Original article

Reproduction of rebel workers in honeybee (*Apis mellifera*) colonies

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Abstract – The honeybee is one of several eusocial species in which the queen is typically the only reproductive member of the colony; worker reproduction is mostly restricted to queenless colonies. Because workers cannot mate, they lay unfertilized eggs, which develop into males. A recent study showed that in queenless colonies, which arise after swarming, worker larvae develop into rebel workers that have greater reproductive potential than do workers reared in queenright colonies, as measured by the number of ovarioles and degree of ovary activation. However, there was no evidence that rebels had an opportunity to produce male offspring. Here, we show for the first time that rebel workers not only activate their ovaries but also produce significantly more male offspring in queenright colonies than do normal workers. Moreover, our results show that the level of rebel reproduction in queenright colonies is similar to the reproduction of normal workers in queenless colonies. This finding suggests that the ultimate factor favouring the evolution of the rebel strategy is the decrease in relatedness between the old-generation workers and the new queen's offspring that occurs after queen exchange at swarming.

Apis mellifera / rebel workers / worker reproduction / kin selection

1. INTRODUCTION

The honeybee, the biology of which is well understood, attracts widespread interest not only as a honey producer and the main pollinator of crops but also as a model organism for testing general biological problems, including the evolution of eusociality, focussing on the altruism of workers caring for siblings (Amdam et al. 2006; Kucharski et al. 2008; Ratnieks and Helanterä 2009; Graham et al. 2011). A recent study showed that in the honeybee, the 'rebel worker' strategy is directly predicted by the assumptions of kin selection theory (Woyciechowski and Kuszewska 2012; Kuszewska and Woyciechowski 2015). Rebel workers develop immediately after swarming, which is the only natural means of colony multi-plication. They have significantly more ovarioles in the ovary and larger mandibular and Dufour's glands than do typical sterile workers, along with underdeveloped hypopharyngeal glands (Woyciechowski and Kuszewska 2012; Kuszewska and Woyciechowski 2015), which synthesize and store brood food (Huang and Otis 1989). The proximate factor that influences rebel caste development is the absence of the queen during the final 4 or more days of the unsealed larva stage (Kuszewska and Woyciechowski 2013) or, more precisely, in the absence of the queen's mandibular gland pheromone (Woyciechowski et al. 2017). However, the decrease in relatedness between old-generation workers and the new queen's offspring appears to be the ultimate factor favouring the shift in the life strategy of workers (Woyciechowski and Kuszewska 2012).

The shift in resource reallocation to reproductive tissue in developing workers suggests that rebel workers, more than normal (non-rebel)

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workers, are physiologically prepared to lay male-destined eggs and thereby produce sons of their own (Woyciechowski and Kuszewska 2012). Moreover, 15-day-old rebel workers have more-active ovaries than those of non-rebel workers whether they live in a colony with or without a queen during their adult life (Woyciechowski and Kuszewska 2012). The appearance of workers with mature ovaries in orphaned colonies is not unexpected. Workers are known to lay eggs if a colony loses its queen and there is no opportunity to rear a new one (Velthuis 1970; Page and Robinson 1994). However, the readiness of rebels to reproduce in queenright colonies is unexpected because of the expectation that the presence of a queen effectively inhibits worker oogenesis (Velthuis 1970; Page and Robinson 1994; Ronai et al. 2015).

The aim of the present study was to determine whether rebel workers can produce their own male offspring. For this purpose, normal and rebel workers housed in queenright colonies throughout their adult life were compared in terms of differences in their anatomy and the number of sons (drones) they produced. Additionally, we investigated whether rebel workers that remained in queenright colonies had greater reproductive success than did normal workers orphaned during their adult life, a condition that generally favours worker reproduction (Velthuis 1970; Page and Robinson 1994). We expected that rebel workers would have more and better-developed ovarioles as well as more sons than would normal workers in both queenright and queenless conditions.

2. METHODS

2.1. General experimental procedures

The research was conducted from May to July 2012 in the experimental apiary of the Institute of Environmental Sciences (Jagiellonian University, Krakow, southern Poland). Eight queenright honeybee (*Apis mellifera carnica*) colonies were studied, each consisting of 20,000–40,000 workers. All colonies were treated the same way, and the procedures were conducted over 8 successive days, one colony per day. The experiment consisted of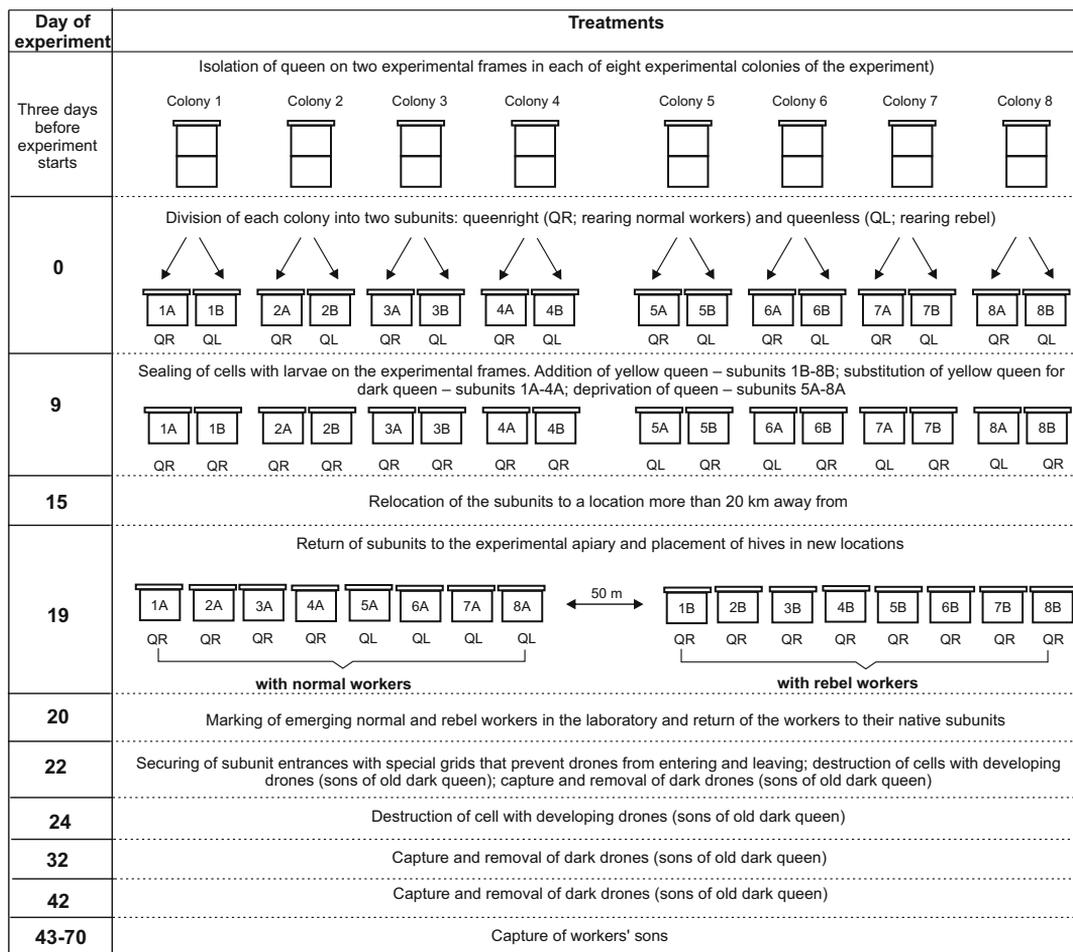 two stages. First, each experimental colony was divided into queenright and queenless subunits to raise normal and rebel workers of the same age. These rebel and normal workers were marked in each subunit. When the marked workers were 15 days old, 30 of them from each experimental group and colony were dissected to assess their anatomical parameters. Second, subunits with rebel and normal workers were searched to collect all of the workers' sons (drones). The two subspecies of *A. mellifera* (*A. m. carnica* and *A. m. ligustica*) were used to distinguish between workers' sons and queens' sons.

2.2. Raising of normal and rebel workers

Initially, the queen (*A. m. carnica*; dark queen) of each colony was restricted to two experimental frames to produce eggs of similar age. Three days later, the colony was divided into queenright and queenless subunits (a total of 16 subunits: 8 queenright, designated 1A through 8A, and 8 queenless, designated 1B through 8B; Figure 1), each with one experimental frame (day 0). When the worker cells on the experimental frames were sealed (day 9) in the eight queenless subunits (1B–8B), queens with a yellow cuticle (yellow queens; *A. m. ligustica*) were introduced (queenright colonies with rebel workers). In addition, in the 4 queenright subunits (1A–4A), the queens (*A. m. carnica*; dark queen) were exchanged for new ones (queenright colonies with normal workers), which were yellow queens (*A. m. ligustica*). The remaining four queenright subunits (5A–8A) were deprived of the queen (Figure 1; queenless colonies with normal workers). This manipulation was designed to permit the queens' sons (yellow queen—yellow drones) and workers' sons (dark workers—dark drones) to be distinguished later.

To avoid drifting of workers, which would confound the results, we ensured that the experimental subunits were separated by 50 m. Because workers are known to return to the original position of their colony when moved only a short distance (Hammer and Menzel 1995; Amdam et al. 2005), the experimental colonies were relocated after sunset (day 15 of the experiments) to a site more than 20 km from the experimental

Day of experiment	Treatments							
Three days before experiment starts	Isolation of queen on two experimental frames in each of eight experimental colonies of the experiment)							
	Colony 1	Colony 2	Colony 3	Colony 4	Colony 5	Colony 6	Colony 7	Colony 8
0	Division of each colony into two subunits: queenright (QR; rearing normal workers) and queenless (QL; rearing rebel)							
	1A 1B	2A 2B	3A 3B	4A 4B	5A 5B	6A 6B	7A 7B	8A 8B
	QR QL	QR QL	QR QL	QR QL	QR QL	QR QL	QR QL	QR QL
9	Sealing of cells with larvae on the experimental frames. Addition of yellow queen – subunits 1B-8B; substitution of yellow queen for dark queen – subunits 1A-4A; deprivation of queen – subunits 5A-8A							
	1A 1B	2A 2B	3A 3B	4A 4B	5A 5B	6A 6B	7A 7B	8A 8B
	QR QR	QR QR	QR QR	QR QR	QL QR	QL QR	QL QR	QL QR
15	Relocation of the subunits to a location more than 20 km away from							
19	Return of subunits to the experimental apiary and placement of hives in new locations							
	1A 2A 3A 4A 5A 6A 7A 8A				50 m ↔	1B 2B 3B 4B 5B 6B 7B 8B		
	QR QR QR QR QL QL QL QL					QR QR QR QR QR QR QR QR		
	with normal workers					**with rebel workers**		
20	Marking of emerging normal and rebel workers in the laboratory and return of the workers to their native subunits							
22	Securing of subunit entrances with special grids that prevent drones from entering and leaving; destruction of cells with developing drones (sons of old dark queen); capture and removal of dark drones (sons of old dark queen)							
24	Destruction of cell with developing drones (sons of old dark queen)							
32	Capture and removal of dark drones (sons of old dark queen)							
42	Capture and removal of dark drones (sons of old dark queen)							
43-70	Capture of workers' sons							

Figure 1. Timeline of the experiment showing the manipulations on particular days. Details are presented in Section 2.

apiary. After 3 days (day 19 of the experiment), before sunrise, the subunits were returned to the experimental apiary and placed in their new location. After 3 days in a different location, the bees had to reset their memory of the site of the subunits and memorize a new location.

On day 20 of the experiment, the experimental frames with newly emerged bees were moved from each colony to an incubator (36 °C). All of the workers that emerged within 24 h were marked on the thorax with a spot of paint (Marabu Brilliant Painter). The combs and all marked bees (600–1200, depending on the colony) were returned to their native subunits. Based on a previous study, it was expected that bees reared in the

queenright condition would develop into normal workers, whereas bees reared in the queenless condition would develop into rebel workers, which have significantly more ovarioles in the ovary (Woyciechowski and Kuszewska 2012). This prediction was tested and proved using 30 dissected 15-day-old workers from each group in each colony (day 34 of the experiment).

2.3. Estimating the number of workers' drones

The development of drones from eggs to adults takes 24 days (Winston 1987); therefore, the sons of the first marked workers were considered

available for sampling on day 43 of the experiment (the marked workers from both groups were 24 days old). Until then, the entrances of all subunits (1A–8A and 1B–8B) were secured with a special grid (day 22 of the experiment) that prevented drones' movement in and out without otherwise limiting the movement of workers. In addition, all drones (dark drones) that originated from dark queens (*A . m . carnica*) were removed, and all cells with developing drones that originated from dark queens were destroyed in all the 16 experimental subunits. The destruction of cells with developing drones was performed on days 22 and 24 of the experiment, whereas the capture and removal of the dark drones (sons of old dark queens) were performed on days 22, 32 and 42 of the experiment. On day 43 of the experiment, the experimental workers' drones were located and captured. The workers' drones (*A . m . carnica*—dark) were differentiated from queen's drones (*A . m . ligustica*—yellow) by colour. The capture of drones was repeated ten times (on days 43, 46, 49, 52, 55, 58, 61, 64, 67 and 70 of the experiment). The total numbers of workers' drones were estimated for all the subunits.

2.4. Examination of anatomical parameters

The criterion for a rebel worker is a higher number of ovarioles in the ovary than normal workers have. Other anatomical parameters also differ, such as the sizes of the mandibular, Dufour's and hypopharyngeal glands; however, the sizes of those structures change with the age and social context of the workers. The number of ovarioles, ovary development (ovary activation) and the sizes of the hypopharyngeal, mandibular and Dufour's glands were determined to confirm that rebel and non-rebel workers were used in this study (Woyciechowski and Kuszewska 2012; Kuszewska and Woyciechowski 2015). The total number of ovarioles in both ovaries of each worker was recorded, and the ovarian development of all dissected bees was assessed. To assess ovarian development, the most developed ovariole of each of the ovaries was selected, and the maximum diameter of these two ovarioles (maximum width) was measured as described by Nakaoka et al. (2008), according to whom ovariole diameter

accurately reflects ovarian activity. The hypopharyngeal gland consists of a great number of lobes, called acini, and their diameter is routinely used as an index of gland size (Nakaoka et al. 2008; Wegener et al. 2009; Kuszewska and Woyciechowski 2013). The size of the hypopharyngeal gland was calculated as the average of ten acini (each acinus was measured as the square root of the longest × shortest diameter, and the average was calculated from five acini from the right gland and five from the left gland). The size of the mandibular gland was calculated as the average of the left and right glands (each gland was measured as the square root of the longest × shortest diameter). The size of Dufour's gland was also calculated as the square root of the longest × shortest diameter. All organs were stained with Giemsa reagent for approximately 10 s before being measured.

2.5. Statistical analysis

Mixed-model two-way ANOVAs were used to compare the parameters (ovariole number, hypopharyngeal gland size, mandibular gland size and Dufour's gland size) between 15-day-old rebel and normal workers, with experimental group (reared in queenright or queenless conditions) as a fixed effect and colony as a random effect. Ovary development was analysed using the generalized linear/nonlinear models (GLZ) module in Statistica 9.0 (Institute 2004), specifying a Poisson distribution and a log link function, which is a semi-parametric statistical test (Härdle et al. 1996). Colony was a random effect and experimental group was a fixed effect. To compare the number of workers' sons between experimental groups, the nonparametric Kruskal-Wallis test was used. The analysis was performed separately for colonies 1–4 (subunits 1A–4A and 1B–4B with yellow queens) and 5–8 (subunits 5A–8A without queens and 1B–4B with yellow queens). All analyses were conducted with Statistica 9.0.

3. RESULTS

Fifteen-day-old workers reared as rebels had significantly more ovarioles than did workers of the same age reared normally (mixed-model two-

way ANOVA; colonies 1–4: $F_{1,3} = 11.77$, $P = 0.041$; colonies 5–8: $F_{1,3} = 23.65$, $P = 0.0165$; Figure 2a). These rebel workers, which remained throughout their adult life in queenright subunits (new yellow queen; subunits 1B–4B and 5B–8B), also had more activated ovaries compared with normal workers, regardless of whether these normal workers remained throughout their adult life in their subunits with the queen (new yellow queen; subunits 1A–4A) or remained in the orphaned subunits (subunits 5A–6A) (GLZ; colonies 1–4; Wald's $\chi^2 = 24.12$; $P < 0.001$; colonies 1–4; Wald's $\chi^2 = 26.01$; $P < 0.001$; Figure 2b).

The rebel workers also had a larger Dufour's gland than did normal workers (mixed-model two-way ANOVA; colonies 1–4: $F_{1,3} = 26.73$, $P = 0.011$; colonies 5–8: $F_{1,3} = 37.84$, $P = 0.008$; Figure 2c). However, the results showed no differences in the size of the mandibular gland between 15-day-old rebel and normal workers (mixed-model two-way ANOVA; colonies 1–4: $F_{1,3} = 1.28$, $P = 0.339$; colonies 5–8: $F_{1,3} = 2.17$, $P = 0.236$; Figure 2d), whereas the size of the hypopharyngeal gland depended on the presence of the queen during the adult life of the experimental workers.
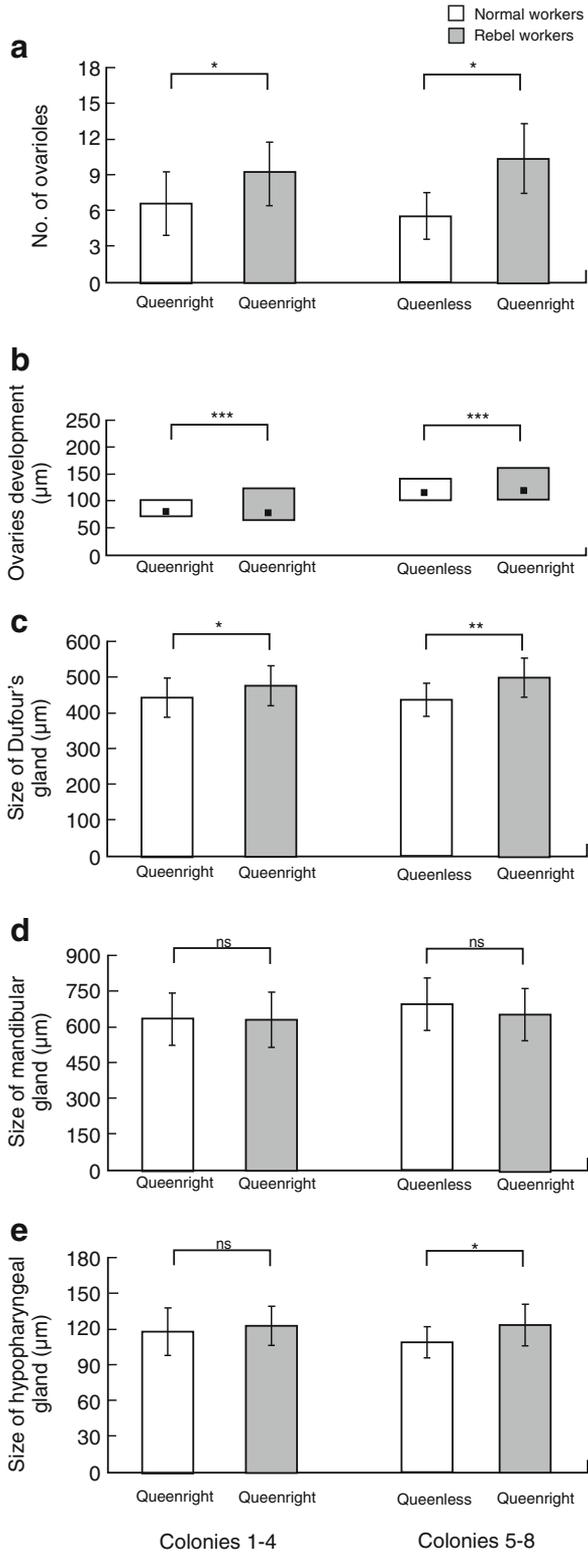
There was no difference in the size of the hypopharyngeal gland between rebel and normal workers when they both remained during their adult life in subunits with the new yellow queen (normal workers—subunits 1A–4A; rebel workers—subunits 1B–4B; mixed-model two-way ANOVA; colonies 1–4: $F_{1,3} = 0.49$, $P = 0.533$; Figure 2e). However, the rebel workers, which remained during their adult life in subunits with the new yellow queen (subunits 5B–8B), had larger hypopharyngeal glands than did the normal workers, which remained during their adult life in subunits without a queen (subunits 5A–8A; mixed-model two-way ANOVA; colonies 5–8: $F_{1,3} = 29.47$, $P = 0.012$; Figure 2e).

Yellow drones (sons of queens) and dark drones (sons of workers) were counted in each tested colony, and the results are shown in Table I. However, to answer to main question in this paper, only the number of black drones (workers' sons) was compared between subunits. The results showed that rebel workers had more sons (drones)

than normal workers when both groups of adult experimental workers remained in subunits with the new yellow queen (subunits 1A–4A and 1B–4B; Kruskal-Wallis: H1 > 5.60, $P < 0.018$; Figure 3; Table I). However, there was no difference in reproductive success between rebel and normal workers when the rebel workers remained in subunits with the substituted yellow queen (subunits 5B–8B) and normal workers remained in orphaned subunits (subunits 5A–8A; Kruskal-Wallis: H1 < 0.34, $P > 0.05$; Figure 3).

4. DISCUSSION

Our results show for the first time that rebel workers, which have high reproductive potential in terms of the number of ovarioles in their ovaries and their more advanced state of ovarian development (Figure 2a, b), have more sons than do normal workers in queenright colonies (Figure 3). Worker reproduction in honeybee colonies is rare and usually limited to queenless colonies. It is well known that workers lay eggs if a colony loses its queen and there is no opportunity to rear a new queen (Velthuis 1970; Page and Robinson 1994). Some studies also suggest that the number of workers with active ovaries is not stable across the season and that this number increases during the period in which most colonies swarm (Kropacova and Haslbachova 1970; Velthuis 1970; Hoover et al. 2006; Holmes et al. 2013). However, the reproduction of workers in queenright colonies is an unexpected phenomenon because the presence of the queen and her pheromones in the colony inactivates the ovaries of almost all of the workers. The curbing of worker reproduction is achieved by two mechanisms: pheromonal suppression by the queen (Hoover et al. 2003) and worker policing (Woyciechowski and Lomnicki 1987; Ratnieks and Visscher 1989), the latter of which includes all of the behaviours of workers or the queen that reduce the reproductive output of other workers, e.g. harassment of reproductive workers (Visscher and Dukas 1995; Monnin et al. 2002) and the selective removal of worker-laid eggs (Ratnieks and Visscher 1989). Until now, worker reproduction in the presence of the queen has been described only in *A. m. capensis* (Cape honeybee) living parasitically in *A. m. scutellata* colonies

a No. of ovarioles — Normal workers / Rebel workers; Queenright, Queenright, Queenless, Queenright (Colonies 1-4, Colonies 5-8) — *, *

b Ovaries development (µm) — Queenright, Queenright, Queenless, Queenright — ***, ***

c Size of Dufour's gland (µm) — Queenright, Queenright, Queenless, Queenright — *, **

d Size of mandibular gland (µm) — Queenright, Queenright, Queenless, Queenright — ns, ns

e Size of hypopharyngeal gland (µm) — Queenright, Queenright, Queenless, Queenright — ns, *

Colonies 1-4   Colonies 5-8

Figure 2. Anatomical parameters of 15-day-old normal and rebel workers. **a** Number of ovarioles (mean ± SD). **b** Ovarian development (median and quartiles). **c** Size of Dufour's gland (mean ± SD). **d** Size of mandibular gland (mean ± SD). **e** Size of hypopharyngeal gland (mean ± SD). Three stars indicate $P < 0.001$. Two stars indicate $0.001 < P < 0.01$. One star indicates $0.001 < P < 0.05$. ns indicates no significant difference ($P > 0.05$).

Figure 3. Number of workers' sons (median and quartiles) produced by subunits with rebel and normal workers. Stars indicate significant differences ($P < 0.05$). ns indicates no significant difference ($P > 0.05$).

(Beekman and Oldroyd 2008; Beekman et al. 2009) and in anarchistic bees, which represent a rare mutant phenotype (Barron and Oldroyd 2001). Workers of *A . m . capensis* can lay unfertilized eggs that develop into females (via thelytoky) and can be reared as either workers or queens (Ruttner 1977), and this type of parthenogenesis is determined by a single recessive gene (Lattorff et al. 2005). Similarly, the anarchistic syndrome is also based on genetic components (Barron et al. 2001; Beekman and Oldroyd 2008), specifically, two independent mutations, and under natural conditions, anarchistic colonies are rare and are eliminated by natural selection (Beekman and Oldroyd 2008). However, these two examples are

biologically different and distinct phenomena from the case of rebel workers. It is possible that our results reflect the ability of workers to recognize the unrelated substituted queen; however, both of our experimental groups (normal and rebel workers) were in nests with the introduced, yellow unrelated queens. Additionally, most studies have shown that in eusocial colonies, kin recognition involves primarily the recognition of nestmates (membership)

Table I. Numbers of queens' sons (yellow drones) and workers' sons (black drones) reared in all experimental colonies

Colony no.	Subunit type	Queen presence	Queens' sons	Workers' sons
1	1A (normal workers)	Queenright	0	0
	1B (rebel workers)	Queenright	0	6
2	2A (normal workers)	Queenright	131	0
	2B (rebel workers)	Queenright	0	4
3	3A (normal workers)	Queenright	99	0
	3B (rebel workers)	Queenright	246	29
4	4A (normal workers)	Queenright	84	0
	4B (rebel workers)	Queenright	128	3
5	5A (normal workers)	Queenless	0	12
	5B (rebel workers)	Queenright	84	0
6	6A (normal workers)	Queenless	0	189
	6B (rebel workers)	Queenright	467	1
7	7A (normal workers)	Queenless	0	0
	7B (rebel workers)	Queenright	0	2
8	8A (normal workers)	Queenless	0	1
	8B (rebel workers)	Queenright	0	4

rather than kin per se (Nonacs 2011; Boomsma and Ettorre 2013). This is a simple classification, in which individuals are classified as either belonging or not belonging to the membership group and, in the former case, are treated as related individuals (Tarpy et al. 2004; Breed 2014).

We also found no significant difference between rebel and normal workers in the number of workers' sons when the rebels remained in subunits with the new yellow queen and normal workers remained in queenless subunits. The reproduction of normal workers in queenless colonies is not surprising; it is well known that up to 30% of workers can activate their ovaries and lay unfertilized, male-destined eggs (Ratnieks 1993). The activation of worker ovarioles in an orphaned nest is determined by declines in the levels of queen and brood pheromones (Hoover et al. 2003; Maisonnasse et al. 2009). The similar number of workers' sons observed between queenright subunits with rebel workers and queenless subunits with normal workers suggests that rebel workers have a higher reproductive capacity than do normal workers. Moreover, when we compared the activity of ovarioles between these two groups of 15-day-old workers, the rebels had more developed ovaries than did normal workers (Figure 2b), which may imply that in queenless colonies, the egg-laying workers come from a different age group.

The rebel workers' strategy is associated with specific changes in their anatomy. To distinguish between the rebel and normal workers, we examined the number of ovarioles in the ovaries and their degree of activation as well as the sizes of the Dufour's, mandibular and hypopharyngeal glands in 15-day-old workers reared in queenless and queenright colonies. In accordance with previous studies, our results showed that workers reared under queenless conditions had more activated ovaries containing more ovarioles (Figure 2a, b) and a bigger Dufour's gland relative to workers in queenright colonies (Figure 2c). However, in contrast to previous studies (Engels et al. 1997; Graham et al. 2011), these two groups of workers did not differ in the size of the mandibular gland (Figure 2d), whereas the size of the hypopharyngeal gland depended on the tested subunits (Figure 2e). It is well known that the sizes of both the mandibular and hypopharyngeal glands can depend on the social context of workers (Huang and Robinson 1996; Engels et al. 1997; Bortolotti and Costa 2014), which in turn depends on the colony's population structure (Robinson 1992; Huang and Robinson 1996). In contrast to previous studies (Woyciechowski and Kuszewska 2012), our two groups of experimental workers remained in separate subunits, with some queenless and some broodless; therefore, we believe that this difference in social context could have affected the sizes of the mandibular and hypopharyngeal glands.

In summary, we have shown that rebel workers, which usually develop after swarming, not only have a higher reproductive potential in the first day of their adult life than normal workers but also produce male offspring under queenright conditions. Thus, the rebel strategy is successful in both queenless and queenright conditions, i.e. after a new queen dies during the mating flight or when a new queen reigns over the colony and begins to reproduce, the latter of which causes a dramatic drop in relatedness between the old-generation workers and the new queen's offspring. Because the latter scenario is realized more often, it appears to be the ultimate factor favouring the evolution of the rebel strategy.

Contributions   KK and MW conceived and designed the experiments. KK and AW performed the experiments. KK analysed the data. KK and MW contributed reagents/materials/analysis tools. KK, MW and AW wrote the paper. All the authors read and approved the final manuscript.

Reproduction des ouvrières 'rebelles' dans des colonies de l'abeille *Apis mellifera*

Reproduction des ouvrières / rebelle / sélection de parentèle / colonies orphelines

Reproduktion von "rebellischen" Arbeiterinnen in Honigbienenvölkern (*Apis mellifera*)

***Apis mellifera* / rebellische Arbeiterinnen / Arbeiterinnenreproduktion / Verwandtenselektion**

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