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Elevated Mating Frequency in Honey Bee (Hymenoptera: Apidae) Queens Exposed to the Miticide Amitraz During Development

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Abstract

Most honey bee (*Apis mellifera* Linnaeus, 1758) (Hymenoptera: Apidae) colonies in the United States have been exposed to the beekeeper-applied miticides amitraz, coumaphos, and *tau*-fluvalinate. Colonies are also often exposed to agrochemicals, which bees encounter on foraging trips. These and other lipophilic pesticides bind to the beeswax matrix of comb, exposing developing bees. We explored whether queen-rearing beeswax containing pesticides affects the reproductive health of mated queens. We predicted that queens reared in pesticide-free beeswax would have higher mating frequencies and sperm viability of stored sperm compared with queens reared in wax containing pesticides. Mating frequency and sperm viability are two traditional measurements associated with queen reproductive health. To test these hypotheses, we reared queens in beeswax-coated cups that were pesticide free or contained field-relevant concentrations of 1) amitraz, 2) a combination of *tau*-fluvalinate and coumaphos, or 3) a combination of the agrochemicals chlorothalonil and chlorpyrifos. We then collected queens once they mated to determine sperm viability, using a dual fluorescent cell counter, and mating frequency, genotyping immature worker offspring at eight polymorphic microsatellite loci. Sperm viability did not differ between control queens and those reared in pesticide-laden wax. However, queens exposed to amitraz during development exhibited higher mating frequency than queens reared in pesticide-free beeswax or beeswax containing the other pesticide combinations. Our results suggest that miticide exposure during development affects queen mating frequency but not sperm viability, at least in newly mated queens. This finding, which has practical implications for commercial queen rearing and overall colony health, calls for further study.

Key words: honey bee, polyandry, mating frequency, queen, pesticide

A landmark survey of commercial beekeeping operations in North America found that nearly all of the 250+ colonies sampled exhibited beeswax that was contaminated with pesticides, some at alarmingly high concentrations (Mullin et al. 2010). Among the 10 pesticides found in highest frequency and abundance were the miticides *tau*-fluvalinate, coumaphos, and amitraz, which were found in 98.1, 98.1, and 60.5 of sampled colonies, respectively (Mullin et al. 2010). Almost a decade later, Ostiguy et al. (2019) obtained similar results after conducting pesticide residue analysis of colonies from six apiaries in different parts of the United States, with coumaphos, fluvalinate, and amitraz products being among the highest detected pesticides in wax in the two different years that the study was conducted. Furthermore, the authors found many co-occurring pesticides in beeswax and pollen, including miticides, which creates the opportunity for many of these compounds to interact synergistically to cause sublethal effects to honey bees (Ostiguy et al. 2019). However, despite the real-world scenario of exposure, whereby bees commonly encounter several pesticides simultaneously, many studies have only examined the effects of individual pesticides in isolation from others. A relatively newer trend in the field of honey bee toxicology has been to examine pesticides that are commonly found in conjunction with each other to gain a more realistic view of pesticide exposure (Johnson et al. 2013a, Traynor et al. 2016), thereby taking hazard risks into consideration.

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Beekeepers typically apply miticides directly to their colonies as a way to manage the ectoparasitic mite, *Varroa destructor*, which feeds on the fat bodies of developing and adult honey bees (Ramsey et al. 2019). *Varroa* mites are the leading drivers of colony mortality in the United States, causing death within 2 yr if infested colonies are left untreated (Guzmán-Novoa et al. 2010, Kulhanek et al. 2017, Steinhauer et al. 2018). In addition, the fungicide chlorothalonil and the insecticide chlorpyrifos, which are agricultural pesticides widely applied to cultivated crops, were found in 49.2 and 63.2% of the colonies sampled by Mullin et al. (2010), respectively. These highly lipophilic pesticides persist and accumulate in the beeswax comb over long periods of time, creating an opportunity for contaminated wax to deliver sublethal doses of multiple pesticides to developing and adult bees. Exposure can be through contact during development, or through consumption of contaminated brood food, or both (Wu et al. 2011). In general, colonies that are exposed to sublethal doses of these and other common bee-encountered pesticides cause delays in worker larval development and adult emergence, in addition to decreased longevity (Wu et al. 2011).

Pesticide contamination of commercial honey bee colonies has been correlated with high colony losses and major queen mortality events in the Eastern United States (Traynor et al. 2016). In particular, queens exposed to wax containing *tau*-fluvalinate and coumaphos during development are smaller and have shorter lifespans than nonexposed queens (Haarmann et al. 2002, Collins et al. 2004, Pettis et al. 2004). More recently, queens reared in wax-coated cups containing field-relevant concentrations of *tau*-fluvalinate, coumaphos, and amitraz were shown to attract smaller worker retinues and had lower egg-laying rates than queens reared in pesticide-free wax (Walsh et al. 2020). Additionally, queens that were exposed during development to wax containing either amitraz (Burley 2007) or a combination of *tau*-fluvalinate and coumaphos (Rangel and Tarpy 2015) had lower spermatozoa viability in their sperm storage organ (i.e., the spermatheca), compared with queens reared in pesticide-free beeswax. Despite these results, queens reared in wax containing a combination of *tau*-fluvalinate and coumaphos were not smaller in size compared with queens reared in pesticide-free wax, even though small body size has traditionally been considered a proxy for poor queen quality (Rangel and Tarpy 2015).

Sexually mature drones exposed to *tau*-fluvalinate, coumaphos, and amitraz during development were shown to have lower spermatozoa viability compared with unexposed drones (Fisher and Rangel 2018). Likewise, adult drones that are exposed to miticides either topically (by brushing their exocuticle with the pesticide) or orally (by feeding them contaminated food) were also shown to have lower spermatozoa viability once they reached sexual maturity compared to unexposed drones (Rinderer et al. 1999, Burley 2007, Shoukry et al. 2013), which begs the question of whether poor mating by queens is in part due to mating with poor quality drones.

In addition to causing individual toxicities, agrochemicals such as fungicides can act synergistically with other pesticides, increasing the lethality of other miticides such as *tau*-fluvalinate and coumaphos (Johnson et al. 2010, 2013a,b; Sanchez-Bayo et al. 2016; Steinhauer et al. 2018). In particular, the fungicide chlorothalonil and the insecticide chlorpyrifos are often applied together in the United States via tank mixes to control pests and pathogens in agricultural crops. Tank mixing of these products is legal and has likely contributed to the application of ~4.75–6.5 million pounds of chlorpyrifos, and 10–11.5 million pounds of chlorothalonil per year in the United States between 2011 and 2017 in overlapping geographic regions (United States Geological Survey 2017). Both chlorothalonil and chlorpyrifos have combinatorial detrimental effects on honey bee health when mixed with other pesticides, sometimes by as much as doubling their lethality (Johnson et al. 2010, 2013a,b; Sanchez-Bayo et al. 2016). This may be due, at least in part, to the high synergistic tendencies of fungicides, which greatly increase pesticides' hazard quotients when bees are exposed to combinations of other products (Sanchez-Bayo et al. 2016, Traynor et al. 2016). For instance, Zhu et al. (2014) found a nearly 30% decrease in survival of larvae after oral exposure to a combination of chlorothalonil and coumaphos, and up to a 71% decrease in survival after oral exposure to a combination of *tau*-fluvalinate and chlorpyrifos. Moreover, sexually mature drones that were reared in wax containing chlorothalonil and chlorpyrifos had significantly lower spermatozoa viability compared with unexposed drones (Fisher and Rangel 2018).

The ubiquitous presence of pesticides in comb is particularly troubling for the health and fitness of honey bee queens, which are typically produced by workers in special wax cells under two circumstances: during supersedure, when the workers replace the mother queen with a new, more vigorous queen, and during swarming, when a colony splits into a swarm colony headed by the mother queen while the remnant colony stays in the old nest and is headed by a new daughter queen (Winston 1987). Queen development from egg to adult takes approximately 16 d, after which a new queen emerges from her pupal cell to roam around the hive for several days until she begins to take orientation flights to learn the landmarks around the hive (Winston 1987). At 5–10 d post-emergence, the queen undertakes nuptial flights to drone congregation areas (DCAs), where thousands of drones gather for a chance to mate. Upon reaching a DCA, drones flock to the queen and mate with her in midflight. Queens typically return to their hives after mating with an average of 12–16 drones from nearby colonies (Tarpy et al. 2004), although hyperpolyandry, or extreme mating frequency by queens, has been shown to occur under certain circumstances (Delaplane et al. 2015, Withrow and Tarpy 2018). High genetic variability obtained through polyandry is considered a fitness gain for the colony, as it is associated with many advantages including greater resistance to pathogens (Seeley and Tarpy 2007), lower *Varroa* mite infestations (Delaplane et al. 2015), and higher colony productivity and better survival (Mattila and Seeley 2007). Furthermore, polyandry increases the queen's attractiveness to caretaking workers (Tarpy 2003, Richard et al. 2007, Seeley and Tarpy 2007, Niño et al. 2012).

After successful mating, a queen's spermatheca can hold over 5 million spermatozoa collected from her drone mates, which she uses to fertilize 1,000–1,500 eggs that she lays every day throughout her 1- to 3-yr lifespan (Winston 1987). As the queen ages and uses up the sperm, each remaining spermatozoon has more space to uncoil and move longitudinally, albeit more slowly, than the spermatozoa in newly mated queens (Al-Lawati et al. 2009). If the spermatheca of a recently mated queen contains three million or fewer spermatozoa, that queen is considered to be poorly mated and is typically replaced by workers within weeks or months (Woyke 1962, Woyke et al. 1995, Cobey 2007). Interestingly, little is known about the effects of pesticide exposure during queen development on the number of drones that a queen mates with, or the viability of the spermatozoa that the queen stores in the spermatheca after mating.

In this study, we exposed queens during development to wax contaminated with field-relevant concentrations of *tau*-fluvalinate, coumaphos, amitraz, chlorpyrifos, and chlorothalonil. We then assessed the viability of spermatozoa in the adult queens' spermathecae and inferred their offspring's paternity to assess each queen's mating frequency. We found that spermatozoa viability remained consistently high in all queen types, regardless of whether they were reared in pesticide-free or pesticide-laden beeswax. Surprisingly, however, we found that the observed and effective mating frequencies of queens reared in wax containing amitraz exceeded those of queens

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reared in miticide-free beeswax. Queens reared in beeswax contaminated with a combination of chlorothalonil and chlorpyrifos were not different from control queens in terms of the observed or effective mating frequency, however. Our findings could have major implications not only for queen health, but also for colony-level productivity, as intracolony genetic diversity can affect resistance to disease and may cause other health challenges in colonies managed by commercial beekeepers.

Materials and Methods

Bee Source and Queen Rearing

The colonies used in this study were kept at the Janice and John G. Thomas Honey Bee Facility of Texas A&M University in Bryan, TX (N 30°38′31.037″W 96°27′39.495″) and the Windy Hill Apiary in Watertown, WI (N 43°7′9.79″W 88°44′30.562). All colonies were headed by queens of Italian descent obtained from Olivarez Honey Queens Inc. (Orland, CA) in the summers of 2016 and 2017.

Experimental queens were reared by transferring first-instar worker larvae into JZBZ Honey Co. plastic cups (Santa Cruz, CA) using a standard queen-rearing procedure known as 'grafting' (Laidlaw and Eckert 1964, Rangel and Tarpy 2015, Walsh et al. 2020). Each plastic cup had been previously coated with approximately 200 mg of molten beeswax. To do this, certified pesticide-free wax pellets (Koster Keunen Inc., Watertown, CT) were melted and the molten wax was either kept untreated or was mixed separately with one of the following: 1) a combination of 204,000 ppb of *tau*-fluvalinate and 91,900 ppb of coumaphos (>98% purity, Thermo Fisher), 2) 43,000 ppb of amitraz (>98% purity, Sigma–Aldrich), or 3) a combination of 9,800 ppb of chlorpyrifos and 53,700 ppb of chlorothalonil (>98% purity, Thermo Fisher). These pesticides and their concentrations were chosen based on their reported frequency and highest concentration in wax samples collected from commercial beekeeping operations in North America (Mullin et al. 2010). In instances where there was a significant amount of a degraded compound detected (e.g., DMPF and DMA) in Mullin et al. (2010), the total detections of both products were added to determine our experimental dose.

All wax-coated cups with grafted larvae were placed into queenless units of bees known as 'cell builders' (Laidlaw and Eckert 1964), where nurse bees cared for queens during development. All cell builders used had grafts from all treatment groups. Although the wax in the cell builders was not tested for pesticide contamination, the cell builders were created using bees previously housed on frames that were at most 2 yr old, had been kept in a nonagricultural area, and had never been subjected to miticide use. There was an approximate 50% initiation rate in grafts from JZBZ cups coated with the treatment wax, although the person performing the grafting typically had a 85–95% initiation rate success in uncoated JZBZ cups. Two to 3 d before the expected queen emergence, each capped cell was put into a queen-holding cage and placed inside a queenless five-frame mating nucleus colony (mating 'nuc') containing approximately 1,000 workers, two frames containing brood of varying ages, one frame containing nectar and pollen, one empty frame, and one frame feeder with sugar syrup (1:1 sugar:water) for bees to feed ad libitum. Upon emergence, the queens were marked, released from their cages, and allowed to mate naturally (Winston 1987). Successful queen mating was verified for every experimental queen by examining the mating nuc for the presence of the queen and/or eggs at least 10–15 d after she was released into the mating nuc, whereupon each queen was introduced to an observation colony for a separate study (Walsh et al. 2020). A 10 cm × 10 cm sample of capped brood was harvested from each mating nuc and stored at −20°C to conduct microsatellite analysis on each queen's female offspring. The offspring of six queens each in the control, *tau*-fluvalinate and coumaphos, and chlorothalonil and chlorpyrifos groups were collected, in addition to the offspring from seven of the queens in the amitraz group.

Spermatozoa Viability Analysis

All queens used for spermatozoa viability analysis were reared in 2016 in Texas and Wisconsin and were also sampled for microsatellite analysis. Upon collection the queens were anesthetized by freezing at −20°C for 3–5 min, and then decapitated, a procedure that was done to carry out a separate but complimentary study (Walsh et al. 2020). We dissected each queen's spermatheca, carefully removed the tracheal net surrounding it, and placed it into a vial containing 100 µl of saline solution (0.24 g of HEPES, 0.88 g of NaCl, and 1 g of bovine serum albumin in 100 ml of deionized water). The spermatheca was ruptured with forceps, and the contents were mixed via tube inversion. To determine the viability of spermatozoa stored in the spermatheca, we used an Invitrogen Live/Dead Sperm Viability Kit (catalog number L7011, Life Technologies, Carlsbad, CA) containing Syber-14, which dyes viable spermatozoa green, and propidium iodide, which dyes nonviable spermatozoa red (Collins and Donoghue 1999, Fisher and Rangel 2018). Briefly, 40 µl of diluted spermatozoa collected from the ruptured spermathecae was placed into a new vial and stained with 3 µl of Syber-14 solution (4 µl of Syber-14 in 196 µl of 0.1 M phosphate buffered saline [PBS]) and 3 µl of propidium iodide solution (derived from diluting 50 µl of propidium iodide in 50 µl of PBS). Vials were then gently vortexed for approximately 2 s at 2,000 rpm to homogenize the samples and the dyes while avoiding damage of the spermatozoa. The homogenization was followed by a dark incubation period of 8 min to ensure uptake of the dyes. We assessed viability by placing 20 µl of the dyed spermatozoa into a Nexcelom Cellometer (Nexcelom Biosciences LLC, Lawrence, MA) cassette. To estimate the numbers of live and dead spermatozoa per slide, we used the Cellometer Vision Software (v. 2.1.2.1) with exposure settings at 1,200 ms for Syber-14 and 7,000 ms for propidium iodide. For each sample, three counts were performed in different locations on the cassette; the resulting viability percentages were averaged to provide a single viability estimate for each queen.

Mating Frequency Analysis

To estimate each queen's mating frequency, we conducted microsatellite analysis from capped brood samples taken from queens reared in Texas in 2017. To do this, approximately 100 worker pupae were harvested from each frozen 10 cm × 10 cm comb section previously collected, which were individually placed into microtubes. Genomic DNA was extracted from each whole body with the Gentra PureGene Tissue Kit using a modified PureGene protocol (Gentra Systems Inc., Minneapolis, MN). These modifications included vortexing each sample at 2,000 rpm rather than inverting the tubes to avoid damage, and using reagent volumes appropriate for 2 mg of tissue, which was sufficient for a bee pupa. DNA concentrations were measured using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) and standardized per sample.

To calculate the mating frequency of each queen, we performed fragment analyses on two multiplex polymerase chain reactions (PCRs) that amplified eight polymorphic microsatellite loci, as done previously (Rangel and Tarpy 2015). The 7.5-µl aliquot for each multiplex PCR included 0.1 µl of Taq polymerase (Bioline Meridian

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Bioscience, London, United Kingdom), 1.5 µl of the accompanying 5× buffer solution (Promega, Madison, WI), and 130 ng of template DNA. The first multiplex reaction consisted of the microsatellites *Am052*, *Am533*, *Am061*, and *Am010* (Estoup et al. 1994, Tarpy et al. 2012). The second multiplex reaction consisted of the microsatellites *Am043*, *Am125*, *Am098*, and *Am059* (Estoup et al. 1995, Garnery et al. 1998, Solignac 2003). All primers, which had been previously designed for use in *Apis mellifera* L. studies (Estoup et al. 1994, 1995; Garnery et al. 1998; Solignac 2003; Tarpy et al. 2012), were fluorescently labeled with 6-FAM, HEX, or NED (New England Biolabs, Ipswich, MA). The multiplex reactions were initially denatured at 95°C for 7 min before running 30 cycles of 30 s at 95°C, followed by 30 s at either 55°C (multiplex 1) or 54°C (multiplex 2), and 30 s at 72°C. The final extension step was 10 min at 72°C for both multiplexes.

We genotyped the resulting PCR products using an ABI3500 Genetic Analyzer (Applied Biosystems, Life Technologies, New York, NY) along with a GeneScan 500 LIZ dye size standard (Applied Biosystems, Life Technologies). We scored each allele with the microsatellite plugin GENEious v6.18 (Kearse et al. 2012). We then used the 'observed mating frequency' (N_o) and subfamily proportions for each mated queen obtained with the software COLONY 1.2 (Wang 2012) to calculate the 'effective mating frequency' (m_e) of the queen's offspring (Nielsen et al. 2003; Tarpy et al. 2004, 2015). The observed mating frequency refers to the total number of drone fathers represented in a queen's worker progeny. The effective mating frequency uses the proportion of each subfamily within a colony and compensates for calculating potentially skewed estimates of paternity (i.e., unequal subfamily proportions in sampled pupae) and intracolony genetic relatedness (Nielsen et al. 2003; Tarpy et al. 2004, 2015). Any individuals without a minimum of six loci amplified were excluded from our analyses, for a total of 78–96 individual offspring analyzed per queen.

Statistical Analysis

We conducted nonparametric analyses due to nonnormal data distribution as determined by a goodness-of-fit test and low sample size. We used a Wilcoxon test to determine whether queens reared in Wisconsin and Texas (nonrandom location variables) had differing sperm viability and, when no differences were detected (χ^2 = 0.14, P = 0.70), we pooled the samples to compare the proportions of viable and nonviable spermatozoa (i.e., % viability) of queen spermathecal contents across treatment groups. We used COLONY 1.2 (Wang 2012) to calculate the observed mating frequency (N_o). We then used the observed mating frequency and subfamily proportions to calculated the effective mating frequency (m_e), as done previously (Tarpy et al. 2004, 2015; Rangel and Tarpy 2015). We conducted Kruskal–Wallis tests on the sperm viability, observed mating frequency, and effective mating frequency data. The observed and effective mating frequency data were also subsequently analyzed with a nonparametric Wilcoxon pairwise comparison, and we report the resulting Z-scores and P-values for that analysis. All tests were conducted using JMP v.13 (SAS Institute Inc., Raleigh, NC). We present all descriptive statistics as mean ± SEM, and we set the level of significance for all tests at α = 0.05.

Results

Spermatozoa Viability

All queen types, regardless of treatment, exhibited exceptionally high spermatozoa viability, ranging from 98.88 ± 0.55% for the *tau*-fluvalinate and coumaphos group to 99.95 ± 0.03% for the chlorothalonil and chlorpyrifos group (Fig. 1). There were no statistical differences in the viability of spermatozoa stored in the spermathecae of mated queens either between rearing locations (χ^2 = 0.14, P = 0.70) or between treatment groups (χ^2 = 1.71, P = 0.63).

Observed Mating Frequency and Effective Paternity Frequency

We encountered a large range in primer amplification efficiency in the eight microsatellites we examined for paternity analysis in offspring from queens reared in Texas in 2017 (Table 1). For example, we obtained amplification sequences from 91% of the brood samples amplified at the *Am052* locus, but only obtained amplification sequences from 40% of the brood samples amplified at the *Am043* locus. Because of these inconsistencies in amplification success, brood samples that yielded amplification results from fewer than six microsatellites were excluded from the analysis.

Overall, we found a significant difference in observed mating frequency based on the queen-rearing treatment group (Table 1; χ^2 = 10.04, P = 0.02). In particular, queens reared in wax containing a combination of *tau*-fluvalinate and coumaphos had a significantly higher observed mating frequency than those reared in wax containing chlorothalonil and chlorpyrifos (Z-score = 2.17, P = 0.03). Queens reared in wax containing chlorothalonil and chlorpyrifos also had significantly lower observed mating frequencies than those reared in wax containing amitraz (Z-score = −2.34, P = 0.02). Observed mating frequency did not differ either between the brood from control queens and those reared in wax containing amitraz (Z-score = −1.66, P = 0.10) or between the brood from control queens and those reared in wax containing *tau*-fluvalinate and coumaphos (Z-score = 1.87, P = 0.06).

We found an overall significant difference in effective mating frequency based on the queen-rearing treatment group (χ^2 = 11.15, P = 0.01; Table 1). Unexpectedly, queens reared in amitraz-laden wax had significantly higher effective mating frequencies than queens reared in pesticide-free wax (Z-score = −2.79, P = 0.005) or those reared in wax containing chlorothalonil and chlorpyrifos (Z-score = −2.93, P = 0.0003). There were no differences in effective mating frequency between queens reared in

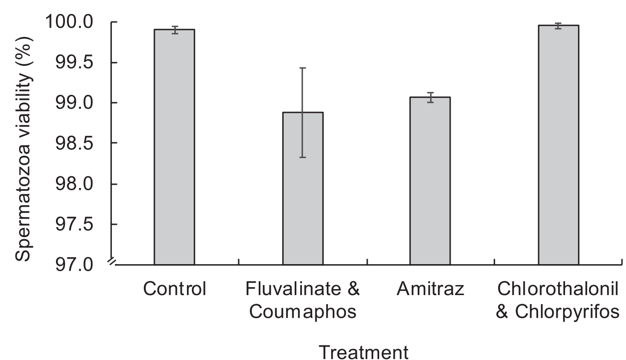

Fig. 1. Mean (± SEM) viability of spermatozoa in the spermathecae of mated queens reared in 2016 in Texas and Wisconsin, which belonged to one of four treatment groups. Queens were reared in plastic cups coated with molten beeswax that was either pesticide free (control group) or contained either (a) a combination of *tau*-fluvalinate and coumaphos, (b) amitraz alone, or (c) a combination of chlorothalonil and chlorpyrifos. Six queens were used per treatment group. See Materials and Methods for details on the queen-rearing process.

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Table 1. The observed and effective mating frequency estimates for honey bee queens in each treatment group

Mating frequency type	Treatment group	*N*	Mean	SEM	χ^2	*P* value
Observed mating frequency (N_o)	Control	6	11.8	1.0	10.04	0.02
	tau-Fluvalinate and coumaphos	6	16.0	2.4		
	Amitraz	7	14.4	0.9		
	Clorothalonil and chlorpyrifos	6	9.5	1.0		
Effective mating frequency (m_e)	Control	6	8.8	0.8	11.15	0.01
	tau-Fluvalinate and coumaphos	6	13.4	2.4		
	Amitraz	7	12.9	0.9		
	Clorothalonil and chlorpyrifos	6	8.2	0.9		

All queens were reared in 2017 at the Texas field location, and the offspring used for microsatellite locus analysis were worker pupae sampled after queen mating success was verified. *N* is the number of colonies tested per treatment group.

wax containing *tau*-fluvalinate and coumaphos and either control queens (*Z*-score = 1.36, *P* = 0.17) or those reared in wax containing chlorothalonil and chlorpyrifos (*Z*-score = 1.68, *P* = 0.09), however. Lastly, queens reared in pesticide-free wax did not have significantly higher effective mating frequencies compared with those reared in wax containing chlorothalonil and chlorpyrifos (*Z*-score = −0.24, *P* = 0.81).

Discussion

In this study, we exposed developing honey bee queens to wax containing either a combination of the miticides *tau*-fluvalinate and coumaphos, the miticide amitraz alone, or a combination of the agrochemicals chlorothalonil and chlorpyrifos. Once those queens were sexually mature, we assessed their mating frequency and the viability of the spermatozoa stored in their spermathecae within a few weeks after mating. We chose to test these pesticides at those specific concentrations and combinations because of their ubiquity in the wax of colonies managed by commercial beekeeping operations across the United States (Mullin et al. 2010, Traynor et al. 2016, Ostiguy et al. 2019). We found no significant differences in spermatozoa viability between queens reared in pesticide-free wax and those reared in any of the pesticide treatment groups. Unexpectedly, however, queens reared in wax containing amitraz had significantly higher effective mating frequencies compared with queens reared in pesticide-free beeswax.

A similar study examining the effects of pesticide exposure on queen mating frequency found that queens reared in wax containing a combination of *tau*-fluvalinate and coumaphos mated with significantly more drones than queens reared in pesticide-free wax (Rangel and Tarpy 2015). The similar results of that study, which was conducted in North Carolina, and our study, done in Texas, support the conclusion that pesticide exposure in the wax matrix can affect the mating behavior of queens regardless of their rearing location. These studies differ somewhat in their findings regarding sperm viability, however. Rangel and Tarpy (2015) found that queens reared in pesticide-free wax had higher sperm viability than queens reared in wax containing *tau*-fluvalinate and coumaphos, which was not the case in our study. This discrepancy could be due to the different methods used to assess sperm viability, as a dual fluorescent microscope was used in Rangel and Tarpy (2015) and a Nexcelom cell counter was used in our study.

The reasons for our unexpected result of higher mating frequency in queens reared in amitraz-laden wax are still unknown. It is possible that queens reared in miticide-contaminated wax mate with more drones because they are compromised physiologically. Perhaps queens exposed to amitraz during development cannot fly at high speeds or altitudes, or are visually impaired (Liberti et al. 2019), both of which may allow more drones to successfully intercept and mate with those types of queens compared with queens reared in pesticide-free wax. Alternatively, queens reared in wax containing miticides may be physiologically unable to detect that they have collected enough semen from drones, and therefore they may take more, or longer, mating flights. Although the mechanisms by which honey bee queens regulate insemination volume is unknown, it has been hypothesized that queens use stretch receptors in their oviducts to provide negative feedback stimuli for further mating attempts, as in other insect systems (Ringo 1996, Richard et al. 2007). In the case of amitraz, this miticide may interfere with the queens' stretch receptors or stretch receptor signaling pathways, potentially leading to queens taking more or longer mating flights, ultimately increasing their mating frequency.

Higher queen mating frequency due to miticide exposure during development could also be a biological form of fitness compensation, in which less reproductively capable queens mate with more males so that they can still head healthy colonies. However, it is unclear whether queens can alter their mating number by regulating the number of mating flights or whether they can assess or control the volume of viable spermatozoa collected from their mates. Hayworth et al. (2009) attached weights to queens and found that heavier queens engaged in fewer mating flights and had lower mating frequencies than queens without weights attached, suggesting that queens alter the number and/or duration of their mating flights in response to energetic demands. More recently, however, Simone-Finstrom and Tarpy (2018) found that weighted queens did not display lower spermatozoa viability or lower effective mating frequencies. The authors speculated that their findings might have differed from those of Hayworth et al. (2009) because of differences in the placement of the weights on the queens' thorax. Furthermore, Schlüns et al. (2005) suggested that the onset of oviposition, as opposed to taking additional mating flights, is governed by mating frequency rather than semen volume. However, other studies have reported that queens can take additional mating flights due to semen volume (Woyke 1962, Kocher et al. 2010), suggesting that spermatozoa volume can sometimes be correlated with other markers of queen quality such as pheromonal signatures or morphological measurements (Kocher et al. 2009, Delaney et al. 2011, Walsh et al. 2020). Interestingly, a study that included measurements from 29 queens found a negative correlation between mating flight duration and spermatozoa volume in the spermatheca, supporting the idea that queens continuously use cues about mating success in flight to adjust the duration of their mating flights (Koeniger and Koeniger 2007).

If miticides are harming the queen by interfering with signaling pathways for stretch receptor function, or are otherwise

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physiologically compromising queens, then other signaling pathways in the nervous system, brain circuitry, and/or reproductive system may also be affected by these pesticides. Additionally, it is unknown whether the higher effective mating frequencies that we found occurred, at least in part, because queens reared in amitraz-laden wax engaged in more or longer mating flights.

Comparing the reproductive behavior of queens exposed during development to ubiquitous agrochemicals may be a fruitful avenue of future research to further explain the findings from all the aforementioned studies. There is still much to discover about the noncorrelative relationships between honey bee queen mating flight behavior, effective mating frequency, semen volume, spermatozoa viability, and oviposition rate (Rueppell et al. 2008, Simone-Finstrom and Tarpy 2018), all of which affect queen reproductive quality and overall colony productivity.

Even though miticide exposure does not always equate with lower spermatozoa viability in mated queens, at least not when they are tested a few weeks after open mating (as was the case in this study), further research on the physiological and behavioral effects of these miticides on queen reproductive physiology is needed. In response to the low initiation rates of grafted larvae into beeswax-coated plastic cups, further studies may be more successful using alternative queen-rearing methods, such as dipping wax onto shaped dowel rods to produce experimental queen-rearing cups. Given that queens reared in wax containing amitraz also had lower egg-laying rates in a separate study (Walsh et al. 2020), research on the prolonged effects of miticide exposure during queen development on colony health deserves high priority in the research community and must be actively communicated to the beekeeping industry so that better pollinator management practices can be implemented, particularly in commercial queen-rearing operations.

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