

## Research article

# Effect of “spraying” by fighting honey bee queens (*Apis mellifera* L.) on the temporal structure of fights

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**Summary.** Young queens start a new colony either without (independently) or with the help of workers (dependently). When colony reproduction is dependent and young queens are produced in excess, conflicts among queens are predicted to occur. Honey bee colonies reproduce dependently by swarming. The mother queen leaves with a “prime swarm” before daughter queens reach adulthood. Several young queens are produced, and often fight to death after emergence. Surviving queen(s) inherit the established nest or a portion of workers which then depart in an “afterswarm”. Honey bee queens show traits considered to be adaptations for fighting and conflict with other queens, such as early venom production and fast development. During fights one of the queens often releases rectal fluid. The function of this “spraying” behaviour is unclear. Possible functions of spraying are to affect worker intervention in fights, to act as a chemical weapon, or to interrupt fights. We staged fights between 24 queen pairs to investigate the temporal pattern of behaviour in spraying and non-spraying fights. Spraying occurred in 67% of the fights, usually upon physical contact, and it resulted in at least temporary separation of the queens in 81% of the spraying fights. Spraying fights were characterized by a significantly lower proportion of time spent in escalated aggression than non-spraying fights and a significantly shorter first escalated bout. This provides quantitative evidence that spraying interrupts fights and suggests that its function is to provide a temporary respite to the spraying queen.

*Key words:* *Apis*, honey bee, conflict, pheromone, fighting behaviour, colony founding.

## Introduction

In social Hymenoptera, queens can either establish a new colony with or without the help of workers. Reproductive

conflicts among queens can occur during the process of colony reproduction. For example, during independent colony founding in some ants, unrelated cofoundress queens fight to death over monopolization of the colony when the first workers emerge (Choe and Perlman, 1997; Bernasconi and Strassmann, 1999). When colony founding is dependent, and the colony produces supernumerary young queens, queen-queen conflicts are expected to occur over the acquisition of the worker force required to establish a new nest or the pre-existing nest (Visscher, 1993). Queen honey bees found colonies dependently as part of a swarm (Winston, 1987). The mother queen leaves with a prime swarm while daughter queens are still immature stages. The colony rears an excess of young queens (10–20, Winston, 1980, 1987; Page and Erickson, 1986). The queen emerging first attempts to kill immature queens still inside their cell (Boch, 1979), and queens emerging at the same time often fight to death (Winston, 1987, p. 188). Depending on the strength of the worker force, the surviving queen(s) inherit either the pre-existing colony (some workers and the nest structure with brood and food stores), or a portion of the workers which then depart on an afterswarm (Winston, 1987). Because a sufficient number of workers are required for winter survival, only one to three daughter colonies are normally produced, each headed by one queen (Winston, 1980). Thus, survival to become an egg-laying queen is a key measure of competition among honey bee queens (Tarpy and Fletcher, 1998). Fights among virgin honey bee queens and among ant foundresses are of particular interest because they represent rare examples of fatal fighting (Enquist and Leimar, 1990; Seger, 1993).

Virgin queens in the honey bee show several adaptations to fights and conflict. They develop faster than workers (16 vs. 21 days), despite their larger adult size (Page and Erickson, 1986), suggesting that selection through hatching asynchrony and age-related fighting advantage have acted to shorten queen developmental time. In agreement with this,

sealed queen cells (containing a larva or pupa) inhibit further queen rearing (Boch, 1979), and audible vibrations (piping) produced by emerged queens prevent other queens from leaving their natal cell (Winston, 1987, p. 188). Venom production begins earlier in queens (immediately after emergence) than in workers (Bachmeyer et al., 1972; Owen and Bridges, 1976; Owen et al., 1997), and queen venom (used in fights) differs in the chemical composition from worker venom (used in nest defense; Owen, 1979).

In this study, we focus on fighting behaviour. In particular, during fights one of the queens often releases a large amount (10–30  $\mu$ l) of rectal fluid with an intense smell of wine grapes (Page and Erikson, 1986; Post et al., 1987; Page et al., 1988; Breed et al., 1992; Tarpy and Fletcher, 1998; Bernasconi et al., 1999). This behaviour (“spraying”) is specific to young queens up to 2 weeks of age, and to agonistic interactions (Post et al., 1987). The fluid released is not a normal waste product (Page et al., 1988; Breed et al., 1992). Spraying has been suggested to be an adaptation to fighting, but the available evidence is equivocal. Several non-exclusive possible functions have been proposed. First, spraying may interrupt aggression by workers against the queen(s), or affect worker intervention in fights. However, the role of workers is unclear. In a study using observation hives workers did not interfere with fights between a newly-mated and a one-year old queen, nor did they exhibit aggression which would result in queen death (Butz and Dietz, 1994). Second, most of the fluid is sprayed on the opponent queen, which may suffer costs, thus providing a relative survival advantage to the spraying queen. Costs to the opponent may arise through toxicity of the fluid, or reduced feeding rates by the workers (Post et al., 1987; Breed et al., 1992). The third hypothesis is that spraying serves to interrupt queen-queen fights, as suggested by observational evidence (Page and Erikson, 1986; Post et al., 1987). The fluid released by queens has a pheromonal effect causing autogrooming behaviour in workers (Post et al., 1987; Page and Erickson, 1986; Page et al., 1988; but see Tarpy and Fletcher, 1998), and this may also interrupt fighting by queens. Interrupting fights may provide a considerable advantage to the spraying queen if it allows her (1) to escape a vulnerable relative fighting position, lowering her risk of injury (Butz and Dietz, 1994), or (2) to flee and hide until other queens first fight each other, thus increasing her individual survival prospects by reducing her overall number of potential aggressive encounters. If contamination with the fluid makes the opponent queen unable to continue fighting effectively, or reverses fighting positions, than retreat after being sprayed upon maybe the safest also for the opponent queen.

None of these hypotheses has been addressed systematically, and background knowledge (such as the lethality of a single fighting encounter under natural conditions) is limited. A problem with the first hypothesis above is that there is little evidence that workers intervene in fights. Similarly, for the second hypothesis, nothing is known about the fluid’s toxicity, and toxicity seems unlikely because the fluid can contaminate the sprayer itself. In this study, we addressed the third proposed function of spraying. We staged fights

between pairs of sister queens of the same age in the presence of workers and compared the temporal structure of the behavioural interactions between queens during spraying and non-spraying fights. This comparison suggests that fighting behaviour is interrupted immediately after release of rectal fluid and that spraying fights had overall lower aggressiveness than non-spraying fights.

## Material and methods

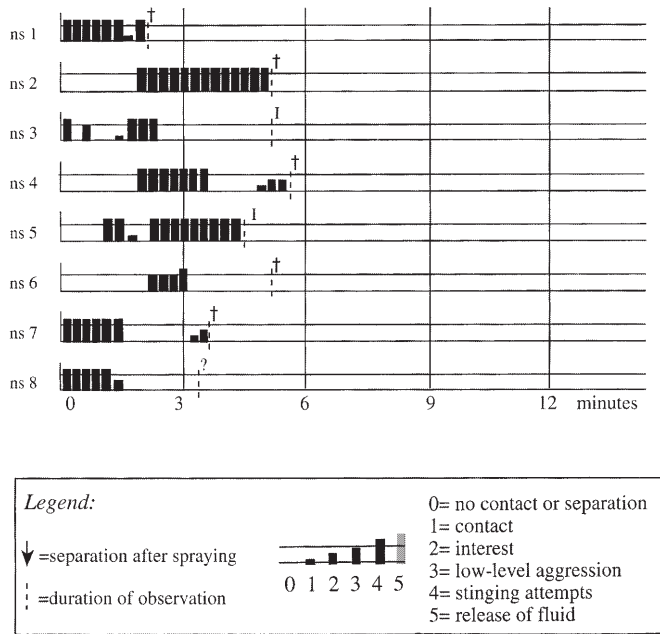
Queens were reared from July 1997 to August 1998 using standard apicultural procedures, i.e., by grafting female larvae (aged  $\leq 24$  hours) obtained from a sample of worker cells into artificial queen cells (Ratnieks and Nowogrodzki, 1987; Tarpy and Fletcher, 1998). After the cells were capped, we individually transferred them to vials (1.5 cm  $\times$  5 cm) and placed them in an incubator (31–34°C) until the adult queen had eclosed from the pupa and left her cell. We provided the vials with sugar candy as a source of food for the queen after emergence from the cell. On the day of emergence, we marked queens on the thorax (von Frisch’ tags, glued with shellac, Bienen Meier AG, Kunten Switzerland), and individually transferred them to mesh cages. Cages were put at a distance of several centimetres from each other in a basket frame back into the mother colony. Larvae for queen rearing were taken from two different colonies. Fights were staged only between sister queens from the same source colony, as occurs naturally (Winston, 1987).

We staged 24 fights between 3-day old queens (age post-emergence). We chose this age because the pheromonal effect of the fluid released during spraying has been recorded for queens aged between one and 14 days (Post et al., 1987). We confronted queens of the same age in the aim to observe fights between evenly matched competitors (age may affect competitive ability, see Discussion). We measured queen size as head width to the nearest  $\mu$ m (measuring table Completron CX, A. Steinmeyer, Albstadt-Ebingen, Germany;  $10 \times 25$ -magnification) on preserved queens. Fights were carried out in the presence of twenty workers in petri-dishes ( $\varnothing$  9 cm, height 1.5 cm) with two entrance holes. Workers were collected on top of the source colony, counted into the petri dish under short CO<sub>2</sub>-treatment, and allowed to recover until their activity seemed normal ( $> 5$  minutes). Queens were placed into the petri dish but were initially kept separated from each other and from the workers by cardboard partitions which we removed as soon as both queens were in place. Queens were not allowed escape. Thus subsequent encounters were possible after fight interruption (queens that are less than 3.5 cm apart can sense each other’s presence, Butz and Dietz, 1994). In the natural situation, the nest provides a three-dimensional structure with thousands of bees, and queens may not meet again within a few minutes after a fight is interrupted (Butz and Dietz, 1994; see Discussion).

Fights were videorecorded inside an incubator (31–34°C, red light). The image was deflected to the camera (camcorder Panasonic NV-M40, lens 1:1.4, 5–40 mm; monitor Sharp 51AT-15H) by a mirror placed at 45° angle. We recorded for 5 minutes following the removal of the cardboard partitions. Queens start fighting and usually one of them is immobile or dead within this time (Butz and Dietz 1994). If not, we continued recording until there was a clear outcome (i.e., one of the queens was injured and/or immobile, and interaction between the queens had ceased; range of duration of observation = 2–15 minutes, median = 5 minutes,  $N = 24$ ; Fig. 1). To ensure that our measure of fight duration was valid, we screened the videotapes a second time measuring to the nearest second the time when injury occurred. These two measures were significantly positively correlated (Spearman rank correlation coefficient:  $r_s = 0.67$ ,  $N = 19$ ,  $p < 0.01$ ). Queens were caged and returned to the colony so that workers could feed them. We checked all queens and recorded whether they were still alive 1 hour and 1 day after the end of the fight.

To quantify behaviour from video playbacks, we recorded every 15 seconds the following interactions: (0) queens not in contact;

a) Non-spraying fights

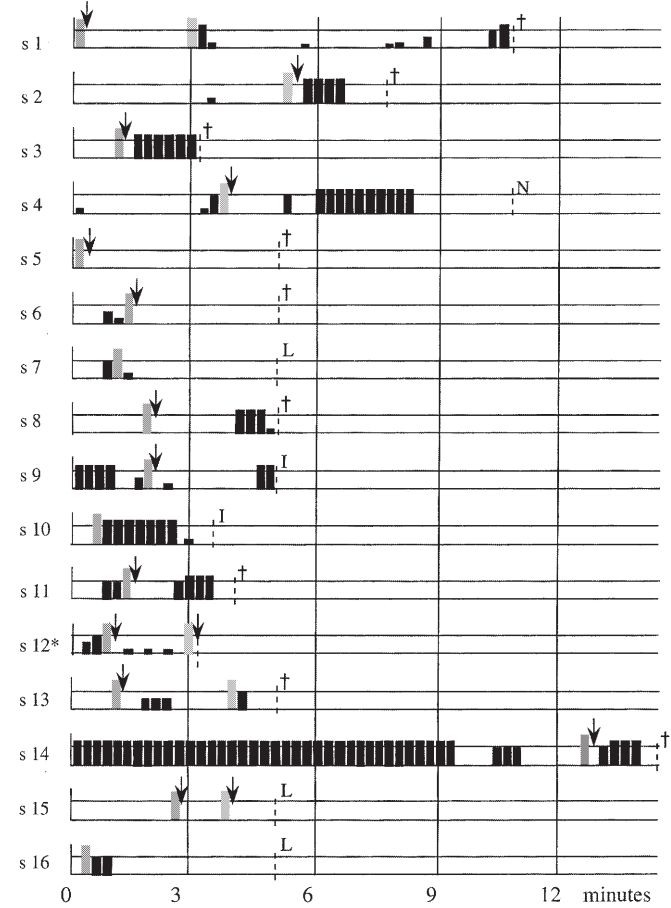


**Figure 1.** Temporal structure of queen-queen fighting behaviour in the presence of workers. Each bar represents one observation interval lasting 15 seconds; for ease of reading, bars are non-contiguous. The height of the bar indicates the behaviour with the highest aggression-value (see Legend) observed in that interval. Grey bars: intervals with spraying event. Loser condition at the end of fight: N = no visible injury, L = dragging one or more legs, I = immobile, † = dead, ? = missing value. Fight *s12* was a draw (no clear winner, see text)

(1) queens in physical contact; (2) queens in contact but non-aggressive to each other (“interest”); (3) displaying low-level aggression, such as walking on top of each other (not stinging or grasping each other); (4) stinging and stinging attempts, with queens maintaining a grip on the rival; (5) whether one or both released rectal fluid (Fig. 1). Levels 4 and 5 represent escalated aggression, with queens being interlocked, and attempting to insert the sting in non-sclerotized regions of the opponent’s body (Butz and Dietz, 1994; Tarpay and Fletcher, 1998). Previous observations have already indicated that spraying occurs during stinging behaviour (Post et al., 1987). We also recorded the loser’s condition after the fight (Table 1; fight no. *s12* was a draw, i.e., both alive, no clear winner, no further interaction; Fig. 1b). For ease of reading, we give these data in seconds. However, it should be noted that accuracy is only to the nearest 15 seconds.

When queens contacted each other, they usually started fighting. This either led to injury and a clear outcome in the first bout, or the queens separated, and then came into contact again and fought a second, or third bout. To quantify the temporal structure, we 1) measured duration and latency of subsequent bouts of fighting behaviour, and 2) calculated the overall proportion of time spent in escalated aggression. We define a bout as a continuous sequence of contact (levels 1 or higher) between queens in which escalated aggression (levels 4–5) occurred in at least one interval. The end of one bout occurs when queens separate (i.e., a no-contact interval occurs). We did not consider a sequence of intervals with only non-escalated behaviour (levels 0–3) to be a bout. For instance, fight no. *s1* (Fig. 1 b) consisted of three bouts of durations 15, 45 and 30 seconds, respectively, with latencies of 2’45” between bouts I and II, and of 6’30” between bouts II and III. Because the artificial environment offered no possibility of escape and so substantially

b) Spraying fights



increased the encounter probability after separation, for analysis we focus on the first bout. Spraying occurred in 16 out of 24 fights (67%). Usually, spraying occurred only once. However, in 4 out of 16 spraying fights it occurred twice, but for the same reason we consider only the first spraying event. In 2 out of these 4 cases we could clearly see that the same queen sprayed twice. For each queen pair, we calculated the proportion of time spent in escalated aggression over the whole encounter as the number of single intervals where levels 4 and 5 occurred, divided by total number of intervals until clear outcome (duration of observation). For instance, queens in fight no. *s1* (Fig. 1 b) spent 7% of the observed time in escalated aggression.

**Table 1.** Loser condition immediately at the end of fights (N = 22; 1 draw and 1 missing value) and delay until death (A = loser dead within 1 hour after the fight; B = loser dead by next morning)

	Non-spraying fights	Spraying fights	A	B
No visible injury	–	1	–	1
Dragging one or more legs but can move	–	3	1	2
Alive but cannot move	2	2	3	1
Dead	5	9	14	–
<b>Total</b>	<b>7</b>	<b>15</b>	<b>18</b>	<b>4</b>

Because of evidence that the rectal fluid has a pheromonal effect (see Introduction), the amount released may correlate with the effectiveness of spraying in modifying queen behaviour. Usually the rectal fluid is ejected on the opponent, but because large quantities are released, part of it also fell on the bottom of the petri dish (which we covered with a standardized, absorbent white cardboard). For 13 of the 16 spraying fights we estimated the area covered on the cardboard as an indirect estimate of the amount released, using Object Image (NIH Image Processing Program, <http://simon.bio.uva.nl/object-image.html>) after scanning the cardboards (Color One Scanner, Ofoto 2.02 for the MacIntosh). Statistical analysis was carried out using JMP 3.1 (SAS Institute Inc. Cary NC, USA). Where appropriate, we give exact probabilities instead of approximations (Mundry and Fischer, 1998). Unless specified, means are given  $\pm$  SD.

## Results

Spraying was significantly more likely to occur during the first escalated bout (14, i.e., 87% out of 16 spraying fights), than in subsequent bouts (binomial test,  $p = 0.002$ ). First contact between the queens and spraying occurred in the same observation interval for 8 out of the 16 spraying fights, and on average after  $68 \pm 155$  seconds (median = 15 seconds;  $N = 16$ ;  $30 \pm 22$  seconds excluding fight no. *s14*, Fig. 1 b) from first contact.

The first escalated bout lasted on average  $66 \pm 121$  seconds ( $N = 24$ ). For fights that had more than one bout ( $N = 13$ ), latency to the next bout was  $96 \pm 65$  seconds, and the second escalated bout lasted  $60 \pm 44$  seconds. The median number of escalated bouts until a clear outcome was recorded was 2 for spraying fights (mean  $\pm$  SD =  $1.8 \pm 0.7$ ,  $N = 16$ ) and 1 for non-spraying fights ( $1.4 \pm 0.5$ ,  $N = 8$ ; Mann-Whitney U-test:  $U_{\min} = 43.5$ ,  $p > 0.20$ ), with 11 out of 16 spraying fights having more than one bout, compared to 3 out of 8 non-spraying fights (Fisher's exact test:  $p = 0.20$ ).

Spraying and non-spraying fights differed in their temporal structure (Fig. 1). In spraying fights, the first escalated bout was significantly shorter ( $22 \pm 28$  seconds,  $N = 14$  spraying fights where spraying occurred in the first bout) than in non-spraying fights ( $79 \pm 64$  seconds,  $N = 8$ , Mann-Whitney U-test:  $U_{\min} = 20$ ,  $p < 0.01$ ). The direction of difference also remained the same when analysis was restricted to the subset of fights with only one escalated bout (spraying fights:  $36 \pm 47$ ,  $N = 5$ ; non-spraying fights:  $99 \pm 71$  seconds,  $N = 5$ ). A shorter first escalated bout in spraying fights is consistent with fight interruption after fluid release. Indeed, in 13 out of 16 spraying fights (81%) the interval of spraying is followed by a no-contact interval (binomial test:  $p = 0.011$ , arrows in Fig. 1 b). Spraying fights were characterized by a significantly lower proportion of time spent in escalated aggression ( $21\% \pm 22\%$ ,  $N = 16$ ) than non-spraying fights ( $43\% \pm 25\%$ ,  $N = 8$ , mean  $\pm$  s.e.; Mann-Whitney U-test:  $U_{\min} = 29$ ,  $p < 0.05$ , Fig. 1). The duration of the first bout was significantly positively correlated with the proportion of time spent in escalated aggression over the entire duration (Spearman rank correlation coefficient:  $r_s = 0.73$ ,  $N = 14$  spraying fights where spraying occurred in the first bout,  $p < 0.01$ ). The proportion of time spent in low-level aggression was of  $12\% \pm 9\%$  ( $N = 16$ ) in spraying fights, and

$9\% \pm 10\%$  ( $N = 8$ ) in non-spraying fights. There was no significant difference in the total duration of non-spraying fights ( $249 \pm 71$  seconds,  $N = 8$ ), and spraying fights ( $362 \pm 193$  seconds,  $N = 16$ ; Mann-Whitney U-test:  $U_{\min} = 44$ ,  $p > 0.20$ ). Injury of the losing queen was recorded after  $202 \pm 95$  seconds from begin of the experiment in non-spraying fights ( $N = 7$ ), and significantly later, after  $557 \pm 327$  seconds, in spraying fights ( $N = 12$ , Mann-Whitney U-test:  $U_{\min} = 6.5$ ,  $0.01 < p < 0.05$ ). The loser was dead at the end of the fight in 14 cases, i.e., 64% of the fights were lethal within the observation period (Table 1).

The released fluid covered on average  $197 \pm 88$  mm<sup>2</sup>. This estimate of the amount released was not significantly correlated to the latency until the next bout, i.e., the length of time during which separation was effective (Spearman rank correlation coefficient  $r_s = 0.35$ ,  $N = 13$ ,  $p > 0.10$ ). There was no significant difference in size (head width) between the winner ( $3964 \pm 262$   $\mu$ m) and the loser ( $3896 \pm 371$   $\mu$ m, paired t-test:  $t = 0.49$ , d.f. = 21,  $p > |t| = 0.62$ ). There was also no significant difference in the size ratio (loser:winner) between non-spraying fights ( $1.01 \pm 0.04$ ) and spraying fights ( $0.98 \pm 0.03$ , unpaired t-test:  $t = 0.58$ , df = 20,  $p > |t| = 0.57$ ).

## Discussion

Our study shows that spraying occurs after physical contact between the queens, and that spraying interrupts fighting. In spraying fights, the first escalated bout was significantly shorter than in non-spraying fights. After spraying queens released their grip and separated, and in many of the spraying fights this first encounter did not end lethally. Overall, spraying fights had a significantly lower proportion of escalated aggression (stinging and stinging attempts, see Methods) than non-spraying fights. This cannot be ascribed to a difference in total duration of fights, and strongly suggests that the release of rectal fluid directly affects the behaviour of queens, without need to invoke worker intervention, by lowering the aggressiveness of the fight. Available evidence of worker aggression against introduced queens indicates that it is usually non-fatal (see Butz and Dietz, 1994, and references in Tarpy and Fletcher, 1998, p. 538), and that it is the queens that kill each other, with the workers finishing the injured one by forming a ball around it (Winston 1987, p. 188). Theoretical models also predict that virgin queens are more likely to favor killing rivals than workers are (Visscher, 1993), although this does not exclude worker intervention, for instance to determine relatedness bias in queen post-emergence survival (Tarpy and Fletcher, 1998). That spraying fights had reduced aggressiveness at the first fighting encounter between queens is important for two reasons. First, in the confined fighting arena queens could easily resume fighting, reducing the information content of subsequent observed bouts. Second, the first encounter is highly aggressive, and often decisive. A study on fights between young and 1-year old queens showed that confrontations lasting up to 20 seconds are usually lethal, with the



longest observed lethal fighting pattern lasting 2–10 minutes (Butz and Dietz, 1994). Therefore, lowering the aggressiveness and potential lethality of the fight, and in particular of the first escalated bout, most likely provides a direct benefit to the spraying queen under natural conditions. That the opponent queen also releases her grip suggests that spraying may reverse the queens' relative fighting positions.

In the artificial situation of a confined fighting arena, queens eventually resumed fighting. Indeed, injury was recorded also in spraying fights, but significantly later than in non-spraying fights. Queens (in particular the spraying queen, which often is the more vulnerable one, Rand, Ratnieks and Bernasconi, unpublished) may escape if they survive the first encounter in the natural situation inside a colony. The outcome of fights staged inside small (nucleus) hives were less often lethal than in our study (Tarpy and Fletcher, 1998). Multiple queens are known to coexist for hours or even days after eclosion (Winston, 1987, p. 188) because of infrequent direct interactions between queens within the large nest (Tarpy and Fletcher, 1998). Theoretical models predict that (non-lethal) fights between closely matched competitors will generally be more prolonged. The longer first escalated bout and overall proportion of time spent in escalation in non-spraying fights may suggest that in these cases queens were more equally matched by competitive ability than in fights where spraying occurred. So far, we have been unable to identify any phenotypic predictor of relative competitive ability (i.e., size) and in this study care was taken to control for potential differences in competitive ability (e.g., we matched queens by age). Also, lethal fights are predicted to occur irrespective of assessment when the individual's entire reproductive success is at stake (Enquist and Leimar, 1984). However, because afterswarming colonies raise supernumerary young queens (Winston, 1987), each individual queen might need to fight several times before gaining the resource (the colony, or a swarm of sufficient size to ensure winter survival), with each encounter bearing a substantial risk of fatal injury. Thus, a mechanism that allows a queen to escape stronger competitors or reduces the aggressiveness of a fight may substantially increase an individual's probability of obtaining a colony. We suggest that one direct advantage of spraying is that, by lowering the lethality of a single encounter, a queen may hide until other queens in the same hive first kill each other, thus reducing the number of opponents it has to fight, or until the workers eventually assign the established nest to one of the queens, which is predicted to occur at random (Visscher, 1993). That spraying occurred in two thirds of the observed fights, and upon contact with the opponent, is in agreement with risk avoidance. Two questions arise. First, whether under natural conditions queens can also break up a fight without necessitating spraying, for example by dropping off the comb. We are unaware of any published evidence that this occurs. A study of fights between newly-mated and one-year old queens (Butz and Dietz, 1994) indicates that the mechanism of queen elimination is stinging by a rival queen, as in the present study. Because the sting can penetrate only non-sclerotized regions, queens must be able to grasp each other and maintain a grip (Butz and Dietz, 1994). Because

queens fight interlocked, if they fell off the comb when fighting they would still remain together. Thus queen fighting requires something active like spraying to cause break up. The second question is why queens do not spray in all fights. This may be due to differences among queens (for instance, age-dependent synthesis of active substances, Post et al., 1987; Page et al., 1988), or if sometimes queens are not in an effective position to spray on the opponent, and this may explain the very short confrontations that end lethally for one queen (within 20 seconds, Butz and Dietz, 1994). It was also the case in the present study that 5 out of 16 spraying fights were lethal in the first bout.

The hypothesis that spraying serves to avoid or reduce the risk of single fighting encounters does not exclude more subtle effects on both the opponent and workers of specific chemicals contained in the fluid released (Post et al., 1987; Page et al., 1988; Breed et al., 1992; Bernasconi et al. 1999), and more studies are required to investigate the pheromonal effects of queen rectal fluid during fights (Tarpy and Fletcher, 1998). Further study of the mechanisms is possible, as most chemicals in the fluid released are commercially available (Page et al., 1988; Bernasconi et al., 1999). However, we found no significant association between an estimate of the amount of rectal fluid released and the latency to the next escalated bout, suggesting that either spraying has a qualitative effect, or that the content of potentially active substance in the released fluid is not directly related to the total amount of fluid sprayed. In this study, we fought queens without prior fighting experience. It is possible that depletion of active substances occurs in subsequent fights.

In conclusion, our data suggest that spraying effectively interrupts fighting behaviour and provides a temporary respite to the spraying queen.

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