

# Matricide and queen sex allocation in a yellowjacket wasp

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**Abstract** In many colonies of social insects, the workers compete with each other and with the queen over the production of the colony's males. In some species of social bees and wasps with annual societies, this intra-colony conflict even results in matricide—the killing of the colony's irreplaceable queen by a daughter worker. In colonies with low effective paternity and high worker-worker relatedness, workers value worker-laid males more than queen-laid males, and thus may benefit from queen killing. Workers gain by eliminating the queen because she is a competing source of male eggs and actively inhibits worker reproduction through policing. However, matricide may be costly to workers if it reduces the production of valuable new queens and workers. Here, I test a theoretical prediction regarding the timing of matricide in a wasp, *Dolichovespula arenaria*, recently shown to have facultative matricide based on intra-colony relatedness. Using analyses of collected, mature colonies and a surgical manipulation preventing queens from laying female eggs, I show that workers do not preferentially kill queens who are only producing male eggs. Instead, workers sometimes kill queens laying valuable females, suggesting a high cost of matricide.

Although matricide is common and typically occurs only in low-paternity colonies, it seems that workers sometimes pay substantial costs in this expression of conflict over male parentage.

**Keywords** Evolutionary conflict · Kin selection · Worker reproduction · Social insects · Sex ratio · Insect surgery

## Introduction

Evolutionary conflict occurs when individuals differ in their optimal outcomes for the same event (Hamilton 1972; Trivers and Hare 1976; Ratnieks and Reeve 1992). How this conflict plays out will depend primarily on two things: the different optima of the individuals in conflict and the relative power the individuals have to influence the outcome of conflict given the biological details of the situation (Ratnieks and Reeve 1992; Beekman and Ratnieks 2003). Reproductive conflict has been studied intensively in the eusocial Hymenoptera, particularly in the contexts of sex allocation and male parentage (reviewed in Queller and Strassmann 1998; Bourke 2005; Ratnieks et al. 2006; West 2009). Conflict over male parentage in single-queen colonies arises because each female (queens and workers) prefers that the colony rears her sons instead of the sons of others. This can be seen as the result of a preference for rearing close relatives: the regression relatedness between a mother and her son is 1, while workers are related to the queen's sons by 1/2 and to the average other workers' sons by 1/4–3/4, depending on the relative frequency of full- and half-sibling workers. In single-queen colonies, worker-worker relatedness is determined by the colony's effective paternity, a measure that accounts for the number of fathers and their relative shares of paternity among workers (Boomsma and Ratnieks 1996; Jaffé 2014). When the colony has an effective

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paternity less than 2.0, worker-worker relatedness is relatively high, and workers are predicted to prefer the sons of other workers to the sons of the queen (Starr 1984; Woyciechowski and Lomnicki 1987; Ratnieks 1988). Workers can influence the outcome of this conflict by eating the eggs of other workers or of the queen (e.g., (Ratnieks and Visscher 1989; d’Ettorre et al. 2004; Wenseleers et al. 2005; Zanette et al. 2012), by aggressing or dominating other workers (e.g., Liebig et al. 1999; Wenseleers et al. 2005; Stroeymeyt et al. 2007), and by killing reproductive competitors, both workers (KJL unpublished observations) and the queen (Bourke 1994).

Matricide, the killing of the mother queen by workers, is likely an extreme outcome of worker-queen conflict over reproduction (Bourke 1994). Matricide is predicted only in eusocial insect species with annual colonies, given the high cost of killing a queen if she is likely to live for many years (Bourke 1994). In most species with annual colonies, workers cannot produce a replacement queen, making the transition to queenlessness irreversible, and possibly quite costly, depending on the timing of the queen killing act in the colony cycle. Workers have been observed killing their queen in at least six species of vespine wasp (Bourke 1994; Loope 2015; T. Wenseleers, personal communication) and at least three species of bumble bee (summarized in Bourke 1994). Here, I test a theoretical model regarding the evolution of matricide using a common North American yellowjacket wasp, *Dolichovespula arenaria* (Vespidae: Vespinae). In this species, and other members of the genus, worker-queen conflict is conspicuous: workers lay eggs in queenright colonies, and workers and queens aggress reproductive workers and eat worker-laid eggs (Greene et al. 1976; Akre and Myhre 1992; Bonckaert et al. 2011a, b; KJL, per sobs). Unlike vespine species with high mating frequency (Foster and Ratnieks 2001a; Bonckaert et al. 2008), mutual policing by workers does not completely suppress worker reproduction, but in queenright colonies, queens still produce the majority of males in *D. arenaria* (Freiburger et al. 2004) and congeners (Foster et al. 2001; Bonckaert et al. 2011a, b). Thus, workers who kill their queen may shift colony investment from the queen’s sons to the worker’s sons, depending on the timing of matricide and the state of the colony.

A kin-selection model of matricide makes several predictions about when matricide should be most favorable to workers (Bourke 1994). First, when an individual worker is unlikely to dominate male production after the queen is dead, matricide should be favored only in colonies with high worker-worker relatedness, because there is a relatedness benefit to the worker of replacing brothers with nephews when producing male reproductives. In *Dolichovespula* colonies, a single worker is unlikely to dominate reproduction: queenright colonies typically contain four to ten reproductive workers, depending on the species (Foster et al. 2001; Bonckaert et al. 2011b). Unpublished data from *D. arenaria*

indicate a median of 11 reproductive workers per colony (F. Ratnieks, personal communication). Furthermore, reproductive worker turnover is likely high, as these workers fight among themselves and with the queen, so their reproductive tenure may be brief relative to the reproductive stage of the colony (Bonckaert et al. 2011a; Loope 2015). As predicted by Bourke’s model, an association between relatedness and matricide is found in my study population of *D. arenaria*: most mature, queenright colonies have effective paternities near 2.0, while most queenless colonies (in which matricide was observed or inferred) have effective paternities near 1.0 (Loope 2015). In the present study, I test an additional prediction of Bourke’s model, that workers should preferentially kill queens who have irreversibly switched to laying male eggs (Trivers and Hare 1976; van der Blom 1986; Bourke 1994). This prediction stems from the fact that workers value the laying of female eggs by the queen: workers are highly related to new queens, and they benefit from increased colony productivity resulting from the addition of new workers. If the queen dies while producing females, workers thus suffer an inclusive fitness cost. But, if the queen is only producing male eggs, then queen-killing involves no cost of lost female production; the colony simply switches from producing brothers to producing sons and nephews, which are more valuable if the colony’s effective paternity is low. Assuming the population sex ratio is in equilibrium, the threshold benefit/cost ratio for matricide by a worker is always lower when toward a male-laying queen than one still producing females regardless of the population fraction of males that are workers’ sons and regardless of whether workers or queens control the sex ratio, though the prediction does not hold if queenless colonies have productivity substantially lower than queenright colonies (Bourke 1994).

The best support for this prediction to date come from *Bombus terrestris*, a bumblebee in which worker-queen conflict has been well studied. Several authors report matricide in colonies whose queens have switched to laying exclusively haploid male eggs, and matricidal workers are reproductively active (van Honk et al. 1981; van Doorn and Heringa 1986), supporting predictions of the kin-selected matricide hypothesis (Bourke 1994). However, unlike *B. terrestris*, most vespine wasps have a relatively long period of producing reproductives, and workers are often produced throughout this reproductive stage (Greene 1984). In most species, male and female reproductives are produced simultaneously, suggesting that a matricidal worker in a typical colony would incur a substantial cost by prematurely ending female production. For these reasons, some have argued against the likelihood of kin-selected matricide in vespine wasps (Martin et al. 2009), though this logic does not explain the observations of matricide, and it does not consider the possibility of facultative matricide in response to colony-specific conditions.

There is suggestive evidence for facultative matricide based on the sex of the queen-laid brood in yellowjackets. Montagner (1966) found, after radiolabeling queens to determine whether queens or workers produce males, that all three queens that produced only male eggs after reintroduction to the colony were killed by workers, while all seven queens that laid only female eggs were not killed. This appears to be facultative matricide in response to queen primary sex ratio, but the methods employed make conclusive interpretation difficult. The male-laying queens may have been more damaged by the radiation than the others, and then died or were killed due to their poor condition; the report provides no description of the queen-killing event, or how this brief, rare act was observed. Additionally, these experiments involved colonies of several species, two of which (*Vespula germanica* and *Vespula vulgaris*) are in a genus with no other reports of matricide (reviewed in Bourke 1994). Thus, although these findings are suggestive, further investigation of the link between the sex of the queen-laid brood and matricide is clearly needed.

To test the brood sex prediction for matricide, I described comb allocation patterns in collected queenless and queenright colonies to determine the context of matricide, and then I used genetic assessments of observed- and inferred-matricide colonies to determine the sex of the last queen-laid brood. I also performed a manipulative experiment similar to Montagner's: some queens were forced to lay only male eggs by surgically removing their spermathecae. Colonies were then observed to document matricide. Counter to theoretical prediction, the results suggest that the laying of male eggs by queens does not trigger matricide in *D. arenaria*.

## Methods

### Study species

*D. arenaria* is an aerial-nesting yellowjacket wasp abundant throughout North America (Greene et al. 1976; Fig. 1a; Akre et al. 1980). In my study population in central New York State, solitary *D. arenaria* queens found new colonies in May and June. The first workers emerge in mid June, and colonies grow rapidly, building one to three combs of worker-producing small cells before building one to three combs of larger, reproductive cells for rearing new queens and males (Greene et al. 1976; this study). Colonies can contain over 800 workers (KJL unpublished data), though the average peak colony size is around 300 (Loope 2015). Typically, colonies are in decline by mid August, and only the new queens survive the winter after mating in late summer and autumn. Worker paternity estimates suggest that queens mate one to three times, and that the harmonic mean effective paternity within colonies is 1.49,

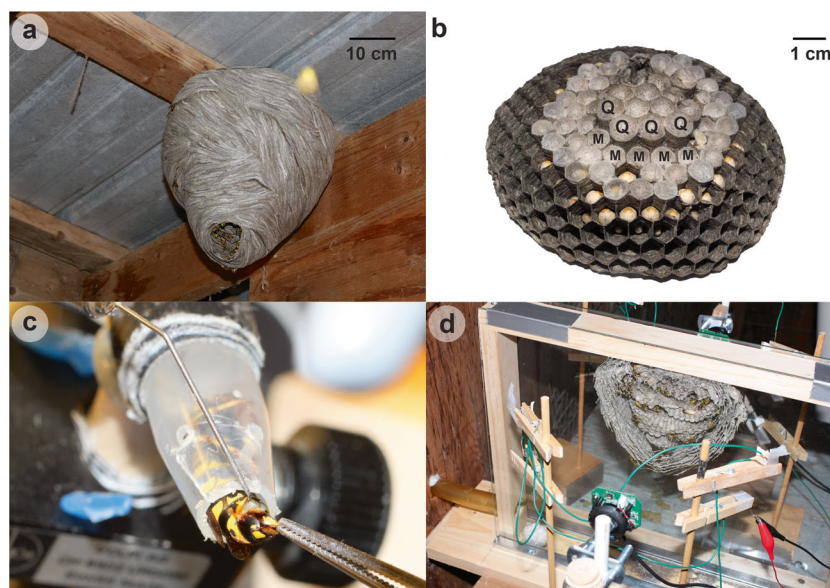
with 70 % of queens (12 of 17) mating more than once (Loope 2015).

### Collecting colonies and inferring matricide

Matricide is very difficult to directly observe, but one can gain information about colonies that exhibit matricide by using queenlessness in mature colonies as a proxy for matricide, under some assumptions. I collected and sacrificed mature colonies in 2010, 2012, and 2013 and used a subset to infer matricide (also see Loope 2015). The subset included colonies with at least four combs but which were not senescing, with many empty cells and shriveled brood. These colonies are all in the reproductive stage and did not include colonies that were queenless and producing males due to queen death in the pre-emergence period when queens forage away from the colony, since such colonies typically do not grow beyond the addition of a third comb (KJL, personal observation). Given the high rate of queenlessness in these mature colonies (13/31 = 42 % in this population; Loope 2015) and the low rates in mature colonies of very similar wasps that lack matricide (4.7 % queenlessness rate in 106 mature colonies across 5 species of *Vespula* spp.: *V. vulgaris*, *V. germanica*, *V. flavopilosa*, *V. vidua*, *V. consobrina*; (Foster and Ratnieks 2001b; Loope et al. 2014), one can calculate that ~89 % of these mature queenless colonies were likely queenless due to matricide, assuming non-matricide queen mortality rate is similar across these species (Loope 2015). Because this implies a very strong correlation between mature-colony queenlessness and matricide, I used these mature, queenless colonies to represent colonies that likely experienced matricide (hereafter "inferred matricide colonies"). From the collected colonies, I measured the surface area of different cell types within each nest. These data revealed how queenright and inferred matricide colonies allocated resources among worker, queen, and male cells. I also collected comb area data for the three observation colonies in which I directly observed matricide (see below).

### Genetic determination of queen sex investment prior to queen death

I used standard Chelex extractions, multiplex PCR, and fragment length analysis to obtain the microsatellite genotypes of brood from six colonies at the loci *Rufa05*, *Rufa13*, *Rufa15* (Thorén et al. 1995), and *List2004* (Daly et al. 2002). Protocols were identical to those reported in a previous study on wasp paternity (Loope et al. 2014). Population allele frequencies and the genotypes of the queens and their mates were known from a companion study of worker paternity in these colonies (Loope 2015). I genotyped 18–24 male pupae from each of the 5 inferred matricide colonies that still contained pupae spanning the transition from queen cells to male cells



**Fig. 1** Investigating matricide in *Dolichovespula arenaria*. **a** A large, mature colony of *D. arenaria*. **b** The third comb from colony 65\_12 illustrates the transition from female production to male production. The 14 central, large queen pupae (marked “Q”) are surrounded by the shorter first cohort of males (marked “M”). **c** A queen ready for spermatheca removal surgery, immediately before the incision is

made in the exposed inter-segmental membrane. CO<sub>2</sub> flowing through the holding tube lightly anesthetizes her. **d** A nest box permitting longitudinal observations of colony development. Wooden clips hold infrared LEDs and a webcam is visible on each side. The tube at lower left connects the box to the outdoors, allowing foragers to come and go freely

(Fig. 1b). The first males laid after the switch to male production can indicate what the queen was laying prior to her death. If the queen was laying females when she died, then the first males laid should all be the sons of workers. In this scenario, the transition from queen production to male production is the result of queen death. Alternatively, the transition from queen production to male production could be the result of the queen switching to laying male eggs. If this is the case, then we expect most or all of the first males laid to be sons of the queen. I inferred parentage of these first males by examining how many of them shared an allele with the queen at all four loci. Given that workers have one queen allele at each locus, there is a chance that a worker-laid male shares alleles with the queen at all four loci. The probability that this happens is  $(1/2)^k$  where  $k$  is the number of informative loci (loci at which the male mate of the queen does not share an allele with the queen). A binomial test can then determine the probability of finding the observed number of all-queen-allele males under the null hypothesis that all males are the sons of workers. If this probability is low, we can infer that the queen laid some male eggs before dying.

An additional correlative data point between queenlessness/matricide and queen sex investment prior to her death was obtained from an (unmanipulated) observation colony in which matricide was observed (see below). In the one thriving observed matricide colony for which I have genetic material (09\_11), I genotyped 24 first-instar larvae and eggs from the edge of the incipient fourth comb (the first reproductive comb) to determine what the queen was laying when she was killed.

These broods were certainly almost all queen offspring, since the colony was collected 12 h after the queen was killed. Determining whether these broods were male or female revealed what the queen was laying when she died.

### Experimental manipulation of queens and direct observation of matricide

Colonies were transplanted to observation boxes when young, typically with fewer than 30 workers and two to three combs. These observation boxes (Fig. 1d) were established in out-buildings at the Liddell Field Station in Ithaca, NY, and the colonies in them were allowed to forage freely outdoors. For details of observation box setup, see ESM 1. A total of 63 colonies were transplanted, but only 31 were used in observations: many early-failing colonies were removed from the study. Some failed due to heavy infestations of *Aphomia sociella*, a brood-eating parasitic moth that destroyed all colony brood. In other colonies, the queen abandoned the nest prior to the initiation of the reproductive phase. Finally, for some colonies, all workers were lost in the transplant and the nest failed to grow. For those colonies that grew and constructed reproductive comb, we observed colony development and manipulated a subset of queens using surgery to test the hypothesis that a queen’s switch to producing only males triggers queen killing by workers.

*Surgical manipulation of queens.* The goal of surgery was to induce matricide by creating queens that only lay male eggs by experimentally removing the spermatheca (spermathectomy).

Because the wasps studied have haplodiploid sex determination, sperm is required to produce female offspring, but not male offspring. If the stored sperms are removed, only males can be produced (Koeniger 1970). Briefly, queens were extracted from colonies constructing their third or fourth comb (i.e., early in the reproductive stage), and anesthetized with CO<sub>2</sub>. Their spermathecae were surgically removed (Fig. 1c) and they were returned to the nest within a few minutes of surgery. Sham surgery queens experienced the same procedure, but the spermatheca was only touched with forceps instead of being pulled out. For a detailed description of surgical methods, see ESM 1.

Three of the four queens survived the spermatheca removal surgery in 2012. Five of the eight queens survived in 2013. The rest died within 3 days of surgery (video observations confirmed that these deaths were not due to matricide and were almost certainly caused by damage during surgery). Five of the six sham surgery queens survived the operation in 2013. We also observed 18 additional colonies that were unmanipulated (also discussed in Loope 2015). Thus, 8 spermatheca removal colonies, 5 sham surgery colonies, and 18 unmanipulated colonies were monitored, for a total of 31 colonies.

**Monitoring colonies for matricide.** After manipulation, colonies were checked daily for queen presence. During these daily checks, I opportunistically noted queen oviposition behavior. Continuous video recording ensured that I could determine the cause of queen death once it was discovered in daily checks. Recordings were made using two Logitech C600 webcams per colony (one on each side). Video from each camera was recorded to hard drives using Eyeline 1.18 (NCH Software). I removed the infrared filters on each camera and illuminated the colonies with infrared LEDs (Fig. 1d).

Spermathectomy colonies were monitored until approximately 30 days after surgery in 2012 and 21 days after surgery in 2013. The egg stage is typically 4–5 days (Edwards 1980), and at this stage in colony development, pupae are often capped within 11–18 days of laying (KJL, unpublished data). Thus, virtually all queen-derived eggs and larvae at the time of collection would be male.

**Sperm counting.** To determine whether an observed-matricide queen in 2012 had run out of sperm, I counted the stored sperm in her spermatheca, as well as those of five spring-caught queens and four mid-season, reproductive-stage queens. I prepared a sperm-counting solution that was 1 part 0.05 % Triton X-100 (Sigma Aldrich) diluted in 19 parts Ringer's solution (Kronauer and Boomsma 2007). Otherwise, I used the method of Stein and Fell (1996). Each spermatheca was dissected in distilled water, transferred with forceps to an Eppendorf tube containing 20  $\mu$ L of sperm-counting solution, and then crushed thoroughly with a plastic pestle. The pestle was rinsed into the tube using four 20- $\mu$ L aliquots of solution, resulting in a final volume of 100  $\mu$ L of sperm in suspension.

After mixing by thorough pipetting, the two sides of a Bright-Line Hemocytometer were immediately filled with sperm in dilution. I allowed the cells to settle before counting the number of sperms in four 100-nL squares on each side, for a total of eight counts. The mean of these counts was multiplied by 1000 to estimate the number of sperm in the total 100- $\mu$ L volume.

## Results

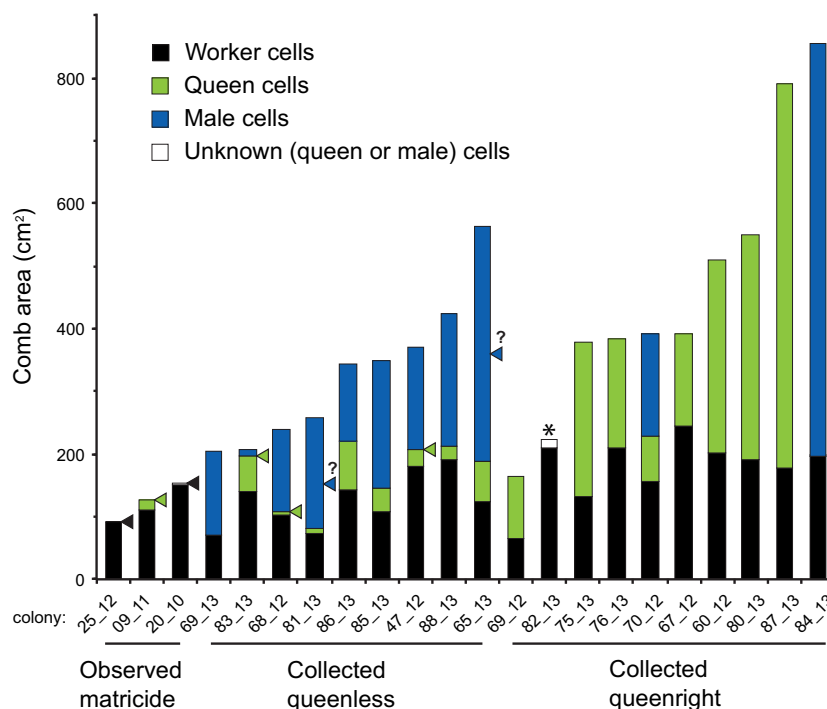
### Comb area and sex allocation

The positions of cell types in all colonies suggest that cells were constructed in the following order: worker, queen, and male, as previously observed in this species (Greene et al. 1976). As also noted by Greene et al. (1976), many colonies specialized on the production of one sex of reproductives (Fig. 2). Seven of ten queenright colonies produced only queen-destined reproductive cells, while two produced mostly male cells, and one colony was just initiating its first reproductive comb and thus the cells were of indeterminate class (Fig. 2). Queenless, inferred matricide colonies were typically male specialists: they produced a greater area of male cells than queen cells (mean  $\pm$  SD queen cell area 33.3  $\pm$  28.3 cm<sup>2</sup>; male cell area 170.1  $\pm$  97.2 cm<sup>2</sup>; paired *t* test *t* = 4.18, *df* = 8, *p* = 0.003) and invested an average of 20.6 %  $\pm$  26.6 SD of reproductive comb area in queen cells.

The three observed matricide colonies had each constructed three combs of worker cells at the time of matricide, and two were starting construction of the first reproductive comb (see Loope 2015 for further description). The area of worker cells in these nests was not different from inferred matricide colonies (observed matricide 117.9  $\pm$  289.0 cm<sup>2</sup>, inferred matricide 125.5  $\pm$  42.7 cm<sup>2</sup>, Welch's *t* = 0.34, *df* = 5.25, *p* = 0.76) but was significantly smaller than the area of worker cells constructed in queenright mature colonies (queenright 178.5  $\pm$  50.4 cm<sup>2</sup>; Welch's *t* = 2.62, *df* = 6.15, *p* = 0.039). Similarly, the area of worker cells constructed in inferred matricide colonies was less than that in queenright colonies (*t* = 2.49, *df* = 16.95, *p* = 0.024). However, inferred matricide- and queenright-collected colonies did not significantly differ in the mean total comb area (queenless 328.9  $\pm$  117.3 cm<sup>2</sup>; queenright 463.5  $\pm$  221.5 cm<sup>2</sup>; Welch's *t* = 1.68, *df* = 13.96, *p* = 0.12).

### Genetic analyses of collected colonies

Genotyping the brood of six colonies (five inferred matricide, one observed matricide) shed light on the sex of the queen-laid brood when the queen died (Table 1). In the two collected colonies, all males sampled from the first cohort of males possessed alleles in common with the queen at all loci. This



**Fig. 2** Colony comb allocation and the timing of queen death. The relative area of combs with different cell types reflects how colonies invest in the production of new males and queens. Colonies often rear males in worker cells as well, making this index an approximate and queen-biased representation of overall sex allocation. Arrows indicate the inferred matricide timing in colony development, in terms of cell construction (Table 1). Green/light gray arrows indicate queens known to be laying females at time of death (determined by genotyping brood;

see Methods). Blue/dark gray arrows indicate queens who had switched to male production before dying (the question mark indicates that the timing of matricide within the male phase is unknown). Black arrows indicate observed matricides for which the sex of the queen’s last brood was unknown. Colony 82\_13, marked with an asterisk, was the only queenright colony with single paternity (Loope 2015). It was also the only queenright colony to have barely initiated the reproductive stage, suggesting it may yet have experienced matricide were it not collected

demonstrates that the queen was alive when the switch to male production occurred, since only approximately three such males were expected if the workers had produced all of these males. In three other collected colonies, most males possessed at least one allele not shared with the queen, indicating they were the sons of workers. In two of the colonies, the number

of all-queen-allele males was close to or less than the number predicted if workers produced all of these males (colonies 83\_13 and 68\_12), suggesting that the queen died before male production began. In one colony, 47\_12, 5 males had all-queen alleles, while only 2.38 were expected if workers laid all of these males; the probability of observing 5 or more such

**Table 1** Genotypes reveal sex of the queen-laid brood at the time of matricide

Colony	No. of broods genotyped	No. of males	No. of informative loci <sup>b</sup>	No. of observed males with all queen alleles	<i>p</i> value <sup>c</sup>	Implication: queen last laid
65_13	20	20	3	20	0.00	Males
81_13	24	24	3	24	0.00	Males
47_12	19	19	3	5	0.079	Females? <sup>d</sup>
68_12	20	20	2	3	0.91	Females
83_13	18	18	3	3	0.39	Females
09_11 <sup>a</sup>	24	0	–	–	–	Females

<sup>a</sup> Matricide was observed directly in this colony

<sup>b</sup> Informative loci are those at which the male mated to the queen has an allele different from both queen alleles, allowing detection of worker-laid brood at that locus

<sup>c</sup> The probability of observing at least this many males with all queen alleles, assuming all workers are workers’ sons

<sup>d</sup> This result is ambiguous; *p* is not low enough to conclusively reject the hypothesis that males are all workers’ sons, though it is possible that a few of the five males with all queen alleles are queens’ sons

males if they were all workers' sons is 0.079 (Table 1). The result is therefore ambiguous: it is possible that the queen laid a few male eggs before dying, or alternatively, the workers may have produced all of the sampled males and the excessive number of queen alleles may be due to chance.

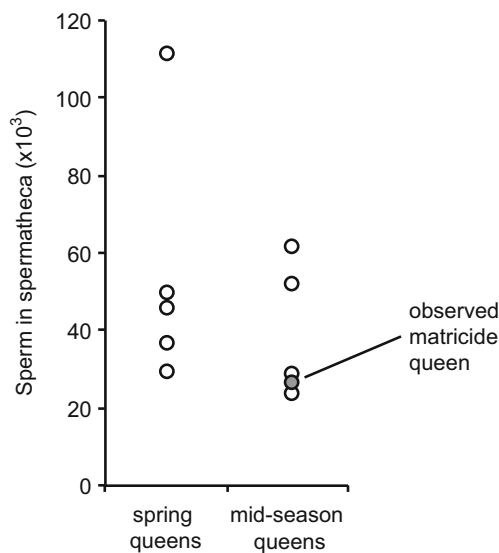
In the observed matricide colony 09\_11, all genotyped young brood from the fourth comb were diploid, indicating that the colony had started a queen-production phase and the queen was killed while laying females (Table 1).

### Spermatheca contents in observed matricide queen 25\_12

The killed queen from colony 25\_12 had ~26,600 sperms in her spermatheca, within the range of other queens collected in 2012 (Fig. 3).

### Surgery experiments

All eight surviving spermathectomy queens were observed laying eggs within a few days of surgery. These observations included seeing the actual egg after oviposition, typically in the shallow cells at the edge of a lower comb. However, two of the eight queens were subsequently observed pumping their abdomens in an unusual manner and partially everting the sting chamber. This eversion was also observed during attempted ovipositions in which these two queens failed to lay an egg. Thus, while these queens did lay some eggs, their egg-laying capacity was reduced after surgery. This behavior was not seen in any other queens.



**Fig. 3** Stored sperm counts for *D. arenaria* queens. Spring queens (those collected on the wing in May and early June) and mid-season queens (those collected inside reproductive-stage colonies in July) had thousands of stored sperm in their spermathecae (white circles). The queen from the observed matricide colony 25\_12 (gray circle) had approximately 26,600 stored sperm

Crucially, matricide was not observed in any of the experimental colonies, even though the eight spermathectomy and five sham surgery queens all survived for at least 21 days after surgery (Table 2). Video observations revealed matricide in 3 of 18 unmanipulated colonies, and 2 additional unobserved queen deaths may have been the result of matricide as well.

### Discussion

The goal of this study was to see if male-laying by queens triggers matricide.

### Queen sex investment and inferred matricide in collected colonies

The data do not support the prediction that workers preferentially kill queens who have switched to male production. This prediction stems from the fact that workers value females highly, so the cost of killing a queen is much higher if she is still producing females than if she is not. In the colonies for which the first males produced were all or nearly all workers' sons (68\_12, 83\_12 and likely 47\_12; Table 1), I interpret this as the death of a female-laying queen, rather than a change in worker reproduction or policing, because there were no female pupae beyond the transition point. If the transition from female production to male production reflected workers reproducing alongside the queen, we would expect there to be some queen pupae among the males on the comb periphery or at least a substantial number of queen-laid males. Therefore, it is probable that the queen was laying queen-destined females when she died in at least three (likely four) of the six queen deaths for which there are data, including the observed-matricide queen with genotyped offspring (Table 1). Furthermore, two of the ten queenright colonies were male specialists (Fig. 2; see also Greene et al. 1976), suggesting that a male-laying queen does not trigger matricide. The latter result can be explained since these colonies had effective paternities near 2.0 (Loope 2015), giving little or no relatedness advantage to replacing brothers with nephews. But, why would workers kill queens producing new queens in low-effective paternity colonies when they are highly related to those new queens? One hypothesis is that workers lack accurate information on the sex of the queen-laid brood, particularly in the egg or young-larvae stages, though they eventually are able to distinguish large male larvae from females because they build cells of intermediate size around male larvae (Greene et al. 1976). Given the high cost of making an error when killing the queen, the lack of accurate information could prevent facultative matricide based on brood sex, even if cues were available that correlate with brood sex.

**Table 2** Outcome of surgery experiments

Treatment	Queens survived	Observed matricides	Unobserved deaths
Spermathectomy	8 <sup>a</sup>	0	0
Sham-surgery	5	0	0
Unmanipulated <sup>b</sup>	15	3	2

<sup>a</sup> Two of these queens had difficulty laying eggs. All survived for >21 days

<sup>b</sup> These observation colonies are reported in Loope (2015)

Another possible explanation is that the strategy of delaying matricide until the queen switches to male production may not be stable if queen behavior co-evolves with worker behavior. If matricides were triggered by the switch to male laying, individual queens could benefit from delaying the switch beyond the point where it is favorable to workers, making killing of female-laying queens beneficial earliest in single-paternity colonies, resulting in the observed facultative matricide based on paternity but not sex allocation. This would be compounded by a relatively low reproductive value of new queens if non-matricide colonies then continue to specialize on queen production, as appears to occur (Fig. 2). Furthermore, female reproductive value is already reduced, given that workers in queenless colonies likely produce a large fraction of males in the population (Pamilo 1991). Even in the extreme case where all males are the sons of workers and there is no conflict over sex allocation, Bourke's (1994) model still predicts a lower threshold for killing male-producing queens, but the reduced reproductive value of females may mean that the threshold is low enough that it also pays to kill queens who are still producing new gynes. No accurate data exist for the fraction of the population's males that are worker-derived, but given the number of queenless colonies in nature and the relative rarity of male-specializing queenright colonies (Fig. 2), the fraction must be substantial. This hypothesis could explain the absence of facultative matricide in response to queen sex allocation but clearly suggests that a formal co-evolutionary game-theoretic model of queen and worker strategies would be helpful in clarifying the predictions regarding the timing of matricide.

Regardless of why workers ignore the sex of the queen's offspring when committing matricide, the outcome appears to be split sex ratios in the direction opposite the classic pattern (Boomsma and Grafen 1990; Boomsma and Grafen 1991; Sundström 1994; Sundström et al. 1996): high-effective paternity colonies produce mostly queens, and low-effective paternity, queenless colonies produce mostly males (Fig. 2; Loope 2015). However, the estimates of sex investment are approximate: they are based on measurements of comb area instead of energetic investment, and they do not include males that are regularly reared in worker cells (Bonckaert et al. 2011b). Furthermore, they do not reflect the fact that the reproductive stage is long enough that a point sample does not accurately capture total sex investment, particularly for colonies collected early in the summer. This being said, the investment

patterns in reproductive cells are strongly bimodal, and these patterns undoubtedly correlate with overall sex allocation. A similar pattern of higher female investment by lower-relatedness/higher-effective paternity colonies occurs in another yellowjacket, *V. maculifrons* (Goodisman et al. 2007; Johnson et al. 2009), though the pattern is among queenright colonies. This species is always highly polyandrous (average effective paternity is ~5) and thus is not predicted to have matricide, and the few queenless mature colonies sampled had high effective paternity (Johnson et al. 2009). Thus, it appears that different selection pressures are probably responsible for the effects of effective paternity on sex investment in these two species.

### Experimental manipulation of queen-laid sex ratio

The results of the spermathectomy experiment also suggest that a queen laying only male eggs does not trigger matricide in this species. This is in some ways a better test of the hypothesis than the correlational data from collected colonies, because the experiment guarantees queens are not laying female eggs in the upper, worker-cell combs; this likely occurs in male-specializing queenright colonies in the reproductive stage (Greene et al. 1976; Greene 1984). However, this experimental advantage is mitigated by the fact that there may be unknown factors in observation colonies that reduce the incidence of matricide: I only observed 3 matricides in 18 unmanipulated colonies (Loope 2015), though 2 additional queen deaths occurred but were not observed on video. This is a substantially lower fraction (17–28 %) than the 42 % of mature, queenless colonies observed in nature (Loope 2015). Regardless, the absence of matricide in manipulated colonies is consistent with observational data from collected colonies, suggesting that workers do not monitor and respond to the sex of the queen-laid brood.

Although it was not confirmed, using genetic or other methods, that the offspring laid by spermathectomy queens were all male, it is likely that such queens were unable to produce daughters following the experiment. It is theoretically possible that queens retained some sperm in their reproductive tract for a short while after spermatheca removal and thus were perhaps still capable of laying female eggs, but observations in other species suggest this is unlikely. It is well documented that female Hymenoptera have clear behavioral control over



the sex of individual offspring in a variety of species (Gerber and Klostermeyer 1970; Cole 1981; Suzuki et al. 1984; Strand 1989; Ratnieks and Keller 1998), which would not be possible if sperm from previous fertilizations remains in the oviduct to possibly fertilize subsequent eggs in the absence of additional contributions from the spermatheca. Thus, I interpret the results under the assumption that the experimental manipulation had the intended effect of restricting queens to the production of male eggs.

The spermathectomy experiment did not replicate the results reported by Montagner (1966). This is somewhat surprising, given that *D. arenaria* is known to have matricide (unlike two of the three species used in his study) and because paternity patterns in *D. arenaria* support the relatedness prediction of kin-selected matricide (Loope 2015). There are several possible interpretations. It is possible that there are species differences in this behavior, though *D. arenaria* seems like a species most likely to exhibit responses to male-laying queens: this species is unusual for yellowjackets in that it has naturally occurring queens that exhibit irreversible male-production phases (Greene et al. 1976; Archer 2006). Alternatively, it is possible that the male-laying queens Montagner reported as killed by the workers instead died from the irradiation treatment that caused the male-egg laying and were mistakenly observed being dismembered and removed from the colony. It is difficult to know exactly what occurred in Montagner's experiments, as the brief report provides no details on how these queens were killed or how this killing was observed.

Finally, the observation that a worker-killed queen in colony 25\_12 had ample sperm in her spermatheca (Fig. 3) suggests that queen killing is not triggered by queens switching to male production because of sperm depletion, a plausible hypothesis for why some queens make this switch. Although sperm use patterns in *D. maculata* suggest that some queens may be sperm-limited (Stein and Fell 1996), there is no evidence that the male production phases of *D. arenaria* result from queens running out of sperm, as queenright male specialist colonies had queens with abundant stored sperm (Greene et al. 1976).

### Comparisons to other species

Several aspects of the reproductive biology of *D. arenaria* are unusual for vespine wasps but are remarkably similar to the bumblebee *B. terrestris*, in which conflict over male production has been studied extensively (Duchateau and Velthuis 1988; Bloch 1999; Bloch and Hefetz 1999; Bourke and Ratnieks 2001; Duchateau et al. 2004; Zanette et al. 2012). First, *D. arenaria* queens appear to have a critical switch point, after which they produce only males, at least in reproductive cells (Greene et al. 1976; this study). To my knowledge, this does not occur in other *Dolichovespula*, with the possible exception of *D. norwegica* (Akre and Myhre 1992;

Akre and Myhre 1994; Archer 2006); instead, other species appear to simultaneously produce both males and queens in large reproductive cells. In two species of *Vespa*, a genus with reports of matricide (Bourke 1994), colonies produce males before females, though their production overlaps extensively (Martin et al. 2009). In contrast, the abrupt switch by *D. arenaria* queens from diploid to haploid egg-laying mirrors the switch point that occurs in the annual colonies of *B. terrestris* (Duchateau et al. 2004). Furthermore, like *B. terrestris* (Bourke and Ratnieks 2001; Duchateau et al. 2004), *D. arenaria* exhibits split sex ratios resulting from an early or late switch to male production in newly constructed combs (even when considering only queenright colonies; Fig. 2; Greene et al. 1976).

Despite these similarities, worker-queen conflict unfolds differently in these two species. Unlike in *B. terrestris* (Bloch 1999; Bourke and Ratnieks 2001), *D. arenaria* workers begin to lay eggs prior to the switch point (KJL, personal observation; also, see description of matricide colony 20\_10 in Loope 2015). Furthermore, although *B. terrestris* workers have been observed to harass and eventually kill the queen after the switch point (van Doorn and Heringa 1986; van der Blom 1986; Bourke 1994), *D. arenaria* workers sometimes kill queens who are still laying queen-destined eggs (Table 1). Worker reproduction in *B. terrestris* does not interfere with queen production, suggesting low costs of conflict for selfish workers (Lopez-Vaamonde et al. 2003), while matricide by workers in *D. arenaria* sometimes stops the production of new queens (Fig. 2; Table 1). Thus, despite many similarities in colony development, the conditions resulting in matricide are not particularly convergent in these two species, beyond the fact that matricide occurs in the reproductive stage and predominantly in colonies with low effective paternity (Schmid-Hempel and Schmid-Hempel 2000).

### Conclusion

Both correlative and experimental evidence suggest that matricide in *D. arenaria* is not a facultative response to the switch to male production by queens, counter to theoretical prediction (Bourke 1994) and a previous study in related species (Montagner 1966). Future theoretical studies should consider the co-evolution of queen reproductive strategies with worker matricide behavior. A detailed, quantitative study of individual worker fitness in this species would be useful to understand how the benefits of matricide outweigh the likely sizeable costs workers experience from killing their queen-producing mothers.

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