtained fro drone co bs above the queen excluder, and the procedure was replicated in 3-day intervals for four ti es in June 2012, resulting in 720 workers being dissected and scored.

# 2.2. Experiment 2. Egg-laying behavior analysis

Each anarchistic colony (=3) was split into two halves (queen excluded half: the area below queen excluder, and queen-excluded half: the area above queen excluder) using a queen excluder, which allows passage freely of workers but not of the queen. In order to easure the reproductive output of the queen and workers in ter s of drones eggs produced within colonies, one drone co b was put in each half. After 8 h, drone co bs were re oved fro the colonies and the eggs in each half were counted.

To co pare the nu ber of worker-laid eggs between the two halves and to evaluate the potential contribution of workers to the eggs laid in the queen right half, we placed one drone co b each in a plastic queen excluder boxes above and below the queen excluder. Again, the nu ber of worker-laid eggs was counted in each half after 8 h, and this was replicated on three or four ti es within 1 week. Analysis for statistical differences of the egg production between queenright half and queenexcluded half was perfor ed using Student's test. Data are presented as eans and standard deviations.

#### 2.3. Experiment 3. Worker policing analysis

### 2.3.1. Sources of eggs

Three wild-type queentight colonies (WT1, WT2, WT3) are the sources of wild-type queen-laid eggs (WTQE), three wild-type queen-less colonies (WTL1,

WTL2, WTL3) as the sources of wild-type worker-laid eggs (WTWE), three anarchistic queenright colonies (AC1, AC2, AC3) as the sources of anarchistic queenlaid eggs (ACQE), and anarchistic worker-laid eggs (ACWE). These colonies ( =9) were divided into three groups; the first group WT1, WTL1, AC1; the second group WT2, WTL2, AC2; and the third group WT3, WTL3, AC3. Each group provided four different types of eggs, including WTQE, WTWE, ACQE, and ACWE.

### 2.3.2. Discriminator colonies

To assay the re oval rates of eggs fro various sources, we used three wild-type colonies (WT1, WT2, WT3) and three anarchistic colonies (AC1, AC2, AC3) as discri inator colonies. Each hive was separated into two parts by a queen excluder, in which the queen was confined to the botto , yielding queenexcluded areas above the queen excluder and queen ight areas below.

#### 2.3.3. Egg removal bioassays

To perfor the assay, we transferred 20 eggs fro each source of eggs; the four different sources (WTQE, WTWE, ACQE and ACWE) into drone cells of the sa e test co b that has been left overnight in their corresponding discri inator colonies. All of these test eggs were collected fro their original drone cells by using sterilized toothpick. A new toothpick was used for each row. After transferring, the test co b with 80 eggs was sandwiched between brood co bs that previously placed in the center of the upper cha ber of each discri inator colony (Pirk et al. 2002). Eggs were not introduced in their colony of origin to avoid any interferences of nest ate recognition (Pirk et al. 2007). Drone co bs with four types of eggs were placed in the discri inator colonies ensuring that co bs and eggs were originating fro other colonies than the discri inator colonies. The nu ber of re aining eggs was counted 1, 2, 4, and 6 h after introduction, and it was repeated four ti es on separate days.

#### 2.3.4. Statistical analysis

Survival data of (WTQE, WTWE, ACQE, and ACWE) were analyzed using a Cox regression survival analysis (Collett 1994) as i ple ented in SPSS. For this analysis, eggs re oved at 1, 2, 4, or 6 h were co plete data points whereas re aining eggs after 6 h were treated as censored data. Egg source, day, and discri inator were used as variables in the Cox regression survival odel, and we used it to calculate the likelihood ratio for the survival rate of the different egg sources after adding the factors of day and discri inator. The survival function was odeled without ("Null") and then with (overall) source of eggs, day, and discri inator colony (overall) as factors, and the i prove ent of the ability of the odel to describe the data tested with a  $\chi^2$  test. The odel then tested the effect of adding source of eggs, day, or discri inator as factors. Lastly, we presented a pairwise co parison between the survival eggs of four different sources. We presented the data graphically as the ean proportion (±standard deviations) of eggs re aining at each ti e period.

# 2.4. Experiment 4. Workers' mandibular pheromone analysis

Phero one co positions analysis was conducted at Pretoria University, South Africa. Ten heads of IAWs and AWs each colony (=3) were re oved and stored in 200 µl dichloro ethane for at least 24 h to extract co pounds of their andibular glands. Half of the extracts were evaporated to dryness under a strea of nitrogen and analyzed by gas chro atography (Zheng et al. 2010). Chro atogra s were recorded using an Agilent 6890 GC and peak areas quantified using HP Che Station software. HOB, 9-ODA, HVA, 9-HDA, 10-HDA, and 10-HDAA were identified based on the retention ti es of synthetic co pounds and their retention ti e relative to the internal standards (Si on et al. 2001). Their relative ass ratios (RMRs) were easured relative to tetradecane. The standard solution containing all of six co pounds was run daily to ensure that RMRs were within the li it of the variability found in the series of standard runs.

We calculated the absolute a ounts ( icrogra s) and relative a ounts (their percentage co position) of HOB, 9-ODA, HVA, 9-HDA, 10-HDA, and 10-HDAA in IAWs and AWs. The ratio of 9-ODA/(9-ODA + 9-HDA + 10-HDA + 10-HDAA) (Moritz et al. 2004; Hoover et al. 2005a; Schäfer et al. 2006) and 10-HDAA/9-HDA (Plettner et al. 1993) were used as indicators of individual fecundity. Analysis for statistical differences between IAWs and AWs were perfor ed using Mann-Whitney test. Data are presented as eans and standard deviations.

# 3. RESULTS

# 3.1. Experiment 1. Ovary activation analysis

A total of 720 workers were rando ly collected fro each colony for ovary activation analysis. The ean percentage of AWs in three anarchistic colonies was 6.25-49.58 %, and the ean percentage of workers with at least one ature egg appeared in their ovarioles (stage 5, Pirk et al. 2010) was 3.00-21.25 % (Table I).

# 3.2. Experiment 2. Egg-laying behavior analysis

According to analysis of variance, the ean rate of egg production of queenright half (222.83 $\pm$ 85.91), to which potentially the queen and workers contributed, was significantly higher than that of the queen-excluded half (11.42 $\pm$ 21.38) within 8 h in anarchistic colonies (= 8.273, =12.358, P<0.001) (Figure 1). The ean rate of egg production of workers in

queenright half  $(13.00\pm20.72)$  was lower than that of queen-excluded half  $(25.67\pm$ 36.88) in anarchistic colonies (Figure 2). Fro that, we can calculate that the contribution of the queen was on average 209 eggs or 94 % of the eggs laid in the queenright section of the hive.

### 3.3. Experiment 3. Worker policing

A Cox regression analysis showed that there were significant differences in the re oval rates for the four different sources of eggs ("Source of eggs" P < 0.001). The experi ental day did not significantly affected the re oval rates ("Day of trial" P > 0.05) nor did discri inator colony ("Discri inator" P > 0.05) (Table II).

In anarchistic discri inator colonies, WTWE were re oved significantly faster than ACWE (P < 0.001); however, ACWE were re oved at a si ilar rate as WTQE (P = 0.182) and ACQE (P = 0.290) (Figure 3a, Table II).

In wild-type discri inator colonies, the pairwise co parisons showed that the four source eggs were treated significantly different: ACQE were re oved significant less than WTQE (P < 0.05) and WTWE were re oved fastest whereas ACQE were re oved significantly slower than WTQE (P < 0.001) (Figure 3b, Table II).

In both anarchistic and wild-type discrininator colonies, WTWE were re oved faster than other

Ti es ( =12)	Colony ( =3)	AWs (%)	$-\pm$ SD	Workers with at least one ature egg (%)	$-\pm$ SD
1 2	AC1	10.00 26.67	16.25±7.50	6.67 23.33	7.25±4.72
3		11.67		6.67	
4		16.67		11.67	
1 2	AC2	3.33 8.33	49.58±14.23	3.33 8.33	21.25±7.59
3		11.67		8.33	
4		1.67		0	
1 2	AC3	58.33 61.67	6.25±4.59	33.33 45.00	3.00±2.45
3		30.00		18.33	
4		48.33		45.00	

**Table I.** The percentage of workers with activated ovaries (AWs) and at least one ature egg appeared in their ovarioles in three anarchistic colonies.



Figure 1. The rate of egg production of queen right half and queen-excluded half in three anarchistic colonies (AC). The two parts were separated by a queen excluder, so the queen was the only individual confined to on art of the hive. Mean and standard deviations are shown.

egg sources, while the survival rates of ACWE were inter ediate between those of queen-laid eggs (WTQE, ACQE) and WTWE. In addition, the anarchistic colonies were less discrininatory than wild-type colonies towards the four sources of eggs (Figure 3).

# 3.4. Experiment 4. Workers' mandibular pheromone analysis

The total a ounts of all identified co pounds were significantly higher in AWs ( $8.88\pm1.71 \mu g$ , =30) co pared to IAWs ( $4.00\pm2.09 \mu g$ , =28) (P < 0.05, Table SI). Further ore, the absolute a ounts of HOB and 9-HDA were significantly higher in AWs co pared to IAWs (P < 0.05), whereas the re aining four co pounds were not significantly different between the two groups (lowest P value=0.565) (Figure 4, Table SI).

The relative a ounts of 10-HDAA and 9-HDA were significantly different between IAWs and AWs (P < 0.001) with 10-HDA ( $34.64\pm8.19$  %) and 10-HDAA ( $22.88\pm4.95$  %) being the do inant co ponents in the profile of IAWs, while AWs was do inated by 9-HDA ( $40.04\pm7.55$  %), the queen substance, 9-ODA was a relatively inor co ponent in both IAWs ( $5.14\pm3.24$  %) and AWs ( $2.09\pm1.14$  %), and was not significantly different between the two groups, si ilar to the re aining three co ponents (lowest P value =0.063) (Figures 4 and 5, Table SI). The ratio of 9-ODA/(9-ODA + 9-



Figure 2. The rate of egg production of workers in queen right and queen-excluded halves of three anarchistic colonies (AC), when the queen was caged onto one drone co b. Mean and standard deviations are shown.

HDA + 10-HDA + 10-HDAA) showed no significant differences between IAWs and AWs (ratio IAWs= $0.057\pm0.074$ , AWs= $0.024\pm0.037$ , P=0.421), whereas the ratio of 10-HDAA/9-HDA was significantly higher in IAWs than in AWs (ratio IAWs= $1.490\pm1.319>1.0$ , AWs= $0.501\pm0.700<1.0$ , P<0.001) (Table<



Figure 3. Re oval rates of eggs laid by wild-type queens (QE) and workers (E) and anarchistic queens (ACQE) and workers (AC E) when introduced into three unrelated anarchistic (a) and wild-type (b) queenright discrimator colonies, respectively. Values were the eans of all 3 or 4 days for all discrimators (=3). The represented the standard errors of the eans.

sources of eggs, especially WTWE were rejected

ore strongly in wild-type discri inator colonies than in anarchistic discri inator colonies. These results strongly support previous studies showing that anarchistic workers evade egg policing by laying ore acceptable eggs (Oldroyd and Ratnieks 2000) and that worker policing see s to be reduced in anarchistic colonies. However, the proxi ate echanis underlying the discriination of worker and queen-laid eggs in honey bees and, in particular, in anarchistic colonies has not been deter ined, besides a potential eggarking phero one (Ratnieks 1995), egg viabil-

ity ight also play a role (Pirk et al. 2004). The analysis of the andibular gland products of workers collected fro these three colonies showed that anarchistic workers had ability to produce queen-like signal to establish their reproductive do inance. When co paring with literature data, the total a ounts in both IAWs and AWs were higher than in *An* queenright workers  $(2.59\pm0.62 \ \mu g)$ , while they were less than *An* queen-less workers  $(10.73\pm$  $2.30 \ \mu g)$  (Tan et al. 2012). Since phero onal do inance can translate into throphallactic do inance and greater ovarian activation potential (Schäfer et al. 2006), it would suggest that our anarchistic workers have a higher reproductive potential than the "standard" *Am* 

queentight workers and pethaps even than laying workers of other anarchistic lines or other European subspecies, but ost likely not laying workers of African subspecies (Zheng et al. 2010; Yusuf et al. 2015). The extracts of IAWs



Figure 4. Co parison of the absolute a ount ( icrogra s,  $ean\pm SD$ ) between workers with inactivated ovary (*IA*) and workers with activated ovaries (*A*) sa pled in three anarchistic honey bee colonies.

had the typical worker-like profiles do inated by

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