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Interactions between honeybees and *varroa* mites influenced by cell sizes and hygienic behaviour

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With 2 figures, 9 tables and 2 pictures

Abstract: Differences in cell sizes used in beekeeping are often proposed to be one of the factors regulating *Varroa* population growth. An in-depth research of the relationship between these factors was undertaken using the 'Carnica Singer population'. The acceptance of foundation with a specific cell size appears to underlay selection. Smaller cell size (4.9 mm) foundation/combs reduce the *Varroa* population growth compared to 5.5 mm. This reduction is also dependent on the presence of the VSH (= *Varroa* sensitive hygienic) – behaviour trait in the worker bee population within the colony. Smaller cell size combs in combination with breeding for the 'right genetics' (cell size and VSH) can be one part of an integrated sustainable treatment concept for *Varroa* control.

Keywords: *Apis mellifera carnica*, *Varroa destructor*, small cell size, VSH (*Varroa* sensitive hygienic behavior), Varroa population growth

1 Introduction

Worldwide the 'new' ectoparasite *Varroa destructor*, Anderson & Trueman 2000, is considered the main cause of colony-losses. Cell size of the foundation is one of the possible solutions beekeepers are experimenting with to help bee colonies to better cope with this parasite.

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In the past the natural cell sizes of European-honeybees (*Apis mellifera*) were smaller than nowadays (Zeissloff 2007). However, beekeepers wanted more productive bees and Baudoux introduced the use of specially produced foundation, with larger cell sizes for colonies to increase performance (Baudoux 1933).

In 1989 a better survival of colonies against *Acarapis woodi*, Rennie 1921, on colonies with combs with cell size 5.1 mm has been observed by Dee & Ed Lusby (Lusby 1996 a). At that time commercial foundation varied between 5.3–5.7 mm in their cell size. The same kind of experiment was started after the arrival of *Varroa destructor* in 1995 (Lusby 1996 a, b.). In the years afterwards many papers on cell size and *Varroa* were published.

A number of studies exist detailing the negative influences of smaller cell sizes on *Varroa destructor* population growth: (Message & Goncalves 1995, Martin & Kryger 2002, Kober 2003, Piccorillo 2003, Forsman et al. 2004, Johnsen 2005, Kleinfeld 2006, Maggi 2009).

On the other hand, scientific works reported no-effect or even larger populations comparing small versus large cell sizes and *Varroa* development: (Fries 2004, Berg 2004, 2005, Dreher 2007, Dreher & Liebig 2007, Liebig & Aumeier 2007, Ellis 2008, Taylor et al. 2008, Berry 2009, Berry et al. 2010, Coffey et al. 2010, Seeley et al. 2011, Khoei 2015).

Beekeepers are using and propagating cell sizes of 5.1 mm and 4.9 mm in order to support the colony to cope better with the ectoparasite. A standardised cell size for *Apis mellifera* foundation does not exist. In Austria the standard foundation offers the bees a cell size of 5.5 mm to draw out their combs.

Since 2004 the removal of mite infested brood by adult bees is described as an inheritable behaviour trait that suppresses the mite reproduction (Ibrahim & Spivak, 2004, 2006, Harbo & Harris, 2002). This mite resistant trait is known as '*Varroa* Sensitive Hygiene' or VSH as it appears to be a form of hygienic behaviour (Harris 2007).

To measure or analyse the presence and expression of the VSH-trait in a colony, the *Varroa* infestation on a comb of sealed brood prior to emergence is used. A standardised number of infested cells are opened and the reproductive success of the mite is recorded. VSH is calculated as the quotient of cells with a non-reproductive *Varroa* / total number of infested brood-cells (n = 50). The theory behind VSH behaviour as a factor in the tolerance of honey bees to *Varroa* shows that an active interruption of the reproductive cycle of a mature *Varroa* (reproductive phase) reduces the population growth. The active interruption is done by worker bees shortly before the young adult bee emerges (elderly pupae) (Harris 2007).

2 Material and methods

2.1 Measuring brood comb sizes

Cell sizes were measured using a digital ruler (calliper). Measurements of ten cells across the foundation were taken along the three axes displaced at 60°, and the cell

sizes were expressed in mean linear distance between two parallel sides of the hexagonal cell-imprint per cell size (Coffey et al. 2010).

2.2 Population build-up, selection of queens

In 2002 1,287 colonies from a Carniolan, *Apis mellifera carnica*, Pollmann, 1879, closed breeding population (Carnica Harald Singer, >1,000 colonies) were selected. In this population, the standard cell size of the foundation given to the colonies was the large cell size (LCS) of 5.5 mm. In order to have the selected 1,287 colonies in the right physiological condition ("summer bees") we conducted following steps after the cherry blooming:

Step 1: During a 10 days' interval three empty frames were positioned in the 12 frames OE-Breitwaben-hive (= a standard bee hive of Austria) type at position '2', 'center' and '12' to allow the bees to build free combs without prescribing a cell size. This allowed the worker bees to show the 'natural cell size preference' of the colony and let them build worker and drone combs.

Step 2: At day 11 the center frame was measured and replaced by the foundation with a small cell size (SCS) of 4.9 mm.

Step 3: 10 to 21 days later the SCS-comb was checked, 9.3% = 120 colonies showed acceptable drown-out cell patterns and were given a frame with a small 'SC start-up foundation' (5 cm wide).

Step 4: 79 of the 120 colonies were selected as they were capable to draw SCS combs in an acceptable way with a regular brood pattern.

Step 5: To see their real acceptance to SCS it was necessary to offer the colonies the SCS foundation twice within a 40-day interval (1.5 generation of worker bees).

Step 6: From the 79 colonies, 8 queens were selected to become foundresses for 8 new lines. They were selected in shook swarms by testing the queens with their own small bees (1.5 kg bees) in swarm boxes (30 × 30 × 30 cm with metal mesh on two sides and on the bottom board and a feeding part on top), which were kept for 48 hours in a dark cool room (15° C). They were fed with 1 litre of syrup (1 : 1/ sugar : water). The natural cell sizes of the built combs were measured. The selection criterion for their burr combs was cell size ≤ 5.1 mm.

For each of the 8 lines 20 daughter queens were artificially inseminated with drones from their own mother colony.

Step 7: The next generations were mated on an isolated mating yard with the 79 group as males.

Steps 8 and 9: 2003 and 2004 more foundresses from the 79 group were used to create 8 more lines (method of selection as described in step 6). The young daughter-queens were brought to the mating yard (79 group males).

In total 16 SCS lines were analysed (8 from 2002, 5 from 2003, and 3 from 2004).

2.3 *Varroa* population growth on LCS and SCS

From 2003 onwards, groups of colonies (see Table 1 for details) were built using young queens with 1.5 kg of worker bees (shook swarming technique). Each 20 kg shook swarm contained *Varroa* infested bees. The worker bees for the shook swarms came from SCS colonies. Samples of each 20 kg bees stocks were taken in order to collect data on the infestation-levels. Out of 20 kg bees 13 shook swarms (1.5 kg each) were formed and given 12 sister-queens, 6 swarms for each cell size (SCS and LCS). Of each series, the thirteenth swarm was used to define the initial *varroa* population number. The test shook swarms contained between 196 and 492 *varroa* mites per swarm (mean number of test shook swarms: 268 mites / swarm).

In season 2003, 205 new colonies were built up. For each line two groups, SCS & LCS colonies, were created and randomly divided on 7 bee-yards in the Danube-March national park, south-east of Vienna. To prevent any contamination with miticide-residues, new hives and bio certified wax foundations were used. The bottom-boards were equipped with mesh-protected drawers to collect the natural mite-fall (Dietemann 2013).

During the active season (May – September) the drawer-contents were collected every 10 days. Colony strength was judged once in August using standard procedures as described by (Imdorf 1987, Delaplane 2013). Colony management was undertaken according to "good apicultural practices" by a "beemaster", but without *Varroa* treatment. All established colonies were overwintered to analyse their winter-survival.

In principle this means the Kefuss way of selection "Bond Test – live and let die" (McNeil 2010). This selection process allows an additional way of determining differences in *Varroa* resistance between the SCS and LCS groups.

In the season 2004, 221 new colonies were created. Five new lines were selected from the 79 group and 4 old lines, these 4 old lines were breeding daughters from the surviving colonies in 2003. The 9 lines were at random divided on 7 bee-yards.

The 2005 experimental group consisted of 3 new lines from the 79 group plus 4 lines (daughters from 2003 and 2004 colonies). In the 2005 season a total of 78 colonies which were developed from shook swarms were analysed. At the end of the 2005 bee-season all colonies (including the surviving colonies – 6 from 2003 plus 126 from 2004) were treated with an oxalic acid sublimation using Varrox®-Andermatt (Andermatt BioVet AG 2012). At that time the colonies were without sealed brood.

From 2006 onwards around 400 colonies on SCS were used to keep 7 lines which were selected for small cell size, low mite development and vitality (honey, swarm tendency, colony strength, etc.). Two mating-periods allowed the use of two different male groups, the first were descendent from the surviving queens in 2003 and their daughter-queens (in total from 4 lines). The second male group consisted of the surviving queens born 2004 and their daughter queens (4 lines).

2.4 Estimating *Varroa* reproduction parameter

From the 7 lines kept, four showed significant differences in *Varroa*-development when comparing LCS & SCS colonies. In May 2007 from these four lines 20 new

colonies per line on LCS and SCS with SCS-bees were created as described in section 2.3, through shook swarms, 4 pairs of sister queens were used in this test, the 80 young queens were all mated on one mating station.

In the middle of August from each colony a comb with elder sealed brood was analysed for reproductive success of *Varroa*, opening cells aged at latest one-day-before-emergence (Harbo & Harris 2009, Harris 2007, Dietemann 2013). From each colony a number of brood-cells were opened, searching for 50 infested cells. From these 50 infested cells the contents were recorded according to the following classification:

A- Cell with one dead *Varroa* mite.
B- Cell with one living *Varroa* mite.
C- Cell with one living *Varroa* mite plus 1 fully developed daughter *Varroa* mite.
D- Cell with one living *Varroa* mite plus 2 young fully developed *Varroa* mites.
E- Cell with one living *Varroa* mite plus 3 young fully developed *Varroa* mites.

These values were used to estimate VSH for each colony. For all 80 colonies the VSH-value was calculated as the quotient of cells with a non-reproductive *Varroa* per total number of infested brood-cells (n = 50), (A+B)/(A+B+C+D+E).

In May 2008 from two 2007 lines (1/2007 & 3/2007) the SCS queens and bees were used to produce 20 new colonies, each fitted with 4 LCS and 4 SCS drawn combs alternatively positioned. Due to supersedure in 2007, 10 original queens and 10 daughters (open mated) were tested. From each colony 400 infested brood cells were analysed, 200 from SCS and 200 from LCS combs. These data were used to compare the expression of the VSH-trait in each colony and on each cell size within that colony.

2.5 Statistical analysis

Data were analysed using IBM SPSS v20. Data are presented as mean and standard deviation. To analyse the impact of factors such as cell size, year, line and bee-yard on mite drop per brood area, a linear mixed model was applied followed by post hoc tests using Scheffe's alpha correction procedure. The assumption of normal distribution was tested using Kolmogorov-Smirnov-test. Differences between SCS and LCS in frequency distribution (e.g. winter losses) were analysed using chi-square tests. The odds ratio (OR) was calculated to evaluate the survival chance according to cell size. For all statistical analyses a p-value below 5% ($p < 0.05$) was seen as significant.

3 Results

3.1 *Varroa* development

Describing the *Varroa* population growth by means of the observed dead mites in the bottom-board drawer gives the following results when SCS (small cell size) colonies

are compared to LCS (large cell size) colonies. The colonies on SCS show a significant (p = 0.001) slower development of *Varroa* populations. This effect is independent of year, line and bee yard. Year, line and bee yard are considered as factors in the statistical analysis. (Data from the years 2003, 2004 and 2005) (Table 1), (Fig. 1).

The drop-outs in Fig. 1 are colonies, which imported high numbers of mites by robbing weaker colonies that were about to collapsed due to the high *Varroa* infes-

Table 1. Mean number of dead mites on *Varroa* board/brood area/year.

Cell size	*Year*	*Mean*	*SD*	*N*[a]
4.9 mm	2003	1416.4	886.9	97
	2004	787.3	653.7	123
	2005	688.3	663.7	39
	total	1008.0	813.0	259
5.5 mm	2003	1721.5	916.9	99
	2004	991.9	680.1	98
	2005	859.5	824.3	38
	total	1277.9	892.8	235
total	2003	1570.5	912.7	196
	2004	878.0	671.7	221
	2005	772.8	747.3	77
	total	1136.4	861.7	494

N[a] = number of colonies in test

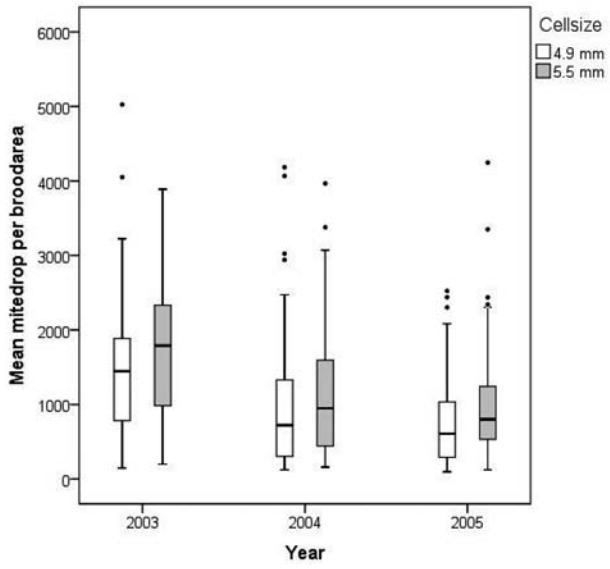

Fig. 1. show SCS colonies compared to LSC colonies (2003, 2004, 2005). The colonies on SCS show a significant (p = 0.001) slower development of the *Varroa* populations. This effect is based on year, line and bee-yard. Year, line and bee-yard are considered as factors in the statistical analysis.

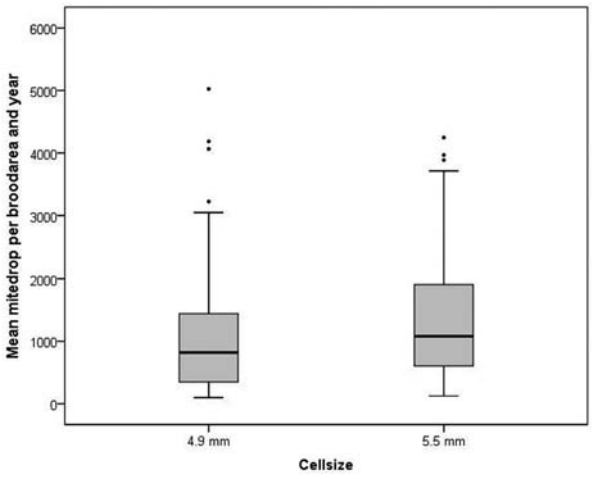

Fig. 2. Mean number of *Varroa* for LCS & SCS per year.

Table 2. Results of the mixed model for all four factors.

Source	***Df***	***F* (*df*, 611)**	***P***
cell size	1	11.0	0.001
year	2	21.5	< 0.001
line	12	13.2	< 0.001
bee yard	9	20.7	< 0.001
cell size x year	2	1.4	0.246
cell size x line	11	2.5	0.005
year x line	17	1.9	0.014
year x bee yard	10	42.9	< 0.001

A clear difference of overall *Varroa* population growth between the LCS and SCS colonies could be observed between lines ($p = 0.001$). The magnitude of growth difference (LCS & SCS) varied between 33% and 0% within lines. Year and bee yard, as they influence colony growth, showed to be significant factors at $p < 0.05$.

tation rate in their hives. The hostile bees dragged the mites with them when they robbed those other infested colonies of their honey. This could be noticed due to the fact that the dead mites-fall on the bottom board increased at the end of season when other colonies collapsed. Moreover a gain of weight (honey from dead colonies) was noticeable in those particular hives.

Table 2 shows the results from the GLM (model I). All main effects and interactions except cell size × year remain significant on mite drop. A clear difference of overall *Varroa* population growth between the LCS and SCS colonies could be observed between lines ($p = 0.001$). The difference between cell sizes among years is not different. Varying differences between cell sizes among years and mother line can be noticed as well as between mother line and bee yard among years. This means, that varying mite drop can be observed with a difference between cell sizes which remains

Table 3. Influence of cell size and year per mean number of dead mites in *Varroa* board/brood area/year (Simplification of analysis above, Table 2).

Factor / Interaction	*Df*	*F*(*df*. 670)	p-value
cell size	1	19.3	< 0.001
Year	2	38.1	< 0.001[a]
cell size x year	2	.1	.931

Supplementary note: [a]Sign. differences (p < 0,0) existed between the years 2003 and 2004 as in 2003 and 2005 (Bonferroni corrected).

Table 4. Mean number of dead *Varroa* on the bottom boards per brood area, separated for line and cell size

Line	SCS (4.9 mm)		LCS (5.5 mm)		*T*	*df*	*p*
	N	*mean* ± SD	*N*	*mean* ± SD			
1	75	1090.8 ± 630.5	59	1558.6 ± 892.9	−2.94	126	0.004
2	29	1517.5 ± 697.4	34	1602.9 ± 622.4	−0.63	58	0.534
3	11	3015.2 ± 1077.9	10	2275.8 ± 552.2	1.81	17	0.087
4	34	1644.2 ± 644.2	26	1977.6 ± 776.7	−1.75	55	0.085
5	37	379.0 ± 300.4	36	511.4 ± 450.9	−1.51	69	0.135
6	47	360.5 ± 315.1	8	1138.4 ± 1146.2	−2.05	8	0.075
7	16	1427.3 ± 554.2	16	1858.2 ± 488.4	−2.52	26	0.018
8	21	1477.3 ± 469.2	31	1467.2 ± 926.3	1.16	46	0.250
9	7	825.8 ± 308.8	8	908.9 ± 384.9	−0.34	13	0.739
10	35	818.1 ± 463.7	35	955.3 ± 694.3	−0.79	67	0.431
12	10	1041.3 ± 493.1	10	1040.3 ± 530.7	0.03	18	0.976
15	32	494.3 ± 273.9	32	493.3 ± 241.4	−0.16	62	0.875
16	17	415.3 ± 272.1					

The lines 1 and 7 were used for further analysis of their significant lower mite development on SCS combs, based on their genetic background. The lines 2 and 6 showed significant lower development at least in one of the three-year period and were also used for the detailed analysis of the *Varroa* reproduction parameters.

constant over the years. Year and bee yard, as they influence colony growth, showed to be significant factors with $p < 0.05$. These results indicate the complexity and variability of honey bee colonies as a result of individual queen genetics and the individual genetic of males mated with (interaction between mother-line, bee yard and year).

In a second analysis, the factors mother line and bee yard were excluded from the model (results shown in Table 3).

As seen in model I the main effects (year and cell size) remain significant. The interaction between cell size and year now disappeared ($F(2, 670) < 1$, $p = 0.931$). This means that the natural mites-fall differs among years (Fig. 2) but the difference between cell sizes remains the same. Small cell size combs have a lower mites' drop rate, whereas large cell size combs feature a higher one.

Table 5. Comparison of the survival rate between the two cell sizes separated by year.

Year			colony		total		
			survived	dead		X^2	p-value
2003	cell size	4.9 mm	29	68	97	1.9	0.163
		5.5 mm	21	78	99		
	Total		50	146	196		
2004	cell size	4.9 mm	85	66	151	5.1	0.023
		5.5 mm	50	68	118		
	Total		135	134	269		
2005	cell size	4.9 mm	107	16	123	1.0	0.302
		5.5 mm	72	16	88		
	Total		179	32	211		

Supplementary note: Odds Ratios (OR) with 95% confidence interval: 2003: OR = 1.58 (0.8, 3.0) 2004: OR = 1.75 (1.1, 2.8), 2005: OR = 1.48 (0.7, 3.2).

The effect of the genetic lines was tested (Table 4). The mean number of dead *Varroa* on the bottom-boards per brood area, separated after line and cell size showed clear differences. The lines 1 and 7 were used for further analysis of their significant lower mite development on SCS combs, based on their genetic background. The lines 2 and 6 showed significant lower development in at least one of the three years' period and were also used for the detailed analysis of the *Varroa* reproduction parameters.

3.2 Wintering

Within SCS 150 out of 371 colonies (40.4%) were lost, compared to 162 (53.1%) out of 305 colonies within LCS, which is significantly different ($X^2 = 10.8$, $p < 0.001$). SCS colonies are significantly more likely to survive the first winter after build-up (OR = 1.69 with a 95% confidence interval ranging from (1.3–2.3)). In 2004 a significant difference between cell sizes can be observed ($X^2 = 5.1$, $p = 0.023$). With an odds ratio (OR) = 1.75 colonies with smaller cell size are more likely to survive the first winter after build up. The OR is similar with 1.58 in 2003 and 1.48 in 2005, but not significant (Table 5).

3.3 *Varroa* reproduction parameter

The results of the variance analysis (Table 6) which were conducted in the year 2007 show that the effect of the line (distinction between the genetic lines) is significant when it comes to reproductive parameters. Line 1 and 7 quite differ from line 2 and 6. Cell size plays no significant factor for these reproduction parameters. There are 3 significant factors: VSH, the percentage of non-reproductive mother mites and the total contingent of non-reproductive mites. That means, that the VSH trait doesn't primary rely on cell size, but on the genetic line. VSH differs vastly between the lines, not between

cell sizes (Table 7). However, the degree of VSH can vary within a line between small and large cell size. Hence the statement, that small cell size exhibit a higher VSH trait, is only right for certain genetic lines, but not for all. Lines that miss the trait of course do not demonstrate the effect of cell size on the expression of the trait.

In 2008 5 test colonies per group (20 colonies) were fitted with SCS combs and LSC combs alternately positioned and inside each colony a more pronounced VSH treatment is observed in the SCS infested combs (Table 8). Even in the line L3 with low VSH activity.

Table 6. Influence of genetic line and cell size on the reproduction parameter of *Varroa* mites in the year 2007. (Results of variance analysis).

QdV	Parameter	df	F(df. 72)	p-value
Line	total of mites	3	23.6	< 0.001[a]
	infestation : wholesome	3	45.3	< 0.001[a]
	degree of infestation (%)	3	48.2	< 0.001[a]
	offspring / infested cells	3	23.6	< 0.001[a]
	ratio non-reproductive / reproductive (VSH)	3	8.2	< 0.001[a]
	percentage of non-reproductive mother mites (%)	3	11.2	< 0.001[a]
	non-reproductive mites	3	10.2	< 0.001[a]
	reproductive mites	3	9.9	< 0.001[a]
Cell size	total mites	1	< 1	0.979
	infested : wholesome	1	< 1	0.488
	degree of infestation (%)	1	< 1	0.508
	offspring / infested cells (50 or 200)	1	< 1	0.979
	ratio non-reproductive / reproductive (VSH)	1	< 1	0.590
	percentage of non-reproductive mother mites (%)	1	< 1	0.387
	non-reproductive mites	1	< 1	0.342
	reproductive mites	1	< 1	0.639
Line x	total mites	3	1.7	0.177
	Cell size	3	1.7	0.180
	infested : wholesome	3	1.6	0.204
	degree of infestation (%)	3	1.7	0.177
	offspring / infested cells (50 or 200)	3	4.1	0.010*
	ratio non-reproductive / reproductive (VSH)	3	3.9	0.013*
	percentage of non-reproductive mother mites (%)	3	3.8	0.014*
	non-reproductive mites	3	2.0	0.116
	reproductive mites			

Supplementary note: [a]Post hoc analysis using Scheffe's alpha correction showed significant differences between line 1 and line 2 and between line 1 and line 3. Line 4 was different from line 2 and line 3. *$p < 0.01$

Table 7. VSH-values of 4 lines.

year 2007	SCS (4.9 mm)				LCS (5.5 mm)			
line	**N**	**max**	**N-VSH ≥ 0.2**	**min**	**N**	**max**	**N-VSH ≥ 0.2**	**min**
L1[a]	10	0.44	60 %	0.08	10	0.26	40 %	0.04
L2 [b]	10	0.14	0 %	0	10	0.14	0 %	0
L6 [b]	10	0.17	10 %	0	10	0.21	10 %	0
L7 [a]	10	0.29	60 %	0	10	0.53	70 %	0.06

Post Hoc analysis (∝-corrected using Scheffé's procedure) showed a significant difference between L1 and L2 ($p < 0.01$), based on 50 infested cells per colony. The VSH calculation for the individual colonies in the test (2007) showed that—using the arbitrarily criteria VSH > 0.2 (e.g. in 20% of the infected cells no reproduction could be observed) - a clear difference between the lines exists, but not between cell-sizes.

Table 8. VSH values of 5 colonies per group with SCS & LCS combs in each colony.

year 2008	SCS – 200 infested cells each hive				LCS – 200 infested cells each hive			
line	**N**	**max**	**N-VSH ≥ 0.2**	**min**	**N**	**max**	**N-VSH ≥ 0.2**	**min**
L1	5	0.54	5	0.29	5	0.29	2	0.09
L1 (F1)	5	0.38	2	0.03	5	0.21	1	0.02
L3	5	0.16	1	0.03	5	0.07	0	0.02
L3 (F1)	5	0.27	1	0.08	5	0.13	0	0.02

In 2008 5 test colonies per group were fitted with SCS combs and LSC combs alternately positioned. Inside each colony a more pronounced VSH treatment is observed in the SCS infested combs. Even in the line L3 with low VSH activity.

Table 9. VSH of single queens and their daughters locally mated 2007, details of L1 & L1(F1) and L3 & L3(F1) in 2008 (Table 8).

number	L1 (2007)	L1(F1) (2008)	+/-	L3 (2007)	L3(F1) (2008)	+/-
1	0.45	0.38	-	0.1	0.14	+ +
2	0.16	0.06	- -	0.09	0.13	+ +
3	0.13	0.19	+	0.18	0.27	+ +
4	0.08	0.12	+	0.06	0.08	+
5	0.09	0.05	- -	0.12	0.14	+

The measured VSH of the original colonies and each daughter colony as measured in 2007 and 2008 to show the natural "drift" of the VSH-trait when queens mate free.

The measured VSH of the original colonies and each daughter colony as measured in 2007 and 2008 to show the natural "drift" of the VSH-trait when queens mate free (Table 9).

4 Discussion

4.1 Cell size

For over 30 generations of the Carnica Harald Singer population the bees were forced to build combs on wax foundation with a 5.5 mm. cell size. Choosing a certain cell-size for a honeybee population under selection, results in a dependency of the controlled population on that cell size to draw impeccable combs. The same non-intentioned result was shown of keeping bees by humans for a "wild" *Apis mellifera mellifera,* Linnaeus 1758 population in Dublin by McMullan & Brown (McMullan & Brown 2006, pers. comm.). To make colonies under selection accept more than one cell size a selection in the population appeared to be necessary.

Only 16 mother queens out of 1.287 = 1.2% fulfilled the criteria "accepting foundation with 4.9 mm. and 5.5 mm". Apparently this group (N = 1287) had unintendedly undergone a selection on the cell size 5.5 mm for at least approximately 40 years. It can be argued that some of the research done on the relationship between cell size and *Varroa* as put forward by A. & D. Lusby just failed to replicate their results because of this fact (Lusby 1996) (Dreher 2007). Coffey used a non-HYG subset of their population and as they describe it: none of the colonies were opening infested cells, and they did not see any effect of cell size on *Varroa* reproduction in undisturbed *Varroa*-infested cells (Coffey et al. 2010). This population showed no signs of the SMR-behavior as described by Harbo and Harris (Harbo & Harris 2002, 2005). It was pointed out that HYG and SMR/VSH targeting different in-cell brood-diseases, might very well be different types of cell cap handling behaviour (Rothenbuhler 1964, Masterman et al. 2000), each with different functional properties and probably involving different parts of the honeybee genome (Mondet et al. 2015). Just being able to draw small size combs does not automatically results in a slower growing *Varroa* population on SCS (Lines 3/2002 and 12/2003).

4.2 Selection

To see differences in *Varroa* reproduction a high *Varroa* pressure is needed. Keeping SCS and LSC groups without any *Varroa* treatment causes this high infestation pressure.

Most lines showed a higher number of survivors in the SCS group of the lines under Kefuss way of selection "live and let die" (Mc Neil 2010), 12 from 16 lines passed the live and let die selection applied on SCS and LCS. Analysing the *Varroa* development 4 lines did not show any influence of the factor cell-size, 2 lines showed

a higher development of *Varroa* in the SCS colonies. The genetics of 6 lines out of the 16 confirmed the Lusby hypotheses (Lusby D. & Lusby A. 1996a).

The step by step selection applied on 1,287 genetically different colonies demonstrates the existence of the variability in dealing with cell-size and *Varroa*. The results help to explain the controversial results found in literature. It clearly shows the genetic variability in a honeybee breeding population after a long time of classical selection on honey-production for traits, not selected for. In this actual project the combination of the traits surviving *Varroa* pressure and differences in cell-sizes are compared. The lines L1 (2002), L2 (2002), L6 (2002) and L7 (2003) showed a clear differentiated *Varroa* development in dependence of cell size, slower on SCS-combs being observed. The negative influences of smaller cell-sizes on *Varroa* population growth was published: (Martin & Kryger 2002, Kober 2003, Piccorillo 2003, Forsman et al. 2004, Johnsen 2005, Kleinfeld 2006, Maggi 2009).

No effects comparing cell size and *Varroa* development were described: (Fries 2004, Berg, 2004, 2005, Dreher 2007, Liebig & Aumeier 2007, Ellis 2008, Taylor 2008, Berry 2010, Coffey 2010, Seeley 2011).

4.3 Mechanism

As not all lines showed the positive effect on *Varroa* reproduction for the colony survival on SCS, the *Varroa* reproduction within the sealed brood cells had to be analysed.

4.3.1 Estimating the *Varroa* reproduction parameter

The division of the young mated daughter sister queens across the cell size groups was always randomized. So a difference by chance of a relevant trait just to appear only in a colony of a specific group (SCS or LSC) within each line can be excluded. The suggested superiority of SCS caused by the not at random division of a specific trait can be excluded.

Looking at the lines 3 and 12 they could eventually miss parts of a relevant trait, as the mother-queens of all lines are the first generation inbreeds descendants of a single artificial inseminated "foundress" queen (Janousek 1992, Brno, personal communication). They can be genetically different. In the whole Carnica Harald Singer group, each generation consists of 5 or more related groups ("lines"), which are always mated with a high number of non-sister queens as fathers, in the old Singer tradition. From generation to generation 7 to 15 selected mother queens are control-mated on isolated mating places with male-producing colonies from the whole population. So differences in the genotype can be expected (Singer 1976, van Praagh 2015, Ebersten 1996).

The daughter-generation from line 3/2002 does not exhibit the traits, which help SCS to be a favourable *Varroa* reproduction inhibitor, three generations were open-mated with the same male pool as the queens from lines 1/2002, 2/2002, 4/2002, 5/2002, 6/2002, 7/2003 and 8/2003. These 7 lines all exhibit the traits. They all show reduced *Varroa* reproduction in the SCS lines when compared to their "LCS-sisters" (Table 4).

Not all lines after the "Bond" selection showed a difference in *Varroa* population growth depending on cell-size. The lines 1/2002, 2/2002, 4/2002, 5/2002, 6/2002, 7/2003 and 8/2003 confirm the hypothesis. Line 3/2002, 12/2005, 15/2005 (Table 4) markedly have a high level of *Varroa* development and no differentiation of this level due to cell-size. These lines apparently miss the VSH-traits. We must conclude that those lines that do not show the VSH-trait, clearly demonstrate that cell-size per se does not influence *Varroa* population growth under the experimental conditions. As group of father queens producing the drones, the four mother colonies (2005, 2006) were used plus the still available sister colonies of each line, building a POOL of father queens (van Praagh 2015). Doing so, the probability of "saving" the bigger part of the genotype of the whole selection is there. The results show, that this way of bee breeding prevented the loss of the hardly understood genetics of VSH behaviour.

At a first glance the expression of the trait showed significant differences between lines. But statistically the expression was not significant in the test comparing daughter queens on the different cell sizes. Due to the genetic make-up of a colony the relationship between sister queens is only 25% (van Praagh 1994). The 4 used lines showed clear differences in *Varroa* reproduction between cell sizes. But expression of the worker-bee trait inside each colony within each line was estimated on 50 infested cells only. Leading to the conclusion that the clear differences found in earlier experiments and based on *Varroa* reproduction (10 days natural mite-fall) could in this experimental make up not be explained just by measuring the difference the classical way. Different worker composition per colony has to be expected. In 2008, using experimental colonies containing both comb types per line the inevitable variability between workers per colony, were overcome. The same kind of experimental set-up was used by (Message & Gonçalves 1995). Offering the different comb-sizes at each side of the bee-spaces gave significant proof, that comb-size influences the observed reproduction rate of *Varroa*. Due to an unknown underlying mechanism, the rate of the VSH cleaning behaviour is higher on the smaller cell size side of a "bee-space". Comparing the infestation rates on each cell size per colony supposing the reproduction on the LCS being undisturbed, allows to estimate the % per cells with reproducing parasites missing (= *Varroa* reproduction being disturbed) on the SCS supposing the original percentage non-reproducing parasites on both comb types were the same. Using the data we calculate a 2.6 x higher chance for a SCS *Varroa* infected cell to be cleaned, compared to the infected LCS cell in the same colony.

Supposing the LCS brood offers the parasite a better chance to reproduce compared to SCS without the VSH trait being present in the worker bee population of the line is unrealistic. The lines (L 2 & L 6) showed no difference in reproduction rate of the parasite between SCS and LCS, a clear indication that the invasion rate of the parasite is not influenced by cell-size, but the more intensive active disruption of the reproduction (VSH) on SCS must be the mechanism that makes SCS supporting the colony survival under *Varroa* pressure. This difference in cleaning behaviour explains the observed difference in reproduction of *Varroa*.

Only colonies headed by mated queens producing worker bees able to build 4.9 mm cell size combs (SCS-able) **and** having the VSH trait have a chance to survive without treatment in an undisturbed environment. Nevertheless being SCS-able &

VSH as colony offers no re-invasion protection. Protection against re-invasion should be a next selection goal, e.g. by selection of intensive guarding as trait.

Dreher & Liebig, Ellis & Berry used genetically unspecified queens (and bees) and demonstrated clearly that cell size as such does not influence *Varroa* population growth (Dreher & Liebig 2007, Ellis et al. 2008, Berry et al. 2009). An even higher number of infested cells in colonies with 4.9 mm as compared to 5.3 mm cell size were found by Dreher & Liebig and Berry (Dreher & Liebig 2007, Berry et al. 2009). This supports our view, that cell size **plus** defined genetics can influence *Varroa* population growth. The VSH trait was unobserved present in the Carnica Harald Singer population (Fries 2004, Berg 2004, 2005, Dreher 2007, Dreher & Liebig 2007, Ellis 2008, Taylor 2008, Berry 2010, Coffey 2010, Seeley 2011).

Piccirillo & De Jong offered Africanised *mellifera* colonies three cell-sizes (4.84 mm "African", 5.16 mm "Italian" and 5.27 mm "Carniolan"). They reported on a significantly higher infestation level in the largest cell size as compared to the other two sizes and suggest: "the use of unnaturally large comb cell size should be re-examined in the light of its effect on parasite levels". A 60% higher infestation rate of adult bees observed in colonies with two cell sizes (4.84 mm and 5.16 mm) as compared to the feral colonies with only 4.84 mm cell size was mentioned (Piccirillo & De Jong 2003). This was already observed in Brazil (Goncalves et al. 1982). Piccirillo & De Jong present the first experimental data on effects of cell size on *Varroa* brood cell infestation rates (Piccirillo & De Jong 2003). Our data on surviving colonies show that for the bee population used, the colonies on smaller cell size (2003–2005) have a 1.3–2.3 times higher survival expectation rate. Cell size can influence the active reproduction of *Varroa* inside capped cells using worker larvae in worker and drone-cell of *Apis mellifera* and *Apis cerana*, Fabricius 1793. The reproduction appeared to be disturbed in the larger cell type (Zhou, Yao, Huang & Huang 2001).

A study on the mite reproduction related to available space in the cell was done by Martin & Kryger. The authors used *Apis mellifera scutellata,* Lepeletier 1836 colonies invaded by a capensis pseudo clone *Apis mellifera capensis*, Eschscholtz 1821. The capensis larvae occupied more of the cell volume. In normally filled cells (scutellata pupae) the measured *Varroa* reproduction rate was higher. They suggest, that the male, as egg laid in the upper part of the cell, cannot reach the feeding site on the pupae and the moulting site in the lower part of the cell, if the cell is "overfilled" by a capensis pupae. The phenomenon as described by Martin & Kryger cannot explain the reduced *Varroa* reproduction rates we found in the SCS group (Martin & Kryger 2002). It is known that in *Apis mellifera* the phenotype body size is regulated by the cell size. Worker bees emerging from SCS cells are expected to be smaller in size compared to those emerging from LCS (Daly et al 1988, Mc Mullan & Brown 2006).

This influence on *Varroa* reproduction cannot hold for the results obtained with the lines 3/2002, 12/2003. Here we could not find the reduced reproduction of *Varroa* in the SCS group as compared to their LCS group of sister queens. These two lines lacked the VSH trait. This meant that the factor of 2.7 on reduction of *Varroa* reproduction due to VSH was not present in the genetics of the lines 3/2002 and 12/2003. The seasonal appearance of outliers in Figs 1 and 2 are most probably caused by a reinvasion, due to active robbing and absconding of weaker colonies (= *Varroa* mites carried into a colony

by drifting bees). We suggest the high numbers of mites within the SCS are the result of active robbery of weak colonies, a process that provides foreign mites with the opportunity to be transferred to a healthy colony under these circumstances.

In this population we found cell size can be used as a management tool for *Varroa* treatment during the active season. The used *Apis mellifera* Carnica Singer population readily accepts the cell size after the selection, as shown by the actual commercial used population of > 1250 colonies wintered 2014/2015. 2010 the commercial Carnica Harald Singer population was completely on SCS > 1000 colonies. Not all "wild mated" (F1) queens produce colonies that readily accept small cells. That is another indication that cell size is a genetically controlled trait. Actually about 10% of the F1-colonies show problems with the correct comb building.

Using the parameter *Varroa* population growth as a selection parameter caused the VSH trait to be kept (or improved) during the selection (2003–2005). The 2007 & 2008 results clearly show this trait was not explicitly available in all lines. As those lines were not scanned for the trait we can only speak about different expression of the trait between the lines. We consider the traits cell size and VSH to be genetically independent. The analysis of the 2007 and 2008 experiments for VSH showed differenced levels of VSH to be correlated with cell size. The results of 2008 – both CS's in a colony – show higher level of VSH for infested brood cells on small cells. The data are statistically convincing – a behavioural explanation is missing.

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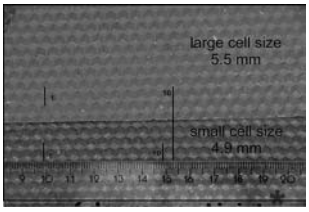
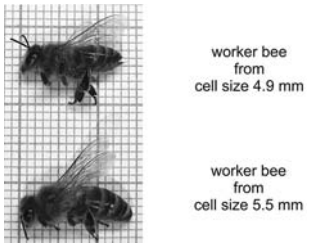


Picture 1. show SCS colonies compared to LSC colonies (2003, 2004, 2005). The colonies on SCS show a significant (p= 0.001) slower development of the *Varroa* populations. This effect is based on year, line and bee-yard. Year, line and bee-yard are considered as factors in the statistical analysis.



Picture 2. Mean number of Varroa for LCS & SCS per year.

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