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A New Stratified Sampling Procedure which Decreases Error Estimation of Varroa Mite Number on Sticky Boards

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ABSTRACT A new procedure of stratified sampling is proposed in order to establish an accurate estimation of *Varroa destructor* populations on sticky bottom boards of the hive. It is based on the spatial sampling theory that recommends using regular grid stratification in the case of spatially structured process. The distribution of varroa mites on sticky board being observed as spatially structured, we designed a sampling scheme based on a regular grid with circles centered on each grid element. This new procedure is then compared with a former method using partially random sampling. Relative error improvements are exposed on the basis of a large sample of simulated sticky boards ($n = 20{,}000$) which provides a complete range of spatial structures, from a random structure to a highly frame driven structure. The improvement of varroa mite number estimation is then measured by the percentage of counts with an error greater than a given level.

KEY WORDS honey bee, sampling, error estimation, sticky board, *Varroa destructor*

Honey bee colonies are almost worldwide infested by the mite *Varroa destructor* which affects the health and global activity of the colonies (Le Conte et al. 2010). The high virulence of the parasite requires colony controls. Several treatment strategies that are available need varroa mite density estimations in the colonies in order to lead methodological adaptations and to compare the results of different strategies of control.

Varroa mite density is generally investigated by two main methods.

The first one is carried out by collecting honey bees on frames inside the hive and applying different treatments to dislodge and count varroa mites from bees by destructive methods. This requires, for assessing low error of estimation, to collect large bee samples, up to 300 bees per hives both from the brood and on the adult bees and to adjust to the total bee population (Lee et al. 2010).

The second method consists of catching dead or knocked out varroa mites on sticky boards at the bottom of the hive and counting the mites. It is an indirect non-destructive method that can be applied without disturbing the colony. This method is often used for long-term surveys in the investigation of varroa mite population dynamics or for estimating the efficiency of treatments in order to control varroa mite populations.

Sampling studies have highlighted two main difficulties in counting varroa mites on sticky boards (Calderone and Lin 1998, 2003, Ostiguy and Sammataro 2000).

First, spatial heterogeneity of varroa mite distribution on sticky boards leads to developing stratified sampling;second, counting large number of varroa mites often encountered on sticky boards (especially after varroa mite control treatments) is time consuming and requires accurate error estimations in order to develop new counting methods.

Two modes of spatial structures were observed for varroa mite distributions on sticky boards:

The distribution of varroa mites within the frame is often described as patchy, varroa mite populations being concentrated at one time on few brood frames regarding the brood development cycle. This leads to a gradient of varroa mite density on sticky boards as if the dead varroa mites had shifted to one side of the sticky board.

The distribution of varroa mites could also be highly dependent on the parallel structure of the frames since dead varroa mites tend to fall vertically in the space between the frames. It is commonly observed that dead varroa mites and debris lie in rows corresponding to inter-frame space (Ostiguy and Sammataro 2000). This spatial structure is even more marked as the number of dead varroa mites increases, especially when sticky boards are used for controlling treatment efficiency.

In addition, sticky boards have proven themselves as useful tools for studying long-term dynamics of varroa mite populations without honey bee colony perturbations (Büchler et al. 2010). During these several month surveys, the varroa mite density on sticky board deeply varies, which requires being able to account for the errors level for further modeling varroa mite population dynamics.

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Sampling patterns and stratification procedures have to be adapted to the spatial heterogeneity of individuals to be counted. Alexander et al. (2005) and Soubeyrand et al. (2009) have pointed out the fact that, in the case of unknown spatial structure of individual distribution, stratified samplings on a regular grid could contribute to decreasing the error in density estimation.

Ostiguy and Sammataro (2000) described an elegant sampling method. They used a sampling grid about two-thirds of the total area of the hive bottom where dead varroa mites could fall. This grid was divided into 12 by 18 cells of 4 cm^2, nested in groups of 3 by 3 (9) cells among which three were randomly selected for counting. The grid was placed on the area where the varroa mite density seemed the highest.

In order to improve on this method and to cope with the problem of spatial structure of varroa mites on sticky boards, we suggest changes to optimize the original sampling procedure described. We applied a grid with centered circles on a regular grid as stratified sampling procedure and we based the error calculation on simulation tests. Simulation procedure provides the opportunity to explore a large range of spatial patterns with different number of varroa mites on sticky board. The error estimation is then based on the exact calculation of the difference between simulated number of varroa mites and actual counted number by means of sampling grid.

Finally, the effect of different patterns of grid on error depending on the varroa mite density is presented in order to allow the experimenter to adapt the method to the needed level of accuracy.

Materials and Methods

Error evaluation on varroa mite density estimation with sampling procedure has been made following four steps: 1) the evaluation of the existence of non-random spatial structures of varroa mite distribution by digitalization of observed sticky boards; 2) the evaluation of the effect of different types of spatially structured simulated sticky boards on error; 3) the evaluation of error risk on a large range of simulated sticky boards with reference to density classes of varroa mite densities; and 4) the evaluation of error with increasing surface of sampling during experiments in order to measure the efficiency of the treatment.

Digitalized Sticky Boards. As a reference for exploring the properties of the methods to be compared and for a first estimation of the error dependence on spatial structure of varroa mites on sticky boards, we digitally mapped the varroa mite positions of 30 by 38 cm sticky boards. One hundred forty-five sticky boards were collected from various sources (mainly from professional beekeeper association experiments) with a range of density from 4 to 1,675 varroa mites on one board. Each sticky board was placed on an A3 digitalizing table and the coordinates X (large side, parallel to frames) and Y (small side, perpendicular to frames) of the position of each varroa mite were collected.

Simulated Varroa Mite Distribution. Three types of simulated spatially structured sticky boards have been achieved:

1) randomly distributed varroa mites: Poisson distributed X and Y coordinates have been simulated. Complete Spatial Randomness (CSR) tests have been used to check the random distribution (Fig. 1; Rcran *kstest*; *package* {`spatstat`});

2) 'shifted' spatially structured varroa mites: as varroa mite distribution on board could be spatially structured in relation to its distribution among the all frames, the position coordinates X and Y have been obtained by the combination of three normal distributions:

1. A normal distribution N_0 ($\mu_0 = 18$, $\sigma_1^2 = 30$), with μ_0 as mean and σ_1^2 as variance, along the width of sticky board (perpendicular to frames), the 10 quantiles q_i, $i = \{5, 15, 25, \ldots, 95\}$ of which give the expected proportion Nq_i, from the total N_{tot} varroa mites, under each frame. For a hive width of 36 cm, the choice of $\mu_0 = 18$ provides a distribution of varroa mites centered along frames. This centered distribution is modified to provide 'shifted" like structure:

$$q_i \sim N_1(\mu_1 = 11, \quad \sigma_1^2 = 30);$$

2. A normal distribution of XNq_i as X coordinates along the length of sticky board (parallel to frames):

$$XNq_i \sim N_2(\mu_2 = 20, \sigma_2^2 = 10);$$

3. A normal distribution of YNq_i as Y coordinates on each side of each frame as a dispersion parameter:

$$YNq_i \sim N_3(\mu_3 = q_i.N_{tot}, \sigma_3^2 = 2).$$

CSR tests have been used to check against the random distribution (Fig. 2).

3) 'frame' spatially structured varroa mites: as varroa mite distribution could be spatially structured in relation to their distribution on each frame, the position coordinates X and Y have been obtained by the combination of three normal distributions:

1. A normal distribution along the width of the sticky board (perpendicular to frames), the 10 quantiles q_i, $i = \{5, 15, 25, \ldots, 95\}$ which give the expected proportion Nq_i, from the total N_{tot} varroa mites, under each frame; this distribution is centered on the middle of the hive width: $q_i \sim N_1(\mu_1 = 18, \sigma_1^2 = 30)$;
2. A normal distribution of XNq_i as X coordinates along the length of the sticky board (parallel to frames):

$$XNq_i \sim N_2(\mu_2 = 25, \sigma_2^2 = 15);$$

3. A normal distribution of $YNqi$ as Y coordinates on each side of each frame as a dispersion parameter (varroa mites can spread over two inter-frame spaces on both sides of the frame i):

$$YNq_i \sim N_3(\mu_3 = q_i.N_{tot}, \sigma_3^2 = 0.2).$$

CSR tests have been used to check against the random distribution (Fig. 3).

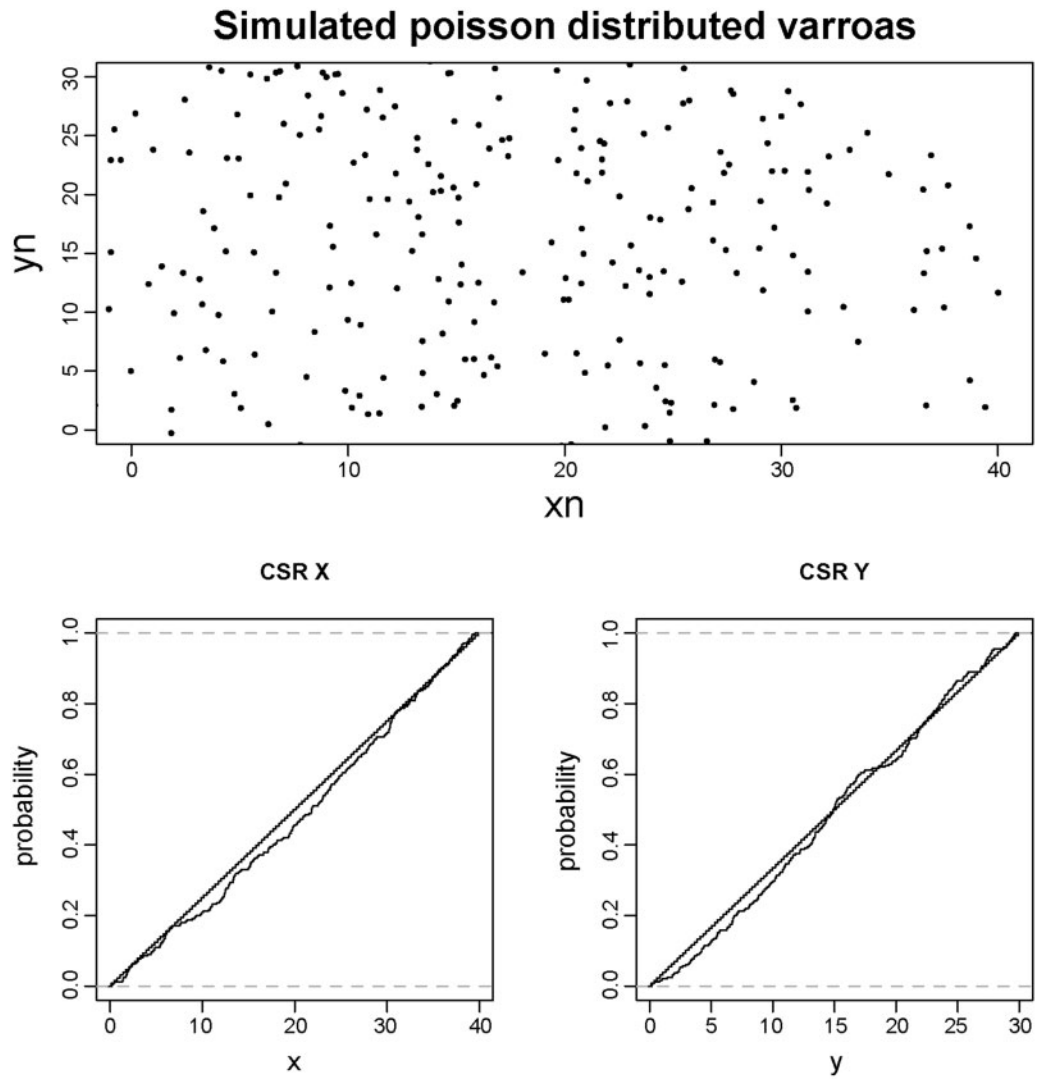

Fig. 1. Randomly simulated Poisson like (random) sticky board (top) and CSR test along X (bottom left) and along Y (bottom right).

For each of the densities N_{tot} of the 145 digitalized sticky boards, the simulation of 100 of the three types of spatially structured sticky boards has been run.

In addition, both methods of stratified sampling have been compared using a large number (20,000) of simulated boards using uniform distribution of the four parameters that control the density and the spatial structure of varroa mites:

- N_{tot}, the number of varroa mites: $N_{tot} \sim U_{nv}$ (50, 3,000);
- the dispersion parameter within frames: $\mu_1 \sim U\mu_1$ (15, 50);
- the dispersion parameter along each frame: $\sigma_2^2 \sim U\sigma_2^2$ (10, 20);
- the dispersion parameter on both sides of a frame: $\sigma_3^2 \sim U\sigma_3^2$ (0.2, 2).

Error Estimation. For each simulated sticky board, the total number of observed varroa mites is noted N_{obs}. If A_t is the total area of the sticky board, A_s the area of a given stratified sampling and N_s the number of varroa mites counted through the stratified sampling, the estimated total number of varroa mites N_{est} is $N_{est} = N_s.\ (A_t/A_s)$.

And the relative error E_{rel} is given by $E_{rel} = |N_{est} - N_{obs}|/N_{obs}$

Stratified Sampling Procedures. Two stratified sampling procedures have been applied

- A sampling procedure named 'Grid', similar to Ostiguy and Sammarato (2000), however with few

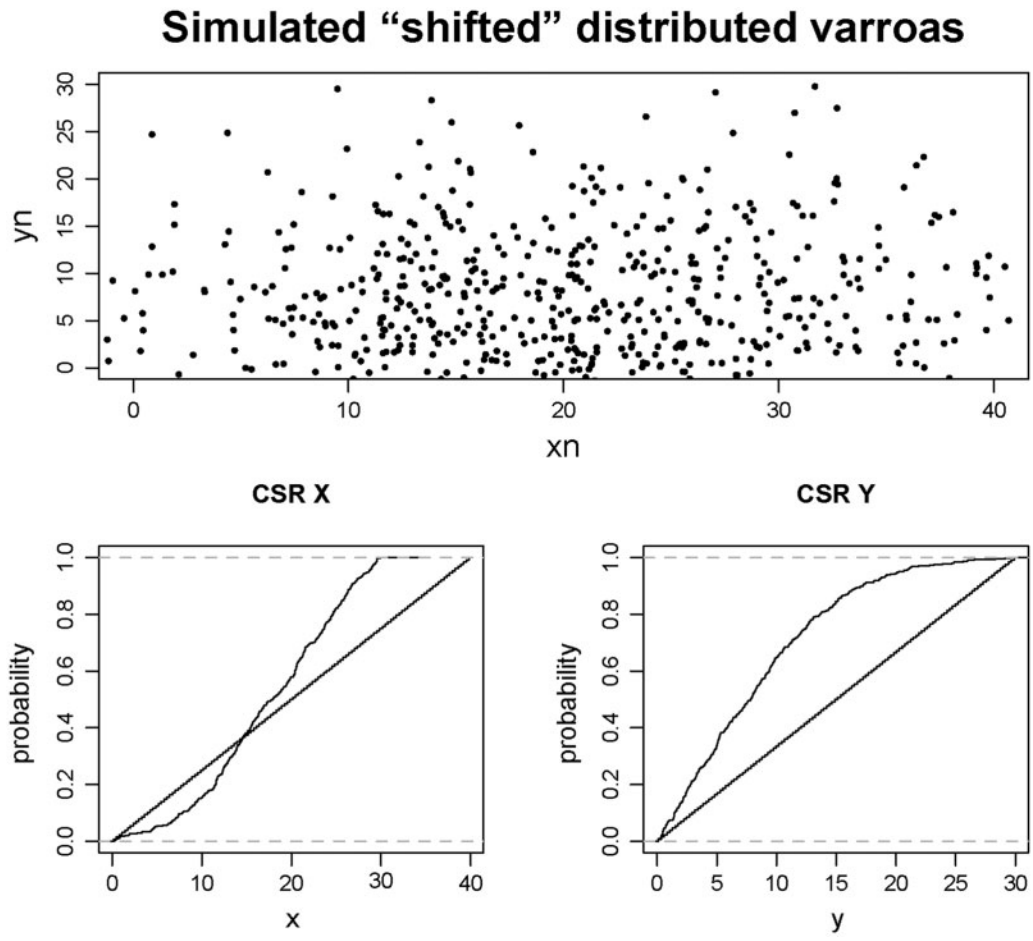

Fig. 2. 'Shifted' simulated dead varroa mites on sticky board (top). CSR test on *X* (bottom left) and *Y* (bottom right) coordinates.

changes. In order to fit the size of the entire sticky board, we designed a grid of 1.26 by 1.26 cm cells, grouped in 3 by 3 cell blocks of 3.78 by 3.78 cm which gives 8 by 10 blocks.

- A sampling procedure named 'Circle' in which circles are centered on each block (Fig. 4).

The increasing proportion of the total sticky board area has been sampled taking 3, 4, 5, 6, and 7 of the nine cells, at random, in each block. The circle areas have been fixed, respectively, to the proportion chosen for the Grid procedure (example of equivalent Grid and Circle sampling designs are given in Fig. 4).

Error Estimation Associated With the Measurement of Treatment Efficiency. One common use of sticky boards is to measure the efficiency of different treatment strategies, by testing various substances or application procedures. During these tests, a count of a usually large number of varroa mites takes place several times. Based on the data that an efficiency test of Apivar with Apistan control provided, we set a model of the counts of dead varroa mite on sticky boards with time.

The measuring experiment employed here as an example is divided into two periods: 1) a treatment period during which the tested substance (Apivar) is applied for 50 days with 10 counts of varroa mites on sticky boards; 2) a control measure during which a reference substance is applied (Apistan) for 80 days with nine counts of varroa mites on sticky boards. For assessing the efficiency error due to Circle sampling methods, a test is built in two phases:

1. A model is fitted on data collected during the aforementioned experiments on 30 hives. The 'treatment' phase is modeled with the hypothesis that the number of dead varroa mites observed on sticky boards (10 times in 50 days), followed a Gamma distribution with parameters α_T (i.e., 'shape') and β_T (i.e., 'rate'), respectively, drawn from a uniform distribution: $\alpha_T \sim U(3.5,6)$ and $\beta_T \sim U(0.25,0.75)$. The 'Control' phase (nine observations in 80 days) is modeled with the same hypothesis and parameters α_C (i.e., 'shape') and β_C (i.e., 'rate'), respectively, drawn from a uniform distribution: $\alpha_C \sim U(0.7,0.99)$ and $\beta_C \sim U(0.1,0.5)$. The range of

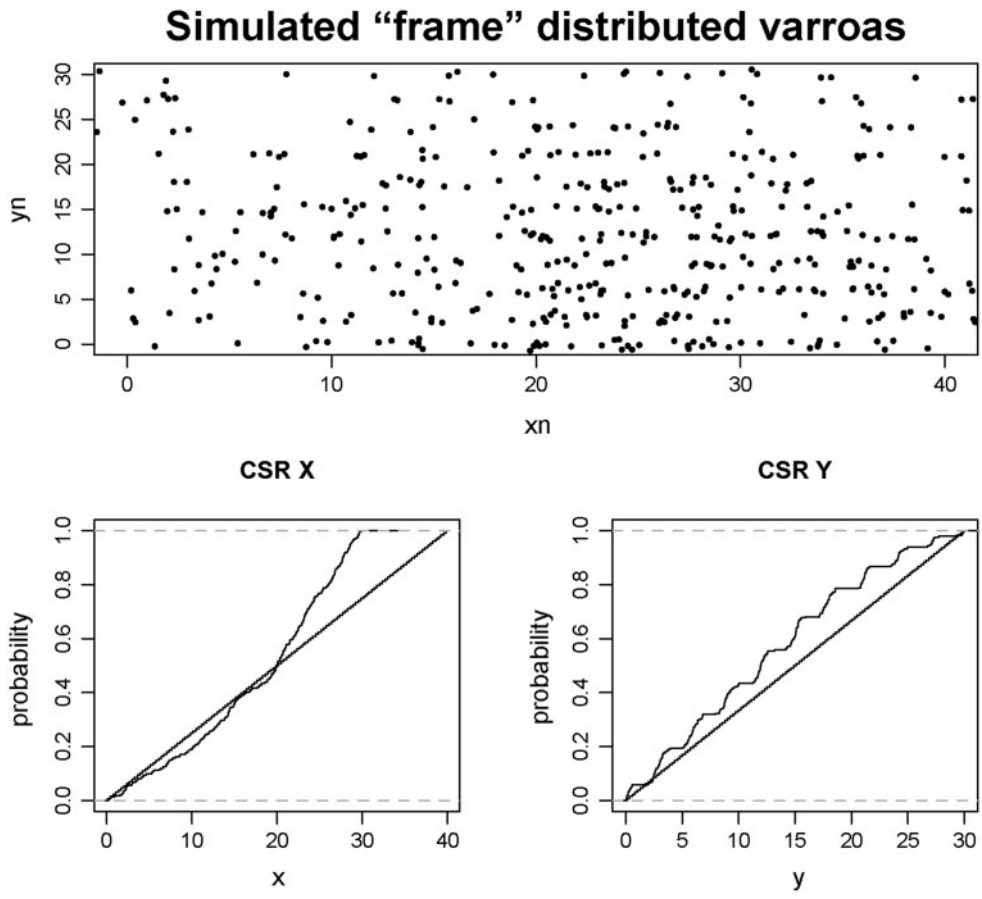

Fig. 3. 'Frame' simulated aggregated dead varroa mites on sticky board (top). CSR test along *X* (bottom left) and along *Y* (bottom right) coordinates.

parameters are chosen to explore a large diversity of quadruplet $\{\alpha_T, \beta_T, \alpha_C, \beta_C\}$ and to confront the Circle sampling method to various densities of varroa mites counted.

2. For each draw of the quadruplet $\{\alpha_T, \beta_T, \alpha_C, \beta_C\}$, 10 simulated 'treatment' numbers and nine simulated 'control' numbers of dead varroa mites are obtained simulating a full efficiency experiment. For each number of varroa mites, a Poisson point process is simulated and, as in the above section, an estimation of this number is then given, using the Circle method on a surface sampling equivalent to 4/9 cells. The efficiency is estimated by:

$$\mathrm{Eff} = \mathrm{Nv_T}/\mathrm{Nv_T} + \mathrm{Nv_C}$$

where

- $\mathrm{Nv_T}$ is the cumulative number of dead varroa mites on sticky board observed during the 10 treatment observations and
- $\mathrm{Nv_C}$ is the cumulative number of dead varroa mites on sticky board observed during the nine control observations.

The efficiency has been calculated using an estimated number of dead varroa mites with the Circle method ($\mathrm{Eff_{est}}$) as well as with the total simulated number ($\mathrm{Eff_{obs}}$), representing the 'true' value of density. The relative error (in percentage) due to the Circle sampling method has been estimated by:

$$E_{\mathrm{rel}} = (|\mathrm{Eff_{est}} - \mathrm{Eff_{obs}}|/\mathrm{Eff_{obs}}) \times 100$$

In addition, the Circle sampling method has been applied with two sampling intensities where sampling areas were 1/3 or 4/9 of the total sticky board area. Altogether, 1,000 simulations have been carried out with each of the sampling intensities.

Results

CSR Tests on Digitalized Sticky Boards. A CSR test was run on each digitalized sticky board. 58.3% have been analyzed as rejecting H_0 test hypothesis (H_0: no difference with random distribution). Most boards accepting H_0 contained <50 varroa mites. The test was then performed on boards with varroa mite number >50. 85.4% of these boards rejected H_0 and were

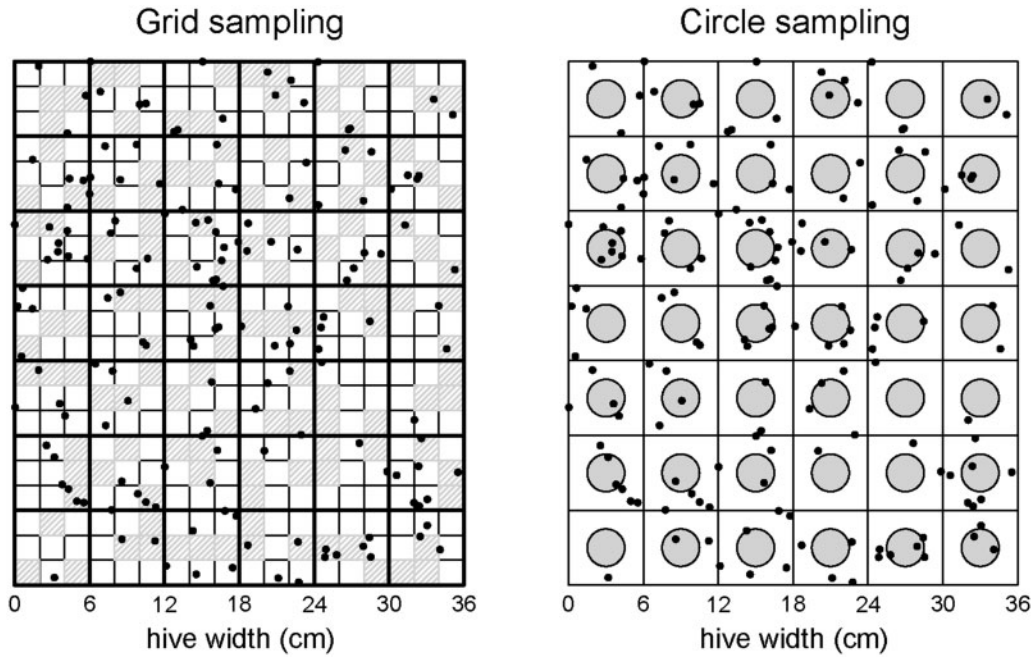

Fig. 4. Examples of Grid sampling and Circle sampling. Grey shaded line area sampled (left) with three cells over nine per blocks. Grey shaded circle area sampled (right) with the equivalent proportion.

therefore expected to present a spatial structure. According to this result, the measures with simulation have then been done on the boards containing more than 50 varroa mites. As a source of varroa mite density on boards (used for simulations), we chose to take the densities >50, observed in the digitalized boards, considering that counts <50 varroa mites are realized, in practice, without requiring sampling procedures.

Effect of the Increasing Sampling Area and of the Structure of Varroa Mite Distribution on Sticky Board. As the proportion of the sampling area increases, the relative error E_{rel} decreases (Fig. 5). In the case of Poisson like and 'Shifted' structure, there is no significant difference between the two methods. When varroa mite distribution depends on the frame structure, the 'Circle' method provides a lesser mean error. The difference between the two methods is all the more significant as the dispersion parameter on each side of the frame is small, i.e., the more varroa mite structure on boards depends on frame structure, the more the 'Circle' method provides a lesser error. When the dispersion parameter s_3^2 is high, thus simulating a large dispersion and a small effect on frame structure, the only significant difference is observed for the sampling area proportion of 3/9. If the dispersion parameter decreases (i.e., the varroa mite fall at the very vicinity of each frame), the difference between the two methods increases and is highly significant for the sampling area proportion of 3/9 and 4/9 (Fig. 5).

Comparison of Error Level. Three levels of relative error have been estimated over the large number of simulated sample. Three classes of varroa mite density have been distinguished: varroa mite density $\leq$500, 500 < varroa mite density $\leq$1,000 and varroa mite density >1,000, following the classification used by Ostygui and Sammataro (2000) (Table 1).

The percentage of boards measured with a relative error E_{rel} less than a given level provides a measure of the risk of error. Practically, the need for beekeepers or technicians in apiculture is not to know the actual number of varroa mites falling on sticky board, but to estimate varroa mite load with a given level of error in order to establish recommendations for treatments (date and products). The relative error less than a given level becomes a useful tool for practitioners working on a large number of sticky boards during varroa mite population surveys over years or during control experiments about the efficiency of a given treatments against varroa mite infestation.

The mean error is the statistical estimation of the actual relative error made over a large number of counting. It is a measure needed for modeling varroa mite population dynamics or for the determination of critical threshold density. It is also a measure for scientific studies especially when modeling is required.

In all classes, at any level of relative error and for all the proportions of sampling area, the Circle method, compared with the Grid method, provides a highly significant improvement of the estimation of varroa mite number (Table 1).

When considering increasing E_{rel}, the difference between the two methods, calculated by the percentage of boards measured with a relative error inferior to a given level, increases as the density of varroa mite increases for all E_{rel} levels. The effect of density on this percentage is less important as the levels of E_{rel}

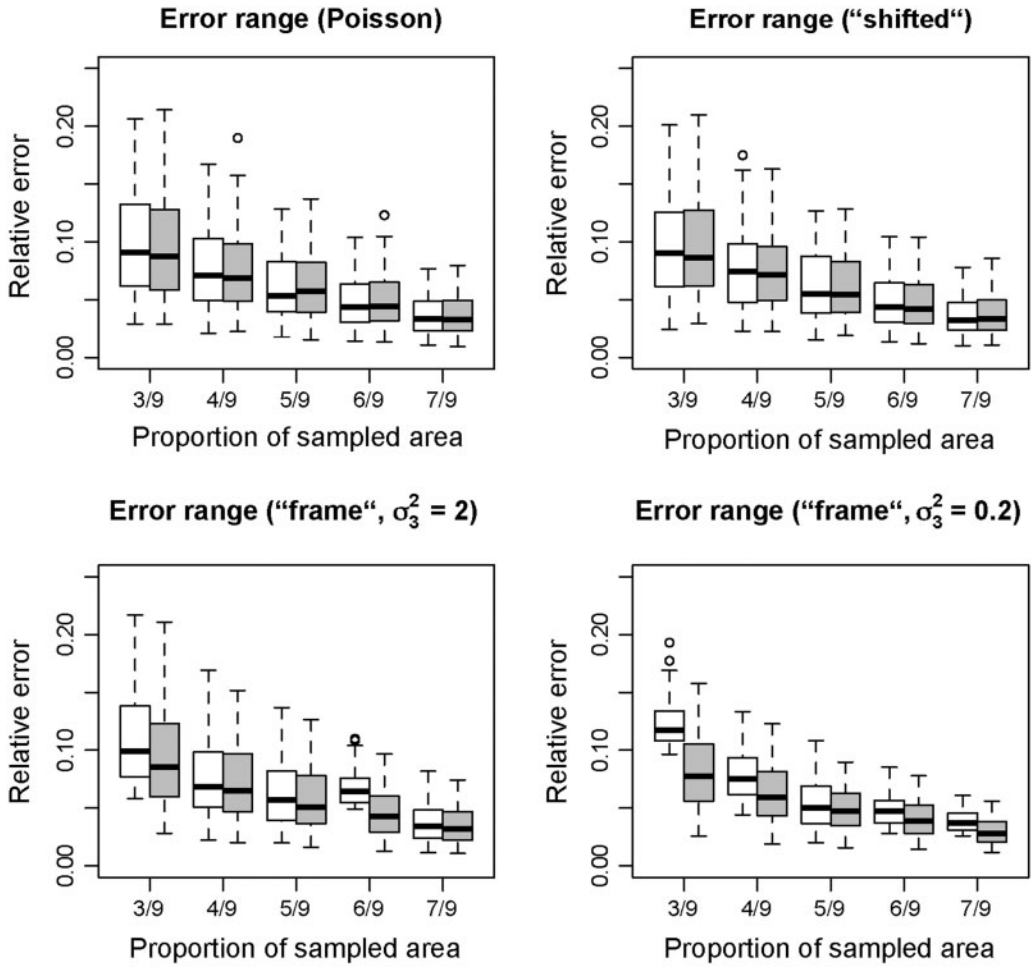

Fig. 5. Range of relative error with different spatial structure of varroa mites (nv >50) on simulated boards. Top left: Poisson-like structure. Top right: 'shifted' structure. Bottom left: 'frame' structure (with dispersion parameter on frame $s_3^2 = 2$). Bottom right: 'frame' structure (with dispersion parameter on frame $s_3^2 = 0.2$). White boxplot for Grid; grey boxplot for Circle.

increase. At the E_{rel} level of 0.2, the percentage difference between the two methods is minor even for the smallest proportion of sampling area (3/9). This level of error should be considered only as useful in preliminary estimation and both methods are suitable in this case. As expected, when the sampling area increases from 3/9 to 5/9, the difference between the two methods decreases to produce the same results. The Circle method provides better results for small sampling areas, saving time as well.

When considering the comparison of the two methods at $E_{rel} \leq 0.05$ and $E_{rel} \leq 0.1$, a clear improvement can be seen at the latter level with the Circle method. This improvement is again better for small sampling areas.

The table showing the variation of the two parameters in the Circle method (percentage of boards with E_{rel} less than a given level and mean E_{rel}) could be used as a guide for the choice of the sampling area depending on the needed level of relative error and the density of varroa mites on boards. For example, if an error ≤ 0.1 is required with the smallest sampling area (i.e., 3/9), the percentage of counts conforming to this specification will be 65.3 and 75.8%, respectively, for Grid and Circle methods, when the number of dead varroa mites on sticky board is <500. When the number of dead varroa mites is >1,000 and if a level of relative error is required to be <0.05, the percentage of counts conforming to this specification is 58.0 and 81.8, respectively, for Grid and Circle methods.

Error on the Estimation of Treatment Efficiency. From the 1,000 simulations of efficiency experiments, the average error associated with the Circle sampling method (with 1/3 and 4/9 of the total board area sampled) is estimated as equal to 0.619 and 0.501% ranging from 0.002 to 3.01% and 0.001 to 2.58%, respectively (Fig. 6) for efficiency ranging from 86.55 to 97.89%. A very poor correlation ($R^2 = 0.045$), although highly significant (P-value $= 7.97e^{-12}$), is noticed between the level of efficiency and the relative error.

Table 1. Percentages of boards measured with an E_{rel} less than a given level ($E_{rel} \leq 0.05$, $E_{rel} \leq 0.1$, and $E_{rel} \leq 0.2$) depending on the density of varroa mites on boards (nv) grouped in three classes (nv $\leq$500, 500 < nv $\leq$ 1,000, and nv >1,000)

	Prop.	All			nv ≤500			500 <nv ≤ 1,000			Nv >1,000		
		Grid	Sign.	Circle	Grid	Sign.	Circle	Grid	Sign.	Circle	Grid	Sign.	Circle
$E_{rel} \leq 0.05$													
% board	1/3	51.20		63.30	36.90		45.30	51.90		67.50	58.00		81.80
	4/9	61.90		77.70	46.00		54.50	61.90		77.20	69.60		89.30
	5/9	71.40		85.80	55.50		63.70	71.90		87.20	79.10		96.10
Mean E_{rel}	1/3	0.024	***	0.022	0.025	**	0.024	0.025	***	0.023	0.025	***	0.023
	4/9	0.023	***	0.021	0.024	NS	0.024	0.023	***	0.022	0.023	NS	0.023
	5/9	0.022	***	0.019	0.024	**	0.030	0.022	***	0.020	0.020	NS	0.022
$E_{rel} \leq 0.1$													
% board	1/3	81.50		92.20	65.30		75.80	82.80		94.90	89.00		99.20
	4/9	89.90		95.40	76.90		84.00	91.40		98.30	95.90		99.90
	5/9	94.90		97.60	85.60		91.40	97.10		99.80	98.70		100.00
Mean E_{rel}	1/3	0.042	***	0.033	0.046	***	0.043	0.042	***	0.036	0.042	***	0.040
	4/9	0.038	***	0.030	0.043	***	0.041	0.039	***	0.031	0.039	***	0.035
	5/9	0.034	***	0.025	0.041	***	0.037	0.034	***	0.026	0.034	***	0.030
$E_{rel} \leq 0.2$													
% board	1/3	97.50		98.70	92.40		95.70	99.20		100.00	98.80		100.00
	4/9	99.00		99.40	96.70		98.20	99.90		100.00	100.00		100.00
	5/9	99.60		99.70	99.00		99.40	100.00		100.00	100.00		100.00
Mean E_{rel}	1/3	0.056	***	0.040	0.073	***	0.063	0.057	***	0.040	0.057	***	0.049
	4/9	0.046	***	0.034	0.062	***	0.054	0.046	***	0.033	0.046	***	0.039
	5/9	0.038	***	0.027	0.053	***	0.045	0.037	***	0.037	0.037	***	0.031

Calculations made on 20,000 simulated boards with three proportions of sampling areas: 1/3, 4/9, and 5/9

Discussion and Conclusion

As *Varroa destructor* is considered as one of the major causes of bee colony weakness, it is necessary to evaluate varroa mite density with precision in the colonies. The sticky board method allows precise counting especially when sticky boards are brought back to the laboratory. But counting varroa mites on sticky boards could be time consuming when the varroa mite density reaches more than several hundreds. As varroa mites spread on boards with various spatial patterns, stratification sampling can help reduce the time spent on counting and increase precision as well.

Using the results of spatial statistic methods, we suggest a procedure to improve the measurement of varroa mite density on boards, starting with the stratified method proposed by Ostygui and Sammarato (2000). A stratification based on regularly distributed sampling circles is compared with the reference method mentioned above. The algorithmic procedure to simulate varroa mite dispersion on boards derived from observations of the heterogeneity of varroa mite distribution on frames (Calderone and Lin 1998, Lee et al. 2010). This is confirmed by CSR tests run on the collected boards, which show that 85% of the boards containing more than 50 varroa mites offer significant spatial structure.

By using this procedure, the estimation of two useful parameters has been highlighted:

- the percentage of boards on which varroa mites density estimation is performed by stratified sampling with a minimum relative error threshold and

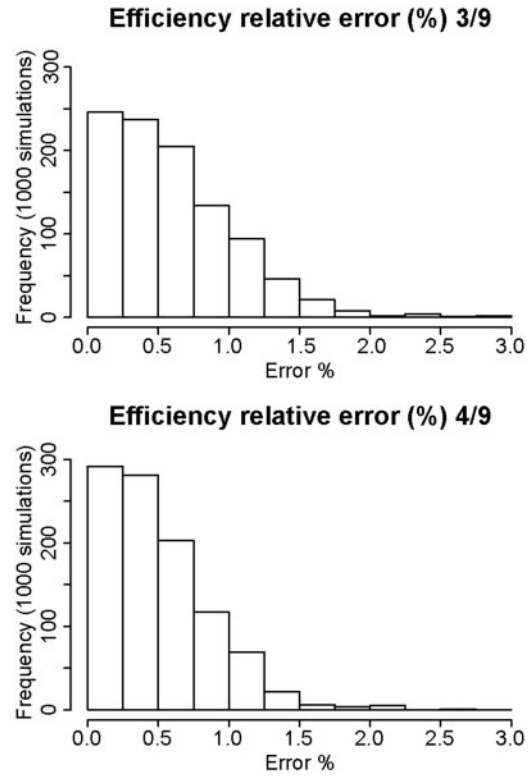

Fig. 6. Distribution of error (%) for efficiency estimation with Circle sampling method. Left: sampling area is 1/3 of the total area of the sticky board. Right: sampling area is 4/9 of the total area of the sticky board.

- the mean relative error which is the statistical estimation of the relative error.

From a practical perspective, the error estimation associated with the Circle sampling method with regards to efficiency experiment reveals that this method can be used without risk in terms of the accuracy of the efficiency evaluation. Nevertheless, during the calculation of efficiency, the very high density of varroa mites in the first days of observation, either in treatment period or in control, strongly affects the final efficiency measure (because cumulative counts are used) as well as supports the minimal error with sampling method. The error with Circle sampling is greater on the last counts at the end of both periods but it is barely relevant for efficiency calculation. In the case of more accurate studies (e.g., when population dynamics is aimed as a result of counting dead varroa mites on sticky board; in this case, the accurate density at any time of counting is requested), it is suggested to the experimenter, balancing between saving time and accuracy, to choose exhaustive counts when the density of dead varroa mites on boards is less than ~100 varroa mites.

Beyond the technical improvement of stratified sampling procedure, aiming at satisfying both the risk evaluation for field work and the statistical estimation for scientific studies, it seems that the design of a sampling mask, with regularly distributed circles covering the whole area of the sticky boards, could bring a real advantage for the standardization of varroa mite counting on boards.

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