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Original article

Reproductive parameters of female *Varroa destructor* and the impact of mating in worker brood of *Apis mellifera*

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Abstract – During a reproductive cycle, not all daughter mites of *Varroa destructor* mate and thus leave the brood cells as virgins. Here, we show that virgin mites are present within both the phoretic (10%) and reproductive (8%) mite population. Most ($n = 29$ of $n = 33$) of these encountered virgins laid unfertilized (= male) eggs, and some ($n = 10$) mated later on with their own son. These findings were verified by tests with artificially reared virgin mites. Obviously, mating is not a prerequisite for *Varroa* reproduction. However, due to the small number of reproductive cycles, the contribution of virgins to the *Varroa* population is regarded as low. This study also confirms conclusively that sex of *V. destructor* is determined via arrhenotokous parthenogenesis and not—as previously assumed—via pseudo-arrhenotoky. Furthermore, reproductive parameters of naturally invaded and artificially introduced *Varroa* females were compared, and artificial infestation was reconfirmed as a suitable method.

***Varroa* / Reproduction / Mating / Spermatozoa / Virgin females**

1. INTRODUCTION

The ectoparasite *Varroa destructor* (Anderson and Trueman 2000) is the predominant threat for apiculture worldwide (Genersch et al. 2010; Rosenkranz et al. 2010; Dietemann et al. 2013). The life cycle of *V. destructor* is divided into two phases: the commonly called phoretic phase on adult honey bees and a reproductive phase inside the sealed brood cell. Here, "phoretic" is not used according to the *sensu stricto* definition (Houck and OConnor 1991), as the *Varroa* mite consumes components of the fat body (Ramsey et al. 2019a, b) from adult bees. The reproductive phase is limited to the capping period of the honey bee host. In *Apis mellifera* (Linnaeus, 1758) worker brood cells, the *Varroa* foundress is capable of producing up to five eggs per brood cycle (Martin 1994). The first egg is haploid and develops into a male mite, while the following eggs are diploid and develop into female mites (Garrido and Rosenkranz 2003; Martin 1994, 1995). After the adult molt, the offspring becomes mature and mates inside the sealed brood cell (Rosenkranz et al. 2010). Mating is triggered by a female sex pheromone (Ziegelmann et al. 2013a, b)

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perceived by the male via the sensory pit organ on the front leg tarsi (Häußermann et al. 2015). During multiple mating, male mites transfer a total of 30 to 40 roundish spermatozoa into the genital opening of each adult daughter (Alberti and Hänel 1986; Donzé et al. 1996; Ziegelmann and Rosenkranz 2014). These spermatozoa are stored inside the spermatheca of female mites and have to undergo a further maturation process, the so-called capacitation, before being able to fertilize oocytes (Alberti and Hänel 1986). During capacitation, a spermatozoon drastically changes its cell shape from roundish to fusiform taking about 5 days (Häußermann et al. 2016). Only adult daughter mites are able to survive outside the brood cell in contrast to younger and more sensitive stages like protonymphs and deutonymphs. Likewise, male mites die soon after hatching of the honey bee host (Rosenkranz et al. 2010).

Beside *Varroa* females producing several eggs per brood cycle, infertile female mites that do not produce any offspring during one brood cycle are a common phenomenon. Such infertility rates vary between 5 and 20% in European honey bees (Martin 1994, 1995; Martin et al. 1997; Rosenkranz 1999; Garrido and Rosenkranz 2003; Garrido et al. 2003; Locke and Fries 2011; Frey et al. 2013; Alattal et al. 2017). The reasons for infertility are still controversial. One of the main questions is whether infertility is caused by the extrinsic factor of the honey bee host or by the intrinsic factor of the parasite itself. Differences in the infertility rate of mites in different honey bee subspecies support the hypothesis of an extrinsic factor (Rosenkranz and Engels 1994; Rosenkranz 1999) as well as the fact that the infertility rate depends on the age of the host larvae (Frey et al. 2013). Both De Ruijter (1987) and Weller (2008) showed that currently infertile mites were able to reproduce if they were transferred into new brood cells, also supporting extrinsic factor(s). Another extrinsic factor that is supposed to affect the infertility rate is the type of phoretic host that was chosen by the mites before brood cell invasion: nurse bees seem to increase the mite fitness (and fertility) in contrast to new bees or foragers (Xie et al. 2016). On the other hand, intrinsic factors seem to be likewise important: Fuchs (1994) compared the infertility rate of *Varroa* mites within the same colony but with different genetic background of the honey bee brood and concluded that the status of the mite is crucial for the infertility rate. In this context, it was frequently supposed that infertile mites are primarily unmated *Varroa* mites. First De Ruijter (1987) and later Martin et al. (1997) claimed that unmated *Varroa* females were not able to produce offspring. Likewise, Harris and Harbo (1999) assumed that infertility is based on unmated females. This was, however, not supported by Kirrane et al. (2011) who could not prove a correlation between reproductive success and the lack of sperm cells. This demonstrates that the relevance of mating of female mites for the further reproduction and the consequences for the *Varroa* population dynamic have not yet been analyzed satisfactorily.

In numerous species, especially in insects, interactions between semiochemicals from male and female are required to start oogenesis (Wolfner 2009). For instance, in the malaria mosquito *Anopheles gambiae*, a male steroid hormone that is transferred during mating interacts with a female protein provoking the start of oogenesis (Baldini et al. 2013). In *Varroa* mites, so far, only a host stimulus from freshly capped brood cells has been confirmed as a trigger for mite's oogenesis (Garrido and Rosenkranz 2003).

Until now, it is not known whether the mating status of *V. destructor* also has an influence on the start of oogenesis. In several acarine taxa, however, mating is not a requirement for a later reproduction. Instead, reproduction by virgin females (parthenogenesis) is a common phenomenon (Oliver 1971; Kiszewski et al. 2001). In certain cases, e.g., the northern fowl mite (McCulloch and Owen 2012), the two-spotted spider mite (Tuan et al. 2016) or the rice tarsonemid mite (Xu et al. 2001) virgin females are able to produce male offspring and even reproduce bisexual offspring after mother-son mating, so-called oedipal mating (McCulloch and Owen 2012). As sex determination in *V. destructor* is regulated via haplodiploidy, it was examined if unmated *Varroa* females might be able to produce haploid (male) offspring without prior mating as well (Martin 1997). If this is feasible, one would expect

unmated female mites being part of both the phoretic and the reproductive mite populations. To analyze this aspect, the reproductive capacity of over 1000 *Varroa* females naturally and artificially infesting honey bee worker brood was analyzed. Females with "abnormal" reproduction such as no offspring (= infertile) or just male offspring (= haploid) were identified in a subset of these brood cells, and their genital tract was analyzed for the presence of spermatozoa. Later on, the results of the females with abnormal reproduction were compared with the reproduction capacity of artificially reared virgin females.

2. MATERIAL AND METHODS

All mites were collected in *A. mellifera* colonies from the Apicultural State Institute during the seasons of the years 2014–2016 at the University of Hohenheim, Germany.

2.1. Dissection of the genital tract of phoretic mites and analysis of spermatozoa

Phoretic *Varroa* females were sampled via the powdered sugar method (Dietemann et al. 2013). Briefly, honey bees were collected from brood combs and put in a special plastic vessel with a mesh that let mites pass but not honey bees. In the laboratory phoretic *Varroa* female mites were dissected, and their genital tract was checked for the presence of spermatozoa.

For dissection, a stereo-zoom microscope (VWR: SZT 100, magnification ×30) was used. Mites were dissected with DUMONT 5 tweezers in phosphate-buffered saline (PBS, ingredients: 1.000 ml H_2O, 8 g NaCl, 0.2 g KCl, 1.25 g $Na_2HPO_4{\cdot}2H_20$, and 0.2 g KH_2PO_4) on top of an object plate. To dissect the genital tract, the ventral shields of *V. destructor* were carefully opened. The intestines were removed, and the spermatheca and rami were isolated. A cover glass was put on the dissected genital tract; then, it was analyzed under a VWR TR 500 light microscope (magnification 100–400). Spermatozoa were counted, and stages were diagnosed according to Häußermann et al. (2016). The pictures were taken with a Canon EOS 60 D camera. In total, 382 phoretic mites from 13 honey bee colonies were analyzed.

2.2. Reproductive parameters of *Varroa* females in honey bee brood cells

2.2.1. Reproductive parameters

Mite-infested brood cells (Sects. 2.2.2 and 2.2.3) were opened, and the numbers and stages of the offspring of the *Varroa* mite were documented. For differentiation of mite sex and stage, see Dietemann et al. (2013).

The following reproductive parameters were recorded:

- *Fertility*: proportion of female mites with at least one offspring
- *Fecundity*: total number of eggs laid per mite. Infertile mites also contribute to fecundity. This parameter does not allow any prediction of the number of successful mated daughter mites.
- *Presumed successful reproduction*: fertile mites that produced at least one living adult male offspring and one living female offspring likely to develop to a mated adult female during the remaining capping period. As brood cells were analyzed 1–3 days before hatching of the bee, only deutonymph, deutochrysalis, or adult daughter mites were considered to develop to a mated adult female.
- *Abnormal reproduction*: female mites without any offspring (= infertile mites) and mites with male offspring only (= encountered virgins; see Sect. 2.2.4)

2.2.2. Naturally infested brood cells

To analyze naturally infested brood cells with *Varroa* mites, honey bee brood combs with host broods in the correct age were examined. The brood comb was brought into the lab, and single brood cells were opened and checked for host worker brood in the correct age and the presence of *Varroa* mites. The age of the honey bee brood was estimated according to Human et al. (2013).

The age of bee brood had to be at minimum 9 days after cell capping, meaning that the eyes of the bee pupae had to be at least dark purple with a light yellow abdomen (Human et al. 2013). At this point in time, the mother mite has laid all the eggs and the adult male is present (Dietemann et al. 2013). In total, we analyzed the reproductive parameters of over 600 worker brood cells from 17 honey bee colonies.

2.2.3. *Artificial introduction of Varroa* mites into brood cells

For artificial introduction of honey bee brood cells with female *V. destructor*, only freshly capped brood cells were used. Brood cells containing L5 worker larvae were marked on transparency sheets. Four hours later, the hitherto-sealed brood cells were used for the experiment. Each brood cell was opened with a scalpel at one edge of the wax cap, and recently collected phoretic *Varroa* mites, which were sampled via the powdered sugar method, were introduced one by one into one honey bee brood cell with a paintbrush; thereafter, the brood cell was closed again (Dietemann et al. 2013). The brood comb was put back into in the honey bee colony for incubation. These artificially infested brood cells were analyzed 10 to 12 days post-capping. In total, we analyzed over 400 worker brood cells from nine honey bee colonies.

2.2.4. *Mating status of Varroa* females with abnormal reproduction

From $n = 402$ brood cells described in Sects. 2.2.2 and 2.2.3, we analyzed 60 mites which displayed signs of abnormal reproduction, like *Varroa* foundresses that were infertile (no offspring) or produced male offspring only. From these mites, we checked the genital tract (= spermatheca and rami) for the presence and developmental stage of spermatozoa (Häußermann et al. 2016, Figure 5). Depending on this developmental stage, one can determine whether the respective female mite has been mated within the current (= by her own son) or during the previous reproductive cycles (= by her brother). As the spermatozoa maturation inside the female genital tract (the so-called spermatozoa capacitation process) takes about 5 days, the presence of capacitated VII spermatozoa indicates that these mites have been mated in the previous reproductive cycles. If younger stage spermatozoa (e.g., I to II spermatozoa) are present, this indicates that the female mated within the current reproductive cycle and can thus be considered as virgin mite (= encountered virgin).

2.3. Artificially reared virgin female mites

The following five steps were performed to analyze the reproductive capacity of artificially reared virgin mites (Figure 1).

2.3.1. *Collection of deutochrysalis*

First of all, female deutochrysalis were collected from infested honey bee brood cells. A "semi in vitro" method that is described in Häußermann et al. (2016) was used to artificially rear virgin female mites starting with deutochrysalis. Substitution for the host brood cell was perforated Eppendorf tubes closed with gauze. In every tube, one bee pupa (purple eyes) was placed, five female deutochrysalis and one adult marked female mite. The adult female mite was marked with a blue marker to distinguish it later from the adult virgin females. The older mite was put into the tubes to facilitate piercing the host. These tubes were put into a special frame inside the honey bee hive for incubation.

2.3.2. *Check for virgin mites*

Two days after collection of the deutochrysalis, the tubes were checked for the presence of living virgin adult females. A new host pupa was put in the tube together with three adult virgin mites and one marked female mite. In the control group we additionally put five adult male mites into the tube. The tubes were put back in the hive for a further 3 days.

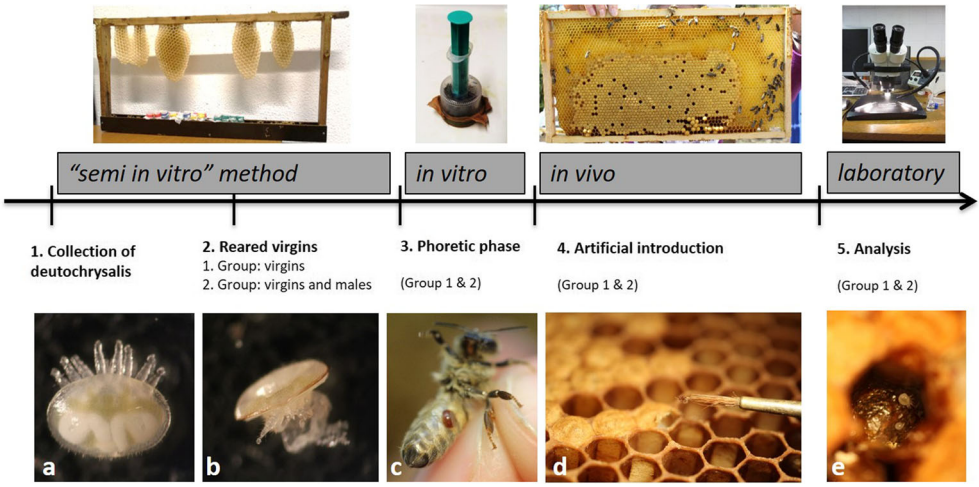

Figure 1. Summary of experimental setup to analyze the reproductive capacity of artificially reared virgin *Varroa* females. Starting with female deutochrysalis (1), virgins were reared artificially and kept "semi in vitro" in tubes in a special frame inside the bee hive (2). During this period, a control group of females was able to mate as male mites were put into the tubes as well. Thereafter, a phoretic phase was simulated on caged adult honey bees in vitro (3). Subsequently, virgin and control mites were artificially introduced into freshly capped brood cells (4). Reproduction and the presence of spermatozoa in female mites were analyzed 11 days post-capping inside the laboratory (5).

2.3.3. Simulation of a phoretic phase

For the simulation of the phoretic phase, virgin and control mites were put on caged adult host bees in the incubator at 30 °C with a relative humidity of about 60% for 3 days. In total, 15 bees were put in one plastic vessel and fed with ApiInvert sugar solution ad libitum. With a fine brush, we infested some honey bees with a total of eight adult virgin mites or eight control mites, respectively.

2.3.4. Artificial introduction into brood cells

Afterwards, the mites were singly introduced into freshly capped brood cell and the comb was put back into the honey bee hive for incubation (see Sect. 2.2.3).

2.4. Analysis of reproductive capacity and dissection of the genital tract

The analysis of the reproductive capacity of the mites was performed 11 days after introduction into the capped brood cells (see Sect. 2.2.1). The genital tract of the introduced "mother" mites was analyzed to ensure the presence (= artificially reared control mite) or absence (= artificially reared virgin mite) of capacitated spermatozoa (= stage VII) in rami and spermatheca (see Sect. 2.1).

2.5. Statistical analysis

Descriptive statistics were calculated in Excel 2010. Mean values are stated as mean ± standard deviation (SD) or standard error (SE). The statistic software SPSS (Vers. 24.0) and SAS (Vers. 9.4) were used for data set analysis. Categorical data were analyzed by chi^2 tests (fertility and presumed successful females). To analyze the mean number of offspring (fecundity), a generalized linear mixed model (GLIMMIX) with colonies as a random factor and assuming the Poisson distribution and using a log link function was used. Differences between groups with $\alpha < 0.05$ were considered statistically significant. For calculation of mean values and categorical data (for naturally and artificially infested brood cells), each colony was considered as a replicate.

3. RESULTS

3.1. Dissection of the genital tract of phoretic mites

In total, 382 phoretic mites were sampled with powdered sugar and their genital tract was analyzed for the presence of spermatozoa inside rami and spermatheca. The mean percentage of phoretic mites without spermatozoa was 9.7% (n = 382, Figure 2).

3.2. Reproductive parameters of *Varroa* females in honey bee brood cells

3.2.1. *Varroa* mites naturally and artificially infesting honey bee brood cells

The rates of fertile mites (number of females with at least one offspring) did not differ between *Varroa* females naturally (91.9% ± 6.7 SD, n = 17 colonies and n = 636 single infested brood cells) and artificially infesting honey bee brood cells (89.5% ± 6.1 SD, n = 9 colonies and n = 446 single infested brood cells; chi^2 2.75; P = 0.097; Figure 3).

However, fecundity rates (mean number of total offspring per infested brood cell) were significantly higher in naturally infested brood cells (3.6 ± 0.4 SD, n = 17 colonies and n = 636 single infested brood cells) compared with artificially infested ones (3.2 ± 0.4 SD, n = 9 colonies and n = 446 single infested brood cells; $F_{1,\ 1058}$ = 10.98, P < 0.001, GLIMMIX). Besides, the parameter "colony" (= random effect) had an impact on the fecundity rate of both groups (covariance 0.004 ± 0.003 SE; GLIMMIX).

Also, the percentage of presumed successful reproduction (at least one living adult male offspring and one living female offspring in the stage of deutonymph, deutochrysalis, or adult daughter) was significantly higher in naturally infested brood cells (62.5% ± 9.3 SD, n = 17 colonies and n = 636 single infested brood cells) compared with artificially infested ones (49.4% ± 15.4 SD; n = 9 colonies and n = 446 single infested brood cells; chi^2 10.4; P = 0.001; Figure 3).

In both approaches, the main reason for unsuccessful reproduction was the absence of a adult male offspring (Table I) followed by the absence of offspring at all, the absence of a prospective adult female, and delayed reproduction (= no adult male and no prospective adult female).

3.2.2. *Varroa* females with abnormal reproduction

From a subset (n = 402) of the brood mites from Sect. 3.2.1, (i) infertile *Varroa* females (n = 16) and (ii) exclusively male producing female mites (n = 44) were dissected and their genital tract was analyzed for the presence of spermatozoa (n = 60). From the 16 infertile *Varroa* females, 75% (n = 12) had capacitated stage VII spermatozoa in their genital tract with an average of 30.2 (± 21.6 SD) and a range of 9 to 81 spermatozoa. The presence of capacitated stage VII spermatozoa clearly indicates that these mites have been mated in a previous reproductive cycle with their brother and for this reason are not virgins (Häußermann et al. 2016).

From the 44 fertile *Varroa* females that exclusively produced male offspring, 15 (34.1%) did have capacitated stage VII spermatozoa in their genital tract. The remaining 29 fertile females must have invaded the honey bee brood cell without previously mating and are therefore considered as virgin mites. Together with the 4 infertile mites without spermatozoa (see above), a total of 33 mites invaded their brood cells as virgin females corresponding to 8.2% of the total analyzed mites (n = 402). Interestingly, 29 of these 33 "encountered virgins" were fertile (87.9%; Figure 4). The mean fecundity rate was 1.7 (± 1.0 SD) male offspring per foundress (n = 33). In total, the encountered virgins produced 21 eggs, 11 protonymphs, and even 23 adult male mites. Oedipal mating by their own son occurred in 34.5% of the fertile mites that invaded the brood cell as virgin female (n = 10 of a total of n = 29), determined by the presence of stage I to II spermatozoa in the genital tract of the respective females. On average, these female mites received 10.5 (± 6.6 SD) spermatozoa through the mating with their own son; most of the spermatozoa (81.0%) had not even reached the spermatheca and were therefore still located inside the rami (Figure 5). This indicates that mating took place

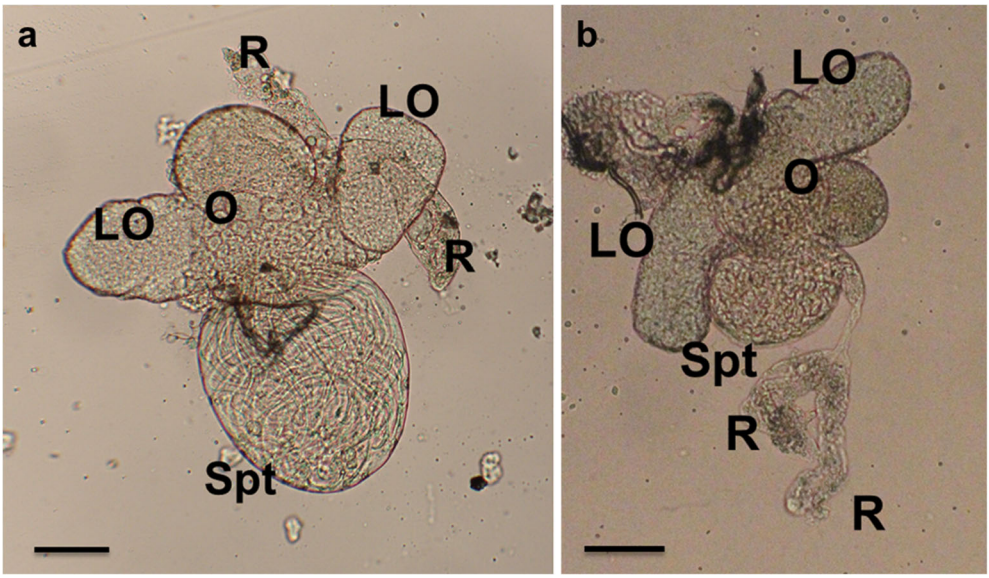

Figure 2. Genital tract of female *V. destructor* (**a**) with and (**b**) without spermatozoa inside the spermatheca. Inside the spermatheca (**a**), several fully capacitated stage VII spermatozoa are visible (arrow). *Spt* spermatheca, *R* ramus, *LO* lyrate organ, *O* ovary. Scale bar is 100 μm.

just several hours ago as spermatozoa migration into the spermatheca takes in most cases (over 70%) 1 day (Häußermann et al. 2016).

3.3. Reproductive capacity of artificially reared virgin female mites

In total, 1525 female deutochrysalis were collected, but only 20% of the female deutochrysalis developed into an adult female that could be transferred for 3 days into cages with adult honey bees (n = 307). Due to the still high mortality, only 115 of these female mites were vital after this procedure and could be introduced into freshly capped brood cells. At the time of inspection of these brood cells 11 days after the artificial infestation, the brood of 31 cells was removed (27%), 15 brood cells were found without the mother mite (13%), 31 brood cells had dead mother mites (27%), and one brood cell was multiple infested and therefore discharged from the analysis. Finally, the reproductive parameters could only be analyzed in 23 virgin mites and 14 mated control mites (reared under the same artificial conditions as the virgin mites).

From 23 virgin females, 17 were fertile (73.9%) whereas the mated control mites had a slightly lower fertility rate (8 from 14 mites; 57.1%,) which did not differ significantly from the virgin group (chi^2 1.12; P = 0.291). The mean fecundity of the fertile virgin females was 1.4 ± 1.0 (SD; n = 23) offspring per foundress compared with 1.6 ± 1.9 (SD; n = 14) offspring per foundress in the mated control mites. The virgin females produced exclusively male offspring (12 eggs, 14 protonymps, 3 deutonymphs, and 2 adult male mites). One of these adult males mated with the virgin mother confirmed by 15 stage II spermatozoa inside her genital tract. The mated control mites produced five protonymphs, seven female deutonymphs, seven adult daughter mites, and four adult male mites.

4. DISCUSSION

We present here for the first time a broad analysis of the presence and reproductive capacity of virgin female *Varroa* mites. We could confirm a high rate of about 10% of virgin females without spermatozoa in the phoretic mite population. This

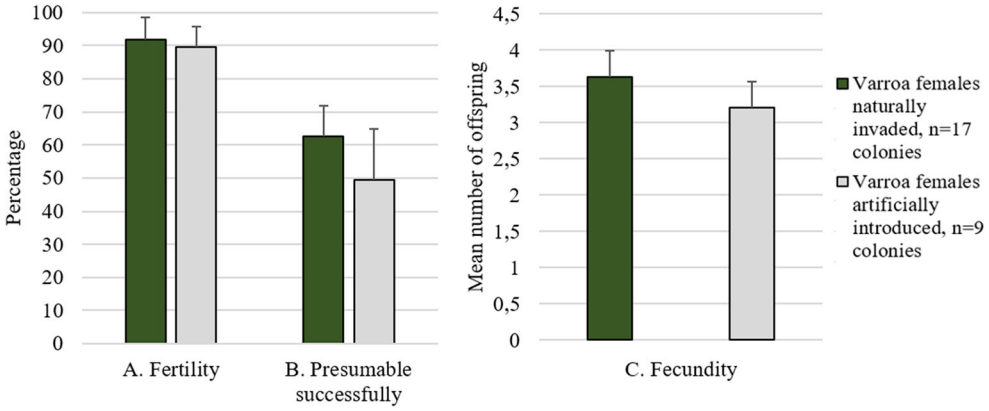

Figure 3. Reproduction parameters of *V. destructor* females naturally invaded versus artificially introduced in honey bee brood cells; each colony was considered as a replicate. **a** Statistical analysis revealed no significant difference in the percentage of fertile (= reproducing) mites (chi^2 2.75; $P = 0.097$, $n = 1082$). The fertility rate of *Varroa* females naturally infesting honey bee brood cells was 91.9% ± 6.7 SD ($n = 17$ colonies and $n = 636$ single infested brood cells) and for artificially infesting honey bee brood cells 89.5% ± 6.1 SD ($n = 9$ colonies and $n = 446$ single infested brood cells). **b** Significant differences were found in the percentage of presumed successful reproducing mites (chi^2 10.4; $P = 0.001$, $n = 1082$). The percentage of presumed successful reproduction (at least one living adult male offspring and one living female offspring in the stage of deutonymph, deutochrysalis or adult daughter) was 62.5% ± 9.3 SD in naturally infested brood cells ($n = 17$ colonies and $n = 636$ single infested brood cells) and 49.4% ± 15.4 SD in artificially infested ones ($n = 9$ colonies and $n = 446$ single infested brood cells). **c** Significant differences were confirmed for fecundity ($F_{1, 1058} = 10.98$, $P < 0.001$, GLIMMIX $n = 1082$). Fecundity (mean number of total offspring per infested brood cell) was 3.6 ± 0.4 SD in naturally infested brood cells ($n = 17$ colonies and $n = 636$ single infested brood cells) and 3.2 ± 0.4 SD in artificially infested ones ($n = 9$ colonies and $n = 446$ single infested brood cells).

result contrasts with earlier results of Garrido and Rosenkranz (2003), which could not find any phoretic female mite without spermatozoa inside their genital tract ($n = 59$). However, the rate is similar to the 7% unmated females of Wendling et al. (2014, $n = 30$). As shown in our study and several others (Martin et al. 1997; Garrido and Rosenkranz 2003; Kirrane et al. 2011; Wendling 2014), unsuccessful reproduction—i.e., the production of at least one mated daughter mite—was caused mainly by the lack of an adult male offspring. Interestingly, in most of these brood cells, an adult but unmated daughter mite is present meaning that such unmated female mites should be abundant within the *Varroa* population. As Garrido and Rosenkranz (2003)—based on a small sample size—did not find such unmated daughters in the phoretic mite population, they assumed that these females are short-living and disappear quickly from the population. Our new results, however, verified that virgin female *Varroa* mites are part of both the phoretic and

Table I. Main causes for unsuccessful reproduction in honey bee brood cells naturally or artificially infested with *Varroa* females

Group	No offspring at all (= infertile)	No adult male	No prospective adult female	No prospective adult female and no adult male
Naturally with *Varroa*-infested brood cells, $n = 237$	16.9% (n = 40)	48.5% (n = 115)	18.1% (n = 43)	16.5% (n = 39)
Artificially with *Varroa*-infested brood cells, $n = 210$	19.0% ($n = 40$)	36.7% ($n = 77$)	16.7% ($n = 35$)	27.6% ($n = 58$)

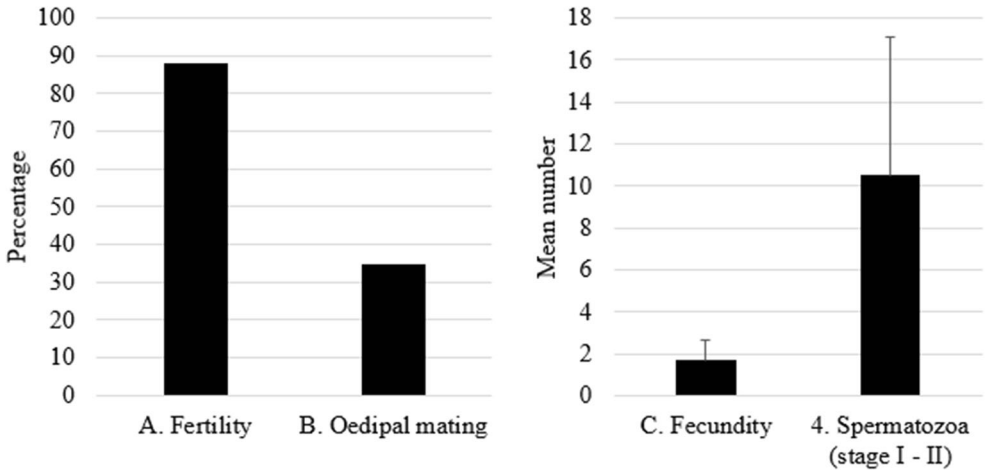

Figure 4. Reproductive parameters from *Varroa* mites that invaded brood cells as virgin females ($n = 33$). **a** Fertility rate of the encountered virgins was 87.9% ($n = 33$). **b** Oedipal mating by their own son occurred in 34.5% of the fertile mites that invaded the brood cell as virgin female ($n = 10$ of a total of $n = 29$), determined by the presence of stage I to II spermatozoa in the genital tract of the respective females. **c** The mean fecundity per foundress was just 1.7 (± 1.0 SD) male offspring ($n = 33$). **d** On average, the female mites that mated with their own son (oedipal mating, $n = 10$) received 10.5 (± 6.6 SD) spermatozoa.

the reproductive mite populations. On the basis of our results, we can theoretically calculate the proportion of unmated female mites leaving the brood cells. Approximately 40–50% of the fertile *Varroa* females do not reproduce successfully (Figure 3), of which about 45% could be referred to females without male offspring (Table I). Using this rough calculation, we come up with theoretically 18 to 23% unmated female brood mites which is in the range of the portions given by Martin et al. (1997) as well as Wendling et al. (2014). This is higher than the determined 10% unmated females in the phoretic population (Sect. 3.1) indicating that a certain proportion of

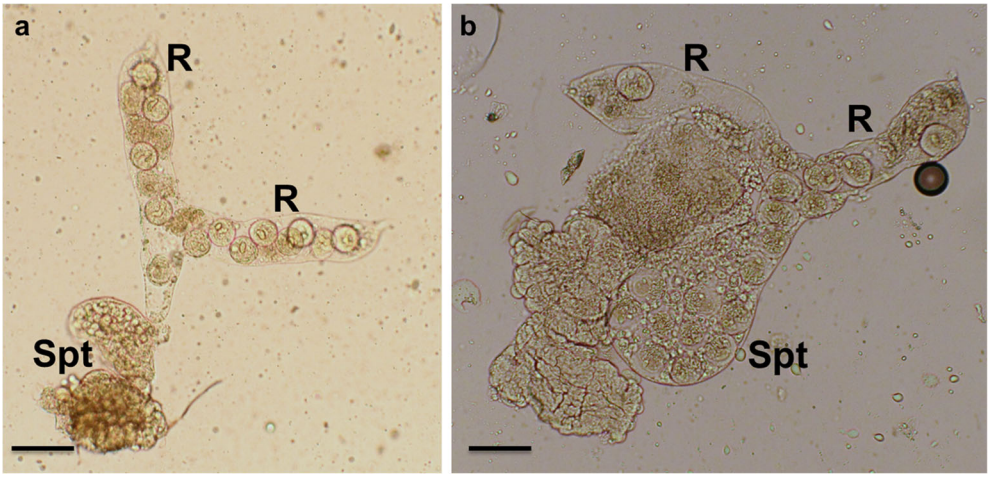

Figure 5. Rami and spermathecae from *Varroa* mites that invaded brood cells as virgin females. The stage II spermatozoa that are present in the rami only (**a**) or in rami and spermatheca (**b**) must derive from mating during the past 2 days (Häußermann et al. 2016). During this time period, the son was the only male present inside the sealed brood cell. *Spt* spermatheca, *R* ramus. Scale bar is 100 μm.

unmated daughter mites disappear from the population. The remaining phoretic virgin female mites seem to invade to a high proportion honey bee brood cells. It has even been shown that the fertility rate of the virgin females (both encountered and artificially reared virgin) was similar to the fertility rates of mated females (from artificially and naturally infested brood cells), although the mean fecundity rate was lower and restricted to produce male mites. Thus, our results confirm the hypothesis that virgin *Varroa* females can produce haploid male offspring which are even capable of developing into adult males. In addition, our study confirms conclusively that sex of *V. destructor* is determined via arrhenotokous parthenogenesis because female mites can definitively produce offspring without previously mating.

During the normal *V. destructor* reproductive program, up to five eggs in the worker brood and up to six eggs in drone brood are laid (Martin 1994; Garrido and Rosenkranz 2003). Usually, the first egg is haploid end develops into a male mite. Encountered and artificially reared virgin *Varroa* females in our experiment often produced more than one male egg; however, the fecundity was significantly reduced. Obviously, virgin females stop reproduction at an earlier time point than their mated sisters. Garrido and Rosenkranz (2003, 2004) as well as Frey et al. (2013) showed that *Varroa* reproduction is triggered by host stimuli. Moreover, Frey et al. (2013) demonstrated that host signals could even trigger a stop of mite reproduction. However, our results also raise the question whether the mating status—i.e., an empty spermatheca—can contribute to an early end of mite oogenesis. Irrespective of the lower fecundity of unmated mites, our results show unambiguously that neither mating nor the number of spermatozoa is crucial for the start of oogenesis and reproduction. About 75% of the infertile *Varroa* females had spermatozoa inside their genital tract which agrees with results from a study of Kirrane et al. (2011) in colonies with VSH behavior. Moreover, several encountered virgin *Varroa* females produced male offspring. Independently from a successful mating, young adult female daughters had a slightly higher infertility rate if one prevents a phoretic phase prior to the reproductive cycle (Häußermann et al. 2016). This is supported by Mondet et al. (2018) showing that the transcriptome profile of young female mites differs significantly from that of phoretic mites indicating their physiological immaturity. Therefore, the maturation of spermatozoa in the genital tract of young female mites might be an intrinsic factor that influences the mite fertility in this case. However, the main causes of infertility remain cryptic, but we can definitively exclude the lack of spermatozoa as a main factor.

Interestingly, including all cases of oedipal mating by the encountered virgins, almost 35% of these virgins had "young" spermatozoa (exclusively stages I and II) in their rami or spermatheca (n = 10 from a total of n = 29). Stage I and II spermatozoa are roundish spermatozoa stages that can only be found in the genital tract of female mites 1 to 2 days after mating. Moreover, the fact that most of the spermatozoa were located inside the rami indicates that mating took place shortly before dissection. Earlier, we could show that most (over 70%) spermatozoa migrate from the rami to the spermatheca within 1 day after mating (Häußermann et al. 2016). During this period, the own son was the only available male inside the sealed honey bee brood cell, and hence, he must be the source of these spermatozoa. On average, male mites transferred about 11 spermatozoa during oedipal mating. This is significantly less than the numbers given in literature for "normal" mating which vary between a total of 30 to 40 spermatozoa (Alberti and Hänel 1986; Donzé et al. 1996; Ziegelmann and Rosenkranz 2014; Häußermann et al. 2018). However, the genital tract of *Varroa* females of our study was analyzed starting at 9 days after brood capping. There were still 1 to 3 days left before hatching of the host bee and therefore time for further mating with their own son.

As mating of *Varroa* mites is triggered by a sex pheromone and attractivity of the female mites decreases already 24 h after the adult molt (Ziegelmann et al. 2013a, b), oedipal mating was expected to be rare events. If male mites have the choice, they clearly prefer freshly molted female mites for mating (Donzé et al. 1996; Ziegelmann and Rosenkranz 2014). In behavioral experiments, it was observed that mother mites not only move faster than freshly molted daughter mites but also turn

away from male mites that try to mount the female's dorsum for mating (Ziegelmann and Rosenkranz 2014). So far, it has not been analyzed whether unmated older female mites change their behavior to facilitate mating. Despite the relatively low number of transferred spermatozoa, oedipal mating might be an adaptive strategy for virgin *Varroa* female mites to successfully reproduce in a next reproductive cycle. Oedipal mating can also be observed in other mites where mating of a virgin female with the son leads to bisexual offspring (Xu et al. 2001; McCulloch and Owen 2012; Tuan et al. 2016).

An additional aspect of our study was the comparison of reproductive parameters of *Varroa* females naturally and artificially infesting honey bee brood cells. Altogether, over 1000 honey bee brood cells infested with *Varroa* females were analyzed. Fertility rates of both, artificially and naturally with *Varroa* -infested brood cells in our study, were similar with values over 89% which is within the fertility range of 80 to 95% in naturally reproducing *Varroa* females in temperate climates (Martin et al. 1997; Garrido et al. 2003; Fries et al. 2011; Frey et al. 2013). Furthermore, the mean number of offspring in our study was within the range of three to four offspring per fertile mother mite in worker brood in temperate climates (Fries et al. 2011; Locke and Fries 2011), but slightly higher in *Varroa* females naturally infesting honey bee brood than in *Varroa* females artificially introduced. However, the absolute number of offspring in both groups was very similar with on average less than 0.5 more offspring in the naturally infested mite group. Moreover, not only the fixed effect (naturally or artificially infested) but also the random effect (colony) seems to have an impact on the results. The percentage of presumed successful mites (at least an adult male and one female offspring, deutonymph, or adult) was significantly higher in naturally versus artificially infested brood cells. But, in both groups, over 49% of female mites produced at least an adult male and one female offspring (deutonymph or adult). Lack of reproductive success was in both approaches mainly due to the lack of an adult male offspring (> 36%) similar to earlier data (Martin et al. 1997; Garrido and Rosenkranz 2003; Kirrane et al. 2011; Wendling 2014).

Altogether, our results show that artificially introduced *Varroa* females can reproduce to a similar rate as naturally invaded mites which may justify that the method is included in standard methods for *Varroa* research (Dietemann et al. 2013). As artificial infestation includes handling and physiological manipulation of *Varroa* females, some differences in the reproductive parameters are not surprising. Despite everything, all reproductive parameters of the artificially introduced *Varroa* females were very similar to those of naturally invaded *Varroa* females. If these differences—mainly for the fecundity—are considered, artificial infestation of *Varroa* mites into honeybee brood cells can be considered a suitable method to analyze reproductive parameters of *V. destructor*.

As it is not possible to distinguish virgin female mites from females that run out of spermatozoa, we additionally tried to analyze artificially reared virgin females. Rearing virgin females was a real challenge of our study. In some—mostly free living—mite species rearing methods have successfully established (Jones et al. 1988; Jagersbacher-Baumann and Ebermann 2012). However, for *V. destructor*, there exists no standard method for rearing under laboratory conditions (Bruce et al. 1988; Dietemann et al. 2013; Tabart et al. 2013). There are only limited reports of rearing *Varroa* mites (Donzé and Guerin 1994; Nazzi and Milani 1994). Up to now, it is almost impossible to rear the complete *V. destructor* life cycle in vitro. In particular, the rearing of sensible mite stages (like nymphal stages or male mites) is only possible with a high rate of individual losses. For rearing virgin females, a semi in vitro method was used starting from the deutochrysalis stage. Even with the inclusion of natural host stages, the mortality rates were extremely high and discouraging which were also experienced by Martin et al. (1997). Fortunately, at least some of the artificially reared virgin females could be introduced into honey bee brood cells and some of these mites were able to produce offspring. Therefore, also these results support our initial hypothesis that virgin female mites are able to produce haploid males. Most of the virgin mite offspring were either eggs or protonymphs indicating a major delay in mite development or delay in egg laying as brood cells were analyzed 11 days after infestation. This

phenomenon was also observed in the encountered virgins sampled from honey brood cells. Certainly, artificial rearing of female mites still needs improvement (Dietemann et al. 2013; Häußermann et al. 2016).

Our study clearly confirms that a previous mating with spermatozoa transfer is not required to start oogenesis and the production of male offspring. This also gives a final answer to the mode of sex determination in *Varroa* which has been discussed for many years (Sabelis and Nagelkerke 1988; Martin et al. 1997). Sex in *V. destructor* is determined via haplodiploidy: males are haploid, and females are diploid. This system also is called arrhenotokous parthenogenesis. However, since Martin et al. (1997) state that unmated females do not reproduce at all, based on just 12 unmated females that did not produce any offspring, it was assumed that sex determination in *Varroa* mites works according to pseudo-arrhenotoky (Sabelis and Nagelkerke 1988). In a pseudo-arrhenotoky system, the sexes are—as in the more common arrhenotoky—determined by haplodiploidy, but female mites need to be fertilized before they can produce offspring. Our results now finally confirm that in *Varroa* mites, sex is determined via arrhenotokous parthenogenesis, because female mites can definitively produce offspring without a previous mating. *Tropilaelaps mercedesae*, another parasitic honey bee mite, likely does not need mating to produce offspring, and unmated females are even capable to produce mature daughters via deuterotoky (Gunzman et al. 2018). While deuterotoky in *Tropilaelaps* might have a huge impact on the population growth, it is questionable to what extent in *Varroa destructor* unmated females contribute to the population growth. As the number of the reproductive cycles of *Varroa* females under natural condition is considered to range between two and three (Fries and Rosenkranz 1996; Martin and Kemp 1997), an unmated female mite needs one of these cycles to mate with her son which reduces her chance for reproduction to one or two reproductive cycles. Therefore, unmated females might only contribute to *Varroa* population growth in a minor scale.

Our study provides new insights into a thus far underestimated aspect of *Varroa* reproduction. The results again demonstrate that the parameter triggering the success of *Varroa* reproduction and therefore mite population dynamic is still poorly understood.

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Paramètres de reproduction des *Varroa destructor* femelles et importance de l'accouplement dans les couvains des ouvrières de *Apis mellifera*.

***Varroa* /reproduction /accouplement /spermatozoïdes /femelles vierges.**

Reproduktive Parameter der weiblichen Varroamilben und die Bedeutung der Paarung in Arbeiterinnenbrutzellen von *Apis mellifera*.

***Varroa* / Reproduktion / Paarung/ Spermatozoen / unbegattete Weibchen.**

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