Investigating RNA Interference as a Method of Varroa Mite Control

Project No.: **11-POLL10-Huang**

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Objectives and Background:

Managed honey bee colonies are currently affected by a syndrome corresponding to an abrupt depopulation during winter. Many biotic and abiotic factors are suspected to be involved into this condition, either alone or combination. Among them, varroa mite (*Varroa destructor*) is considered the greatest threat. The varroa mite has developed resistance to synthetic acaricides in many countries (reviewed by Milani, 1999), and acaricide residues have appeared in honey and beeswax (reviewed by Wallner, 1999). These residues decrease bee vitality and survival (Haarmann et al, 2002, Ali et al, 2003, Melathopoulos and Gates, 2003), and may be one of the factors contributing to the recent colony collapse disorder (Huang, 2009). The issues of acaricide resistance and residues are of pressing concern to U.S. beekeepers, as the varroa mite has become resistant to the two most effective acaricides registered in the U.S.: Apistan (active ingredient fluvalinate), and Checkmite+ (active ingredient coumaphous). It is urgent that we learn more about varroa mite biology and use this knowledge for developing new and improved control methods. In this project we propose to use RNAi (RNA interference) technology to disrupt the varroa life cycle by either causing death immediately (preferred) or causing sterility in mites. Our preliminary study has successfully caused an 87% reduction in gene expression by injecting double stranded ribonucleic acid (dsRNA) of the sodium channel gene into varroa mites.

In this study, we proposed to determine the effect of injecting a few selected genes on varroa mite survival and reproduction.

Interpretive Summary:

The importance of four different genes was studied in the mite *Varroa destructor,* using a cutting edge molecular technique, RNA interference (RNAi) – interfering or “silencing” gene expression. One gene, Proteasome 26s subunit ATPase, affected mite survival significantly. The other three genes, ribosomal proteins S13, L8, L11, all affected mite reproduction and did not affect mite survival significantly. After we

know the importance of these genes in mite survival and reproduction, we can find ways to introduce the double stranded RNA of these genes to mites and can manage this mite with high specificity (not affecting honey bees) and low toxicity (non toxic to bees or humans).

Materials and Methods:

RNA interference assays

RNA interference (RNAi) based gene silencing was performed using a modification of the standard procedure used for mosquitoes (Xi et al, 2008). Briefly, we first extracted mRNA from 5-6 mites using RNeasy Mini Kit (QIAGEN), then we reverse transcribed them to DNA using QuantiTect Reverse Transcription Kit (QIAGEN). We search for a candidate gene in the mite genome first (http://www.ncbi.nlm.nih.gov/genome?term=varroa%20destructor), if found, then we PCRed for the gene in our mite cDNA. Once confirmed that the gene had 50% identity in our mites, we used SnapDragon (http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl) to design double stranded (ds) RNA primers (20 bp). dsRNA (500-550 bp) was synthesized using a T7 MEGAscript kit (Ambion, Austin, Texas, United States) in our lab and verified to be correct by polymerase chain reaction (PCR).

To conduct the dsRNA injection, varroa mites were glued with their ventral side up using a thin layer of honey on a glass slide. Another piece of glass slide was offset about 2 mm so that the mites had no room to move during injection. Approximately 20 pl dsRNA (at a concentration of 4 µg/µl) in water was injected into the idiosoma of mites between the dorsal and ventral plates just behind the capitulum using a Femtojet Injector (Eppendorf, Hamburg, Germany). Injection was performed using a Picospritzer II under a microscope at x40 magnification. The injection time was set to 195 minisecond and the injection pressure provided by a nitrogen tank was set to 15 pounds per square inch. Injected mites were then cleaned off the honey using a moistened brush and enclosed inside a small petri dish, with about 20 mites per dish with 3-4 honey bee drone pupae as food. The injected mites were incubated at 27°C and a relative humidity of 75%.

Assessment of varroa mite reproduction

We assessed the survival of mites during the 4 days post injection. If dsRNA injected mites did not show significant reduction in survival, then we assessed whether mite reproduction was affected. We selected recently sealed (within 6 hours) brood cells as transfer hosts. This was done by mapping the brood that was nearly capped at one time and then remapping it again 6 hours later. We obtained phoretic mites from adult honey bee workers and transferred them into recently capped brood cells using a paint brush after each cell was opened with a small pin. The opening was immediately sealed with melted beeswax after mite introduction. The brood frames were incubated at 34°C (50% RH) for 9 days after which each cell was opened and mite progenies scored.

Results and Discussion:

We developed a gentle protocol to fix the Varroa mite for microinjection and the survival rate was 81.59 ± 2.59 % for dsRNA of GFP (green fluorescent protein, as a control injection of dsRNA, which is absent in honey bees). **Figure 1** shows how we used honey to glue mites and how micro-injection was done under a microscope.

We assessed the effects on mite survival and reproduction of 4 candidate genes: proteasome 26s subunit ATPase (Pts26.4), ribosomal protein S 13 (RPS13), ribosomal protein L8 (RPL8), and ribosomal protein L11 (RPL11). For each gene, 22-61 mites were injected each time (about half for the gene, and half for control), and 3-4 batches of mites were injected, to be hosted by brood from three different colonies as replicates. A total of 171-283 mites were injected for each gene and its control.

1. Proteasome 26s subunit ATPase (Pts26.4) gene affected mite survival and we did not assess their effect on mite reproduction. ds-Pts26.4 injection caused a significantly reduction in mite survival compared to the GFP control (**Figure 2**, Log-Rank = 36.19, $P < 0.0001$, Survival Analysis by SAS).
2. Ribosomal Protein S13 (RPS13) gene: injection of dsRNA showed no difference in survival, compared with the GFP control group (Log-Rank = 1.71, $P = 0.19$). The mean (± SE) fecundities of mites that were injected with dsRNA of RPS13 gene and of GPF (control) were 1.30 ± 0.18 (N= 129) and 2.69 ± 0.24 (N=102), respectively (T-test, $P < 0.001$). RPS13 therefore seems to be affecting reproduction in *Varroa destructor* too.
3. Ribosomal protein L8 (RPL8) gene: injection of dsRNA showed no difference in survival, at 96 h post-injection compared with the GFP control group. The mean (± SE) fecundities of mites which were injected with dsRNA of RPL8 gene and of GPF (control) were 1.51 ± 0.20 (N=146) and 2.15 ± 0.23 (N=137), respectively (T-test, $P = 0.035$). RPL8 therefore seems to be affecting reproduction in Varroa mites.
4. Ribosomal protein L11 (RPL11) gene: injection of dsRNA showed no difference in survival, compared with the GFP control group. The mean (± SE) fecundities of mites which were injected with dsRNA of Ribosomal Protein L 11 (RPL11) gene and of GPF (control) were 0.20 ± 0.10 (N=94, some of the transferred mites died due to wax moth damage, so it was less than the number of injected ones) and 2.27 ± 0.20 (N=77), respectively (T-test, $P < 0.001$). RPL11 has a very strong effect in reducing the reproduction of mites (the injected mites had less than 1/10 of the control mites in the number of offspring).

Future work

We will continue to screen for more genes and test the same ds-RNA on honey bees to make sure that the selected dsRNAs affect mite survival or reproduction but do not adversely affect honey bees.

Because RPL11 gene caused a majority of mites (57 out of 64 mites) to have no offspring, we are very interested in this gene. We would like to clone RPL11 in the Varroa mite and study its functions in the future.

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Figure 1. Method for mite injection. Varroa mites were glued ventral side up using honey (left) and a varroa mite was injected with dsRNA (right).

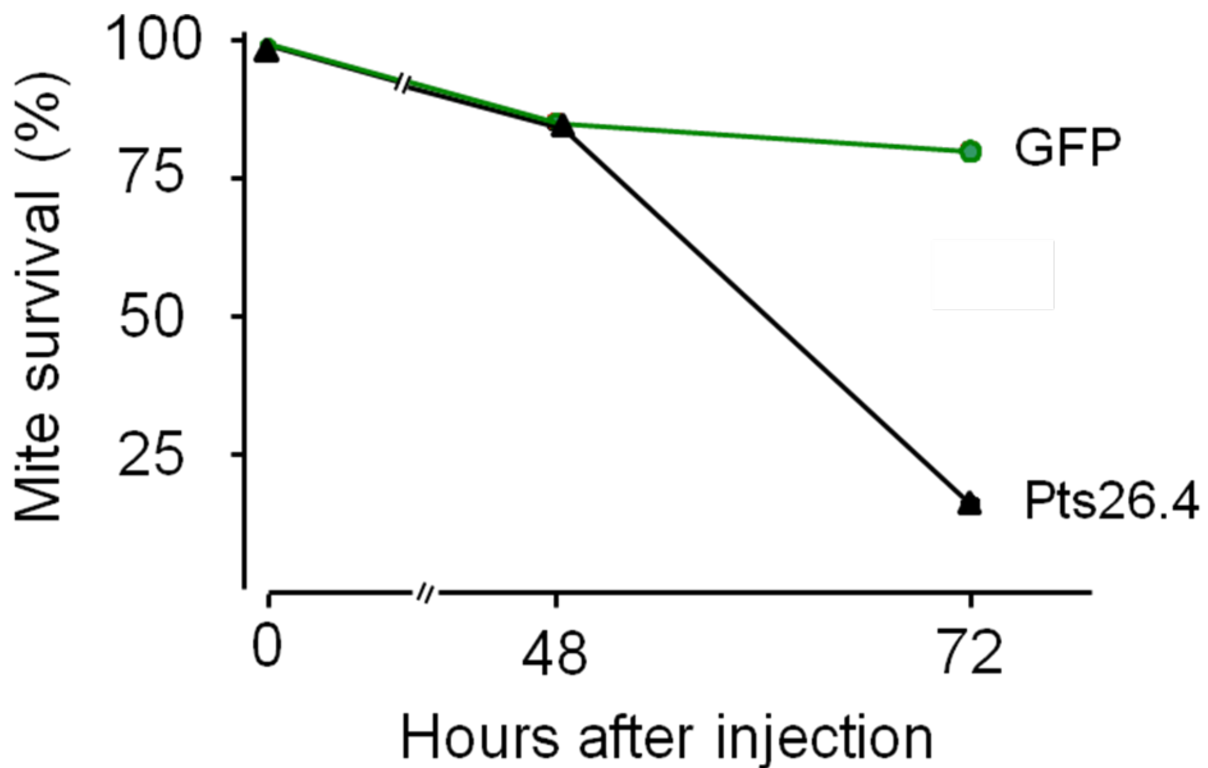

Figure 2. Survival of varroa mites after injection of double stranded RNA of proteasome 26s subunit ATPase gene (black, N = 105) or green fluorescent protein (GFP) gene as a control (green, N=125).