

Practical Sampling Plans for *Varroa destructor* (Acari: Varroidae) in *Apis mellifera* (Hymenoptera: Apidae) Colonies and Apiaries

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ABSTRACT The parasitic mite *Varroa destructor* Anderson & Trueman (Acari: Varroidae) is arguably the most detrimental pest of the European-derived honey bee, *Apis mellifera* L. Unfortunately, beekeepers lack a standardized sampling plan to make informed treatment decisions. Based on data from 31 commercial apiaries, we developed sampling plans for use by beekeepers and researchers to estimate the density of mites in individual colonies or whole apiaries. Beekeepers can estimate a colony's mite density with chosen level of precision by dislodging mites from ≈ 300 adult bees taken from one brood box frame in the colony, and they can extrapolate to mite density on a colony's adults and pupae combined by doubling the number of mites on adults. For sampling whole apiaries, beekeepers can repeat the process in each of $n = 8$ colonies, regardless of apiary size. Researchers desiring greater precision can estimate mite density in an individual colony by examining three, 300-bee sample units. Extrapolation to density on adults and pupae may require independent estimates of numbers of adults, of pupae, and of their respective mite densities. Researchers can estimate apiary-level mite density by taking one 300-bee sample unit per colony, but should do so from a variable number of colonies, depending on apiary size. These practical sampling plans will allow beekeepers and researchers to quantify mite infestation levels and enhance understanding and management of *V. destructor*.

KEY WORDS honey bee, parasitic mite, pest management

European-derived honey bees, *Apis mellifera* L., are vital pollinators in many ecological and agricultural systems throughout the world. Yet, honey bee colonies are in decline, particularly in the United States, probably due to combined effects of pesticides, pathogens, and parasites (Johnson et al. 2009a, VanEngelsdorp et al. 2009). The most destructive parasite of honey bees is the mite *Varroa destructor* Anderson and Trueman (Acari: Varroidae), which was introduced inadvertently into the United States in 1987 (reviewed in Martin 2001). This mite has had a significant negative impact on honey bees colonies and on beekeeping in North America and Europe. Controlling this parasite has been hindered by the lack of a standardized sampling plan to estimate abundance of mites in individual colonies or whole apiaries. Such a sampling plan would help beekeepers make informed treatment decisions.

Adult female mites are phoretic and feed on adult worker and drone bees. They leave their adult hosts to invade brood cells occupied by mature bee larvae just before worker bees seal the cells with wax. An individual female mite (foundress) trapped in a brood cell feeds and reproduces on the host pupa, producing on average 1.14 mated female offspring per worker (fe-

male) bee pupa (Martin 1994a). The foundress and mated female offspring exit the cell with the adult bee and continue the phoretic phase of the mite's life cycle.

Colonies infested by *V. destructor* will eventually suffer debilitating effects. Feeding by mites on bee pupae can reduce the resulting adult bees' body weights, suppress their immune systems, and reduce their life spans (De Jong et al. 1982a, Schneider and Drescher 1987, Yang and Cox-Foster 2005). Infested colonies often die within 1–2 yr. Mites also can transmit viruses during feeding, which can have devastating effects on colony health (Chen and Siede 2007, Cox-Foster et al. 2007, Johnson et al. 2009a). Previous research indicated treatment would be warranted if $\approx 10\%$ or more of adult bees were infested (Delaplane and Hood 1997, 1999; Martin 1998). That threshold was based on mite levels in stationary colonies and may not apply to colonies transported by migratory beekeepers, who move their colonies to different states during the year for pollination services, or for production of honey, bulk bees, and queens.

Many beekeepers apply acaricides to their colonies to prevent potentially large losses and may treat all of their colonies once or twice a year, irrespective of mite levels. Acaricidal treatments increase operating expense, increase risk of contamination of hive-products

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(Johnson et al. 2009b Mullin et al. 2010), and pose health risks to bees (Currie 1999, Rinderer et al. 1999, Haarmann et al. 2002, Collins et al. 2004). Mites have developed acaricide resistance (Baxter et al. 1998, Elzen et al. 1998, Elzen and Westervelt 2002, Pettis 2004), and resistance results in beekeepers increasing treatment doses and application frequencies. By treating only when economic risks are real, beekeepers may be able to reduce acaricide use, and thereby make beekeeping more sustainable.

Most beekeepers that sample for *V. destructor* do so to determine whether the mite is present in their colonies. Three techniques (reviewed by Webster 2001) have been used: sampling for mites on adult bees, sampling for mites on pupae in brood combs, and sampling of colony floors with sticky boards for mites that have fallen from bees and combs. Unfortunately, sample sizes to achieve desired levels of precision with these methods have not been examined or incorporated into standard sampling plans for beekeepers.

In contrast, two sampling plans have been proposed for researchers needing to estimate the total mite population in an individual colony. Martin (1999) developed a plan based on a life-stage structured model of a *V. destructor* population in a honey bee colony (Martin 1998). Martin suggested the total number of female mites in a colony could be extrapolated from estimated densities of mites per adult bee and per pupa, the estimated number of adult bees or pupae, and a correction factor (MAFF 1998, Martin 1999). Alternatively, Branco et al. (2006) developed a sticky board method, based on an empirical regression estimator, converting number of mites caught per day on sticky boards into numbers of mites concurrently on adult bees and pupae. Neither the plan of Martin (1999) nor Branco et al. (2006) is practical for sampling entire apiaries. Estimation of numbers of adult bees or pupae in a few colonies may be feasible for researchers or hobby beekeepers, but those tasks are impractical on a commercial scale. The sticky board method requires special equipment and two trips to place and retrieve the sticky board, and it is time-consuming to estimate bee populations.

The objective of the present research was to develop efficient methods to estimate the density of *V. destructor* on bees in an individual honey bee colony and in an apiary, and to evaluate sample unit sizes and numbers of units required to achieve desired levels of sampling precision. Based on extensive samples from colonies and apiaries spanning multiple seasons and locations, we found that measures of colony-level mite density (mites per bee, adults, and pupae combined) can be estimated with satisfactory precision by sampling only adult bees. Our aim is to reduce in-hive pesticide use to control *V. destructor* by providing a standard sampling method so that beekeepers can make informed treatment decisions.

Materials and Methods

Extensive Sampling for Mites on Adult Bees. Adult bees were sampled from colonies in 31 apiaries owned

by five commercial beekeepers based in four states: Minnesota, North Dakota, California, and Texas (Table 1). The sizes of the five operations ranged from 1,000 to 20,000 colonies, and numbers of colonies at sampled apiaries ranged from 24 to 84. Sampling was done in March (California and Texas), May–June (Minnesota and North Dakota), or August–September (Minnesota and North Dakota) in 2005, 2006, or 2007. These were times when these migratory beekeepers would normally sample and possibly treat their apiaries for *V. destructor*.

Colonies were maintained in Langstroth style equipment with eight to 10 frames of wax comb in each box, depending on whether the beekeepers used in-hive feeders to provide supplemental syrup feedings. Each colony consisted of one or two brood boxes, where the queen and brood were confined. Some colonies had additional boxes above for honey storage, which were not sampled. A recommendation to sample bees from honey supers would be a less sensitive test compared with sampling bees from brood boxes, as bees in brood boxes are approximately twice as infested (Calderone and Turcotte 1998). All colonies were placed in sets of four on wooden pallets for transport, and pallets were arranged in different configurations, depending on beekeeper style and local landscape features.

Upon arrival at each of the 31 apiaries, a map was drawn by hand to detail the apiary layout, including colony and pallet placement and numbers. Each colony's entrance direction was measured with an electronic compass (63-1223, Radio Shack, Ft. Worth TX). Individual colonies were opened, and frames were processed sequentially to score each one for comb content and to obtain a sample of adult bees from the comb. Frames were numbered sequentially, starting from an arbitrary end, and each comb (two sides combined) was scored as being empty, or having predominantly at least one of the following components: honey (nectar, sealed honey, or both), pollen, open worker brood (eggs and larvae), sealed worker brood (pupae), or sealed drone brood. The precise location of each sample was made explicit to determine if infested workers congregated in predictable locations.

Adult bees on the comb of each frame were sampled in groups of ≈ 20 –50 by scooping them into a 20-ml screw-cap vial containing 70% ethanol. Hereafter, this sample unit is designated as a "small-vial unit." Small-vial units were collected from every frame in each colony's brood box, regardless of comb content, and origins were noted for all collections.

Because it would be operationally convenient to sample bees from a single frame, we also collected a larger sample of 200–400 adults at the same time the small-vial units were collected, to evaluate correspondence between the two methods. The larger samples were taken from $\approx 30\%$ of the colonies in each apiary, by collecting bees from the first frame with uncapped brood inward from the edge of the uppermost brood box. Each larger collection was scooped with a 100-ml screw-cap vial containing 70% ethanol and is referred to as a "large-vial unit."

Table 1. Characteristics of an extensive sample of 31 apiaries in five commercial beekeeping operations, grouped by year, time of year, state, and beekeeper

Apiary group	Apiary	Yr	Mo(s)	State	Beekeeper	No. colonies (N)	No. colonies sampled (n)	Avg. mites per 100 adult bees ^a	SD
1	1	2005	May–June	MN	1	40	40	1.3	1.5
1	2	2005	May–June	MN	1	32	32	1.2	1.7
1	3	2005	May–June	MN	1	24	22	6.2	4.7
2	4	2005	May–June	ND	2	32	31	1.0	1.2
2	5	2005	May–June	ND	2	32	32	0.1	0.4
3	6	2005	Aug.–Sept.	MN	1	26	25	3.1	2.2
3	7	2005	Aug.–Sept.	MN	1	29	29	3.2	2.7
3	8	2005	Aug.–Sept.	MN	1	36	34	6.1	4.0
4	9	2005	Aug.–Sept.	ND	2	32	31	1.3	1.7
4	10	2005	Aug.–Sept.	ND	2	32	30	3.2	2.9
4	11	2005	Aug.–Sept.	ND	2	32	29	3.5	3.5
4	12	2005	Aug.–Sept.	ND	2	32	31	1.0	1.4
5	13	2006	March	CA	3	32	30	2.8	3.2
6	14	2006	Aug.–Sept.	MN	1	40	26	15.5	10.8
6	15	2006	Aug.–Sept.	MN	1	37	29	2.1	2.5
6	16	2006	Aug.–Sept.	MN	1	84	57	1.3	1.6
7	17	2006	Aug.–Sept.	ND	2	38	30	1.9	2.3
7	18	2006	Aug.–Sept.	ND	2	50	31	0.6	1.0
8	19	2006	Aug.–Sept.	ND	4	30	30	1.7	2.6
8	20	2006	Aug.–Sept.	ND	4	39	31	1.5	2.2
9	21	2007	May–June	MN	1	40	30	0.2	0.5
9	22	2007	May–June	MN	1	36	30	0.3	0.7
10	23	2007	March	TX	2	52	30	0.5	1.0
10	24	2007	March	TX	2	24	24	1.0	1.4
10	25	2007	March	TX	2	50	30	0.8	1.4
11	26	2007	May–June	ND	5	44	30	0.1	0.3
11	27	2007	May–June	ND	5	56	30	0.1	0.7
11	28	2007	May–June	ND	5	56	30	0.1	0.3
11	29	2007	May–June	ND	5	52	30	0.1	0.3
12	30	2007	Aug.–Sept.	MN	1	40	30	1.5	2.0
12	31	2007	Aug.–Sept.	MN	1	36	30	1.9	2.2

^a Averaged across colonies in each apiary.

Bees and mites in units of both sizes were processed to extract mites with an alcohol wash method (De Jong et al. 1982b). Each collection was poured into a shallow dish filled with enough 70% ethanol to cover the bees, shaken for ≈ 60 s, and then strained through coarse hardware cloth to separate the mites from the bees and count numbers of each.

Intensive Sampling for Mites on Adults and Pupae.

Sixty-two colonies were sampled more intensively to examine the distribution of mites among adult bees on combs, and worker pupae and drone pupae in brood cells. Fifty-three of the 62 colonies were sampled from apiaries 21, 22, 26, 27, 28, 29 in May–June and from apiaries 30 and 31 in August–September (Table 1). For comparison, the other nine colonies were sampled more thoroughly in August 2006 and August and September 2007 from a stationary apiary at the University of Minnesota (i.e., university colonies).

In each colony, we estimated total number of adult bees, number of mites per adult bee, number of sealed worker and drone brood cells containing pupae, proportion of worker brood cells infested by one or more foundress mites, and proportion of bees that were pupae. One of two methods was used to estimate total number of adults (*A*): comparing visually each side of every comb to a calibrated set of images (MAFF 1998) or by shaking all bees from all frames into a screened box, weighing them, and then multiplying by 0.12 bees

per g to obtain the total number of adult bees. Density of mites per adult bee (M_a) was estimated from the collection of small-vial units taken from individual frames if the colony was among those sampled extensively or by collecting three large-vial units from colonies in the stationary apiary (see below). Total number of mites on adult bees was obtained by multiplying the number of mites per adult bee by the corresponding number of adult bees.

The number of worker pupae in each colony (*P*) was estimated by placing a wire grid (4 cm² per grid cell) over every comb's surface, counting the number of grid cells containing sealed brood, multiplying by 63.5, the number of brood cells per occupied grid cell, and then totaling over frames.

Number of pupae infested with at least one foundress mite in the 53 migratory colonies was estimated by opening a minimum of 200 individual sealed cells containing worker pupae (50 cells on each of four combs) and examining them for the presence of mites. Among the stationary colonies, we examined every comb with brood in 2006 by opening 200 pupal cells on every frame in the colony, or all cells if a frame had <200 cells present. In 2007, we examined pupal cells on one side of just three combs in each brood box: the left-most brood comb, the center comb, and the second brood comb in from the right-most brood comb. From each comb's side, we examined 20 pupal cells if there

were ≤ 200 cells, 10% of the cells if there were 201–1,999 cells, and 200 cells if there were $\geq 2,000$ cells. For both the migratory and stationary colonies, total number of mites on pupae was estimated by multiplying the estimated number of mites per pupa by the number of pupae in the colony.

We also estimated the number of drone pupae and presence or absence of mites on drone pupae in three of the commercial colonies and seven of the university colonies.

Variation in Mite Density. Mite density on bees from individual small- or large-vial sample units was calculated as follows:

$$x = m/b, \quad [1]$$

where m is number of mites and b is number of adult bees in a vial. Density among frames in each colony was estimated as follows:

$$\bar{x}_c = \sum_{i=1}^f x_i/f, \quad [2]$$

where f was number of frames from which vials of bees were taken. Finally, density among colonies in an individual apiary was estimated as follows:

$$\bar{x}_a = \sum_{i=1}^c \bar{x}_c/c, \quad [3]$$

where c was number of colonies examined. For convenience in presentation, mean densities at the colony and apiary level were multiplied by 100 and are expressed as mites per 100 bees.

We examined the relations between sample means and variances for samples from frames with and without brood comb, using Taylor's variance-mean relation (Taylor 1961). Mean (equation 2) and variance (s^2) in mites per bee among vials from frames with each comb type were analyzed using

$$\log(s^2) = a + b \log(\bar{x}_c), \quad [4]$$

with logs in base 10. Intercept a is scaled by choice of sample unit, whereas slope b is a measure of statistical aggregation. A value of $b = 1$ would indicate mites were randomly distributed among sample units, $b < 1$ would indicate they were more uniform, and $b > 1$ would indicate they were more aggregated.

We applied an analysis of covariance (ANCOVA) to determine whether variance-mean relations differed among frames with or without brood comb. Least-squares regression models were fit with `lm` in R (R Development Core Team 2009), and residuals were examined graphically for nonconstant variance and normality.

We next examined variation in mite density among apiaries, and among hierarchically arranged pallets within apiaries, colonies in pallets, boxes in colonies, and frames with or without brood in boxes, by using a nested analysis of variance (ANOVA) (PROC VARCOMP, SAS Institute 2005). Separate analyses were applied to 12 subsets of the 31 apiaries, grouped by year, time of year, beekeeper, and state (Table 1).

To determine whether mean mite density varied with a colony's entrance orientation and presence or absence of brood, we analyzed densities with a split-plot treatment design, treating apiaries as blocks. Colonies were analyzed as main plots, classified by quadrant of entrance orientation, and frames within colonies were treated as split plots, cross-classified by presence or absence of brood. Models were fit with `lm` in R, residuals were examined graphically, and densities were log transformed and reanalyzed to better meet assumptions of equal variance and normality among residuals.

We also compared mite densities among colonies in different apiary positions, and among apiaries with different pallet arrangements. Positions were at ends or centers of pallet rows, and arrangements were with pallets in one or two lines, or in U, J, or L-shaped patterns. Results from six of the 31 apiaries were omitted in this analysis because their pallet arrangements had no clear ends. Densities were analyzed with a two-factor ANOVA, where individual colonies were cross-classified by position and apiary shape. Models were fit with `lm` in R, and residuals were examined graphically. The data were log transformed to better meet the assumptions of constant variance and normality.

Estimating Colony-Level Density From One Large Sample. To determine whether colony mite density could be estimated from the single large-vial unit taken from the first frame with brood comb in a colony's top brood box, we used sequential ANCOVA to analyze the relation between colony-matched measures of mean mite density, calculated from the eight to 18 small vials per colony (equation 2), and predictor variables of density as calculated from bees in the single large vial (equation 1), and categorical dummy variables for season (March, $n = 28$ colonies; May–June, $n = 59$; August–September, $n = 50$). Models were fit with `lm` in R, and residuals were examined graphically for nonconstant variance and normality. If colony density could be estimated from a single large-vial unit, then the regressions of mean density from small vials on density from large vials would have zero intercept and unit slope, and would be the same for all seasons.

Extrapolation From Adults to Whole-Colony Density. To account for the *V. destructor* population on pupae, we analyzed data from the 62 intensively sampled colonies to determine how well density of mites as measured on adult bees could predict density of mites in an entire colony, adults and pupae combined, and whether the relation was the same in May–June and August–September. Statistically, we used ANCOVA to examine the relationship between whole-colony density and adult density, with an additional coefficient for proportion of bees in a colony that were pupae ($P/[A + P]$), a term for August–September versus May–June, and possible pairwise interactions, for a total of eight coefficients. Because this full model was unnecessarily complicated, we used backwards elimination to evaluate progressively simpler models, first disregarding time of year, and

then disregarding proportion of bees that were pupae, to see how well a simple linear correction factor could extrapolate from mites per 100 adults to whole-colony density, adults and pupae combined.

Our intensive sampling protocol scored pupae as infested or not and did not count multiple foundresses. To determine how much inclusion of multiples would influence the correction factor numerically, we assumed the average number of mites per cell was 1.14 (Martin 1994a, 1995), and refit the linear model using adjusted mite densities on pupae.

Development of Colony-Level Sampling Plans. Results from the 31 extensively sampled apiaries were used to develop sampling plans to estimate mites per bee in a colony. Required sample size (RSS), the number of small-vial units needed to achieve a specified level of precision over an arbitrary range of anticipated mite densities (m), was calculated as follows:

$$RSS = n = \frac{s^2}{h^2} = \frac{s^2}{C^2 m^2}, \quad [5]$$

where n is required sample size, s^2 is anticipated sample variance, h is chosen absolute precision level (=SE, in mites per 100 bees), C is relative precision (=SE/mean), and m is anticipated mean mite density (after Karandinos 1976, Ruesink 1980). Because sample variance was expected to be related to mean density, s^2 was estimated from the variance-mean relation (Taylor 1961) as follows:

$$s^2 = 10^a m^b. \quad [6]$$

Following Pedigo and Rice (2009), we propose that a relative precision level of $C = 0.25$ would be appropriate for beekeepers, whereas a more precise $C = 0.1$ may be more appropriate for researchers. Corresponding levels of absolute precision would be $h = 1.25$ and $h = 0.5$ mites for beekeepers and researchers, respectively, if a colony's mean density were five mites per 100 bees. It should be understood that precision of $\pm h$ or $\pm C$ would correspond with a 66% confidence interval for an estimated mean. Values of h or C would need to be halved approximately to achieve 95% confidence intervals. Following Cochran (1977), RSS was adjusted downward for use in colonies up to 60,000 workers, as follows:

$$RSS' = n' = \frac{n}{1 + ([n - 1]/N)}, \quad [7]$$

where n is from equation 5, and N is the (finite) number of sample units actually available to be examined.

Development of Apiary-Level Sampling Plans. An apiary-level sampling plan also was developed from the extensively sampled apiaries. An apiary-level plan would specify the size of sample unit to be taken from a colony, as well as the number and location of colonies to be sampled. Different unit sizes were evaluated by combining and analyzing counts of mites and bees from different numbers of small vials taken from frames in the top brood boxes of colonies in 20 of the 31 extensively sampled apiaries. Recombined sample

units of increasing size were 1, vial units from the fifth frame in each brood box; 2-vial units from frames 4 and 5; 3-vial units from frames 1, 5, and 8; 4-vial units from 1, 4, 5, and 8; 5-vial units from frames 1, 4–6, and 8; 6-vial units from frames 1, 3–6, and 8; 7-vial units from frames 1 and 3–8; and 8-vial units from frames 1–8. Once recombined, variances and means among colonies in the 20 apiaries were analyzed with Taylor's variance-mean relation to estimate values of a and b for each sample unit size. Colonies with missing data, and apiaries with fewer than 15 colonies were omitted from this analysis. An apiary, number 23, was removed from analysis of 6-, 7-, and 8-vial units because its colonies had too few bees.

The second step was to estimate numbers of colonies that would be required to achieve a desired level of precision. This was accomplished by resampling the data sets from the remaining 10 apiaries, using resampling for validation of sample plans (RVSP) (Naranjo and Hutchison 1997). The RVSP software implements bootstrap sampling, which requires no assumption about the underlying sampling distribution, and can be used where sample sizes are small (e.g., only 10 apiaries). We used RVSP to simulate use of the fixed precision, sequential sampling plan Green (1970), and thereby derive the number of colonies that would be needed on average to estimate apiary-level mite density with a stated level of precision. Four of the 10 apiaries had infestations lower than one mite per 100 adult bees. A low apiary infestation requires more colonies to be sampled to achieve the same precision. Because these low infestation levels are not critical in a sampling plan for beekeepers, the apiaries with less than one mite per 100 bees were excluded from analysis of Green's sequential plan for beekeepers but were included in analysis of Green's plan for researchers.

To illustrate with 1-vial units, we defined Green's sequential stop lines with Taylor's a and b for 1-vial units (as above), and set nominal precision at $C = 0.25$. In turn, we sequentially sampled with replacement the redefined (1-vial) colony-level densities from each apiary, beginning with a minimum of five colonies, and recorded the number of colonies that were eventually required to achieve the nominal precision. Results from the 10 apiaries, or six apiaries in the case of the beekeeper sampling plan, were then averaged. The same procedure was repeated for a total of 500 iterations to obtain the average number of colonies required to achieve $C = 0.25$, by using 1-vial units.

An equivalent procedure was repeated with the seven remaining sample unit sizes, using appropriate values for Taylor's a and b (as above), and for all eight sizes with the more rigorous $C = 0.1$. Because average colony numbers needed to achieve the latter level of precision would be appropriate for apiaries with an effectively infinite number of colonies, we also used equation 7 to adjust average colony numbers downward for smaller apiaries having 80, 40, and 20 colonies.

Results

Extensive Sampling for Mites on Adult Bees. In total, 954 colonies in 31 apiaries were sampled by scooping $\approx 12,000$ small-vial units of bees from individual frames (Table 1). These units contained an average of 35 adult bees ($SD = 15$, $n = 11,997$). Colony-level mite densities ranged from 0 to 223 mites per 100 bees and had a mean of 5.6 ($SD = 11.2$, $n = 11,997$). A subset of 142 of the same colonies was sampled with a single large-vial unit taken from an upper brood-box frame (These larger units contained an average of 338 bees ($SD = 163$). Apiary-level densities ranged from 0.2 to 50.2 mites per 100 bees and had a median of 3.8 and mean of 6.2 ($SD = 9.1$, $n = 31$) mites per 100 bees. Thus, the extensive sample of apiaries had a wide range of mite densities, and densities in many of colonies and apiaries exceeded plausible economic injury levels.

Intensive Sampling for Mites on Adults and Pupae. The average colony (migratory and stationary colonies together) contained 24,500 adult bees ($SD = 11,600$, $n = 62$) and 14,000 worker pupae in sealed brood comb ($SD = 4,900$). For all the migratory colonies intensively sampled, the mean number of cells containing drone pupae was 173.7 ($SD = 173.5$, $n = 20$) for colonies in spring with one box, 800.8 ($SD = 481.3$, $n = 39$) for colonies in spring with two boxes, and 348.8 ($SD = 457.2$, $n = 20$) for colonies with one box in late August.

The amount of drone pupae relative to worker pupae was relatively small in our study. Drone brood was $3.2 \pm 1.9\%$ (mean \pm SD , $n = 15$) of the total pupae. Furthermore, an average (\pm SD) of $6.7 \pm 2.1\%$ of all mites were on drone pupae, $40.0 \pm 16.1\%$ were on worker pupae, and the remaining 46% were on adult bees ($n = 7$).

Variation in Mite Density. The statistical relationship between sample variances and means among frames in colonies was independent of presence of brood ($F = 0.11$; $df = 1, 1,428$; $P = 0.74$). Combined, Taylor's variance-mean parameters were $a = 0.0003$ ($SE = 0.07$) and $b = 1.01$ ($SE = 0.02$). These results indicated mites were distributed approximately at random among bees on brood box frames.

The initial split-plot ANOVA for differences in densities among colonies facing different directions, and among frames with or without brood comb, indicated results needed to be log-transformed to counteract unequal variances and non-normality in residuals. Back-transformed least-squares means ranged from 1.6 mites per 100 bees among colonies facing NW (271 – 360°) to 2.0 among colonies facing SE, but those differences were not significant ($F = 0.21$; $df = 3, 83$; $P = 0.89$). In contrast, densities on frames with and without brood comb were statistically different ($F = 91.0$; $df = 1, 11,017$; $P < 0.001$). However, the difference was modest, with 1.8 mites per 100 bees on frames without brood comb, and 2.4 on frames with brood comb.

The nested ANOVAs of mite densities among the 12 groups of apiaries (Table 2) indicated an average of

Table 2. Results of nested analysis of variance in mite densities among small-vial sample units of adult bees in the extensive sample from 31 apiaries

Apiary group ^a	% overall variance, by source					
	Apiary	Pallet	Colony	Box	Comb content ^b	Residual
7	0.0	11.0	27.6	0.2	0.9	60.2
4	0.5	7.4	21.2	NA ^c	0.4	70.6
5	NA	4.2	17.1	0.9	8.6	69.3
3	15.6	6.0	16.5	NA	3.0	59.0
6	16.9	12.4	13.0	NA	1.3	56.0
2	0.4	15.4	10.5	NA	0.8	72.9
10	2.8	8.3	9.8	0.9	0.8	77.4
8	0.2	7.4	8.6	NA	0.0	83.8
12	NA	21.8	7.4	0.2	0.6	70.0
1	50.2	3.0	6.9	0.1	2.3	37.6
9	17.0	1.4	4.8	0.3	1.8	74.6
11	0.1	0.9	1.4	0.1	0.2	97.3
Avg.	10.4	8.3	12.1	0.4	1.7	69.1

^a Apiaries grouped by year, months sampled, state, and beekeeper (see Table 1), and ranked in descending order by relative amount of variation among colonies within apiaries. Apiary 16 in group 6 was omitted from analysis because its colonies had been imported from multiple apiaries the day before sampling.

^b Frames with or without brood comb.

^c Not applicable, due to singularities.

69% ($SD = 15\%$, $n = 12$ groups) of overall variance was from individual frames within colonies. The next largest component was from colonies within apiaries, which averaged 12.1% ($SD = 7.4\%$). Variance components from apiaries, pallets, brood boxes, and comb types (with or without brood) were consistently smaller, although apiary explained relatively high levels of variance in four of the groups, making apiary the third largest source of overall variance (average = 10.4%, $SD = 15.9\%$, $n = 15$).

Colony-level mite densities did not vary substantially with colony position. Densities among colonies in end and center positions were not different from each other ($F = 0.36$; $df = 1, 760$; $P = 0.55$), and effect of position was independent of apiary arrangement (interaction between position and arrangement: $F = 1.65$; $df = 4, 760$; $P = 0.160$). However, colony-level densities did differ significantly among apiaries with pallets in different arrangements ($F = 7.20$; $df = 4, 760$; $P < 0.001$). This phenomenon was probably due to apiaries in a straight line happening to have higher mite levels and not apiary shape influencing mite levels.

Estimating Colony-Level Mite Density From One Large Sample. Colony-level densities could be estimated with reasonable accuracy from single, large-vial sample units. Initial analysis indicated slope of the regression of colony-level mean densities (calculated from small-vial units, equation 2) on matching large-vial units varied slightly among months of sampling (interaction $F = 2.71$; $df = 2, 123$; $P = 0.07$; $R^2 = 0.78$). Subsequent analyses with progressively simpler models without terms for interactions, and then without months, reduced R^2 from 0.77 to 0.76, respectively. The simplest regression, disregarding months, had an intercept of 0.004 ($SE = 0.002$), a slope of 0.960 ($SE = 0.048$), and a standard error of prediction (SE_{pred}) of

Table 3. Statistics from three progressively simpler models for predicting whole-colony mite density from density on adults (M_a), proportion of adults and pupae combined that were pupae (PP), and month in the intensive sample of 62 colonies

Model	Term ^a	Coefficient	SE	P value ^b	SE pred ^c	R ²
1	Constant	-0.10	1.93	0.96		
	M_a	0.85	2.61	0.75		
	PP	0.52	5.42	0.92		
	Aug.-Sept.	1.52	3.64	0.68		
	$PP \times M_a$	-0.27	6.58	0.97		
	Aug.-Sept.* M_a	-1.43	2.68	0.60		
	Aug.-Sept.* PP	0.01	9.11	1.00		
	M_a * PP *Aug.-Sept.	5.09	6.72	0.45		2.13
2	Constant	0.55	1.32	0.68		
	M_a	-0.29	0.43	0.50		
	PP	-1.33	3.43	0.70		
	M_a * PP	4.57	0.97	<0.01	2.27	0.88
3	Constant	-0.39	0.45	0.39		
	M_a	1.77	0.10	<0.01	2.75	0.83

^a Months coded as categorical dummy variables, May–June implied.

^b Test of hypothesis the coefficient = 0.

^c Standard error for estimated mites per 100 pupae and adults combined.

2.3 mites per 100 bees. Thus, there was good agreement between estimates from eight to 18 small-vial units and single, large-vial units from the same colonies, and estimates from the larger vials were acceptably precise over the range of seasons and locations.

Extrapolation From Adults to Whole-Colony Density. Analysis indicated mite density on adult bees (M_a) could estimate whole-colony mite density (mites on adults and pupae) with reasonable precision, but inclusion of a measure of proportion of bees that were pupae ($PP = P/[A + P]$), where A is adult bees and P is worker pupae, if available, would improve precision. Among the 62 intensively sampled colonies, density averaged 2.8 (SD = 3.4) mites per 100 adult bees, 5.2 (SD = 7.8) mites per 100 adults and pupae, and PP averaged 0.38 (SD = 0.11). The initial ANCOVA (Table 3, model 1) with eight coefficients had an $R^2 = 0.91$, was statistically significant overall ($F = 77.26$; $df = 7, 54$; $P < 0.01$), and had $SE_{pred} = 2.1$ mites per 100 adults and pupae. However, none of the coefficients was significant, presumably due to overparameterization and colinearity with excess terms for sampling months.

To determine how well whole colony densities could be estimated with a simpler model, we omitted terms involving months and fit a second model with just M_a , PP and the pairwise interaction (model 2, Table 3). The simpler model had an $R^2 = 0.89$, was statistically significant ($F = 155.7$; $df = 3, 58$; $P < 0.01$), and had $SE_{pred} = 2.3$ mites per 100 adults and pupae, slightly greater than with model 1, but coefficients were still insignificant. Finally, we fit a simple linear regression to see how well whole-colony density could be estimated from M_a alone. This regression (model 3, Table 3) had an $R^2 = 0.83$, was significant ($F = 298.6$; $df = 1, 60$; $P < 0.01$), and $SE_{pred} = 2.7$. This regression had an intercept of -0.4 and a slope of 1.8. Rounding up from 1.8, this final regression model indicates beekeepers could simply double mite density on adults to estimate whole-colony density across a broad range of adult bee infestation levels. Disregarding the trivial intercept of -0.4 will err on the side of slight over-

estimation. Where greater precision is required and a measure of PP is available, a more precise estimate of whole-colony density could be obtained using the coefficients for model 2 (Table 3).

To determine how inclusion of multiple foundresses would alter the correction factor, we assumed the number of mites per cell was 1.14 (Martin 1994a, 1995), rescaled the data, and used a regression to compare adjusted whole-colony densities with adult bee mite densities. This analysis resulted in an intercept of -0.6 (SE = 0.6) and a slope of 2.2 (SE = 0.1) ($R^2 = 0.68$, $F = 138.3$; $df = 1, 66$; $P < 0.001$), which was not different than the slope of 1.8 with an intercept of -0.4 from model 3.

Development of Colony-Level Sampling Plans. The variance-mean relation among sampled frames in individual colonies was used to estimate the number of small vials that would be needed to estimate mean density with a chosen level of precision. Sample size curves were calculated with equation 5, using Taylor's $a = 0.0003$ and $b = 1.01$, and solved for colony-level densities ranging from near zero to 10 mites per 100 bees ($\approx 2 \times$ the observed mean in the intensively sampled apiaries) (Fig. 1).

Where precision was expressed as $SE = h$ mites, calculated sample sizes increased with mean density, because sample variances were proportional to sample means. In the case of a beekeeper who wanted to estimate mean density $\pm h = 1.25$ mites, a minimum of two small-vial samples would be needed if density were three mites or lower, but seven or more would be needed if density exceeded 10 mites. A researcher desiring a more rigorous $h = 0.5$ would need a minimum of two vials if density were 0.5 mites or lower, but 41 or more if density were 10 or greater. A sample size of two vials may be practical for both beekeepers and researchers where colony-level density is low, but sample sizes much larger than two vials are probably impractical, at least for beekeepers.

A complementary situation occurred when precision was defined in relative terms ($C = SE/\text{mean}$) (Fig. 1). Required sample sizes for a beekeeper using

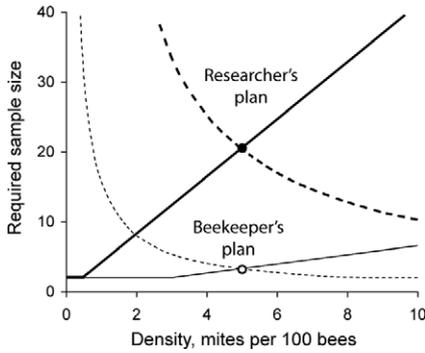


Fig. 1. Numbers of small-vial sample units (≈ 35 bees) needed to estimate colony-level density of *V. destructor* in individual colonies infested at different mite densities. Sample size curves calculated with equation 5, $a = 0.0003$, $b = 1.01$. Criteria for beekeeper plan: $C = SE/\text{mean} = 0.25$ (thin dashed line), $h = 1.25$ mites per 100 bees (thin solid line). Intersection (open circle) at five mites is crossover density, above or below which a compromise five sample units will meet or exceed one criterion or the other. Researcher plan: $C = 0.1$ (thick dashed line), $h = 0.5$ mites (thick solid line), compromise 21 units.

$C = 0.25$ were ≤ 3 vials when density was >6 mites per 100 bees, but exceeded five vials when density was below three mites. Required sample sizes for a researcher using $C = 0.1$ were ≤ 17 vials when density was >6 mites, but exceeded 30 vials when density was below three mites. Clearly, these sample sizes will be impractical when mite densities are low.

The dilemma presented by impractical sample sizes for each precision definition, as applied over the full range in density, can be resolved by compromise. Rather than setting specific numerical values for either h or C , we redefined acceptable precision as being either less than or equal to h , or less than or equal to C . In turn, a worst-case fixed sample size for chosen values of h and C could be determined from the crossover point of the corresponding h and C sample size curves (Fig. 1).

To illustrate, a beekeeper using $h = 1.25$ and $C = 0.25$ would achieve one or the other goal if colony-level density were five mites per 100 bees and would do so with a fixed sample size of four small-vial units (rounded up from calculated size 3.3) (open circle, Fig. 1). Precision with that sample size would be better (smaller) than $h = 1.25$ if true density were below five mites and would be better than $C = 0.25$ if density were greater. A researcher's standard of $h = 0.5$ mites and $C = 0.1$ would be achieved at the same crossover density of five mites per 100 bees but would require a fixed sample size of 21 vials (rounded from 20.3). Adjustment of these sample sizes (equation 7) to correct for finite numbers of bees in a colony would be trivial in almost all situations, except where numbers of possible 35-bee sample units were limited by severe depopulation.

Development of Apiary-Level Sampling Plans. Coefficients for Taylor's variance-mean relation among the 20 extensively sampled apiaries changed with sample unit size (Table 4). When units from individual colonies were redefined by combining data from progressively larger sets of small-vial units, the variance-mean intercepts (a) decreased and slopes (b) increased with increasing unit size. All intercepts were significantly different from zero, and all slopes were significantly different from 1.0. Slopes >1.0 indicated mites were aggregated among colonies within the sampled apiaries. The net effect of increasing sample unit size from one 35-bee sample from the central frame to a ≈ 280 -bee sample (eight vials) from all frames was to reduce the variance in estimated apiary-level density, because intercepts declined to a greater extent than slopes increased.

Numbers of colonies that would be needed to estimate apiary-level mite density, as estimated through resampling of data from the 10 remaining apiaries, declined when increasingly larger sample units were used to sample the colonies (Table 4). For $C = 0.25$, average numbers of colonies estimated from 10,000 resamplings ranged from a maximum of 16 colonies if they were represented by singular small-vial units to

Table 4. Results of Taylor's variance-mean regressions for sample units recombined from different numbers of small-vial sample units in 20 apiaries, and numbers of colonies required to estimate apiary-level mite density at two precision levels (C), as quantified by resampling data from 10 separate apiaries using the fixed precision, sequential sampling plan of Green (1970)

Sample unit size ^a	Regression coefficient		Mean no. colonies required				
	<i>a</i>	<i>b</i>	$C = 0.25$	$C = 0.1$, for apiaries of given size ^b			
				∞	80	40	20
1	2.08	1.34	16	295 ^c	63	35	19
2	1.45	1.50	12	184	56	33	18
3	1.09	1.55	10	142	51	31	18
4	1.07	1.58	9	135	50	31	18
5	0.97	1.62	9	117	48	30	17
6	0.92	1.64	9	109	46	29	17
7	0.85	1.70	8	96	44	28	17
8	0.80	1.64	8	96	44	28	17

^a Number of small-vial units (≈ 35 bees each) combined from original units collected individually from all frames in brood boxes of chosen colonies in 20 separate apiaries (see text).

^b Average sample sizes adjusted downward from infinite no. of colonies to 80, 40, and 20 colonies (using equation 7).

^c Apiary 26 was excluded because we were unable to estimate its apiary-level density of 0.2 mites per 100 bees with the smallest sample unit size. The precision for estimate of its mite density with one small-vial unit was $C = 0.13$.

a minimum of eight colonies if represented by aggregates of eight small-vial units. Numbers of colonies needed to achieve the more rigorous $C = 0.1$ were substantially greater.

Discussion

The main goals of this research were to develop general sampling plans that will allow beekeepers and researchers to quantify the abundance of *V. destructor* mites in individual colonies and whole apiaries. This is the first time that any sampling plan has been designed to estimate apiary-level mite infestation, a process that will be especially helpful to commercial beekeepers who need information to manage *Varroa* mites in their apiaries but have little time for sampling. Standardization will allow comparison of results within and among different beekeeping operations and research studies.

Sampling Plans for Beekeepers. Our results indicate that beekeepers would be able to estimate colony-level mite density with a precision of $C \leq 0.25$ or $h \leq 1.25$ if they were to dislodge and count mites from at least five, small-vial units, or an equivalent single sample of 175 adult bees (Fig. 1). These units could be obtained from separate frames in the uppermost brood box. However, for convenience, and to increase sampling precision and chance of detecting mites when they are rare, we recommend beekeepers take a single large-vial sample of 300 adult bees from any frame in the uppermost brood box. Sampling 300 adult bees has been recommended to beekeepers (Delaplane 1997, Strange and Sheppard 2001). The present research confirms statistically that this recommendation will yield adequate precision. Beekeepers needing estimates of whole colony totals can most simply double numbers of mites per bee to adjust for mites in sealed brood.

Mean density in a whole apiary can be estimated with $C = 0.25$ if one large-vial unit is examined from one frame in each of eight or fewer colonies in the apiary (Table 4). For small apiaries, number of sampled colonies can be adjusted downward (via equation 7). For example, a sample size of only six would be needed from an apiary with $N = 20$ colonies, five would be needed from 10 colonies, and three would be needed from a single pallet of four colonies. The requisite number of colonies should be selected from across the apiary, starting at any edge, without regard to colony orientation or position, because mite densities in our extensive samples were independent of orientation and position.

Beekeepers with colonies in multiple apiaries should sample each apiary separately, because apiary-level infestations can vary widely within a single operation (e.g., Table 1, group 6). If mite treatments were applied to individual apiaries, as opposed to blanket treating an entire operation, then there would be potential to save money and retard resistance by reducing unnecessary treatments.

Sampling Plans for Researchers. Comparable sampling plans for researchers needing to achieve greater

precision will differ only in the amount of sampling effort. To achieve $C \leq 0.1$ or $h \leq 0.5$ mites per 100 bees in an individual colony, researchers should examine 21 small-vial units, for 735 adult bees in total. However, for sake of simplicity, researchers could examine three large-vial units (total of ≈ 900 bees) from three separate frames in the upper brood box to account for frame-to-frame variation and err on examining more bees. Sample size of $n = 3$ will allow researchers to estimate sample variance and calculate a confidence interval for a mean mite density in a colony. If the mites are dislodged from the bees in a way that does not kill the bees (e.g., using powdered sugar, Macedo et al. 2002), sampling this large of number of bees is feasible. Sampling 900 bees in alcohol can be destructive to some colonies. Researchers will need to determine the costs and benefits of sampling to achieve a higher precision. If desired precision is greater than $C > 0.1$ and a confidence interval is not needed, then a single sample of 300 bees from any frame should be acceptable. If researchers need to estimate mite density in a whole colony, adults and pupae combined, then they may need to estimate numbers of adult bees and sealed brood cells in the colony (Table 3).

To estimate apiary-level mite density with $C \leq 0.1$, researchers should examine one large-vial unit per colony and do so from numbers of colonies that will depend on apiary size (N , Table 4). Numbers of colonies can be calculated directly with equation 7.

Extensive Sampling for Mites on Adult Bees. These proposed sampling plans were developed from data describing infestation levels in 31 commercial beekeeping apiaries (Table 1). The apiaries were located in four states (Minnesota, North Dakota, Texas, and California), were sampled at different times of year and encompassed a wide range of bee populations and mite infestation levels. The breadth of commercial conditions represented in our data sets provides confidence that results with the proposed sampling plans will meet or exceed intended levels of sampling precision.

Intensive Sampling for Mites on Adults and Pupae. Colonies sampled intensively provided additional information on the relationship between adult bee mite density and colony mite density, and resulted in conversion factors that will allow extrapolation to colony mite density from adult bee mite density.

In general, our intensively sampled colonies differed in bee populations and thus number of brood boxes. There were also differences in size of colonies (numbers of boxes) within the migratory colonies and between the migratory and university colonies. These differences reflected management practices unique to each beekeeper.

Variation in Mite Density. Knowledge of sources of variation in mite density within a colony and apiary provided guidance on the most effective locations to sample. The nested ANOVA indicated most of the variation arose from frames within colonies, but variation among colonies contributed the second greatest amount of variation. This finding makes biological sense. Colonies are independent groups of related

bees that live in and protect the same nest. Moreover, colonies probably have different susceptibilities to mites, based on genetics and behavior (Boecking and Spivak 1999), and they probably also differ demographically and in mite colonization history.

Independence of mite density and both colony entrance direction and colony position in different apiary layouts indicated that colonies can be chosen without regard to orientation and position in an apiary.

Bees on frames containing brood comb had significantly more mites than frames without brood, but the difference is small biologically. Greater levels on brood combs were probably due to mites preferring nurse bees, which tend to stay on brood combs (Pernal et al. 2005). The differences may have been underestimated in our study, to the extent that bees may have been redistributed among frames when colonies were opened for sampling.

The Taylor's power law parameters from the analysis comparing brood and nonbrood combs implied the mites were randomly distributed among bees within brood boxes. In contrast, the same parameters from individual or recombined 1-vial units taken from different colonies indicated mites were aggregated among colonies (Table 4, row 1). The variance-mean regressions for samples within colonies and among apiaries were different because the former described variation in mite numbers among groups of bees within the same colonies, based on $n = 8-18$ vials per colony, and the latter described variation in mite numbers among colonies within apiaries, based on $n = 1$ or 2 (middle frame) units per colony.

Estimating Colony-Level Mite Density From One Large Sample. Data from our extensively sampled apiaries indicated estimates of colony-level mite densities with single, large-vial units were equivalent to matching estimates from eight to 18 small-vial units, yet took much less time and effort to obtain. To simplify the sampling process, we recommend that beekeepers use single, large-vial units to estimate colony mite density. Although a sample variance cannot be calculated from a single sample unit of any kind, equivalence of estimated densities from one large-vial unit and eight small-vial units from colonies in our extensively sampled apiaries indicated that on average, results from large-vial units will be unbiased and equally precise. Researchers needing greater precision should examine three large-vial units per colony, and will be able to calculate sample variances if needed.

Extrapolation From Adults to Whole-Colony Density. Our data showed that amount of pupae relative to number of adult bees influenced the number of mites found on pupae: colonies with relatively more pupae had more mites on pupae, and colonies with relatively more adults had fewer mites on pupae. These results are supported by Boot et al. (1995) who demonstrated that the relative amounts of pupae and adults influenced the rate of mite invasion in pupal cells. Researchers have used estimates of sealed brood, adult bees, and their respective infestations to obtain the mite population in a colony (Strange and Shepard 2001, Branco et al. 2006, De Guzman et al. 2007).

Martin's (1998) method only requires either the measurement of brood or adults and their corresponding infestation rates, but precision is unknown. To achieve a higher level of precision, this study recommends researchers convert the adult bee infestation to colony-level infestation by including the proportion of pupae. However, the brood infestation does not need to be estimated. For beekeepers to estimate colony mite density, we recommend multiplying adult bee density by a correction factor of two. However, if beekeepers know colonies have little or no brood, then no correction is necessary.

Adjustment of pupal mite densities to correct for 1.14 foundresses per infested cell did not change the conversion factor substantially. Martin (1994a, 1995) found that 29% of worker cells were infested, which was higher than 9.8% in the current study. If mites invade brood cells at random, then it is likely there were fewer than 1.14 mites per infested pupa in our intensively sampled apiaries, which would further reduce the extent of overestimation by doubling.

Mite densities, rather than totals per colony, were considered in this study, because the same number of mites probably has different implications for colonies of different sizes. For example, 1,000 mites will have more impact on a colony of 10,000 bees than on a colony of 40,000 bees.

Importance of Drone Brood. *V. destructor* foundresses are more attracted to drone brood than worker brood, and foundress mites produce more offspring on drone brood (Martin 1994b). Because of these differences, it is widely thought that mites in drone brood are a substantial fraction of a colony's mite population. Martin (1998) predicted 10% of mites in a colony would occur on drone pupae, 55% on worker pupae, and 35% on adult bees. Mondragón et al. (2005) found exclusion of mites in drone brood made little difference in estimates of mite population growth in a whole colony. Results from our intensively sampled colonies indicated only a small fraction of brood contained drones; consequently, a small percentage of the average colony's mites were in drone brood.

Processing Individual Samples. Sampling an individual colony or apiary with multiple colonies will require collecting individual units of adult bees. We used 20- and 100-ml vials filled with alcohol to scoop samples of bees from chosen frames. These unit sizes were based on convenience. We have developed a sampling device that will collect and measure a desired volume of adult bees with little effort (Walter T. Kelly Company Catalog 2010). However, each sample unit is obtained, mites could be dislodged from the adult bees by using an alcohol wash (De Jong et al. 1982b), which we used, or they could be dislodged with a powdered sugar shake method (Macedo et al. 2002). Alcohol extraction may be more accurate, but powdered sugar does not kill bees. Because the mites can be counted in the field using either method, beekeepers can sample and treat during a single visit to an apiary.

Sampling for Treatment and Breeding. Previous research has indicated that a colony should be treated

to control *Varroa* mites if $\approx 10\%$ or more of adult bees are infested (Delaplane and Hood 1997, 1999; Martin 1998). That threshold was based on mite levels in stationary colonies and may not apply to colonies transported by migratory beekeepers. Migratory transport increases horizontal transmission of bee pathogens and pests and can extend the mite reproduction season in colonies that are moved from colder to warmer climates.

There is probably an absolute lowest threshold, where no beekeeper should need to treat. But, between that low threshold and the 10–12% level is a gray area where a beekeeper will need to make a treatment decision, based on the nature of the beekeeping operation and the beekeeper's own limits for acceptable colony loss. With further research, it could be possible to use sequential decision sampling (Moon and Wilson 2009) to determine whether treatment is warranted. Presuming a treatment threshold can be established, benefits of sequential sampling could include reductions in sampling time compared with a fixed sample-size approach, and reductions in erroneous decisions when densities are near the treatment threshold.

The sampling plans proposed here, along with good record keeping, may help beekeepers obtain a better estimate of mite density below which colonies can survive to the next treatment opportunity and above which treatment would be justified. Beekeepers and researchers also could use the sampling plans to monitor mite densities in individual colonies, and use that information to select queens for breeding and thereby increase heritable resistance to *Varroa* mites.

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