

Original Article

BALLING BEHAVIOR OF WORKERS TOWARD HONEY BEE QUEENS RETURNING FROM MATING FLIGHTS

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Received: 19 April 2018; accepted: 30 October 2018

Abstract

During natural mating honeybee queens can get lost due to drifting, predators or other cases. In this work, the balling of queens returning from flights by worker bees originating from the same colony was observed. Three subspecies of bees Carniolan, Caucasian and European Black Bee were tested. Research was conducted in both spring and summer, but in the former in newly created colonies, while in the latter in new and earlier used ones. Generally 15.2% of queens were balled and in total 30.2% of queens were lost during mating flights. 269 queens performed 785 mating flights, and 5.2% of those finished with balling. Three times more queens were balled when returning from mating flight rather than orientation flight. Subspecies matches or mismatches of queens and workers in nucleuses did not significantly affect the balling or its frequency. Additionally, no bee subspecies characterized stronger tendencies to ball a queen. Worker bees from newly created nucleuses treated queens similarly to the ones in nucleuses earlier used. However, significantly more queens had been balled during the spring in comparison to summer. There were days with higher balling of queens. During some days the weather was very unstable and unpredictable with such anomalies as heat waves, thunderstorms or sudden drops in insolation. Most of the queens were balled at the entrance while returning from flight and only a few inside the hive. In the research, clear causes of balling were not found, but some factors can be excluded.

Keywords: balling of queens, mating flights, queen losses

INTRODUCTION

Balling of queens is an aggressive behavior of worker bees related to queens. Usually of workers crowd around a queen to cover completely and sting her. Sometimes, worker bees only tear her body with mandibles, then the queen looks black, shiny and hairless. Even if the queen survives, her quality is most likely to decrease in the further period of life (Robinson, 1984; Gerula & Bieńkowska, 2008). In popular science publications, it worker's balling behavior is described to generally happen when new queens are introduced by a beekeeper into a colony. Apart from that, in beekeeping balling most often occurs in times of stress for example during either poor weather in spring is or robbing. Moreover, old or infertile queens are superseded shortly after the emergence of

their successor. Incompatibility between queens and workers subspecies in a colony is also listed as possible cause of balling (Ostrowska, 1980; Gerula et al., 2007).

The balling of young queens by bees from their own colonies after a mating flight has not been thoroughly studied so far, even though Alley mentioned it a long time ago in 1883. It is difficult to indicate the exact causes of queens' losses, but balling is one of them. Also losses of the queens in commercial apiaries widely ranges between 3 to over 90% (Skowronek, 1976; Al-Ghzawi & Zaitoun, 2008; Woyke, Fliszkiewicz, & Jasiński, 2001; Schlüns et al., 2005; Heidinger et al., 2014; Czekońska, 2000; Al-Ghzawi & Zaitoun, 2008; Gąbka, 2018).

In most conditions, queens are recognized by worker bees for their pheromones produced mainly in the mandibular gland. Pettis, Westcott,

& Winston (1998) and Yadava & Smith (1971) have indicated that the odor may be responsible for balling and applied the substance on a worker bee that caused her to be balled by other bees. Young queens that produce little of the substance and introduced directly to colony are rarely balled, but those that are ten or more days old are balled every time. In turn, Lensky et al. (1991) stated that workers treated with ethanol extract from Koschevnikov glands of queens were balled by other workers. According to Szabo (1974) and Yadava & Smith (1971), older and egg-laying queens are more attractive to worker bees than younger ones, which is reflected in a higher number of bees interested in the queen. Bees are supposedly less attracted to virgin and not to egg-laying queens which could explain the balling after mating flights. Queens' behavior was also noticed to may have induced aggression in workers. According to Woyke's personal information (2018), some bee queens returning from a mating flight sat at once on the landing board at the nucleus entrance. However, others were hovering in the air up and down, left and right like a hornet or robber bees. When the hovering queens landed at the entrance, they were balled by the workers. On the other hand, queens' losses may be higher if beehives are close together as a result of drifting (Gąbka, 2018).

The aim of the study was to investigate the phenomenon of balling queens after a mating flight by workers from the mating colonies, which had been created for the time of mating, and evaluate its significance in queen production.

MATERIAL AND METHODS

Experimental set-up

The study was performed in the years 2013-2015 at the Institute of Horticulture, Apiculture Division in Puławy, Poland. The apiary is located in a suburban area (51°24'59"N, 21°58'09" E, altitude 155 m a.s.l.). Each year two series of observation were performed in spring in the first ten-day period of June and in summer in the first ten-day period of July. Three subspecies of bees were used:

Apis mellifera carnica Pollm. (Carniolan), *Apis mellifera caucassica* Gorb. (Caucasian) and *Apis mellifera mellifera* L. (European Dark Bee). All of the queens were marked after emergence with unique numbers on plastic discs (Fig. 1, 3). They were introduced into mating colonies with bees of the same subspecies and with the others who were not directly related.

Every year of the study only two subspecies were tested in every possible combination. Carniolan and Caucasian bees were tested in 2013, Caucasian and European Dark bees in 2014 and Carniolan and European Dark bees in 2015. Virgin queens on emergence day were individually introduced into a three-skewer trapezoid Styrofoam mating nuclei with a capacity of about 1.5 dm³ and colonized with about 1000 bees taken from the brood nests during sunny days when most foragers were outside. Nucleuses were placed in two rows at the apiary in a forest clearing, and the entrances were arranged in opposite directions. The distance between entrances was about 120 cm and between the rows 5 m. By the summer, half of the nucleuses were used a second time for the study. Workers in these colonies were one month older and the sealed brood was present. The mating nucleuses were equipped with a transparent covered runway and a queen excluder at the entrance (Fig. 1). This protection made easier both overseeing mating flights and determining the time of a queen's mating flight and return and possible mating sign after her copulation. Queens were freed for the flights when an entrance protection was raised at the nucleus entrance, and let back in with the same procedure. Mating flights were observed starting from the fifth day of queens' lives and continued to the start of oviposition of all the queens. Daytime observations were carried out from 1 pm to 5:30 pm. Afterwards, all nucleuses were checked for queen presence. Some queens did not perform any flights during our experiment, and observation of these ended when the rest of the flying ones started to lay eggs (they were at least nineteen days old). After that, the queen excluders were removed and brood presence was checked ten days later.



Fig. 1. Nucleus with runway and queen excluder at the entrance which allows the observation of workers and queens' behavior

Testing hypotheses

Many factors influence queens and workers' behavior and the balling during the season, and this study design allowed us to test several hypotheses about their possible impact. One of the hypotheses was the subspecies' influence considered in three variants: subspecies incompatibility of queens and workers in

nucleuses, vulnerability of queens of particular subspecies towards balling and the tendency of workers from different subspecies to ball. During the three years of the study, queens were introduced to mating colonies with bees of the same subspecies and line (n=144) or another (n=125) (Tab. 1). Repeating observation during spring and summer allowed us to check the hypothesis about the probable impact of seasons when physiology and bee behavior of bees differ. The hypothesis about the impact of the uncontrolled factors mating behavior and flight performance, duration and number of flights, type of flights and weather and foraging conditions was also worked out.

Statistical analysis and environmental data

Depending on the tested hypothesis, the experimental object was a queen or nuclei. In every case, the number of balled queens was analyzed

Table 1. Annual experiment design for testing effect of subspecies compatibility and physiological state of nucleuses on balling behavior of workers

Time of season	Subspecies of workers in nucleuses	Subspecies of introduced queens	Racial compatibility	Physiological state of nucleuses	N
Spring 1 st decade of June	A	B	No	new	12
	B	A	No		12
		A	Yes		12
		B	Yes		12
Summer 1 st decade of July	A	B	No	new	6
	A	B	No	early used	6
	B	A	No	new	6
	B	A	No	early used	6
		A	Yes	new	6
		A	Yes	early used	6
		B	Yes	new	6
		B	Yes	early used	6

A, B- does not indicate a specific subspecies

in relation to non-balled ones with the use of the Pearson χ^2 test. Differences in the frequency of mating success as well as the number of queens falling out of the experiment in subsequent cycles were analyzed using a contingency tables based on χ^2 test. For analyzing the length of flights, we used the non-parametric median Kruskal-Wallis test and Mann-Whitney tests at $\alpha = 0.05$. Meteorological data were taken from the Davis Vantage Pro 2 local meteorological station. The CAPAZ GSM 200 Beehive Scales were used to calculate nectar flow during the experiment. We performed all analyses using the Statistica package v.13.1.

RESULTS

Structure of losses of experimental queens

During the three years of research, 269 queens were observed (accepted in nucleuses), and 188 started laying eggs, 69.8% of the starting number. The reasons for queen losses were balling, not returning from mating flights, drifting and not flying until the end of the observation (therefore not mated) (Fig. 2). Bees balled forty-one queens, 15.2% of the total. The majority of queens were attacked at the entrance before entering the nuclei (Fig. 3). Some were caged in Zander cages in order to be checked if they survived attacks

from bees. Some percent of queens survived balling, hence the difference in number of observed incidents to number of killed queens (Fig. 2). Some of the queens were attacked just in nucleuses. Taking into account all the incidents which caused death of queen, the total losses reached 30.2%. Therefore, the loss of balled queens would be similar to losses caused by other factors together. Significant amount of queens (16.3%) was drifting. Forty-four queens tried to enter foreign nucleuses but finally found their nucleus and successfully ended their flights, and thirty of them started to lay eggs. A relatively high number of queens, twenty-two and 8.2% of total, did not perform any flights; some fell to the ground and others did not even try to fly. After removal of the entrance protection, only four of the twenty-two queens started to lay eggs. The others not flying were not in the hive or not laying eggs.

Testing hypotheses of subspecies compatibility and subspecies preferences to balling queens

Comparing subspecies compatibility and incompatibility (according to study design presented in Tab. 1), the number of queens that were balled in such configurations was similar- twenty-two queens of the same origin as bees, 15.3%, and

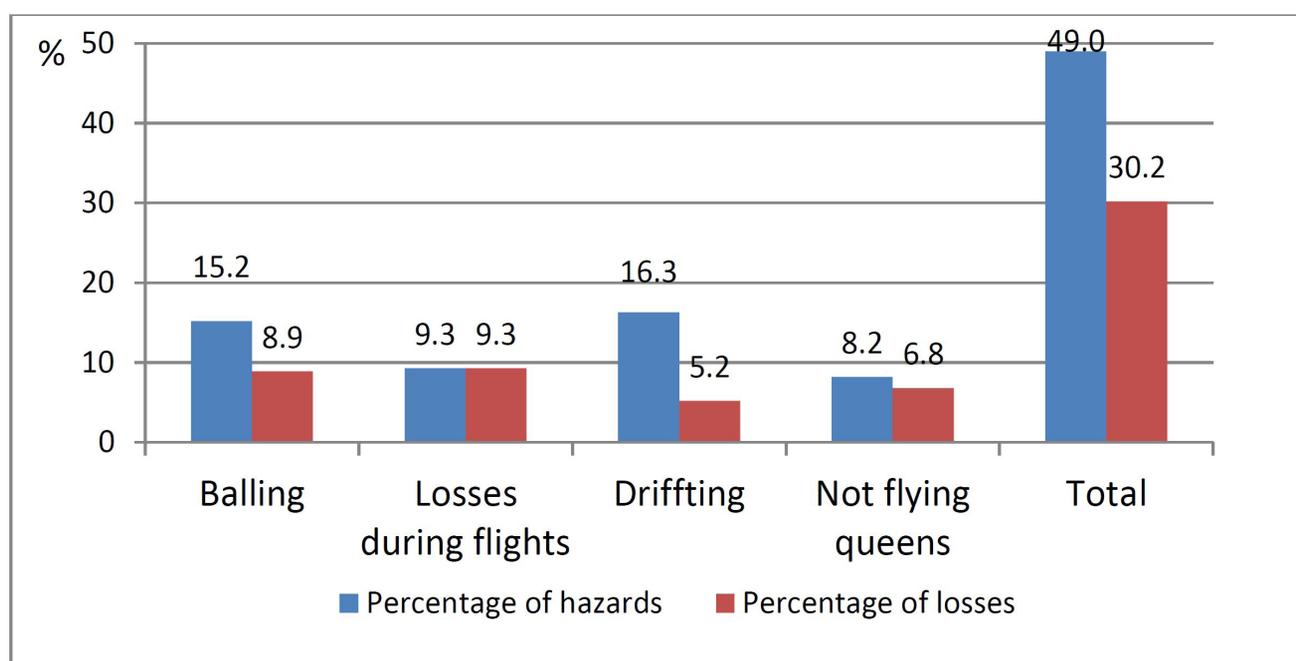


Fig. 2. Structure of risky incidents and behaviors when the queens were lost including queens unable to fly.



Fig. 3. Balling cluster. Balling of queen returning from nuptial flight by workers from her own nucleus at the entrance. Balled queen is crowded by the bees, only the plastic disc number 93 lets the queen to be recognized.

nineteen queens of different subspecies, 15.2% ($\chi^2 = 0.0003$, $df=1$, $p=0.98$). Queens of different subspecies were not balled more frequently ($\chi^2=4.5$, $df=2$, $p=0.1$): Carniolan 9.3%, Caucasian 15.2 and European Black Bee and 21%. Thus no significant difference was found in the tendency to balling: Carniolan 15.4%, Caucasian 17.5 and European Black Bee 14%.

Testing hypotheses of seasonal conditions and status of nucleuses to balling behavior of workers

The percentage of balled queens during spring observations ($N=129$) was nearly two times higher than that in summer ($N=140$), 20.1% and 10.7% respectively ($\chi^2=4.63$, $df=1$, $p=0.03$). However, for balling in newly created nucleuses during spring and summer seasons, the difference was even higher, 20.1% and 7.3% respectively ($\chi^2=5.5$, $df=1$, $p=0.02$). There were no significant differences in the balling rate according to the biological status of nucleuses. In newly created nucleuses ($N=166$) bees balled 18.6% of queens, whereas 16.1% in those which were used again ($N= 62$) ($\chi^2=0.13$, $df=1$, $p=0.71$).

Testing hypothesis of effect of type of flight performed by queen and flight duration to balling

269 queens flew out of nucleuses 785 times,

and twenty-five of them did not return. The average number of nuptial flights per one queen was 2.9 and with range of 1 to 10 per queen. The average number of mating flights was 1.42. Some started to lay eggs after one nuptial flight, whereas the others never started laying eggs despite the ten flights performed. Ten queens (3.7%) started to lay eggs despite no noticeable no mating signs. The number of orientation flights was higher ($N=498$) than that of mating flights ($N=262$). The frequency of balled queens returning from mating flights was 9.1% and significantly higher compared to orientation at 3.4% ($\chi^2=11.1$, $df=1$, $p<0.01$) (Tab. 2).

Queens were balled more often after their first or second orientation flight but when balled after mating, it was usually their third or fourth flights. Successful mating flight, that queen was not balled, lasted 21.7 minutes but ended with balling lasted 22.0 minutes. Successful orientation flights lasted significantly shorter (14.7') than ones ending with balling (22.5') and both mating flights - successful and unsuccessful (Kruskal-Wallis: $H_{(3,720)} = 107,5452$, $p<0.01$). The average age of queens and time when the flight started during the day was similar for all type of flights. (Tab.2). Regardless of the type of flight, the average length of a flight that ended with balling was significantly longer than the others, 22.3 and 17.0 minutes respectively (Mann-Whitney-test; $U=8688$, $p\leq 0.01$).

Out the 760 registered returns of queens from their flights, fifty-three (6.7%) drifted and seven were balled (13.2%). The number of queens balled after non-drifted flights ($N=707$) was significantly lower (4.8%) (Yates corr. $\chi^2=4.36$, $df=1$, $p=0.03$). The average length of flights of drifting queens ($N=53$) was similar for those balled or not balled after a mating flight, 32.0 and 34.3 min respectively, and for those balled or not after an observation flight, 34.0 and 30.9 min respectively (Kruskal-Wallis: $H_{(3,51)} = 1.79$, $p=0.078$).

Table 2.

Balling of queens depending on type of nuptial flight

Type of flight	No	No of flights ended with balling	Percent of flights ended with balling	Flight data			
				Flight category	Flight day - age of queens (Average)	Start time during Day (Mode)	Time duration (Minutes)*
Mating	262	24	9.1 b	Successful	9.8	14:45	21.7 a
				Ended with balling	9.9	15:09	22.0 a
Orientation	498	17	3.4 a	Successful	9.0	14:40	14.7 b
				Ended with balling	8.9	15:15	22.5 a

*different letters indicate significant differences at $\alpha=0.05$

External conditions during experiment-detailed description of subsequent experimental series

The start of observations and its finish were strictly dependent on the weather conditions during the day and trial. The queens were flying between days 6 and 19 of age but most often between days 7 and 11 (Fig. 4) when 78% of all flights were performed and 80% of balling cases were noticed. Depending on the series, the period from the first queen's mating flight to the start of laying eggs by all flying queens differed and was closely related to weather conditions,

as no queens flew on rainy or bad weather days (Tab. 3). The shortest period was eight and the longest was twelve days. The number of flights varied highly on individual days with a maximum of sixty-one a day, including twenty-five mating, but the other day, only one observation flight was noted. The above data show that the queens were not balled during most flying days, but on some days 2.1% to 50% of flights ended with balling. The balling frequencies was correlated with the flights' frequencies on a particular day (Sperman $R=0.4$, $p \leq 0.01$). For example, one day the total number of queens'

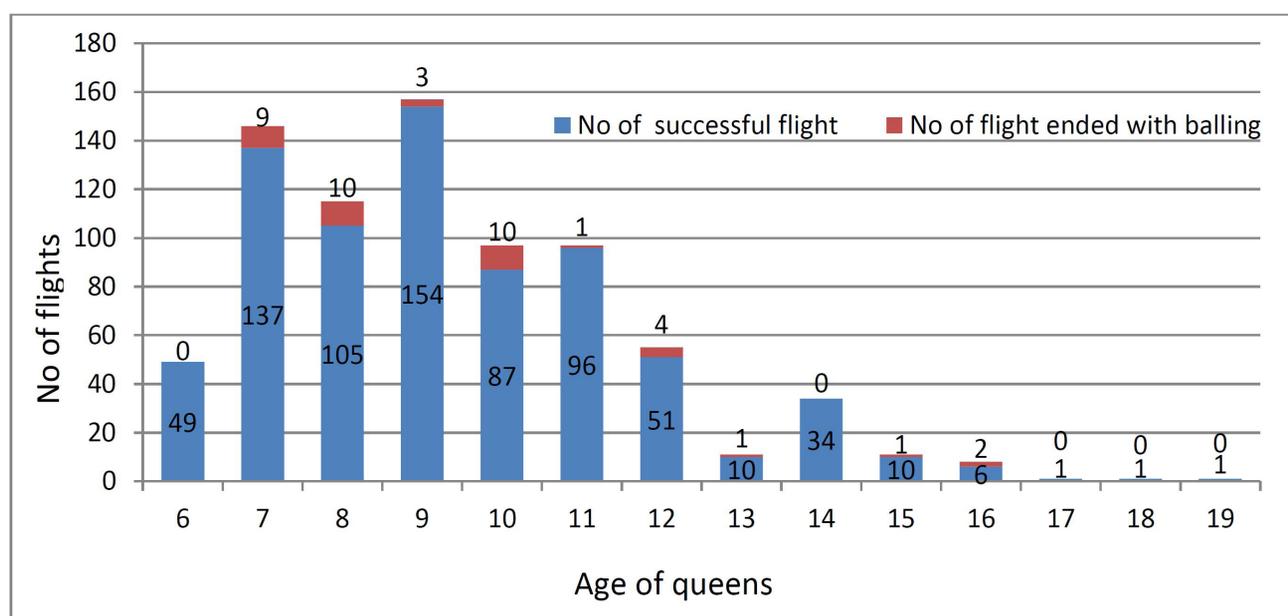


Fig. 4. Number of flights made by queens and structure of successful and unsuccessful flights in following days of their lives of all experimental series.

Table 3.

Detailed data concerning number of observed queens. Balling incidences and number of flights in each experimental series

Experimental series	No of queens	No of flying days	No of balling days	Queen's age during flight	No of balled queens	No of nuptial flight	No of lost queens	No of mating flight	No of nuptial flight per queen
2013 Spring	44	9	5	6-16	6	152	12	52	3.45
2013 Summer	47	8	1	6-15	1	166	6	49	3.53
2014 Spring	43	8	4	7-15	8	107	10	39	2.48
2014 Summer	50	9	4	7-17	7	113	20	43	2.26
2015 Spring	42	8	3	6-12	12	119	16	38	2.83
2015 Summer	43	7	3	8-19	7	128	20	34	2.97
Total	269	49	20	6-19	41	785	84	262	2.90

flights reached 48, including 15 mating and none of the queen was balled. On the other hand, we observed two days that queens performed only two flights, including one mating, after which they were balled.

Queens were flying during 49 days in total (Tab.3) for the three-year study. Forty-one queens were balled during twenty days, which indicates days with intensified balling. The balling of queens was observed either only one day or for a maximum of 5 days in each series, and they were not always the consecutive days. The increased frequency of hostile behavior was observed in 2015, when bees injured twelve queens in only three days, and during one of these days bees damaged six queens in two hours.

A comparison of the weather conditions during these three days, indicates that the high air temperature might have increased balling behavior. The temperature during the flights hours exceeded 30°C and the THSW index (Temperature, Humidity, Solar, and Wind) reached 40°C. Such a high THSW index was observed during thirteen of the twenty days when queens were balled. The analysis of individual meteorological parameter does not show explicitly that behaviour was connected with high temperatures because balling was also observed at

19.7°C and THSW index of 23.8°C. In the spring series of 2013, five out of six cases of balling occurred when solar radiation decreased considerably by more than 300 W/m², but this repeated only in two cases in the other years of research. Other cases of balling happened on days with slightly decreased or increased insolation.

In 2013 poorer nectar flow was observed, and the percentage of balled queens was lower than in subsequent years when the flow had been better (Fig. 5). A summing up the three years of results shows no correlation between balling and nectar flow. In individual years and series, total losses of queens ranged from 12.7 to 45.9% and differed significantly ($\chi^2=29.76$, $df=5$, $p\leq 0.01$). There were no significant differences in the number of queens balled in each series ($\chi^2=7.87$, $df=5$, $p\leq 0.16$) which ranged from 2.3 to 27.2% (Tab. 3, Fig. 5).

Unusual behavior in both workers and queens was observed during the study. Three queens were balled when a storm was approaching, but without raining yet and lower insolation and air temperature and approaching atmospheric discharges. Two other queens after the initial aggression of workers flew out but afterwards did not return to any nucleus. The bees in another nucleus killed their own accepted queen and accepted a foreign one returning from flight and

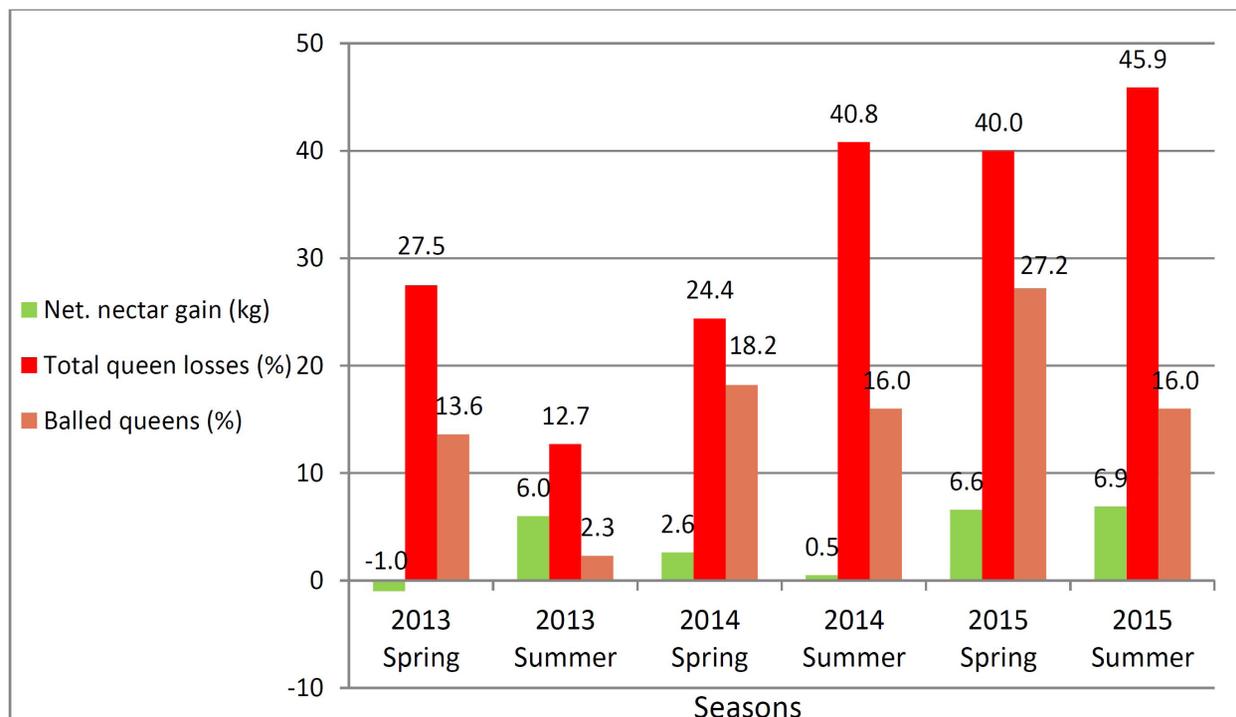


Fig. 5. Net nectar flow (kg) and total losses and balled queens (%) in subsequent experimental series.

tried to enter into their nucleus. The strangest was when bees absconded their nucleus but without their queen. After about thirty minutes they returned with another mated queen (with a mating sign), that had attracted them in the air. After separating this queen from the bees and moving her into the proper nucleus, she was balled there. And at the same time, the first bees upon entering their nucleus balled the queen that had been absconded earlier.

DISCUSSION

Total queens losses during natural mating reached 30.2% (Fig. 2), and about a third of these was caused by balling. Some queens were saved but only because they were caged when attacked by bees, but if they had been left with bees and not caged, the losses would probably have been higher. Such losses can be estimated as medium; Skowronek (1976) reported a similar result of 30.5% in observations performed in the same place but forty years earlier. Many researchers have observed smaller losses: Al-Ghzawi & Zaitoun (2008) 3-4%, Woyke, Fliszkiewicz, & Jasiński (2001) 21.7%, Schlüns et al. (2005) 14%, Heidinger et al. (2014) 17.9%, Gąbka

(2018) 18%, while others higher: Czekońska (2000) 50%, Al-Ghzawi & Zaitoun (2008) 80-99%. However, the latter research was done in very high heat in a different climate, but both the amount of losses and especially their cause are difficult to compare due to lacking literature data. Many queens in our study were unable to fly by the 19th day of their life (8.2%); some fell to the ground when trying to fly and others did not attempt to fly at all. Skowronek (1976) had similar results (10.2%). The hypothesis that bees of different subspecies ball queens more frequently was not confirmed, nor was it confirmed that queens of any origin were balled more frequently or workers of individual breeds had a higher tendency to balling. More queens were balled in the spring than in the summer, but it was not connected with the amount of nectar income, regardless the research year. Furthermore, the hypothesis that older worker bees ball queens more frequently was not confirmed. Queens which copulated with drones were significantly more balled often than those returning from orientation flights, which suggests such factors as a mating sign, the smell of ejaculate or the length of time when queens stayed outside the hive prompted workers to be aggressive.

Queens which prolonged their orientation flights were noted to be also balled. These cases suggest a relationship between prolonged flights and the drifting of queens during their return to the nucleus (Tab. 2). Regardless of the type of flight, queens first drifted and then were balled in 13.2% of flights, and it concerned only to seven flights by queens (one queen one flight) which is 17% of all balled individuals. The average flight by queens which drifted and were balled lasted thirty-four minutes, while in queens non-drifting but balled the flight duration was eighteen minutes. Therefore, drifting or staying outside longer the hive is often followed by balling but do not seem to be the most important factors. The length of time for flights did not differ from that of by Woyke (1964), Skowronek (1976), Schlüns et al. (2005) and Koeniger & Koeniger (2007). However, the average number of flights performed was lower than that in the studies by Heidinger et al. (2014), Skowronek (1976) and Verbeek (1976). This may be the result of controlled conditions when queens were forced to cross the entrance protection while returning to the nucleus. The average number of flights with copulation was 1.42 per single queen. Skowronek (1974) had a similar result reaching 1.62. In our research the mating sign was not noted in 3.7% of queens that began oviposition, which is a much lower value than 6-8% in studies by Skowronek (1976) and Woyke (1960).

Balling was not evenly distributed throughout the entire observation period. It was not noticed for 61% of the observation days, and during the days of intensive flights, balling did not intensify. More intensive attacks on queens with might have been caused by weather conditions on those days. Despite laborious research, no clear reasons could be indicated for the balling of queens that returned from mating flights by workers from the same colony, and only some factors were excluded. At the same time, some facts suggest that the workers' aggression were initiated by queen pheromones coming from one or more secretory glands. In the case of mandibular glands pheromones, it is not known whether their high concentration protects

queens against workers' attacks or prompts workers to attack. It seems more likely that workers attack queens when alarm pheromones releases. Workers' pheromones secreted from Koshevnikov glands signalled the balling (Lensky et al., 1991). However, queens during mating flights do not contact workers but only drones as they do not have Koshevnikov glands. At the same time, the role of Koshevnikov gland secretion of queens and workers varies (Free, 1987). Stress pheromones in a queen may be activated by either such specific weather conditions as heat or an approaching thunder storm or the prolonged staying of queen in a foreign and potentially hostile environment outside the beehive.

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