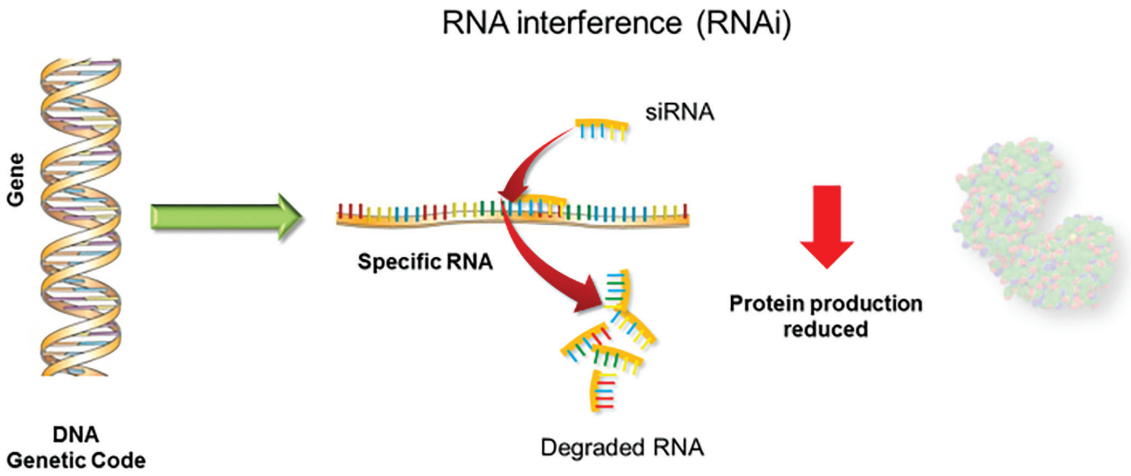


Developing Double-stranded RNA as a New Varroa Control Product

by JAMES D. MASUCCI

Varroa destructor mites are the major pest of the European honey bee and, together with the viruses they vector, are one of the major causes of high colony losses. Over the years, several management strategies have been developed to manage varroa levels, including screened bottom boards, induced brood breaks, drone brood collection, and chemical treatments (Rosenkrantz, et al., 2010, Honey Bee Health Coalition, 2018). The chemical treatments are often referred to in two categories. The so-called "soft" treatments are the fumigants, organic acids, and essential oils, and the "hard" treatments are the synthetic compounds that act as contact miticides. Having choices with multiple modes of action allows for Insect Resistant Management (IRM). IRM slows the development of resistance to any one product so that if a mite develops resistance to one miticide, it gets killed by another. If only a single miticide is used, then those mites that start to develop resistance to it can propagate and will lead to a population resistant to that single miticide.

Although there are several mite treatments on the market, various factors limit the choices to just a couple. Many of the soft treatments have temperature limitations for safe use, making them difficult to time appropriately, especially in Southern locations. As for the hard treatments, varroa in the U.S. has developed resistance to all options but amitraz. Therefore, new miticides are needed to increase the durability of all miticides by enabling IRM strategies.

Over the past several years, the Bee Health Team at Bayer has been exploring the potential for varroa control using RNA interference (RNAi) technology (see inset, next page). RNAi is one of the cell's natural mechanisms to regulate the expression of genes. The beauty in this technology is its specificity — what is lethal to the varroa mite will be harmless to the bee. If this approach is successful, it would offer a completely new mode of action for use in an IRM system. In addition, our approach targets varroa during reproduction, a different life stage than most other treatments.

A female varroa mite has two stages in her life: the time she spends on adult bees and the time she spends reproducing. During this reproductive phase, when the foundress mite and her developing progeny are inside the capped cell, they are protected from most chemical treatments because the wax cappings act as a barrier to most miticides (there are some reports that formic acid can penetrate the cappings). That's why most treatments work best when the colony is broodless or when used as continuous treatment over several weeks.

The double-stranded RNA (dsRNA) active ingredient targets reproductive mites so it is applied differently than current mite control products. The dsRNA is delivered to the colony in sugar syrup where nurse bees deliver it directly to the brood food. When the foundress mite enters the brood cell to reproduce, she buries herself in the brood food and is exposed to the dsRNA. Although some of the dsRNA gets eaten by adult bees, its specificity to mites allows for no detrimental effects to the bees. A mite treatment that is active inside the brood cell provides beekeepers with a novel treatment strategy.

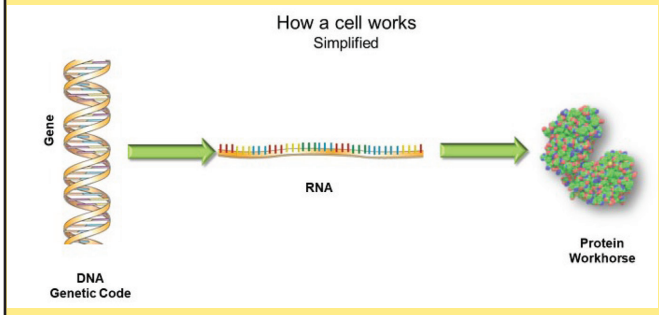
Developing a new varroa-control product requires a lot of work and investment. Not only is there a need to show that it is effective against mites and brings value to the beekeeper, but also to show that it is safe for humans, bees, and the environment. Since 2016, we have been conducting multi-location field trials to develop a dsRNA-based varroa-control product. Some of the things we learned about mite management and trial design were shared in the December 2019 issue of the American Bee Journal (Masucci, 2019). The goal of this article is to share what we've learned so far about the effectiveness of dsRNA in protecting colonies from mite damage and to provide a glimpse into the world of product development.

Field Season 2016: The importance of trial design

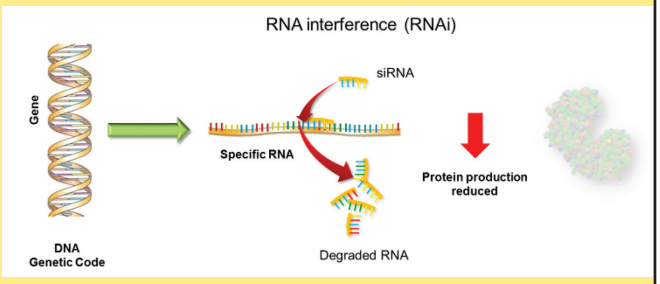
To develop a dsRNA product, we have been generating large data sets looking at the dsRNA's impact on mites, mite control, and colony survival. The year 2016 marked the first time we performed a field trial of more than 2500 colonies. Our goal was to test our product's ability to protect bees from the negative impact of mites in different environments and in different beekeeping operations. The colonies used in the trials were managed by the beekeepers for all typical beekeeping practices except varroa treatments. This approach al-

RNA interference (RNAi):

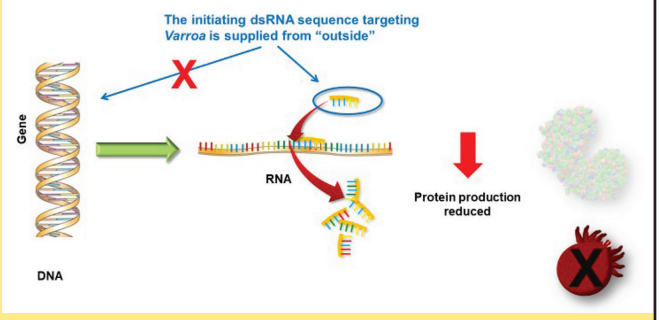
The central dogma of biology, DNA to RNA to Protein: Genes store the instructions for the form and function of all living organisms. The genes are found in the genome, which is made of DNA (deoxyribonucleic acid). The information stored in genes corresponds to the proper expression of proteins. Proteins are the workhorses of all living creatures. They play structural, sensing and enzymatic roles where they are responsible for building all the materials a cell needs to function. Therefore, each protein must be expressed in the right place, at the right time, and at the right level. When a gene needs to be expressed — that is, the protein encoded by that gene is needed — the code within the gene is used to make a gene-specific messenger RNA molecule (ribonucleic acid). It is that molecule that acts as the template for the cell to produce the protein. So, the flow of information goes from DNA (the gene) to RNA (the messenger) to protein (the workhorse).



RNA interference (RNAi) – to regulate gene expression: Cells have developed several mechanisms to regulate gene expression. Many of them are designed to alter the levels of the messenger RNA because without the messenger, no protein can be made. RNA interference (RNAi) is one mechanism that cells use to regulate the levels of the messenger. It's based on the idea of small, interfering RNAs (siRNAs). When siRNA's are present, they are used to scan all the messengers for a specific, matching sequence. When that sequence is found, that RNA is degraded. When siRNAs are present for a specific gene, messages from that gene get degraded and the corresponding protein is no longer made. When a cell recognizes a double-stranded RNA molecule, it cleaves that molecule into siRNAs. Therefore, providing a cell with double-stranded RNA that matches a messenger from a gene-of-interest will knock down the expression of that gene.



Inducing the RNAi pathway in varroa through dsRNA: dsRNA designed to match the messenger RNA from a specific varroa gene that is essential for survival is provided to varroa cells. When the double-stranded RNA enters the varroa cells, it is converted to siRNAs, recognizes the messengers for that specific gene, and causes their degradation. Without that necessary protein, the varroa dies. The beauty of this approach is its specificity. The sequence is specific to varroa and not found in honey bees. In addition, when most organisms ingest double-stranded RNA, they digest it. Pharmaceutical companies have been trying to use double-stranded RNA for years to target human diseases, with little to no success as there are many barriers that prevent the double-stranded RNA from being active. This is true for most organisms. However, there are a select few that have a specialized uptake mechanism that allows orally digested double-stranded RNA to enter cells where they can be active. Luckily, varroa is one of these organisms.



lowed us to test our product under real life conditions. We had to decide how to design the field trial in a way that incorporates the beekeeper's routine management while providing consistency in product delivery and data collection.

We initially compared the dsRNA treatment to an untreated control and demonstrated that dsRNA is better than no treatment, but that doesn't give an indication on the level of varroa control. We needed to compare the dsRNA treatment to a commercial treatment that beekeepers normally use, because if a new product can't match the efficacy of what's already on the market, it brings little value to the beekeeper. We used Apivar, a commercially available strip impregnated with amitraz for this purpose. We also tested the dsRNA at two different concentrations to test the effect of dsRNA use (the "treatment effect"). Seeing a dose effect, that is, showing a higher dose works better than a lower dose, provides confidence that the treatment effect is real.

It was important to minimize the time the researcher needed to spend on each colony collecting data, to minimize disruption to the colony. In 2016, there were three key parameters to measure: mite load, colony survival, and colony strength (this would change in subsequent years). Mite loads were determined by the alcohol wash method (De Jong, 1982). Approximately 300-400 bees were collected from each colony and sent to our lab for mite counts by alcohol washes. Although we target brood mites, evaluating mites on adult bees is the common practice and, we reasoned, an impact on brood mites should, eventually, impact the number of mites detected on adult bees. In addition, available methods to monitor brood mites are very labor intensive and time consuming and were not practical for a trial this size. Colony survival was determined as the number of surviving colonies at the end of the trial compared to the num-

ber of starting colonies. A colony was deemed dead if it lacked a queen and young brood or contained less than a frame of bees. The strength of the colony was determined by counting the number of frames covered with bees. In addition, we kept track of evidence of disease or pests.

The decision of how to randomize treatments within the trial was complicated by the possibility of mites moving between colonies. Most field trials use a design in which every treatment is randomly placed at every location thus minimizing location effects (Protocol 1, Figure 1). However, there are several reports indicating that mites can migrate between colonies (Sakofski, et al., 1990, Greatti, et al., 1992, Seeley and Smith, 2015) and mite migration could impact our research conclusions if untreated colonies are located next to treated colonies. Presumably, mites from the untreated controls could contaminate the other treatment groups. Separating colonies by treatment could reduce mite migration among colonies but also could result in differences caused by location effects.

We set up a trial to test both designs (see Figure 1). The trial used 11 different locations around the U.S. with 240 colonies at each location. A traditional design (Protocol 1) was used at six locations with five apiaries per location and 48 colonies in each apiary. Colonies were on pallets of four and all treatments were randomized by pallet. This design minimized location effects but allowed mite migration between treatments due to their proximity to each other. A modified protocol (Protocol 2) was used at five locations with the aim to minimize mite migration but allowed for location effects (see Figure 1). Treatments were randomized by apiary (Protocol 2). Each of the Protocol 2 locations had seven apiaries with each colony in the apiary receiving the same treatment. The five treated apiaries had 40 colonies and the 2 untreated apiaries had 20 colonies each. The untreated controls were separated into two apiaries to determine how much the different apiaries could vary in their results (see Figure 2 in Masucci 2019). For both protocols, apiaries were separated by a minimum of 100 yards and a maximum of 5 miles.

The schedule of the 2016 trial is shown in Figure 2. Two weeks prior to the start of the trial, an assessment was done to determine colony strength and mite loads. These data were used to randomize pallets to different treatments (Protocol 1) to ensure each treatment started with similar mite loads and colony strengths. This was not done for Protocol 2 hives because hives could not be moved once the apiaries were established. The untreated control did not receive any mite treatments for the duration of the trial. The colonies treated with Apivar received strips according to manufacturer's instructions at the start of the trial and they remained in the colonies for 8 weeks. Three dsRNA treatments were given throughout the season at 8-week intervals.

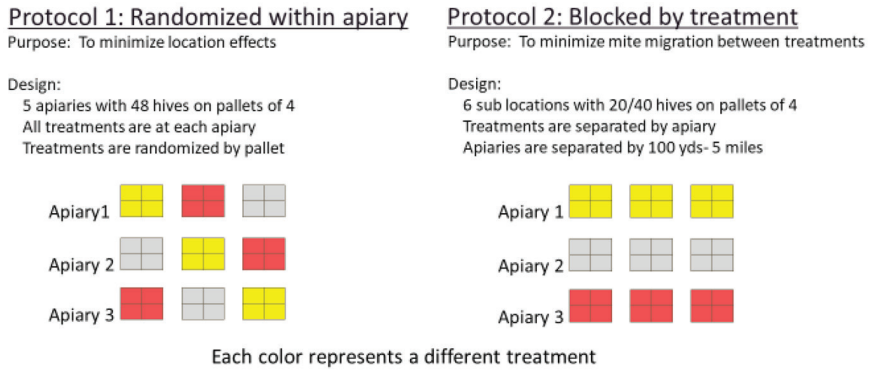


Fig. 1 *Pictorial description of how treatments were randomized in the 2016 trial. In protocol 1, all treatments were present in each apiary and they were randomized by pallet. The goal of this design was to minimize location effects. In Protocol 2, each apiary had only a single treatment. The goal of this design was to minimize the potential impact of mite migration between treatments.*

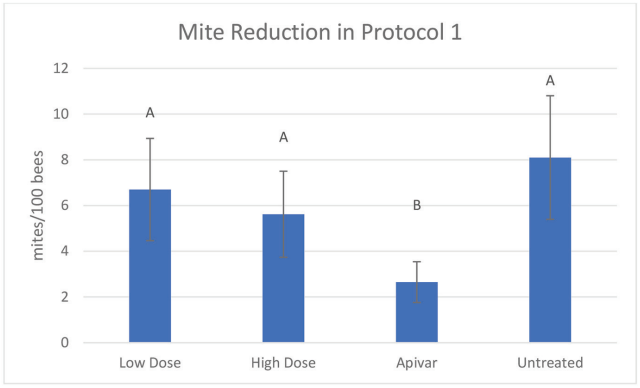
What do the data tell us?

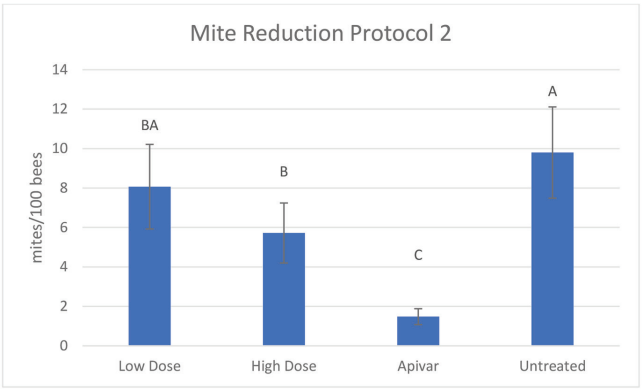
In the data figures of this article, you will see the data associated with letters. Data with like letters were not statistically different. Those with different letters were statistically significant. Data associated with multiple letters, such as AB, are not statistically different from data with either an A or a B. What does statistically different mean? Statistics is used to determine the probability that a particular outcome is the result of chance. For example, just because you get heads five times in a row during a coin toss does not mean you have a two-headed coin. The alpha level used in statistical analyses indicates the probability that a result occurs by chance. Something statistically significant at an alpha level=0.1 would have a 10% probability or less of occurring by random chance. Similarly, an alpha level=0.05 would have less than 5% probability of occurring by chance. In the case of Figure 3A, there is a greater than 10% chance that any differences observed between the dsRNA-treated colonies and the untreated control was caused by random variation.

The overall results of the 2016 field trial were quite informative. Mite infestation levels and colony survival data are shown in Figures 3 and 4, respectively. The first conclusion drawn from these data is that the trial design mattered as the data look different between the two protocols. Mite levels were not statistically different ($\alpha = 0.1$) between the untreated control and dsRNA treated colonies in Protocol 1 when all treatments were present in the same apiary (Figure 3A). Only

	Week																					
Treatment	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Low Dose dsRNA	RNA			RNA					RNA			RNA					RNA			RNA			
High Dose dsRNA	RNA			RNA					RNA			RNA					RNA			RNA			
Apivar	Apivar																						
Untreated Control																							
Assessments	1			2			3		4			5			6		7			8			9
Mite Samples	x						x		x						x		x						x

Fig. 2 *Schedule for the 2016 trial: The trial ran for 22 weeks. A dsRNA treatment consisted of two doses, three weeks apart. There were three dsRNA treatments in the trial, spaced eight weeks apart. Apivar strips were placed in the colonies at the beginning of the trial and remained in the colonies for eight weeks. The untreated control colonies did not receive any mite treatments over the course of the trial. Because the dsRNA was applied in 500 mLs of sucrose syrup, colonies not treated with dsRNA received 500 mLs of syrup only at the same time as the dsRNA treatments.*



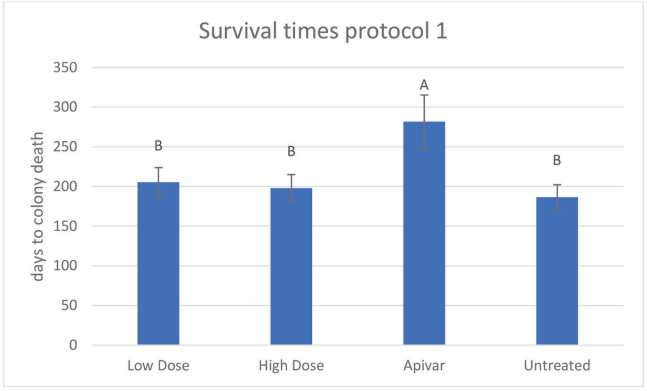


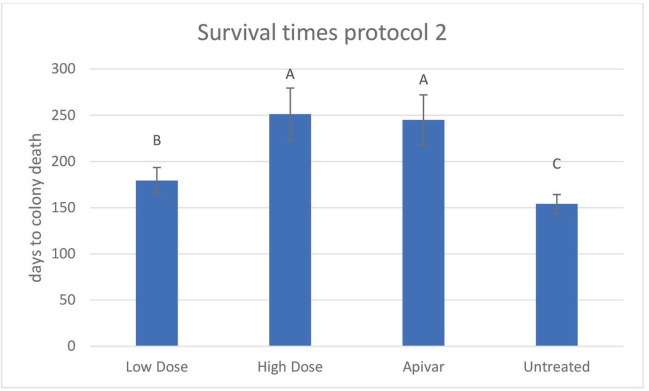
(L) Fig. 3A *Across location analysis of mite levels where treatments were randomized by pallet (Protocol 1). Mite levels were analyzed at the last assessment for each location where no differences in colony survival were detected between treatments. This was done because colonies with the highest mite levels died first, thus artificially reducing mite levels in subsequent assessments. Different letters indicate statistical significance between treatments at α = 0.1.* **(R) Fig. 3B** *Across location analysis of mite levels where treatments were randomized by apiary (Protocol 2). Mite levels were analyzed at last assessment for each location where no differences in colony survival were detected between treatments. This was done because colonies with the highest mite levels died first, thus artificially reducing mite levels in subsequent assessments. Different letters indicate statistical significance between treatments at α = 0.1.*

the positive control Apivar treatment resulted in significantly lower mite levels (67% reduction). However, we did observe statistically different mite levels between the dsRNA-treated and untreated colonies in Protocol 2 when treatments were randomized by apiary (Figure 3B). Mite levels were statistically lower with the high dose dsRNA treatments compared to the untreated control. In both cases, however, mite levels were significantly lower in the Apivar treatment relative to the dsRNA treatments.

From an experimental design point of view, results from the trial highlight how field data can be influenced by design. In this case, under Protocol 1, we hypothesize that mite migration from control colonies to treated colonies, due to the close proximity among treatments, overrode the ability to detect the contribution of the dsRNA treatments to mite loads. We hypothesize that the commercial strip Apivar worked in both trial designs because it acts on the mites on the adult bees and can target mites dispersing among colonies during the eight-week treatment duration. The dsRNA product, however, works on reproductive mites inside the brood cells. As mites migrate into colonies treated with dsRNA, the exposure is limited, and yields little control. Based upon our results, dsRNA treatments can be effective when an entire apiary is treated for varroa.

The differences in study design are even more pronounced when looking at colony survival (Figure 4). Like what was seen with the level of mites on adult bees, the data in Figure 4A indicate no statistical differences between dsRNA-treated colonies and the untreated control when treatments were randomized by pallet within the same apiary (Protocol 1) and the Apivar treatment had a significantly longer survival time than any of the other treatment groups. In locations where treatments were randomized by apiary (Protocol 2), both the low dose and the high dose dsRNA treatments had significantly longer survival times compared to the untreated control (Figure 4B). In addition, a statistically-significant dose response was seen within the dsRNA treatments. The high-dose dsRNA treatment had a survival time that was significantly longer than the low dose treatment and had equally high survival as the





(L) Fig. 4A *Across location survival analysis of colonies in Protocol 1 (treatments randomized by pallet). The colony survival data was used to generate a parametric failure time model assuming a Weibull distribution fit for the survival times. The model provides the estimated time to colony death based on the treatments. Higher numbers indicate longer colony survival times. Different letters indicate statistical significance between treatments at α = 0.1.* **(R) Fig. 4B** *Across location survival analysis of colonies in Protocol 2 (treatments randomized by apiary). The colony survival data was used to generate a parametric failure time model assuming a Weibull distribution fit for the survival times. The model provides the estimated time to colony death based on the treatments. Higher numbers indicate longer colony survival times. Different letters indicate statistical significance between treatments at α = 0.1.*

Apivar treatment. We also measured colony strength and observed treatment differences in a dose-dependent manner when treatments were separated by apiary (Protocol 2, Figure 5). Colonies that received the high dose of dsRNA had significantly more bees than both the untreated control and colonies that received the low-dose. More importantly, the strength of the colonies treated with the high dose of dsRNA were equal ($\alpha = 0.1$) to the strength of the Apivar-treated colonies (Figure 5).

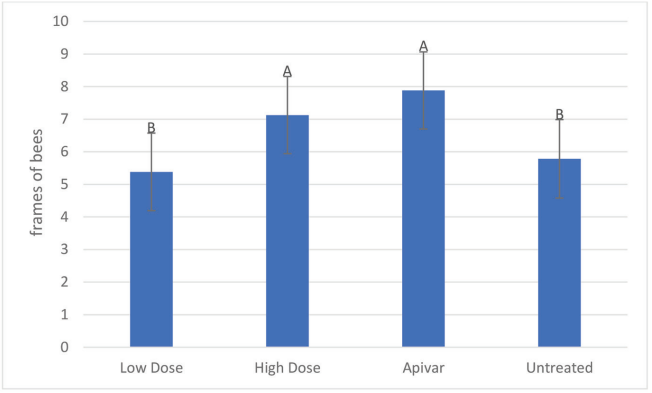


Fig. 5 *Frames of bees at the last assessment for colonies in Protocol 2 (treatments randomized by apiary). Numbers represent frames covered more than 75% with bees. Different letters indicate statistical significance between treatments at α = 0.1.*

One interesting component to this data is that Apivar provided greater mite control than dsRNA yet the survival rates and colony strength between the two treatments were comparable. Our data raises questions on how. dsRNA treatments result in colonies as healthy as Apivar-treated colonies without showing the same level of mite control. One possibility is that the effect it has on brood mites is not captured by measuring the mite load on adult bees. Alternatively, dsRNA may not be killing the mites outright but rendering them less harmful to the bees. These are questions yet to be answered.

We continued our trials in 2017 and made three changes to the trial design based on what we learned in 2016. First, we eliminated the untreated control since we hypothesized the untreated colonies in 2016 spread mites and disease to colonies within the trial. Second, we randomized by apiary. In 2016 we learned that mite migration effects could be larger than location effects and we saw that location effects occur even with colonies in close proximity (Masucci, 2019). Third, we extended the duration of our trials through the overwintering period. The 2016 data showed an impact on colony survival with only a moderate impact on mite levels. Therefore, the trial was designed with a spring and fall treatment and progressed through the winter to mid-January to determine if the "colony health" effects would continue through the winter. Colony health in January/February is paramount to commercial beekeepers as it determines how many hives are suitable for pollination.

The trial was designed to repeat the 2016 result of dsRNA-only treatments resulting in similar survival to the commercial control Apivar. Because the 2017 trial was extended through winter, the Apivar-treated colonies received two six-week treatments (spring and fall) instead of the single eight-week treatment in 2016. The Apivar label states treatments should last 6-8 weeks. In addition, a combined Apivar + dsRNA treatment was added to provide better mite control than either treatment alone based upon our observation that mite populations can recover quickly once Apivar strips are removed (Masucci, 2019). We hypothesize that a population of reproductive mites that is not sufficiently exposed to the Apivar strips allows for a quick recovery in the mite population. The combined treatment would target both mites on adult bees and reproductive mites, thereby reducing the number of mites that are not exposed during single treatments.

The results of combined treatments are shown in Figure 6. A six-week Apivar treatment occurred in the spring with dsRNA treatments happening at the start and end of the Apivar treatment. Mite levels were similar between all treatments when the strips were removed (data not shown). However, just prior to the fall treatment the levels of mites in colonies that received both treatments were significantly less ($\alpha = .05$) than those that received either dsRNA or Apivar alone. These data indicate that the ability to target both mite populations, those on adult bees and those within the capped brood cells, could provide beekeepers with better mite management options.

Overwintering survival was the same whether the colonies were treated with dsRNA, Apivar strips, or both (Figure 7A). This finding supports what was initially observed in our 2016 trial of increased survival from colonies receiving the dsRNA treatment and suggests that dsRNA has the potential to be part of IRM for varroa control. Frames of bees as the measure of colony health, was analyzed at the end of the trial (Figure 7B). The strength of the dsRNA-treated colonies was no different than the Apivar-only control. The Apivar + dsRNA treatment had, on average, nearly an extra frame of bees than the Apivar-only or dsRNA-only treatment groups, although this number was not statistically significant and needs to be repeated to evaluate its relevance.

The results of the two separate field trials showed that dsRNA-treated colonies survive as well as Apivar-treated colonies and with as many bees. We also showed in a single trial that a combination treatment of Apivar and dsRNA provided better mite control than either product alone. The consistent performance of the dsRNA over multiple years under "real life" beekeeping conditions, indicates that

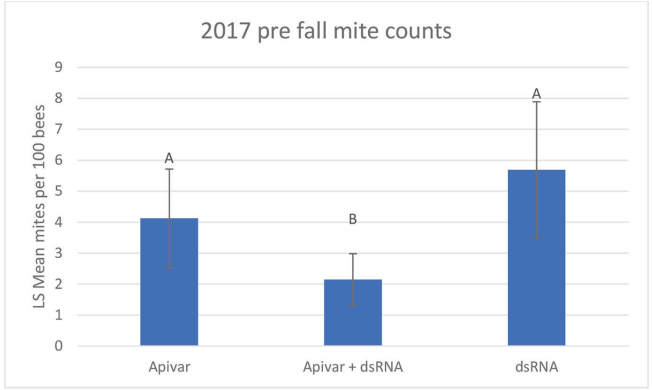
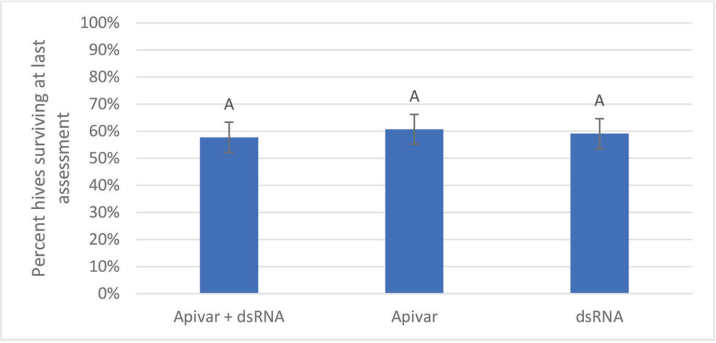
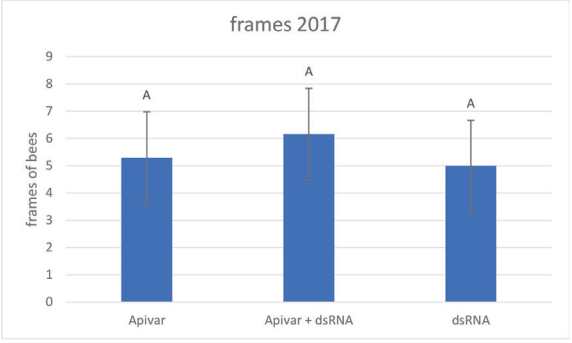


Fig. 6 *Pre-fall mite levels for 2017 trial: Mite levels just prior to the fall treatments showed that Apivar + dsRNA-controlled mites significantly better than either treatment alone. Different letters indicate statistical significance between treatments at α 0.05.*





(L) Fig. 7A *Overwinter survival rates for 2017 trial. The same level of colony survival was seen with the dsRNA treatment, the Apivar treatment, and the combination treatment.* **(R) Fig. 7B** *Overwinter frame counts for 2017 trial. The same frame counts were seen with the dsRNA treatment, the Apivar treatment, and the combination treatment.*

dsRNA has potential as a tool in an IRM system for varroa control.

Developing and testing dsRNA in the field has taught us a great deal about mite management and conducting honey bee field trials. Whether making colony management decisions or designing colony-level experiments, beekeepers need to be aware of the complexity of honey bee colonies and their interactions with each other and the environment.

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James D. Masucci - I am a molecular biologist who found a way to combine my day job with my hobby. I've been with Monsanto/Bayer for 22 years where I've worked in Biotechnology, Regulatory, and Chemistry. I also currently run about 100 hives of my own and have my little sideliner business selling bees and honey. Being both a beekeeper and a bee researcher gives me the perspective of understanding what's important scientifically and what's meaningful to the beekeeper. In 2014, I joined the Bee Health Team to run the field trial program with the aim of developing a novel varroa-control biological product. Since 2016, I've been running some of the largest honey bee field trials ever performed to evaluate how our RNAi-based product (BioDirect™) compares with what's commercially on the market. This has given me the opportunity to work with some of the top commercial beekeepers in the U.S. and Canada and to evaluate large data sets under different management systems. I've not only learned a lot about how our BioDirect™ product works in the hives, but also a lot about beekeeping and mite management in general.